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Application of fluorine-19 nuclear magnetic resonance to the determination of plasma-protein binding of 5′-deoxy-5-fluorouridine, a new antineoplastic fluoropyrimidine

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Abstract: Two distinct fluorine-19 nuclear magnetic resonance (^{19}F NMR) signals have been observed in human serum for free and plasma-protein bound 5′-deoxy-5-fluorouridine (5′dFUrd). The binding of this drug was studied directly in serum using ^{19}F NMR. To evaluate the validity of this method, a parallel study was conducted with equilibrium dialysis as the reference method. Two assay methods were applied after equilibrium dialysis, UV spectrophotometry and ^{19}F NMR spectrometry, the UV assay being used to validate the ^{19}F NMR assay. A study of the binding of 5′dFUrd to human serum albumin was also reported. The reliability of ^{19}F NMR as a technique to measure directly the binding of the drug and as an assay after equilibrium dialysis was demonstrated. The percentage of 5′dFUrd bound to plasma proteins is low and concentration-dependent in the 0.04–3.5 mmol l^{-1} range.

Keywords: *^{19}F NMR; plasma-protein binding; 5′-deoxy-5-fluorouridine; antineoplastic fluoropyrimidine.*

Introduction

5′-Deoxy-5-fluorouridine (5′dFUrd) is a new antineoplastic fluoropyrimidine that is active against several animal and human tumours [1, 2]. In a previous paper, a new method was described for the analysis of 5′dFUrd metabolite pools in human biological fluids by fluorine-19 nuclear magnetic resonance (^{19}F NMR) [3]. All the fluorinated metabolites of the catabolic process of this drug were detected (Fig. 1). Moreover, two signals were observed for 5′dFUrd in plasma samples of patients treated with this drug (Fig. 1), the narrow resonance at $\delta = -88.4$ ppm corresponding to unbound 5′dFUrd and the broad resonance at $\delta = -89.95$ ppm to plasma-protein bound 5′dFUrd since it disappeared after deproteinization [3]. It was thought therefore that it might be possible to determine the percentage of this drug bound to plasma proteins directly in plasma or

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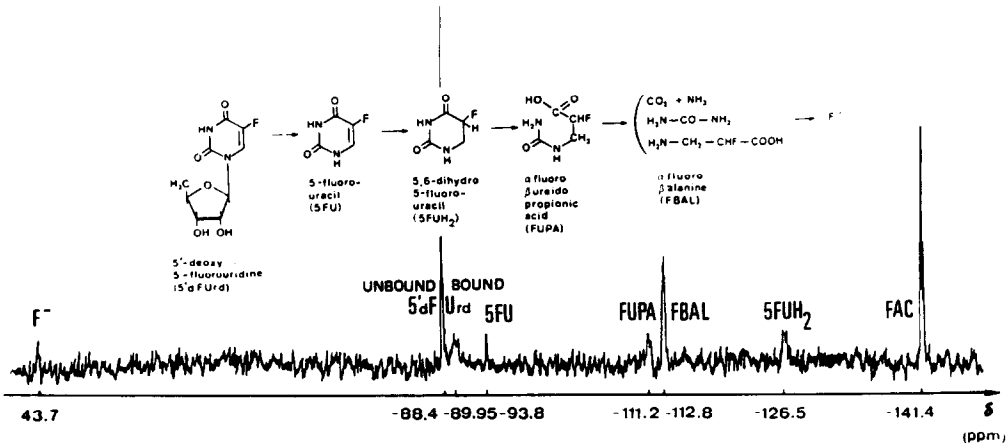

Figure 1
^{19}F NMR spectrum of a plasma sample of a patient treated with 5'dFUrd. The resonance positions are upfield from an external reference CF_3COOH (5 g l^{-1} aqueous solution) resonance peak. FAC is an internal standard for assay. The signals were attributed by comparing their chemical shifts (δ) and coupling constants with those of authentic samples [3]. F^-, fluoride ion. Number of scans: 36,000.

serum samples by ^{19}F NMR. This technique has often been used to study the interactions between a fluorinated substrate and a protein [see for example 4–7]. In weak complexes where the ligand is exchanged rapidly between its bound and free states, a single enlarged ^{19}F NMR signal is observed, the linewidth of which is the weighted mean of the intrinsic linewidth for the bound and free substrate [4, 6]. In tightly bound complexes where the ligand is exchanged slowly, two peaks are detected, a narrow signal that arises from the free substrate and a broad signal that arises from the bound substrate [4–7]. However, ^{19}F NMR has not been used previously to study quantitatively the binding of a fluorinated drug to plasma proteins. Therefore a parallel study was conducted on the binding of the drug with plasma proteins by equilibrium dialysis as the "reference method" [8]. The results obtained from the direct method (^{19}F NMR) were then compared with those from the indirect method (equilibrium dialysis).

Materials and Methods

Reagents

Chemicals. 5'dFUrd was a gift from Hoffmann-La Roche (Basel, Switzerland). Sodium monofluoroacetate (FAC) was a product of Fluka (Buchs, Switzerland). The relaxation reagent, chromium (III) acetylacetonate [$Cr(acac)_3$] was obtained from Spectrométrie Spin Techniques (Paris, France). Human serum albumin (HSA) (No. A-1887; fraction V essentially fatty acid free (less than 0.005%)) was purchased from Sigma Chemical Co. (St Louis, MO).

Two buffer solutions were used for equilibrium dialysis: a phosphate buffer (67 mmol l^{-1}, pH 7.4, ionic strength 0.329 with NaCl 0.9%) and a Tris-HCl buffer (197 mmol l^{-1}, pH 7.4, ionic strength 0.350 with NaCl 0.9%).

Preparation of serum and serum ultrafiltrate. Pooled human serum was prepared from healthy subjects' blood freshly collected in dry Vacutainer tubes. The tubes were kept at

37°C for 35 min, centrifuged at 2000 g for 15 min at 4°C and the serum was then carefully removed. This serum was sampled into 5-ml tubes, frozen at −20°C and thawed by aliquot fraction just before use. To prevent the conversion of 5'dFUrd into 5-fluorouracil (5FU) due to the enzymatic content of blood cells [9], it was verified by Coulter counter and Malassez cell counts that this serum contained a negligible amount of residual blood cells. The total protein concentration in the serum pool (67 g l^{-1}) was measured by use of the biuret reagent (Biopack®-protéines, Biotrol, Paris, France).

The serum ultrafiltrate was prepared using an ultrafiltration apparatus (type UH 100/2b, Schleicher and Schuell, Dassel, West Germany) equipped with membranes having a molecular weight-cutoff of 25,000 (Schleicher and Schuell) and operating under reduced pressure (30 mm Hg). The ultrafiltration process was stopped when the volume of the collected ultrafiltrate was about 70% of the initial volume of the serum. A protein assay in the serum ultrafiltrate showed the absence of trace amounts of protein.

Binding techniques

The binding of 5'dFUrd to HSA and serum was measured by equilibrium dialysis and direct ^{19}F NMR measurements.

Equilibrium dialysis. Two sets of experiments were done: (i) equilibrium dialysis of 5'dFUrd in buffer (phosphate or Tris-HCl) against HSA in the same buffer; (ii) equilibrium dialysis of 5'dFUrd in serum ultrafiltrate against serum. Two initial concentration ranges of 5'dFUrd were used, 6–0.06 mmol l^{-1} for HSA-phosphate or Tris buffer equilibrium dialysis and 6.5–0.37 mmol l^{-1} for serum–serum ultrafiltrate equilibrium dialysis. HSA was dissolved in phosphate or Tris buffer to give a concentration of 0.6 mmol l^{-1}. 5'dFUrd was dissolved in either the buffer or the serum ultrafiltrate depending on whether the equilibrium dialysis concerned HSA-buffer or serum–serum ultrafiltrate. The 5'dFUrd solution was introduced into the protein-free compartment. Teflon macrocells (Dianorm®; Diachema, Ruschlikon, Zurich, Switzerland) were used with two 2-ml chambers separated by a semipermeable membrane (molecular weight-cutoff 5000; Diachema AG, Langnau, Zurich, Switzerland). Samples were stirred continuously at 20 rotations per min, at 37°C. The dialysis chambers were filled to 1.50 or 1.80 ml using microsyringes accurate to 0.01 ml. Each experiment was run in duplicate.

Preliminary experiments showed that 5'dFUrd did not bind either to the membrane or to the dialysis cell and equilibrium was attained in less than 1 h of dialysis; therefore all samples were measured after 1 h of dialysis. Since the magnitude of the volume shift from the protein-free to the protein compartment was negligible up to 2 h (the volumes of the compartments were measured with a microsyringe [10]), there was no need to correct the results.

The 5'dFUrd concentrations were measured in both compartments by ^{19}F NMR and in the buffer compartment only by UV assay.

Direct ^{19}F NMR measurements. Varying quantities of two 5'dFUrd solutions (11.2 and 1.12 mmol l^{-1}) containing $Cr(acac)_3$ (1 mmol l^{-1}) in serum were added to 0.7 ml of serum to cover a 5'dFUrd concentration range of 2–0.1 mmol l^{-1}. The relaxation reagent, $Cr(acac)_3$, was used in order to decrease the T_1 relaxation times and therefore the time required for the NMR recordings. It was verified that $Cr(acac)_3$ did not modify the binding of 5'dFUrd to plasma proteins by comparing the percentages bound with and

without this relaxation reagent. Each experiment was run in duplicate or triplicate. The assay was made directly by ^{19}F NMR.

In equilibrium dialysis and in direct ^{19}F NMR measurements, no conversion of 5'dFUrd into 5FU was detected by ^{19}F NMR within the limits of sensitivity of this method.

Assay methods

UV assay. This method allows the determination of the free 5'dFUrd concentration in the protein-free compartment. As for all the compounds in the uracil family [11], the molar absorptivity (ε) of this drug depends on the equilibrium between the neutral form (lactam) and the ionized form (lactim); it is therefore a function of pH. In order to conduct the UV assay at a pH where one form was predominant, it was necessary to determine the pK_a of 5'dFUrd; this was done by following the variations of the 5'dFUrd ^{19}F chemical shift (which is sensitive to the ionization state of the drug) as a function of pH and by potentiometry. The pK_a determined by these methods (7.45 and 7.55, respectively) is within the physiological pH zone. The 5'dFUrd molar absorptivity is also a function of the drug concentration in the concentration range studied and the nature of the medium in which the drug is dissolved.

In the light of these observations, the following assay protocol was chosen: (i) determination of the molar absorptivity of 5'dFUrd for each concentration to be measured; measurements were made in an acidified blank dialysate (pH $\simeq$ 5 where molar absorptivity does not depend on pH because the drug is in the lactam form only) to which a known quantity of 5'dFUrd was added to reach a concentration near that of the dialysate to be measured; (ii) assay of the equilibrium dialysis samples after acidification (pH $\simeq$ 5).

UV measurements were made with a Beckman spectrometer UV 5260 (Beckman Instruments, Gagny, France) connected with a Hewlett–Packard HP 87 computer, at a constant temperature (25°C). UV cells of different thicknesses (0.1, 0.5 and 1 cm) were used to measure absorbances between 0.2 and 1.2.

^{19}F NMR assay. This method was used for the assay of 5'dFUrd in both compartments after equilibrium dialysis and for the direct ^{19}F NMR measurements.

^{19}F NMR spectra were recorded at 250 MHz with a Cameca 250 FT spectrometer (Cameca, Courbevoie, France) connected with a 16-K memory Nicolet 80 computer. Proton decoupling and frequency field lock were not used. Samples were placed in NMR tubes 5 mm in diameter. The resonance positions were measured from the H_2O proton signal which is always positioned arbitrarily for any sample at the same frequency; the resonance peak of CF_3COOH (5 g l^{-1} aqueous solution, 25°C) was used as an external reference. The instrument settings for quantitative analysis were as follows: probe temperature, 25°C; sweep width, 33,333 Hz; pulse width, 1.6 μs ($\alpha \simeq 45°$); recycling time, 2 s; number of scans, 6000–40,000; computer resolution, 4.1 Hz per point; receiving filter, out. The magnetic field was skimmed by using the ^1H NMR resonance of water observed in the continuous wave mode. To ensure that the comparison of peak intensities was valid, the T_1 relaxation times of free 5'dFUrd (longer than that of bound 5'dFUrd) and of FAC (the internal standard for the assay) were measured in water and plasma with and without the relaxation reagent $Cr(acac)_3$ (data not shown and [12]). The spectral acquisition parameters were optimized for the longest T_1 in the presence of $Cr(acac)_3$, that of the FAC resonance.

^{19}F NMR measurements were made at 25°C. It was verified that the percentage bound of 5'dFUrd was not significantly modified at 25°C compared with that at 37°C (the temperature at which the equilibrium dialysis was conducted). For example, for a 5'dFUrd concentration of 1 mmol l^{-1}, the percentage bound was 22% at 25°C and 23% at 37°C (mean of 3 experiments).

In the equilibrium dialysis experiments, samples taken at the end of the dialysis were immediately frozen at −20°C. Just before ^{19}F NMR analysis, a solution of $Cr(acac)_3$ (1 mmol l^{-1}) and the internal standard FAC (which does not bind to plasma proteins) was added to the thawed sample so that the concentration was near that of the drug in the sample to be measured. This added volume never exceeded 10% of the volume of the solution to be measured. The free 5'dFUrd concentration was determined from the intensity of its NMR signal, estimated by comparison of the NMR signal area with that of the internal standard FAC. The areas, on an expanded scale of 20 Hz cm^{-1}, were determined after cutting out and weighing the chart recordings for the different signals. The bound 5'dFUrd was evaluated from the integration of the expanded resonances of bound 5'dFUrd/(free 5'dFUrd + bound 5'dFUrd) (Fig. 2). The percentage bound of 5'dFUrd was determined by considering the total drug concentration in the protein compartment.

For the direct ^{19}F NMR measurements, the addition of the internal standard solution was unnecessary since the total concentration of 5'dFUrd was known. The percentage bound was established directly by integration as described above.

The quantitative evaluation of bound 5'dFUrd by ^{19}F NMR required the comparison of the areas of a sharp signal with that of a broad signal (Fig. 2). To minimize the risk of error, the analysis of each ^{19}F NMR spectrum was done at least four times.

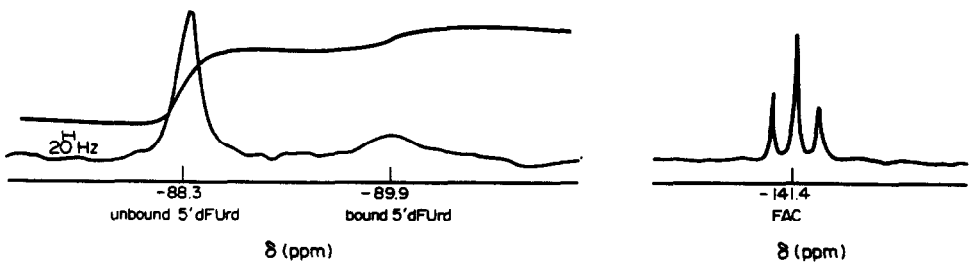

Figure 2
Expanded ^{19}F NMR signals of bound and unbound 5'dFUrd and internal standard (FAC).

Calculations of percentage 5'dFUrd bound

The protein and protein-free compartments are labelled 1 and 2, respectively. The relationships detailed below are valid only because (i) identical volumes were introduced in each dialysis compartment, (ii) the short dialysis time produced no change in the volume.

UV assay. The concentration of free drug $C_{F.2}$ was determined experimentally. The concentration of bound drug (C_B) was calculated by the relationship

$$C_B = C_I - 2C_{F.2} \quad (1)$$

where C_I is the initial 5'dFUrd concentration, i.e. the drug concentration introduced in the compartment 2 at the start of equilibrium dialysis. The percentage bound was determined by the relationship

$$\% \text{ bound} = [C_B/(C_I - C_{F,2})] \times 100 \quad (2)$$

^{19}F NMR assay. The concentrations $C_{F,1}$, $C_{F,2}$, C_B, i.e. the free drug concentrations in compartments 1 and 2, and the bound drug concentration in compartment 1 were determined experimentally. The percentage bound can be expressed as

$$\% \text{ bound} = [C_B/(C_B + C_{F,1})] \times 100 \quad (3)$$

Another relationship was used to determine the percentage bound by ^{19}F NMR. C_B was not determined directly, but indirectly from $C_{T,1} - C_{F,2}$; $C_{T,1}$ being the sum of the free drug and bound drug concentrations in compartment 1. The percentage bound was then calculated as follow

$$\% \text{ bound} = [(C_{T,1} - C_{F,2})/C_{T,1}] \times 100 \quad (4)$$

With direct ^{19}F NMR measurements, the percentage bound can be expressed as

$$\% \text{ bound} = [C_B/(C_B + C_F)] \times 100 \quad (5)$$

Verification of the concentration balance after equilibrium dialysis and ^{19}F NMR assay. The percentage error in the concentration balance was determined as follow

$$\% \text{ error} = [C_I - (C_{F,1} + C_{F,2} + C_B)/C_I] \times 100 \quad (6)$$

where C_I is the initial concentration and $C_{F,1}$, $C_{F,2}$, C_B are the concentrations evaluated by ^{19}F NMR.

Evaluation of the precision of the assay methods

The reproducibility in the estimation of the free drug concentration after dialysis experiments was about 5% by UV and 5–10% by ^{19}F NMR. The reproducibility in the ^{19}F NMR estimation of the 5'dFUrd percentage bound was assessed with six serum samples spiked with a 5'dFUrd concentration of 1.12 mmol l^{-1}. The mean percentage bound was 23.4% (± standard deviation 2.4%), thus the relative standard deviation was 10%.

Results and Discussion

The binding of drugs to plasma proteins is usually determined by equilibrium dialysis, ultracentrifugation and ultrafiltration. These methods provide only an indirect evaluation of the binding of a drug since, to calculate this parameter, it is necessary to establish the free drug concentration in the protein-free compartment of the equilibrium dialysis, the ultrafiltrate or the ultracentrifugation supernatant. Additionally, the use of a labelled drug has proved to be preferable to obtain greater precision. The fact that two distinct ^{19}F NMR signals are observed in human plasma for free and plasma-protein

bound 5'dFUrd has been used to study the binding of this drug which has never been reported in the literature. The ^{19}F NMR method that does not require a labelled drug was used; this provides a direct estimation of the binding. To evaluate the validity of this method, the results obtained through direct ^{19}F NMR measurements were correlated with an equilibrium dialysis study of 5'dFUrd binding. A study of the binding of 5'dFUrd to HSA was made also to establish any possible difference in the binding of 5'dFUrd between HSA and serum. Since no labelled 5'dFUrd was available, two assay methods were applied after equilibrium dialysis, UV spectrophotometry and ^{19}F NMR spectrometry; the UV assay was used to validate the ^{19}F NMR assay.

For the serum equilibrium dialysis experiments (Table 1), serum was used rather than plasma since anticoagulant additives may influence the protein binding of drugs [13]. Moreover, these experiments were conducted with serum ultrafiltrate as the dialysis fluid, which has been reported to give free drug concentration values very close to those *in vivo* [13].

Validity of ^{19}F NMR as an assay method and as a technique to directly measure the percentage bound

Results are presented in Tables 1–4. Only Table 1 is presented in detail to provide the information required for a discussion of the methods used in our study. For clarity, Tables 2 and 3 are presented in simplified form. A study of these tables confirms that ^{19}F NMR is a valid assay method. This is demonstrated by three points: (i) the sum of the concentrations of free and bound drug in both compartments is close to the initial concentration introduced into the dialyzer (Table 1); (ii) free drug concentrations measured in both compartments are similar and also very close to those obtained by UV assay in compartment 2 (Table 1); (iii) the percentages bound obtained directly from measurements in compartment 1 [relationship (3)] agree with indirect measurements [relationship (2)] obtained by UV assay (Tables 1–3). ^{19}F NMR is therefore a dependable assay method when the drug has two distinct signals for its bound and free forms. It should be noted that the indirect estimation of the percentage bound by ^{19}F NMR assay [relationship (4)] is less accurate although such results are generally acceptable (Table 1).

The validity of ^{19}F NMR as a technique to directly measure the binding was verified by comparing the binding results obtained by direct ^{19}F NMR measurements (Table 4) with those obtained from serum–serum ultrafiltrate equilibrium dialysis (Table 1). The results from the direct method are comparable to those obtained from the reference method. Additionally, two direct ^{19}F NMR measurements made on rat plasma for 5'dFUrd concentrations of 0.6 and 0.3 mmol l^{-1} did not allow detection of a signal corresponding to bound 5'dFUrd; this agreed with the equilibrium dialysis study reported by Au [14] which showed that 5'dFUrd binds only very weakly to rat plasma proteins (for a concentration range of 4–0.02 mmol l^{-1}, the percentage of free drug was 93 ± 3%). It is therefore understandable that such weak concentrations of bound 5'dFUrd ($\leq$0.04 mmol l^{-1}) are not detected by ^{19}F NMR since the bound 5'dFUrd signal is very broad.

Over the concentration range studied (0.1–2 mmol l^{-1}), the relative standard deviation for the percentages bound determined by direct ^{19}F NMR measurements was about 10% which agrees with the precision of the ^{19}F NMR methodology [12].

Table 1
Percentages of 5'dFUrd bound in serum as determined by equilibrium dialysis (serum against serum ultrafiltrate) and subsequent UV and ^{19}F NMR assays

C initial*	6.51	2.96	1.02	0.88	0.74	0.37
UV assay†						
$C_{F,2}$‡	3.18	1.26	0.42	0.36	0.28	0.14
C_B§	0.14	0.43	0.18	0.16	0.17	0.09
Bound percentage (indirect)‖	4	25	30	31	37	39
NMR assay†						
Compartment 2‡						
$C_{F,2}$	3.21	1.25	0.38	0.35	0.28	0.15
Compartment 1‡						
C_B‡	0.44	0.37	0.19	0.15	0.18	0.09
$C_{F,1}$‡	3.17	1.43	0.42	0.38	0.31	0.16
$C_{F,1} + C_{F,2} + C_B$	6.82	3.05	0.99	0.88	0.77	0.40
Percentage error in the concentration balance**	+5%	+3%	−4%	<+1%	+4%	+9%
Bound percentage (direct)††	12	21	31	29	36	36
Bound percentage (indirect)‡‡	12	30	38	33	43	40

*All concentrations (C) are expressed in mmol l^{-1}.
†UV data represent the average of two equilibrium dialysis experiments. NMR data represent the mean of two equilibrium dialysis experiments with at least four analyses of each ^{19}F NMR spectrum.
‡The protein and the protein-free compartments are designated 1 and 2 respectively; F = free, B = bound.
§Determined according to relationship (1).
‖Determined according to relationship (2).
**Determined according to relationship (6).
††Determined according to relationship (3).
‡‡Determined according to relationship (4).

Table 2
Percentages of 5'dFUrd bound to HSA as determined by equilibrium dialysis (HSA (0.6 mmol l^{-1}) in phosphate buffer against phosphate buffer) and subsequent UV and ^{19}F NMR assays

C initial*	6.11	3.06	1.07	0.70	0.30	0.09	0.06
UV assay†							
$C_B + C_{F,2}$‡	3.50	1.70	0.64	0.43	0.19	0.06	0.04
Bound percentage (indirect)§	17	20	31	34	44	52	57
^{19}F NMR assay†							
$C_B + C_{F,1}$‡	3.56	1.56	0.57	0.42	0.17	‖	‖
Percentage error in the concentration balance**	+7%	−5%	−7%	−3%	+1%	‖	‖
Bound percentage (direct)††	14	22	28	37	40	‖	‖

* All concentrations (C) are expressed in mmol l^{-1}.
† UV data represent the mean of two equilibrium dialysis experiments. NMR data represent the mean of two equilibrium dialysis experiments with at least four analyses of each ^{19}F NMR spectrum.
‡ The protein and the protein-free compartments are designated 1 and 2 respectively; F = free, B = bound.
§ Determined according to relationship (2).
‖ Because of the low initial concentration, these experiments were not studied with ^{19}F NMR.
** Determined according to relationship (6).
†† Determined according to relationship (3).

Table 3
Percentages of 5'dFUrd bound to HSA as determined by equilibrium dialysis (HSA (0.6 mmol l^{-1}) in Tris-HCl buffer against Tris-HCl buffer) and subsequent UV and ^{19}F NMR assays

C initial*	6.74	3.37	2.02	1.18	0.77	0.34	0.11	0.07
UV assay†								
$C_B + C_{F,2}$‡	3.54	1.82	1.08	0.63	0.44	0.19	0.08	0.05
Bound percentage (indirect)§	10	15	12	15	22	26	51	60
^{19}F NMR assay†								
$C_B + C_{F,1}$‡	3.72	1.50	0.96	0.52	0.44	0.22	‖	‖
Percentage error in the concentration balance**	+1%	−8%	−9%	+2%	+2%	<+1%	‖	‖
Bound percentage (direct)††	9	13	13	18	27	33	‖	‖

* All concentrations (C) are expressed in mmol l^{-1}.

† UV data represent the mean of two equilibrium dialysis experiments. NMR data represent the mean of two equilibrium dialysis experiments with at least four analyses of each ^{19}F NMR spectrum.

‡ The protein and the protein-free compartments are designated 1 and 2 respectively; F = free, B = bound.

§ Determined according to relationship (2).

‖ Because of the low initial concentration, these experiments were not studied with ^{19}F NMR.

** Determined according to relationship (6).

†† Determined according to relationship (3).

Table 4
Percentage of 5'dFUrd bound in serum as determined by direct ^{19}F NMR measurements

5'dFUrd (mmol l^{-1})	Bound percentage*,†
2.09	16.6 (±1.7)
1.12	23.4 (±2.4)
0.86	32.3 (±1.0)
0.78	35.7 (±2.3)
0.70	32.5 (±3.3)
0.61	33.1 (±3.0)
0.52	38.0 (±7.0)
0.34	37.3 (±4.0)
0.20	41.3
0.10	47.2

*Each result is the mean (±SD) of 2 or 3 NMR measurements (except for the two lowest 5'dFUrd concentrations for which a single measurement was performed owing to the time required for the NMR recording) with at least four analyses of each NMR spectrum.
†Determined according to relationship (5).

Characteristics of 5'dFUrd binding

The observation of two distinct ^{19}F NMR signals for free and bound 5'dFUrd is characteristic of a slow exchange process between the two forms; this implies a high affinity between the drug and the plasma proteins.

The percentages bound of 5'dFUrd to serum (determined by equilibrium dialysis or direct ^{19}F NMR measurements) and to HSA in phosphate buffer are identical (Fig. 3). The percentages bound are, however, lower for HSA in Tris-HCl buffer, at least for initial concentrations of >0.1 mmol l^{-1} of 5'dFUrd (Fig. 3). This demonstrates that chloride ions, the concentration of which is much higher than in phosphate buffer or serum (and maybe also the Tris buffer) compete with 5'dFUrd in binding to HSA [15]. 5'dFUrd binding is concentration-dependent; in the 0.1–3.5 mmol l^{-1} range in serum, the percentage bound decreased from approximately 50% to 5% as the 5'dFUrd concentration increased; in the 0.04–3.5 mmol l^{-1} range, at an HSA concentration of 0.6 mmol l^{-1}, it decreased from approximately 60% to 10% as the 5'dFUrd concentration increased. This shows that the binding of 5'dFUrd to plasma proteins or to HSA is a saturable process in the concentration range studied.

5'dFUrd is therefore a drug with a low percentage bound to plasma-proteins. Compared with other fluoropyrimidines used clinically, the percentage bound of 5'dFUrd is similar to that of 1-tetrahydro-5-fluorouracil (ftorafur) but much higher than those of 5FU and 5-fluorocytosine. In the range 0.03–0.5 mmol l^{-1}, ftorafur is 30–50% bound to human plasma proteins [16]; the percentage bound of 5FU on human plasma proteins is approximately 10% at pH = 7.2 over the range 0.0008–0.8 mmol l^{-1} [17]; it is 3.5% for 5-fluorocytosine (concentration range 0.015–0.4 mmol l^{-1}) [18].

The 5'dFUrd binding results obtained in this study do not allow definition of the binding parameters of this drug with HSA or serum since the concentration range studied is too narrow. This is because at the low 5'dFUrd concentrations studied, the assay

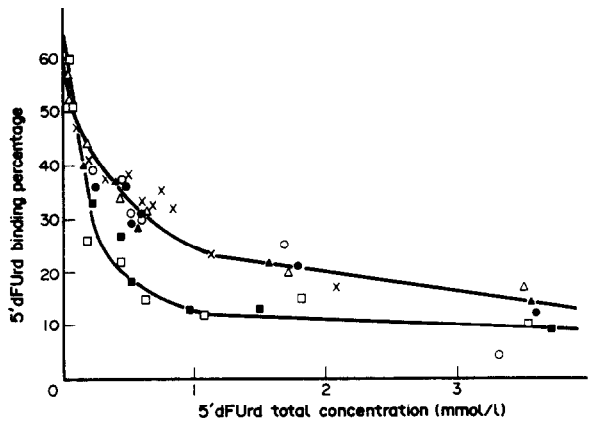

Figure 3
Binding percentages of 5'dFUrd (4–0.04 mmol l^{-1}) to HSA (0.6 mmol l^{-1}) or to serum as determined by equilibrium dialysis and direct ^{19}F NMR measurements.

For equilibrium dialysis, the 5'dFUrd total concentration is the drug concentration in the protein compartment; for direct ^{19}F NMR measurements, it is the drug concentration in the NMR tube.

The 5'dFUrd binding percentage is determined according to relationship (2) for the equilibrium dialysis experiments with subsequent UV assay, to relationship (3) for the equilibrium dialysis experiments with subsequent ^{19}F NMR assay, and to relationship (5) for the direct ^{19}F NMR measurements.

Each point is the mean of 2 equilibrium dialysis experiments (with at least 4 analyses of each ^{19}F NMR spectrum) and of 2 or 3 direct ^{19}F NMR measurements with at least 4 analyses for each ^{19}F NMR spectrum.

5'dFUrd equilibrium dialysis experiments (i) against HSA in phosphate buffer: ▲ ^{19}F NMR assay. △ UV assay; (ii) against HSA in Tris-HCl buffer: ■ ^{19}F NMR assay, □ UV assay; (iii) against serum in serum ultrafiltrate: ● ^{19}F NMR assay, ○ UV assay. Direct ^{19}F NMR measurements in serum: ×.

methods used are limiting. This is particularly true for ^{19}F NMR, where the sensitivity limit (with the spectrometer used in the present work) is ≈0.01 mmol l^{-1} for a sharp signal (free 5'dFUrd and FAC) and ≈0.05 mmol l^{-1} for a broad signal (bound 5'dFUrd). With high 5'dFUrd concentrations, the problem arises from the low percentage bound of the drug. The study does, however, show that ^{19}F NMR allows the determination of the percentage of 5'dFUrd bound to plasma proteins without physical separation of the unbound drug from the bound form, without a labelled drug and under conditions which are as physiological as possible. In this way, ^{19}F NMR allowed the measurement of bound 5'dFUrd in plasma samples of patients treated with the drug. Using acellular plasma samples in order to avoid 5'dFUrd conversion into 5FU [9], the results were in complete agreement with *in vitro* studies (5'dFUrd percentages bound: ≈30% for a 5'dFUrd concentration of 0.6 mmol l^{-1}, ≈35% for 0.3 mmol l^{-1}, and ≈40% for 0.1 mmol l^{-1}) [3].

Even when they are bound to plasma proteins, fluorinated drugs do not necessarily have two distinct ^{19}F NMR signals for their free and bound forms [4, 6]. In this case, ^{19}F NMR may be used, but only as an assay method after equilibrium dialysis (with, however, a lower accuracy for the estimation of binding percentages). This was verified on 5FU, the binding of which to plasma proteins does not give rise to two separate signals but simply an enlarged ^{19}F NMR signal. For example, after equilibrium dialysis and subsequent ^{19}F NMR assay of the two compartments, the 5FU binding percentage was 10% for a concentration of 0.57 mmol l^{-1} in the protein compartment, which agrees with published data [17].

The determination of binding parameters for other fluorinated drugs by ^{19}F NMR seems possible provided that the percentage of the drug bound to plasma proteins is high and that an NMR spectrometer is used of sufficiently high performance to examine concentrations in the order of 0.001 mM.

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