Journal of Chromatography, 566 (1991) 147–154
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5787

High-performance liquid chromatographic method for the simultaneous measurement of floxuridine and fluorouracil in human body fluids

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(First received April 10th, 1989; revised manuscript received December 14th, 1990)

ABSTRACT

A method for the simultaneous measurement of floxuridine (5-fluorodeoxyuridine) and fluorouracil in human plasma and peritoneal fluid has been developed. This method utilizes high-performance liquid chromatographic analysis with a Zorbax RX column (25 cm × 4.6 mm I.D.) plus a guard cartridge of the same material. The sensitivity limits for this assay are 0.25 μmol/l in a 20-μl sample. The detection limit at a signal-to-noise ratio of 3 is 2.5 nmol/l. This procedure has been used to quantitatively measure concentration *versus* time profiles of floxuridine and fluorouracil in plasma and peritoneal fluid of human patients after receiving intraperitoneal administration of floxuridine.

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0378-4347/91/$03.50

INTRODUCTION

Floxuridine, a pyrimidine antagonist, is a nucleoside consisting of the pyrimidine base fluorouracil and the sugar deoxyribose [1,2]. The effects of fluorouracil and floxuridine in carcinomas principally confined to the peritoneal cavity have been studied [3,4]. The advantage of this route of administration is that it allows for increased drug exposure to the tumor for prolonged time periods prior to drug metabolism by the liver [5–7]. Thus, side-effects associated with high systemic concentrations of the drug are decreased. Following the intraperitoneal administration of floxuridine, rapid catabolism to fluorouracil may occur. The same antimetabolic and toxic effects are seen with floxuridine as with fluorouracil, although the former drug is much more potent when given as an intravenous infusion. The action of fluorouracil on cell metabolism occurs through two pathways: one involves the incorporation of fluorouracil into RNA, and the other involves conversion of fluorouracil into fluorodeoxyuridylate (FdUMP), which inhibits thymidylate synthetase. The latter results in the suppression of DNA synthesis by depletion of thymidine triphosphate [8,9]. In contrast, unless threre is significant metabolism of floxuridine to fluorouracil, floxuridine acts primarily by inhibiting thymidylate synthetase. To date there is limited information available on the pharmacokinetics of floxuridine when administered intraperitoneally. Measurement of plasma levels and peritoneal levels of both floxuridine and fluorouracil and the evaluation of these pharmacokinetic data may provide a better understanding of both the local and systemic toxicity of floxuridine administered by the intraperitoneal route.

Presently available high-performance liquid chromatographic (HPLC) methods for measuring floxuridine and fluorouracil usually require separate assays. Those methods which do simultaneously measure floxuridine and fluorouracil lack the sensitivity required to allow for pharmacokinetic analysis of both these drugs in plasma after intraperitoneal administration [4,10–13]. This report describes a sensitive HPLC method for the simultaneous detection of floxuridine and fluorouracil from plasma and peritoneal fluid. Preliminary pharmacokinetic data of patients receiving floxuridine by the intraperitoneal route are also presented.

EXPERIMENTAL

Materials and reagents

Floxuridine was supplied as FUDR® by Quad Pharmaceuticals (Indianapolis, IN, U.S.A.). Fluorouracil was suplied as 5-FU® by SoloPak Labs. (Franklin Park, IL, U.S.A.). The internal standard bromouridine was purchased by Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.), and ammonium phosphate monobasic from J.T. Baker (Phillipsburg, NJ, U.S.A.). All other reagents were of analytical grade.

Deionized water was prepared by a Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.).

Chromatographic conditions

The HPLC system was manufactured by Spectra Physics (Piscataway, NJ, U.S.A.). It consisted of a ternary pump (Model SP 8800), an automatic dynamic mixer (Model SP 8500), and an autosampler (Model SP 8775), with a sample capacity limit of 80. The system was interfaced with a programmable integrator (SP 4290), and a UV–visible variable-wavelength detector (SP 8450). The raw data were collected and stored with a Spectra Physics WINner system interfaced with an Epson Equity 1+ computer (manufactured by Epson America, Torrance, CA, U.S.A.). Analyses were performed on a Zorbax RX column, 5 μm particle size (25 cm × 4.6 mm I.D.), plus 12.5 mm × 4.6 mm I.D. guard cartridge of the same material and manufacturer. This assay utilized a gradient mobile phase system. Using the gradient method, mobile phase A consisted of 25 mmol/l ammonium phosphate (pH 2.5), and mobile phase B of 25 mmol/l ammonium phosphate with 7% (v/v) acetonitrile (pH 7.5). HPLC was performed at 20°C with a flow-rate of 1 ml/min. The gradient was run with 100% mobile phase A for the first 5 min, then a linear gradient to 100% mobile phase B over the next 10 min. The system was then held for 10 min in 100% B and finally returned to 100% mobile phase A in 1 min. The HPLC apparatus was then allowed to equilibrate for 20 min in mobile phase A before making the next injection. Column eluates were monitored at 270 nm with a recording sensitivity of 0.005 a.u.f.s. Retention times for fluorouracil were 6–7 min and for floxuridine 16–17 min. Bromouridine, the internal standard, had a retention time of 18 min. Injection volumes were 20 μl for plasma and peritoneal fluid.

Sample prepration

All samples utilizing human plasma were prepared in a similar manner. Samples (1 ml) of drug-free plasma, plasma containing unknown amounts of fluorouracil and floxuridine or plasma standards were pipetted into Eppendorf tubes. An amount equal to 10 μmol of the internal standard bromouridine was added to all samples with the exception of drug-free plasma. After the addition of 70 μl of perchloric acid, all samples were thoroughly mixed with a Fisher Scientific touch mixer (Model 231) and allowed to remain at least 12 h at 4°C to allow for maximum protein prccipitation of the plasma. The samples were then centrifuged (Eppendorf centrifuge Model 5412) for 5 min.

The supernatant, which contained the drug, was saved for future analysis and the precipitate was discarded. Prior to HPLC analysis, the samples were neutralized carefully to a pH of 7 with 5 mol/l potassium hydroxide and placed on ice for an additional 2 h. It is important to note that adjustment to an alkaline pH will result in interference with plasma peaks during HPLC analysis.

Peritoneal samples were diluted 1:100 with double-deionized water and injected directly onto the column without any further preparation.

Preparation of a standard curve

A standard curve was prepared in water, plasma, and peritoneal fluid. The standard samples in water were prepared by adding six known concentrations of fluorouracil and floxuridine to double-deionized water at a concentration range of 0.25–10 μmol/l. The internal standard bromouridine was added at a constant concentration of 10 μl of an 100 μmol/l stock solution. This procedure was repeated with drug-free plasma and peritoneal fluid samples which were then precipitated and/or diluted using the procedure as described previously.

Recovery and assay precision

The absolute recoveries of floxuridine and fluorouracil in plasma and peritoneal fluid samples were determined by comparing peak areas of each precipitated or diluted sample to the peak areas of the standard solutions in water at each concentration employed in the standard curve (0.25–10 μmol/l). This assay procedure was performed on three consecutive days to assess system reproducibility. A set of ten samples of the same concentration (5 μmol/l) were also run each day to determine the within-day precision of the assay.

Patient studies

Twenty-five patients (sixteen male and nine female, median age of 50 years) were entered onto a clinical study which consisted of an intraperitoneal infusion of floxuridine 2 g/m^2 daily for three consecutive days (this study was reviewed and approved by an investigatinal review board). Following a single course of therapy, all patients remained drug-free for four weeks before the next course of therapy. The drug was administered in 1 l of 0.9% (w/v) sodium chloride over 1 h. Peritoneal fluid samples were taken before therapy (time 0 h), and at time intervals after the drug was given. Blood samples were also taken at time 0 h, and at time points corresponding with the peritoneal sample schedule thereafter. All blood and peritoneal samples were centrifuged for 10 min at 1000 *g* and the plasma was removed for HPLC analysis. All samples were then stored at −20°C for future HPLC analysis.

RESULTS AND DISCUSSION

The problems encountered with previous simultaneous detection methods for these drugs were the coelution of floxuridine and fluorouracil peaks or failure to have baseline separation between the two peaks, thus making quantitation difficult [4,11,13]. This method provides excellent separation of fluorouracil from floxuridine and bromouridine without interfering with plasma peaks. The method is sensitive enough to measure plasma levels of 0.25 μmol/l with a 20-μl sample. The detection limit at a signal-to-noise ratio of 3 is 2.5 nmol/l.

Typical chromatograms of extracted plasma and peritoneal fluid are shown in Figs. 1 and 2. The retention times for fluorouracil, floxuridine, and bromouridine

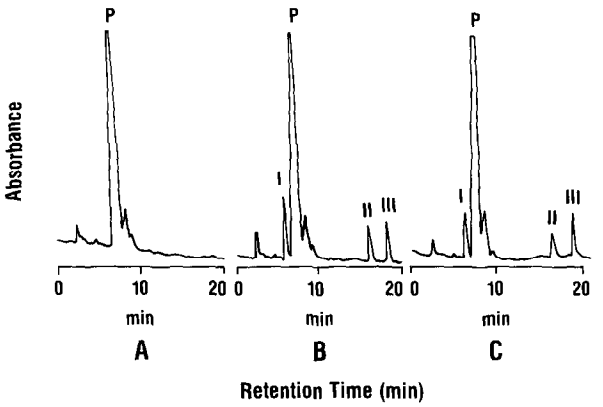

Fig. 1. Chromatograms obtained after a 20-μl injection of (A) blank plasma, (B) blank plasma containing 10 μmol/l fluorouracil (I), 10 μmol/l floxuridine (II) and 10 μmol/l bromouridine (internal standard, III); and (C) plasma 6 h after administration of 2 g/m^2 floxuridine. P = drug-free plasma.

were 6, 16, and 18 min, respectively. There were no interfering drug-free plasma or drug-free peritoneal fluid peaks noted to occur at these time points.

The recoveries of fluorouracil and floxuridine from standard plasma samples, following the specified assay extraction procedures, were assessed by comparing peak areas of these plasma extracts with those of the standard preparations at the same concentrations in water. These results are summarized in Table I. Peritoneal fluid recovery was quantitated in the same manner as the plasma recoveries,

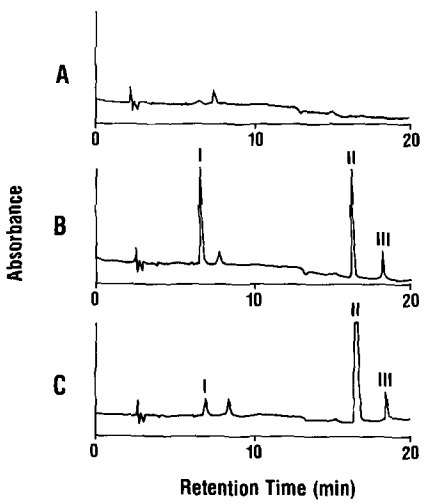

Fig. 2. Chromatograms obtained after a 20-μl injection of (A) blank peritoneal fluid, (B) blank peritoneal fluid containing 10 μmol/l fluorouracil (I), 10 μmol/l floxuridine (II) and 10 μmol/l bromouridine (I.S., III), and (C) peritoneal fluid containing 2 g/m^2 floxuridine 6 h after administration.

TABLE I

INTER-ASSAY PRECISION FOR FLOXURIDINE AND FLUOROURACIL IN PLASMA

Internal standard recovery = 98.7 ± 0.687%.

Theoretical concentration (μmol/l)	Observed concentration (mean ± S.D., n = 10) (μmol/l)	C.V. (%)	Accuracy[a] (%)
Fluorouracil			
1.9	1.84 ± 0.05	8.6	96.0
3.85	3.92 ± 0.08	2.0	102.0
7.69	7.46 ± 0.16	6.6	97.0
19.23	18.92 ± 0.46	3.0	98.4
38.45	38.99 ± 0.96	2.1	101.4
76.9	76.21 ± 1.84	3.9	99.1
153.8	148.42 ± 0.18	8.9	94.9
Floxuridine			
1.01	0.95 ± 0.14	9.1	88.0
2.03	1.95 ± 0.31	6.8	96.0
4.06	4.18 ± 0.35	2.1	103.0
10.15	10.03 ± 0.35	3.6	98.8
20.3	20.46 ± 0.26	2.7	100.8
40.6	40.15 ± 1.55	3.3	98.9
81.2	78.20 ± 0.80	6.8	96.3

[a] Accuracy = (amount found × 100)/(amount introduced).

following the appropriate dilution procedures of this assay. Resulting values ranged from 92 to 100% recovery for both fluorouracil and floxuridine.

To assess the precision and accuracy of the assay, ten plasma samples were spiked with the standard curve concentrations of each drug, ranging from 0.25 to 20 μmol/l. These samples were assayed on three consecutive days to determine day-to-day variation of the assay. Results indicate that the inter-assay coefficient of variation (C.V.) was 5.7%, with a recovery of 97% of fluorouracil at a concentration of 10 μmol/l (n = 10). Floxuridine had a recovery of 98% at 10 μmol/l concentration with an inter-assay coefficient of variation at 4.6% (n = 10). The intra-assay coefficients of variation of plasma samples containing fluorouracil concentrations of 20, 5, and 0.5 μmol were 2.0 1.5, and 1.1%, respectively (n = 10); and the coefficients of variation for floxuridine at the identical concentrations were 1.9, 2.0, and 0.9%, respectively (n = 10). The detection limits for the assay were 0.025 μmol/l in a 100-μl sample size. The detection limit at a signal-to-noise ratio of 3 was 2.5 nmol/l.

To demonstrate the pharmacokinetic capabilities of this assay for human patient samples, initially forty drug-free plasma samples were subjected to this assay

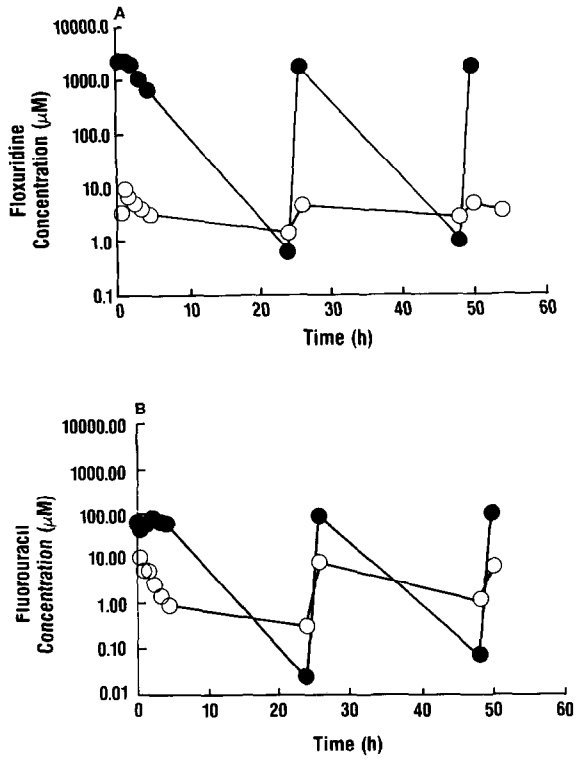

Fig. 3. (A) Plasma and peritoneal concentration *versus* time curves in a patient receiving 2 g/m^2 fluoxuridine intraperitoneally. (○) Plasma; (●) peritoneal fluid. (B) Plasma and peritoneal concentration *versus* time curves of fluorouracil in a patient receiving 2 g/m^2 floxuridine intraperitoneally. (○) Plasma; (●) peritoneal fluid.

procedure with a standard injected every fourth run. In all cases the peaks of floxuridine, fluorouracil, and bromouridine were clearly separated from the plasma peaks. This portion of the study provided assurance that the plasma peaks of forty different human subjects did not interfere with the compounds studied.

Fig. 3A and B illustrate preliminary pharmacokinetic data obtained from a patient receiving intraperitoneal floxuridine at a dose of 2 g/m^2 at 0, 24, and 48 h after the dose was given. It is of interest to note that only a small amount of floxuridine and fluorouracil are present in the systemic circulation as compared to the levels present in the peritoneal fluid. This assay is sensitive and reproducible enough to provide the information necessary to help make pharmacokinetic predictions of drug metabolism and distribution after intraperitoneal administration. It is currently utilized in monitoring patients on the previously described protocol.

ACKNOWLEDGEMENTS

This research was supported in part by Quad Pharmaceuticals (Indianapolis, IN, U.S.A.). The technical assistance of Justino Vasquez is greatly appreciated.

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