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COMMUNICATION

Determination of Flavopiridol (L 86 8275) in Rat Serum Using Ion-Pair Reversed-Phase High-Performance Liquid Chromatography

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ABSTRACT

A specific, sensitive, and simple high-performance liquid chromatographic method has been developed for assaying flavopiridol in rat serum for preliminary bioavailability and pharmacokinetic studies. The drug and internal standard were extracted with dichloromethane from serum. After evaporating the solvent, the extract was reconstituted with mobile phase. Chromatography was performed on a 10-μ Bondapak C_{18} *column (30 cm × 3.9 mm ID), using methanol:water (50:50, v/v) containing 0.05 M ion-pairing reagent PIC B-6 as a mobile phase at a flow rate of 1.1 ml/min, with ultraviolet detection at 278 nm. The drug and internal standard were well resolved from other serum constituents. Due to high sensitivity and reproducibility the method can be adapted very well for assaying flavopiridol in higher animals.*

INTRODUCTION

Flavopiridol (L 86 8275), (–)*cis*-5,7,-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)piperidinyl]-4*H*-1-benzopyran-4-one hydrochloride, is a novel anticancer agent under clinical study in human volunteers.

The molecule was developed from the lead compound rohitukine, an alkaloid obtained from the plant *Amoora rohituka* (1), initially developed as an anti-inflammatory-cum-immunomodulatory agent. In vivo preliminary studies revealed that it inhibits the growth of LX 529 and A 549 lung carcinomas (2). It is a more potent inhibitor of cell proliferation than the polyhydroxylated flavone quercetin or the polyhydroxylated isoflavone genistein. The drug has reversible cytostatic effect which indicates the need of continuous presence of the drug for most antitumor action in vivo.

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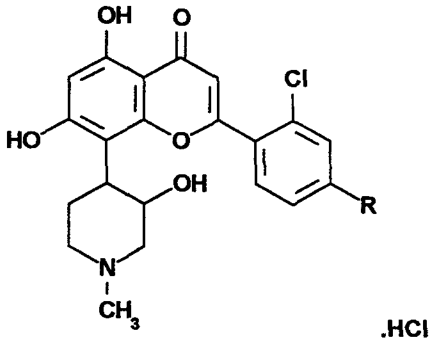

Flavopiridol, R = H; I.S., R = Cl

Structure of flavopiridol and internal standard (IS).

The work described here was undertaken to evaluate oral bioavailability of flavopiridol in rats. A very sensitive and accurate method was needed for assaying flavopiridol because of small dose of the drug (5 mg/kg of body weight) and limited volume of serum withdrawn from the animal. We describe, herein, a liquid-liquid extraction followed by the high-performance liquid chromatographic (HPLC) method and report their application to the bioavailability of flavopiridol in rats.

EXPERIMENTAL

Reagents and Chemicals

Analytical reference samples of flavopiridol and the internal standard were provided by the Chemistry Department of our Research Center. Methanol, chloroform, and dichloromethane were HPLC grade (E. Merck, India); and double-distilled water was used. PIC B-6 from Millipore, Waters Chromatography Division, was used.

Apparatus and Instruments

Instruments used were: Shimadzu's high-performance liquid chromatograph equipped with an LC-6A solvent delivery system, SIL-9A autoinjector, SPD-6AV UV-VIS spectrophotometric detector, and C-R4A as computing integrator, Sartorius 2432 balance (Sartorius GMBH, Germany), Nuova II stir plate (Thermolyne Corporation, USA), and Labaids Minor V vortex mixer (Lab Equipments Private Limited, India).

Analytical Solutions

Stock solution of flavopiridol was made by dissolving accurately weighed (1.832 mg) amount of drug in methanol (10 ml). This solution was further used for making solutions for construction of the calibration curve.

Stock solution of internal standard was made by dissolving accurately weighed (1.579 mg) amount in methanol (10 ml). This solution was further diluted with water to obtain a concentration of 3.158 μg/ml.

Sample Preparation

The serum samples stored at -20°C were thawed to room temperature. Serum (1 ml) was accurately pipetted out into a stoppered conical flask (25 ml). Internal standard solution, 200 μl (631.6 ng), was added to the serum. The serum was extracted with dichloromethane (5 ml) by vortexing for 5 min. The organic layer was separated by decantation and transferred (4 ml) to a stoppered vial. The solvent was evaporated to dryness under nitrogen at 40°C. The residue was dissolved in mobile phase (200 μl) and the aliquot was injected in triplicate on HPLC.

Chromatographic Conditions

Chromatography was performed at ambient temperature on 30 cm × 3.9 mm ID stainless steel column packed with 10-μ Bondapak C_{18} (Waters Associates). A mobile phase consisting of 0.05 M PIC B-6 in equal volumes of methanol and water was employed at 1.1 ml/min flow rate. The chromatographic separation was monitored at 278 nm at 0.002 aufs detector sensitivity. Injection volume used was 20 μl.

Calibration

Calibration samples were prepared by adding increasing volumes of the above-described working standard solution to drug-free rat serum (1 ml) and a constant volume of internal standard (200 μl). These calibration samples were processed in the same way as described in the sample preparation.

Quantitation

The concentration of the drug in the rat serum samples was determined by comparing peak-area ratios of the drug to internal standard, to the known peak-area ratios obtained from calibration samples.

Recovery Study

The extraction recovery of the compound was determined by spiking blank serum with a known amount of the drug (conc. range 0.3–2.0 μg/ml) and keeping concentration of internal standard (IS) constant. These samples were treated as unknown blood samples. Each sample was assayed in triplicate by the described assay method. The absolute recovery of drug and internal standard was calculated by comparing the peak area of the drug and IS to the paired reference solutions (a set of solutions of the same concentration range without serum).

The accuracy and precision was determined by calculating standard deviations and coefficient of variance from the results obtained from the recovery study.

Dosing and Sample Collection

Rats (5/group) weighing in the range of 100–120 g were fed orally with the drug (dose 5 mg/kg). Blood was withdrawn at 0.0, 0.25, 0.5, 0.75, 1.00, 1.5, 2.0, 2.5, 3.0 hr by killing one group at each interval. The zero-hour sample was considered as blank. The blood collected was immediately centrifuged. The serum was transferred to glass vials (5 ml), sealed with rubber caps, and stored at -20°C until assayed.

RESULTS AND DISCUSSION

Flavopiridol as a free base could be very well isolated from biological fluid by liquid-liquid extraction using water-immiscible organic solvents such as chloroform or dichloromethane. The dichloromethane gave cleaner chromatograms. Flavopiridol was efficiently extracted from serum throughout the concentration range of 0.3–2.0 μg/ml. The absolute recovery (mean ± *SD*, $n = 4$) ranged from 94.82 ± 2.80 to 104.99 ± 5.21. Aggregate mean value (± standard error) for the absolute recovery of flavopiridol at the four concentrations was 97.90 ± 4.77. The results of the extraction recovery are presented in Table 1.

The HPLC chromatogram of drug-free rat serum without addition of internal standard is shown in Fig. 1(a). Comparison with the chromatogram of rat serum with the drug and internal standard, as depicted in Fig. 1(b), clearly demonstrates the absence of endogenous components in serum which could affect the assay of drug in rat serum.

The peak area ratios of the flavopiridol to the internal standard were directly proportional to the drug con-

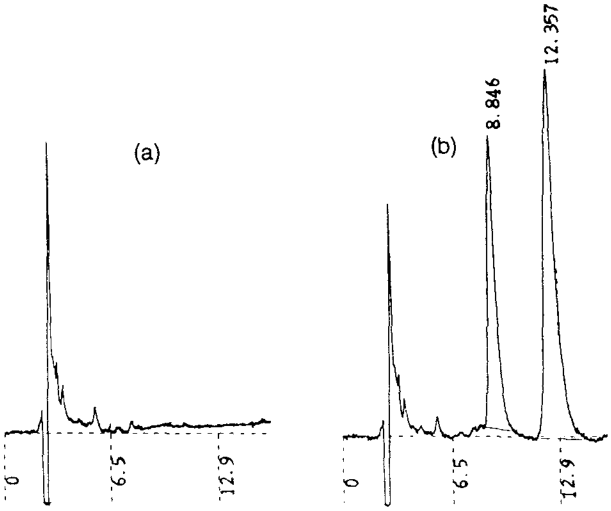

Figure 1. HPLC chromatogram of drug-free rat serum (a) and chromatogram of rat serum with drug and IS (b).

Table 1

Recovery Data for Flavopiridol from Rat Serum

Concentration Taken (μg/ml)	Concentration Found (μg/ml)	% Recovery	*SD* ($n = 3$)	% *CV*
0.293	0.282	96.46	3.79	3.82
1.025	0.978	95.33	9.47	9.93
1.612	1.692	104.99	5.21	4.96
1.905	1.806	94.82	2.80	2.96
Mean		97.90	4.77	4.87

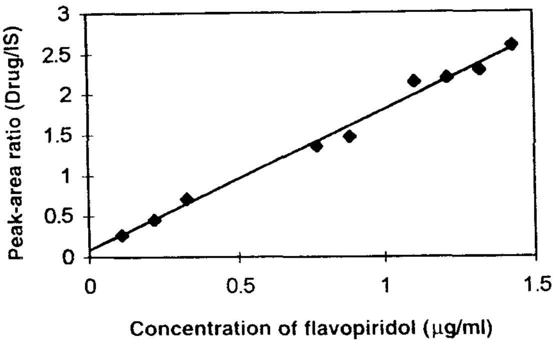

Figure 2. Standard curve plot showing the linearity between concentration of drug and ratio of peak-area of drug to IS.

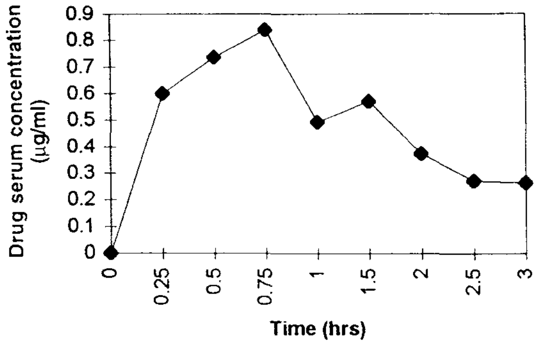

Figure 3. Graph of drug serum concentrarions at various time intervals after oral administration.

centration for the standard solutions in the concentration range 0.1 to 1.5 μg/ml. The standard curve exhibited linearity, and the regression analysis showed correlation coefficient 0.995, with intercept values that did not deviate significantly from the origin (Fig. 2).

The lower limit of quantitation was found to be 20 ng/ml of serum, which is adequate to study the bioavailability of flavopiridol in rats.

The serum levels of the drug at different time intervals are shown in Fig. 3. The peak serum concentration ($C_{\max}$) was 0.84 μg/ml at 45 min ($T_{\max}$). The area under the serum concentration-time curve, $AUC_{0-\infty}$ calculated by the trapezoidal rule (3), was 122.86 μg · min/ml.

CONCLUSIONS

A specific, sensitive, and simple assay method based on isocratic reversed-phase ion-pair HPLC has been developed for biological fluid. The simplicity of the extraction method reduces the total analysis time required. Although the limit of quantitation is 20 ng/ml of serum, sensitivity can be increased by increasing the injection volume from 20 μl upwards. The method described for the analysis of flavopiridol from rat serum can be extrapolated for human serum; it will be useful for preclinical and clinical studies as well as monitoring levels of the drug in cancer patients.

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