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Note

High-performance liquid chromatographic analysis of fluoropyrimidine nucleosides and fluorouracil in plasma

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5-Fluorouracil (FUra) and its nucleoside analogue 5-fluoro-2′-deoxyuridine (FdUrd) are antimetabolites used in the treatment of malignant neoplasms [1,2]. In the body FdUrd is extensively metabolized to FUra in reactions catalyzed by at least two different nucleoside phosphorylases [3,4]. The possibility of using selective nucleoside phosphorylase inhibitors to enhance the anticancer activity of FdUrd has also been investigated [5–7]. To date, however, there have been few in vivo studies on the kinetics of FdUrd breakdown to FUra in the presence or absence of inhibitors, probably due in part to the lack of suitable analytical methods. Early studies on FdUrd elimination were based on microbiological [8] or combined anion-exchange and paper chromatographic [9] methods which lacked specificity or sensitivity. A sensitive radioimmunoassay for FdUrd, developed by Schreiber and Raso [10], also had a low specificity. De Leenheer and Gelijkens [11,12] described gas chromatographic (GC) methods for fluoropyrimidine nucleoside analysis which involved laborious extraction procedures that differed for FdUrd and 5-fluorouridine (FUrd). Buckpitt and Boyd [13] described a sensitive high-performance liquid chromatographic (HPLC) method based on the use of radioactively labeled FdUrd and FUra as internal standards, but large plasma volumes were required for the measurement of low concentrations.

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A simple and rapid method for extraction of fluoropyrimidine nucleosides and FUra from plasma by sequential cation-exchange and anion-exchange chromatography, and subsequent analysis by GC, was described in a recent report from this laboratory [14]. The fluoropyrimidines were extracted with an efficiency of 71–95% and were well separated from potentially interfering endogenous compounds such as uracil (Ura), uridine (Urd), and 2′-deoxyuridine (dUrd) on the GC columns. A disadvantage of the method was the requirement of different GC columns for the nucleosides and FUra. Another problem was the partial breakdown of FdUrd to FUra on the GC column. Although the amount of FUra formed was predictable from the amount of FdUrd injected, the measurement of FUra in the presence of FdUrd required correction for this breakdown. In the present investigation the ion-exchange chromatographic method of fluoropyrimidine isolation was also found to be suitable for preparing plasma samples for HPLC analysis. The HPLC method overcomes the above disadvantages of the GC method but has the same level of sensitivity and specificity.

EXPERIMENTAL

Chemicals

FdUrd and FUrd were kindly provided by Dr. W.E. Scott of Hoffmann-LaRoche (Nutley, NJ, U.S.A.). Other pyrimidine bases and nucleosides were obtained from Sigma (St. Louis, MO, U.S.A.), ion-exchange resins from Bio-Rad Labs. (Richmond, CA, U.S.A.), and HPLC columns from Hewlett-Packard (Palo Alto, CA, U.S.A.). HPLC-grade solvents were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.) and were filtered through polyester membranes with a pore size of 0.4 μm (Nucleopore, Pleasanton, CA, U.S.A.) before experimental use.

Sample preparation

Human plasma was used in the development of the HPLC method. Standards were prepared by the addition of varying known amounts of test compound and a constant amount of internal standard to blank plasma. Experimental samples were routinely diluted with blank plasma to produce fluoropyrimidine concentrations below 20 μg/ml. The preparative procedure has been described in detail previously [14]. 5-Chlorouracil (ClUra), the internal standard, dissolved in 80 μl of 0.3 *M* ammonium formate buffer, pH 5.0, was added to 0.72 ml of plasma. The sample was loaded onto a cation-exchange column (Bio-Rad AG 50W-X4, 200–400 mesh, hydrogen form, 4.5×0.7 cm) and the eluate was discarded. Next, 4.8 ml of 0.03 *M* ammonium formate buffer, pH 5.0, were added; the initial 0.8 ml of eluate were discarded and the remaining 4 ml collected. To the eluate 1 ml of 0.5 *M* carbonate–bicarbonate buffer, pH 10.7, was added and the sample was loaded onto an anion-exchange column (Bio-Rad AG 1-X4, 100–200 mesh, chloride form, 2.0×0.7 cm). The column was washed sequentially with 4 ml of 0.1 *M* carbonate–bicarbonate buffer (pH 10), 10 ml of water, and 10 ml of methanol, then eluted with 10 ml of 0.3 *M* acetic acid in methanol. The eluate was evaporated to dryness under air in a 50°C water-bath. The residue was redissolved in 0.5 ml of mobile phase. A 20- or 100-μl aliquot was injected onto the HPLC column.

High-performance liquid chromatography

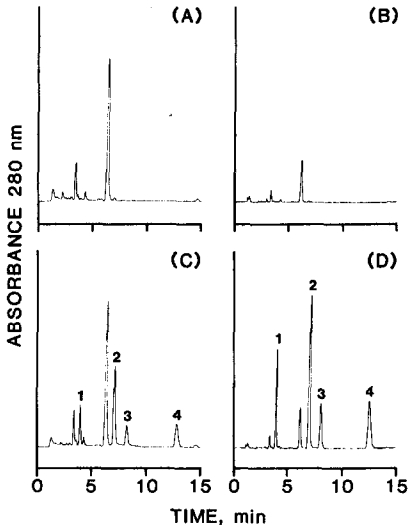
The chromatographic system consisted of a Beckman Model 110A solvent delivery pump (Beckman Instruments, Fullerton, CA, U.S.A.), a Rheodyne Model 7125 syringe loading injection valve (Rheodyne, Cotati, CA, U.S.A.), a Beckman Model 160 ultraviolet detector operated at 280 nm, and a Hewlett-Packard Model 3390A reporting integrator. Chromatography was performed on a reversed-phase C_8 analytical column (Hewlett-Packard Hypersil-MOS, 5 μm, 200×4.6 mm I.D.). The mobile phase, 0.86 m*M* sodium acetate buffer, pH 4.8, was delivered at 1.2 ml/min. Standard curves were plotted as the fluoropyrimidine-to-internal standard peak-area ratio versus concentration of standard samples; the linear regression line was calculated by the method of least squares. The concentration of fluoropyrimidine in experimental samples was calculated from the equation of the standard curve and the peak-area ratio of the sample.

Pharmacokinetic studies

Livers from male Sprague–Dawley rats were isolated and perfused by the method of Miller [15]. The apparatus described by Miller was modified by incorporation of a flow-meter to allow maintenance of a constant hepatic blood flow. Under diethyl ether anesthesia, the portal vein, hepatic vein, and bile duct were cannulated, and the liver was excised. The isolated liver was perfused at 20 ml/min at 37°C with 100 ml of recirculating fluid composed of bovine erythrocytes (hematocrit 20%) suspended in Krebs–Ringer bicarbonate solution containing bovine albumin (3 g/dl), glucose (200 mg/dl), heparin (20 U/ml), and amino acids at physiological concentrations. The pH of the fluid was maintained at 7.4 by autotitration with sodium bicarbonate. After a 30-min equilibration period, 10 mg of FdUrd, dissolved in 1 ml of 0.9% sodium chloride, were added to the perfusion reservoir and serial blood samples (0.5–1.0 ml) were collected over 90 min. Kinetic constants [maximal rate of elimination (V_{max}), concentration at half-maximal elimination rate (K_m), and volume of distribution (V_d)] for FdUrd elimination were estimated with the NONLIN84 curve-fitting computer program [16]. Initial estimates of V_{max} and K_m, required by the program, were obtained by the method of Van Rossum et al. [17]. The initial estimate of V_d was calculated from dose divided by initial FdUrd concentration. The elimination rate constant (k_e) was obtained from the slope of the linear (first-order) portion of a semilogarithmic plot of FdUrd concentration versus time. Clearance during the first-order phase of elimination was calculated from $k_e \cdot V_d$ [18].

RESULTS AND DISCUSSION

The sequential cation-exchange and anion-exchange chromatographic procedure originally developed for use in extracting fluoropyrimidines for GC [14] was also suitable for preparing samples for HPLC analysis. Chromatograms of blank and fluoropyrimidine-containing plasma extracts are shown in Fig. 1. Blank plasma contained several unidentified peaks but none interfered with the fluoropyrimidines or the internal standard at the concentrations employed in these studies. FdUrd, FUrd, and FUra were well separated from each other and from



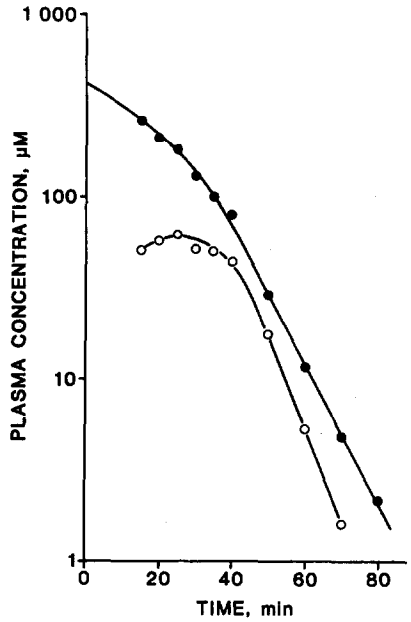


Fig. 1. Chromatograms of blank and fluoropyrimidine-containing plasma extracts. (A) Blank, 100 µl injected. (B) Blank, 20 µl injected. (C) Plasma containing 0.05 µg/ml FUra (peak 1), 0.10 µg/ml ClUra (peak 2), 0.10 µg/ml FUrd (peak 3), and 0.10 µg/ml FdUrd (peak 4), 100 µl injected. (D) Plasma containing 0.50 µg/ml FUra, 1.0 µg/ml ClUra, 1.0 µg/ml FUrd, and 1.0 µg/ml FdUrd, 20 µl injected. Detector: 0.007 a.u.f.s. for chromatograms A and C, 0.05 a.u.f.s. for chromatograms B and D.

Fig. 2. Time course of FdUrd elimination (●) and FUra formation (○) by the isolated perfused rat liver. FdUrd dose: 10 mg.

Ura, Urd, and dUrd. The retention times of the fluoropyrimidines and structurally related compounds were (in min): FUra, 4.0; ClUra, 7.2; FUrd, 8.4; FdUrd, 13.2; Ura, 3.7; Urd, 6.4; and dUrd, 10.4. Using these procedures, twenty samples were routinely extracted and analyzed simultaneously for FdUrd and FUra in 8 h. The problem of decomposition of FdUrd to FUra, observed during GC analysis [14], did not occur with the HPLC method.

There was a linear relationship between peak-area ratio (fluoropyrimidine to internal standard) and plasma concentration for each compound. Concentrations as low as 0.01 µg/ml (77 nM) for FUra and 0.02 µg/ml (77–81 nM) for the nucleosides were readily quantified. In routine experiments, six-point standard curves were generated from plasma samples containing 0.10 µg/ml ClUra, 0.01–0.50 µg/ml FUra, and 0.02–1.0 µg/ml of the nucleosides. The equations obtained from six replicate curves with each compound were (in units of µg/ml): FUra, $y=7.289x-0.028$ ($r=1.0000$); FUrd, $y=2.801x-0.0001$ ($r=0.9996$); and FdUrd, $y=4.744x+0.041$ ($r=1.0000$). The standard error of the peak-area ratio was less than 15% of the mean at each concentration. The range of linearity of the standard curves was also investigated, using samples containing 1.0 µg/ml ClUra and concentrations of fluoropyrimidines varying over a wide range. The curves were linear over a concentration range of 0.05–50 µg/ml (0.384–384 µM)

for FUra and 0.1–100 μg/ml (about 0.4–400 μM) for FdUrd and FUrd. There was a deviation from linearity above these ranges. The equations for these standard curves, which were each run in triplicate, were: FUra, $y=0.642x+0.141$ ($r=0.9998$); FUrd, $y=0.330x+0.102$ ($r=0.9999$); and FdUrd, $y=0.479x+0.026$ ($r=0.9998$).

Application of the HPLC method to the analysis of FdUrd pharmacokinetics in the isolated perfused rat liver is illustrated in Fig. 2. The decrease in plasma FdUrd concentration from an initial value of about 400 μM (98 μg/ml) was linear with time (zero-order); at concentrations below ca. 80 μM (20 μg/ml) the decline became exponential (first-order). This pattern of elimination is typical of a Michaelis–Menten process and suggests saturable elimination [19]. V_{max}, the apparent maximal rate of FdUrd elimination, was 14 nmol/ml/min and K_m, the concentration at the half-maximal elimination rate, was 127 μM (31 μg/ml). The V_d of FdUrd was 98 ml, which was close to the volume (100 ml) of perfusion fluid. During the exponential phase of FdUrd elimination, the half-life was 7.7 min and the clearance was 8.8 ml/min. The concentration of FUra, derived from the metabolism of FdUrd, increased to a peak value of about 60 μM (7.8 μg/ml) at 20–30 min. After 40 min, FUra concentration decreased exponentially with a half-life of 5.8 min.

REFERENCES

1 C. Heidelberger, N.K. Chaudhuri, P. Danenberg, D. Mooren, L. Griesbach, R. Duschinsky, R.J. Schnitzer, E. Pleven and J. Scheiner, Nature (London), 179 (1957) 663.
2 C.E. Myers, Pharmacol. Rev., 33 (1981) 1.
3 C. Heidelberger, in A.C. Sartorelli and D.G. Johns (Editors), Antineoplastic and Immunosuppressive Agents, Part II, Springer-Verlag, Berlin, 1975, p. 193.
4 P.W. Woodman, A.M. Sarrif and C. Heidelberger, Cancer Res., 40 (1980) 507.
5 B.R. Baker, Design of Active-Site Directed Irreversible Enzyme Inhibitors, Wiley, New York, 1967, p. 70.
6 P.W. Woodman, A.M. Sarrif and C. Heidelberger, Biochem. Pharmacol., 29 (1980) 1059.
7 M.Y.W. Chu, F.N.M. Naguib, M.H. Iltzsch, M.H. el Kouni, S.H. Chu, S. Cha and P. Calabresi, Cancer Res., 44 (1984) 1852.
8 B. Clarkson, A. O'Connor, L.R. Winston and D. Hutchison, Clin. Pharmacol. Ther., 5 (1964) 581.
9 K.L. Mukherjee, J. Boohar, D. Wentland, F.J. Ansfield and C. Heidelberger, Cancer Res., 23 (1963) 49.
10 R. Schreiber and V. Raso, Cancer Res., 38 (1978) 1889.
11 A.R. De Leenheer and C.F. Gelijkens, J. Pharm. Sci., 67 (1978) 417.
12 A.R. De Leenheer and C.F. Gelijkens, J. Chromatogr. Sci., 16 (1978) 552.
13 A.R. Buckpitt and M.R. Boyd, Anal. Biochem., 106 (1980) 432.
14 W.M. Williams, B.S. Warren and F.-H. Lin, Anal. Biochem., 147 (1985) 478.
15 L.L. Miller, in I. Bartosek, A.G. Guaitani and L.L. Miller (Editors), Isolated Perfused Liver and its Applications, Raven Press, New York, 1973, p. 11.
16 C.M. Metzler and D.L. Weiner, NONLIN84 User's Guide, Statistical Consultants, Lexington, KY, 1984.
17 J.M. van Rossum, C.A.M. van Ginneken, P.Th. Henderson, H.C.J. Ketelaars and T.B. Vree, in J.M. van Rossum (Editor), Kinetics of Drug Action, Springer-Verlag, Berlin, 1977, p. 125.
18 M. Gibaldi and D. Perrier, Pharmacokinetics, Marcel Dekker, New York, 2nd ed., 1982, p. 16.
19 E.J. O'Flaherty, Toxicants and Drugs: Kinetics and Dynamics, Wiley, New York, 1981, p. 186.