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

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-butyldimethyl 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\textsubscript{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 nM (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 nM 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\textsuperscript{2} per day, resulted in mean steady-state plasma concentrations of from 200 to 300 nM. Levels declined in a biexponential manner following termination of the infusion, falling to approximately 10 nM after 48 h. Conclusions: An analytical method for the assay of flavopiridol in human plasma was developed with sensitivity to at least 10 nM. 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
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 nM [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 cytotstatic activity revealed that flavopiridol strongly inhibits cyclin-dependent kinases cdk1, cdk2, cdk4 and cdk7, arresting cell cycle progression in both G1 and G2 [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 nM (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 nM (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-benzoxypyridine (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 x 150 mm analytical column containing YMC-Pack Polymer C18 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 x 150 mm stainless steel column containing Nova-Pac C18 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-aided 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 mM were prepared in DMSO. The stock solution was appropriately diluted with drug-free plasma to yield a 500 nM plasma standard. Serial dilution of this standard with drug-free human plasma yielded plasma standards ranging in concentration from 10 to 500 nM (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