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Gas chromatography–mass spectrometry identification of a novel N^3-methylated metabolite of 5′-deoxy-5-fluorouridine in plasma of cancer patients undergoing chemotherapy

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Abstract

Evidence of in vivo biomethylation of the anticancer pro-drug 5′-deoxy-5-fluorouridine (5′-dFUR) is presented for the first time. Biomethylation seems to occur specifically at the N^3 site on the pyrimidine ring. This novel metabolic product was detected by gas chromatography–mass spectrometry of the trimethylsilylated extract of plasma samples from cancer patients undergoing doxifluridine chemotherapy. Considering the observed electron impact fragmentation pattern, the metabolic product was tentatively identified as N^3-Me-5′-dFUR. Definite confirmation of the proposed structure was achieved by comparison of the mass spectra and chromatographic characteristics of the suspected metabolite with those of a synthetically prepared reference standard.

Keywords: Cancer; Doxifluridine; Drug metabolism; Gas chromatography; Mass spectrometry

1. Introduction

5′-Deoxy-5-fluorouridine (doxifluridine, 5′-dFUR) is a pro-drug of 5-fluorouracil (5-FU) [1,2] synthesized [3,4] in an attempt to improve the therapeutic index of this well-known anticancer agent. By using the deoxyribofuranosyl moiety as a carrier into neoplastic tissue, 5′-dFUR is cleaved [5–8], by an intracellular thymidine phosphorylase, to the active 5-FU. After this step the metabolic scheme of 5′-dFUR (see Fig. 1) is assumed to be strictly similar to that of 5-FU which is anabolised by two possible pathways [4,8–10]. It can be reversibly converted, by uridine phosphorylase, to 2′-deoxy-5-fluorouridine (2′-dFUR, FUDR) and subsequently phosphorylated (by thymidine kinase) to 2′-deoxy-5-fluorouridine-5′-monophosphate (2′-dFUMP), which inhibits DNA synthesis by blocking the thymidilate synthetase system. The second anabolic pathway involves conversion of 5-FU to 5-fluorouridine monophosphate (FUMP) either directly, by a phosphoribosyltransferase, or stepwise via 5-fluorouridine (FUR). FUMP is then phosphorylated to the corresponding diphosphate followed by reduction to 2′-deoxy-5-fluorouridine-

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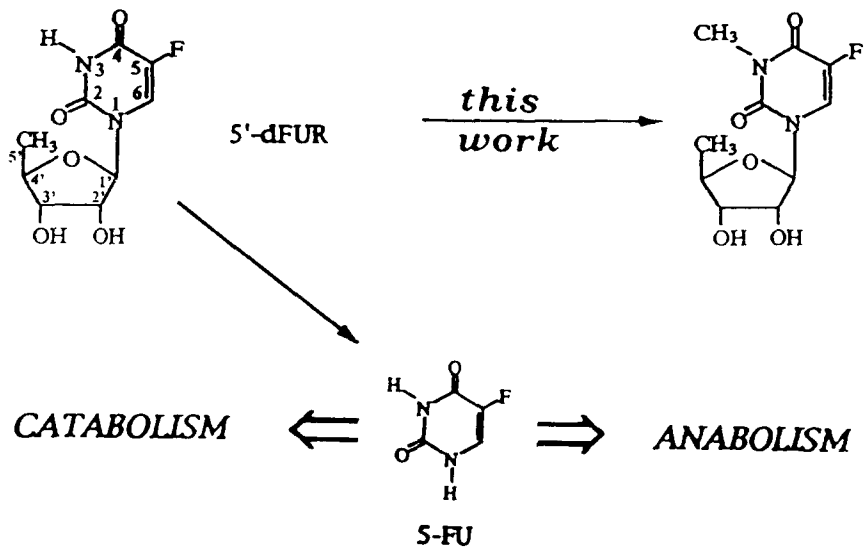


Fig. 1. Schematic representation of the biotransformation routes of 5'-dFUR. Enzymatic cleavage to 5-FU is paralleled by a biomethylation route leading to N^3-methyl-5'-dFUR.

diphosphate (2'-dFUDP) which is finally dephosphorylated to 2'-dFUMP.

The liver seems to be the major site of 5-FU catabolism, the initial step being reductive degradation to 5,6-dihydro-5-fluorouracil (5-FUH_2) by dihydrouracil dehydrogenase (DUD), followed by ring splitting to α-fluoro-β-ureidopropionic acid (FUPA), which is then catabolized to α-fluoro-β-alanine with final release of ammonia, urea and carbon dioxide.

Evidence that the above scheme is not an exhaustive description of 5'-dFUR biotransformation, has been given by Palmisano et al. [11]. Plasma extracts of cancer patients treated with doxifluridine were analysed by HPLC and found to contain a metabolic product of 5'-dFUR with a retention time different from that of the known anabolites mentioned above. This unknown metabolite was systematically absent in blank control plasma samples and was found to reach its peak concentration about 1 h after the end of i.v. infusion of doxifluridine. The only information available at that time was the UV spectrum collected by diode array detection during gradient elution HPLC. The possibility that the unknown product could originate from drug catabolism (e.g. 5-FUH_2) can also be excluded based on more recent chromatographic and spectral evidence [12]. Evidently the "unknown" must be a metabolite never observed or identified before.

The present paper describes the gas chromatography–mass spectrometry (GC–MS) detection of this new metabolite in the trimethylsilylated extract of plasma samples from cancer patients undergoing doxifluridine chemotherapy. Based on the observed electron impact fragmentation pattern, the metabolic product was tentatively identified as N^3-Me-5'-dFUR. Definite confirmation of the proposed structure was achieved by comparison of the mass spectra and chromatographic characteristics of the suspected metabolite with those of a synthetically prepared reference standard.

2. Experimental

2.1. Chemicals

Isopropanol, methanol (Carlo Erba, Milan, Italy), ethyl acetate (Merck, Darmstadt, Germany) and methylene chloride (Labscan, Dublin, Ireland) were HPLC grade. Other chemicals were analytical-grade reagents. The HPLC mobile phase was filtered through a 0.45 μm membrane

(Whatman Limited, Maidstone, UK) before use. 5-FU and 5-bromouracil (5-BrU) were obtained from Sigma Chemical Co. (St. Louis, MO). 5′-dFUR and 5-FUH[2] were kindly provided by Roche SpA (Milan, Italy) and Hoffmann-La Roche AG (Basle, Switzerland) respectively. Derivatization was performed with *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA; one-shot silyl reagent, 0.1 ml ampoule; Supelco, Bellefonte, CA) in silylation-grade pyridine (Pierce, Rockford, IL). Reaction vessels or syringes and the chromatographic column were silanized by Sylon CT and Rejuv-8 silylating agent (Supelco) respectively.

The carrier gas for GC–MS analysis was helium; residual contaminants (water, hydrocarbons, etc.) were removed on-line by a drying tube, a Supelpure-HC trap and a OM-1 indicating purifier trap (all Supelco).

2.2. Apparatus

The HPLC system consisted of a Waters (Milford, MA) 600-MS multisolvent delivery system equipped with a Waters U6K injector with a 1 ml loop and a Partisil 10 ODS-1 M/9-25 (250 mm × 9.5 mm) semipreparative column (Whatman, Clifton, NJ) The detector was an HP 1040A photodiode array spectrophotometer (Hewlett-Packard, Palo Alto, CA), interfaced to an HP 85 computer equipped with an HP 9121 dual disk drive and an HP 7470 plotter.

GC–MS analysis was performed with an HP 5890 series II gas chromatograph equipped with a "cool on-column" injector, and interfaced, by a GC transfer line, to a VG Trio-2000 quadrupole mass spectrometer (VG Biotech, Altrincham, UK).

The chromatographic column consisted of a Supelco fused silica SPB-5 capillary column (30 m length, 0.20 mm i.d. with 0.25 μm film thickness). A deactivated fused silica capillary tube (1 m length, 0.32 mm i.d.), connected to the GC column through a "Glass Seal" connector (Supelco), was used as retention gap.

A home-made, temperature-controlled, aluminum heating block was used for sample silylation.

The proton NMR spectrum of synthetically prepared N^3-Me-5′-dFUR was obtained using a Bruker 300 MHz NMR spectrometer.

2.3. Chromatographic and detection conditions

The HPLC mobile phase was composed of phosphate buffer (pH 6.5; 0.05 M) and methanol (60/40 v/v). The flow rate was 3 ml min^{-1}, the injection volume was 0.5 ml and the temperature was ambient. Spectra were acquired in the 210–400 nm range at the apex and on the ascending or descending part of each peak. Detection was at 269 nm (4 nm bandwidth; reference signal 450 nm, 80 nm bandwidth). The following oven temperature program was used: 130°C (2.5 min) to 250°C at 25°C min^{-1} with 10 min hold time. The GC transfer line was maintained at 250°C. The injection volume was 2 μl. The mass spectrometer was operated in the electron impact positive ion (EI^+) mode. The source temperature was 200°C and the operating pressure was typically 3×10^{-6} mbar (analyzer chamber typically at 2×10^{-8} mbar). The electron energy was 70 eV and the filament current 200 μA. Mass spectra were acquired by scanning over a 50–500 range with a scan time of 1 s and an interscan time of 0.1 s. Unless otherwise specified, MS acquisition was started 4 min after the GC injection. Chemical ionization (CI^+) mass spectra were collected using methane as reagent gas at a pressure of $\approx 10^{-4}$ mbar. The source temperature was 100°C and the electron energy was 30 eV.

2.4. Sample collection and sample pretreatment

Collection and storage of plasma samples has already been described [11,12]. The sample pretreatment procedure (deproteinization/extraction) was the same as that described in Refs. [11,12]. Briefly, 250 μl of plasma and 20 μl of 5-bromouracil (5-BrU) solution (the internal standard), were transferred to a tapered tube, and 0.5 ml of a saturated ammonium sulfate solution was added. After a brief vortex mixing, 5 ml of ethyl acetate–isopropanol mixture (90/10, v/v) was added, followed by 5 min vigorous shaking. The resulting mixture was then centrifuged for 5 min

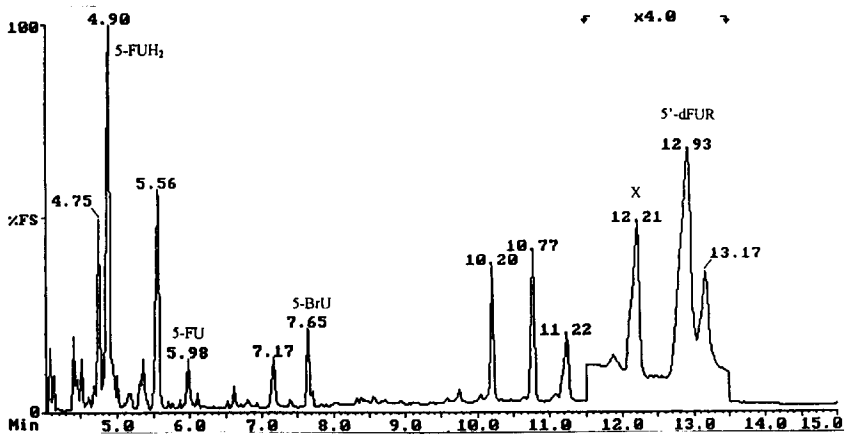


Fig. 2. Total ion current GC–MS chromatogram of the TMS derivative of a plasma extract from a patient undergoing doxifluridine treatment (4 g m^{-2} i.v. over 60 min period). Sampling time: 120 min from the start of infusion. The time window from 11.5 to 13.5 min has been amplified by a factor of four. Injected (on-column) volume: 2 μl. MS acquisition started 4 min after the GC injection. Peak legend as specified in the text. Peak denoted by X (retention time 12.21 min) refers to a suspected unknown 5′-dFUR metabolite.

at 4000 rev min^{-1} and the organic phase was carefully transferred to a microreaction vial. The extract thus 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).

Derivatization for GC–MS analysis was performed as follows. To the dry sample (obtained by the above procedure) 0.5 ml of methylene chloride was added and evaporated, under a nitrogen stream, to azeotropically remove residual traces of water (the procedure was repeated twice). Dry extracts thus obtained (or dry analyte standards) were combined with 100 μl of pyridine and 100 μl of BSTFA and silylated at 150°C for 15 min. After cooling at room temperature the reaction mixture was ready for injection.

2.5. Synthesis and characterization 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. The reaction yield, also estimated by HPLC, was ≈35%. Semipreparative liquid chromatography was used to separate milligram amounts of N^3-Me-5′-dFUR from unreacted 5′-dFUR and reaction by-products. LC fractions containing the compound of interest (purity ascertained by the technique of UV spectra overlaying after normalization) were combined and transferred to a rotary evaporator to eliminate most of the methanol and reduce the volume to ≈10 ml; N^3-Me-5′-dFUR was extracted with ethyl acetate–isopropanol (90/10 v/v) mixture. Alternatively the aqueous phase of the reaction mixture could be simply extracted with the ethyl acetate–isopropanol mixture (3 × 8 ml). In this case the compound of interest (extraction yield ≈75%) was 98% pure (as ascertained by HPLC) which is suitable for most purposes; contaminants are represented by 5′-dFUR and an unknown compound. The extract was evaporated to dryness and the residue was silanized for GC–MS analysis and/or redissolved in D_2O for collection of the ^1H NMR spectrum.

Chemical shifts, δ, coupling constants, J, and proton assignments relevant to the NMR spectrum of the synthetic product, are summarized as follows: δ 7.86 (d, $J = 5.9$ Hz, CHCF); δ 5.87 (dd, $J = 1.0$ and 3.4 Hz, C_1·H); δ 4.33 (dd, $J = 3.6$ and 5.3 Hz, C_2·H); δ 4.18 (quintet, $J = 6.5$ Hz, C_4·H); δ 3.93 (dd, $J = 5.5$ and 6.4 Hz, C_3·H); δ 3.33 (s, CH_3N); δ 1.44 (d, $J = 6.5$ Hz, CH_3CH). These data are clearly consistent with the expected structure of N^3-methylated 5′-dFUR.

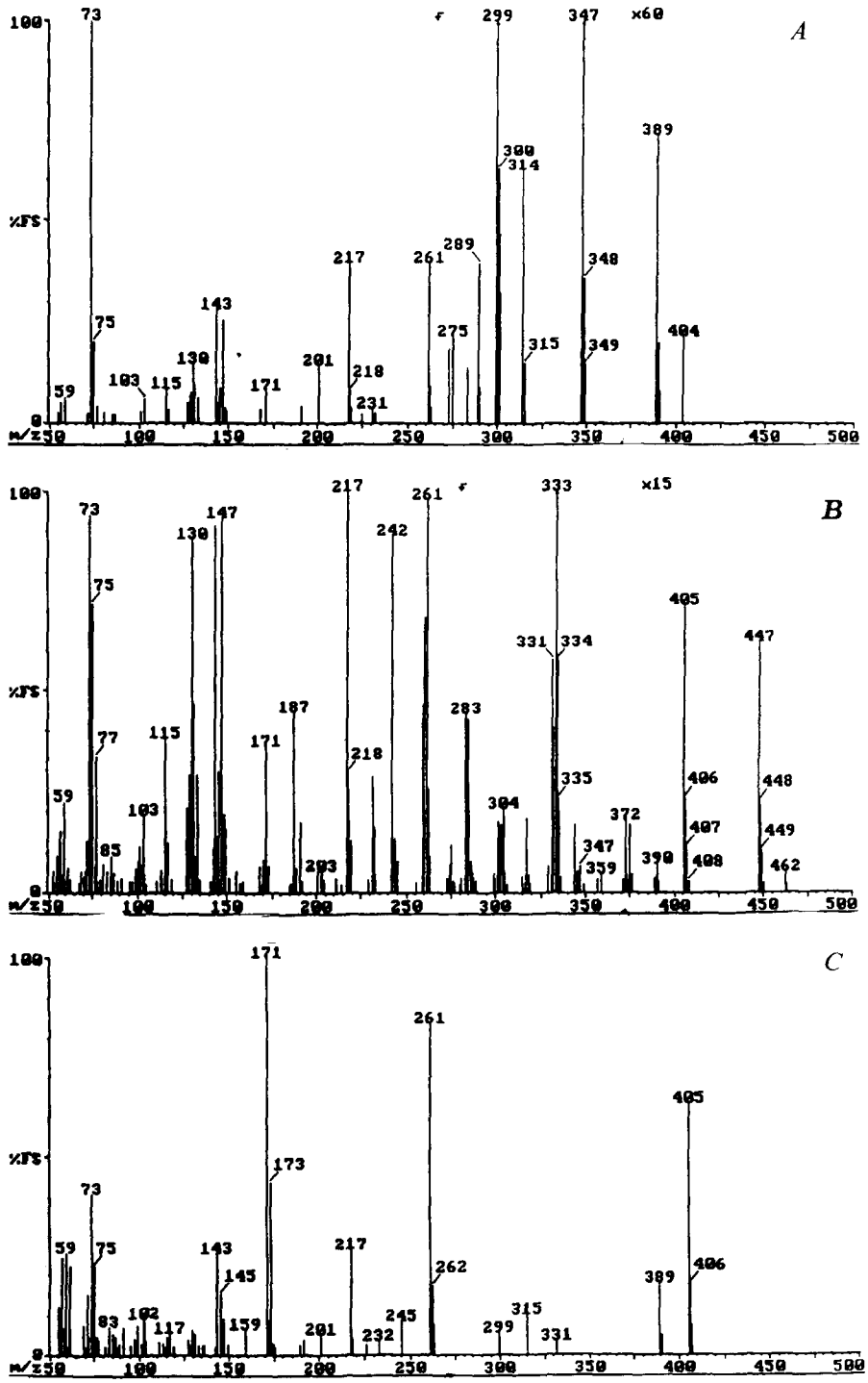


Fig. 3. (A), (B) EI mass spectra of the TMS derivative of the suspected metabolite and 5′-dFUR respectively. The portion from m/z 270–500 in (A) has been amplified by a factor of 60. The portion from m/z 280–500 in (B) has been amplified by a factor of 15. (C) Methane CI mass spectrum of the TMS derivative of the suspected metabolite.

The UV spectrum of N^3-Me-5′-dFUR, collected by diode array detection during LC separation, showed an absorption maximum at 269 ± 2 nm.

3. Results and discussion

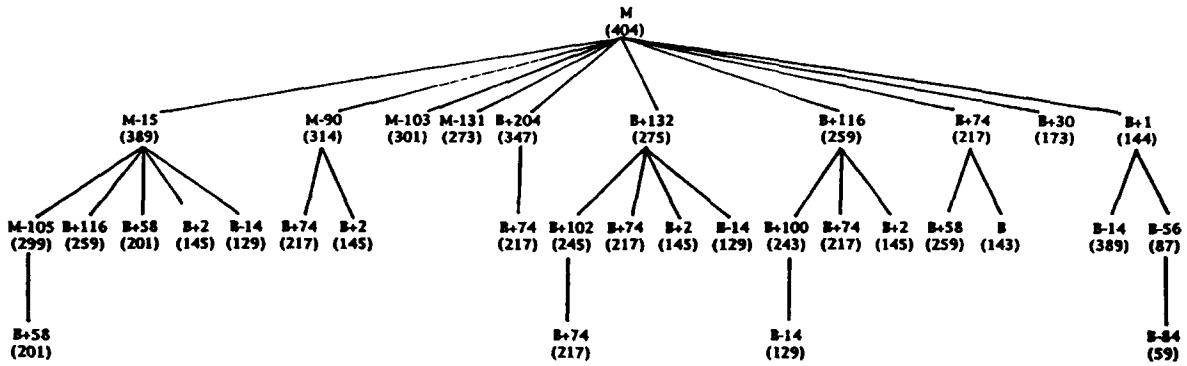
A typical GC–MS chromatogram relevant to the silylated extract of a plasma sample taken from a patient undergoing 5′-dFUR chemotherapy (4 g m^{-2} by i.v. infusion over a 60 min period) is shown in Fig. 2. As can be seen the described extraction/derivatization procedure is the first one permitting a simultaneous GC–MS determination of 5′-dFUR, 5-FU, and 5-FUH_2 (5-BrU as internal standard). The absence of interferences arising from endogenous compounds was ascertained by comparison with a control plasma. The usefulness of such a finding and its analytical use are outside the scope of this paper and will be presented and discussed elsewhere. In the present context the most relevant finding, inferred from a careful inspection of the MS spectra recovered from the reconstructed total ion chromatogram of Fig. 2, is the presence of a peak (denoted by X) at a retention time (RT) of 12.21 min, possessing an EI fragmentation pattern (see later) typical of a TMS derivative of a pyrimidine nucleoside. Considering the highest mass present in the relevant MS spectrum, the possibility that the peak could originate from one of the known anabolites of 5-FU was excluded. Moreover, this peak was absent in a drug-free plasma sample and its height was dependent on the time of collection of plasma samples after drug infusion. This finding strongly suggests that the GC peak identified by a RT of 12.21 min could originate from an unknown metabolite.

Table 1
Composition of some observed fragments in the EI^+ mass spectra of the trimethylsilyl derivative of N^3-Me-5′-dFUR

Composition	Ion	Mass	RA (%)
Ions related to the molecular ion M			
M	M	404	≤1
$M-CH_3$	M−15	389	1.2
$M-C_3H_{10}OSi$	M−90	314	≤1
$M-C_4H_{11}OSi$	M−103	301	≤1
$M-C_4H_{13}OSi$	M−105	299	1.3
$M-C_5H_{11}O_2Si$	M−131	273	≤1
Ions containing the base B plus portions of the sugar			
$B+C_8H_{20}O_2Si_2$	B+204	347	2.4
$B+C_5H_{12}O_2Si$	B+132	275	≤1
$B+C_5H_{12}OSi$	B+116	259	≤1
$B+C_4H_{10}OSi$	B+102	245	1.6
$B+C_4H_8OSi$	B+100	243	≤1
$B+C_3H_{10}Si$	B+74	217	40.2
$B+C_2H_6Si$	B+58	201	14.4
$B+CH_2O$	B+30	173	1.4
B+CH	B+13	156	≤1
B+2H	B+2	145	8.9
B+H	B+1	144	4.9
B	B	143	28.7
$B-CH_2$	B−14	129	7.5
$B+H-C_2H_3NO$	B−56	87	2.1
$B+H-C_3H_3NO_2$	B−84	59	6.3
Ions related to sugar S			
S	S	261	40.6
S−H	S−1	260	1.5
$S-C_3H_{10}OSi$	S−90	171	8.3
$S-CH_4-C_3H_{10}OSi$	S−106	155	1.2
$C_3H_3O_2(C_3H_9Si)_2$	217	217	40.2
$S-2(C_3H_{10}OSi)$	S−180	81	2.3
$C_3H_9OSiCH_2$	103	103	5.9

The 70 eV EI mass spectra of the TMS derivatives of the unknown compound and 5′-dFUR are shown in Figs. 3A and 3B respectively. The mass fragmentation patterns of nucleosides and their TMS derivatives have been studied extensively [13–15] and have been shown to possess considerable structural information. The major routes of fragmentation of nucleosides produce three groups of structurally informative ions: molecular ion (M)-related fragments, sugar (S)-related ions and those fragments (base ion series) containing some portion of the sugar attached to the intact base (B). Table 1 shows the main fragments observed in the mass spectrum of the new metabolite and Scheme 1 reports a plausible fragmentation pattern for the main observable ions.

The simultaneous presence of fragment ions at m/z 404, m/z 389 (loss of a $-CH_3$ group), m/z 314 (loss of a $-C_3H_{10}OSi$ group) and m/z 273 (loss of a $-C_5H_{11}O_2Si$ group) permits the identification of molecular ion, M^+, as the fragment at m/z 404. This molecular ion assignment is strongly reinforced by the methane CI mass spectra (see Fig. 3C) showing an intense (even if not dominant)



Scheme 1. Fragmentation pattern for the main observable ions in the EI^+ mass spectra of the trimethylsilyl derivative of N^3-Me-5′-dFUR.

$[M+H]^+$ ion at m/z 405 (the use of a milder reagent gas would probably give rise to a more intense quasi-molecular ion and reduced fragmentation). The occurrence of sugar-related fragment ions at m/z 261, 260, 171, 155, 217, 81, and 103 (see Table 1 for their assignments), which are also present in the EI^+ MS spectrum of the TMS derivative of 5′-dFUR, strongly suggests the presence of an unmodified deoxyribose moiety. Subtraction of the mass of the derivatized deoxyribose (261 Da) (deoxyribose + two TMS groups) from the mass of the molecular ion gives an aglycone mass of 143 Da which can be reasonably assigned to methylated 5-FU. Further evidence that the mass of the aglycone is 143 Da is provided by the presence of other base $[B]^+$-related fragment ions such as those at m/z 144 $[B+H]^+$, 145 $[B+2H]^+$, 201 $[B+58]^+$, 217 $[B+74]^+$, 275 $[B+132]^+$ and 347 $[B+204]^+$ (see also Table 1). It is worthy of note that in the MS spectrum of 5′-dFUR the $[B]^+$, $[B+H]^+$, and $[B+2H]^+$ fragments (m/z 201, 202, 203) are all below 5% relative abundance, compared to 30, 10 and 25% for m/z 143, 144 and 145 respectively. The higher abundance observed (particularly for the $[B]^+$ ion) in the case of the unknown metabolite, once more reinforces the idea of a base methylated derivative. In the intact 5′-dFUR nucleoside, the $[B]^+$, $[B+H]^+$ and $[B+2H]^+$ group of ions is

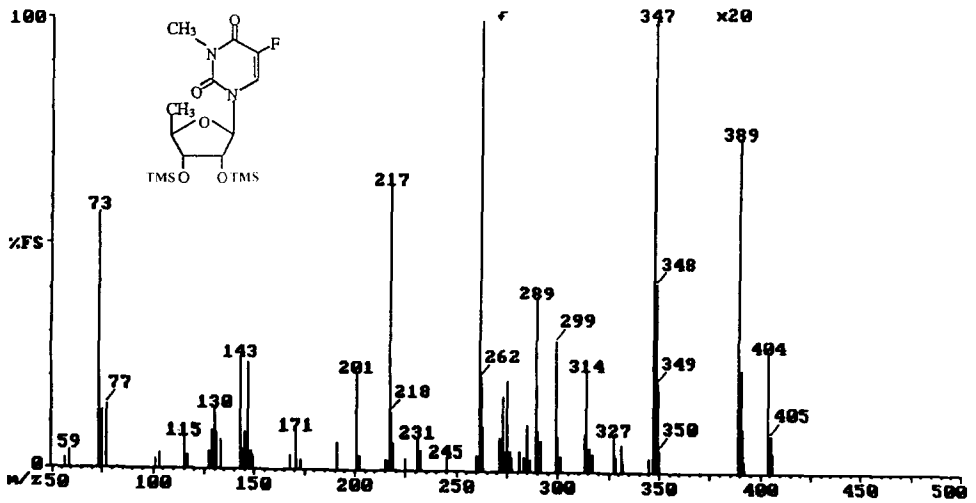


Fig. 4. EI mass spectrum of the TMS derivative of a synthetic standard of N^3-Me-5′-dFUR.

of very low abundance which means that once $[B]^+$ is formed (cleavage of the glycoside bond) it rapidly converts to other fragments (see Scheme 1). The presence of a methyl group on the base moiety produces an inductive stabilization of the positive charge which increases the abundance of the $[B]^+$ ion concomitantly with $[B+H]^+$ and $[B+2H]^+$ ions (proton abstraction from the sugar to the base). The fragmentation pattern and relative abundance of other base-related ions change accordingly (see e.g. $[B+74]^+$, $[B-14]^+$, $[B+41]^+$).

There are three possible methylation sites on the pyrimidine ring: O^2, O^4 and N^3; considering that O-methylation is a rare event and that $-OCH_3$-related fragments are absent in the mass spectrum, it must be concluded that the GC peak at a RT of 12.21 min should be assigned to the di-TMS derivative of N^3-Me-5′-dFUR (see Fig. 1).

For a definitive structure assignment, a standard of N^3-Me-5′-dFUR was synthesized and characterized as described in Section 2. The EI mass spectrum of the synthetically prepared standard (see Fig. 4) exhibited a fragmentation pattern and ion abundance essentially the same as the plasma component. Furthermore, GC retention times for the unknown and the standard are, within experimental error, essentially the same. This last finding rules out the possibility of epimeric sugar hydroxyl groups or the presence of anomers (in this case a difference in retention time would have been observed).

Unfortunately, an NMR spectrum of the circulating metabolite, which would be the definitive evidence, could not be obtained due to the limited amount of available plasma, preventing the isolation of a sufficient quantity of N^3-Me-5′-dFUR. Despite this, all the above findings strongly support the conclusion that the unknown metabolite is the product of a bio-methylation process of 5′-dFUR, probably competing with pro-drug cleavage to 5-FU, catalyzed by an intracellular thymidine phosphorylase. The mechanism by which methylation occurs in vivo, as well as the cytotoxicity of the new metabolite are, at the moment, unknown but appear worthy of further investigation. Work in this direction is in progress in this laboratory.

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References

[1] R. Duschinnsky, E. Pleven and C. Heidelberger, J. Am. Chem. Soc., 70 (1957) 4559–4560.
[2] C. Heidelberger, N.K. Chandhuri, P. Dannenberg, D. Mooren, L. Griesbach, R. Duschinscky, R.J. Schnitzer, E. Pleven and J. Scheiner, Nature, 179 (1957) 663–666.
[3] A.F. Cook, M.J. Holman, M.J. Kramer and P.V. Trown, J. Med. Chem., 22 (1979) 1330–1335.
[4] R.D. Armstrong, J. Gesmonde, T. Wu and E. Cadman, Cancer Treat. Rep., 67 (1983) 541–545.
[5] H. Ishitsuka, M. Miwa and K. Takemoto, Gann, 71 (1980) 112–123.
[6] S. Suzuki, Y. Hongu, H. Fukazawa, S. Ichihara and H. Shimizu, Gann, 71 (1980) 238–245.
[7] A. Kono, Y. Hara and S. Sugata, Chem. Pharm. Bull., 31 (1980) 112–116.
[8] R.D. Armstrong and R.B. Diasio, Cancer Res., 41 (1981) 4891–4894.
[9] R.D. Armstrong and R.B. Diasio, Cancer Res., 40 (1980) 3333–3338.
[10] R.D. Armstrong, K.M. Connolly, A.M. Kaplan and E. Cadman, Cancer Chemother. Pharmacol., 11 (1983) 102–105.
[11] F. Palmisano, F. Berardi, M. DeLena, A. Guerrieri, V. Lorusso and P.G. Zambonin, Chromatographia, 33 (1992) 413–417.
[12] A. Guerrieri, F. Palmisano, C.G. Zambonin and M. DeLena, Anal. Chim. Acta, 296 (1994) 43–50.
[13] H. Pang, K.H. Schram, D.L. Smith, S.P. Gupta, L.B. Townsend and J.A. McCloskey, J. Org. Chem., 47 (1982) 3923–3932.
[14] J.M. Rice and G.O. Dudek, J. Am. Chem. Soc., 89 (1967) 2719–2725.
[15] J.M. Rice, G.O. Dudek and M. Barber, J. Am. Chem. Soc., 86 (1964) 1444–1449.