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New approach to metabolism of 5′-deoxy-5-fluorouridine in humans with fluorine-19 NMR

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Summary. *The metabolism of 5′-deoxy-5-fluorouridine (5′dFUrd), an antitumor fluoropyrimidine, has been investigated in human biofluids (blood, plasma, urine) using a new method: fluorine-19 NMR spectrometry. This method allows direct study of the biological sample and simultaneous identification of all the fluorinated metabolites. In the blood of a patient treated with 5′dFUrd during a 6-h continuous perfusion, we observed unmetabolized 5′dFUrd, 5-fluorouracil, 5,6-dihydrofluorouracil, and another metabolite which has not previously been reported α-fluoro-β-alanine. The two major metabolites in urine are unmetabolized 5′dFUrd and α-fluoro-β-alanine.*

Introduction

5′Deoxy-5-fluorouridine (5′dFUrd), a novel fluoropyrimidine, is an antitumor agent with a higher therapeutic index and lower toxicity than 5-fluorouracil (FUra), 5-fluorouridine (FUrd), 2′-deoxy-5-fluorouridine (dFUrd), and ftorafur [3, 5, 6, 8, 11, 13, 14]. 5′dFUrd produces less leukopenia [8, 11] and is less immunosuppressive than the other fluoropyrimidines [11, 17]. It has been suggested that 5′dFUrd is a prodrug from which FUra is generated by cellular nucleoside phosphorylase [4, 5, 13]. The mechanism of action and toxicity of 5′dFUrd are dependent upon its plasmatic and cellular metabolic pathway. Up to now, only chromatographic methods, viz. high-performance liquid chromatography (HPLC) [4–6, 9, 12, 18–20], thin-layer chromatography (TLC) [21], and combined gas chromatography and mass spectrometry (GC/MS) [7, 20] have been described for the determination of cellular or plasmatic metabolites of 5′dFUrd. These methods are very sensitive, but they require the use of labeled drugs [4–6, 12, 19, 21]; or, if cold drugs are used, they need extraction [9, 18, 20] or extraction plus derivatization [7, 9, 20].

The present communication reports an analysis of 5′dFUrd metabolite pools in human biofluids using a new and direct method: fluorine-19 NMR (^{19}F NMR). With this NMR method we have been able to identify a 5′dFUrd metabolite in the blood that has not previously been reported so far as we are aware, and to study 5′dFUrd metabolites in urine for the first time.

Materials and methods

Drugs and chemicals. 5′dFUrd, FUra, and 5,6-dihydrofluorouracil ($5FUH_2$) were generously supplied by Hoffmann-La Roche, Basel, Switzerland; α-fluoro-β-alanine (FBAL) was purchased from Koch-Light Labs.

Patient protocol. One patient, being treated for a carcinoma of the endometrium metastasizing to the lung, was evaluated in this study. She was randomly recruited from a group of patients treated in a phase II trial run by the EORTC Clinical Screening Group [2]. According to the trial protocol, 5′dFUrd (10 g/m^2 = 14 g) was dissolved in 500 ml 5% glucose isotonic solution and infused IV at a constant rate over 6 h. The patient had never received chemotherapy before and she did not receive any other drug, particularly fluorinated medication, during the study.

Blood sampling. Venous blood samples (5 ml) were taken from the contralateral arm and collected into heparinized tubes at specified times: $t = 0$ (pretreatment blood sample), 1 h, 3 h, 6 h (end of the perfusion), 7 h, 9 h, 12 h, 15 h, and 18 h after the beginning of the perfusion. A 2-ml aliquot of blood was immediately frozen and stored at −20° C until analysis. The 3-ml aliquot remaining was centrifuged at 4° C (4,000 rpm for 5 min). The plasma was then immediately frozen and stored at −20° C until analysis. The packed red cells were washed twice in 0.9% sodium chloride solution and, after centrifugation, were immediately frozen and stored at −20° C until analysis. An aliquot of the blood sample taken at 3 h was immediately analyzed without freezing.

Urine collection. Urines were collected over 24 h. A sample of urine was retained after each spontaneous micturition, and was frozen and stored at −20° C. The time of micturition was noted.

Analytical method. After thawing, the sample (blood, plasma, packed red cells, or urine) was placed into a 5-mm NMR tube. ^{19}F NMR spectra were recorded at 250 MHz on a CAMECA 250 FT spectrometer, without proton decoupling, and interfaced to a NICOLET 80 computer with 16 K memory.

For patient samples, typical spectral accumulation conditions were as follows: spectral width, 31,249.9 Hz (125 ppm); pulse, 60° (6 μs); delay between pulse and data acquisition, 100 μs; receiving filter out; probe temperature, regulated at 25° C. The spectra were acquired in 300 scans with a 4s repetition rate for urine samples, and in 6 blocks of 5,000 scans with a pulse interval of 1 s for the blood, plasma, and red cell samples. The computer resolution is 3.8 Hz/point in the former case and only 7.6 Hz/point in the latter. These conditions produce no saturation effects in the spectra.

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For reference compounds in H_2O or biological fluids (blood, plasma, urine), the spectra were recorded in 100 scans with a repetition rate dependent on their spin-lattice relaxation times. The computer resolution is 0.3–1 Hz/point. The stability of the magnetic field was such (deviation 8 Hz/24 h) that field frequency lock was not required.

Table 1. ^{19}F Chemical shifts[a] (δ, ppm) and H-F coupling constants (J, Hz) of authentic samples of 5'dFUrd, FUra, FBAL, and $5FUH_2$

		5'dFUrd	FUra	FBAL	$5FUH_2$
H_2O	δ	− 88.45	− 93.8	− 112.9	− 126.5
		dd	d	ddd	td
	J	$^3J = 6.3$	$^3J = 5.4$	$^2J = 50.4$	$^2J = 46.6$
		$^5J = 1.4$		$^3J = 27.95$	$^3J = 12.65$
				$^3J = 18.0$	
Blood	δ	− 89.5	− 93.9	− 112.6	− 126.4
		d	d	ddd	td
	J	$^3J = 5.1$	$^3J = 4.7$	$^2J = 50.5$	$^2J = 45.8$
				$^3J = 28.0$	$^3J = 11.8$
				$^3J = 18.5$	
Plasma	δ	− 89.3	− 93.8	− 112.9	− 126.6
		Broad	d	ddd	td
	J	signal	$^3J = 3.9$	$^2J = 50.6$	$^2J = 46.3$
				$^3J = 28.6$	$^3J = 10.7$
				$^3J = 18.2$	
Urine	δ	− 90.0	− 93.9	− 113.0	− 126.8
		d	d	ddd	td
	J	$^3J = 5.2$	$^3J = 5.2$	$^2J = 50.6$	$^2J = 46.8$
				$^3J = 28.5$	$^3J = 12.3$
				$^3J = 19.3$	

[a] Chemical shifts are related to external CF_3COOH (0.5% aqueous solution); negative numbers indicate upfield direction from the reference

d, doublet; t, triplet

The magnetic field was shimmed by using the ^1H NMR resonance of H_2O, which is always positioned arbitrarily for any sample (including the aqueous 0.5% CF_3COOH reference sample) at the same frequency.

The limit of sensitivity of the NMR technique was about 10^{-5} *M*.

Some preliminary experiments were accomplished at 188.3 MHz on a BRUKER AM 200 spectrometer (Bruker Spectrospin, Wissembourg) with proton decoupling in 5-mm diameter tubes. In particular, spin-lattice relaxation times of authentic samples of 5'dFUrd, FUra, $5FUH_2$, and FBAL were determined in the biofluids.

Results

Identification of 5'dFUrd and its metabolites in biofluids

^{19}F chemical shifts (δ) and H-F coupling constants (J) of authentic samples of 5'dFUrd, FUra, FBAL, and $5FUH_2$ in H_2O, blood, plasma, and urine are given in Table 1. The NMR characteristics of the different compounds found in the patient's biofluids are shown in Table 2.

5'dFUrd, FUra, $5FUH_2$, and FBAL were identified by comparison of the values determined for δ and J (when computer resolution and line-broadening due to biological medium allowed determination of J) with those of authentic samples. For example, in Fig. 1A, a spectrum of authentic FBAL in blood is compared with the signal obtained in a blood sample at $t = 6$ h. Their δ and J are similar and agree with data in the literature [1].

The signal at −111.05 ppm in blood and plasma and −111.1 ppm in urine was presumably attributable to α-fluoro-β-ureidopropionic acid (FUPA), a compound of the catabolic pathway of FUra (Fig. 2), because δ and multiplicity are in keeping with this proposed structure (Fig. 1B); it was not attributed to α-fluoro-β-guanidinopropionic acid (FGPA),

Table 2. ^{19}F Chemical shifts[a] (δ, ppm) and H-F coupling constants (J, Hz) of 5'dFUrd metabolites in patient biofluids samples

		5'dFUrd	FUra	FUPA[b]	FBAL	$5FUH_2$
Blood	δ	− 89.1	− 93.7	− 111.05	− 112.7	− 126.1
		Broad signal	Broad signal	ddd	ddd	d
	J			$^2J = 50$	$^2J = 47.2$	$^2J = 43$
				$^3J = 25$	$^3J = 24.4$	^3J ND
					$^3J = 18.3$	
Plasma	δ	− 88.4	− 93.75	− 111.05	− 112.7	− 126.2
		− 89.95				
		Broad signals	Broad signal	Broad signal	ddd	Broad signal
	J				$^2J = 50.0$	
					$^3J = 28.0$	
					$^3J = 18.0$	
Packed red cells	δ	− 89.2	Not detected	Not detected	− 112.6	Not detected
		Broad signal			Broad signal	
Urine	δ	− 89.9	− 93.8	− 111.1	− 112.8	Not detected
		Broad signal	Broad signal	ddd	ddd	
	J			$^2J = 48$	$^2J = 50.0$	
				$^3J = 24$	$^3J = 28.3$	
				$^3J = 24$	$^3J = 18.0$	

[a] Chemical shifts are related to external CF_3COOH (0.5% aqueous solution); negative numbers indicate upfield direction from the reference

[b] Presumed structure

ND: not determined with accuracy

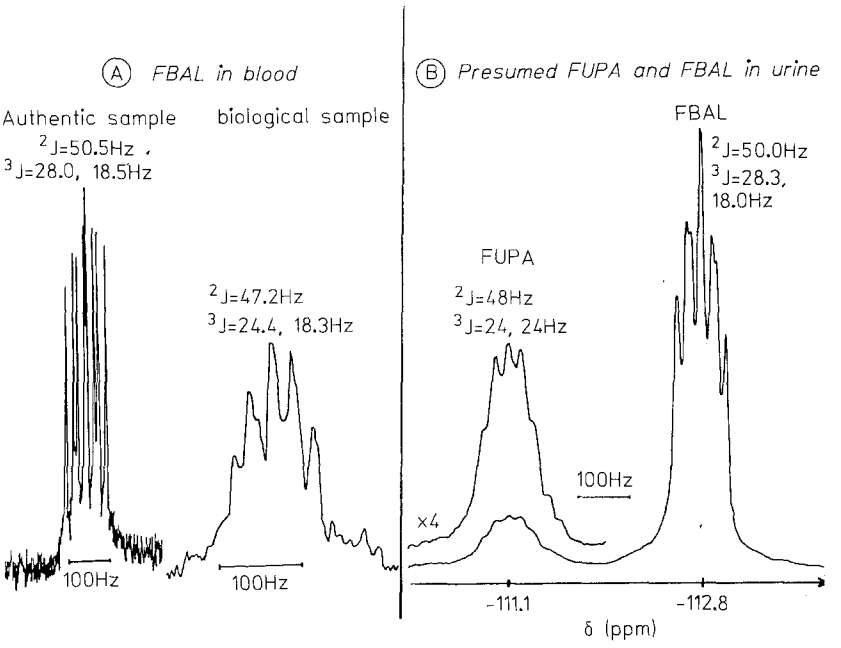

Fig. 1. ^{19}F NMR spectra: **A** FBAL in blood: authentic sample and biological sample (t = 6 h, end of perfusion). **B** FBAL and presumed FUPA in urine (end of perfusion)

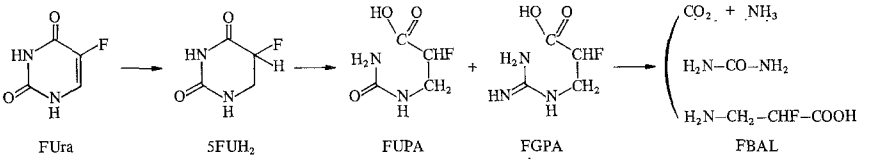

Fig. 2. Catabolic pathway of 5-fluorouracil

because this latter catabolite, which it is suggested is formed simultaneously with FUPA, was observed at a lower level than FUPA [10, 15, 16].

5'dFUrd chemical shifts are not identical in the different biofluids (Tables 1 and 2). We have shown that the 5'dFUrd chemical shift is sensitive to the pH of the medium. We therefore attributed the variability of the 5'dFUrd chemical shift to the different pH values of the biological media, which involve a modification in the uracil acid-base equilibrium. From the literature [22], we think that in urine (acidic pH) and in blood samples (near-neutral pH), the neutral and the monoanionic forms, respectively, are predominant.

The signal observed at −89.95 ppm in patient plasma (Table 2 and Fig. 4A) is assigned to 5'dFUrd bound to plasmatic proteins. We have shown that after 20,000 scans, 5'dFUrd added to a blank plasma gave two signals: a sharp signal (δ = −88.6 ppm), which corresponds to unbound 5'dFUrd, and a broad signal (δ = −90.05 ppm) for bound 5'dFUrd.

5'dFUrd metabolic pathway during and after perfusion

In blood. As the chemical instability of $5FUH_2$ is well known [19], a fresh blood sample (3 h after the beginning of the perfusion) immediately stored in ice was studied about 90 min after sampling. The ^{19}F NMR spectrum (Fig. 3A) shows the presence of 5'dFUrd, FBAL, and $5FUH_2$. The different levels of metabolites, particularly that of $5FUH_2$, estimated every 10,000 scans, seem not to change during the spectrum recording (30,000 scans, about 11 h). Therefore the presence of FBAL in this sample is not due to breakdown of $5FUH_2$ after sampling but arises from $5FUH_2$ catabolism during perfusion.

Assuming that the decomposition of 5'dFUrd metabolites in blood (pH 7.3–7.7) stored at −20° C for 2 weeks is relatively limited [19], we studied the evolution of 5'dFUrd metabolites as a function of time. Some examples of frozen blood ^{19}F NMR spectra are presented in Fig. 3.

At t = 0 (pretreatment blood sample) and t > 12 h, no fluorinated compound was detected (in the sensitivity limit of our NMR method). Unmetabolized 5'dFUrd was found at t = 1 h, 3 h, and 6 h. Except for blood at t = 1 h, FUra has never been detected at the beginning of spectrum recording; FUra shown on each spectrum was produced by 5'dFUrd enzymatic splitting (enzymatic content of red cells disrupted at freezing), which occurs and can be followed throughout spectrum recording. FUra was not indeed observed in fresh blood sample (Fig. 3A) or in plasma (except in the first hour) (Fig. 4A). $5FUH_2$ was detected at t = 1 h and 3 h. FBAL was found from t = 1 h to t = 12 h; its level increased steeply up to 6 h then decreased slowly; FBAL was the only metabolite observed in samples at 7, 9, and 12 h. The presumed FUPA was present in samples at 3 and 6 h.

Comparison of fresh and frozen blood samples taken at 3 h revealed a slight decrease in $5FUH_2$ with the appearance of FUPA, documenting slight $5FUH_2$ instability during storage at −20° C [19].

The 3-h plasma spectrum (Fig. 4A) is similar to the fresh blood sample spectrum (Fig. 3A), except that a signal appears at δ = −89.95 ppm (5'dFUrd bound to plasmatic proteins) and the $5FUH_2$ level is lower (pH of this sample was not adjusted to 7, thus involving a more rapid breakdown of $5FUH_2$) [19].

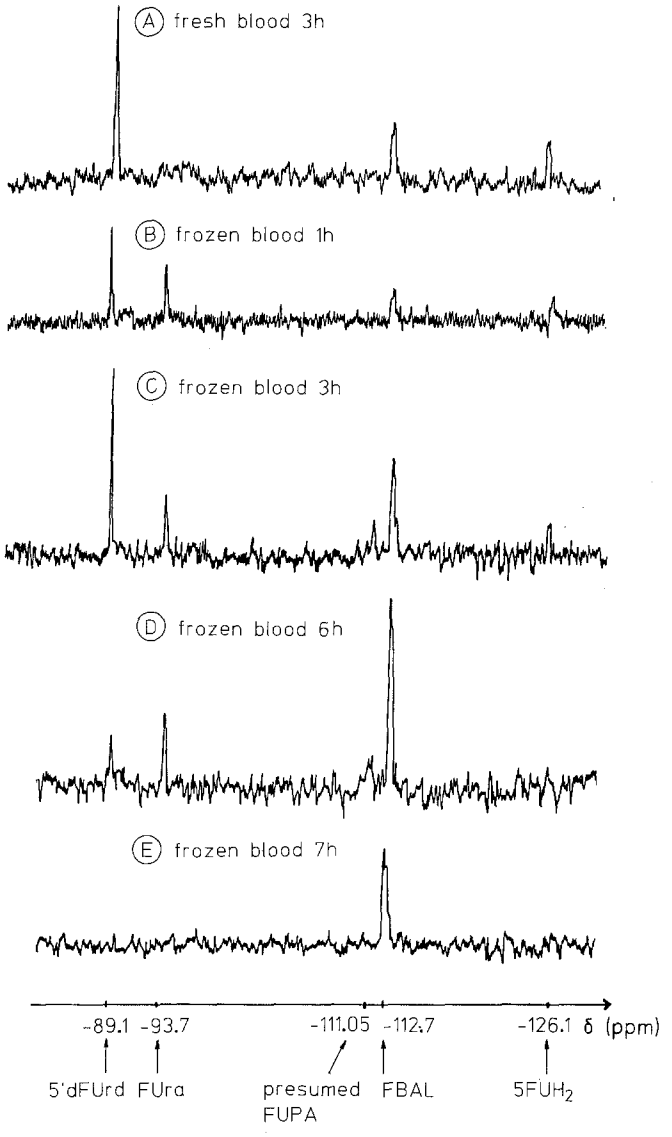

Fig. 3. ^{19}F NMR spectra: **A** fresh blood, **B–E** frozen blood

The 3-h packed red cell spectrum shows the presence of 5'dFUrd and FBAL in small amounts (Fig. 4B).

These results indicate that the compounds detected in the blood of a patient treated with 5'dFUrd during a 6-h continuous perfusion are unmetabolized 5'dFUrd, $5FUH_2$, FBAL and presumably FUPA. These three last products are FUra catabolites [16, 19] (Fig. 2).

In urine. Figure 5 shows the spectrum recorded for urine [in a specimen collected 5 h 30 min to 6 h 45 min after the beginning of the perfusion (pH 6.6)].

At $t = 0$, no fluorinated compound was detected. For all urine samples analyzed, the two major products were unmetabolized 5'dFUrd and FBAL. FUra, presumed FUPA, and another metabolite (not yet identified, $\delta = -43.7$ ppm) were detected at a very low level. $5FUH_2$ was not observed in the experimental recording conditions used (300 scans): this is consistent with the fact that an acidic pH induces its rapid breakdown [19].

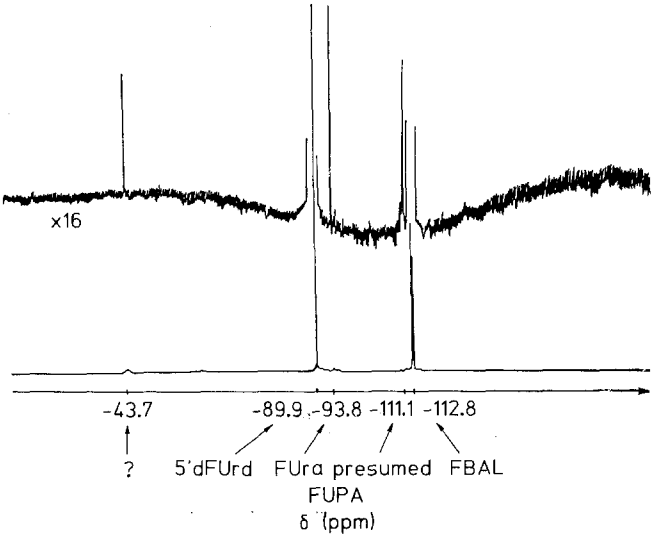

Fig. 5. ^{19}F NMR spectrum of urine (fraction 5 h 30 min to 6 h 45 min after the beginning of the perfusion)

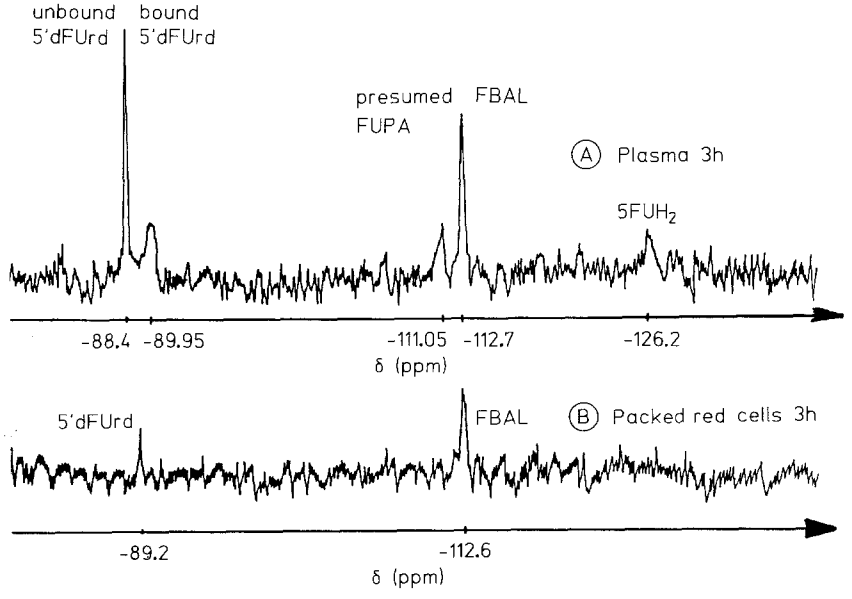

Fig. 4. ^{19}F NMR spectra: **A** plasma, **B** packed red cells

5'dFUrd elimination seems to increase rapidly for 2 h 30 min and then to decrease gradually between 2 h 30 min and 12 h and rapidly thereafter. FBAL reaches a maximum concentration in 6 h 45 min and then declines rapidly to a low level comparable with that of 5'dFUrd at 22 h. FUra and presumed FUPA are scarcely detected within the first 12 h. The presumed FUPA elimination profile is similar to that of FBAL: the maximum of elimination is reached in 6 h 45 min. FUPA decreases rapidly thereafter.

A quantitative determination of these different metabolites in blood, plasma, and urine is in progress.

Discussion

In these studies the formation of 5'dFUrd metabolites in blood and urine during a 6 h continuous perfusion was analyzed using a new ^{19}F NMR methodology.

^{19}F NMR was chosen for the following reasons: (1) fluorine-19 is an attractive nucleus for biological NMR work, since, occurring at 100% natural abundance, it has spin 1/2 and a relative sensitivity 0.833 that of proton; its chemical shift is highly sensitive to changes in its environment and has a wide range (960 ppm), this latter property allowing the various fluorinated compounds to be clearly distinguished in spite of signal line-broadening due to the biological system; (2) quantitative follow-up of the evolution of any fluorinated drug is possible with no confusion with biological compounds, because of the quasi-absence of fluorine in the organism; (3) heterogeneous biological samples such blood can be directly analyzed with no need for separation, extraction, or derivatization; moreover the NMR is noninvasive and nondestructive so it could be possible to use biological samples more than once; and (4) simultaneous identification of the fluorinated metabolites is easy without any differential treatment. (In previous studies, for the same sample analysis HPLC was used for 5'dFUrd and a GC/MS method for FUra and $5FUH_2$ [20].)

The main limitation of NMR applied to biological problems is its low intrinsic sensitivity compared with GC or HPLC. With our FT spectrometer the detectable concentration is about 10^{-5} *M*. The sensitivity of the method could be increased by using a spectrometer equipped with a proton decoupler and/or a ^{19}F NMR probe larger than 10 mm.

This new ^{19}F NMR analysis method has permitted the identification in blood of 5'dFUrd metabolites already described, FUra and $5FUH_2$ [20] and the detection of an additional one that had not previously been reported, FBAL, a FUra catabolism compound. In urine, the major products excreted were unmetabolized 5'dFUrd and FBAL.

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