ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 291–297

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Determination of N^3-methyl-5′-deoxy-5-fluorouridine, a novel metabolite of doxifluridine, in body fluids by high performance liquid chromatography

Carlo Giorgio Zambonin [a,*], Silvio Mastrolitti [b], Francesco Palmisano [b]

[a] *Dipartimento di Chimica, Università degli Studi della Basilicata, Via N. Sauro 85, 85100-Potenza, Italy*
[b] *Dipartimento di Chimica, Università degli Studi di Bari, Via Orabona 4, 70126-Bari, Italy*

Received 22 April 1997; received in revised form 6 August 1997

Abstract

A high performance liquid chromatography method for the determination of N^3-methyl-5′-deoxy-5-fluorouridine, a possible metabolic product of the anticancer pro-drug 5′-deoxy-5-fluorouridine, in human serum and urine is described. Sample treatment involved addition of internal standard (5-bromouracil) and protein precipitation with ammonium sulphate (serum samples) followed by liquid–liquid extraction with ethyl acetate–isopropanol (90:10, v/v). The average recovery at 0.5 mg ml^{-1} level was ($80 \pm 4\%$). A linear response extending over two decades of concentration was observed. Detection limits of 50 and 100 ng ml^{-1} were obtained in serum and urine, respectively.

Keywords: Anticancer drugs; HPLC; N^3-Me-5′-dFUR

1. Introduction

5′-Deoxy-5-fluorouridine (doxifluridine, 5′-dFUR) is a fluoropyrimidine derivative that has been synthesized [1] in an attempt to improve the therapeutic index and to minimize the toxic effects of the anticancer drug 5-fluorouracil (5-FU) [2,3], which is widely used for the treatment of some solid tumours of breast, colon and rectum. Using the deoxyribofuranosyl moiety as a carrier into the neoplastic tissue, 5′-dFUR is cleaved, preferentially in tumour cells, to the active 5-FU under the action of an intracellular thymidine phosphorylase [4–7].

5-FU is then anabolized, by two possible pathways, to 2′-deoxy-5-fluorouridine-5′-monophosphate, which blocks the thymidylate synthetase system. 5-FU catabolism occurs mainly in liver where it is degraded by dihydrouracil dehydrogenase to 5,6-dihydro-5-fluorouracil (5-FUH_2), which then undergoes further biotransformation with the release of ammonia, urea and carbon dioxide. 5-FUH_2 seems to produce inhibition of thymidylate synthetase activity in Ehrlich ascites tumour cells [8], suggesting that it may also contribute to 5-FU toxicity.

* Corresponding author.

0731-7085/98/$19.00
PII S0731-7085(97)00200-8

Recently, evidence of in vivo biomethylation of 5′-dFUR with formation of a novel metabolite of the pro-drug has been presented for the first time by Zambonin et al. [9]. Plasma extracts of cancer patients undergoing 5′-dFUR chemotherapy were analysed by gas chromatography–mass spectrometry and found to contain a metabolic product of 5′-dFUR with a retention time different from that of the known metabolites mentioned above and absent in blank control plasma samples.

Based on the observed electron impact fragmentation pattern and comparison of the mass spectra and chromatographic characteristics of the suspected metabolite with those of a synthetically prepared reference standard, the metabolic product was identified as N^3-methyl-5′-deoxy-5-fluorouridine (N^3-Me-5′-dFUR).

Furthermore, in a recent in vitro study [10], N^3-Me-5′-dFUR was found to be non-toxic for different cancer cell lines; the biomethylation process leading to the new metabolite could then be considered as a possible detoxification pathway, altering the drug bioavailability, in competition with 5′-dFUR cleavage to the active 5-FU.

Thus, a sensitive method permitting a fast and simple determination of N^3-Me-5′-dFUR in body fluids could be a very useful tool to study 5′-dFUR metabolism and to optimize the pro-drug dosage.

In this paper, high performance liquid chromatography (HPLC) with UV-diode array detection was used to develop an analytical procedure for the determination of N^3-Me-5′-dFUR. To demonstrate the usefulness of the proposed method some applications on spiked serum and urine samples are presented.

2. Experimental

2.1. Chemicals

Stock solutions were prepared in tridistilled water and stored at 4°C in the dark. More dilute solutions were prepared just before use.

Isopropanol, methanol (Carlo Erba, Milan, Italy) and ethyl acetate (Merck, Darmstadt, Germany) were HPLC grade. The HPLC mobile phase was filtered through a 0.45 μm membrane (Whatman Ltd., Maidstone, UK) before use. 5-FU and 5-bromouracil (5-BrU) were obtained from Sigma (St. Louis, MO). 5′-dFUR and 5-FUH_2 were kindly provided by Roche (Milan, Italy) and Hoffmann-La Roche (Basel, Switzerland), respectively.

2.2. Apparatus

The HPLC system consisted of a Waters (Milford, MA) 600-MS multisolvent delivery system equipped with a Rheodyne injector with a 20 μl loop and a 5-μm Supelcosil LC-18-S ODS column (250 mm × 4.6 mm i.d.; Supelco, Bellefonte, PA). A 5-μm Supelguard LC-18-S precolumn (20 mm × 4.6 mm i.d.; Supelco) was used to protect the analytical column. The detector was an HP 1040A photodiode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA) interfaced to an HP 85 computer equipped with an HP dual disk drive and an HP 7470A plotter. Otherwise an 875-UV intelligent UV/vis detector (Jasco, Tokyo, Japan) interfaced with an HP 3395 integrator (Hewlett-Packard) were used.

2.3. Chromatographic and detection conditions

A binary gradient composed of phosphate buffer ($I = 0.05$ M, pH 6.5) methanol (4060, v/v) (solvent A) and phosphate buffer ($I = 0.05$, pH 6.5) (solvent B) was used. The gradient programme was: 15 min concave from 7% A to 67% A; 5 min isocratic at 67% A; 2 min linear to 7% A; equilibration time 10 min. The concave segment was generated using the gradient curve 'number 7,' available in the software of the HPLC pump used, which could be fitted by the following equation:

$$\%A(t) = M_0 + M_1 t + M_2 t^2$$

where $M_0 = 7$, $M_1 = 0.024$, $M_2 = 0.266$ and t is expressed in min.

The flow rate was 1 ml min^{-1} and the temperature was ambient. Detection wavelength was

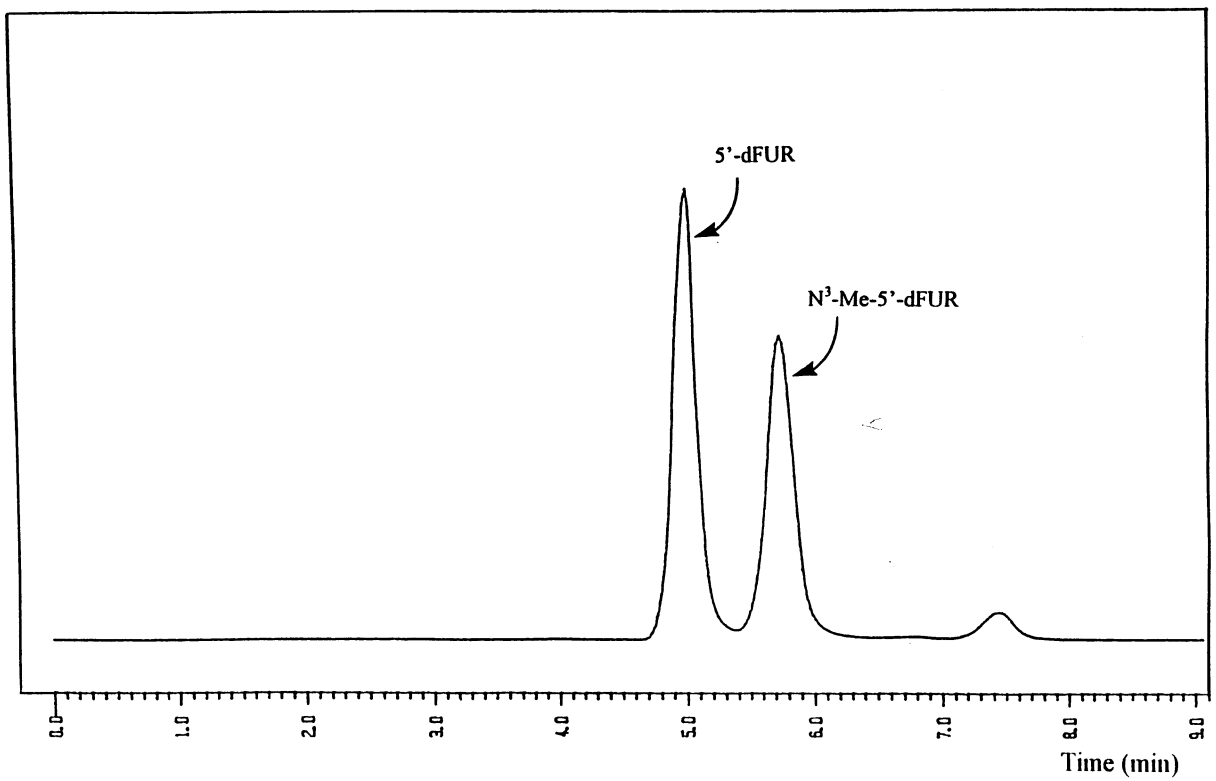

Fig. 1. Chromatogram relevant to the reaction mixture taken at the end of the synthesis of N^3-Me-5′-dFUR. Detector employed: HP 1040 diode-array. Detection wavelength: 269 nm. Absorbance axis: 950 mAU full scale.

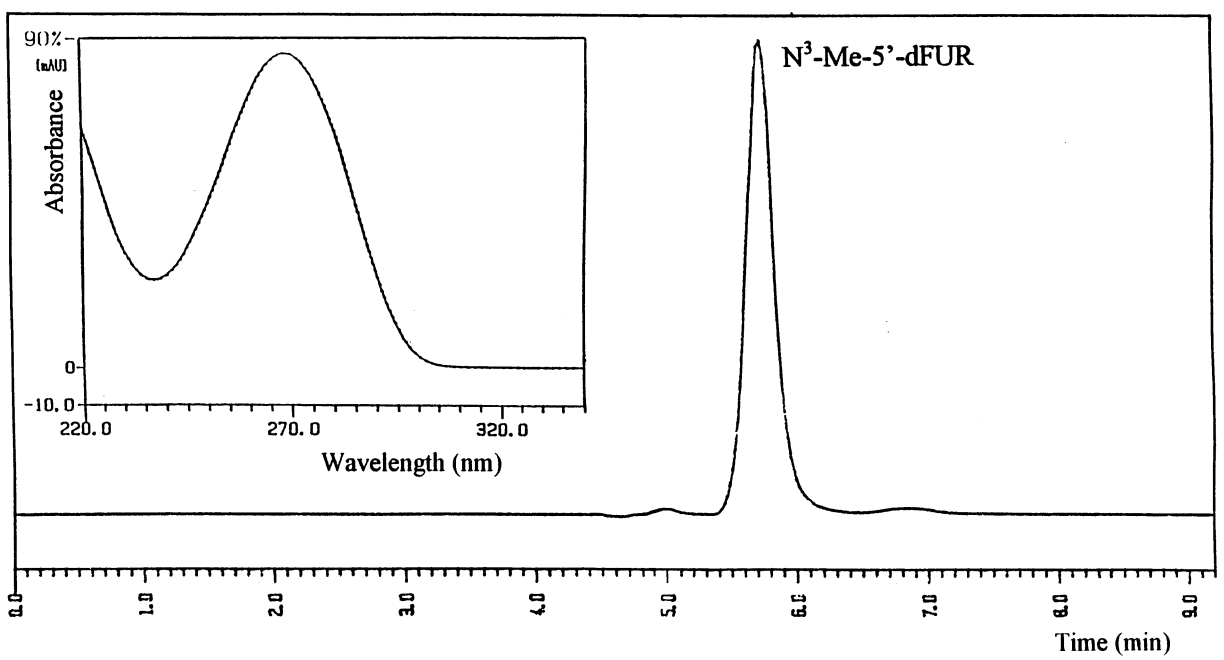

Fig. 2. Chromatogram relevant to the final extract of the reaction mixture of the synthesis of N^3-Me-5′-dFUR. Detector employed: HP 1040 diode-array. Detection wavelength: 269 nm. Absorbance axis: 950 mAU full scale. The inset reports the UV spectra acquired at the apex and on the ascending and descending part of the N^3-Me-5′-dFUR peak.

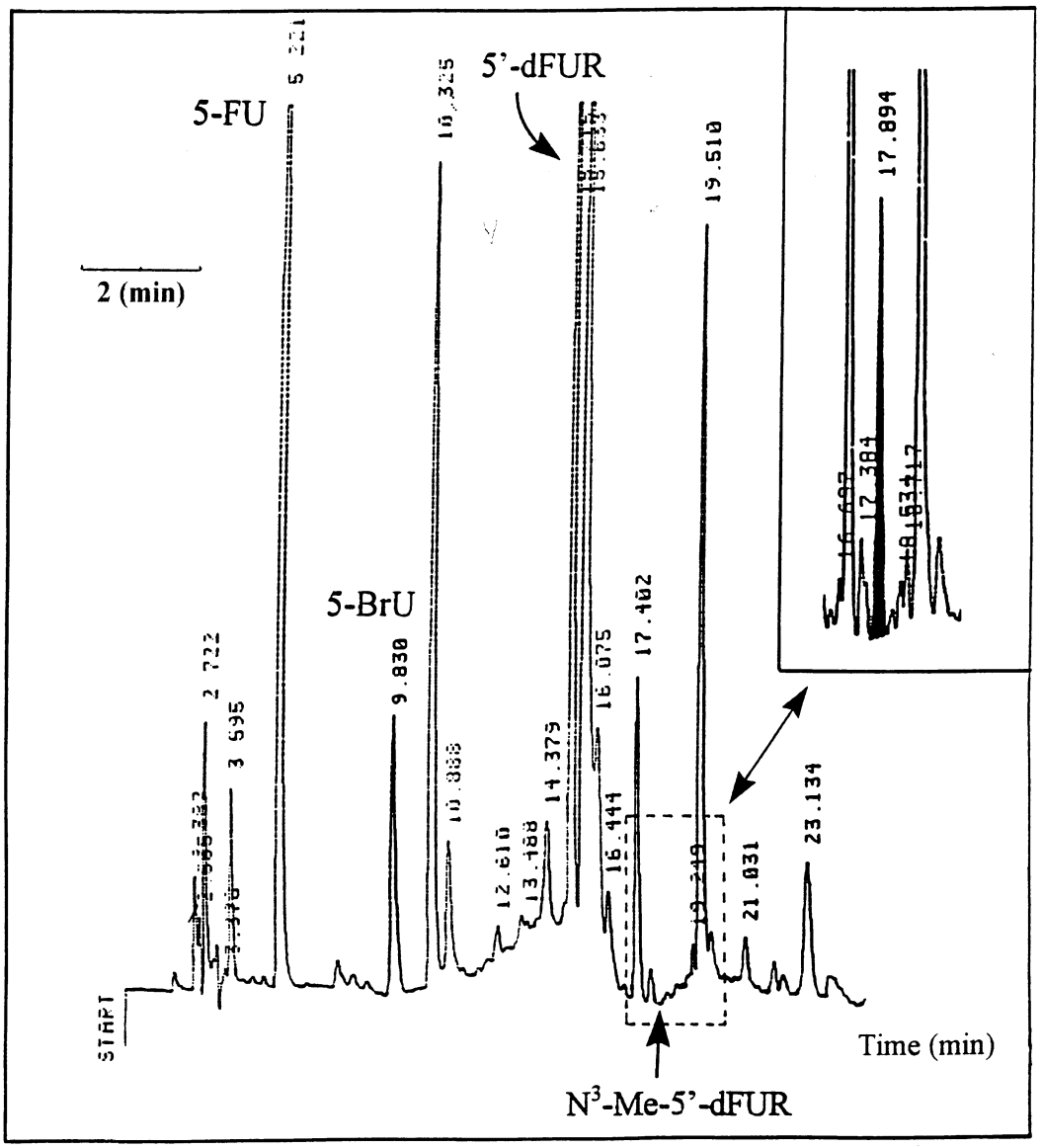

Fig. 3. Chromatogram of the extract of a drug-free serum sample spiked with known amounts of 5-FU, the internal standard 5-BrU, and 5′-dFUR standard. The arrow indicates the zone of retention time of N^3-Me-5′-dFUR. The inset reports a portion of the chromatogram of the same serum extract spiked with N^3-Me-5′-dFUR standard at a known concentration level (16 ng injected). Detector employed: Jasco UV/vis spectrophotometer. Detection wavelength: 269 nm. Absorbance axis: 64 mAU full scale.

269 nm. Spectra were acquired in the 210–400 nm range at the apex and on the ascending or descending part of each peak. Peak purity was checked by the technique of spectra overlaying after normalization.

2.4. Sample collection and pretreatment

Serum sample deproteinization and liquid–liquid extraction (LLE) procedures have already been described [9–12]. Briefly, 250 μl of serum

and 20 μl of the internal standard (5-BrU) solution were mixed in a tapered tube and 500 μl of a saturated ammonium sulphate solution were added, followed by a brief vortex mixing. Then 5 ml of an ethyl acetate–isopropanol (90:10, v/v) mixture were successively added and the resulting mixture was vigorously shaken for 5 min, centrifuged at 4000 rpm for 5 min and the organic phase carefully transferred into a tapered tube. The extract so obtained was evaporated to dryness at room temperature under a gentle stream of nitrogen with a Visiprep vacuum manifold coupled to a Visidry drying attachment (Supelco).

The LLE procedure was also used for urine samples, with an initial volume of 500 μl.

Finally, the extract was simply reconstituted with 100 ml of mobile phase and 20 μl were injected.

2.5. Synthesis of N^3-Me-5′-dFUR

The synthesis of N^3-Me-5′-dFUR was accomplished by refluxing 8 ml of carbonate buffer (pH 10.7, 0.1 M), containing 30 mg of 5′-dFUR with 3 ml of CH_3I for 1 h at 70°C. The reaction time course was followed by HPLC (see Section 3). Semipreparative liquid chromatography can be used to separate the fractions after the synthesis; in this work, the aqueous phase of the reaction mixture was simply extracted with ethyl acetate–isopropanol mixture (3×8 ml). In fact, at the working pH value of 10.7, 5′-dFUR ($pK_a = 5$) is almost completely dissociated, having an acid proton in the N^3 position on the pyrimidine ring. However, no acidic proton in N^3-Me-5′-dFUR exists due to the presence of a methyl group in the same position; as a consequence, the methylated product can be easily extracted by the organic mixture (extraction yield $\approx 75\%$) while 5′-dFUR remains mainly in the aqueous phase.

3. Results and discussion

Fig. 1 shows the chromatogram of the reaction mixture at the end of N^3-Me-5′-dFUR synthesis; as can be seen, together with N^3-Me-5′-dFUR, 5′-dFUR and an unknown reaction by-product are also present. The extimated reaction yield was $\approx 35\%$.

Fig. 2 gives the chromatogram of the final extract, showing that N^3-Me-5′-dFUR was 98% pure, which was suitable for most purposes. The purity of the peak of the reaction product is demonstrated in the inset of Fig. 2, showing the UV spectra acquired at the apex and on the ascending and descending part of the peak due to N^3-Me-5′-dFUR. As can be seen, the relevant UV spectra overlap completely.

The N^3-Me-5′-dFUR response was linear over about two concentration decades. The calibration curve showed a correlation coefficient better than 0.999 and an intercept not significantly different from zero at 95% confidence level. A typical calibration curve for N^3-Me-5′-dFUR gave a slope of 2.95 ± 0.06 mAU ml^{-1} μg^{-1} and an intercept of 0.09 ± 0.16 mAU.

The LLE procedure already developed for 5′-dFUR, 5-FU and other metabolites [11,12] also gave satisfactory results for the extraction of N^3-Me-5′-dFUR from serum and urine with a percentage recovery of ($80 \pm 4\%$) at the 0.5 μg ml^{-1} level. Recovery remained practically unchanged in the range 5–50 μg ml^{-1}.

Fig. 3 reports the chromatogram of a drug-free serum sample spiked with known amounts of a mixture of standard fluoropyrimidine and shows the capability of the gradient programme to resolve the drug 5-FU, the internal standard 5-BrU and the pro-drug 5′-dFUR; furthermore, no significant interference from endogenous components in serum was observed for all the analytes, including N^3-Me-5′-dFUR (arrow in Fig. 3). The inset in Fig. 3 reports a portion of the chromatogram of the same serum sample spiked with N^3-Me-5′-dFUR standard at a known concentration level (16 ng injected).

At a signal-to-noise (S/N) ratio of 3 (noise calculated peak to peak in a blank chromatogram at the elution time of N^3-Me-5′-dFUR), the estimated detection limit in spiked serum for N^3-Me-5′-dFUR was around 50 ng ml^{-1} using a 250 μl sample size, 100 μl reconstitution volume and a 20 μl injection volume.

Quantitation was performed by the internal standard method. A calibration curve in serum

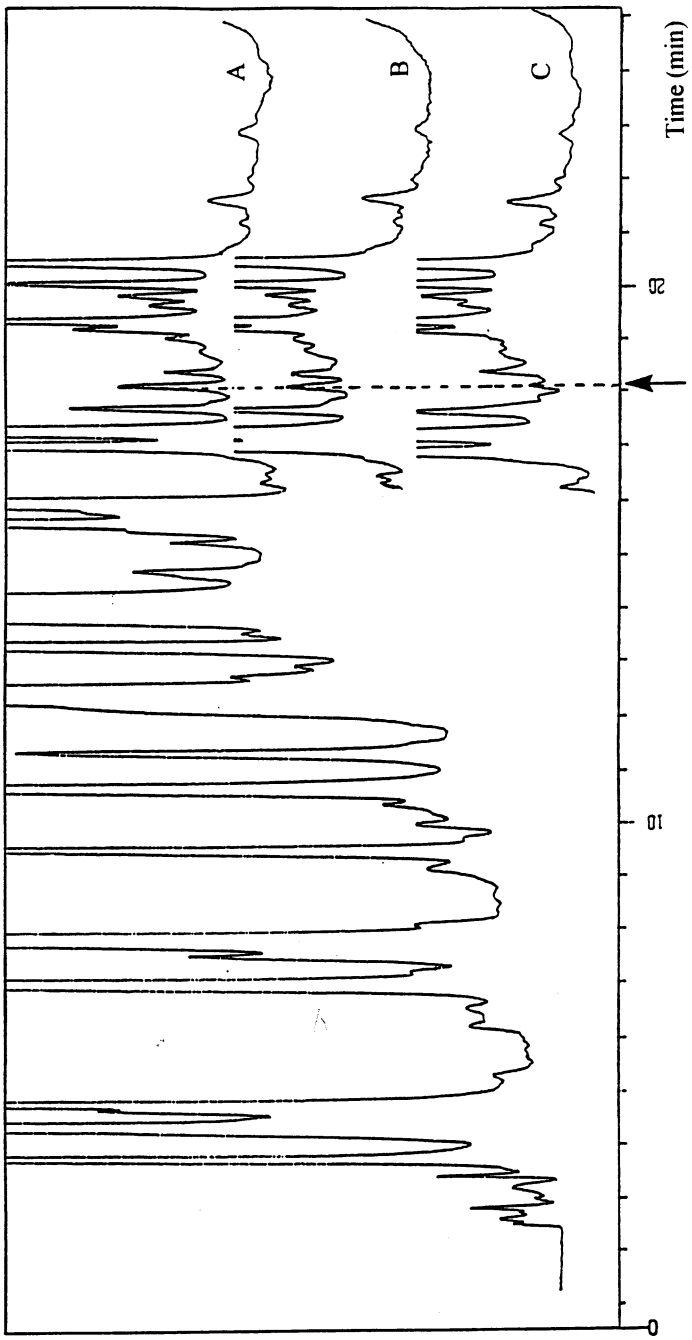

Fig. 4. Chromatograms relevant to a drug-free urine sample (C) and spiked with known amounts (10 and 20 ng injected) of N^3-Me-5′-dFUR (B and A, respectively). Detector employed: HP 1040 diode-array. Detection wavelength: 254 nm. Absorbance axis: 10 mAU full scale.

was obtained using standards prepared by spiking drug-free plasma with 2 μg ml^{-1} of the internal standard (5-BrU) and variable amounts of N^3-Me-5′-dFUR in order to cover the concentration range from 50 ng ml^{-1} to 5 μg ml^{-1}. The curve was linear over the investigated range with a correlation coefficient better than 0.997 and the intercept not significantly different from zero at 95% confidence level.

The applicability of the present method to urine samples is shown in Fig. 4, which shows chromatograms of a drug-free urine sample (Fig. 4C) and urine spiked with known amounts (10 and 20 ng injected) of N^3-Me-5′-dFUR (Fig. 4B, A), respectively. The detection limit obtained in this case was around 100 ng ml^{-1} (S/N = 3) and was mainly dictated by 'chemical noise' originating from matrix complexity. It is worth noting that for the analysis of urine samples the detection wavelength was changed from 269 to 254 nm in order to reduce interferences from some endogenous compounds eluting at a retention time similar to that of N^3-Me-5′-dFUR. Unfortunately, the use of an internal standard is not possible for urine samples due to their great complexity. In this case, an external calibration curve has to be performed.

In conclusion, the method described in this paper is the first one permitting the determination of the new metabolite N^3-Me-5′-dFUR in serum and urine. Furthermore, it is also uniquely capable of the simultaneous determination of N^3-Me-5′-dFUR, 5′-dFUR and 5-FU in serum samples.

Acknowledgements

This work was carried out with financial support from the Associazione Italiana per la Ricerca sul Cancro (AIRC, Milan) and MURST. The authors thank Antonella Aresta for her experimental help.

References

[1] A.F. Cook, M.J. Holman, M.J. Kramer, P.W. Trown, J. Med. Chem. 22 (1979) 1330–1335.
[2] R. Duschinsky, E. Pleven, C. Heidelberger, J. Am. Chem. Soc. 79 (1957) 4559–4560.
[3] C. Heidelberger, N.K. Chandhuri, P. Dannemberg, D. Mooren, L. Griesbach, R. Duschinsky, R.J. Schnitzer, E. Pleven, J. Scheiner, Nature 179 (1957) 663–666.
[4] H. Ishitsuka, M. Miwa, K. Takemoto, Gann 71 (1980) 112–123.
[5] S. Suzuki, Y. Hongu, H. Fukazawa, S. Ichihara, H. Shimizu, Gann 71 (1980) 238–245.
[6] A. Kono, Y. Hora, S. Sugata, Chem. Pharm. Bull. 31 (1980) 112–116.
[7] R.D. Armstrong, R.B. Diasio, Cancer Res. 41 (1981) 4891–4894.
[8] R.B. Diasio, J.D. Schuetz, H. J Wallace, J.P. Sommadossi, Cancer Res. 45 (1985) 4900–4906.
[9] C.G. Zambonin, F. Palmisano, J. Pharm. Biomed. Anal. 14 (1996) 1521–1528.
[10] C.G. Zambonin, A. Aresta, M. Grano, J. Pharm. Biomed. Anal. (in press).
[11] F. Palmisano, F. Berardi, M. De Lena, A. Guerrieri, V. Lorusso, P.G. Zambonin, Chromatographia 33 (1992) 413–417.
[12] A. Guerrieri, F. Palmisano, C.G. Zambonin, M. De Lena, Anal. Chim. Acta 296 (1994) 43–50.