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Note

High-performance liquid chromatographic method for the determination of bromodeoxyuridine and its major metabolite, bromouracil, in biological fluids

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Administration of the halogenated nucleoside, 5-bromodeoxyuridine (BUDR) to mammalian systems is followed by its incorporation into DNA resulting in an increased susceptibility of mammalian cells to the lethal effects of x-irradiation [1—4]. Because tumor cells generally have higher turnover and therefore a higher DNA synthesis rate than the surrounding normal tissues, this radiosensitization effect of BUDR makes it a useful agent in the treatment of neoplastic disorders.

Several clinical trials have been conducted to evaluate the efficacy of BUDR [5—8]. The initial human study utilized ^{82}Br-radiolabeled BUDR and demonstrated the drug's rapid plasma clearance. Subsequent trials [6, 7] using continuous intracarotid arterial infusions of BUDR in the treatment of gliomas did not include drug level data. In a recent study [8] BUDR was administered by intravenous infusion for 12 h per day for fourteen days. Plasma BUDR

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levels were determined using a high-performance liquid chromatographic (HPLC) method which necessitated washing the column with methanol following each injection.

This paper describes a rapid, accurate and sensitive HPLC procedure for the simultaneous measurement of BUDRand bromouracil (BU) in plasma and urine samples.

MATERIALS AND METHODS

Reagents

Ethyl acetate and methanol were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), monobasic ammonium phosphate from J.T. Baker (Phillipsburg, NJ, U.S.A.), potassium hydroxide and ammonium sulfate from Mallinckrodt (St. Louis, MO, U.S.A.), BUDR, bromouracil and iodouracil from Sigma (St. Louis, MO, U.S.A.).

Stock solutions

Bromodeoxyuridine solution (0.5 mg/ml in methanol), bromouracil solution (0.5 mg/ml in methanol) and iodouracil (IU) solution (1.0 mg/ml in methanol) were prepared and stored at −20°C. Saturated ammonium sulfate solution and 0.5 *M* potassium hydroxide solution were prepared and stored at room temperature.

Standards

BUDR and BU stock solutions were mixed 1:1 and diluted with water to yield the first BUDR+BU standard solution (standard I = 100 μg/ml of each). Standard I was further diluted to 10 μg/ml of each to give the second standard solution (standard II). Iodouracil stock solution was diluted with water to yield the internal standard solution containing 10 μg/ml IU.

Quality control samples at three concentrations (200, 800 and 1600 ng/ml each of BUDR and BU) were prepared by spiking blank plasma with the appropriate volumes of the BUDR+BU standards. After mixing, 1.0-ml aliquots were transferred to glass tubes, tightly capped, and stored frozen at −30°C. Two quality control samples of each concentration were included with every group of experimental samples to be analyzed.

Chromatographic conditions

The HPLC unit included a Waters Assoc. Model 6000A pump, a Waters Assoc. Model 440 UV/VIS absorbance detector with a 280-nm filter, a Waters Assoc. Model 710B WISP autosampler and a Hewlett-Packard Model 3390A integrator. The column used was a Dupont Zorbax C_8 reversed-phase column (25 cm × 4.6 mm I.D., spherical, 6 μm particle size). The mobile phase was a 0.05 *M* ammonium phosphate buffer (pH = 7.3)—methanol mixture with a final methanol concentration of 12%. The flow-rate was set at 1.0 ml/min.

Under these conditions the retention times of BU, IU and BUDR were 5.1, 7.0 and 8.0 min, respectively. Fig. 1 depicts the chemical structures of BU, BUDR and the internal standard, IU.

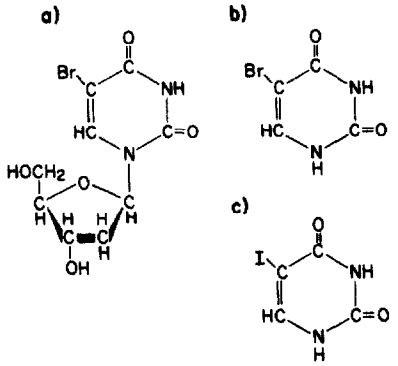

Fig. 1. Chemical structures of (a) 5-bromodeoxyuridine (BUDR), (b) 5-bromouracil (BU) and (c) 5-iodouracil (IU), the internal standard.

Sample preparation

To glass tubes (15 ml capacity) were added 1.0 ml plasma, 100 μl internal standard solution (1000 ng IU), 2.0 ml saturated ammonium sulfate solution, 100 μl ammonium phosphate buffer, pH 6.7, and 8.0 ml ethyl acetate. The tubes were tightly capped and shaken for 15 min at room temperature. In unknown plasma samples where drug concentrations were expected to exceed 2000 ng/ml, a smaller volume was utilized with the difference to 1.0 ml made up with an appropriate volume of blank plasma. After centrifugation (room temperature) for 10 min at 1200 *g*, the ethyl acetate phase was transferred to clean conical-bottomed glass tubes and concentrated to approximately 1.0 ml by evaporation in a waterbath under a stream of air. To each sample were added 400 μl of 0.5 *M* potassium hydroxide and the drugs and internal standard back-extracted by shaking for 15 min at room temperature. After centrifugation (room temperature) at 1000 *g* for 10 min, the ethyl acetate phase was removed by aspiration. A 3.0—30.0 μl aliquot of the alkaline aqueous phase was injected for HPLC analysis. The analysis of urine samples was conducted in an identical fashion.

Standard calibration curve

Blank plasma samples were spiked in duplicate with the appropriate volumes of BUDR+BU standard solutions I or II to concentrations ranging from 0 to 2000 ng/ml and subjected to the sample preparation procedure described above. Calibration curves were run with each set of experimental samples.

Calculations

Calibration curves were constructed by plotting the ratio of the peak height of each drug to that of the internal standard as a function of the plasma drug concentration. The best-fit straight line was determined using the method of least squares. The BU and BUDR concentrations of unknown samples were calculated from the least-squares regression line of the calibration curve.

RESULTS AND DISCUSSION

Under the described conditions, the retention times of BU, internal standard

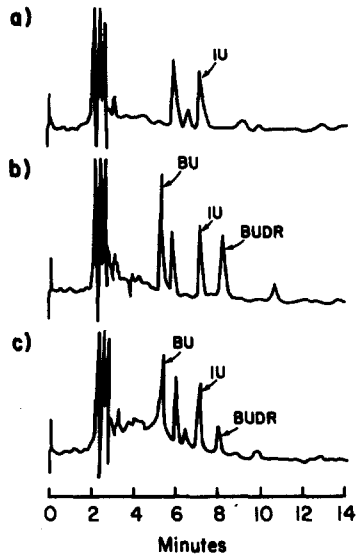

Fig. 2. Typical chromatograms of (a) control plasma, (b) a quality control sample containing 800 ng/ml BU and 800 ng/ml BUDR in plasma and (c) a plasma sample obtained from a dog 15 min post-administration of 4.0 mg/kg BUDR as an intravenous bolus injection. Internal standard (IU) was added to each sample.

and BUDR were 5.1, 7.0 and 8.0 min, respectively. Fig. 2 illustrates typical chromatograms for control plasma, a quality control plasma sample containing 800 ng/ml BU and 800 ng/ml BUDR, and a plasma sample obtained from a dog 15 min post-administration of 4.0 mg/kg BUDR as an intravenous bolus injection. The plasma samples elicit no interfering peaks. The total analysis time required for each run was 15 min. The validity of the assay procedure was established through a careful study of the linearity of response, reproducibility, accuracy and precision.

The peak height ratio was directly proportional to the BU and BUDR concentrations over a range of 50—2000 ng/ml and 100—2000 ng/ml, respectively. The best-fit lines were obtained using linear regression analyses. The results of the regression analyses for BU and BUDR were: $y = 0.00184x + 0.0171$ ($r = 0.9980$) and $y = 0.00105x + 0.094$ ($r = 0.9940$), respectively.

The accuracy and precision of the method were assessed by seeding plasma at BU and BUDR concentrations of 200, 800 and 1600 ng/ml of each. Triplicate quality control samples at each concentration were assayed on each of three consecutive days. Table I gives the results of this experiment. The precision of the assay was found to have coefficients of variation (C.V., %) ranging from 6.2% to 10.6% and 5.8% to 10.3% for BU and BUDR, respectively. The concentration means for the seeded control samples were found to be with −2.2% to +0.4% and −12.2% to −4.8% of the theoretical values for BU and BUDR, respectively.

Application of the method developed was demonstrated by measuring the plasma BU and BUDR levels in a dog following the intravenous bolus administration of a 4.0 mg/kg BUDR dose. Peripheral venous blood samples were withdrawn at regular intervals and the plasma obtained was used for drug

TABLE I

BROMOURACIL AND BROMODEOXYURIDINE CONCENTRATIONS IN SEEDED CONTROL SAMPLES ASSAYED OVER A THREE-DAY PERIOD

Day	Concentration (ng/ml)					
	Bromouracil			Bromodeoxyuridine		
1	213.2	809.8	1511.1	191.6	770.3	1455.3
	197.5	784.7	1517.6	163.1	737.6	1499.7
	204.4	739.6	1406.3	162.1	692.5	1382.8
2	216.2	802.2	1628.8	190.9	702.6	1453.1
	221.3	860.6	1721.5	190.9	929.8	1604.5
	221.3	806.2	1705.4	185.0	699.2	1656.0
3	173.2	820.7	1576.0	163.8	831.4	1618.2
	199.2	718.8	1571.0	183.6	737.8	1510.2
	160.8	699.4	1576.0	149.6	716.1	1534.5
Mean	200.8	782.4	1579.3	175.6	757.5	1523.8
S.D.	21.3	52.5	98.1	15.9	77.8	89.0
C.V. (%)	10.6	6.7	6.2	9.1	10.3	5.8
Percent from theoretical	+0.4	−2.2	−1.3	−12.2	−5.3	−4.8

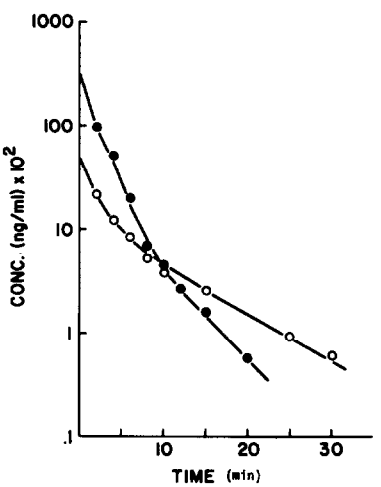

Fig. 3. The plasma BUDR and BU concentration—time profiles in a dog following the intravenous bolus administration of 4.0 mg/kg BUDR. (•) BUDR; (○) BU.

and metabolite analysis. Fig. 3 illustrates the plasma BUDR and BU concentrations versus time plots from this experiment. The BUDR data are well described by the function:

$$C_{BUDR} = 1539e^{-0.164t} + 28728e^{-0.541t}$$

The distribution half-life ($t_{½\alpha}$) of BUDR was 1.28 min and the elimination half-life ($t_{½\beta}$) was 4.23 min. The BU data are also well described by a biexponential function:

$$C_{BU} = 3472e^{-0.560t} + 1336e^{-0.105t}$$

Here, the $t_{½\alpha}$ of BU was 1.24 min and the $t_{½\beta}$ was 6.6 min.

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