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Note

Determination of 5-fluoro-2′-deoxyuridine in human plasma by high-performance liquid chromatography with pre-column fluorimetric derivatization

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Fluorinated pyrimidines, such as 5-fluorouracil (FU), 5-fluoro-5′-deoxyuridine (a prodrug, which is converted into FU) and 5-fluoro-2′-deoxyuridine (FUDR), are widely used in the chemotherapy of a variety of human carcinomas [1, 2]. The antitumor activity of these cytostatic agents is thought to be primarily due to inhibition of thymidine synthetase by 5-fluoro-2′-deoxyuridine monophosphate (FdUMP) (for review see ref. 3), a common metabolite.

For the treatment of colorectal carcinomas metastatic to the liver, good response rates can be achieved by constant infusion of FUDR into the liver artery using a totally implantable infusion pump [4, 5]. For this therapeutic approach, relatively low systemic FUDR concentrations and low toxicity could be expected because high hepatic extraction ratios of FUDR were observed in humans [6]. The benefit of the palliative therapy seems to be limited by the recently reported serious biliary toxicity during regional chemotherapy [7]. Inter-individual variations of the hepatic elimination capacity, as well as intra-individual changes of the pharmacokinetic parameters throughout the therapy, may be a cause of severe hepatic and biliary toxicity. Therefore, monitoring of individual FUDR plasma concentrations during chemotherapy is desirable.

High-performance liquid chromatographic (HPLC) methods with UV detection have been reported for the determination of 5-fluoro-5′-deoxyuridine, FU, FUDR, their nucleosides and nucleotides [8–10]. In addition, an HPLC analysis with pre-column derivatization and fluorescence detection has been published for FU and an FU derivative, 1-(tetrahydro-2-furanyl)-5-fluorouracil [11]. More recently, newly developed spectroscopic analysis of fluoropyrimidine metabolism

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by ^{19}F nuclear magnetic resonance spectrometry was reported [12, 13]. During regional chemotherapy, FUDR plasma concentrations of less than 0.01 μM can be expected [6]. Determination of FUDR plasma concentrations using radioimmunoassay gives reliable results down to the 0.02 μM range [6], but this assay is not suitable for routine use. The extraction of FU and FUDR from 10-ml samples by anion-exchange chromatography followed by additional steps (lyophilization, extraction into organic solvents [9]), has allowed the determination of plasma concentrations with a detection limit of 0.05 μM.

The specific aim for this study was to develop a more sensitive method for the determination of FUDR plasma concentrations using a modification of the procedure described for the determination of FU [11]. FUDR was quantitated by fluorescence detection after pre-column derivatization with 4-bromomethyl-7-methoxycoumarin (Br-Mmc). Br-Mmc is widely used for pre- or post-column derivatization [14] of, among others, fatty acids [15, 16], thiouridine and thiouracil [17], FU and the FU-derivative 1-(tetrahydro-2-furanyl)-5-fluorouracil [11]. The method (extraction and derivatization procedure) was controlled by the addition of 5-bromo-2'-deoxyuridine (BUDR) to the plasma samples, which served as an internal standard.

EXPERIMENTAL

Reagents and chemicals

5-Fluoro-2'-deoxyuridine, 5-fluorouracil and 5-bromo-2'-deoxyuridine were purchased from Sigma (Munich, F.R.G.); 5-fluoro-2'-deoxy[6-^{3}H]uridine (specific activity: 14.7 Ci/mmol = $5.4 \cdot 10^{11}$ Bq/mmol) from DuPont de Nemours, NEN (Dreieich, F.R.G.); 4-bromomethyl-7-methoxycoumarin and 18-crown-6 from Fluka (Neu-Ulm, F.R.G.). Extraction was performed using Sep-Pak C_{18} cartridges. Purification was achieved with silica gel cartridges from Waters/Millipore (Eschborn/Taunus, F.R.G.). Methanol and acetonitrile (HPLC grade) were from Baker (Gross-Gerau, F.R.G.) and ethanol, chloroform (Uvasol), acetone (LiChrosolv), ammonia (25%) and potassium carbonate, as well as all other chemicals, were obtained from Merck (Darmstadt, F.R.G.) and were of the highest purity available.

Apparatus and HPLC conditions

The HPLC system consisted of a Gynkotek Model 600/200 constant-flow pump, a Model 190 six-port universal injector (Negretti & Zambra, Hampshire, U.K.) and a Hibar LiChrospher Si 100/II column, particle size 5 μm, 250×4 mm I.D., purchased from Merck. The fluorescence signals were recorded using a Gynkotek RF 530 fluorescence spectromonitor (Gynkotek, Munich, F.R.G.) fitted with a 12-μl flow-cell operated at an emission wavelength of 400 nm and an excitation wavelength of 320 nm. In some experiments, the radioactivity was recorded on-line with the fluorimetric measurements, using a Berthold LB 503 HPLC radioactivity monitor equipped with a Model 1024/System BS 27/N multichannel analyzer (Berthold, Wildbad, F.R.G.).

The mobile phase for the HPLC assay consisted of chloroform–ethanol–25%

ammonia (95:5:0.1, v/v/v); the flow-rate was 1.2 ml/min and the column temperature was 22°C.

Reagent solutions and derivatization reaction

To prepare the reagent solution, 50 mg of Br-Mmc, 10 mg of 18-crown-6 and 100 mg of freshly powdered potassium carbonate were dissolved in 100 ml of acetone–acetonitrile (1:2, v/v) under stirring (5 min). The solution was stored at 4°C in the dark for several days.

The optimal conditions for the derivatization reaction were established using FUDR, FU and BUDR solutions, each 100 μg/ml in acetone–acetonitrile (1:2, v/v). The calibration curves were first registered using FUDR and BUDR (100 μg/ml) in doubly distilled water. These stock solutions were stored at 4°C in the dark for several weeks and were occasionally diluted as required. The extraction medium was a 0.5 *M* sodium phosphate buffer prepared by dissolving 17.8 g of disodium hydrogen phosphate dihydrate in 200 ml of doubly distilled water, with the pH adjusted to 7.0 with 25% sulphuric acid. FUDR dissolved in acetone–acetonitrile (0.1 ml organic solution) was derivatized with 0.9 ml of the reagent solution. For the derivatization of FUDR extracted from human plama, 0.5 ml of the reagent solution was directly added to the plasma extract. The mixture was sonicated for 5 min in a supersonic waterbath (Bandelin Sonorex RK 106, Bandelin Electronic, Berlin, F.R.G.). For purification of the derivatization product(s) from impurities, the organic solvent was evaporated under nitrogen (80°C), and the residue was redissolved in 1 ml of chloroform and placed on a silica-gel cartridge. Impurities from the reagent mixture, which would interfere with the HPLC analysis, were washed out with 5 ml of chloroform followed by 2 ml of chloroform–ethanol–25% ammonia (95:5:0.1). The FUDR derivative could be eluted with 2 ml plus twice 1.5 ml of ethanol. Prior to HPLC these products were dissolved in 0.1 ml acetonitrile after evaporation of the ethanol. The injection volume was 20 μl.

Plasma sample treatment

The extraction of FUDR from human plasma samples was essential for the derivatization reaction. This can be done using Sep-Pak C_{18} cartridges activated with 10 ml of methanol and 10 ml of water. Excess water was removed with air before use. A 1-ml plasma sample spiked with 10 ng of BUDR in 10 μl of doubly distilled water as internal standard was diluted with 0.2 ml of extraction buffer (pH 7.0). The solution was slowly passed through the C_{18} cartridge, and washing was performed with 5 ml of a 0.05 *M* phosphate buffer (pH 7.0, prepared as a 1:10 dilution of the extraction buffer). The cartridges were dried for 15 min by aspirating air preheated (95°C) with a hair-dryer by means of a vacuum pump installed at the bottom of the column. Elution of FUDR from the cartridges was performed with acetonitrile (three times 1.5 ml). The eluates were combined, evaporated to dryness under nitrogen (heating to 80°C is possible without degradation of FUDR). The residue was derivatized as described above, with 0.5 ml of the reagent mixture that was added directly to the residue. The following steps,

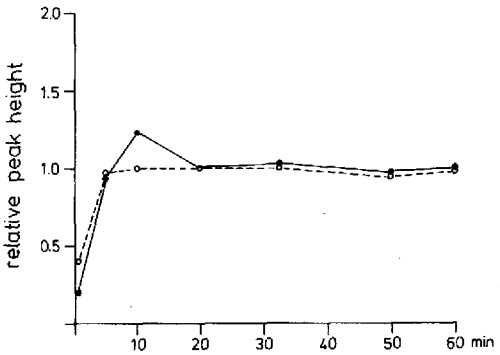

Fig. 1. Rate of FUDR derivative formation at 23°C (●) and 80°C (○) from 100 ng of FUDR in 1 ml of acetone–acetonitrile (1:2), derivatized with 450 μg of Br-Mmc and 90 μg of 18-crown-6 in the presence of potassium carbonate. The values given are results taken from a typical experiment, and are depicted as relative fluorescence intensity compared with the fluorescence intensity after 20 min of reaction time.

including the purification from excess reagents, were performed as described above.

RESULTS AND DISCUSSION

Derivatization reaction and HPLC conditions

The HPLC quantification of FUDR plasma concentrations in the range below 0.1 *μM* by UV detection is not sensitive enough for clinical application [8, 9, 10, 13]. Higher sensitivities can be achieved using fluorescence detection after appropriate fluorescence labelling with specific reagents in a pre- or post-column derivatization [14].

With slight modifications of the method reported for FU derivatization [10], we achieved a rapid reaction between Br-Mmc and FUDR in organic solvents with 18-crown-6 acting as a catalyst in the presence of potassium carbonate. The fluorescence spectra of the FUDR derivative after purification by HPLC showed wavelengths of maximum excitation and maximum emission of 320 and 400 nm, respectively.

The reaction is complete after 5–10 min (Fig. 1). No difference in reaction yield was observed between the reaction temperatures of 23 and 80°C (quantified as peak height of the fluorescence signal).

Before the HPLC analysis it was necessary to remove the excess reagent and its impurities, which exhibited strong fluorescence. In contrast to the method reported for FU, where the addition on *n*-valeric acid was used for the treatment of excess reagent, this procedure was not successful for FUDR. Appropriate extraction of the FUDR derivative from the reagent mixture was obtained by commercially available silica-gel cartridges. Impurities were washed out with chloroform and chloroform–ethanol–25% ammonia (95:5:0.1), and the FUDR derivative was subsequently eluted with ethanol.

The HPLC conditions for a satisfactory FUDR determination were achieved

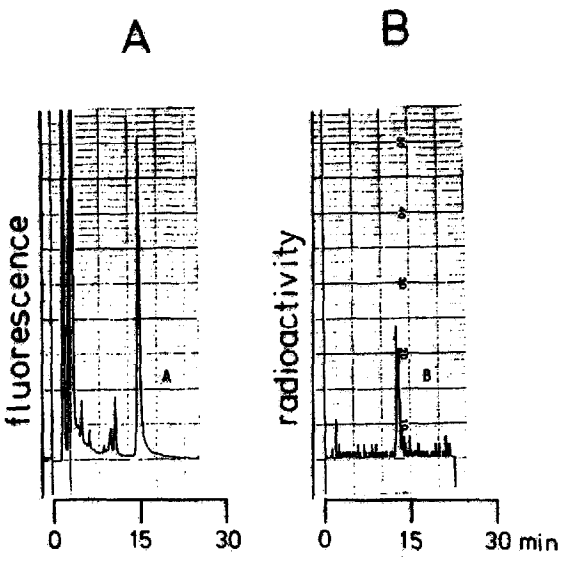

Fig. 2. HPLC separation of a human plasma sample spiked with [^{3}H]FUDR (80 μCi, 2.9·10^6 Bq, 1.34 μg). In parallel to the fluorescence signal (A) the radioactivity was recorded (B) with a radioactivity monitor equipped with a multichannel analyzer.

using a normal-phase analytical column with a freshly prepared mobile phase of chloroform–ethanol–ammonia. Other HPLC modes, including reversed-phase C_{18} and phenyl columns combined with a variety of mobile phases, were less effective.

Specificity of the method

The identity of the FUDR derivative was verified using human plasma spiked with [^{3}H]FUDR. The radioactivity was monitored on-line with the fluorescence signal. The sample preparation was performed as described for non-labelled FUDR. Fig. 2 shows the identical retention times for the fluorescence and the radioactive signal.

The difference of 6 min between the retention times of FU and FUDR derivative in the HPLC analysis (with the FU derivative eluting first) ensured that there was no interference between the two compounds.

Analysis of FUDR in plasma samples

The relationship between peak height of the fluorescence signal and the FUDR concentration was linear in the range between 2.5 ng/ml and 10 μg/ml when the reaction was performed after the addition of FUDR in organic solutions [regression line: y (peak height) $= 1.62x$ (concentration value) $+ 21.5$; correlation coefficient $r = 0.999$]. The calibration curve of FUDR in human plasma was obtained using samples spiked in vitro with FUDR and BUDR as the internal standard. The peak-height ratios of FUDR to BUDR showed linearity over the range from 5 ng/ml to 10 μg/ml [regression line: y (peak-height ratio) $= 0.017x$ (concentration value) $- 1.87$; $r = 0.997$]. The chromatogram of blank plasma showed no constituents eluting at the retention time of the BUDR derivative.

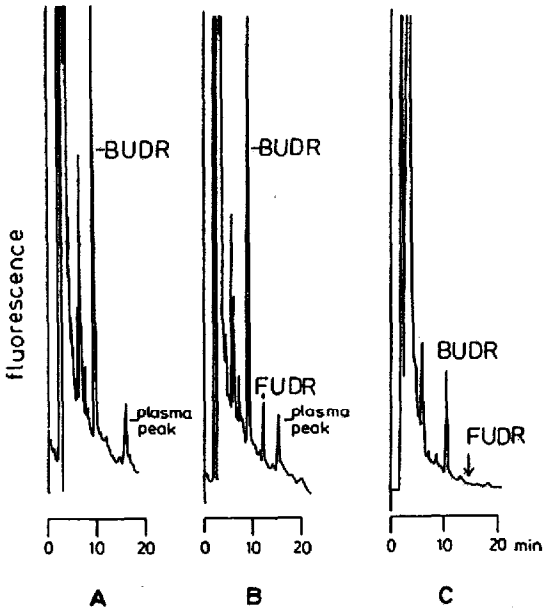

Fig. 3. HPLC separation of plasma samples. (A) Plasma blank; (B) plasma spiked with 10 ng/ml FUDR, from which the FUDR was extracted using Sep-Pak C_{18} cartridges prior to the derivatization; (C) chromatogram of a plasma sample from a patient during regional chemotherapy for colorectal carcinomas metastatic to the liver, registered at a four-fold attenuation of the detector signal (compared with A and B). The latter sample was obtained during a four-week period without FUDR dosing for clinical indication. This chromatogram is representative of the separation of other plasma samples ($n=11$) and shows that there were no plasma constituents in the patient during regional chemotherapy that might interfere with the FUDR assay. The plasma samples were spiked with the internal standard BUDR (100 ng/ml of plasma, dissolved in 10 μl of doubly distilled water) prior to the extraction and derivatization procedure.

The applicability of the described method to human plasma samples depends on the separation of FUDR from other plasma constituents before the derivatization reaction. The extraction of FUDR from human plasma by anion-exchange chromatography has been reported [9]. For the isolation of the hydrophilic compound FUDR from plasma we used Sep-Pak C_{18} cartridges, which were eluted with organic (acetonitrile) solutions. The recovery of FUDR by this technique was $72 \pm 4\%$ determined at a concentration of 100 μg/ml ($n=5$).

A representative HPLC profile of a human plasma sample spiked with 10 ng/ml FUDR (0.04 μM) is shown in Fig. 3B. The signal-to-noise ratio at a concentration of 3 ng/ml was 12.2 dB, referred to the detector signal of the mobile phase [coefficient of variation (C.V.) = 8.7%, $n=3$] for FUDR in aqueous solutions, and was 7.7 dB (C.V. = 6.5%, $n=3$) for spiked human plasma. The maximum sensitivity of the method would suggest a detection limit of ca. 1 ng/ml for spiked plasma, which may be further improved regarding the signal-to-noise ratio for aqueous solutions. In a control group of patients in the FUDR-free interval of regional chemotherapy of at least four weeks ($n=11$), no interference with the plasma constituents was detected (Fig. 3C).

An advantage of our method is seen in an increased sensitivity and, in comparison with the radioimmunoassay, in the dependency on reagents that are commercially available. Its application will be useful, e.g. for the surveillance of the rather low systemic concentrations of FUDR during regional cytostatic chemotherapy.

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