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Simultaneous determination of 5′-deoxy-5-fluorouridine, 5-fluorouracil and 5,6-dihydro-5-fluorouracil in plasma by gas chromatography–mass spectrometry

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Abstract

A gas chromatography–mass spectrometry (GC–MS) method for the simultaneous determination of 5′-deoxy-5-fluorouridine (doxifluridine, 5′-dFUR), 5-fluorouracil (5-FU) and its main catabolite 5,6-dihydro-5-fluorouracil (5-FUH_2) in human plasma has been developed. Sample preparation consisted of protein precipitation with ammonium sulphate followed by analyte extraction with ethyl acetate/isopropanol (90/10, v/v) mixture. Extracts were then analysed by GC–MS in positive electron impact mode after derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide/pyridine (1/1, v/v). The trimethylsilyl (TMS) derivative of 5′-dFUR was proved to be stable so that no interference in 5-FU determination was observed. Mass spectra of TMS derivatives of 5′-dFUR, 5-FU and 5-FUH_2 are discussed. Analyte response was linear over two decades of concentration and detection limits were typically 20 ng/ml of plasma.

Keywords: Gas chromatography–mass spectrometry; 5-Fluorouracil; Doxifluridine; Fluoropyrimidine drugs

1. Introduction

Fluorinated pyrimidines and related nucleosides have a significant anti-cancer activity [1,2]. Among these compounds, 5-fluorouracil (5-FU) is one of the most active anti-cancer drugs [3,4], clinically useful against tumours of gastrointestinal, breast and female genital tract origin. Unfortunately, 5-FU also causes undesired toxic effects [1,2] so that fluorinated pyrimidine analogues, such as 5′-deoxy-5-fluorouridine (doxifluridine, 5′-dFUR) [5], have been tested as 5-FU pro-drugs to improve its therapeutic index [6]. Using the deoxy-ribofuranosyl moiety as a carrier into neoplastic tissue, 5′-dFUR is cleaved into the active 5-FU preferentially [7–10] in tumour cells under the action of intracellular pyrimidine nucleoside phosphorylase.

The anti-cancer activity of 5-FU (and hence of 5′-dFUR) is mainly due to the ability of its anabolites [6,10–12] to block DNA synthesis causing "thymineless death" of neoplastic cells; however, the role of catabolic pathways on 5-FU cytotoxicity also seem to be important. 5-FU catabolism occurs mainly in

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liver where it is degraded [13–17] by dihydrouracil dehydrogenase to 5,6-dihydro-5-fluorouracil (5-FUH_2) which then undergoes further biotransformation with the final release of ammonia, urea and carbon dioxide. 5-FUH_2 has shown a significant thymidilate synthetase inhibition in *Ehrlich ascites* tumour cells [18] suggesting that it may also contribute to 5-FU toxicity, possibly by being anabolized to 5-FU.

Although a large number of analytical procedures have been developed for fluoropyrimidine analysis in biological matrices (see [19–22] for a review and a critical discussion), reversed phase-liquid chromatography (RP-LC) remains the most widely applied technique. In particular, some RP-LC procedures capable of a simultaneous determination of 5′-dFUR, 5-FU, 5-FUH_2 and some other anabolites have been developed [20–22] in our laboratory.

Gas chromatography coupled to mass spectrometry (GC–MS) is a very powerful analytical technique, combining the resolving power of gas chromatography with the ability of mass spectrometry, to identify the separated compounds. A large number of GC–MS methods have been reported for the determination of 5-FU [23–36] in several biological matrices, mainly as alkyl [23–29] or trialkylsilyl [30–33] derivatives; N-perfluoroaryl derivatives of 5-FU have also been used in GC-(negative ion chemical ionization (NICI))MS [34–36] to improve sensitivity. Pantarotto et al. [23] and Sato et al. [37] described GC–MS procedures for the determination of 2′-deoxy-5-fluorouridine (FUDR), a 5-FU related metabolite; however, the possibility of a 5-FU codetermination was not mentioned. Aubert et al. [27] as well as Odagiri et al. [29] developed GC–MS methods for the determination of 5-FU and for the simultaneous determination of 5-FU and 5-FUH_2.

The development of GC–MS methods for fluoropyrimidine nucleoside determination is hampered by the decomposition of these compounds to 5-FU during the derivatization step and/or the chromatographic separation. For example, Williams et al. [38] reported that FUDR was partially decomposed to 5-FU in the GC column, requiring a correction of 5-FU concentrations found in the presence of FUDR; Min and Garland [24] and Odagiri et al. [29] reported 5′-dFUR conversion to dimethyl 5-FU during derivatization with diazomethane. Because of the greater stability [39] of bulky alkylsilyl derivatives, N-methyl-N-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) has been recently proposed [33] as a derivatizing agent for 5-FU in the presence of FUDR; however, the possibility of a FUDR codetermination was not reported. Hence, a simultaneous GC–MS determination of fluoropyrimidine derivatives has yet to be achieved.

This work describes the first GC–MS method permitting simultaneous determinations of 5′-dFUR, 5-FU and 5-FUH_2. Since silylation of bases, nucleosides and nucleotides usually provide thermally volatile derivatives whose electron-impact (EI) mass spectra are useful for structural characterization [40], analyte derivatization was accomplished with N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA). Mass spectra of trimethylsilyl (TMS) derivatives of 5′-dFUR, 5-FU and 5-FUH_2 are discussed. The method proved useful for the determination of 5′-dFUR, 5-FU and 5-FUH_2 in plasma from patients receiving doxifluridine chemotherapy, requiring only a simple liquid–liquid extraction (LLE) step before analyte derivatization.

2. Experimental

2.1. Chemicals

The fluoropyrimidine source has been described elsewhere [20–22]. Stock solutions were prepared in tridistilled water and stored at 4°C in the dark. More dilute solutions were prepared just before use. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, One-Shot Silyl Reagent, 0.1 ml/ampoule) was supplied by Supelco (Bellefonte, PA, USA). Other derivatizing reagents, i.e. N,O-bis(trimethylsilyl)acetamide (BSA), N-methyl-N-(*tert*-butyl dimethylsilyl)trifluoroacetamide (MTBSTFA) and 1-(*tert*-butyl dimethylsilyl)imidazole (TBDMSI) were purchased from Aldrich (Milan, Italy). Pyridine and acetonitrile, both silylation grade, were obtained by Pierce (Rockford, IL, USA). Reaction vessels, syringes and chromatographic columns were silanized by Sylon CT and Rejuv-8 Silylating Agent (Supelco), respectively. Other chemicals were of analytical reagent grade.

2.2. Apparatus

A HP 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a HP 5890 GC split/splitless injector and a HP 5890 GC cool on-column inlet was interfaced by a direct GC transfer line to a VG Trio-2000 quadruple mass spectrometer (VG BIOTECH, Altrincham, UK). The carrier gas was helium; contaminants were removed by using a Drying tube, a Supelpure-HC Trap and an OMI-1 Indicating Purifier (all from Supelco) in series. Chromatographic separations were performed either by a J&W DB5 fused silica capillary column (15 m, 0.25 mm i.d., 0.25 μm film thickness, Folson, CA, USA) connected to the split/splitless injector or a SPB-5 fused silica capillary column (30 m, 0.20 mm i.d., 0.20 μm film thickness, Supelco) connected to the on-column inlet through a 1 m fused silica deactivated capillary tubing (0.53 mm i.d., Supelco) used as a retention gap.

2.3. Chromatographic and detection conditions

In the splitless mode (J&W DB5 column, 15 m), a column head pressure of 5 psi, a flow rate of 30 ml/min, an injection temperature of 250°C and an injection volume of 2 μl were used. The temperature program was 70°C (2 min) to 250°C at 20°C/min and then hold for 10 min. In the "on-column" injection mode (SPB-5 column, 30 m), the column head pressure was 15 psi and the injection volume was 2 μl. The oven temperature program was 130°C (2.5 min) to 250°C at 20°C/min and then hold for 15 min. In both cases, MS acquisition was delayed by 3 min from the GC injection and the transfer line temperature was 250°C.

The mass spectrometer was operated in positive EI (EI+) ionization mode (70 eV) with a source temperature of 200°C. Source and analyser pressures were typically 3×10^{-6} and 2×10^{-8} mbar, respectively. Mass spectra were acquired in scan mode by using start and end mass values of 20 and 500 m/z, respectively, a scan time of 1 s and an interscan time of 0.1 s. Detection of analytes was accomplished by selected ion monitoring (SIM) mode using the fragment ions at 447, 405, 333, 275, 261, 242 and 187 m/z for 5′-dFUR, 274, 273 and 259 m/z for 5-FU, 276, 275 and 261 m/z for 5-FUH_2 and 319 m/z for 5-BrU; the dwell time and the mass span were 0.08 s and 0.6 amu respectively, for each fragment.

2.4. Sample extraction procedure

To prevent 5-FUH_2 decomposition, plasma specimens were stored and processed according to the method of Van Den Bosch et al. [41]. In particular, plasma samples were immediately frozen after collection. Before analysis, plasma samples were thawed at 4°C and extraction was performed on ice. Once thawed, samples were not re-used for further analysis. The deproteinization and LLE procedures previously described [20,22] were used for analyte extraction. Briefly, 20 μl of an internal standard (5-BrU) solution and 500 μl of a saturated ammonium sulphate solution were added to 250 μl of plasma followed by a brief vortex mixing. 5 ml of an ethyl acetate–isopropanol mixture (90:10, v/v) were then added, the resulting mixture vigorously shaken for 5 min, centrifuged at 4000 rpm for 5 min and the organic phase carefully transferred into a microreaction vessel. Since 5-FUH_2 is stable in ethyl acetate [41], the extract 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) that permitted simultaneous processing of up to 12 extracts.

2.5. Derivatization procedure

Dichloromethane (0.5 ml) was added to the dry samples in the microreaction vessel and evaporated as described above to remove azeotropically any residual trace of water. This procedure was repeated twice. Dry extracts and dry analyte standards were silylated with 100 μl of pyridine and 100 μl of BSTFA in a microreaction vessel tightly sealed with a Teflon-faced rubber septum and cap (Supelco) at 150°C for about 15 min. After cooling at room temperature, samples were ready for injection.

2.6. Quantitation

Quantitation was performed by the internal standard method. Calibration curves were obtained using standards prepared by spiking drug-free plasma with 1 mg/ml of 5-bromouracil (5-BrU, the internal

standard) and variable amounts of 5′-dFUR, 5-FU and 5-FUH_2 in order to cover the desired concentration ranges.

3. Results and discussion

Preliminary experiments indicated that BSA and TBDMSI were both ineffective for the simultaneous derivatization of 5′-dFUR, 5-FU and 5-FUH_2, even in the presence of trimethylchlorosilane, a well-known catalyst for silyl derivatization. MTBSTFA was successful for 5-FU, 5-FUH_2 and 5-BrU (the internal standard) derivatization but failed with 5′-dFUR (very long reaction times, detrimental for pyrimidine derivatization, were required for an appreciable conversion). On the contrary, 5′-dFUR, 5-FU, 5-FUH_2 and 5-BrU were converted to their respective TMS derivatives in about 15 min at 150°C when BSTFA was employed for their derivatization. Chromatographic and mass-spectroscopic evidence (vide infra) indicated the formation of the di-TMS derivative for 5-FU and 5-FUH_2, and of the tri-TMS derivative for 5′-dFUR. The derivatives proved stable for many hours at room temperature and for more than 5 days if stored below 0°C in the dark. More importantly no thermal decomposition of 5′-dFUR to 5-FU was observed, despite the relatively high temperatures required during derivatization and chromatographic steps.

Fig. 1 shows a typical total ion current (TIC) chromatogram relevant to a mixture of TMS derivatives of 5′-dFUR, 5-FU, 5-FUH_2 and 5-BrU standards: well-defined and resolved peaks were observed for each analyte. Moreover, trimethylsilylation gave fluoropyrimidine derivatives with sufficient volatility permitting complete elution of all analytes in a few minutes.

Fig. 2 shows the EI+ mass spectra of TMS derivatives of 5-FU, 5-FUH_2 and 5-BrU; m/z values and the relative abundances of the main fragment ions are listed in Table 1. In all the three cases mass spectra were relatively simple, reflecting the resistance of the aromatic ring towards fragmentation [42]. Molecular ion (M) m/z values and fragmentation patterns were consistent with the formation of di-TMS derivatives for all the three analytes. Mass spectra consisted mainly of M and intense fragment ion peaks at m/z values of M-15, corresponding to the loss of a methyl group from the molecular ion. Further common fragments were those derived from the expulsion of halogen from the molecular ion.

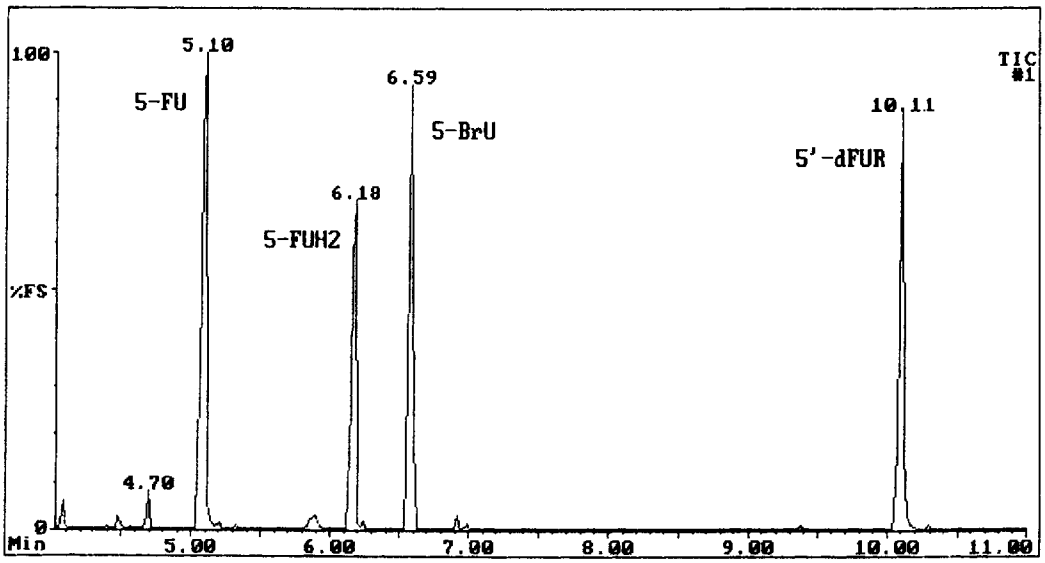


Fig. 1. Total ion current (TIC) chromatogram relevant to a mixture of di-TMS derivatives of 5′-dFUR, 5-FU, 5-FUH_2 and 5-BrU standards. Amounts injected, 125 ng each; Column length, 15 m; Splitless injection; Other conditions as in Section 2.

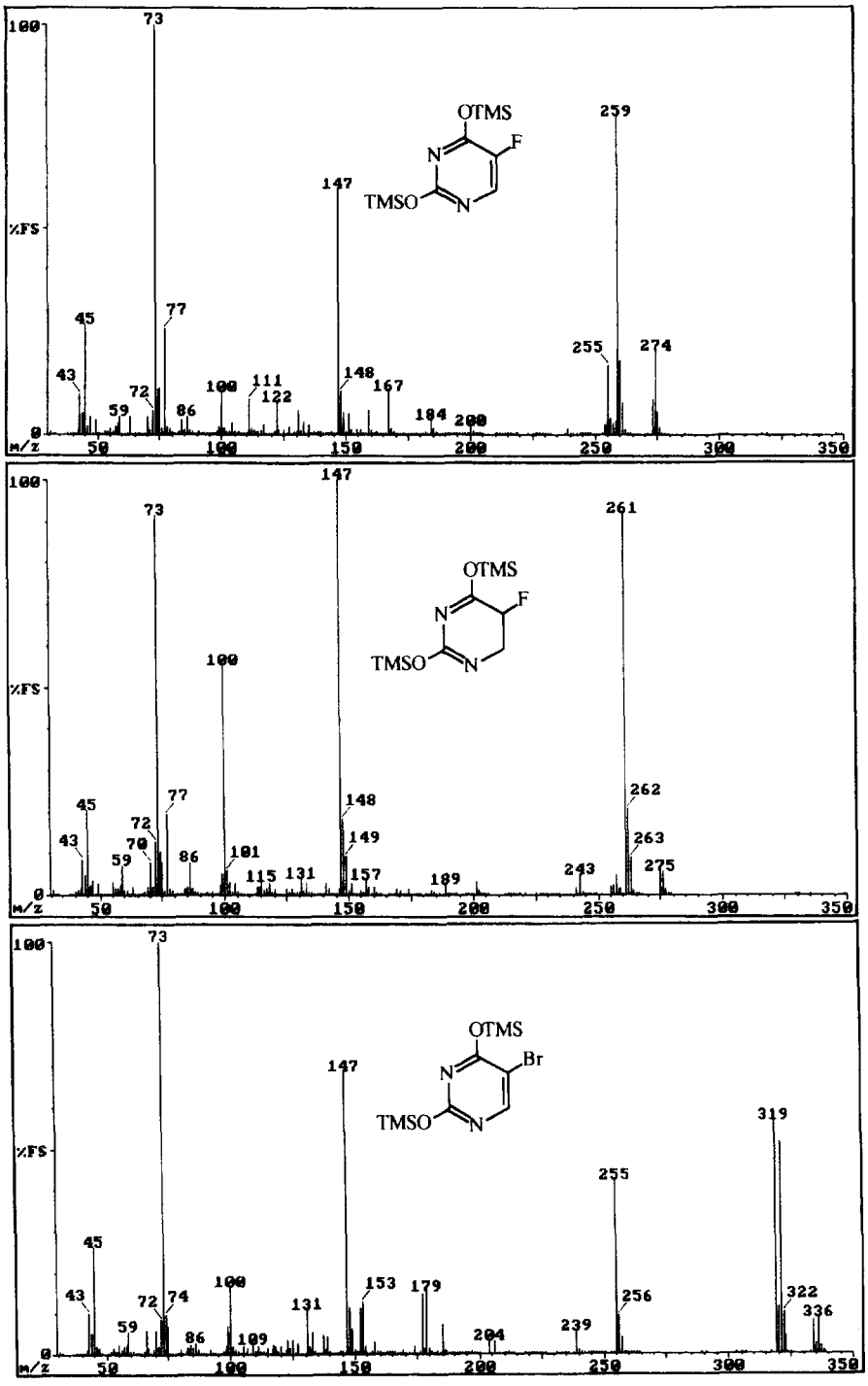


Fig. 2. EI+ mass spectra relevant to di-TMS derivatives of 5-FU (a) 5-FUH_2 (b) and 5-BrU (c). Column length, 15 m; Splitless injection; Other conditions as in Section 2.

Table 1
Main ions observed in the EI+ mass spectra (70 eV) of the di-TMS derivatives of 5-FU, 5-FUH_2 and 5-BrU

Parent compound	Main ions in mass spectra[a]				
	M	M-H *[M-1]*	M-CH_3 *[M-15]*	M-X[b]	Other ions
5-FU	274 (20)	273 (8)	259 (77)	255 (17)	184[c] (4)
5-FUH_2	276 (6)	275 (6)	261 (92)	257 (5)	201[d] (3)
5-BrU	334 (9)	—	319 (62)	255 (45)	204[e] (3)
	336 (10)	—	321 (58)		206[e] (3)
					177[f] (14)
					179[f] (15)

[a]Relative abundances are shown in parentheses.
[b]X=F or Br.
[c]M-TMSOH: *[M-90]*.
[d]M-DMSOH: *[M-75]*.
[e][M-CH_3]-TMSOCN: *[M-15-115]*.
[f][M-CH_3]-$TMSOC_2N_2H$: *[M-15-142]*.

Fragments at m/z values of M-1 corresponding to the loss of hydrogen had a significant abundance only in spectra of di-TMS derivatives of 5-FU and 5-FUH_2, suggesting the greater instability of the corresponding fragment in the case of the 5-BrU derivative. Further significant fragments were those derived from loss of dimethylsilanol DMSOH (M-75) and trimethylsilanol TMSOH (M-90) and, in the case of 5-BrU derivative, those deriving from the M-15 fragments, i.e. [M-15]-TMSOCN and [M-15]-$TMSOC_2N_2H$, characteristic of mass spectra of simple pyrimidines [43].

Fig. 3 shows the EI+ mass spectrum of the TMS derivative of 5′-dFUR. Mass fragments can be conveniently grouped into three categories [40]: fragments resulting from simple losses from the molecular ion M, fragments containing the intact

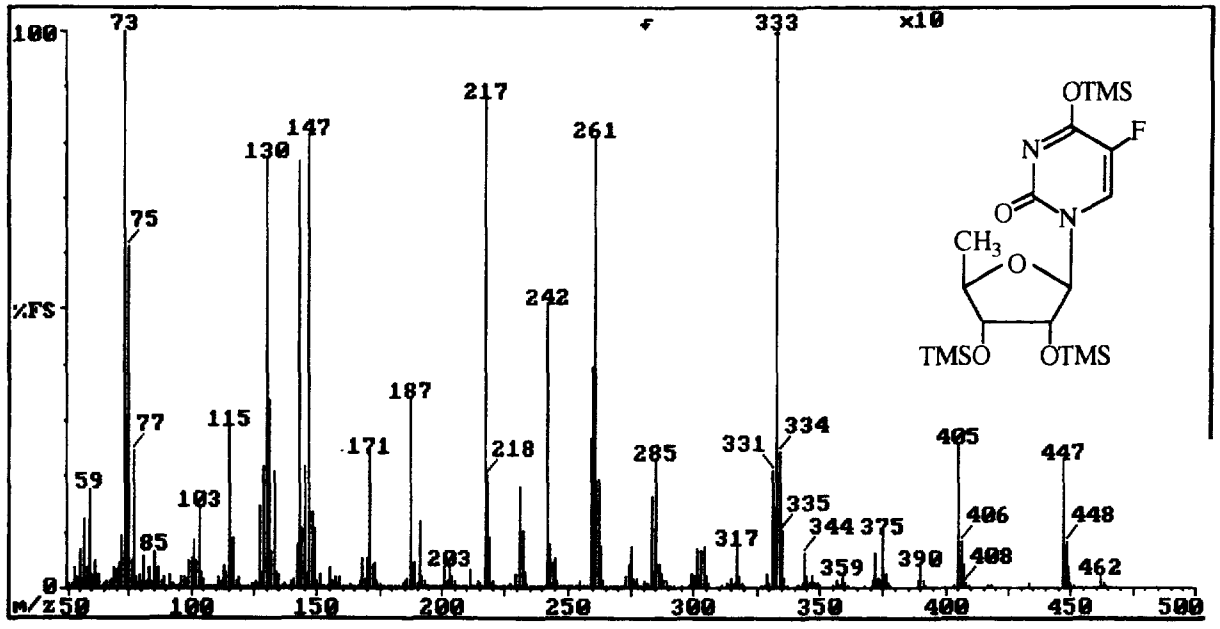


Fig. 3. EI+ mass spectrum relevant to the tri-TMS derivative of 5′-dFUR. Column length, 15 m; Splitless injection; Other conditions as i Section 2.

Table 2
Composition and relative abundance % (RA%) of some ions observed in the EI+ mass spectra (70 eV) of the tri-TMS derivative of 5′-dFUR

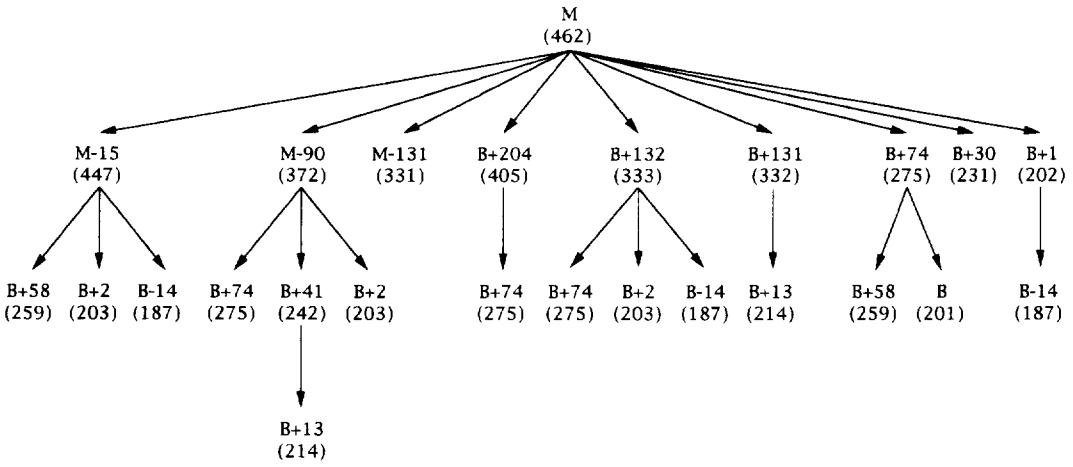
Composition	Ion	Mass	RA%
M	M	462	≤1
M-CH_3	M−15	447	2.2
M-TMSOH	M−90	372	≤1
M-CH_3-TMSOH	M−105	357	≤1
M-CO-TMSOH	M−118	344	≤1
M-C_2HO-TMSOH	M−131	331	2.1
B+TMS+$C_2H_2O_2$TMS	B+204	405	2.5
B+TMS+C_2H_2OTMS	B+188	389	≤1
B+H+$C_2H_2O_2$TMS	B+132	333	11.1
B+$C_2H_2O_2$TMS	B+131	332	1.4
B+H+C_3H_2OTMS	B+128	329	≤1
B+H+C_2H_2OTMS	B+116	317	≤1
B+H+$C_2H_2O_2$TMS-CH_2O	B+102	303	≤1
B+H+C_2H_2OTMS-CH_4	B+100	301	≤1
B+H+TMS	B+74	275	7.3
B+DMS	B+58	259	26.6
B+C_2HO	B+41	242	50.5
B+H+CHO	B+30	231	18.0
B+CH	B+13	214	1.3
B+H_2	B+2	203	3.7
B+H	B+1	202	1.2
B	B	201	3.6
B-CH_2	B−14	187	33.8
S	S	261	80.7
S-H	S−1	260	39.6
S-H-CH_2O	S−31	230	≤1
S-CH_3CHO	S−44	217	87.5
S-TMSOH	S−90	171	23.7
S-H-TMSOH	S−91	170	5.2
S-CH_4-TMSOH	S−106	155	3.9
CH_2OTMS	103	103	14.4

M – Ions related to the molecular ion M.
B – Ions containing the base B plus portions of the sugar.
S – Ions related to sugar S.

pyrimidine base B plus portions of the sugar, and the sugar moiety S and its fragmentation products. The m/z values and relative abundances of some significant fragment ions grouped according to these categories are listed in Table 2. According to Pang et al. [40], Scheme 1 suggests a possible fragmentation pattern for main ions listed in Table 2. The m/z values relative to the molecular ion, as well as the fragmentation pattern, indicated the formation of the tri-TMS derivative of nucleoside. Fragments M (462 m/z), M-15 (447 m/z), B+102 (303 m/z), B+74 (275 m/z), B+41 (242 m/z), B+30 (231 m/z), B+13 (214 m/z), B+2 (203 m/z) and B+1 (202 m/z) are useful for identification of members of the base series and for recognition of the nucleoside linkage, whereas fragments B+188 (389 m/z), B+132 (333 m/z), B+116 (317 m/z), S (261 m/z) and S-H (260 m/z) are important in nucleoside structural characterization for recognition of sites of sugar modification [40]. Other fragments, e.g. 103, 147 and 217 m/z, are common sugar ions [44].

Ions at 259 and 261 m/z were chosen for quantitative purposes in selected ion monitoring (SIM) of 5-FU and 5-FUH_2, respectively; an evaluation of chromatographic peak purity was accomplished by also monitoring 274 and 273 m/z ions, for 5-FU, and 276 and 275 m/z ions, for 5-FUH_2. Although useful for structural characterization, the mass spectrum of 5′-dFUR derivative did not show specific fragments with high abundance, even at the lower electron impact energy; fragments at values of 261, 242 and 187 m/z were selected for quantitation whereas those at 447, 405, 333 and 275 m/z were also monitored as a test of peak purity. Finally, the 319 m/z ion was selected for internal standard monitoring. In SIM mode, detection limits (at a signal-to-noise ratio S/N=3, noise calculated peak-to-peak in a blank chromatogram at the elution time of the analyte of interest) were 500 pg for both 5-FU and 5-FUH_2 and 100 ng for 5′-dFUR. Calibration curves were linear up to 2 μg for each analyte with correlation coefficients greater than 0.999. The repeatability ranged from 2.5% to 4.7%.

The applicability of the present method to biological samples is demonstrated in Fig. 4 showing typical TIC and SIM chromatograms of a plasma specimen from a cancer patient receiving doxifluridine chemotherapy. As can be seen, 5′-dFUR, 5-FU and 5-FUH_2 can be easily separated and quantitated. Analyte recoveries from plasma, calculated by comparison with standards, were essentially the same as those already reported [20,22], ranging from 83% to 96%. Detection limits in spiked plasma were higher than those observed for a synthetic solution of standards. The use of a longer capillary column and of an "on-column" injection technique permitted a tenfold decrease in the limits of detection. As an example, by using a 250 μl sample size, a reaction volume of 200 μl and a 2 μl injection volume,



Scheme 1. Fragmentation pattern for main ions displayed in EI+ mass spectrum of tri-TMS derivative of 5′-dFUR.

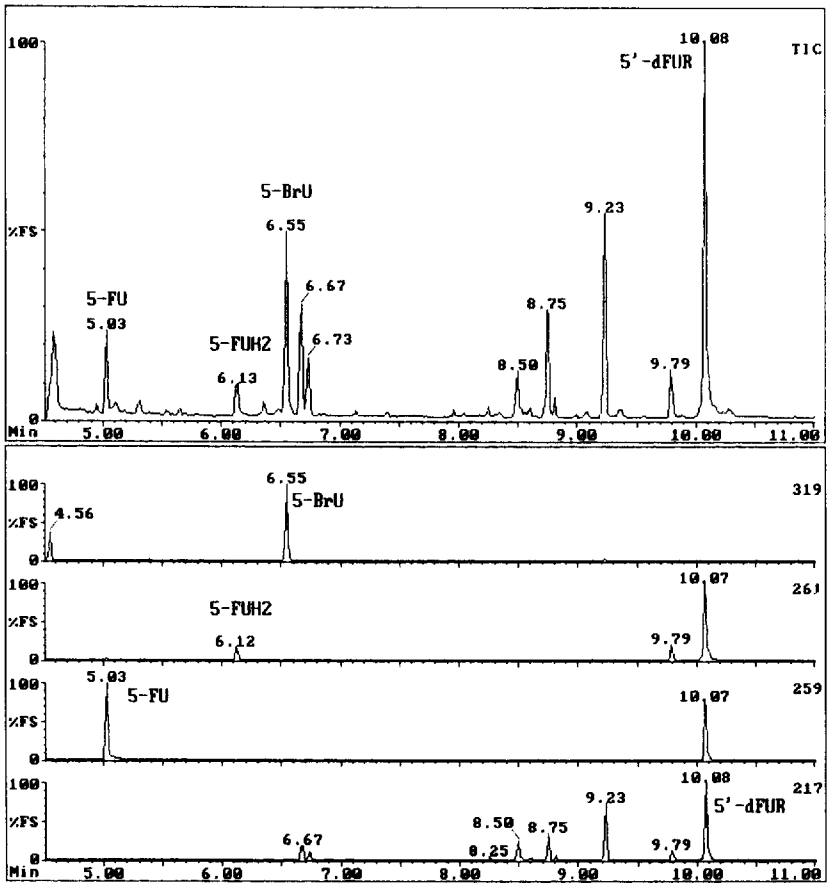


Fig. 4. Total ion current (*top*) and selected ion monitoring (*bottom*) chromatograms relevant to a derivatized extract of a plasma specimen from a patient treated with doxifluridine (4 g/m^2 by i.v. infusion over a 60 min period). Column length, 15 m; Splitless injection; Other conditions as in Section 2.

the limit of detection (S/N=3) in spiked plasma samples was 20 ng/ml for 5-FU and 5-FUH_2 and 100 ng/ml for 5′-dFUR. Detection limits for 5-FU and 5-FUH_2 compared well with those reported by Aubert et al. [27] but with the significant advantage of 5′-dFUR codetermination. Calibration curves were linear over about two decades of concentration; for example, calibration curves for 5-FU were linear in the range 0.02–5 mg/ml with correlation coefficients greater than 0.999 and intercepts not significantly different from zero at the 95% confidence level. Between- and within-days coefficients of variation were in the range of 4.0%–5.9% and 9.1%–16.9%, respectively.

In conclusion, the GC–MS procedure here described is first permitting a simultaneous determination of 5′-dFUR, 5-FU and 5-FUH_2 in plasma. Optimization of the analytical procedure resulted in an improved analyte stability so that, for example, interference in 5-FU analysis originating from 5′-dFUR decomposition, was not observed. Application in the analysis of 5′-dFUR, 5-FU and 5-FUH_2 in real samples has been demonstrated. In comparison to analytical methods previously developed for fluoropyrimidine analysis (see [19–22] for a review), this GC–MS procedure has comparable detection limits but requires analyte derivatization and is intrinsically more complex from an instrumental point of view. For example, capillary electrophoresis coupled to UV detection [45] permits the analysis of several fluoropyrimidines including 5′-dFUR, 5-FU and 5-FUH_2 without analyte derivatization, but until now applications to real samples are not reported. Determination of 5′-dFUR, 5-FU and 5-FUH_2 in biological samples can be achieved [46] by isocratic LC-UV for 5′-dFUR and 5-FU and capillary GC coupled to electron-capture detection for 5-FUH_2. Although both techniques do not need derivatization and are at least as sensitive as the presently described GC–MS method, two different sample pretreatments and extraction procedures are required. The GC–MS method herewith described and the LC–UV procedure already reported [22] seems at the present the unique method capable of a simultaneous determination of 5′-dFUR, 5-FU and 5-FUH_2 in real samples. The apparent drawbacks showed by GC–MS are compensated for by the very high selectivity obtained in the SIM mode of detection, simplifying the chromatographic requirements, and reducing the interference effects of coeluting compounds. Furthermore, MS spectra produce a considerable amount of structural information not obtainable by UV detection.

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