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Isolation of an unknown metabolite of capecitabine, an oral 5-fluorouracil prodrug, and its identification by nuclear magnetic resonance and liquid chromatography–tandem mass spectrometry as a glucuroconjugate of 5′-deoxy-5-fluorocytidine, namely 2′-(β-D-glucuronic acid)–5′-deoxy-5-fluorocytidine

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Abstract

A new metabolite of capecitabine, a prodrug of 5-fluorouracil, was detected by ^{19}F NMR in bile and liver of rats treated with this anticancer drug. Crude bile and perchloric acid extract of liver was subjected to liquid–liquid separation followed by a pre-purification step on a preparative octadecyl silane column (C_{18}). The compound was purified by HPLC optimised to allow the detection of the unknown metabolite and its assumed precursor 5′-deoxy-5-fluorocytidine (5′-DFCR). Treatment with β-glucuronidase from three sources showed that it was a glucuroconjugate of 5′-DFCR. HPLC–TIS-MS–MS and ^{1}H NMR allowed identification of the unknown metabolite as 2′-(β-D-glucuronic acid)–5′-deoxy-5-fluorocytidine.

Keywords: Capecitabine; 5′-Deoxy-5-fluorocytidine; 5-Fluorouracil

1. Introduction

Capecitabine (CAP) is a new oral antineoplastic drug mainly used for treating colorectal cancers [1]. This fluoropyrimidine carbamate was designed to be readily absorbed from the human gastro-intestinal tract and take advantage of the differential enzymatic levels in tumours to achieve better targeting of 5-fluorouracil (5-FU), the active moiety of CAP. Indeed, CAP metabolism into 5-FU requires three sequential enzyme-mediated reaction steps. First, CAP is converted in the liver to 5′-deoxy-5-fluorocytidine (5′-DFCR) by carboxylesterase. The subsequent conversion of 5′-DFCR into 5′-deoxy-5-fluorouridine (5′-DFUR) is mediated by cytidine deaminase, an enzyme found in liver and tumour tissues. Finally, 5′-DFUR is converted to 5-FU by thymidine phosphorylase whose activity is increased in tumour tissue [2].

To get new insight into the CAP metabolic pathway, ^{19}F NMR analysis of plasma, urine, bile and perchloric acid (PCA) extracts of liver from rats treated with CAP was performed. An unknown compound (metabolite X) was detected in bile and

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PCA liver extracts [3], suggesting that it results from a pathway of CAP detoxification. The recent trend for the combination of CAP with other drugs active in advanced colorectal cancer, e.g., irinotecan (CPT-11) [4], emphasises the need for identifying this new metabolite. Indeed, a better knowledge of CAP metabolic pathways will enable any drug interactions to be avoided or at least moderated.

This study describes the chromatographic and analytical methods that were used for the isolation and identification of this new CAP metabolite found in the bile and liver of treated rats.

2. Experimental

2.1. Chemicals

5-FU, phenolphthalein glucuronic acid, D-saccharic acid 1,4-lactone, β-glucuronidase (EC 3.2.1.1.31) from bovine liver (type B-10), *Helix pomatia* (type H-1), *Escherichia coli* (type IX) were purchased from Sigma, and chromium (III) acetylacetonate ($Cr(acac)_3$) from Aldrich (all from Sigma–Aldrich, Saint-Quentin Fallavier, France). α-Fluoro-β-alanine (FBAL) hydrochloride was provided by Tokyo Kasei Chemicals (Tokyo, Japan). 5,6-Dihydro-5-fluorouracil (5-FUH_2), 5′-DFUR and 5′-DFCR were generously supplied by Hoffmann-La Roche (Basel, Switzerland). CAP was a gift from Professor W. Wolf, University of Southern California, Los Angeles, CA, USA. Matrex silica C_{18} was obtained from Millipore (Danvers, MA, USA). All other chemicals were reagent grade and obtained from standard commercial sources.

2.2. Sample collection

Male Wistar rats weighing between 400 and 550 g received an oral dose of 80 mg/kg b.w. of CAP dissolved in 1.5 ml of water. One hour later they were anaesthetised with pentobarbital (60 mg/kg, i.p.). The abdomen was opened, bile duct cannulated, and bile collected over 2 h. At the end of the experiment, liver and kidneys were excised and immediately frozen in liquid nitrogen. Urine was drawn from the bladder. All samples were maintained at −80 °C until further treatments. The storage period did not exceed 1 week.

2.3. NMR spectroscopy

2.3.1. ^{19}F NMR

^{19}F NMR spectra were recorded at 282.4 MHz with ^1H-decoupling on a Bruker WB-AM 300 spectrometer (Bruker, Wissembourg, France) using 10-mm diameter NMR tube. The recording conditions were: probe temperature, 25 °C; sweep width, 41 667 Hz; 32 768 data points zero-filled to 65 536; pulse width, 7 μs; pulse interval, 1.4 s for quantification of liver and kidney PCA extracts or 3.4 s for quantification of bile and urine samples; number of scans, 10 000–50 000; line broadening caused by exponential multiplication, 5 Hz. The chemical shifts (δ) were reported relative to the resonance peak of CF_3COOH (5%, w/v, aqueous solution) used as external chemical shift reference ($\delta = 0$ ppm).

The concentrations of fluorinated compounds were measured by comparing the expanded areas of their respective NMR signals with those of the external standard for quantification, placed in a coaxial capillary, namely a solution of sodium parafluorobenzoate (FBEN) in D_2O doped at saturation with $Cr(acac)_3$. The areas were determined after the different signals were cut out and weighed. The detection limit for our spectrometer after 15–18 h recording was about 2 μ*M*.

2.3.2. ^1H NMR

^1H NMR spectra were recorded in D_2O at 400.1 MHz on a Bruker ARX 400 spectrometer (Bruker SA, Wissembourg, France) equipped with a 5-mm inverse multinuclear probe. 1D ^1H NMR spectra were acquired with a spectral width of 8064 Hz. Free induction decays were recorded in blocks of 128 scans with 65 536 K real data points and a repetition time of 1 s. Processing parameters were: zero filling to 131 072 data points and line broadening 0.2 Hz. 2D ^1H–^1H COSY and ^1H–^1H long-range COSY spectra were acquired with a spectral width of 3448 Hz in both dimensions. The $f2$ dimension was acquired with 2048 real data points and recorded in blocks of 128 scans per experiment. The $f1$ dimension contained data points from 256 experiments. Prior to processing, the $f1$ dimension was filled to

1024 data points. Fourier transform was done after applying an exponential apodisation in both dimensions. 3-(Trimethylsilyl)-2,2,3,3-tetra-deuteropropionic acid ($TSPd_4$) was used as internal shift reference ($\delta = 0$ ppm).

The identification of ^{1}H NMR signals from metabolite X was performed from one- and two-dimensional experiments on the purified compound, and from some considerations including proton characteristics and assignments of standard 5′-DFCR and comparison with literature data of proton signal attributions of glucuronide sugar moieties [5].

2.4. HPLC apparatus and chromatographic conditions

The HPLC system was a Waters 2960 Alliance model with a Waters 996 diode array detector. The analysis conditions were: reversed-phase column Prontosil C_{18} (250×4-mm I.D.; 50-μm particle size; Bischoff Chromatography, Stuttgart, Germany); column temperature, 35 °C; mobile phase, (A) acetonitrile (isocratic grade for LC) and (B) demineralised water (Milli-Q quality; Millipore, Eschborn, Germany) containing 0.1% trifluoroacetic acid (TFA) (v/v); flow-rate, 1.0 ml/min; volume of injection, 10 μl unless otherwise indicated; detection, UV absorbance between 210 and 350 nm. The elution profile was: 0–8.5 min, isocratic elution with A:B mixture in 5:95 ratio; 8.5–11 min, linear gradient from 5:95 to 90:10 A:B ratio; 11–16 min, column washing out with 90:10 A:B mixture; 16–18 min, return to 5:95 A:B mixture ratio; and, finally, the system was left to stabilise for 12 min between consecutive injections.

2.5. Isolation of metabolite X

2.5.1. Sample pre-treatment

Liver and kidneys were immersed in liquid nitrogen, powdered, and sequentially extracted with cold PCA. Briefly, powdered liver was extracted twice with 4 volumes of ice-cold 1 *M* PCA. The suspension was centrifuged, the supernatant collected, the pellet resuspended in PCA and recentrifuged. The pH of the supernatant was adjusted to 5.5 with a KOH solution. For ^{19}F NMR analysis, lyophilised materials were resuspended in ≈3 ml of H_2O containing 30 m*M* EDTA and the extract centrifuged at 1000 *g* for 5 min. Crude bile samples were diluted with H_2O to 2.5 ml.

Bile and liver extract samples of two in vivo experiments were separately pooled and each subjected to a cleaning procedure. In brief, 3 volumes of dichloromethane were added. After extraction, the aqueous phases were collected and re-extracted with ethyl acetate. The aqueous phases were lyophilised and stored at −80 °C.

2.5.2. Column chromatography

A preparative column (10×0.8-cm I.D.) packed with octadecyl silane (C_{18}, 50-μm particle size and 60 Å pore diameter) was successively rinsed with 40 ml methanol–water (80:20, v/v) and 25 ml H_2O. Dry residues resulting from the cleaning procedure of bile or liver extract were diluted in 400 μl H_2O and then placed on the top of the column. Elution used a mixture of methanol–water according to the following scheme: first 5 ml 0:100 (v/v), followed by 4 ml 20:80 (v/v), 5 ml 50:50 (v/v), and 4 ml 100:0 (v/v). Seventeen effluent fractions of 1 ml were successively collected and analysed with ^{19}F NMR. Fractions 4 and 8 (methanol–water, 0:100 and 20:80, v/v) contained metabolite X and 5′-DFCR, respectively, as sole fluorinated compounds. 5′-DFCR was identified by spiking with authentic standard.

2.5.3. Subsequent purification by HPLC

Fraction 4 was lyophilised and the residue dissolved in 600 μl of H_2O. Further purification was carried out with HPLC under the conditions previously described except that the volume of each injection was 100 μl, and the detection wavelength 280 nm. The volume corresponding to the elution of the metabolite X (ca. 500 μl) was collected. The effluents of six successive injections were gathered, and the purity of the isolated compound was checked by HPLC. The absorbance of the purified metabolite X accounted for 98% of the total absorbance of the peaks in the chromatogram recorded over 12 min, whereas its absorbance in fraction 4 represented only 56% of the total absorbance. The effluents were freeze-dried and stored at −80 °C until enzymatic hydrolysis and ^{1}H NMR analysis.

2.6. Enzymatic hydrolysis

Twenty-five μl of a solution of metabolite X purified from liver or bile (corresponding to approximately 60 μmol as determined from ^{19}F NMR quantification of this compound in fraction 4) were diluted with 175 μl of acetate buffer (0.2 *M*, pH 5.0). Incubations were run in duplicate overnight in a shaking water bath at 37 °C with 1000 units of β-D-glucuronidase from bovine liver or *Helix pomatia* (50 μl, 0.1% NaCl). For *Escherichia coli* β-D-glucuronidase experiments (1000 units, 50 μl, 0.1% NaCl), acetate buffer was changed for phosphate buffer (0.2 *M*, pH 6.8). Control experiments, i.e., incubation of the glucuronide with β-D-glucuronidase and its inhibitor D-saccharic acid 1,4-lactone (0.2 *M*), and incubation with a denatured β-D-glucuronidase (80 °C for 20 min) were done concurrently. The pH value of the acid solution of D-saccharic acid 1,4-lactone was adjusted to 5 prior to its addition to test vials. Following overnight incubation, aliquots were injected directly into the HPLC column. Incubations verifying enzyme viability with phenolphthalein glucuronide were run concurrently.

2.7. HPLC–turbo ion spray (TIS)-MS–MS experiments

The HPLC system used consisted of a PE series 200 LC pump, a Rheodyne 8125 injector valve (40-μl loop) and a PE 785 UV–Vis detector (λ=280 nm). A Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer, equipped with a Turbo Ion Spray interface, was used for detection. Both were controlled by a PE Sciex MassChrom data system (version 1.1.1). HPLC conditions were identical to those reported above except that TFA was replaced by formic acid. The mass spectrometer was operated in positive ionisation mode with TIS heater set at 450 °C. Nitrogen served both as auxiliary and collision gas, air as nebuliser gas. The operating conditions for TIS interface were: (i) in MS mode: mass range 250–450 and 400–600 u; step size 0.15 u; dwell time 2 ms; Q1 TIS MS spectra were recorded in profile mode, IS 5500 V, OR 15 V, RNG 170 V; (ii) in MS–MS mode: precursor mass 422 u; mass range 50–430 u; step size 0.3 u; dwell time 2 ms; LC–MS–MS spectra were recorded in profile mode, IS 5500 V, OR 15, RNG 260 V, Q0 −11 V, RO2 −30 V. The flow-rate was 1 ml/min with a split of 500 μl to mass spectrometer and 500 μl to waste.

3. Results and discussion

3.1. Fluorinated metabolites of CAP

Typical 1D ^{19}F NMR spectra of bile, urine and liver and kidney extracts from rats treated with CAP are presented in Fig. 1. Signals of CAP, 5′-DFCR, 5′-DFUR, 5-FUH$_2$ and FBAL were assigned by adding the standard compound. Spectra of samples spiked with 5′-DFUR were recorded at various pH as the ^{19}F δ of this compound is pH-dependent near physiological pH since its pK_a is 7.5 (unpublished data).

In liver extract, the major compound was 5′-DFCR accounting for 42% (29.7 μmol/g liver w.w.) of total fluorine-containing compounds, metabolite X representing 38% (26.9 μmol/g liver w.w.). In bile, metabolite X was the major compound accounting for 90% (37 μmol/g liver w.w.) of fluorinated compounds, whereas it was undetected in kidney extract and urine. The ^{19}F NMR signals of metabolite X in liver extract and bile were not differentiated by cross check at various pH or ionic strengths suggesting that these compounds could be identical. When acquired without proton decoupling, the ^{19}F NMR signal of metabolite X in liver extract as well as in bile was a doublet with a proton–fluorine coupling constant (J_{HF}) of 6.1 Hz like CAP and 5′-DFCR whose J_{HF} were 5.2 and 6.4 Hz, respectively (Table 1). This indicated that metabolite X has a pyrimidine ring with a double bond in the 5,6-position and a hydrogen atom in position 6. Moreover, the very close δ of metabolite X and 5′-DFCR ($\Delta\delta$<0.1 ppm) (Fig. 1) indicated that the immediate neighbourhood of the fluorine atom was the same in the two compounds. In order to determine its structure, metabolite X was isolated from bile and liver extract.

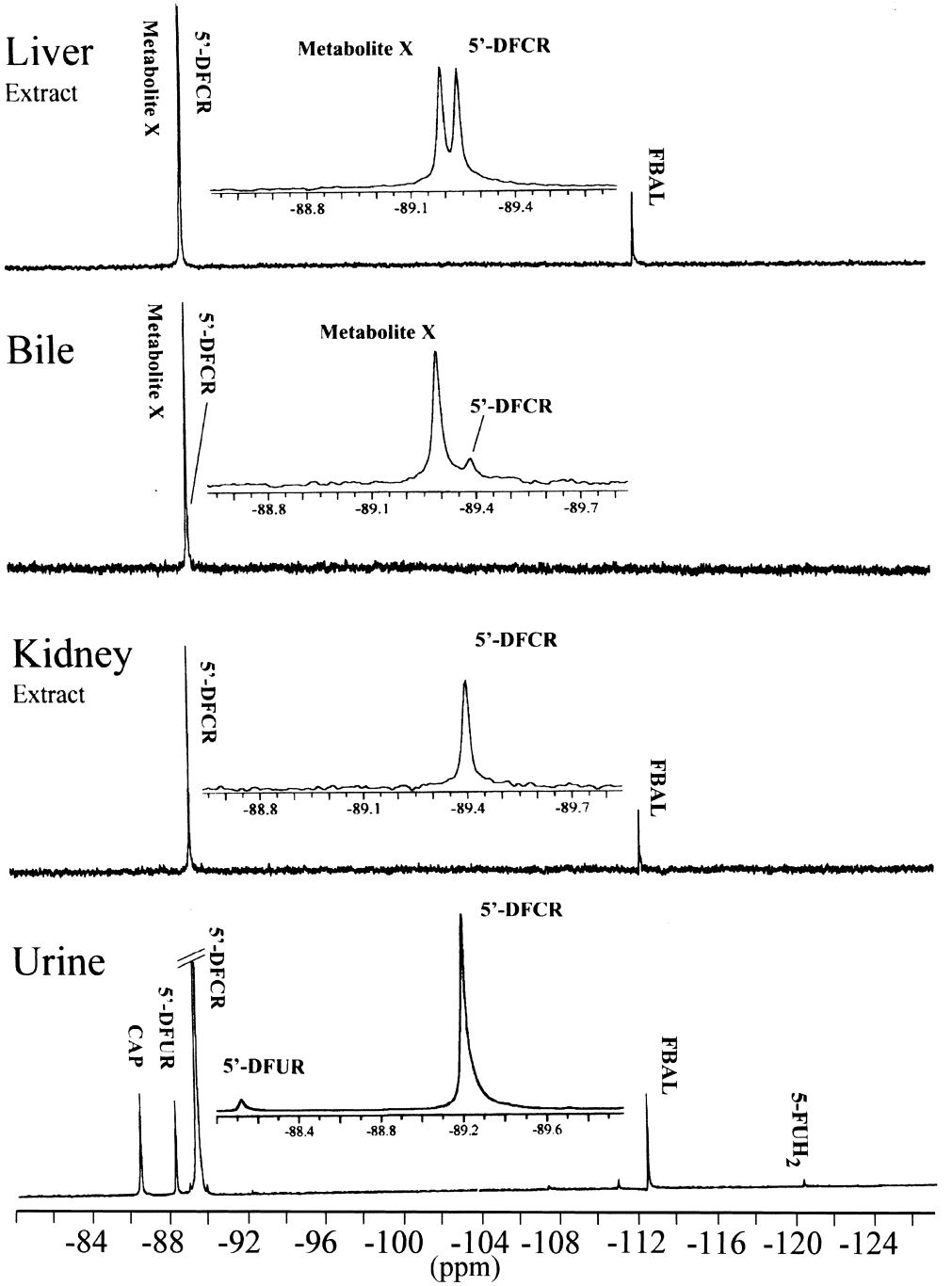

Fig. 1. Typical ^{1}H-decoupled ^{19}F NMR spectra of liver extract, crude bile, kidney extract and crude urine from a rat treated with capecitabine. Spectra were recorded at pH 5.5, 8.4, 5.6, 8.3 for liver extract, crude bile, kidney extract and urine, respectively. The region of interest surrounding metabolite X was expanded to discriminate its signal from that of 5′-DFCR.

Table 1
^{19}F and ^{1}H NMR characteristics of the glucuronide conjugate of 5′-DFCR (5′-DFCR-G) and 5′-DFCR

	5′-DFCR-G		5′-DFCR	
	δ (ppm)[a]	Multiplicity[b], J (Hz)	δ (ppm)[a]	Multiplicity[b], J (Hz)
Cytosine ring				
F5	−89.45	d, 6.1[c]	−89.52	d, 6.4[c]
H6	7.76	d, 6.2	7.74	d, 6.3
Ribose ring				
H1′	5.76	dd, 1.3; 3.3	5.77	dd, 1.4; 3.2
H2′	4.36	dd, 3.4; 5.2	4.25	dd, 3.3; 5.3
H3′	4.00	dd, 5.3; 6.6	3.88	dd, 5.5; 6.6
H4′	4.24	app quin, 6.5	4.13	app quin, 6.5
H5′	1.41	d, 6.4	1.41	d, 6.4
Glucuronic acid moiety				
H1″	5.53	d, 7.8		
H2″	3.36	m		
H3″	3.49	m		
H4″	3.78	m		
H5″	3.47	d, 6.85		

[a] ^{19}F NMR spectra were recorded in H_2O at pH 5.0 without proton decoupling. δ are expressed in ppm relative to CF_3COOH (aqueous solution 5%, w/v) used as external reference. ^{1}H NMR spectra were recorded in D_2O at pD 4.4. δ are expressed in ppm relative to $TPSd_4$. Complete assignments of the protons of the sugar moieties were obtained from ^{1}H–^{1}H COSY experiments.
[b] d, doublet; dd, doublet of doublets; app quin, apparent quintuplet; m, multiplet.
[c] J_{HF} coupling constant.

3.2. Isolation of metabolite X from bile and liver extract

The ^{19}F NMR analysis of samples obtained after dichloromethane cleaning and C_{18} column chromatography allowed HPLC isolation of metabolite X from prepurified fractions containing essentially the compound of interest (Fig. 2) or 5′-DFCR. The assignment to metabolite X of the peak detected at a retention time of 6.1 min in fraction 4 of the C_{18} column chromatography was based on the following considerations: (i) it exhibited the characteristic UV absorption spectrum of a 5′-DFCR pyrimidine ring with a maximum absorbance at 285 nm; (ii) it was only present in that fraction; (iii) the ^{19}F NMR δ of the purified compound corresponding to this peak and that of metabolite X in fraction 4 were identical. The compound detected in fraction 8 with a retention time of 6.6 min was assigned to 5′-DFCR as its retention time matched with that of standard 5′-DFCR, and its UV absorption spectrum with a maximum absorbance at 285 nm was the same as that of the authentic standard. Chromatographic conditions were optimised to obtain good resolution of metabolite X and 5′-DFCR within the first 8 min of the experiment. Thus, the capacity factors (k') for metabolite X and 5′-DFCR were 0.3 and 0.5, 2.05 and 2.3, and 0.3 and 0.7 when the mobile phase consisted of 1% TFA-water–acetonitrile (90:10, v/

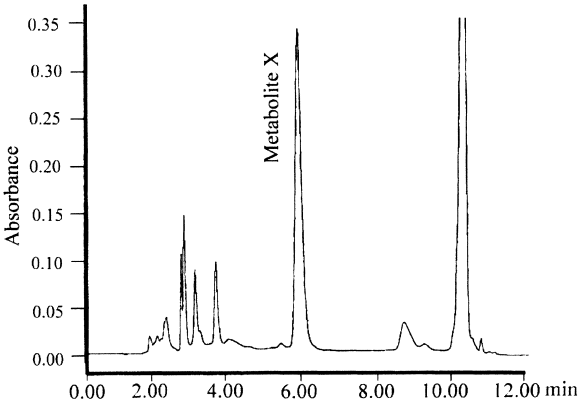

Fig. 2. HPLC–UV (detection at 280 nm) chromatogram of fraction 4 of C_{18} column-chromatographed liver extract. Retention time of metabolite X: 6.1 min.

v), 1% TFA-water–acetonitrile (95:5, v/v), and water–acetonitrile (90:10, v/v), respectively.

3.3. Structural determination of metabolite X

3.3.1. Biochemical characterisation of metabolite X as a β-D-glucuronide

The attribution of metabolite X purified from bile or liver extract to a β-D-glucuronide was supported by the results of the HPLC analysis of the following experiments. The peak at a retention time of 6.1 min disappeared after incubation of purified bile or liver extract with β-D-glucuronidase from bovine liver or *Helix pomatia* and a new peak with a retention time of 6.6 min was detected and identified as 5′-DFCR since its retention time was identical to that of authentic 5′-DFCR. Fig. 3A, B and E illustrate these findings for metabolite X purified from bile. No change in the chromatographic profile was observed (i) in control experiments without enzyme, (ii) in

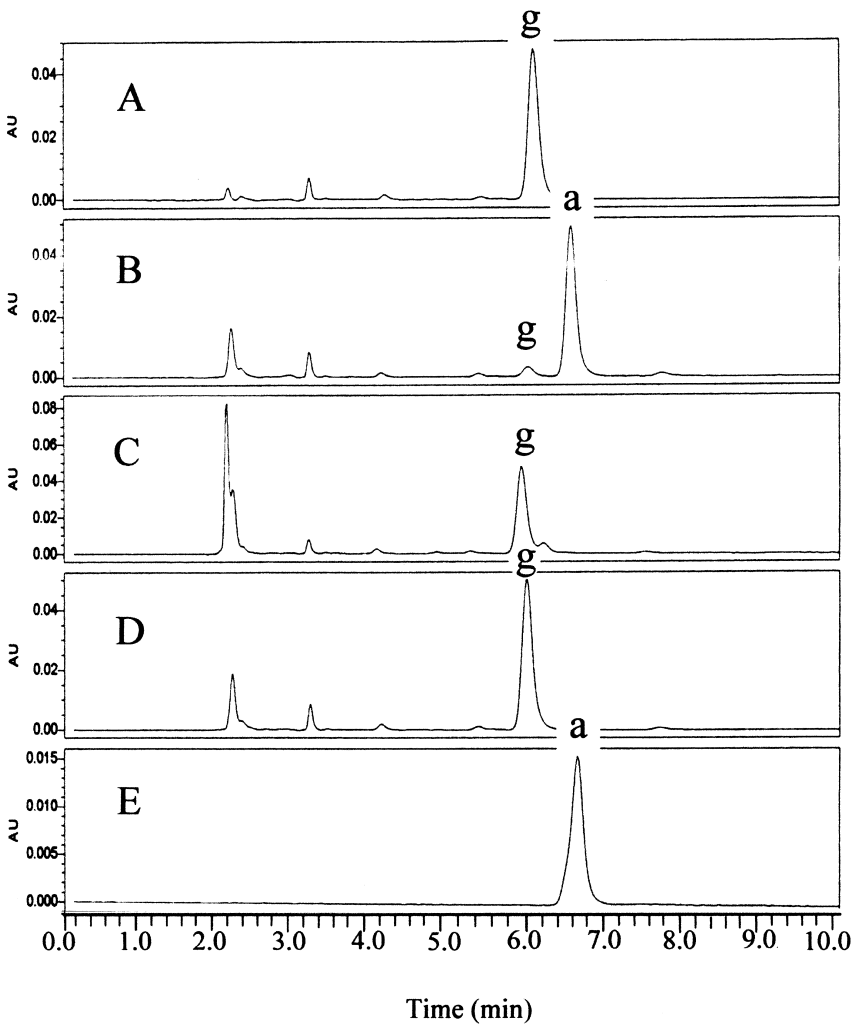

Fig. 3. Effect of β-D-glucuronidase from bovine liver on the HPLC chromatograms of metabolite X isolated and purified from bile (UV detection at 280 nm). (A) Control experiment (peak **g** (glucuronide), retention time 6.1 min); (B) incubation with β-D-glucuronidase (appearance of peak **a** (aglycone), retention time 6.6 min); (C) incubation with β-D-glucuronidase+D-saccharic acid 1,4-lactone; (D) incubation with β-D-glucuronidase previously inactivated by heating; (E) authentic standard of 5′-DFCR (retention time 6.6 min).

experiments in which purified bile or liver extract was treated with enzyme in the presence of its specific inhibitor (saccharic acid 1,4-lactone), and (iii) in samples containing the enzyme previously denatured by heating (Fig. 3A, C and D). When treated with β-D-glucuronidase from *Escherichia coli*, the peak of metabolite X disappeared but that of its aglycone, 5′-DFCR, was not detected. The presence of deaminase- and pyrimidine phosphorylase-like enzymatic activities in this *Escherichia coli* β-D-glucuronidase batch might be the cause of the further reactivity of 5′-DFCR as previously observed for 5-fluorocytidine [6]. It must be pointed out that all the β-D-glucuronidases used were ineffective on metabolite X in crude bile or liver extract.

3.3.2. HPLC–TIS-MS–MS characterisation of 5′-DFCR-glucuronide (5′-DFCR-G)

The TIS mass spectrum (positive ionisation mode) obtained from metabolite X isolated from bile or liver extract exhibited a pseudomolecular ion $[MH]^+$ at m/z 422 suggesting a molecular mass of 421 u, namely that of 5′-DFCR-G. The HPLC–TIS-MS–MS product ion scan of molecular ion at m/z 422 is presented in Fig. 4. Characteristic ion fragments were found at m/z 130 (100%), 141, 159, 177, 246, 257, 275, 293. Neutral loss of FC produced the ion at m/z 293. Two successive losses of H_2O from this last ion afforded the ions at m/z 275 and 257. Neutral loss of the glucuronic acid moiety by glycosidic cleavage gave an ion with a low relative intensity (1%) at m/z 246 $[aglycone+H]^+$ characteristic of protonated 5′-DFCR. The ion at m/z 177 with a relative intensity of 9% was the characteristic fingerprint of a glucuronic acid moiety. The ion at m/z 130 was assigned to protonated FC. The fragmentation pathways leading to all these ions are reported in Fig. 4.

3.3.3. ^{1}H NMR characteristics of 5′-DFCR-G

First of all, ^{19}F and ^{1}H NMR spectra of both 5′-DFCR-G purified from bile or liver extract were

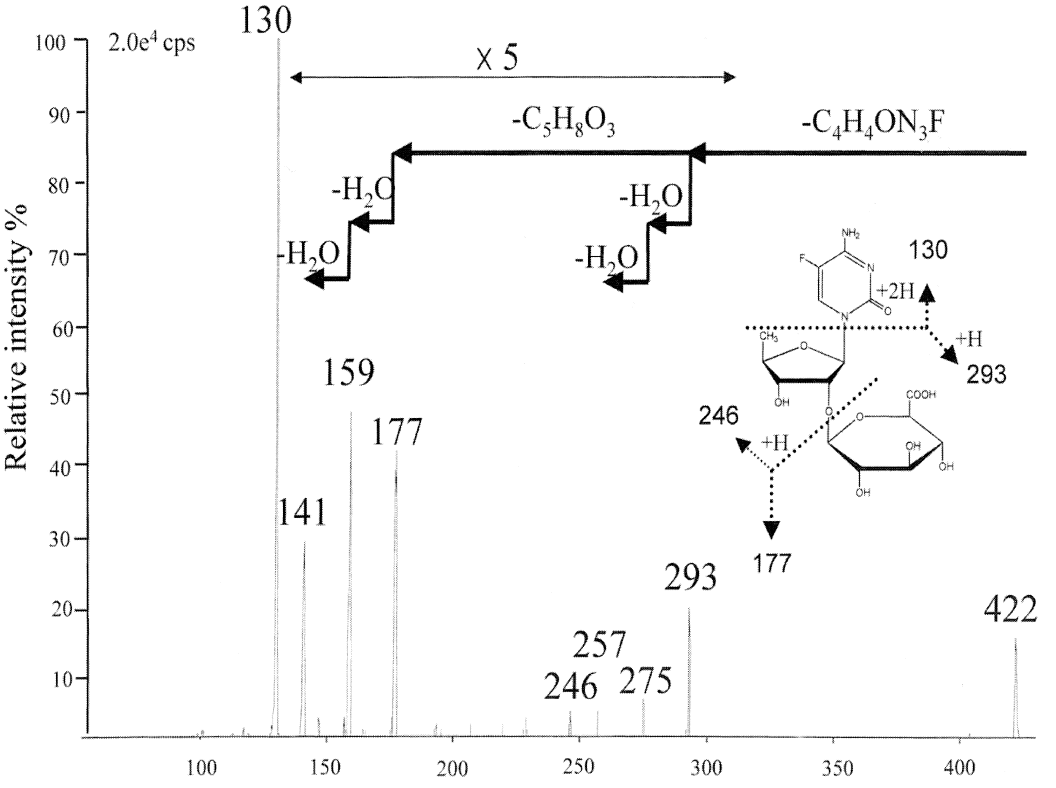

Fig. 4. Positive mode HPLC–MS–MS product ion scan of $[MH]^+$ ion at m/z 422 of metabolite X purified from liver extract. The fragmentation pathway was established from the 5′-DFCR-G structure determined by ^{1}H NMR.

identical. ^{19}F and non-exchangeable proton ^{1}H NMR characteristics of 5′-DFCR-G and 5′-DFCR are reported in Table 1. The ^{1}H NMR spectrum of 5′-DFCR-G purified from bile is presented in Fig. 5A. The δ, multiplicity, and J_{HF} values of ^{19}F and ^{1}H NMR signals of the cytosine rings of 5′-DFCR-G and 5′-DFCR were very close (Table 1). The ^{1}H–^{1}H COSY correlation of 5′-DFCR-G showed that its ^{1}H NMR resonances belonged to two different coupled spin systems. From comparison of the 1D and 2D ^{1}H–^{1}H COSY spectra of 5′-DFCR-G with those of 5′-DFCR, the system H1′–H2′–H3′–H4′–H5′ corresponds to the ribose ring protons (Table 1, Fig. 6). From comparison with literature data [5], the system H1″–H2″–H3″–H4″–H5″ corresponds to the protons of the glucuronic acid ring (Fig. 6). Further characterisation of 5′-DFCR-G as a β-D-glucuronide was obtained from the coupling constant of the anomeric proton H1″ of the glucuronic acid moiety at 5.53 ppm. Indeed the $^{3}J_{H1''-H2''}$ of 7.8 Hz (Table 1) was characteristic of a diaxial configuration of H1″ and H2″ and thus of a β-anomeric configuration for the glucuronic acid [5].

The coupling constants of the ribose ring protons of 5′-DFCR-G and 5′-DFCR were similar as well as the δ of H1′ and H5′. On the other hand, the δ of H2′, H3′ and H4′ of 5′-DFCR-G were deshielded by about 0.1 ppm relative to the corresponding protons of 5′-DFCR (Table 1). Thus, the position of the glucuroconjugation could concern 2′, 3′ or 4′ ribose ring carbons. The proof of glucuronidation at position 2′ was finally achieved by a long-range ^{1}H–^{1}H COSY experiment that showed a long-range coupling constant (^{4}J) between the anomeric proton H1″ of glucuronic acid and proton H2′ of the ribose ring (Fig. 5B). Thus, the structure of 5′-DFCR-G corresponds to 2′-(β-D-glucuronic acid)-5′-DFCR (Fig. 6).

4. Conclusion

In this investigation, a new metabolite of CAP, a recent 5-FU prodrug, was isolated and its structure defined. 2′-(β-D-Glucuronic acid)–5′-deoxy-5-fluorocytidine was isolated from bile and liver

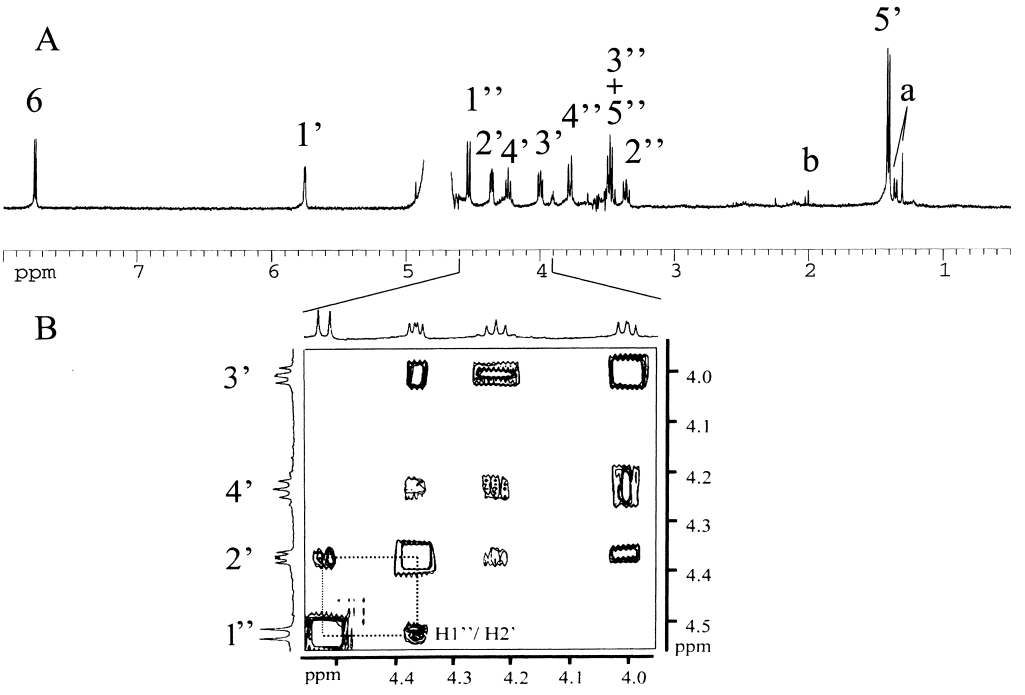

Fig. 5. 1D ^{1}H NMR (A) and 2D ^{1}H–^{1}H long-range COSY (B) partial spectra of metabolite X purified from bile. Region of interest, i.e., 3.9–4.6 ppm of 2D ^{1}H–^{1}H long-range COSY spectrum has been expanded. Cross-peaks correlation between H1″ and H2′ have been pinpointed. **a** and **b** are signals of impurities.

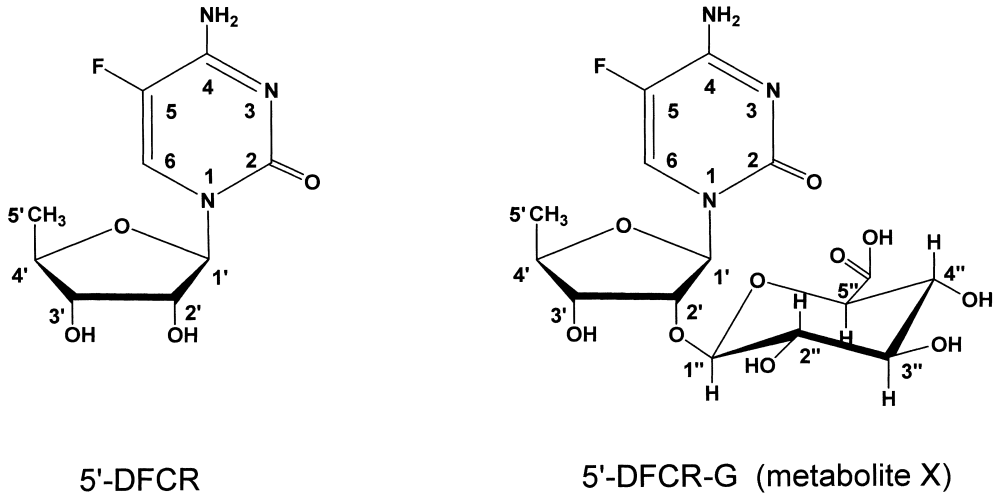

Fig. 6. Structure of 5′-deoxy-5-fluorocytidine (5′-DFCR) and 2′-(β-D-glucuronic acid)-5′-deoxy-5-fluorocytidine (5′-DFCR-G; metabolite X).

extract of rats treated with CAP. This compound diverges from the CAP activation pathway at the level of 5′-DFCR. This new metabolite was first discovered with ^{19}F NMR which allows the simultaneous detection and quantification in a single run of all fluorine-containing compounds in a crude sample. The determination of its exact structure needed isolation and characterisation by classical analytical techniques. The high sensitivity of the HPLC–TIS-MS–MS technique could be useful to investigate human CAP metabolism in in vitro models such as hepatic microsomes. This work is in progress and could confirm or invalidate the existence of such a glucuronidation pathway in humans.

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