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Note

Rapid and simple high-performance liquid chromatographic assay for 5′-fluorouracil in plasma for bioavailability studies

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The antineoplastic agent 5′-fluorouracil (5FU, Fig. 1) has been established successfully in the chemotherapy of tumours for many years. In contrast to most other cytostatics, the drug has sufficient bioavailability to be administered via the peroral route [1]. Peroral administration makes drug handling easier for the oncologist as well as for the patient.

On the one hand, the plasma levels of 5FU after peroral administration should be monitored in patients with renal or liver impairment to provide the oncologist with information about the extent of the resorption from the gut and also about plasma levels. On the other hand, drug monitoring is necessary in the case of the use of 5FU pro-drug formulations such as tetrahydrofurylfluorouracil (FTO, Fig. 1), because the extent of 5FU release from the pro-drug after peroral administration may differ greatly from patient to patient.

In both cases there is a need for information about the bioavailability. The control of 5FU plasma levels should be performed to optimize dosage regimens for each patient individually.

A number of gas chromatographic (GC) [2,3] and high-performance liquid chromatographic (HPLC) assays for 5FU have been reported. These methods are relatively sensitive [4–6], but have a time-consuming extraction step [4] and/or use pre-column derivatization (*e.g.* with 4-bromomethyl-7-methoxycoumarin) to obtain the desired sensitivity. This is necessary in the case of prolonged (24 h) intraarterial infusion owing to the low plasma levels of 5FU. In our studies, there

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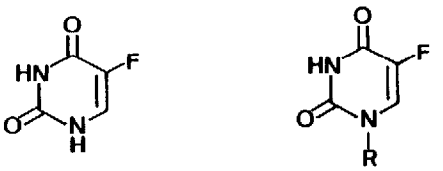

Fig 1. Structures of 5FU (left) and FTO (R = tetrahydrofuryl) (right)

is less a need for extremely high sensitivity than for high accuracy and rapid results. This paper describes a simple HPLC assay for studies of the absolute and relative bioavailability of 5FU.

EXPERIMENTAL

Chemicals

5FU was supplied by Hoffmann la Roche (Basle, Switzerland) and FTO was supplied form Schoeller Pharma/Cehasol (Vienna, Austria). For preparation of the mobile phase, deionized and distilled water and methanol (LiChrosolv, Merck, Darmstadt, F.R.G.) were used. The HPLC solvent was degassed (Erma degasser, SRD, Vienna, Austria) and filtered prior to use through a 0.45-μm Fluoropore membrane filter from Millipore (Bedford, MA, U.S.A.). The mobile phase was acidified with 0.01 *M* acetic acid. HPLC-grade ethanol (Merck) was used to precipitate plasma proteins. Liquemin (Hoffmann la Roche) served as a stabilizer for plasma samples.

HPLC equipment

The liquid chromatograph was from Kontron (Vienna, Austria), consisting of a Model 420 pump, a Model 460 autosampler, a Model 830 column oven and a Uvikon 430 photometric detector. A LiChrosorb RP-8 (10 μm) guard column (10 mm × 4.6 mm I D.) and two coupled Spherisorb RP-8 (5 μm, 300 mm × 4.6 mm I.D.) analytical columns were used in series. Integration of chromatograms was performed by a Model 450 data system.

Chromatographic conditions

The mobile phase was water–methanol (90:10, v/v). The water was adjusted to pH 5.5 with acetic acid before being mixed with the methanol. The HPLC system was operated at a flow-rate of 0.8 ml/min (pressure 105 bar) and thermostatted at 42°C. Detection was performed by measuring the absorbance of the eluate at 276 nm (0.005 a.u.f.s. with a response time of 2 s).

Sample handling

5FU was administered perorally as a drinking ampoule (1000 mg in 10 ml of aqueous solution), and 1600 mg of FTO, containing an equivalent amount of

5FU (1032 mg/10 ml), was given as an aqueous suspension. Blood samples were obtained by venepuncture (left cubital vein) of 5FU-treated patients 0, 5, 15, 30, 45, 60, 90, 120 and 180 min after administration, mixed with liquemin (10 U/ml) and centrifuged at 1500 *g* for 5 min. The supernatant was stored at −35°C until analysis (within fourteen days).

Standard samples

Six standard samples were prepared for the calibration curve by mixing 0.990 ml of fresh plasma with 10 μl of different aqueous stock solutions of 5FU, giving final concentrations of 0.5, 1.0, 2.0, 5.0, 10.0 and 25.0 μg/ml 5FU. These spiked plasma standard samples were stored at −35°C until analysis.

Sample clean-up

A 1.0-ml volume of thawed plasma or 5FU-spiked standard sample was shaked vigorously with 3.0 ml of absolute ethanol (Paramix II, Julabo, Seebach, F.R.G.) for 30 s and centrifuged for 5 min at 1500 *g* (Haereus Labofuge II, Christ, Austria). The supernatant was filtered through a FP003 Spartan filter (0.45 μm, Schleicher & Schuell, Dassel, F.R.G.), and an aliquot of 40 μl was directly injected into the liquid chromatograph by the autosampler.

RESULTS AND DISCUSSION

Extraction procedure

According to the literature, the most wideley used sample clean-up for 5FU is the extraction by solvent-partitioning with ethyl acetate (and/or 2-propanol). The reported detection limits range from 0.005 to 0.05 μg/ml, and the coefficients of variation (C.V.) are *ca.* 10% [5,6].

The main advantage of the proposed 5FU assay is as follows: the method is simple and accurate owing to the rapid extraction procedure of protein precipitation by ethanol. Thus the disadvantage of variable batches of solid-phase extraction cartridges can be avoided. Protein precipitation by trifluoro- or trichloroacetic acid (as described by Kao *et al.* [8]) was unfavourable, because 5FU seemed to be coprecipitated by these chemicals and the recovery was lowered (recovery <85%) [8]. Recovery in the case of protein precipitation with ethanol was 98% in plasma samples spiked with 10 μg/ml 5FU (S.E.M. 3.9%, $n = 8$) and 95% in plasma samples spiked with 25 μg/ml 5FU (S.E.M. 2 8%, $n = 8$). The pre-column had to be renewed after *ca.* 80 injections. This was indicated by an increase of the back-pressure to over 160 bar, caused by clogging of the pre-column by non-precipitated matrix components.

Chromatography

Fig. 2. shows the chromatographic separation of 5FU from FTO (in aqueous buffer, pH 3.0) during a degradation kinetic study [7]. The compounds elute in the

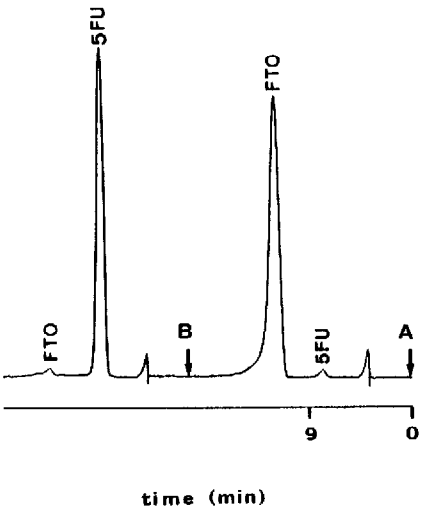

Fig. 2 Chromatograms of 5FU and FTO from an aqueous buffer (pH 3 0) recorded (A) 5 min and (B) 60 min after the start of an FTO degradation experiment. Arrows indicate the start of the chromatogram For chromatographic conditions see text.

order of their lipophility at 8.2 min (5FU) and 11.9 min (FTO). In plasma samples only traces of FTO have been detected owing to the rapid degradation of FTO in acidic solutions.

As can be seen from Fig. 3, the 5FU peak is well separated from the matrix peaks (t_R = 8.2 min), and there is no overlap with coadministered drugs such as cimetidine (t_R = 10.5 min, detection wavelength 228 nm) or nitrazepam (t_R = 17.1 min, peak plasma levels < 100 ng/ml). The protein precipitation method was facilitated by the detection wavelength of 276 nm and by the use of two analytical columns in series, thus making the chromatography sufficiently selective. When Nucleosil C_8 was used as packing material insufficient separation of 5FU (>30 μg/ml) from the matrix peaks in front of the chromatogram has been observed at high concentrations.

Quantitation and limit of detection

Quantitation was effected via the external standard method, by comparing the peak heights of 5FU standard plasma samples (six-point calibration graph) with the peak height of sample chromatograms. The use of an internal standard, such as 5-chlorouracil, was not possible owing to overlap with matrix peaks. After a series of 24 samples (three patients) a six-point calibration graph was run for quantitation. The calibration graph was linear over the range 0.3–32 μg/ml ($y = 7.69x + 7.0$, $r = 0.997$, $n = 6$) where y is the peak height in mV and x is the drug concentration in μg/ml.

The limit of detection was at 0.15 μg/ml by injection of a 40-μl aliquot of

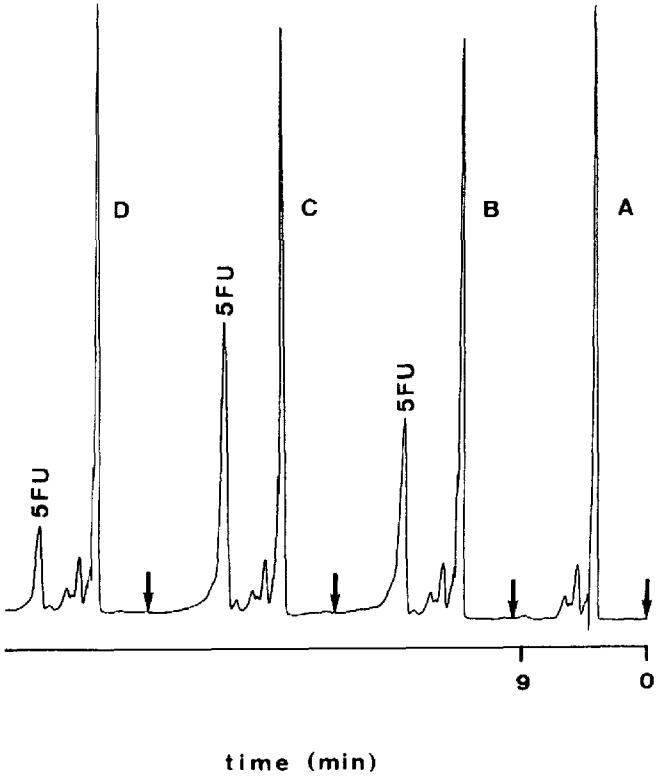

Fig 3 Typical chromatograms of 5FU in an FTO-treated patient. (A) Plasma sample before 5FU administration (blank), (B) plasma sample collected 15 min after administration, containing 14.2 μg/ml 5FU, (C) plasma sample collected 45 min after administration, containing 21.8 μg/ml; (D) plasma sample collected 90 min after administration, containing 5 2 μg/ml Arrows indicate the start of the chromatogram. For chromatographic conditions see text.

sample. This limit is adequate, because of the high plasma levels of 5FU after peroral administration; it could be improved by injection of larger volumes of samples.

Precision of assay

Analysis of eighteen standard curves over seven weeks indicated that the intra-assay C.V. was 1.9% and the inter-assay C.V. was 2.3% (concentration range 1–25 μg/ml).

Clinical application

Fig. 4 depicts the mean (three patients) plasma concentration–time curve of 5FU after peroral administration of pure 5FU and FTO, respectively. As can be seen, 5FU is absorbed rapidly into the blood from the gut ($t_{1/2}$ = 4 min). Administration of peroral FTO leads to higher plasma levels of 5FU and shows a

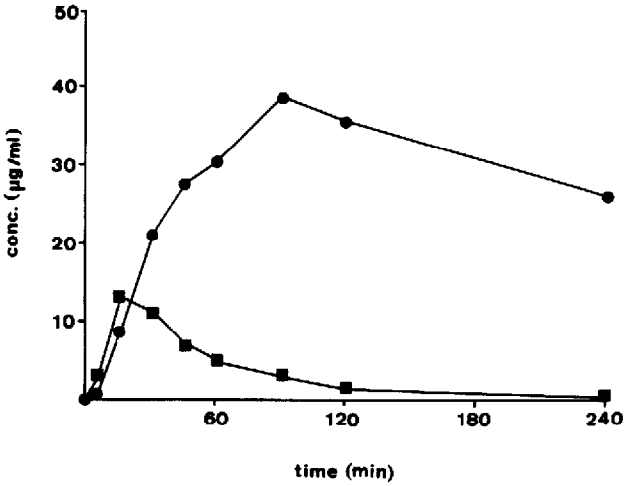

Fig. 4 Plasma levels of 5FU after peroral administration of 5FU (■) and after peroral administration of FTO (●), values are the mean of three patients

prolonged (and desired) absorption phase ($t_{1/2}$ = 17 min), indicating a better bioavailability of 5FU. Table I compares the pharmacokinetic parameters of 5FU obtained after peroral administration of 5FU and FTO. As can be seen from Fig 4, the peroral administration of FTO leads to higher 5FU plasma levels than the administration of peroral 5FU, thus indicating a higher bioavailability of the drug.

TABLE I

PHARMACOKINETIC PARAMETERS OF 5FU AFTER 5FU AND FTO PERORAL ADMINISTRATION

Values are means from three patients.

Parameter	5FU	FTO
Half-life of absorption (min)	4	17
Area under plasma concentration–time curve (µg/ml/min)	922	17 076
Half-life of elimination (min)	24	20
Peak plasma concentration (µg/ml)	13	39
Peak plasma time (min)	15	90
Volume of distribution (l)	38	33
Total clearance (ml/min)	1085	89
Coefficient of partition (ml/g)	0.6	0 5

CONCLUSIONS

With the proposed assay preliminary data are available to the oncologist within 30 min after blood sample collection. Thus the bioavailability after peroral administration can be determined rapidly and further dosage regimens can be calculated individually for each patient.

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