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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE QUANTIFICATION OF 5-BROMO-2′-DEOXYURIDINE AND 5-BROMOURACIL IN PLASMA

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SUMMARY

A sensitive and specific procedure using high-performance liquid chromatography (HPLC) was developed for the quantification of 5-bromo-2′-deoxyuridine (BUdR) and 5-bromouracil (BU) in plasma. BUdR and BU were first extracted with a mixture of ethyl acetate and 2-propanol from plasma presaturated with solid ammonium sulfate. Following evaporation of the organic extract, the remaining residue was reconstituted in saturated ammonium sulfate solution, washed with a mixture of *n*-pentane–methylene chloride and re-extracted with the original solvent mixture. The organic extract was evaporated, reconstituted in mobile phase and chromatographed on a regular-bore ODS HPLC column using ultraviolet absorbance detection. The BUdR and BU quantification limits were both 0.1 μM, the mean intra-assay coefficients of variation were 5.0 and 5.6%, respectively, and the mean inter-assay coefficients of variation were 5.4 and 10.7%, respectively. This method was used to determine steady-state femoral arterial and hepatic venous plasma concentrations of BUdR and BU in a patient receiving a continuous intravenous infusion of BUdR (20 mg/kg per day).

INTRODUCTION

In 1960, the thymidine analogue, 5-bromo-2′-deoxyuridine (BUdR, Fig. 1) was reported to enhance the radiosensitivity of cells [1]. Recently, BUdR has been used clinically in the radiation treatment of osteosarcoma [2] and glioma [3,4]. BUdR is incorporated into DNA during the S-phase of the cell cycle [5]. Radiosensitization is presently thought to result from the reaction of incorporated

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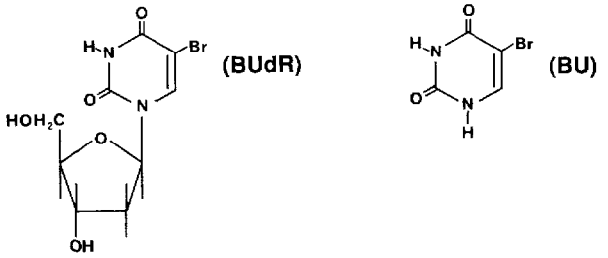

Fig. 1. Structures of 5-bromo-2′-deoxyuridine (BUdR) and 5-bromouracil (BU).

BUdR with radiation-induced hydrated electrons, producing uracilyl radicals and halide ions. These reactive intermediates abstract hydrogen atoms from adjacent deoxyribose moieties causing DNA strand breakage [6]. BUdR incorporation has also been used to estimate in vivo tumor growth fractions and cell cycle time following its intravenous administration to patients [5,7]. Recently developed monoclonal antibodies against BUdR have facilitated its use for the detection of DNA replication [8–10].

BUdR is primarily metabolized by pyrimidine phosphorylases in man to form 5-bromouracil (BU, Fig. 1) [11,12]. These enzymes have also been reported to catalyze the conversion of a related compound, 5-(2-bromovinyl)uracil, to form 5-(2-bromovinyl)-2′-deoxyuridine [13]. However, there have been no reports of the regeneration of BUdR from BU in man.

Analytical methods employing high-performance liquid chromatography (HPLC) have been previously reported for the determination of BUdR in serum or plasma [3,14,15]. However, these methods required complicated HPLC column washing [14] and were insensitive [3,15] and confounded by endogenous components at low BUdR plasma concentrations [15].

An improved HPLC assay for the quantification of BUdR and BU in plasma has been developed.

EXPERIMENTAL

Reagents and chemicals

Methylene chloride, 2-propanol and ammonium sulfate were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Ethyl acetate, *n*-pentane, 1 *M* sodium hydroxide and anhydrous sodium acetate were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Methanol was purchased from Mallinckrodt (Paris, KY, U.S.A.). Tetrabutylammonium hydrogen sulfate was obtained from Aldrich (Milwaukee, WI. U.S.A.).

High-performance liquid chromatography

The HPLC instruments included a Model M-6000A pump and a Model 990 photodiode array detector (Waters Assoc., Milford, MA, U.S.A.) and a Model 7125 injector (100-μl loop; Rheodyne, Cotati, CA, U.S.A.). The HPLC column–mobile phase combination presently reported was previously described for the quantification of theophylline [16]. An Ultrasphere® ODS HPLC column was used (5 μm particle size, 250 mm $\times$ 4.6 mm I.D.; Beckman Instruments, Berkeley,

CA, U.S.A.). The mobile phase consisted of aqueous 10m$\tilde{M}$ sodium acetate and 5 mM tetrabutylammonium hydrogen sulfate (adjusted to pH 4.75 with 1 M sodium hydroxide or glacial acetic acid)–methanol (88:12, v/v). The aqueous solution and methanol were separately filtered (Nylon-66 filters, 0.45 μm pore size; Rainin Instruments, Woburn, MA, U.S.A.) prior to being mixed. The column temperature was maintained at 23 °C with a water jacket connected to a temperature-controlled recirculation apparatus (MGW, Lauda RC3, Model T-2, Brinkman, Westbury, NY, U.S.A.). Other HPLC conditions included a mobile phase flow-rate of 1.0 ml/min and UV absorbance measurement at 290 nm.

Extraction

Ammonium sulfate (1 g), sample plasma (1 ml) and the aqueous internal standard 5-iodouracil (IU) (0.1 ml, 5 μg) were added to PTFE-lined screw-capped glass tubes (15 ml). The aqueous plasma layers were extracted twice with 5 ml of the solvent mixture ethyl acetate–2-propanol (80:20, v/v) by mixing (10 min, shaker, Eberbach, Ann Arbor, MI, U.S.A.) and centrifuging (2000 g, 10 min, Sorvall Model RC-3, Ann Arbor, MI, U.S.A.). The organic layers were decanted into clean glass tubes (15 ml) and evaporated to dryness at 23 °C, under vacuum (Speed Vac concentrator, Model SVC 200h-115, Savant Instruments, Hicksville, NY, U.S.A.). The remaining residues were reconstituted in saturated ammonium sulfate solution (0.5 ml) and twice washed with the solvent mixture methylene chloride–n-pentane (50:50, v/v) by mixing for 10 min. After each wash the organic layers were separated from the aqueous layers by standing for 1 min, then aspirated and discarded. The remaining aqueous layers were subsequently twice re-extracted with 5 ml of the original solvent mixture [ethyl acetate–2-propanol (80:20, v/v)] by mixing (10 min) and centrifuging (10 min). The organic layers were decanted into clean glass tubes (15 ml) and evaporated to dryness under vacuum at 23 °C. The tubes were capped and stored at −20 °C. The residues were reconstituted in mobile phase (0.1 ml) prior to HPLC analysis.

Extraction efficiencies

The percent recoveries of BUdR, BU and IU from plasma were determined subsequent to the HPLC analysis of spiked plasma extracts (0.1–50 μM) comprising the BUdR and BU calibration curves. The peak areas of BUdR, BU and IU were compared to their respective peak areas following the direct HPLC analysis of an organic solution containing an equivalent amount of BUdR, BU and IU.

Application of the method

A 66-year-old male (70 kg) with a colonic malignancy and hepatic metastases was administered BUdR by continuous intravenous infusion (20 mg/kg per day, IMed-960 continuous infusion pump). Blood samples (10 ml) were obtained, via catheters, simultaneously from the femoral artery and hepatic vein at 75, 90, 105 and 120 min after the start of the infusion. All blood samples were collected in polypropylene syringes containing EDTA (10 ml, Monovette, Sarstedt, Princeton, NJ, U.S.A.) and centrifuged (2000 g, 10 min, 4 °C). The plasma was trans-

ferred to polypropylene tubes (5 ml, serum/plasma filter for Monovette, Sarstedt) and stored at −20°C until analysis.

RESULTS AND DISCUSSION

Chromatograms

Control blank plasma, blank plasma spiked with BUdR and BU (both varying between 0.1 and 50 μM) and plasma samples from a 66-year-old male patient (70 kg) administered BUdR by continuous intravenous infusion (20 mg/kg per day) were extracted and analyzed by HPLC. Typical chromatograms (Fig. 2) show that there were no significant endogenous components in the blank plasma extract (Fig. 2A) which had retention times similar to that of BUdR in either the spiked or patient plasma extracts (peak c, Fig. 2B and C). There were two minor endogenous peaks in the blank plasma extract (Fig. 2A) which had retention times similar to that of BU in both the spiked and patient plasma extracts (peak a, Fig. 2B and C). Interference by these two endogenous components in the quantitation of BU was minimized by measuring the UV absorbance of the HPLC eluent at 290 nm. The wavelengths of maximum UV absorbance for BUdR and BU were 280 and 276 nm, respectively. However, the decreased absorptivity (i.e. sensitivity of detection) of BUdR and BU at 290 nm was marginal (19 and 36%, respectively) compared to the decreased absorptivity of these interfering components (66%). There were also several endogenous components in the blank plasma extract (Fig. 2A) which coeluted with the internal standard IU (peak b,

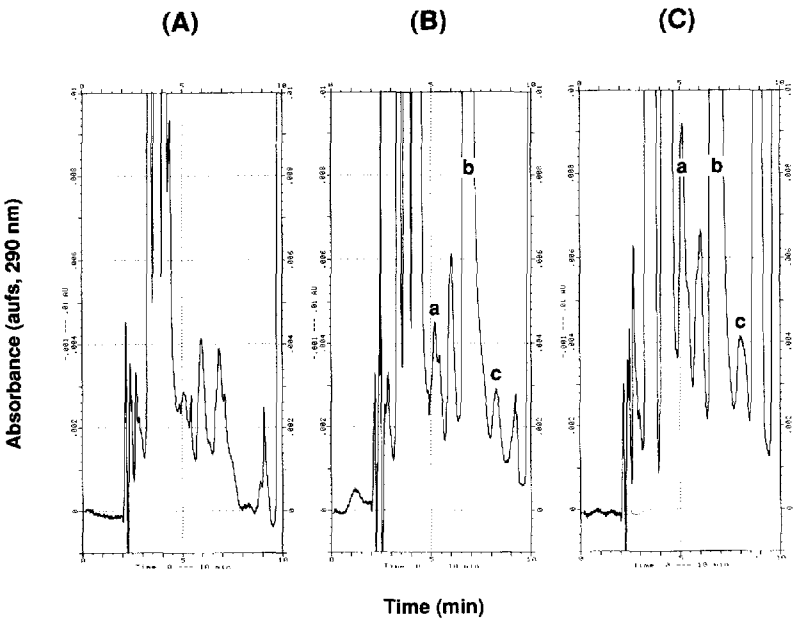

Fig. 2. Chromatograms of plasma extracts. (A) Blank plasma; (B) plasma spiked with BUdR and BU, both 0.1 μM; (C) hepatic venous plasma ($t = 75$ min) from a patient infused intravenously with BUdR (20 mg/kg per day). Peaks: a = BU; b = IU (internal standard, 5 μg); c = BUdR.

Fig. 2B and C). Since the amount of IU (5 μg) was much greater compared to the amount of these endogenous components, any interference was negligible.

Extraction efficiencies

The mean percentage recoveries of BUdR and BU as well as the internal standard IU from plasma were determined after the HPLC analysis of spiked plasma extracts containing BUdR and BU (0.1–50 μM) and IU (5 μg) for three consecutive calibration curves. The peak areas of BUdR, BU and IU (corrected for the fraction of the extract injected) were compared to their respective peak areas following the direct HPLC analysis of an organic solution containing an equivalent amount of BUdR, BU and IU. Mean recoveries of BUdR and BU (0.1–50 μM) and IU from plasma were determined to be 90.7, 82.1 and 71.7%, respectively, with coefficients of variation (C.V.) less than 15% (Table I). However, the recovery of BUdR was found to range between 83.2% (0.5 μM) and 93.6% (50 μM) and that of BU between 75.6% (0.5 μM) and 91.1% (50 μM). The extraordinarily high apparent recovery of 101.0% for BUdR at 0.1 μM is difficult to explain considering that there were no apparent endogenous components interfering with the determination of BUdR, as previously discussed. We attributed

TABLE I

MEAN PERCENTAGE RECOVERIES (DAYS 1–3) OF BUdR, BU AND THE INTERNAL STANDARD IU FROM PLASMA

Analyte	BU or BUdR concentration (μM)	Recovery (%)	Coefficient of variation (%)	n
BUdR	0.1	101.0	13.6	8
	0.5	83.2	12.4	9
	1	83.5	7.7	9
	5	90.8	9.9	9
	10	92.1	9.9	9
	50	93.6	6.5	9
	Mean	90.7	10.0	
BU	0.1	58.2	42.6	9
	0.5	75.6	9.5	8
	1	85.1	12.1	9
	5	90.4	5.8	9
	10	91.9	8.4	9
	50	91.1	6.7	9
	Mean	82.1	14.2	
IU (5 μg)	0.1	66.6	5.0	9
	0.5	66.5	5.3	9
	1	69.9	11.5	9
	5	76.5	7.2	9
	10	75.3	6.9	9
	50	75.5	9.8	9
	Mean	71.7	9.6	54

the apparent low recovery of 58.2% for BU at 0.1 μM to result from the underestimation of the BU peak area due to the interfering endogenous components.

Calibration curves

Calibration curves for BUdR and BU were obtained by fitting a quadratic function, $y=a+bx+cx^2$, to the values of plasma concentration x (BUdR or BU, 0.1–50 μM) and peak-area ratio y [(BUdR or BU peak area/IU peak area)$\times$100] using weighted $1/y_i^2$) non-linear regression [17]. The regression coefficients a, b and c for three consecutive BUdR and BU calibration curves are shown in Table II. All three calibration curves for BUdR showed positive a coefficients and po-

TABLE II

PARAMETERS OF THE QUADRATIC FIT TO THE VALUES OF PEAK-AREA RATIO AND PLASMA CONCENTRATION FOR BUdR AND BU

$y=a+bx+cx^2$, where y is the peak-area ratio ($\times$100), x is the plasma concentration (0.1–50 μM) and a, b and c are regression coefficients. Values in parentheses are standard deviations.

Analyte	Day	a	b	c
BUdR	1	0.2116	7.124	0.006805
		(0.02302)	(0.09533)	(0.007370)
	2	0.1118	5.913	0.001940
		(0.02256)	(0.08457)	(0.003398)
	3	0.1596	4.889	0.01120
		(0.02048)	(0.08387)	(0.003558)
BU	1	0.05107	4.276	−0.002360
		(0.01654)	(0.07910)	(0.00309)
	2	−0.1310	3.720	0.002360
		(0.01640)	(0.1054)	0.004454)
	3	−0.2406	4.040	−0.0006339
		(0.007614)	(0.06016)	(0.002507)

TABLE III

GOODNESS-OF-FIT STATISTICS FOR THE QUADRATIC FIT TO THE VALUES OF PEAK-AREA RATIO AND PLASMA CONCENTRATION FOR BUdR AND BU

$y=a+bx+cx^2$, where y is the peak-area ratio ($\times$100), x is the plasma concentration (0.1–50 μM) and a, b and c are regression coefficients.

Analyte	Day	Standard deviation	Coefficient of determination
BUdR	1	0.07988	0.9995
	2	0.07711	0.9995
	3	0.07120	0.9992
	Mean	0.0761	0.9994
BU	1	0.05528	0.9992
	2	0.04820	0.9982
	3	0.01796	0.9995
	Mean	0.0405	0.9990

TABLE IV

INVERSELY ESTIMATED PLASMA CONCENTRATIONS OBTAINED FROM THE QUADRATIC FIT TO THE VALUES OF PEAK-AREA RATIO AND PLASMA CONCENTRATION FOR BUdR AND BU

Inversely estimated plasma concentrations were estimated by the equation $x=\{-b+\sqrt{[b^2-4c(a-y)]}\}/2c$ where y is the peak-area ratio ($\times 100$), x is the plasma concentration (0.1–50 μM) and a, b and c are regression coefficients. The quadratic fit was $y=a+bx+cx^2$, where y is the peak-area ratio ($\times 100$), x is the plasma concentration (0.1–50 μM), and a, b and c are regression coefficients.

Day	Actual concentration (μM)	Mean predicted concentration (μM)	Mean percentage of theoretical plasma concentration	C.V. (%)	n
BUdR					
1	0.1	0.1002	100.2	3.0	3
	0.5	0.4920	98.4	8.4	3
	1	1.011	101.1	3.2	3
	5	4.923	98.5	1.7	3
	10	10.20	102.0	0.7	3
	50	49.89	99.7	0.7	3
			100.3	5.7	18
2	0.1	0.1019	101.9	14.8	2
	0.5	0.5073	101.5	0.6	3
	1	0.9729	97.29	1.7	3
	5	5.069	101.4	2.0	3
	10	10.10	101.0	2.1	3
	50	50.65	101.3	1.5	3
			100.6	4.2	17
3	0.1	0.1008	100.8	7.6	3
	0.5	0.5061	101.2	10.1	3
	1	0.9867	98.7	4.7	3
	5	4.939	98.8	0.2	3
	10	10.34	103.4	1.7	3
	50	49.77	99.5	0.9	3
			100.4	5.0	18
		Mean	100.4	5.0	
BU					
1	0.1	0.09884	98.9	2.9	3
	0.5	0.4760	105.5	0.5	2
	1	1.066	106.6	6.4	3
	5	4.7551	95.1	0.4	3
	10	9.728	97.3	1.3	3
	50	50.51	101.0	0.5	3
			100.4	4.9	17
2	0.1	0.1007	100.7	5.4	3
	0.5	04886	97.8	1.2	3
	1	1.078	107.8	20.5	3
	5	5.057	101.1	5.6	3
	10	9.940	99.4	5.5	3
	50	50.06	100.1	1.3	3
			101.2	8.6	18
3	0.1	0.1005	100.5	3.5	3
	0.5	0.4974	99.5	1.4	3
	1	0.9750	97.5	2.0	3
	5	4.932	98.6	0.3	3
	10	10.34	106.4	1.9	3
	50	49.55	99.1	1.0	3
			100.3	3.4	18
		Mean	100.6	5.6	

TABLE V

ESTIMATED PLASMA CONCENTRATIONS OF QUALITY CONTROL SAMPLES FOR BUdR AND BU

Day	Actual concentration (μM)	Mean predicted concentration (μM)	Mean percentage of theoretical plasma concentration	C.V. (%)	n
BUdR					
1	0.25	0.2536	101.4	4.4	2
2	0.25	0.2591	103.6	7.8	3
3	0.25	0.2140	85.6	1.7	3
			96.3	10.5	8
1	2.5	2.580	103.2	0.3	3
2	2.5	2.409	96.4	0.6	3
3	2.5	2.455	99.6	4.2	3
			99.3	3.3	9
1	25	25.32	101.3	1.3	3
2	25	24.07	96.3	1.3	3
3	25	25.53	102.1	3.2	3
			99.0	2.4	9
		Mean	98.2	5.4	
BU					
1	0.25	0.1827	73.0	8.2	3
2	0.25	0.2522	100.9	20.4	3
3	0.25	0.2487	99.5	0.9	3
			91.1	19.0	9
1	2.5	2.471	98.8	1.5	3
2	2.5	2.696	107.8	17.4	3
3	2.5	2.549	101.9	2.3	3
			102.9	10.0	9
1	25	24.44	97.7	1.7	3
2	25	24.55	98.2	3.4	3
3	25	25.62	102.4	2.0	3
			99.5	3.1	9
		Mean	97.8	10.7	

sitive c coefficients. The positive y-axis intercepts were probably due to what appeared to be greater recovery of BUdR at 0.1 μM, while the positive curvature of the regression lines could be readily explained by the observed increase in percentage recovery of BUdR with increased plasma concentration. Two of the three calibration curves for BU showed negative a coefficients (y-axis intercepts) and negative c coefficients (negative curvature). This was likely the result of the apparent decreased percentage recovery of BU at 0.1 μM, as previously discussed.

The BUdR and BU calibration curves displayed excellent quadratic fits, as shown by the goodness-of-fit statistics in Table III. The mean standard deviations of the data for three consecutive BUdR and BU calibration curves were 0.0761 and 0.04505, respectively, while the mean coefficients of determination were 0.9994 and 0.9990, respectively.

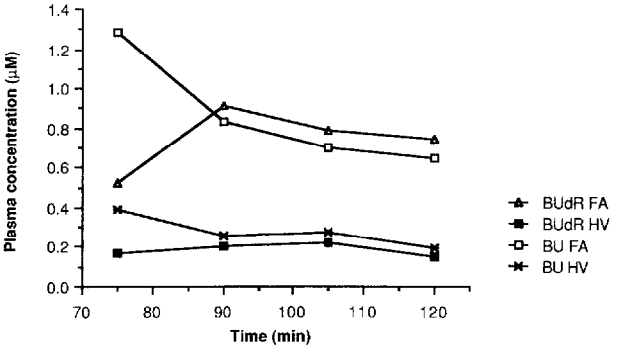

Fig. 3. Plots of BUdR and BU plasma concentration in the femoral artery (FA) and hepatic vein (HV) versus time for a patient infused intravenously with BUdR (20 mg/kg per day).

Intra-assay and inter-assay variation

Inversely estimated plasma concentrations for BUdR and BU calibration curves were calculated with the equation

$$x=\{-b+\sqrt{[b^2-4c(a-y)]}\}/2c$$

where x is the plasma concentration (μM), y is the peak-area ratio ($\times 100$) and a, b and c are regression coefficients. The inversely estimated plasma concentrations were subsequently expressed as a percentage of the theoretical spiked plasma concentrations. The mean values (days 1–3) of the mean percentages of the theoretical plasma concentrations for BUdR and BU (0.1–50 μM) were 100.4 and 100.6%, respectively (Table IV). Both BUdR and BU exhibited acceptable intra-assay (within-day) variation, since the mean values (days 1–3) of the mean coefficients of variation of the percentages of the theoretical plasma concentrations (0.1–50 μM) were less then 10% (5.0 and 5.6%, respectively, Table IV).

The accuracies of BUdR and BU quantification were verified by estimating plasma concentrations of several quality control samples (0.25, 2.5 and 25 μM). Again, the estimated plasma concentrations were expressed as percentages of the theoretical spiked plasma concentrations. The means (0.25–25 μM) of the mean percentages of theoretical for estimated plasma concentrations of BUdR and BU quality control samples were 98.2 and 97.8%, respectively (Table V). Coefficients of variation of the mean percentages of theoretical for the estimated plasma concentrations of the quality control samples were used to express the inter-assay (between-day) variations for BUdR and BU determination. The mean values (0.25–25 μM) of the mean coefficients of variation (days 1–3) were 5.4 and 10.7% for BUdR and BU, respectively (Table V).

The analytical method was applied to the quantification of BUdR and BU in plasma obtained from the femoral artery and hepatic vein of a patient infused intravenously with BUdR (20 mg/kg per day). There was sufficient sensitivity to determine steady-state plasma concentrations of BUdR and BU in both the femoral artery and hepatic vein (Fig. 3). This method could be applied to studying the pharmacokinetics of BUdR in patients receiving BUdR for radiosensitization.

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