METABOLISM OF CAPECITABINE, AN ORAL FLUOROURACIL PRODRUG: $^{19}$F NMR STUDIES IN ANIMAL MODELS AND HUMAN URINE

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ABSTRACT:

Capecitabine (Xeloda; CAP) is a recently developed oral antineoplastic prodrug of 5-fluorouracil (5-FU) with enhanced tumor selectivity. Previous studies have shown that CAP activation follows a pathway with three enzymatic steps and two intermediary metabolites, 5'-deoxy-5-fluorocytidine (5'-DFCR) and 5'-deoxy-5-fluorouridine (5'-DFUR), to form 5-FU preferentially in tumor tissues. In the present work, we investigated all fluorinated compounds present in liver, bile, and perfusate medium of isolated perfused rat liver (IPRL) and in liver, plasma, kidneys, bile, and urine of healthy rats. Moreover, data obtained from rat urine were compared with those from mice and human urine. According to a low cytidine deaminase (3.5.4.5) activity in rats, 5'-DFCR was by far the main product in perfusate medium from IPRL and plasma and urine from rats. Liver and circulating 5'-DFCR in perfusate and plasma equilibrated at the same concentration value in the range 25 to 400 μM, which supports the involvement of es-type nucleoside transporter in the liver. 5'-DFUR and α-fluoro-β-ureidopropionic acid (FUPA) + α-fluoro-β-alanine (FBAL) were the main products in urine of mice, making up to 23 to 30% of the administered dose versus 3 to 4% in rat. In human urine, FUPA + FBAL represented 50% of the administered dose, 5'-DFCR 10%, and 5'-DFUR 7%. Since fluorine-19 nuclear magnetic resonance spectroscopy gives an overview of all the fluorinated compounds present in a sample, we observed the following unreported metabolites of CAP: 1) 5-fluorocytosine and its hydroxylated metabolite, 5-fluoro-6-hydroxycytosine, 2) fluoride ion, 3) 2-fluoro-3-hydroxypropionic acid and fluoroacetate, and 4) a glucuroconjugate of 5'-DFCR.

Capecitabine (CAP), N\(^4\)-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine (capcitabine); 5'-DFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil; 5'-DFCR, 5'-deoxy-5-fluorocytidine; IPRL, isolated perfused rat liver; 5'-DFCR-G, 2'β-D-glucuronide of 5'-deoxy-5-fluorocytidine; 5-FUH\(_2\), 5,6-dihydro-5-fluorouracil; FUPA, α-fluoro-β-ureidopropionic acid; FHPA, 2-fluoro-3-hydroxypiperidine acid; PCA, piperidinic acid; AS, acid-soluble; AI, acid-insoluble; B.W., body weight; LD, low-dose; HD, high-dose; CPT-11, irinotecan; FBAL, 4-pentyloxycarbonyl-5-fluoro-β-alanine; 5-FCOH, 5-fluorocytosine; 5-FC, 5-fluorouracil; 5-FU, 5-fluorouracil; 5'-DFUR, 5'-fluoro-6-hydroxycytosine; Cr(acac)\(_3\), chromium (III) acetylacetonate; FBAL, α-fluoro-β-alanine; 5-FUH\(_2\), 5,6-dihydro-5-fluorouracil; FUPA, α-fluoro-β-ureidopropionic acid; FHPA, 2-fluoro-3-hydroxypiperidine acid; PCA, piperidinic acid; AS, acid-soluble; AI, acid-insoluble; B.W., body weight; LD, low-dose; HD, high-dose; CPT-11, irinotecan; FBEN, sodium parafluorobenzoate; Ucap, unknown compound 0.03 ppm downfield from the CAP signal; Ufu, unknown compound close to the 5-FU signal; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; LC-MS, liquid chromatography/mass spectrometry; COSY, correlation spectroscopy; a.d., administered dose; OFBAL, N-carboxy-α-fluoro-β-alanine; δ, chemical shift; τ\(_T\), longitudinal relaxation time; τ\(_R\), retention time.

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Novel metabolites of capecitabine are shown in boxes.

Materials and Methods

Chemicals. 5-FU, 5-FC, fluorooacetate (FAC), hydroxypropylmethyl-cellulose 2910, phenolphtalein glucuronic acid, d-saccharic acid 1,4-lactone, -D-glucuronidase (EC 3.2.1.31) from bovine liver (type B-10) and from Helix pomatia (type H-1) were purchased from Sigma and chromium (III) acetylacetonate (Cr(acac)₃) from Aldrich (all from Sigma-Aldrich Chimie, Saint Quentin Fallavier, France). -Fluoro-alanine (FBAL) hydrochloride was provided by Tokyo Kasei Chemicals (Tokyo, Japan). 5,6-Dihydro-5-fluorouracil (5-FUH₂), 5'-DFUR, 5'-DFCR and 5-FCOH were generously supplied by F. Hoffmann-La Roche (Basel, Switzerland). CAP was a gift of Professor Walter Wolf, University of Southern California (Los Angeles, CA). -Ureidopropionic acid (FUPA) was prepared by chemical opening of 5-FUH₂ pyrimidine ring in 1 M NaOH (Malet-Martino et al., 1986). 2-Fluoro-3-hydroxypropionic acid (FHPA) was synthesized as described by Arellano et al. (1998). All other chemicals were reagent grade and obtained from standard commercial sources.

IPRL Experiments. Male Wistar rats (Iffa Credo, Lyon, France) were used. The IPRL experiments have been described previously (Arellano et al., 1997). CAP was injected into the perfusate after 1 h of liver equilibration, and the experiments were continued for 3 h. At the end of the experiments (n = 5), the perfusate was freeze-dried, stored at −80°C, and resuspended in H₂O to a known volume close to 3 ml immediately before ¹⁹F NMR analysis. Bile samples were gathered (1.12 ± 0.26 ml) and diluted with H₂O to a known volume close to 2.5 ml, then stored at −80°C until analysis. Liver was weighed (13.6 ± 1.2 g), immersed in liquid nitrogen, powdered, and sequentially extracted with cold and hot 1 M perchloric acid (PCA) by using the method of Wain and Staatz (1973). The acid-soluble (AS) and acid-insoluble (AI) fractions thus obtained were lyophilized to dryness and stored at −80°C until analysis. The lyophilized materials were resuspended in a known volume of H₂O containing 30 mM EDTA close to 3 ml and centrifuged immediately before ¹⁹F NMR analysis. The pH of the supernatant was adjusted to 5.5.

In Vivo Experiments, Rat and Mice Urine. CAP was dissolved in an aqueous solution of 0.5% hydroxypropylmethyl cellulose immediately before oral administration. Six rats simultaneously treated with 80 mg/kg B.W. of CAP (1 ml of the above-mentioned solution) were sequentially anesthetized per pair at 1, 2, or 3 h. The abdomen was then opened and whole blood collected. Bloodless liver and kidneys were excised and immersed in liquid nitrogen, then maintained at −80°C until PCA extractions were performed. Three experimental groups were named rats, low-dose (LD) mice and high-dose (HD) mice. Each treated rat was considered as an individual experiment of the rat group, whereas a cohort of 3 to 4 mice treated and maintained together to collect a sufficient volume of urine was considered as an individual experiment of the mice groups. Numbers of experiments were 4, 4, and 3 in rats, LD mice, and HD mice groups, respectively. Rats weighing between 380 and 440 g and mice weighing 35 to 4 g were orally administered with 1 and 0.5 ml of CAP solution, respectively. Rats and LD mice received a dose of 80 mg/kg B.W. and HD mice 500 mg/kg B.W. The animals were housed in metabolism cages and urine collected into containers after 6, 24, 48, and 72 h.
following CAP administration. The volumes were recorded and the samples stored at \(-80^\circ\text{C}\) until \(^{19}\text{F}\) NMR analysis. Urine was freeze-dried and resuspended in \(\text{H}_2\text{O}\) to a known volume close to 3 ml immediately before \(^{19}\text{F}\) NMR analysis.

**Human Urine.** Eleven colorectal cancer patients received an intravenous injection of 200 to 250 mg/m\(^2\) irinotecan (CPT-11) over 30 min followed 24 h later by CAP administered orally at a dose of 1000 to 1250 mg/m\(^2\) twice daily at 12 h interval. Among these patients, four received a second treatment three months later, and the urine was included in the study. A part (33 \(\pm\) 10 ml) of the total volume of urine (500 \(\pm\) 300 ml) collected over 12 h after the first CAP dose was freeze-dried, stored at \(-80^\circ\text{C}\), and resuspended in \(\text{H}_2\text{O}\) to a known volume close to 3 ml immediately before \(^{19}\text{F}\) NMR analysis.

**Control Experiments.** To check that the formation of fluorinated compounds was a metabolic process rather than a chemical transformation of CAP taking place during the perfusion time, several control experiments were carried out. CAP or \(^5\text{D}-\text{DFCR}\) was added to a perfusate collected from a blank IPR5 experiment and maintained under standard warming and gassing for 3 h. Only the signal arising from CAP or \(^5\text{D}-\text{DFCR}\) was detected when perfusates were analyzed.

We did not observe any changes in the \(^{19}\text{F}\) NMR spectra of solutions of CAP, \(^5\text{D}-\text{DFUR}\), \(^5\text{F}-\text{FU}\), or FBAL, when submitted to conditions of cold and hot PCA extractions.

\(^{19}\text{F}\) NMR Spectroscopy and Quantification. \(^{19}\text{F}\) NMR spectra were recorded at 282.4 MHz with \(^1\text{H}\)-decoupling on a Bruker WB-AM 300 spectrometer using 10-mm diameter NMR tubes (Bruker S.A., Wissembourg, France). The recording conditions were: probe temperature, 25\(^\circ\text{C}\); sweep width, 41,667 Hz; 32,768 data points zero-filled to 65,536; pulse width, 7 \(\mu\text{s}\) (flip angle \(\approx 40^\circ\)) in bile and urine, \(\sim 30^\circ\) in PCA extracts, and \(\sim 20^\circ\) in concentrated perfusates; pulse interval, 1.4 s for quantification of perfusates, AS and AI extracts, or 3.4 s for quantification of bile and urine samples; number of scans, 10,000 to 50,000; line broadening caused by exponential multiplication, 5 Hz. The chemical shifts (\(\delta\)) were reported relative to the resonance peak of CF\(\text{COO}^-\) (5% w/v aqueous solution) used as external chemical shift reference (\(\delta = 0 \text{ ppm}\)).

The concentrations of the fluorinated compounds were measured by comparing the expanded areas of their respective NMR signals with that of the external standard for quantification placed in a coaxial capillary, namely a paring the expanded areas of their respective NMR signals with that of the external standard compound for CAP, \(^5\text{D}-\text{DFCR}\) (see below), since cross-saturation of these three media led to a single \(^{19}\text{F}\) signal. Two minor signals could not be attributed; they have been named Ucap and Ufu according to their vicinity with the signal of a known compound.

**Isolation and Purification of \(^5\text{D}-\text{DFCR-G.}\)** Prior HPLC analysis, crude bile (3 ml) and liver AS extract (3 ml) samples of two in vivo experiments were separately pooled and each subjected to a cleaning procedure. In brief, three volumes of dichloromethane were added; after extraction, the aqueous phases were collected and reextracted with ethyl acetate. The aqueous phases were then lyophilized. The dry residues were diluted in 400 \(\mu\text{l}\) of \(\text{H}_2\text{O}\) and then placed at the top of a 10 cm-length glass column of 0.8-cm diameter, which had already been packed with Matrix Silica C\(\text{as}\) (50 \(\mu\text{m}\) particle size and 60 \(\AA\) pore diameter; Millipore Corp., Danvers, MA). Elution of the column was carried out by water (5 \(\times\) 1 ml fractions) and then by a water-methanol (80:20 \(\text{v}/\text{v}\)) mixture (6 \(\times\) 1 ml fractions). Each fraction was analyzed by \(^{19}\text{F}\) NMR. Fractions 4 and 8 contained the unknown metabolite and \(^5\text{D}-\text{DFCR}\), respectively.

Fractions 4 were used for further fine purification by HPLC with a Waters 2695 Alliance chromatographic system (Waters, Milford, MA) under the following conditions: column, C\(\text{as}\) ProntoSIL (250 \(\times\) 4 mm i.d., 50 \(\mu\text{m}\) particle size; Bischoff Chromatography, Leonberg, Germany) maintained at 35\(^\circ\text{C}\); mobile phase, mixture of acetonitrile/water with 0.1% trifluoroacetic acid (TFA) in 5:95 ratio for the first 8.5 min and then in 90:10 ratio for 3 min to wash out the column; flow rate, 1.0 ml/min; detection, UV absorbance at 280 nm with a diode array detector (Waters 996). Several collections of the major peak at a retention time (\(R_t\)) of 6.1 min were done. After checking the purity of the isolated compound by HPLC, the effluents were freeze-dried and stored at \(-80^\circ\text{C}\) until enzymatic hydrolysis and \(^3\text{H}\) NMR.

**Enzymatic Hydrolysis of \(^5\text{D}-\text{DFCR-G.}\)** \(\beta\)-\text{d-Glucuronidase from bovine liver or } \(H. \text{ pomatia}(1000 \text{ units}) \text{ was added to the purified compound from liver or bile dissolved in 0.2 ml of 0.2 M acetate buffer (pH 5.0). Incubations were run overnight in a shaking water bath at 37\(^\circ\text{C}\). At the end of the incubation period, remaining \(\beta\)-glucuronidase activities were checked with phenolphthalein glucuronic acid. Parallel incubations were conducted in the absence of \(\beta\)-glucuronidase (control), and in the presence of \(\beta\)-glucuronidase previously inactivated by heating (20 min at 80\(^\circ\text{C}\)), or in which \(\alpha\)-saccharic acid 1,4-lactone was included in the incubation medium. The HPLC analysis showed that the unknown compound at \(R_t = 6.1\) min disappeared when subjected to \(\beta\)-glucuronidase. No change in the chromatogram was observed in assays without enzyme or with enzyme denatured by heating or inhibited by \(\alpha\)-saccharic acid 1,4-lactone. In the presence of \(\beta\)-glucuronidase, the peak at 6.1 min disappeared in favor of a compound giving a peak at \(R_t = 6.6\) min and identified as \(^5\text{D}-\text{DFCR}\) with authentic standard. Some chromatograms are presented in Fig. 2.

**LC-MS and \(\text{H}^3\text{NMR Analysis of } \(^5\text{D}-\text{DFCR-G.}\)** LC-MS was carried out in positive mode on a PerkinElmer Sciex API 365 triple quadrupole mass spectrometer (PerkinElmerSciex Instruments, Boston, MA) equipped with a turboionspray source operating at 450\(^\circ\text{C}\). Nitrogen served both as auxiliary and collision gas and air as nebulizer gas. The HPLC system used consisted of a PE series 200 LC pump, a Rheodyne 8125 injector valve (40-\(\mu\text{l}\) loop; Rheodyne, Rohnert Park, CA) and a PE 785 UV-Vis detector (\(\lambda = 280\) nm), both 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. MS analysis showed that the glucuronide with a \(R_t = 6.1\) min exhibited a characteristic ion at \(m/z\) 422 [M + H\(^+\)], suggesting a molecular mass of 421 Da, namely that of a glucuronide of \(^5\text{D}-\text{DFCR}\).

\(^3\text{H}\) NMR analysis was performed on a Bruker 400 MHz spectrometer with 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid as internal chemical shift reference (\(\delta = 0.00\) ppm). \(^{19}\text{F}\) NMR spectra of both the glucuronides purified from bile or liver dissolved in \(\text{D}_2\text{O}\) were identical. The signal at 7.76 ppm (d, \(J = 0.00\) ppm) was not assigned because the difference in chemical shift at \(1223\) ppm downfield from the signal of \(^5\text{D}-\text{DFCR\) is compared to the same compound, which is a glucuron conjugate of \(^5\text{D}-\text{DFCR\} (see below), since cross-}

\text{spiking of these three media led to a single } ^{19}\text{F signal. Two minor signals could not be attributed; they have been named Ucap and Ufu according to their vicinity with the signal of a known compound.}\n
\text{2.4 Hz) of the ribose moiety. In conclusion, data
Results

Isolated Perfused Rat Liver and in Vivo Experiments. IPRL were treated with CAP for 3 h at 80 mg/kg B.W., which corresponds to a mean value of 6.8 ± 0.5 nmol/g of liver wet weight. Typical 19F NMR spectra of perfusate, bile, and acid extract samples are presented in Fig. 3. Data of the quantitative analysis are reported in Table 1.

In perfusates, 5′-DFCR was by far the main product making up 86% of fluorinated compounds. Signals of 5′-DFUR, 5-FC, 5-FCOH, CAP, 5′-DFCR-G, and an unknown compound (Ucap) at 0.03 ppm downfield from the CAP signal were also observed (Fig. 3A).

In AS fractions of liver extracts (Fig. 3B), the major compounds were 5′-DFCR and 5′-DFCR-G with mean concentrations of 254 and 162 nmol/g wet weight, respectively. CAP, 5-FC, FBAL, 5-FCOH (observed in 1 experiment of 5) and two other unknown compounds, Ucap and Ufu, were measured in those fractions. In AI fractions, only very low amounts of 5′-DFCR, 5′-DFCR-G and 5-FU were measured. AS fractions accounted for 7 ± 1% of administered dose (a.d.), whereas AI fractions represented 0.1 ± 0.05% a.d. Concentration values of 5′-DFCR measured in the perfusates collected at the end of the experiments (394 nmol/ml) and estimated in the liver (362 nmol/ml of tissue total water) were not significantly different (p > 0.4) (Table 1).

A typical spectrum of a bile sample collected over 3h after the addition of CAP to the perfusion medium is presented in Fig. 3C. Four fluorinated compounds were excreted in bile, 5′-DFCR-G, 5′-DFCR, CAP, and Ucap. In contrast to perfusate and PCA extracts, 5′-DFCR-G was the main fluorinated compound making up 64% of the biliary fluorinated compounds, above 5′-DFCR which amounted to 22% (Table 1).

To confirm that the metabolism of CAP was similar in rat under in vivo conditions, liver, bile, plasma, and kidneys of rats killed at 1, 2 or 3h following the administration of 80 mg/kg B.W. of CAP were analyzed. The metabolic profile was simpler than in IPRL experiments. 5′-DFCR, FBAL and 5′-DFCR-G were observed in liver AS fractions with mean concentrations of 24, 10, and 37 nmol/g wet weight, respectively after 3h. Bile contained 5′-DFCR-G as the main compound (91%) and a low amount of 5′-DFCR (9%). 5′-DFCR and FBAL were detected in kidney AS fractions at mean concentrations of 125 and 26 nmol/g wet weight. (i.e., 162 and 34 nmol/ml, respectively after 3h). Only 5′-DFCR was detected in plasma. In both liver and plasma, concentration of 5′-DFCR markedly decreased with time (Table 2). When the plasma 5′-DFCR concentration value for each rat was plotted against the corresponding estimated liver 5′-DFCR concentration value in nanomoles per milliliter, a linear relationship was observed (Fig. 4). The high correlation factor (r = 0.992) and the slope (1.00) demonstrated that, at equilibrium, 5′-DFCR concentration was equal in liver and plasma.

Analysis of Urine from Rats and Mice. Urine from rats and mice treated with CAP have been fractionally collected over 72 h. The dose was 80 mg/kg B.W. for rat and LD mice groups and 500 mg/kg B.W. for the HD mice group. Typical spectra of urine from the 6 to 24 h collections are presented in Fig. 5. A–C. CAP and its metabolites, 5′-DFCR and 5′-DFUR, were found in all urine samples. 5-FU and its first catabolite, 5-FUH₂, were detected in three of four experiments for LD mice and in all experiments for HD mice, whereas 5-FU was barely detected and 5-FUH₂ never detected in urine of rats. FUPA was barely detected in the 0 to 6 h urine collections of rats whereas it...
was detected in all urine collections of mice. FBAL was found in all urine samples whatever the species or the urine fraction. N-carboxy-\(\alpha\)-fluoro-\(\beta\)-alanine (CFBAL) was detected mainly in urine of rats because they were alkaline (Martino et al., 1987). Indeed, urine collections of rats had a mean pH value of 8.2 ± 0.8 (\(n = 18\)), which was significantly higher than the mean pH values of 7.2 ± 0.8 (\(n = 15\)) and 7.3 ± 0.6 (\(n = 16\)) for urine of LD mice and HD mice groups, respectively (\(p < 0.001\)). FHPA was detected in one of the four experiments for rats or LD mice but in all experiments for HD mice. 5-FC and its hydroxylated metabolite, 5-FCOH, were found in urine of rats and mice. 5-FCOH was never detected when 5-FC was not excreted in urine. Two unknown compounds, one in rat urine and the other in mice urine, giving rise to weak signals at -107.2 ppm and -108.8 ppm, respectively, were also detected in some samples. Table 3 gives the amounts of fluorinated metabolites excreted in the successive collections of urine from rats and mice expressed as percentages of a.d. In urine of rats, as observed in IPRL experiments, 5'-DFCR provided the bulk of fluorinated compounds, making up 78% of the excreted metabolites over 72 h. CAP, 5'-DFUR, FU, FUPA + FBAL + CFBAL, and fluoride ion (F\(^-\)) represented 5, 4, 0.1, 5, and 7%, respectively. The sum 5-FC + 5-FCOH contributed to 0.7% with a 5-FCOH/5-FC ratio of 1.7 ± 0.5 (\(n = 3\)). 5-FC and 5-FCOH were only present in the 0 to 6 and 6 to 24 h urine collections. 84% a.d. were excreted in 72 h, with 45% a.d. in the first 6 h. In contrast, 5'-DFUR and FUPA + FBAL were the main CAP metabolites in urine of mice, making up 35 and 28% of the excreted compounds over 72 h in LD mice and 32 and 33% in HD mice. 5'-DFCR accounted for 19 and 20% in the LD and HD mice groups, respectively. CAP, FU, FUH\(_2\), and F\(^-\) represented, respectively, 14, 0.2, 0.2, and 4% of excreted metabolites in LD mice and 11, 0.2, 0.4, and 2% in HD mice. Contribution of FHPA was minor with a maximum observed in the 6 to 24 h urine collection for HD mice yielding 0.05% of excreted compounds. 5-FC was detected in 2 of 4 experiments for LD mice and only in the two first fractions, whereas 5-FCOH and 5-FC were detected in all fractions in HD mice. The sum 5-FC + 5-FCOH accounted for 0.3 and 1% of compounds excreted over 72 h with a 5-FCOH/5-FC ratio of 1.8 (\(n = 1\)) and 1.8 ± 0.6 (\(n = 3\)) for LD and HD mice, respectively. In LD mice, 86% a.d. were

**Fig. 3.** Typical 1H-decoupled 19F NMR spectra from an IPRL treated with capecitabine (80 mg/B.W.) for 3h: A, perfusate, pH = 8.2; B, acid-soluble extract, pH = 5.5; C, bile, pH = 8.4. Region of interest surrounding the 5'-DFCR peak was expanded to discriminate the signal of 5'-DFCR and 5'-DFCR-G. S, doublet arising from the 13C-19F coupling.
excreted over 72 h with 59% a.d. in the 0 to 6 h fraction. In the HD mice group, excretion of fluorinated compounds was 18% a.d. in the 0 to 6 h collection then peaked at 39% a.d. in the 6 to 24 h urine collection for a total of 73% a.d. over 72 h.

Data are means ± S.D. expressed as % a.d. Concentrations of compounds in perfusate are expressed relative to the volume of perfusate recovered at the end of the IPRL experiment before lyophilization. Concentrations of compounds in liver extracts and bile are expressed as nanomoles per gram wet weight; for AS extracts, concentrations are also given as nanomoles per milliliter of tissue water (μM); see Materials and Methods section for further details. Significance of differences from the concentration measured in the perfusate, * p < 0.04; † p < 0.005.

**TABLE 1**

Metabolites quantification carried out by [19F NMR in perfusate, bile, AS and AI fractions from IPRL experiments (n = 5)

![Image](https://via.placeholder.com/150)

**FIG. 4.** Correlation between 5'-DFCR concentrations measured in liver versus plasma.

Blood and liver from six rats submitted by pair to the same dose of CAP for 1, 2, or 3 h were taken to measure the amounts of 5'-DFCR in the plasma and AS extract, respectively. Values of 5'-DFCR concentration determined in the AS extract of liver are estimated in nanomoles per milliliter of tissue water (μM).

**TABLE 2**

Metabolite concentration versus time in liver and plasma of rats after administration of capecitabine (80 mg/kg B.W.) (mean of two experiments)

![Image](https://via.placeholder.com/150)

**FIG. 5.** Typical ¹H-decoupled ¹⁹F NMR spectra of urine samples.

A and B, fractions 6 to 24 h from rats and mice treated with oral capecitabine at 80 mg/B.W. A, rat (pH = 8.5); B, mice (pH = 6.25); C, fraction 6 to 24 h from mice treated with oral capecitabine at 500 mg/B.W. (pH = 6.8); and D, fraction 0 to 12 h of urine from a patient treated with a single oral dose of 1900 mg of capecitabine (pH = 5.5).
TABLE 3
Metabolites of capecitabine in urine from rats or mice collected over 72 h after administration of capecitabine at a dose of 80 mg/kg B.W. for rat and LD mice groups and 500 mg/kg B.W. for the HD mice group

<table>
<thead>
<tr>
<th></th>
<th>0–6 h</th>
<th>6–24 h</th>
<th>24–48 h</th>
<th>48–72 h</th>
<th>Over 72 h</th>
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<tr>
<td>CAP</td>
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<tr>
<td>Rat</td>
<td>3.5 ± 0.7</td>
<td>0.79 ± 0.44</td>
<td>0.02 ± 0.03</td>
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<tr>
<td>LD mice</td>
<td>10 ± 3*</td>
<td>1.4 ± 0.4*</td>
<td>0.09 ± 0.08*</td>
<td>12 ± 2*</td>
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<tr>
<td>HD mice</td>
<td>3.3 ± 3.8†</td>
<td>3.5 ± 0.6†</td>
<td>0.73 ± 0.26†</td>
<td>7.7 ± 2.6†</td>
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<tr>
<td>5'-DFCR</td>
<td>37 ± 15†</td>
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<td>2.9 ± 1.0</td>
<td>65 ± 11</td>
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<tr>
<td>Rat</td>
<td>14 ± 2*</td>
<td>1.7 ± 0.8*</td>
<td>0.09 ± 0.08*</td>
<td>16 ± 1*</td>
<td></td>
</tr>
<tr>
<td>HD mice</td>
<td>5.4 ± 5.4†</td>
<td>6.9 ± 1.9†</td>
<td>1.3 ± 0.7†</td>
<td>14 ± 2</td>
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<tr>
<td>5'-DFUR</td>
<td>1.9 ± 1.6</td>
<td>1.2 ± 0.3</td>
<td>0.16 ± 0.09</td>
<td>3.3 ± 2.1</td>
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</tr>
<tr>
<td>Rat</td>
<td>23 ± 2*‡</td>
<td>6.2 ± 1.7*</td>
<td>0.45 ± 0.26*</td>
<td>30 ± 1*</td>
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<tr>
<td>HD mice</td>
<td>6.1 ± 5.1†</td>
<td>13 ± 3†</td>
<td>3.5 ± 1.9</td>
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<td>5-FU</td>
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<td>0.10 ± 0.12</td>
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<tr>
<td>LD mice</td>
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<td>0.19 ± 0.24</td>
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<tr>
<td>HD mice</td>
<td>0.01 ± 0.01</td>
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<td>0.14 ± 0.02</td>
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<tr>
<td>5-FUH₂</td>
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<td></td>
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<tr>
<td>Rat</td>
<td>0.12 ± 0.11</td>
<td>0.07 ± 0.06</td>
<td>0.06 ± 0.04</td>
<td>0.19 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>HD mice</td>
<td>0.05 ± 0.06</td>
<td>0.15 ± 0.02</td>
<td>0.26 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUPA + FBAL</td>
<td>1.5 ± 0.5</td>
<td>2.4 ± 1.1</td>
<td>0.35 ± 0.12</td>
<td>4.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>11 ± 1*</td>
<td>11 ± 0.5*</td>
<td>1.2 ± 0.4*</td>
<td>24 ± 2*</td>
<td></td>
</tr>
<tr>
<td>HD mice</td>
<td>3.0 ± 2.8†</td>
<td>14 ± 2*</td>
<td>6.2 ± 2.9†</td>
<td>24 ± 1</td>
<td></td>
</tr>
<tr>
<td>5-FC + 5-FCOH</td>
<td>0.14 ± 0.04</td>
<td>0.47 ± 0.35</td>
<td>0.61 ± 0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>0.06 ± 0.08</td>
<td>0.22 ± 0.17</td>
<td>0.28 ± 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HD mice</td>
<td>0.07 ± 0.04</td>
<td>0.50 ± 0.07†</td>
<td>0.77 ± 0.19†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F⁻</td>
<td>0.64 ± 0.26</td>
<td>1.8 ± 0.4</td>
<td>1.8 ± 1.3</td>
<td>1.5 ± 0.5</td>
<td>5.7 ± 1.3</td>
</tr>
<tr>
<td>Rat</td>
<td>0.64 ± 0.37</td>
<td>1.0 ± 0.4*</td>
<td>1.2 ± 0.7</td>
<td>3.4 ± 0.7*</td>
<td></td>
</tr>
<tr>
<td>HD mice</td>
<td>0.05 ± 0.06†</td>
<td>0.73 ± 0.25</td>
<td>0.48 ± 0.15†</td>
<td>1.7 ± 0.2†</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>45 ± 17</td>
<td>31 ± 8</td>
<td>5.3 ± 1.7</td>
<td>2.3 ± 0.7</td>
<td>84 ± 10</td>
</tr>
<tr>
<td>Rat</td>
<td>59 ± 6</td>
<td>22 ± 3</td>
<td>3.0 ± 0.7</td>
<td>1.8 ± 1.2</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>LD mice</td>
<td>18 ± 17†</td>
<td>39 ± 4†</td>
<td>13 ± 6†</td>
<td>3.3 ± 1.9</td>
<td>73 ± 6†</td>
</tr>
</tbody>
</table>

Data are expressed as % a.d. Data in bold characters represent the main metabolite in each urine fraction or in total collection.

‡ Significant difference between rat and LD mice groups (p < 0.05).
† Significant difference between LD mouse and HD mice groups (p < 0.05).

Analysis of Human Urine. Urine (n = 14; pH 5.7 ± 0.3) of patients collected over 12 h after the first daily dose of CAP were analyzed. Urinary recovery of CAP and its metabolites accounted for 71 ± 17% a.d. The major metabolite was by far FBAL accounting for 46 ± 4% a.d. The other main compounds were CAP (3 ± 1% a.d.), 5'-DFCR (10 ± 3% a.d.), 5'-DFUR (7 ± 2% a.d.), and FUPA (4 ± 1% a.d.). 5-FU (0.6 ± 0.2% a.d.) and capecitabine, 5-FUH₂ (0.3 ± 0.2% a.d.), F⁻ (0.2 ± 0.3% a.d.), FHPA (0.3 ± 0.1% a.d.), and FAC (in 4 of 14 samples analyzed; (0.004 ± 0.002% a.d.) were minor metabolites. 5-FC and 5-FCOH were observed in 4 over 14 urine samples accounting for 0.01 ± 0.002% a.d. and 0.02 ± 0.002% a.d., respectively. A spectrum of a concentrated urine where 5-FC and 5-FCOH were detected is presented in Fig. 5D.

Discussion

The main metabolites of the activation pathway of CAP are 5'-DFCR and 5'-DFUR that finally yields 5-FU (Bajetta et al., 1996; Reigner et al., 2001). Up to now, 5'-DFCR has been described as a simple intermediary metabolite. However, the formation of 5-FC, 5-FCOH, and 5'-DFCR-G supports that several metabolites diverge from the CAP activation pathway at the level of 5'-DFCR.

5-FC and 5-FCOH are found in the liver and perfusion medium of IPRL experiments, in urine of rats and mice, and in some human urine samples. The presence of 5-FC raises the question of the mechanism responsible for its synthesis from 5'-DFCR. A spontaneous formation of 5-FC from 5'-DFCR can be excluded regarding the stability of 5'-DFCR (see Materials and Methods). The metabolism of 5'-DFCR into 5-FC is rather the result of a non-specific liver oxidation system than that of the action of uridine nucleoside phosphorylase for which cytidine is not a substrate (Yamada, 1978). A participation of the intestinal flora to the formation of 5-FC is probably minimal as this metabolite is observed in IPRL experiments. Nevertheless, it cannot be ruled out as 5-FC is detected in urine. 5-FCOH is a direct catabolite of 5-FC as it is observed only if 5-FC is formed, therefore excluding a chemical or enzymatic process from 5'-DFCR. 5-FCOH was initially observed in urinary gravel, then in urine of patients treated with 5-FC (Williams et al., 1981; Vialaneix et al., 1987).

5'-DFCR-G is formed in rat liver and exclusively excreted in bile. Cellular leak inherent to damages induced during the perfusion is likely responsible for the small amount of 5'-DFCR-G found in the perfusate medium from IPRL experiments (Table 1). The low biliary excretion found in IPRL experiments (2.2% a.d.) and the low recovery of radioactive dose measured in the feces after administration of 14C-CAP to humans (2.6%) (Judson et al., 1999) suggest that, if glucuronidation occurs in humans, it would be a minor CAP detoxification pathway. Since cholestasis has not been described as a side effect of CAP treatment, it is likely that 5'-DFCR-G does not affect
the physicochemical properties of bile in contrast to what has been observed with a biliary conjugate of FBAL (Sweeny et al., 1987).

The third activation step of CAP leads to the formation of 5-FU that occurs mainly via uridine phosphorylase in rodents and thymidine phosphorylase in humans (Ninomiya et al., 1990). FBAL, the main catabolite of 5-FU, is formed since dihydroxyirimidine dehydrogenase (E.C. 1.3.1.2), the rate-limiting enzyme of the catabolic pathway of 5-FU, has a high activity in the liver. In healthy animals, 5-FU catabolites account for ~10% and ~26% to 28% a.d. in rats and mice treated with CAP, respectively. In contrast, 5-FU catabolites account for ~50% a.d. in human urine. A higher cytidine deaminase activity in humans (Ho, 1973) combined with an enhancement of the thymidine phosphorylase level in tumor tissue (Mriwa et al., 1998) could account for the high formation of 5-FU and catabolites observed in human urine. It should be pointed out that CAP, as 5-FU (Lemaire et al., 1996; Arellano et al., 1997), leads to the formation of low amounts of two FBAL metabolites, FHPA and FAC, even in humans.

Our results support the involvement of a plasma-membrane transporter for CAP and 5′-DFCR as the Na+-independent equilibrative nucleoside transporter, es-type transporter (Balimane and Sinko, 1999). Indeed, in vivo experiments confirm that 5′-DFCR is present at the same concentration in liver and plasma (Fig. 4). In contrast, we observed that 5′-DFCR concentrations 3 h after CAP administration were four to five times higher in kidneys than in plasma supporting another type of transporter specific to that organ allowing an efficient secretory function (Chen and Nelson, 2000).

In IPRL experiments, CAP is forced only toward the liver metabolism whereas under in vivo conditions CAP administered orally must pass the intestinal barrier to reach the liver and then spread out the body. Therefore, CAP excreted in the urine depends on gastrointestinal, hepatic, extra-hepatic metabolisms, and renal clearance efficiency. Recoveries of drug-related materials in the urine are not significantly different between rats and mice when administered at the same dose, 84 ± 10 and 86 ± 1% a.d., respectively (p > 0.4) (Table 3), which is consistent with a nearly complete and reliable absorption of CAP from the gastrointestinal tract in these species as already observed in other studies with animals or humans (Verweij, 1999; Schüller et al., 2000). Even for a six-fold higher dose (500 mg/kg), the mean urinary recovery is 73 ± 6% a.d. in mice (Table 3).

The remaining of drug-related compounds could be eliminated via feces as intact drug, 5′-DFCR and 5′-DFCR-G since these compounds are detected in the bile from IPRL and in vivo experiments. CAP excreted in urine over 72 h is significantly higher in mice than in rats (p < 0.005). Discrepancies of susceptibility of CAP to carboxylesterase between tissues, particularly intestine and liver, and species which have been described (Shimma et al., 2000) obviously accounts for such a difference with likely a higher carboxylesterase activity in rats than in mice.

In rats, however, in spite of a higher initial activation process of CAP, the subsequent activation step is limited. 5′-DFCR accumulates in the perfusion medium of IPRL experiments whereas the amount of 5′-DFUR is weak, leading to a high 5′-DFCR/5′-DFUR concentration ratio in the liver (Table 1). The same phenomenon is observed in urine where this ratio is close to 20 over 72 h (Table 3). The high 5′-DFCR/5′-DFUR ratio due to a very weak concentration of 5′DFUR leads to a low reaction rate through the uridine phosphorylase, regarding the $K_m$ value for uridine, which is 140 and 240 μM in mice and rat livers, respectively (Yamada, 1978; Naguib et al., 1987). As a result, a weak amount of FBAL, the main catabolite of 5-FU, is found in liver and urine of rats. Therefore, CAP activation appears to be blocked at the cytidine deaminase step. This confirms the low cytidine deaminase activity in rat liver (Camener and Smith, 1965) and suggests a lack of an extra-hepatic cytidine deaminase activity in rat in agreement with previous studies on other 5′-DFCR-related compounds such as galocytabine (Ninomiya et al., 1990; Funaki et al., 1993). On the contrary, the high activity of cytidine deaminase is revealed by the low value of the 5′-DFCR/5′-DFUR ratio (<1) in mice treated with a LD or a HD of CAP. The 5′-DFCR/5′-DFUR ratio could therefore be used as an indirect indicator of cytidine deaminase activity.

Pretreatment of patients with CPT-11 does not sensibly affect the pattern of metabolites excreted in urine. Indeed, our results are in agreement with the sole $^{19}$F NMR study of CAP metabolism that reports urine analysis from 6 patients treated with a single oral dose of 2 g of CAP (Judson et al., 1999). The same metabolites (CAP, 5′-DFCR, 5′-DFUR, 5-FU and its classical metabolites FUH$_2$, FUPA and FBAL) in close percentages of a.d., except for FBAL, are detected. Our analysis is limited to a period of 12 h after CAP administration versus 48 h in the Judson’s study. This can explain the difference between the amount of FBAL found here (46 ± 4% a.d.) compared with 57 ± 5% a.d. as FBAL has a long half-life time. Regarding schedule treatment (administration of CPT-11 and CAP at 24 h interval), and CPT-11 pharmacokinetic parameters (Rivory et al., 1997), a possibility of drug interaction seems limited. Moreover, although both drugs share an esterase-mediated activation via a carboxylesterase, there is no interference of CAP on the formation of SN-38, the active metabolite of CPT-11, in human liver microsomes (Charasson et al., 2002). In addition, the tissue distribution of carboxylesterase activities toward CPT-11 and CAP suggest that the enzymes responsible for CAP (Shimma et al., 2000; Tsukamoto et al., 2001) and CPT-11 (Guichard et al., 1999; Khanna et al., 2000) activations are different. However, although CAP has limited effect on the formation of the glucuronide of SN-38 (Charasson et al., 2002), a competition between drugs at the level of the glucuronidation reaction should be reinvestigated in the light of a possible formation of a glucuronide of 5′-DFCR.

This study illustrates the advantages of $^{19}$F NMR applied to fluorinated drug metabolism and disposition. It allows a direct study of any biological medium without prior treatment avoiding the problems encountered in extraction recovery and chemical derivatization. It leads to the simultaneous detection and quantification in a single run of all-fluorine-containing compounds even unexpected substances. This contrasts with chromatography that usually requires some prior knowledge of metabolite structure to optimize sample preparation and/or detection. Set against these advantages, the method presents a main drawback, which is its relatively low sensitivity compared with chromatographic and other spectroscopic techniques (∼2 μM with a currently available spectrometer). Moreover, the unequivocal elucidation of the structure of unknown metabolites requires their isolation if a nonhydrophenated method such as HPLC-NMR is not employed.

In conclusion, several new metabolites of CAP have been detected using $^{19}$F NMR spectroscopy. Three of them are 5-FU catabolites already observed in the 5-FU metabolic pathway (F−, FHPA, and FAC) (Martino et al., 2000). 5-FC and 5-FCOH are also minor CAP metabolites. A detoxification pathway, reported for the first time, leads to a glucuronide of 5′-DFCR in rat. A complemented metabolic pathway of CAP is thus proposed in Fig. 1. The extent of involvement and pharmacological consequences of these new catabolic ways on the treatment of patients remains to be clarified.

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References


