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Note

Determination of a novel fluoropyrimidine, 5'-deoxy-5-fluorouridine, in plasma by high-performance liquid chromatography

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5'-Deoxy-5-fluorouridine (5'-dFUR, Ro21-9738) is a fluoropyrimidine recently synthesized by Cook et al. [1] with antineoplastic activity. The therapeutic potential has been tested both in vitro and in vivo against several rat and murine tumour lines [2—4]. Up to now, only isotopic methods using labelled drugs with high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) have been described in the literature [5, 6]. Also, it appeared of interest to develop a new sensitive and specific HPLC method not requiring labelled substances and utilizable in phamacokinetic studies.

EXPERIMENTAL

Apparatus and operating conditions

A high-performance liquid chromatograph (Hewlett-Packard 1084B) was equipped with automatic injector, variable-wavelength spectrophotometer and chromatograph terminal (Hewlett-Packard 79850 ALC). Detection was performed at 269 nm. The column used was LiChrosorb RP-18 (5 μm), 125 mm × 4 mm I.D. (E. Merck, Darmstadt, G.F.R.).

The mobile phase was of water—methanol—acetonitrile (97:1.5:1.5) with a flow-rate of 1 ml/min. After degassing, the mobile phase was maintained at a temperature of 80°C for the water and at 40°C for the mixture methanol—acetonitrile.

A mass spectrometer, Model 5980A, with data system 5934A (Hewlett-Packard) was also used to establish identity and purity of the 5'-dFUR HPLC peak.

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Reagents

Methanol (HPLC grade; Merck), acetonitrile (HPLC grade; Carlo Erba, Milan, Italy), diethyl ether (analytical grade; Solvant Documentation Synthèse, Valbonne, France), acetic acid 99.7% (analytical grade; Riedel de Haën, Hannover, G.F.R.) and isopropanol (analytical grade; Prolabo, Paris, France) were used without further purification. Double-distilled water was filtered through a 0.22-μm pore membrane filter (Millipore, Bedford, MA, U.S.A.).

The stock solutions of 5-dFUR (Ro21-9738) and 3-methylxanthine (No. 69772; Fluka, Buchs, Switzerland) were prepared in water at 100 μg per 100 μl and 10 μg per 100 μl, respectively. The same solvent was used for standard solutions.

Operating procedures

The blood samples were collected in oxalated tubes (Venoject T 200 × F 105) and then centrifuged for 15 min at 2400 *g*. The plasma should then be immediately frozen until analysis.

Place 50—100 μl of an internal standard solution (in the range 20—0.2 μg/ml) into a 10-ml cylindro-conical centrifuge tube. The concentration used depends on the level of 5'-dFUR to be analysed in the sample. Add 0.2—1 ml of plasma

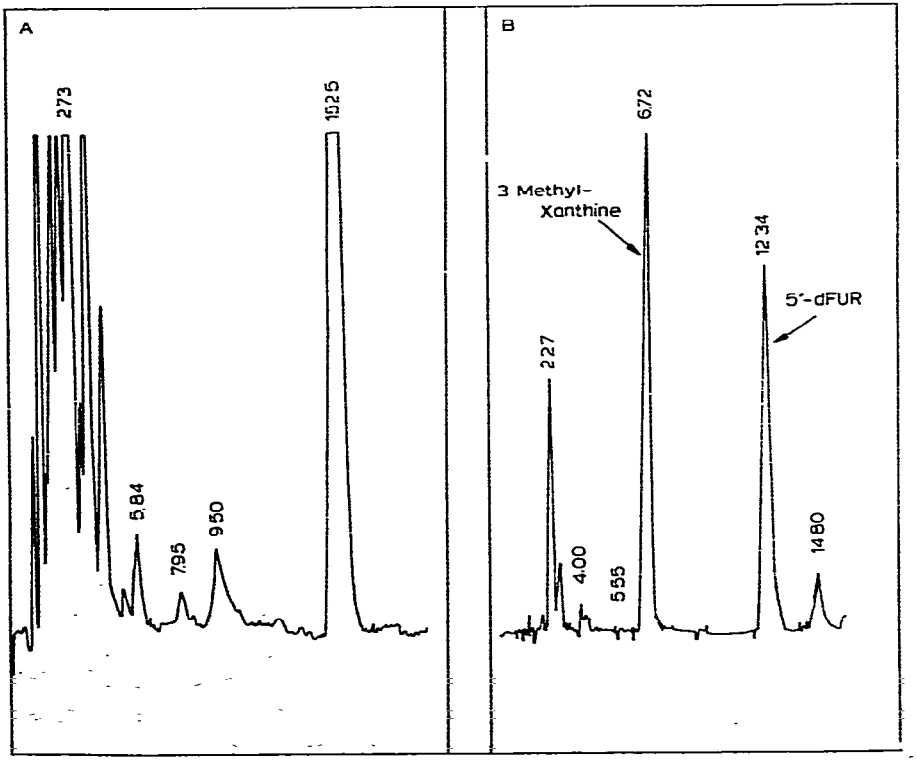

Fig. 1. (A) Plasma control after extraction. (B) Chromatogram of a patient's plasma containing 19 μg/ml of 5'-dFUR with 3-methylxanthine as internal standard (10 μg/ml).

and mix on a vortex-type mixer for a few seconds. Add 1 ml of a 0.3 *M* solution of acetic acid in methanol and mix for about 20 sec to obtain a homogeneous mixture. Place the tube in a water-bath (100°C) for 1 min and during this time mix the contents twice for 2 sec. Cool the tube with ice and centrifuge at 2800 *g* for 20 min. Collect the supernatant, add 20 ml of the diethyl ether—isopropyl alcohol mixture (8:2) and mix the contents vigorously; then agitate mechanically for 30 min. Centrifuge at 2800 *g* for 20 min. Recover the organic phase, evaporate to dryness at 45°C under a stream of nitrogen and re-dissolve the residue in 50—100 μl of water while vortex-mixing for 15 sec. Then, inject 10—25 μl of this solution into the chromatograph for analysis.

Under the conditions defined above, the retention times for internal standard (I) and 5′-dFUR (II) were 6.72 min and 12.34 min, respectively. In Fig. 1 are presented chromatograms of extracts of control plasma (A) and plasma from a patient (B).

Standard calibration curves (ratios of the 5′-dFUR to internal standard peak areas versus concentrations of 5′-dFUR) were obtained after analysis of plasma samples to which increasing quantities of 5′-dFUR [either (a) 0.05—1 μg/ml, or (b) 1—20 μg/ml] were added together with a constant quantity of internal standard [either (a) 0.22 μg/ml, or (b) 2.2 μg/ml]. The following values were obtained for the regression curves: (a) $y = 2.835x - 0.018$, and (b) $y = 0.313x - 0.058$. Both have a correlation coefficient of 0.999.

RESULTS AND DISCUSSION

Internal standard

3-Methylxanthine was chosen as internal standard. This compound is not structurally similar to 5′-dFUR, but its maximum absorption wavelength (272 nm) and its percentage recovery were nearly the same. (The recovery relative of 5′-dFUR to the internal standard was about 90%.) In addition, 3-methylxanthine is neither a drug nor a metabolite of 5′-dFUR. Although 3-methylxanthine is not used as a therapeutic agent, it is a metabolite of both caffeine and theophylline. Therefore, a control plasma sample is examined prior to each pharmacokinetic study to make sure that the retention times corresponding to compounds I and II are free from any possible interference.

Specificity

The maximum absorbance of 5′-dFUR occurred at 269 nm with the instruments used. Each morning the column was conditioned by flushing for 1 h with water—acetonitrile—methanol (90:5:5) at a rate of 1 ml/min followed by the analytical mobile phase at 1 ml/min for 30 min.

No interference from such compounds as uric acid, 5-fluorouracil, 5-fluorouridine, 5-fluoro-2′-deoxyuridine, thymine, thymidine, uracil and uridine was found. However, some samples of plasma, in this study, presented an unknown interference and the complete baseline separation of this peak and 5′-dFUR was only possible by changing the percentage composition of the eluting solvent. Thus, the percentage of methanol—acetonitrile in the mobile phase was decreased from 3% to 2.5% or 2.2%.

Plasma from a patient was checked using mass spectrometry and it was shown that the HPLC peak with a retention time of 12.34 min could be attributed to 5′-dFUR itself. This study was carried out in the chemical ionization mode using 50 eV ionisation energy, an emission current of 200 μA, source temperature of 180°C and pressure of 0.5—1 Torr. Under these conditions, 3 μl of a methanol standard solution containing 10 μg per 100 μl were injected into the mass spectrometer via the direct insertion probe, heating progressively until 250°C. The mass spectrum of 5′-dFUR showed a small amount of $[M^+ + H]$ at m/e = 247 (relative intensity = 1.5%), an abundant m/e = 117 corresponding to the sugar fragment (relative intensity = 100%), and a peak at m/e = 131 corresponding to the base, 5-fluorouracil, plus hydrogen arising from the loss of the sugar fragment from 5′-dFUR (relative intensity = 30%). These results, excluding the relative intensity of the fragments, were in agreement with those obtained for parent compounds, such as adenosine [7].

Thus, the drug-containing fraction of a patient plasma was collected and evaporated completely at 45°C under a stream of nitrogen. The residue was dissolved in 100 μl of methanol and 3 μl of this solution were analysed by direct probe chemical ionization mass spectrometry. Under the analysis conditions defined above, mass spectral fragments identical to those of the standard solution were obtained.

Extraction procedure

The performance of the proposed method was influenced by the extraction pH and the nature of the solvents. The best results were obtained using a mixture of methanol—acetic acid (0.3 *M*) at pH 3.4, which precipitated serum proteins (and reduced the significance of the interferences), and performing the extraction with the ether—isopropanol mixture (8:2). Under these conditions, the percentage recovery for 5′-dFUR quantities between 0.05 and 20 μg/ml was approximately 95%.

Sensitivity, reproducibility and accuracy

Similar, for routine assays, the quantitative limit of sensitivity was about 50 ng/ml plasma. Repeatability was investigated by analysing a plasma pool containing 1 μg of 5′-dFUR and 2 μg of internal standard per ml. The coefficient of variation (s = 0.95) within tests, for 10 successive extractions and assays was 4%.

Application

The proposed technique was used to carry out a pharmacokinetic investigation of 5′-dFUR after intravenous infusion. We studied the decrease with time of 5′-dFUR plasma levels in a pancreatic carcinoma patient with liver metastasis, who had received 1.5 g by short infusion (20 min). The drug disappeared from the plasma rapidly and no measurable residues were present after 2 h (Fig. 2).

A preliminary pharmacokinetic approach showed that this substance appeared to follow a two-compartment open model. For this patient, the pharmacokinetic parameters of elimination half-life and plasma clearance were 10.89 min and 0.879 l/min, respectively.

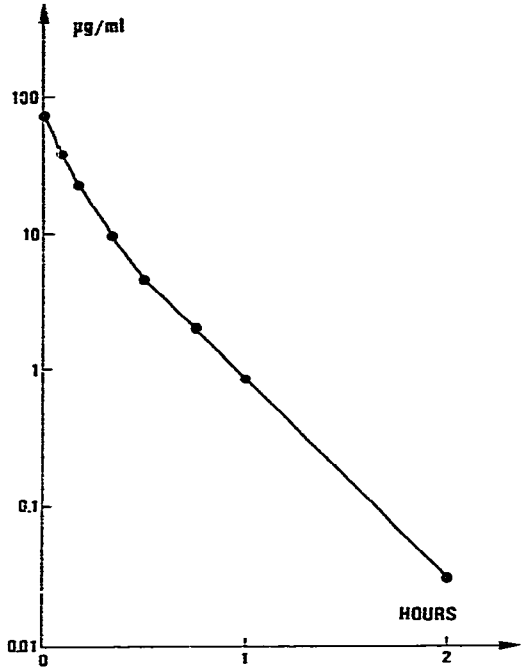

Fig. 2. 5'-dFUR plasma levels in a patient after continuous, 20-min intravenous infusion. Dose administered = 1.5 g.

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