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ANALYSIS OF 5'-DEOXY-5-FLUOROURIDINE AND 5-FLUOROURACIL IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

L.J. SCHAAF*

Department of Pharmacy, University of Otago, P O. Box 913, Dunedin (New Zealand)

D.G. FERRY

Medical Research Council of New Zealand, Toxicology Research Unit, Dunedin (New Zealand)

C.T. HUNG and D.G. PERRIER

Department of Pharmacy, University of Otago, P.O Box 913, Dunedin (New Zealand)

and

I.R. EDWARDS

Medical Research Council of New Zealand, Toxicology Research Unit, Dunedin (New Zealand)

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SUMMARY

A relatively simple and sensitive high-performance liquid chromatographic (HPLC) method is described for measuring the two anticancer drugs 5'-deoxy-5-fluorouridine (5'dFUR) and 5-fluorouracil (5-FU) in human plasma and urine. The procedure for plasma includes solvent extraction using ethyl acetate—isopropyl alcohol (85.15) followed by silica gel column chromatography to separate these compounds from constituents normally occurring in plasma. The analysis by reversed-phase HPLC is performed on a phenyl column using an aqueous mobile phase with ultraviolet detection (280 nm). The overall recovery from plasma was 61% and 65% for 5'dFUR and 5-FU, respectively The sensitivity limit of the assay for both compounds was 50 ng/ml of plasma. Analysis of these compounds in urine did not require the silica column chromatography isolation step.

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INTRODUCTION

The recently synthesized fluoropyrimidine, 5'-deoxy-5-fluorouridine (5'dFUR, doxifluridine, Ro 21-9738), is under investigation for use in the treatment of carcinomas of head and neck, ovary, breast and colon/rectum [1—5]. It is believed that 5'dFUR is a prodrug of 5-fluorouracil (5-FU), and is converted to 5-FU by intracellular enzymatic hydrolysis [1, 2, 6—9]. The enzyme responsible for the conversion, thymidine phosphorylase, is found in many normal tissues but appears to be present in higher activity in neoplastic cells [1, 2, 5, 8, 10]. This would be consistent with the fact that 5'dFUR exhibits a higher therapeutic index [1, 2, 5—7, 9, 11—15], and has been reported to be less immunosuppressive [16, 17] and cardiotoxic [18] than other fluorinated pyrimidines.

Detailed studies investigating the disposition kinetics of 5'dFUR and 5-FU following 5'dFUR administration are limited [4, 19—21]. This may be due to the lack of a simple and sensitive method to quantitate both compounds in biological fluids. Methods have been described for the determination of 5'dFUR in biological fluids using thin-layer chromatography [10] and high-performance liquid chromatography (HPLC) in conjunction with either labelled drugs [2, 7] or ultraviolet (UV) detection [22]. The novel HPLC method of Sommadossi and Cano [22] based on spectrophotometric detection was highly specific, however, the simultaneous quantitation of 5-FU was not possible according to the authors. In addition, its application to urine analysis has not been demonstrated.

Gustavsson et al. [23] recently reported a method for the simultaneous analysis of both compounds in plasma. The procedure involved deproteinization with picric acid followed by ion-exchange chromatography and analytical isotachophoresis. The complexity of the procedure and the instrumentation involved will probably result in the method having limited utility.

More recently, Malet-Martino et al. [24] have described a fluorine-19 NMR assay for measuring both compounds as well as major metabolites in whole blood, plasma and urine. While this method has the advantage of not requiring extraction, it appears to be less sensitive than reported HPLC procedures.

The purpose of this paper is to describe a relatively simple and sensitive HPLC method with UV detection for the quantitation of 5'dFUR and its metabolite, 5-FU, in both human plasma and urine.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. They included methanol (HPLC grade, Fisher Scientific, Fair Lawn, NJ, U.S.A.), ethyl acetate (Baker Analyzed Reagent, J.T. Baker, Phillipsburg, NJ, U.S.A.), isopropyl alcohol (HPLC grade, Waters Assoc., Milford, MA, U.S.A.), orthophosphoric acid (BDH, Poole, U.K.), 50—100 mesh silica gel (Koch-Light, Colnbrook, U.K.) and dimethyldichlorosilane (Fluka, Buchs, Switzerland).

Analytical standards

5-Fluorouracil, 5'-deoxy-5-fluorouridine and other metabolites were kindly supplied by Hoffman-La Roche (Basel, Switzerland) and used without further purification. 5-Bromouracil (5-BrU) was purchased from Sigma (St Louis, MO, U.S.A.) and used as the internal standard (I.S.). Aqueous stock solutions (1 and 4 mg/ml 5-FU and 5'dFUR; 200 μg/ml 5-BrU) were prepared in silanized glassware and stored at 4°C. These solutions were stable for at least four months.

HPLC instrumentation

The chromatographic system consisted of a reciprocating piston pump (Model 6000A, Waters Assoc.), a syringe loading sample injector (Model U6K, Waters Assoc.), and a Spherisorb phenyl column (12.5 cm × 4.9 mm I.D., particle size 5 μm, Hichrom House, Berkshire, U.K.). All chromatography was performed at ambient temperature. The column effluent was monitored at 280 nm using a selectable-wavelength UV detector (Model 441, Waters Assoc.). The output from the detector was connected to a 10-mV potentiometric integrator (Model 3380A, Hewlett-Packard, Avondale, PA, U.S.A.) set at a chart speed of 1 cm/min.

Mobile phase

The isocratic mobile phase was distilled water which was purified through a Milli-Q system (Millipore, Bedford, MA, U.S.A.), filtered through a 0.45-μm Nylon 66 filter (Alltech, Deerfield, IL, U.S.A.) and degassed ultrasonically under vacuum. The mobile phase was pumped at a flow-rate of 1.5 ml/min (73 bar) and not recycled.

Analytical procedures

All centrifuge tubes and clean-up columns were silanized by placing them in a dessicator containing dichlorodimethylsilane (DMCS). Following exposure to DMCS vapor for 4—8 h, the tubes were placed in a methanol desiccator overnight, rinsed with water and then dried at 110°C.

Plasma

The flow diagram for the extraction procedure is shown in Fig. 1. Aliquots of plasma (0.5 ml) were pipetted into 15-ml silanized glass centrifuge tubes (Kimax, 100 × 16 mm, Kimble, Vineland, NJ, U.S.A.) fitted with PTFE-lined screw caps. After addition of internal standard (50 μl of 4 μg/ml aqueous stock for the 50—1000 ng/ml range; 50 μl of a 40 μg/ml aqueous stock for the 1—75 μg/ml range), 50 μl of 3% (v/v) aqueous orthophosphoric acid was added to adjust the pH to approximately 5.5. The tubes were gently shaken to ensure mixing and 5 ml of ethyl acetate—isopropyl alcohol (85:15) were added, then vortexed for 30 sec on a Vortex-Genie mixer (Model K-550-GE, Scientific Industries, Springfield, MA, U.S.A.). Following centrifugation (Model HN, International Equipment, Needham Heights, MA, U.S.A.) for 6 min to separate the phases, the entire organic layer was pipetted into a silanized, conical glass centrifuge tube and evaporated to dryness on an evaporator (N-EVAP, Organomation Assoc., Northborough, MA, U.S.A.) at 45—50°C under a gentle stream of nitrogen.

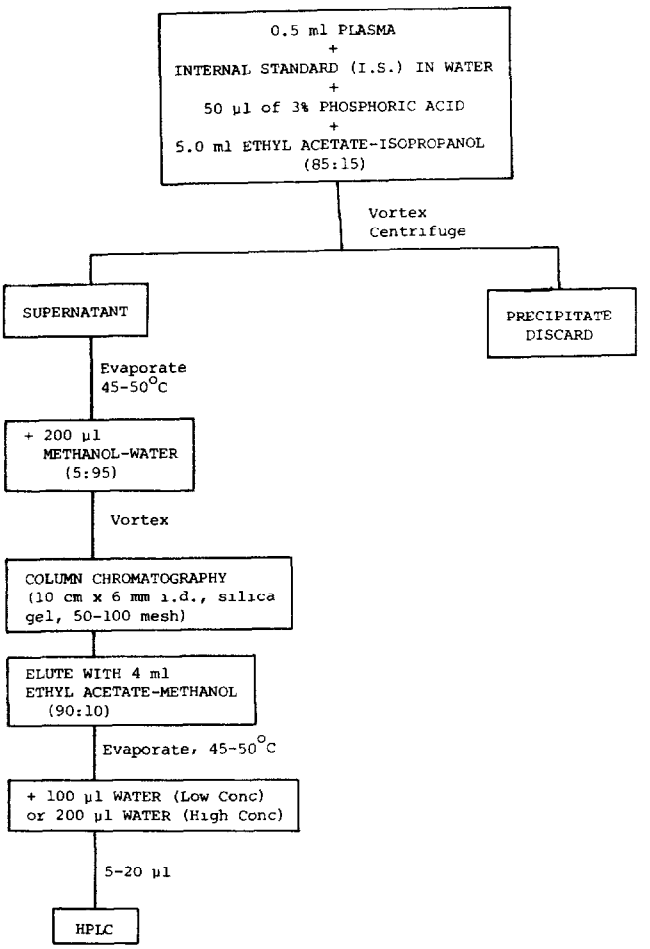

Fig. 1. Flow diagram for the isolation procedure of 5'dFUR and 5-FU from plasma.

The dried residue was dissolved in 200 µl methanol—water (5:95), vortexed for 30 sec and then subjected to column chromatography (10 cm × 6 mm I.D., silanized glass columns packed with 2.56 g 50—100 mesh silica gel). The gravity packed columns were rinsed with 10 ml of ethyl acetate—methanol (90:10) and then the entire residue mixture was placed on the top of the column using a micropipet (Pipetman, Gilson France, Villiers-le-Bel, France). The column was eluted with 4 ml of ethyl acetate—methanol (90:10) and the eluate fraction evaporated to dryness as described above. The resulting residue was dissolved in 100 µl (low concentration range) or 200 µl (high concentration range) deionized water, vortexed for 30 sec, and 5—20 µl injections were made into the HPLC system.

Urine

A 100-µl aliquot of a 1:100 dilution (5-FU) or 1:1000 dilution (5'dFUR) of urine was pipetted into a silanized glass centrifuge tube (Kimax, 100 × 13 mm, Kimble). A 40-µl (5-FU assay) or 50-µl (5'dFUR assay) volume of a

4 μg/ml aqueous solution of internal standard was added and the mixture acidified to a pH of approximately 5.5 with orthophosphoric acid. A 2-ml volume of ethyl acetate—isopropyl alcohol (85:15) mixture was added and the tubes were vortexed for 30 sec. After centrifuging for 6 min, the organic phase was removed with a Pasteur pipet and evaporated as described under plasma samples. The dried sample was redissolved in 100 μl deionized water and vortexed for 20 sec to facilitate dissolution. A volume of 10– 20 μl was injected onto the column.

Calculations

Standard plasma calibration curves of peak height ratio versus plasma concentration were constructed using plasma samples to which increasing quantities of both drugs were added to give concentrations in the range 1—1000 ng/ml or 1—75 μg/ml. Similarly, urine calibration curves were prepared by adding 5-FU in concentrations of 0—250 μg/ml or 5′dFUR in concentrations of 0—10 mg/ml to blank urine. Concentrations of 5-FU and 5′dFUR were obtained from the peak height ratios and the regression equation of the appropriate calibration curve.

RESULTS AND DISCUSSION

Internal standard

5-Bromouracil was chosen as an internal standard because of its similarity in structure, maximum absorption wavelength and percentage recovery when compared to 5-FU. In addition, 5-BrU is not used as a therapeutic agent and is not a metabolite of either 5-FU or 5′dFUR.

Extraction and isolation procedure

Plasma. The performance of the extraction and isolation procedure was dependent on the nature of the extracting solvent mixture, extraction pH and inclusion of the additional silica column isolation step. The physical recovery of 5-FU and 5′-dFUR was determined by comparing the peak heights measured from the final extracts of plasma containing known concentrations (100 ng/ml) of both compounds with the peak heights measured from unextracted aqueous solutions supplemented with known concentrations of 5-FU and 5′dFUR. Recoveries of 5-FU and 5′dFUR determined in this manner using ethyl acetate—isopropyl alcohol (85:15) were found to be approximately 66 and 61%, respectively. Less satisfactory recoveries were found when ethyl acetate, diethyl ether, chloroform, pentane or lower percentages of isopropyl alcohol in ethyl acetate were used.

Since the pK_a values of these acidic compounds are approximately 8 [25], an extraction pH of approximately 5.5 was chosen to ensure that they would exist in the unionized form. Extraction at lower pH values (2 or 4) did not alter the extraction efficiency of 5-FU or 5′dFUR, but chromatograms of blank plasma resulted in additional peaks which interfered with 5-FU. In addition, these chromatograms demonstrated late peaks with retention times greater than 10 min. While these peaks did not directly interfere with analysis, they did prolong the time between injections.

Typical chromatograms from blank human plasma and plasma spiked with known amounts of 5-FU and 5'dFUR are shown in Fig. 2. While a small peak still eluted just before 5-FU, it did not interfere with the quantitation of 5-FU. Chromatograms using the silica column clean-up procedure were cleaner than those obtained by using the procedures of Christophidis et al. [26] or Sampson et al. [27].

The optimal quantity of silica gel for purifying 0.5 ml of plasma was approximately 2.6 g. Smaller amounts resulted in high blank readings while larger amounts did not result in cleaner chromatograms. Similarly, 4 ml of ethyl acetate—methanol (90:10) provided good recoveries of all compounds from the silica column. The use of larger volumes resulted in higher quantities of interfering substances.

Silica gel purchased from different sources demonstrated higher blank readings. This was apparently due to polar contaminants in the silica gel since pre-washing with methanol generally produced clean traces. However, with certain lots, use of 5 m*M* potassium dihydrogen phosphate, adjusted to pH 4, was required to separate unknown peaks from the compounds of interest. In addition, the use of silica gel which had been activated by heating at 110°C introduced interfering peaks. Therefore, unactivated silica gel was used for all analytical procedures.

The sample preparation and chromatography present many opportunities for selective loss of 5'dFUR and 5-FU. To avoid adsorption of 5-FU to glass [28, 29], all glassware was silanized as described previously. Similarly, the temperature used for evaporation was maintained at 45—50°C since temperatures higher than 60°C resulted in losses of up to 25% while temperatures lower than 40°C were not sufficient to permit acceptable evaporation times.

Urine. Typical chromatograms from blank human urine and urine spiked with known amounts of 5-FU and 5'dFUR are shown in Fig. 3. Since drug concentrations in urine are much higher than those found in plasma, no

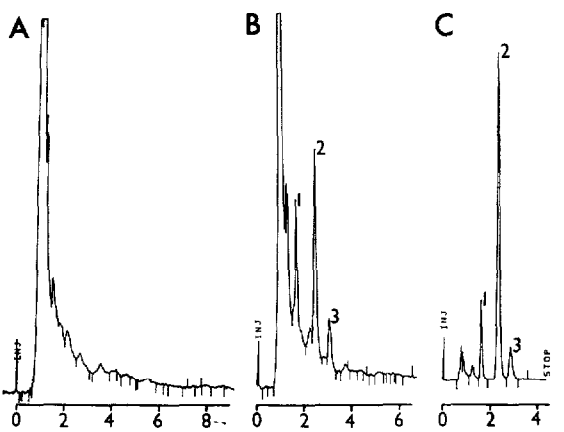

Fig. 2. Representative chromatograms from a 0.5-ml plasma extract showing retention times (min) for (1) 5-FU, (2) 5-BrU (internal standard) and (3) 5'dFUR. (A) Plasma control after extraction; (B) plasma spiked with 100 ng/ml 5-FU and 5'dFUR; (C) plasma spiked with 1 μg/ml 5-FU and 5'dFUR. The detector settings were 0.005 a.u.f.s. for A and B and 0.02 a u.f.s. for C.

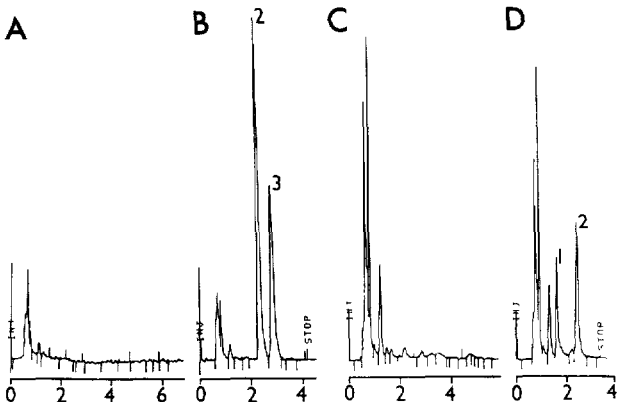

Fig. 3. Representative chromatograms from urine showing retention times (min) for (1) 5-FU, (2) 5-BrU and (3) 5'dFUR. (A) 1 1000 dilution of blank urine; (B) 1:1000 dilution of urine initially spiked with 2 mg/ml 5'dFUR; (C) 1:100 dilution of blank urine, (D) 1:100 dilution of urine initially spiked with 100 μg/ml 5-FU. The detector settings were 0 005 a.u.f.s. for A and B and 0.01 a.u.f.s. for C and D.

interference from normal urinary constituents were observed and thus, the silica column purification step was not necessary. The physical recovery of 5-FU (50 μg/ml) and 5'dFUR (2 mg/ml) from urine using this method was 83 and 85%, respectively.

Chromatographic behavior

The pH and ionic strength of the mobile phase had a profound effect on the resolution of 5-FU, 5'dFUR and I.S. from unknown plasma components. In general, as pH was lowered or ionic strength increased, the retention times of the three compounds were unaltered but blank chromatograms demonstrated peaks which could not be resolved from 5-FU. In contrast, when the pH of phosphate buffer was greater than 6.5, resolution was improved but column performance deteriorated rapidly. This may be related to precipitation of a plasma component in the column since flushing with 0.06% orthophosphoric acid (pH 2.3) and methanol restored column performance.

The Spherisorb phenyl (12.5 cm × 4.9 mm I.D.) column was chosen because it allowed adequate resolution of both 5'dFUR and 5-FU and provided optimal sensitivity since all peaks were eluted within 4 min. Other reversed-phase columns (C_8 and C_{18}) were tried but resulted in decreased sensitivity for 5'dFUR and longer assay times (20 min or longer per sample).

Linearity and precision

The standard curves for both compounds in plasma were linear over the concentration ranges studied, 50—1000 ng/ml and 1—75 μg/ml ($R^2 > 0.990$). The within-day coefficient of variation (C.V.), based on triplicate determinations, was less than 10% for both compounds at all concentrations. The between-day variation was calculated by performing triplicate analyses of plasma samples on three to four different days. This was done in samples containing both compounds at three or four concentrations. A summary of the

analysis is presented in Table I. Between-day C.V. values were all less than 10% except for the 100 ng/ml concentration of 5-FU for which we have no apparent explanation. Mean analytical recovery (accuracy), expressed as the ratio of compound added to that measured, was 102% (S.D. 3.8%) for 5-FU and 100% (S.D. 3.1%) for 5'dFUR.

Standard curves for 5-FU and 5'dFUR in urine over the range 50—250 μg/ml and 1—10 mg/ml, respectively, exhibited good linearity ($R^2 > 0.998$). Within-day variation was less than 4% for both compounds. Between-day variation was determined by analyzing triplicate samples on three different days (Table II). All C.V. values were less than 3%. Mean analytical recovery was 100% (S.D. 0.5%) for 5-FU and 100% (S.D. 0.4%) for 5'dFUR.

TABLE I

BETWEEN-DAY VARIABILITY OF PLASMA ASSAY

Concentration added	Mean concentration measured	*n*	Coefficient of variation (%)
5-FU			
50.0 ng/ml	53.9 ng/ml	4	3.7
100 ng/ml	107 ng/ml	4	13.5
400 ng/ml	401 ng/ml	4	4.3
1000 ng/ml	1012 ng/ml	4	1.9
1.00 μg/ml	0.99 μg/ml	3	4.4
5.00 μg/ml	4.98 μg/ml	3	2.0
50.0 μg/ml	49.6 μg/ml	3	1 7
5'dFUR			
50.0 ng/ml	50.1 ng/ml	4	9.6
100 ng/ml	98.4 ng/ml	4	8.7
400 ng/ml	389 ng/ml	4	6.3
1000 ng/ml	954 ng/ml	4	3.3
1.00 μg/ml	1.05 μg/ml	3	0.9
5.00 μg/ml	5.01 μg/ml	3	4.8
50.0 μg/ml	50.8 μg/ml	3	2.2
75.0 μg/ml	77.0 μg/ml	3	4.4

TABLE II

BETWEEN-DAY VARIABILITY OF URINE ASSAY

Concentration added	Mean concentration measured	*n*	Coefficient of variation (%)
5-FU (μg/ml)			
50.0	49.5	3	1.6
100	99.4	3	2.2
250	250	3	0.7
5'dFUR (mg/ml)			
1.00	1.00	3	0.9
2.00	2.01	3	0.7
10.0	9.99	3	0.7

Sensitivity

Using a signal-to-noise ratio of 3, the minimum detectable quantity on the column was 0.6 ng for 5-FU and 1.6 ng for 5'dFUR. By injecting 20 μl of the 100-μl reconstituted plasma extract at a sensitivity setting of 0.005 a.u.f.s., both of the compounds could be quantitated with acceptable precision at concentrations of 50 ng/ml in 0.5 ml of plasma.

Selectivity

The maximum UV absorbance of 5-FU, 5'dFUR and I.S. occurred at 266, 270 and 276 nm, respectively. Consequently, the chosen wavelength of 280 nm was near the maximum for all three compounds.

No interference was seen for the 5-FU anabolites 5-fluorouridine and 5-fluoro-2'-deoxyuridine or for the catabolic metabolite 2-fluoro-β-alanine. The inactive metabolite, 5,6-dihydrofluorouracil (DHFU), does exist at low μg/ml levels in plasma following the administration of 5-FU and 5'dFUR [20, 21, 30—32]. However, a pure sample of this compound in water does not demonstrate any absorbance at 280 nm, and thus cannot be detected using the present method.

Pharmacokinetic application

The application of the present method to the determination of 5'dFUR and 5-FU in plasma is shown in Fig. 4. The sensitivity of the assay was such that 5'dFUR and 5-FU plasma concentrations could be quantitated for 240 and 180 min, respectively, following the infusion of 2.0 g/m^2 5'dFUR to a patient with colorectal carcinoma.

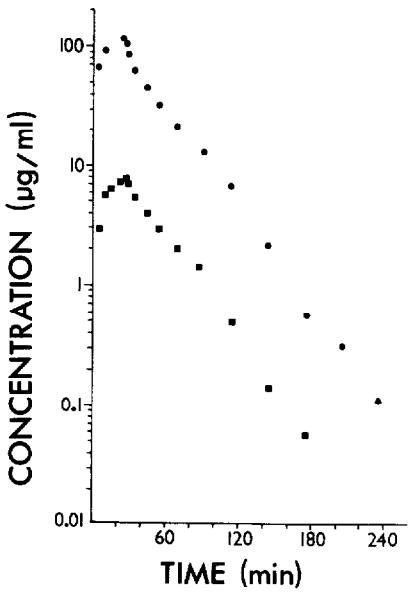

Fig. 4 Plasma concentration—time curves of 5'dFUR (•) and 5-FU (▪) in one patient after infusion of 2.0 g/m^2 5'dFUR over 25 min.

CONCLUSION

In summary, the present method enables the quantitation of both 5'dFUR and 5-FU in human plasma and urine. The sensitivity of the method is adequate for the analysis of both compounds following the administration of single doses of 5'dFUR. Consequently, the procedure should permit more detailed pharmacokinetic investigations of this novel fluoropyrimidine.

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