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Short Communication

High-performance liquid chromatographic analysis of 5-ethylpyrimidines and 5-methylpyrimidines in plasma

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method employing a C_{18} reversed-phase column, a mobile phase of sodium acetate and methanol, and an ultraviolet detector was developed for the analysis of 5-ethylpyrimidines and 5-methylpyrimidines in plasma. Samples were prepared for HPLC by sequential cation-exchange and anion-exchange column chromatography. Linear standard curves were obtained for samples containing 0.05–50 μg/ml 5-ethyl-2′-deoxyuridine and 5-ethyluracil, 0.05–10 μg/ml 5-(1-hydroxyethyl)uracil, and 0.1–50 μg/ml thymidine, thymine and 5-hydroxymethyluracil. Applicability of the method to determination of the kinetics of 5-ethyl-2′-deoxyuridine elimination by the isolated perfused rat liver was demonstrated; clearance of the drug was 1.29 ml/min.

INTRODUCTION

The pyrimidine nucleosides 5-ethyl-2′-deoxyuridine (EdUrd) and thymidine (dThd, 5-methyl-2′-deoxyuridine) are investigational drugs used in the treatment of viral infections and cancer, respectively. EdUrd is effective in the topical treatment of herpes simplex virus infections in animal models [1,2] and humans [3], and is undergoing evaluation for topical use in the treatment of genital herpes infections [4]. dThd can enhance the anticancer activity of 5-fluorouracil [5,6] and cytarabine [7,8] in animal tumor models, and has been used clinically to rescue bone marrow cells from the effects of high-dose methotrexate therapy [9,10]. EdUrd and dThd are rapidly metabolized *in vivo* to the corresponding pyrimidine

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bases 5-ethyluracil (EUra) [2,11] and thymine (Thy) [12,13] in reactions catalyzed by pyrimidine nucleoside phosphorylases [14,15]. The recent identification of 5-(1-hydroxyethyl)uracil (HEUra) in the urine of rats receiving EdUrd [16] and 5-hydroxymethyluracil (HMUra) in the plasma and urine of humans receiving dThd [17] indicates that hydroxylation of the 5-alkyl group is an additional route of EdUrd and dThd metabolism.

Little detailed information is available on the kinetics of EdUrd or dThd elimination or metabolite formation, possibly due in part to a lack of suitable analytical methods. A modification of a method described by Hempel [18], for measuring plasma levels of EdUrd and EUra in the rat, required organic extraction of large volumes of plasma and a time-consuming high-performance liquid chromatographic (HPLC) separation of the test compounds by gradient elution [11]. Many brief descriptions of HPLC methods for analysis of dThd and Thy in plasma have been reported [13,17,19], but few experimental details have been provided. A sensitive and specific radioimmunoassay for measurement of serum dThd has been described, but is not suitable for analysis of Thy or HMUra [20].

Simple and efficient procedures for the isolation of fluoropyrimidine nucleosides and bases from plasma by ion-exchange chromatography [21] and subsequent analysis by reversed-phase HPLC [22] have previously been reported. In the present investigation, these procedures were modifed for the determination of plasma concentrations of the 5-alkylpyrimidine nucleosides EdUrd and dThd and the bases EUra, HEUra, Thy, and HMUra. The biological applicability of the method was demonstrated in a study of EdUrd elimination by the isolated perfused rat liver.

EXPERIMENTAL

Chemicals and supplies

EdUrd, EUra, and HEUra were kindly provided by Ortho Phamaceutical (Canada). The EdUrd used in preliminary experiments was kindly provided by Dr. R. F. Schinazi, Emory University. The following pyrimidines were purchased from Sigma (St. Louis, MO, U.S.A.): 2′-deoxyuridine (dUrd, Product No. D3251), dThd (T9250), 5-fluoro-2′-deoxyuridine (FdUrd, F0503), 5-hydroxymethyl-2′-deoxyuridine (HMdUrd, H8631), HMUra (H2627), Thy (T0376) and uracil (Ura, U0750).

Ion-exchange resins were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) and included the cation-exchange resin AG 50W-X4, 200–400 mesh, hydrogen form (Product No. 140-1341) and anion-exchange resin AG 1-X4, 100–200 mesh, chloride form (142-1351). Solutions for ion-exchange chromatography included 0.03 and 0.3 *M* ammonium formate buffer, pH 5.0, and 0.1 and 0.5 *M* sodium carbonate, pH approximately 11.3–11.5. C_{18} Reversed-phase analytical HPLC columns (Hypersil-ODS, 5 μm, 200 mm × 4.6 mm I.D.; Product No. 799160D-574) were obtained from Hewlett-Packard (Palo Alto, CA, U.S.A.).

The HPLC mobile phases were prepared by combining methanol and sodium acetate buffer, pH 4.6. The final sodium acetate concentration was 1 m*M*. Mobile phases A and B contained 8 and 3% methanol, respectively. HPLC-grade glacial acetic acid was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.), while all other HPLC-grade solvents were from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Sample preparation

Human plasma was used in the development of the HPLC methods. Standards were prepared by addition of different known amounts of test compounds and a constant amount of the internal standard to blank plasma. Sample preparation for HPLC involved sequential cation-exchange and anion-exchange chromatographic extractions of the test compounds. The procedure was modified from a method previously described for extraction of fluoropyrimidines [21]. The internal standard, FdUrd, in 70 μl of 0.3 *M* ammonium formate buffer, was added to 0.63 ml of plasma containing the test compounds. The mixture was loaded onto a cation-exchange column (4.5 cm × 0.7 cm I.D.), which had been equilibrated with 0.03 *M* ammonium formate buffer, and the eluate was discarded. Next, 4 ml of 0.03 *M* ammonium formate buffer were added and the eluate was collected. To the eluate, 1 ml of 0.5 *M* sodium carbonate was added and the sample was loaded onto an anion-exchange column (2.0 cm × 0.7 cm I.D.), which had been equilibrated with 0.1 *M* sodium carbonate, and the eluate was discarded. The column was washed sequentially with 10 ml of 0.1 *M* sodium carbonate and 10 ml of water, and then eluted with 10 ml of 0.3 *M* glacial acetic acid in methanol. The eluate was evaporated to dryness under nitrogen in a 50°C water bath. The residue was redissolved in 0.5 ml of mobile phase and a 20-μl aliquot was injected onto the HPLC column.

High-performance liquid chromatography

The HPLC system consisted of a Model 110A solvent delivery pump (Beckman Instruments, Fullerton, CA, U.S.A.), a Rheodyne Model 7125 syringe loading injection valve (Rheodyne, Cotati, CA, U.S.A.), a Beckman Model 160 ultraviolet detector operated at 254 nm, and a Waters Model 740 recorder and reporting integrator (Millipore, Milford, MA, U.S.A.). Mobile phase was delivered to the analytical column at 1.2 ml/min; mobile phase A was used for analysis of 5-ethylpyrimidines and mobile phase B was used for 5-methylpyrimidines. Standard curves were plotted as test compound-to-internal standard peak-height ratio *versus* the concentration of test compound. The equation of the standard curves was calculated by linear regression, using the method of least squares. The concentration of test compound in experimental samples was calculated from the equation of the standard curve and the peak-height ratio of the sample.

Sample recovery and stability

The recovery of compounds from plasma was determined by comparing the

HPLC peak heights of extracted and non-extracted samples. Samples of uniform volume and containing known amounts of the pyrimidines were extracted by the ion-exchange chromatographic procedure described above, and injected onto the HPLC column. Control samples, containing identical amounts of test compounds in the mobile phase, were directly injected onto the HPLC column. Recovery studies were performed at two plasma concentrations of each test compound: 0.1 and 10 μg/ml for pyrimidine nucleosides (EdUrd, dThd), and 0.05 and 5.0 μg/ml for pyrimidine bases (EUra, HEUra, Thy, HMUra). To assess the stability of the extracts, plasma samples containing internal standard and either pyrimidine nucleoside (0.1 and 10 μg/ml) or base (0.05 and 5.0 μg/ml) were extracted as described above and analyzed by HPLC on the same day (day 0) and after storage at 0°C for seven days.

Pharmacokinetic studies

The technique and apparatus for isolated rat liver perfusion were similar to those of Miller [23] and have been described in detail previously [24]. Under diethyl ether anesthesia, the portal vein, hepatic vein and bile duct were cannulated. The liver was then excised and transferred to the perfusion apparatus. The liver was perfused at 20 ml/min with 100 ml of recirculating fluid composed of bovine erythrocytes (hematocrit 20%) suspended in Krebs–Ringer bicarbonate solution containing albumin (0.03 g/ml), glucose (2 mg/ml), heparin sodium (10 U/ml) and physiological concentrations of amino acids [25,26]. The fluid was aerated with 95% O_2–5% CO_2 and the pH was maintained at 7.4 by titration with 0.375 *M* sodium bicarbonate. The temperature was maintained at 37°C. After a 30-min equilibration period, 78 μmol (20 mg) of EdUrd in 1 ml of 0.9% saline were injected into the perfusion reservoir, and serial perfusion fluid samples (0.5–1.0 ml) were collected over 120 min. The volume of distribution (V_d) of EdUrd was calculated from dose/C_0, where C_0 is the plasma concentration extrapolated to time zero. The apparent first-order elimination rate constant, k_e, was obtained from the terminal slope of the plasma EdUrd concentration *versus* time curve, and half-life ($t_{\frac{1}{2}}$) was calculated from ln $2/k_e$. The total area under the plasma concentration *versus* time curve (AUC)) was calculated as the sum of AUC_{0-t} (the area from time zero to the time of the last plasma sample) and $AUC_{t-\infty}$ (extrapolated area from the last sample to infinite time). AUC_{0-t} was estimated by the trapezoidal rule and $AUC_{t-\infty}$ was estimated from C_{last}/k_e, where C_{last} is the concentration of the last sample [27]. Plasma clearance was calculated from dose/AUC.

RESULTS AND DISCUSSION

A modification of the ion-exchange chromatographic technique for extraction of fluoropyrimidines from plasma [21] provided a simple and rapid method for extraction of 5-ethylpyrimidines (EdUrd, EUra, and HEUra) and 5-methylpyri-

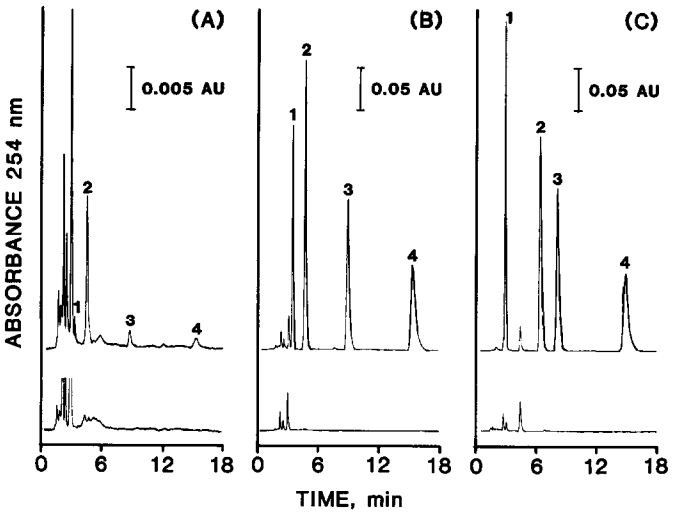

Fig. 1. Chromatograms of blank and pyrimidine-containing plasma extracts, the lower tracing in each panel obtained with blank plasma and the upper tracing with drug-containing plasma extracts; 20 μl injected. (A) Plasma containing 5-ethylpyrimidines: 0.05 μg/ml HEUra (peak 1), 0.6 μg/ml FdUrd (peak 2), 0.05 μg/ml EUra (peak 3) and 0.1 μg/ml EdUrd (peak 4). (B) Plasma containing 5-ethylpyrimidines: 5 μg/ml HEUra (peak 1), 10 μg/ml FdUrd (peak 2), 5 μg/ml EUra (peak 3) and 10 μg/ml EdUrd (peak 4). (C) Plasma containing 5-methylpyrimidines: 5 μg/ml HMUra (peak 1), 5 μg/ml Thy (peak 2), 10 μg/ml FdUrd (peak 3) and 10 μg/ml dThd (peak 4). AU, absorbance units.

midines (dThd, Thy, and HMUra) from 0.5–1.0 ml plasma samples before analysis by HPLC. The entire procedure, when applied to eight standards and fifteen experimental samples, was routinely accomplished in 3 h.

The test pyrimidines are weak acids, with pK_a values of about 9.8 [28,29], enabling their isolation by ion-exchange chromatography. At pH 11.3, the test compounds existed primarily in an anionic form and were presumed to interact with the anion-exchange resin through ionic bonding. The initial cation-exchange procedure was required to remove interfering substances not removed by the anion-exchange procedure. The recovery after the two ion-exchange chromatographic procedures was 75–81% for the nucleosides EdUrd and dThd (0.1 and 10 μg/ml), 88–100% for the bases EUra and Thy (0.05 and 5 μg/ml) and 64–65% for the hydroxylated bases HEUra and HMUra (5 μg/ml). The recovery of HEUra at a concentration of 0.05 μg/ml was 62%; a small unidentified peak that was not separated from HMUra prevented an accurate determination of HMUra recovery at this concentration.

HPLC chromatograms of blank and 5-ethylpyrimidine-containing plasma extracts are shown in Fig. 1A and B. Blank plasma extracts contained several unidentified peaks, but none interfered with the test compounds or the internal standard over the concentration range examined. Retention times of the test compounds, using mobile phase A, were (in min): HEUra, 3.5; FdUrd, 4.7; EUra,

9.0; and EdUrd, 16. The retention times of the structurally similar compounds Ura and dUrd were 2.5 and 3.8 min, respectively. Chromatograms of blank and 5-methylpyrimidine-containing plasma extracts are shown in Fig. 1C. Blank plasma extracts contained several unidentified peaks; one of these was not completely resolved from the HMUra peak and prevented determination of HMUra concentrations below 0.1 μg/ml. Retention times for this group, using mobile phase B, were (in min): HMUra, 2.9; Thy, 6.6; FdUrd, 8.4; and dThd, 15. The retention times of some structurally similar compounds were (in min): Ura, 3.0; dUrd, 5.7; and HMdUrd, 9.3.

A linear relationship between peak-height ratio (test compound to internal standard) and plasma concentration was obtained with each compound. For 5-ethylpyrimidines, eight-point standard curves generated with an internal standard concentration of 1 μg/ml, were linear over the range 0.05–50 μg/ml with EdUrd and EUra and 0.05–10 μg/ml with HEUra. Above these ranges, there was a deviation from linearity. The equations of the curves ($n = 3$) were, in units of μg/ml: EdUrd, $y = 0.240x - 0.017$ ($r = 1.0000$); EUra, $y = 0.927x - 0.033$ ($r = 1.0000$); and HEUra, $y = 1.398x - 0.194$ ($r = 0.9999$). For 5-methylpyrimidines, eight-point standard curves, generated with an internal standard concentration of 1 μg/ml, were linear over the range 0.1–50 μg/ml. The equations of

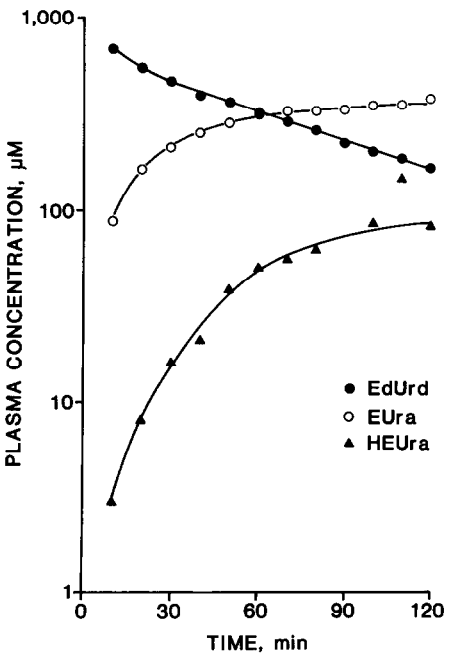

Fig. 2. Time course of elimination of EdUrd and formation of EUra and HEUra by the isolated perfused rat liver. EdUrd dose: 78 μmol (20 mg).

these curves (n = 3–4) were, in units of μg/ml: dThd, $y = 0.477x + 0.011$ (r = 1.0000); Thy, $y = 2.677x - 0.095$ (r = 1.0000); and HMUra, $y = 3.820x + 0.009$ (r = 1.0000). The maximum standard error of the peak-height ratio was 8.2% for 5-ethylpyrimidines and 13.9% for 5-methylpyrimidines. In addition to the above results with human plasma, linear standard curves were obtained for each test compound dissolved in blank plasma from liver perfusion experiments. In routine analyses, six-point standard curves were generated from perfusion plasma samples containing 0.10–5.0 μg/ml test compounds and 1 μg/ml internal standard. Experimental samples containing higher concentrations of the pyrimidines were diluted with blank plasma to a concentration below 5.0 μg/ml before extraction. Storage of the dried anion-exchange column eluates for up to seven days had no effect on peak-height ratios, indicating a lack of decomposition. The HPLC peak-height ratios obtained with extracts injected on day 7 differed by ≤ 6% from those obtained with extracts injected on day 0.

Application of the HPLC method to the analysis of 5-ethylpyrimidine elimination by the isolated perfused rat liver is illustrated in Fig. 2. The decrease in plasma concentration of EdUrd from an initial value of about 800 μ*M* (205 μg/ml) was an apparent exponential process. The kinetic pattern was similar to the pattern of *in vivo* plasma disappearance in rats [11]. The calculated V_d of EdUrd was 94.8 ml, which was close to the volume of perfusion fluid. During the terminal phase of EdUrd elimination, the $t_{\frac{1}{2}}$ and k_e were 63.6 min and 0.0109 min^{-1}, respectively. Total clearance was 1.29 ml/min. The concentrations of the EdUrd metabolites EUra and HEUra increased steadily with time and appeared to be approaching plateau levels of about 400 μ*M* (56 μg/ml) and 100 μ*M* (15.6 μg/ml), respectively, after 2 h of perfusion.

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