*Journal of Chromatography*, 575 (1992) 63–67
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6212

# Quantification of 5-fluoro-2′-deoxyuridine in plasma by gas chromatography and negative-ion chemical ionization mass spectrometry

Junichi Sato*, Kenzo Watanabe, Shigehiro Takahama and Tokutaro Makita

*Bio-Medical Research Laboratories, Teijin Ltd., 4-3-2 Asahigaoka, Hino, Tokyo 191 (Japan)*

(First received August 13th, 1991; revised manuscript received November 12th, 1991)

ABSTRACT

A sensitive and specific method using gas chromatography and negative-ion chemical ionization mass spectrometry is described for the determination of 5-fluoro-2′-deoxyuridine (FdUrd) in plasma. The method is based on the formation of the pentafluoropropionyl derivative of FdUrd and of its stable isotope as internal standard after sample clean-up by solid-phase extraction and purification by high-performance liquid chromatography. Quantification in plasma was possible down to 300 pg/ml. The method was applied to the analysis of plasma levels of FdUrd in mice and dogs.

## INTRODUCTION

2′-Deoxy-5-fluoro-5′-tetradecylphosphouridine (TEI-6170, I) is a potent antitumour agent synthesized by the Bio-Medical Research Laboratories (Teijin, Tokyo, Japan) [1,2]. The compound is a prodrug aimed at raising the tumour concentration of 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), which is a potent inhibitor of thymidylate synthetase and, consequently, of DNA synthesis [3]. In our preliminary study, we found high concentrations of FdUMP in tumour tissue and 5-fluoro-2′-deoxyuridine (FdUrd) in plasma after oral administration of I to mice. It is known that FdUMP is formed in a single reaction from FdUrd [4]. Therefore, the determination of FdUrd in plasma is important for analysis of the antitumour effect. Several analytical methods have been reported for the determination of FdUrd in biological samples [5–11]. However, because of inadequate sensitivity and specificity these methods seemed inappropriate for our present study, which was intended to clarify differences in plasma concentrations of FdUrd between species.

This paper described a gas chromatographic and negative-ion chemical ionization mass spectrometric (GC–NICIMS) method using a selected-ion monitoring (SIM) technique for the determination of FdUrd in plasma. We measured FdUrd as a pentafluoropropionyl (PFP) derivative, which improved the sensitivity and specificity of the method by producing a better response and a cleaner baseline at focused channels monitored during the assay.

## EXPERIMENTAL

### *Materials*

Pentafluoropropionic anhydride (PFPA) was purchased from Gasukuro Kogyo (Tokyo, Japan), FdUrd was from Sigma (St. Louis, MO, USA) and [1,3-$^{15}N_2$]-5-fluorouracil was from MSD Isotopes (Quebec, Canada). TEI-6170 (I) and [1,3-$^{15}N_2$]FdUrd were synthesized in our laboratories. Bond Elut $C_{18}$ and SCX cartridges

0378-4347/92/$05.00 

were purchased from Analytichem International (Harbor City, CA, USA). All other solvents and reagents used were of analytical-reagent grade.

*I and [1,3-$^{15}N_2$]FdUrd*

Compound I was synthesized as previously described [2]. [1,3-$^{15}N_2$]FdUrd was obtained by the method of Aoyama [12] from [1,3-$^{15}N_2$]-5-fluorouracil.

*High-performance liquid chromatography (HPLC)*

The HPLC system consisted of a Shimadzu LC-6A pump (Shimadzu, Kyoto, Japan), a Rheodyne 7125 injector valve (Rheodyne, Cotati, CA, USA), a Shimadzu Model SPD-M6A photodiode array UV–VIS detector and a Shimadzu Model UP2000 recorder. A LiChrosorb RP-18 reversed-phase column (250 mm × 4 mm I.D.; particle size 7 μm; Merck, Darmstadt, Germany) was used for the separation of FdUrd. The mobile phase was acetonitrile–distilled water–acetic acid (30:1000:1, v/v) used at a flow-rate of 1 ml/min.

*Gas chromatography-negative-ion chemical ionization mass spectrometry*

GC–NICIMS was performed with a Hitachi M-80B mass spectrometer (Hitachi, Tokyo, Japan) interfaced to a Hitachi 663-30 gas chromatograph. An M-0101 data system was used to control the mass specrometer and for data acquisition and processing. GC separations were achieved by use of a DB-1 (J & W Scientific, Rancho Cordova, CA, USA) methyl silicone fused-silica capillary column (30 m × 0.32 mm I.D.; phase thickness, 0.25 μm). The temperature of the column oven was programmed initially to be 90°C for 30 s and was then increased at a rate of 25°C/min until a final temperature of 250°C was reached for elution of derivatized FdUrd. The carrier gas was helium at a flow-rate of 2 ml/min. A cooled on-column injector (Gasukuro Kogyo) was used. The mass spectrometer was operated in the NICI mode, and methane was used as the reagent gas at a CI chamber pressure of *ca.* 1 Torr. The ionization energy was 100 eV and the filament emission current 100 μA. The source temperature was 140°C. SIM was accomplished by electrical cycling at constant magnetic field between masses *m/z* 537 for the FdUrd derivative and *m/z* 539 for the corresponding [1,3-$^{15}N_2$]FdUrd derivative.

*Extraction, purification and derivatization of FdUrd*

A plasma sample (0.05 or 1 ml) was introduced into a 10-ml glass-stoppered centrifuge tube, and 50 μl of acetonitrile containing 40 ng/ml [1,3-$^{15}N_2$]FdUrd as internal standard (I.S.) and 6 ml of acetonitrile were added. The mixture was shaken for 20 min in a mechanical shaker, and then centrifuged at 1500 *g* for 15 min. The supernatant liquid was transferred to a new tube and evaporated to dryness in a VC-96 centrifugal concentrator (Taiyo Scientific Industrial, Tokyo, Japan). The residue was dissolved in 2 ml of 0.05 *M* Tris–HCl buffer (pH 6.0) and then applied to a Bond Elut $C_{18}$ (500 mg of sorbent) that had been previously washed with 3 ml of methanol followed by 9 ml of water. After the cartridge had been washed with 2 ml of the same buffer, the Bond Elut $C_{18}$ was connected directly to a Bond Elut SCX (500 mg of sorbent) that had been prewashed with 3 ml of methanol and 9 ml of 1% acetic acid solution. Then FdUrd was eluted with 4 ml of 1% acetic acid solution into a 10-ml centrifuge tube. The eluate, mixed with 4 ml of ethanol, was evaporated to dryness in the centrifugal concentrator and the residue taken up in 0.6 ml of HPLC mobile phase by vortex-mixing and centrifuged. The volume injected into the HPLC system was 0.6 ml. Peak identification was achieved by reference to the HPLC profile of authentic FdUrd. FdUrd was obtained with a retention time of 9 min. The effluent fractions corresponding to FdUrd were corrected in a 10-ml glass-stoppered tube and evaporated to dryness in the centrifugal concentrator. The residue of the eluate was dissolved in 50 μl of acetonitrile and 100 μl of PFPA. After the mixture had been allowed to stand for 30 min at 60°C, the excess reagent was evaporated to dryness under a stream of nitrogen at room temperature. The res-

idue was dissolved in 20 μl of ethyl acetate, and then an aliquot (2 μl) of it was injected into the GC–MS system.

*Calibration curves*

Solutions containing 0, 0.3, 0.5, 0.8, 1, 2, 5 and 10 ng/ml FdUrd were prepared by dissolving FdUrd in methanol. After 1 ml of each solution had been placed into a centrifuge tube, the solvent was evaporated off, and then 1 ml of drug-free plasma was added to each residue. These samples were assayed according to the method described above. The peak-height ratios of *m/z* 537 for the PFP derivative of FdUrd to *m/z* 539 (I.S.) were calculated and plotted against the known amounts of FdUrd.

*Animal studies*

Male BALB/c mice (18–22 g) and male beagle dogs (9–13 kg), maintained with free access to food and water, were fasted overnight prior to the oral administration of I dissolved in 150 m*M* phosphate buffer (pH 7.0). After dosing, blood samples were collected into a heparinized syringe from the inferior vena cava of the mice and the antecubital vein of the dogs at scheduled time periods. Plasma samples obtained by centrifugation were kept frozen at −20°C until analysed.

## RESULTS AND DISCUSSION

In the present derivatization pocedure, FdUrd was converted to a tri-PFP derivative (Fig. 1). The identity of the derivative was confirmed with the mass spectrometer used in the electron-impact ionization (EI) mode.

Fig. 2a shows the EI mass spectrum of the PFP derivative of FdUrd. The appearance of the molecular ion (*m/z* 684) and the fragment ion at *m/z* 409, with low relative intensity, was sufficient to confirm the structure of the derivative.

Fig. 2b shows the mass spectrum of the PFP derivative of FdUrd in the NICI mode with methane used as a reagent gas. In contrast to the EI mass spectrum, the NICI mass spectrum of the PFP derivative gave high-intensity ions in the high-mass region. Although a quasi-molecular or

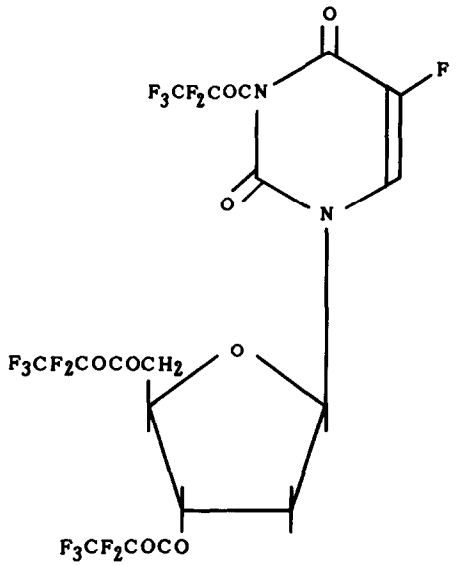


Fig. 1. Structure of PFP derivative of FdUrd (MW = 684).

adduct ion near the region of the molecular ion of the PFP derivative was not recognized, the characteristic negative ion $[M - 147]^-$ at *m/z* 537, which was produced by the elimination of a PFP

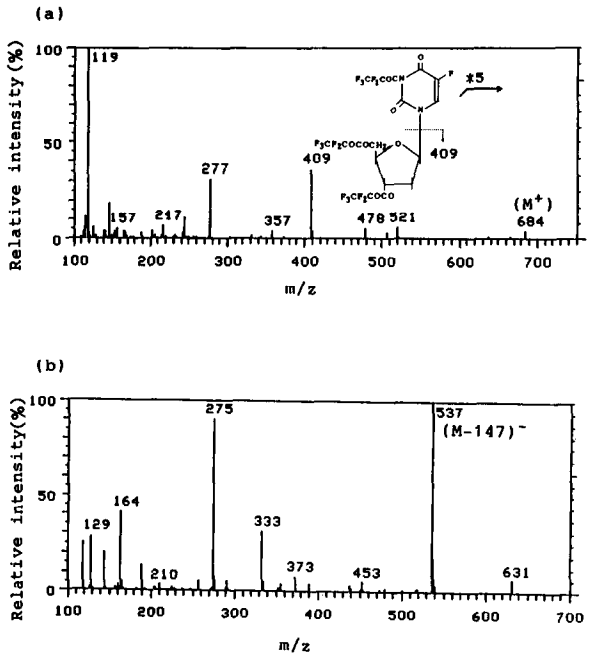


Fig. 2. (a) Electron-impact (70 eV) and (b) negative-ion chemical ionization mass spectra of the PFP derivative of FdUrd.

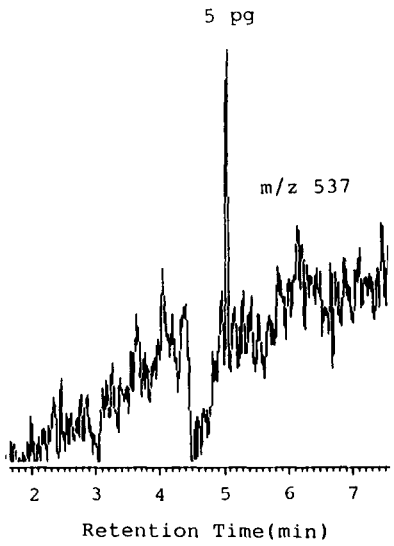


Fig. 3. Selected-ion monitoring profiel of the PFP derivative of FdUrd showing the lower detection limit.

from the molecule, was observed as a base peak. The PFP derivative of [1,3-$^{15}N_2$]FdUrd as I.S. gave a similar mass spectrum. Thus, ions of *m/z* 537 for the PFP derivative of FdUrd and of *m/z* 539 for that of I.S. were chosen for the SIM analysis of FdUrd.

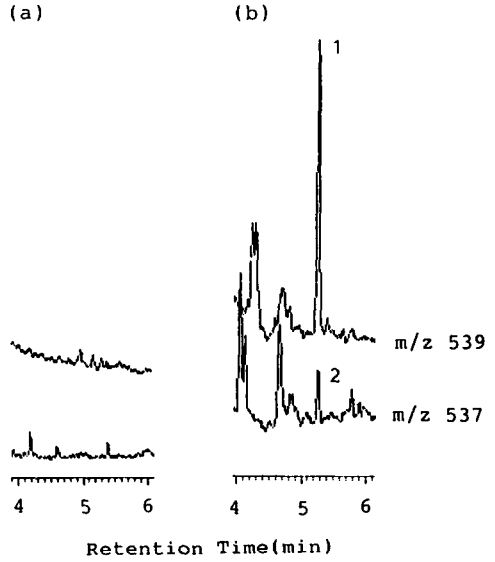


Fig. 4. Selected-ion monitoring chromatograms of (a) blank plasma sample and (b) plasma sample containing 300 pg/ml FdUrd. Peaks: 1 = PFP derivative of [1,3-$^{15}N_2$]FdUrd (internal standard); 2 = PFP derivative of FdUrd.

SIM was carried out to examine the detection limit of the PFP derivative of FdUrd in the NICI mode. As judged from Fig. 3, the minimum detectable amount of the PFP derivative of FdUrd was estimated to be 5 pg with a signal-to-noise ratio of 5.

Typical chromatograms obtained from drug-free human plasma and human plasma supplemented with FdUrd and the I.S. are shown in Fig. 4. No interfering peaks were observed when blank human plasma was analysed without addition of the I.S.

Calibration curves were constructed by addition of known amounts of FdUrd and I.S. to drug-free plasma. The peak-height ratios of *m/z* 537 and 539 were calculated by measurement of the peak heights of the respective selected-ion current profiles. The resultant data exhibited good linearity over the range 0.3–10 ng/ml for FdUrd (regression equation: $y = 0.326x + 0.108$; $r = 0.998$). The coefficients of variation of the method were 9.2, 4.1 and 3.4% at 0.3, 5 and 10 ng/ml, respectively ($n = 5$). The recovery test was performed with 2 ng of FdUrd added to drug-free human plasma (1 ml). The overall recovery of FdUrd was 63 ± 18% (mean ± S.D., $n = 3$).

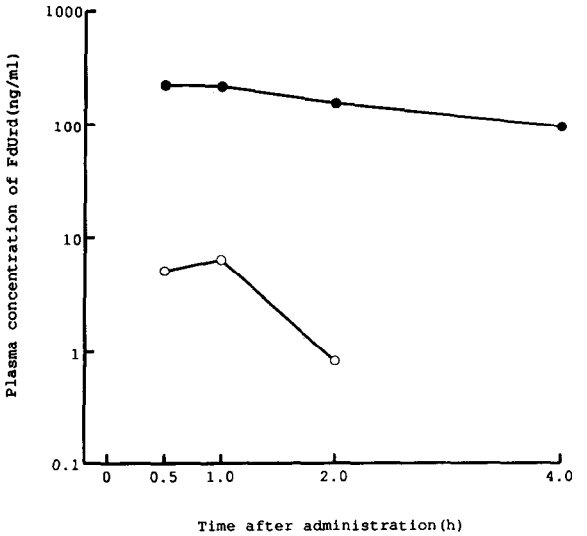


Fig. 5. Plasma concentration of FdUrd after the oral administration of I (20 mg/kg) to mice (●) and dogs (○). Each point represents the mean ($n = 2$).

The PFP derivative used for the GC–MS analysis was unstable after drying of the reaction mixture. So we recommend that sample injections into the GC–MS system should be made immediately after the drying step.

The method described here was applied to the quantification of FdUrd in plasma after oral administration of I (20 mg/kg) to mice and dogs. The results are shown in Fig. 5. As seen, there were large differences in plasma concentrations of FdUrd between the two species. These data indicate that the described assay method is appropriate for pharmacokinetic studies of FdUrd in different species.

In our laboratory, this method is currently being applied to the determination of FdUrd in patients with cancer after I administration. The results will be published later.

REFERENCES

1 F. Kanzawa, Y. Matsushima, J. Ishihara, A. Hoshi, T. Ohba and K. Watanabe, *J. Pharmacobio-Dyn.*, 9 (1986) 688.
2 M. Saito and K. Watanabe, in preparation.
3 S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb and J. Lichtenstein, *Proc. Natl. Acad. Sci. U.S.A.*, 44 (1958) 1004.
4 E. Harbers, N. K. Chaudhuri and C. Heidelberger, *J. Biol. Chem.*, 234 (1959) 1255.
5 B. Clarkson, A. O'Connor, L. Winston and D. Hutchison, *Clin. Pharmacol. Ther.*, 5 (1964) 581.
6 R. A. Jones, A. R. Buckpitt, H. H. Londer, C. E. Myers, B. A. Chabner and M. R. Boyd, *Bull. Cancer (Paris)*, 66 (1979) 75.
7 R. Schreiber and V. Raso, *Cancer Res.*, 38 (1978) 1889.
8 M. Iigo, Z. Yamaizumi, Y. Nakajima, S. Nishimura and A. Hoshi, *Drugs Exp. Clin. Res.*, 14 (1988) 257.
9 W. M. Williams, B. S. Warren and F. Lin, *Anal. Biochem.*, 147 (1985) 478.
10 F. P. LaCreta, *J. Chromatogr.*, 414 (1987) 197.
11 S. Yoshida, K. Urakami, M. Kito, S. Takeshima and S. Hirose, *J. Chromatogr.*, 530 (1990) 57.
12 H. Aoyama, *Bull. Chem. Soc. Jpn.*, 60 (1987) 2073.