Cancer Chemother Pharmacol (1998) 42: 261–265

ORIGINAL ARTICLE

Sherman F. Stinson · Kimberly Hill
Timothy J. Siford · Lawrence R. Phillips
Tracy W. Daw

Determination of flavopiridol (L86 8275; NSC 649890) in human plasma by reversed-phase liquid chromatography with electrochemical detection

Received: 2 September 1997 / Accepted: 15 January 1998

Abstract *Purpose*: Flavopiridol is a flavone which inhibits several cyclin-dependent kinases, and exhibits potent growth-inhibitory activity against a number of human tumor cell lines both in vitro, and when grown as xenografts in mice. It is currently being evaluated in a phase I clinical trial at the National Cancer Institute. The objective of this project was to develop and validate an analytical method for the assay of flavopiridol in human plasma, with sufficient sensitivity to permit the plasma pharmacokinetics of flavopiridol to be studied during clinical trials. *Methods*: Flavopiridol was isolated from human plasma samples by extraction with *t*-butylmethyl ether following alkalinization with borate buffer (pH 8.0). The extract was evaporated, the residue was dissolved in mobile phase, and analyzed by reversed-phase high-pressure liquid chromatography. Chromatography was accomplished with a polymer-based C_{18} column eluted with a mobile phase consisting of methanol-phosphate buffer, pH 11.0 (53:47 v/v). Electrochemical detection (ECD) was employed. *Results*: Flavopiridol was recovered from human plasma with an efficiency of 85–87%. Calibration curves were linear over the concentration range 10–500 n*M* (4.4–219 ng/ml). Plasma standard concentrations were measured with an accuracy and precision ranging from 3.2% to 10%. Regression analysis of flavopiridol concentrations of 15 clinical trial plasma samples ranging in concentration from approximately 50 to 4000 μ*M* quantitated by both ECD and mass spectrometry showed close agreement. The equation of the regression line was $y = 1.02x + 8$ with a correlation coefficient of 0.969. Continuous infusion of flavopiridol in four patients for 72 h at a rate of 50 mg/m^2 per day, resulted in mean steady-state plasma concentrations of from 200 to 300 n*M*. Levels declined in a biexponential manner following termination of the infusion, falling to approximately 10 n*M* after 48 h. *Conclusions*: An analytical method for the assay of flavopiridol in human plasma was developed with sensitivity to at least 10 n*M*. The assay is accurate, precise and specific, and is suitable for determination of plasma flavopiridol concentrations for pharmacokinetic studies during clinical trials.

Key words Flavopiridol · L86-8275 · NSC 649890 HPLC

Introduction

Flavopiridol (L86 8275, NSC 649890, Fig. 1) is a novel flavone currently in a phase I clinical trial at the National Cancer Institute (NCI) [7]. It was originally synthesized based on the chemical structure of the alkaloid Rohitukine, which emerged from a research program directed at discovering natural products with both antiinflammatory and immunomodulatory properties [5, 6]. A number of Rohitukine derivatives were also screened for their ability to inhibit EGF receptor kinase activity. Flavopiridol displayed potent activity, kindling

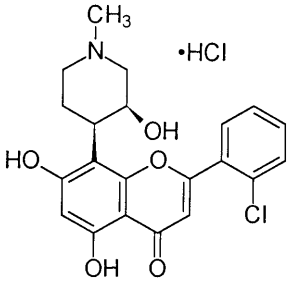

Fig. 1 Chemical structure of flavopiridol

S.F. Stinson (✉) · L.R. Phillips
Laboratory of Drug Discovery Research and Development,
Developmental Therapeutics Program,
Division of Cancer Treatment Diagnosis and Centers,
National Cancer Institute – FCRDC,
Frederick, Maryland 21702, USA
Tel. +1-301-846-1118; Fax +1-301-846-6910

K. Hill · T.J. Siford · T.W. Daw
SAIC Frederick, National Cancer Institute – FCRDC,
Frederick, Maryland 21702, USA

interest in the compound as a potential anticancer therapeutic agent.

Flavopiridol was tested against a panel of 60 human tumor cell lines in the NCI primary in vitro anticancer drug screen. It exhibited a unique pattern of differential growth inhibitory activity, completely inhibiting the growth of certain lung, colon, ovarian and prostate cell lines at concentrations below 200 n*M* [6]. In addition, flavopiridol retarded the growth of various human tumor xenografts implanted subcutaneously in nude mice [2, 6]. Investigations into the mechanism of its cytostatic activity revealed that flavopiridol strongly inhibits cyclin-dependent kinases cdk1, cdk2, cdk4 and cdk7, arresting cell cycle progression in both G_1 and G_2 [1, 3, 4, 6, 8]. Because of these unique properties, flavopiridol was selected for clinical trial by the NCI, to be administered as a 72-h continuous intravascular (IV) infusion.

An analytical method for the assay of flavopiridol was required for the clinical trial to allow quantitation of steady-state plasma concentrations during the infusion, as well as postinfusion levels. Preclinical pharmacokinetic studies conducted in mice, rats and dogs [6], suggested that steady-state plasma concentrations of 20 to 90 n*M* (depending on the species used for the extrapolation) might be anticipated in humans at the initial dose level. Postinfusion levels, of course, would be much lower. The assays used for the preclinical studies did not possess sufficient sensitivity to accurately quantitate flavopiridol concentrations in this range. It was, therefore, necessary to develop a method with the required sensitivity for the clinical trial. This communication describes an analytical method for the assay of flavopiridol in human plasma employing high-performance liquid chromatography coupled with electrochemical detection, with accuracy and precision to concentrations of at least 10 n*M* (4.4 ng/ml).

Materials and methods

Reagents and chemicals

Flavopiridol was synthesized by Hoechst Marion Roussel, and supplied by the Pharmaceutical Resources Branch, Developmental Therapeutics Program, Division of Cancer Treatment, Diagnosis and Centers, NCI.

Methanol, acetonitrile and *t*-butylmethyl ether were all HPLC grade and were obtained from various sources. Sodium tetraborate (99.99%), the internal standard (IS) 2-amino-3-benzyloxypyridine (99%), dibasic sodium phosphate (ACS grade), sodium hydroxide (ACS grade), ammonium formate (97%) and formic acid (96%), were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). All chemicals were used without additional purification.

Apparatus and analytical conditions

Chromatography was performed using a Hewlett Packard (HP) (Palo Alto, Calif.) model 1050 pump and autosampler, and a HP 1049A series electrochemical detector (ECD). The ECD was equipped with a glassy-carbon electrode operated in the oxidation mode, with an operating potential of 0.75 V. The analytical system was interfaced to a computer with HP software installed, which was responsible for module control and data acquisition and analysis. The system utilized a stainless steel, 4.6 × 150 mm analytical column containing YMC-Pack Polymer C_{18} packing (YMC, Wilmington, N.C.). A 0.5-μm filter was installed before the column. The column was eluted isocratically with a mobile phase consisting of methanol/0.01 *M* sodium phosphate buffer, pH 11.0 (53:47, v/v), at ambient temperature, using a flow rate of 1 ml/min.

To verify assay specificity, a similar pump and autosampler were connected in series to a HP 1050 variable wavelength ultraviolet (UV) detector and a HP 5989A mass spectrometer (MS). A 3.9 × 150 mm stainless steel column containing Nova-Pac C_{18} packing (Millipore, Milford, Mass.) was eluted isocratically at a flow rate 0.7 ml/min, with a mobile phase composed of acetonitrile/0.05 *M* ammonium formate buffer, pH 3.0 (45:55, v/v), at ambient temperature. The column effluent was first monitored for UV absorbance at a wavelength of 275 nm (6.5 nm bandwidth), and then it was introduced directly into the thermospray interface of the MS. The thermospray ion source was operated in the filament-assisted ionization mode with positive ion detection. The operating temperatures for the ion source, probe stem and probe tip were 250 °C, 114 °C, and 228–232 °C, respectively. Nominal resolution mass spectra (150–500 u were acquired at the rate of 0.82 scan/s with the electron multiplier set at 2500 V. Selected-ion monitoring was performed by measuring the ions at m/z 402 and m/z 404 sequentially, using a mass width of 0.07 u and a dwell time of 1000 ms, with the electron multiplier set at 2400 V. Signals were monitored and analyzed as described above.

Calibration standards

Flavopiridol stock solutions with a concentration of 1 m*M* were prepared in DMSO. The stock solution was appropriately diluted with drug-free plasma to yield a 500 n*M* plasma standard. Serial dilution of this standard with drug-free human plasma yielded plasma standards ranging in concentration from 10 to 500 n*M* (4.4 to 219 ng/ml). Plasma standards were stored at −70 °C for up to 1 month without apparent deterioration.

Sample preparation

A 200 ng/ml solution of IS in 0.0125 *M* sodium borate buffer (pH 8.0) was prepared from a 1 mg/ml stock of the IS in DMSO. A 100-μl aliquot of calibration standard or patient plasma and 100 μl of the borate buffer containing IS was mixed by vortexing for 15 s in a 15-ml glass conical-bottomed screw-top centrifuge tube, following which 7.5 ml of *t*-butylmethyl ether was added. The tubes were mixed vigorously for 5 min, then centrifuged at approximately 3000 *g* for 10 min. The organic (upper) layer was transferred to a similar tube, and evaporated to dryness in a centrifugal vacuum concentrator (Jouan, Winchester, Va.). The residue was reconstituted in 250 μl mobile phase, transferred to a glass autosampler vial, and 200 μl of this was sampled for analysis.

Quantitation

Calibration curves were constructed by plotting the ratio of the peak areas of flavopiridol to the IS against the theoretical flavopiridol concentration of the plasma standards. Linear least squares regression analysis was performed using a weighting factor of 1, $1/y$, and $1/y^2$, and the line of best fit was determined from analysis of residuals, the regression coefficient and the standard error of the fit. Based on these considerations, the line of best fit was usually obtained with a weighting factor of $1/y^2$. The concentrations of plasma samples were determined from the calibration curve by interpolation.

Recovery, precision and accuracy

The absolute recovery of flavopiridol from human plasma was determined from comparison of the peak areas of standards

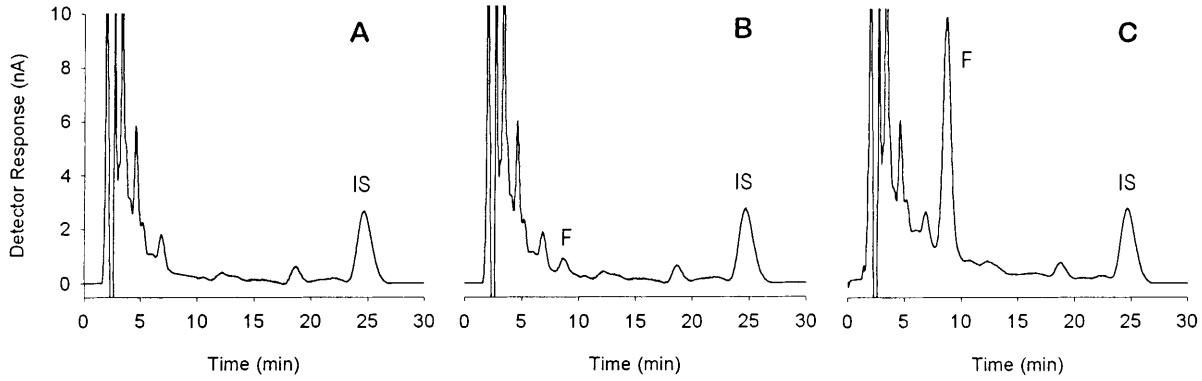

Fig. 2A–C Selected HPLC chromatograms obtained from extracted samples of (**A**) blank human plasma, **(B)** blank human plasma with flavopiridol added to a concentration of 10 n*M*, and (**C**) blank human plasma with flavopiridol added to a concentration 250 n*M* (*IS* internal standard, *F* flavopiridol)

prepared in mobile phase, to extracted plasma samples containing the same amount of drug. Precision and accuracy were assessed by regression and statistical analysis of calibration curves assayed under routine laboratory conditions on 20 randomly selected days over a 4-month period. The predicted concentration for each standard was calculated from its observed peak area ratio using the slope and intercept of the regression line. The precision of the assay was indicated by the coefficient of variation of the mean of the predicted concentrations for each standard. The ratio of the mean of the predicted concentration for each standard to the theoretical concentration provided the estimate of accuracy.

Specificity

Fifteen plasma samples from patients treated with flavopiridol, with concentrations determined by HPLC with ECD from approximately 50 to 4000 n*M*, were prepared for analysis as described above, and re-assayed by HPLC with MS detection. Plasma concentrations quantitated using the two different methods were compared by linear least-squares regression analysis.

Stability of flavopiridol in human plasma

The stability of flavopiridol in human plasma was evaluated by conducting kinetic studies with initial concentrations of 50 and 500 n*M* at temperatures of −20 °C and 5 °C, and at room temperature (approximately 23 °C). The stability in heparinized whole blood was determined in a similar manner at 5 °C and at room temperature.

Applicability

Plasma samples from a group of four patients given 50 mg/m^2 per day flavopiridol as a 72-h continuous IV infusion were analyzed to demonstrate the applicability of the analytical method to pharmacokinetic studies of patients enrolled in the clinical trial. Plasma samples were initially assayed in duplicate. Samples whose replicate determinations deviated from their average by more than 10% were re-assayed until acceptable agreement was obtained.

Results and discussion

Chromatography

Acceptable chromatography, defined in terms of peak shape and separation from endogenous interfering substances, and sensitivity, were the primary considerations dictating the choice of the chromatographic system used for the assay of flavopiridol in human plasma. To obtain acceptable chromatography, buffers at or below a pH of 3, or above a pH of 11 were required. To achieve the sensitivity essential for the clinical trial, ECD was selected. Efficient electrochemical oxidation of flavopiridol was accomplished only at a pH above 9. These constraints led to the selection of a mobile phase with a high pH, which necessitated the use of a column packing

Table 1 Accuracy and precision for the assay of flavopiridol in human plasma

Theoretical concentration (n*M*)	Number of replicates	Mean assayed concentration (n*M*)	Accuracy (% assayed/ theoretical)	Precision (% CV)
Interday				
10	20	11.0	110	9.5
25	20	24.5	98	6.9
50	20	47.8	96	6.8
75	20	73.4	98	5.5
100	20	97.8	98	5.7
250	20	251.8	101	6.0
500	20	506.6	101	3.2
Intraday				
25	10	25.6	102	8.2
250	10	250.5	100	5.3

which was stable under alkaline conditions. A column with a polymer-based stationary phase was selected for this purpose. Typical HPLC chromatograms of blank human plasma and plasma with flavopiridol added at concentrations of 10 and 250 n*M* are shown in Fig. 2. The retention times for flavopiridol and the IS were approximately 9 and 25 min, respectively, with a run time of 30 min. No peaks were detected in blank human plasma which coeluted with either flavopiridol or the IS. Variation in chromatographic properties between unused columns was insignificant, but with continued use of a column, minor adjustment of the relative proportions of mobile phase constituents was necessary to maintain optimal separation of flavopiridol from interfering peaks. While acceptable separation of flavopiridol and the IS from endogenous substances was achieved, the peak widths were greater than ideal. Peak widths at half-height were approximately 0.7 and 1.0 min for flavopiridol and the IS, respectively. This is probably related to the chromatographic properties of the polymer-based stationary phase, resulting from the large particle size (6 μm) and low carbon load (10%) relative to most silica-based analytical C_{18} columns, and to the high pH, as the peak width of flavopiridol, when chromatographed at low pH using a silica-based column, was generally less than 0.3 min with a similar retention time. While the chromatography of flavopiridol at low pH with a conventional column packing was superior, the sensitivity with UV or MS detection was only approximately 100 n*M*. While this was sufficient for verification of assay specificity for samples possessing concentrations of flavopiridol above this limit, it was inadequate for the lower concentrations of drug encountered in the pharmacokinetic studies.

Assay validation

The analytical method was validated in terms of recovery, accuracy, precision, linearity and specificity. Recovery was assessed at plasma concentrations of 25 and 250 n*M*. The extraction efficiencies were 85% and 87%, respectively. Accuracy and precision for the assay of flavopiridol in human plasma are summarized in Table 1. Accuracy of the assay over the range of concentrations analyzed varied from 98% to 110% of the theoretical concentration, and the precision ranged from 3.2% to 9.5%. Linear regression analysis of the data indicated that the peak area ratio of flavopiridol to the IS was linear with the theoretical plasma concentration over the concentration range of 10 to 500 n*M*. The mean regression coefficient was 0.996 (± 0.003).

The specificity of the method was demonstrated by close agreement of the plasma concentrations assayed using two different chromatographic systems employing either ECD or MS detection. The slope of the regression line comparing the two sets of values was 1.02 (y intercept = 8) with a regression coefficient of 0.969 (Fig. 3).

Stability of flavopiridol in human plasma

Human plasma with flavopiridol added at concentrations of 50 and 500 n*M* showed no detectable change in flavopiridol concentration for up to 1 month at −20 °C, 1 week at 5 °C and 24 h at room temperature. In human heparinized whole blood samples, flavopiridol levels showed no detectable change for up to 24 h at 5 °C or at room temperature.

Applicability

The mean plasma concentrations during and after a 72-h continuous IV infusion of four patients treated with

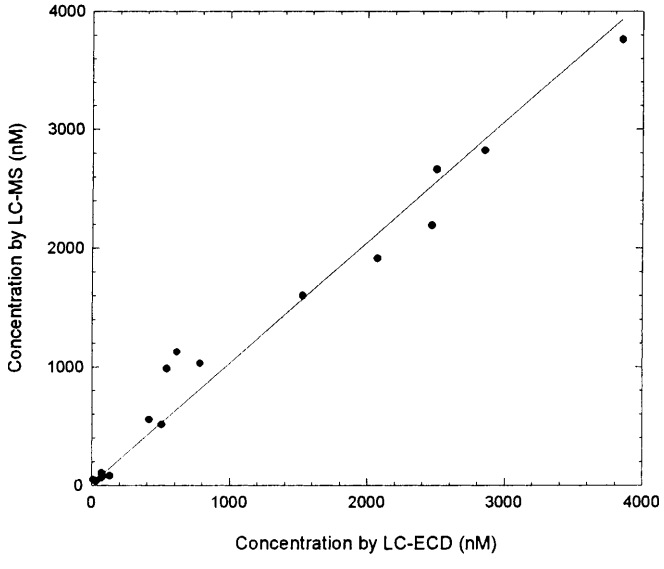

Fig. 3 Comparison of flavopiridol plasma concentrations determined using HPLC with electrochemical (LC-ECD) and MS (LC-MS) detection. Shown are the data points and the line of best fit determined by linear regression analysis

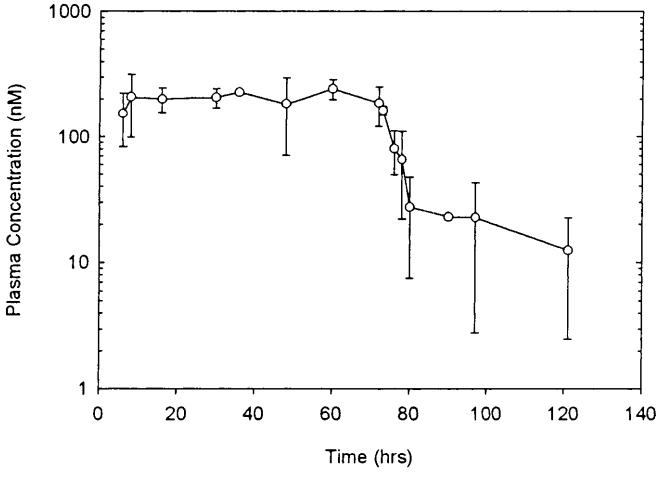

Fig. 4 Mean plasma concentrations of flavopiridol from four patients during and after a 72-h continuous IV infusion at a dose of 50 mg/m^2 per day. *Error bars* represent the range of concentrations assayed

flavopiridol at a dose 50 mg/m^2 per day are shown in Fig. 4. Mean concentrations of flavopiridol during the infusion were between 200 and 300 n*M*. Following the infusion, levels declined in a biphasic manner. There was considerable variability between the patients in the levels observed during the terminal phase. Terminal half-lives also varied widely, ranging from 3 to 28 h. A few samples contained concentrations below the limit of the standard curve, but as replicate determinations reproduced within 10%, they were included for illustrative purposes.

Conclusions

An analytical method employing HPLC with ECD for the assay of flavopiridol in human plasma was developed. The stability of flavopiridol in human plasma is sufficient to allow routine and convenient collection, handling and storage of samples. Flavopiridol was efficiently isolated from plasma by simple liquid-liquid extraction. The assay is specific for flavopiridol, and possesses acceptable linearity, accuracy and precision over a concentration range of 10 to 500 n*M*. The sensitivity of the method is adequate to permit quantitation of flavopiridol at concentrations encountered during and after drug administration in clinical trials.

References

1. Carlson BA, Dubay MM, Sausville EA, Worland PJ (1996) Flavopiridol induces G1 arrest with inhibition of cyclin-dependent kinase (cdk 2 and cdk 4 in human breast carcinoma cells. Cancer Res 56: 2973
2. Czech J, Hoffman D, Naik R, Sedlacek H-H (1994) Antitumoral activity of flavone L 86-8275. Int J Oncol 6: 31
3. Kaur G, Stetler-Stevenson M, Sebers S, Worland P, Sedlacek H, Myers C, Czech J, Naik R, Sausville E (1992) Growth inhibition with reversible cell cycle arrest of carcinoma cells by flavone L86-8275. J Natl Cancer Inst 84: 1736
4. Losiewicz MD, Carlson BA, Kaur G, Sausville EA, Worland PJ (1994) Potent inhibition of cdc2 kinase activity by the flavonoid L86-8275. Biochem Biophys Res Commun 201: 589
5. Naik R, Kattige SL, Bhat SV, Alreja B, de Souza NJ, Rupp RH (1988) An antiinflammatory cum immunomodulatory piperidinylbenzopyranone from *Dysoxylum binectariferum*. Tetrahedron 44: 2081
6. Sedlacek HH, Czech J, Naik N, Kaur G, Worland P, Losiewicz M, Parker B, Carlson, B, Smith A, Senderowicz A, Sausville E (1996) Flavopiridol (L86 8275; NSC 649890), a new kinase inhibitor for tumor therapy. Int J Oncol 9: 1143
7. Senderowicz AM, Headlee D, Stinson S, Lush RM, Figg WD, Pluda J, Sausville EA (1997) A phase I trial of flavopiridol (FLA), a novel cyclin-dependent kinase inhibitor, in patients with refractory neoplasms. Proc Am Soc Clin Oncol 16: 226A #793 (Abstract)
8. Worland PJ, Kaur G, Stetler-Stevenson M, Sebers S, Sartor O, Sausville EA (1993) Alteration of the phosphorylation state of $p34^{cdc2}$ kinase by the flavone L86-8275 in breast carcinoma cells. Biochem Pharmacol 46: 1831