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Review

Fluorine-19 or phosphorus-31 NMR spectroscopy: A suitable analytical technique for quantitative in vitro metabolic studies of fluorinated or phosphorylated drugs

Robert Martino, Véronique Gilard, Franck Desmoulin, Myriam Malet-Martino *

Groupe de RMN Biomédicale, Laboratoire SPCMIB (UMR CNRS 5068), Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France

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Abstract

Fluorine-19 or phosphorus-31 NMR (^{19}F NMR or ^{31}P NMR) spectroscopy provides a highly specific tool for identification of fluorine- or phosphorus-containing drugs and their metabolites in biological media as well as a suitable analytical technique for their absolute quantification. This article focuses on the application of in vitro ^{19}F or ^{31}P NMR to the quantitative metabolic studies of some fluoropyrimidine or oxazaphosphorine drugs in clinical use. The first part presents an overview of the advantages (non-destructive and non-selective direct quantitative study of the biological matrices) and limitations (expensive cost of the spectrometers, limited mass or concentration sensitivity) of NMR spectroscopy. The second part deals with the criteria to be considered for successful quantification by NMR (uniform excitation over the entire spectral width of the spectrum, resonance signals properly characterised by taking into account T_1 values and avoiding NOE enhancements, optimisation of the data processing, choice of a suitable standard reference). The third and fourth parts report some examples of quantification of 5-fluorouracil, its prodrug capecitabine, 5-fluorocytosine and their metabolites in bulk solutions (biofluids, tissue extracts, perfusates and culture media) and heterogeneous media (excised tissues and packed intact cells) as well as cyclophosphamide and ifosfamide in biofluids. These two parts emphasise the high potential of in vitro ^{19}F or ^{31}P NMR for absolute quantification, in a single run, of all the fluorine- or phosphorus-containing species in the matrices analysed. The limit of quantification in bulk solutions is 1–3 μM for ^{19}F NMR and approximately 10 μM for ^{31}P NMR. In heterogeneous media analysed with ^{19}F NMR, it is 2–5 nmol in excised tissues and cell pellets.

Keywords: ^{19}F NMR; ^{31}P NMR; In vitro quantification; Biological fluids; Excised tissues; Cell pellet; Fluoropyrimidine drugs; Oxazaphosphorine drugs

Contents

* Corresponding author. Tel.: +33 5 61 55 68 90; fax: +33 5 61 55 76 25.
E-mail address: martino@chimie.ups-tlse.fr (M. Malet-Martino).

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1. Introduction

Today, about 60 years after the observation of proton nuclear magnetic resonance (^{1}H NMR) in liquid water and paraffin wax by research groups led by Bloch at Stanford University and Purcell at Harvard University, NMR has become a major tool for determining molecular structures and has found many applications not only in chemistry but also in other fields such as materials science, biology and medicine [1]. Indeed the last 30 years have seen considerable development of biological NMR since it is the only physical method used routinely for the direct study at the molecular level of biological samples, from biofluids, cell or tissue extracts, excised tissues, packed intact cells (in vitro studies) to isolated living cells or isolated perfused organs (ex vivo studies), and finally animal models and human subjects (in vivo studies). Consequently, NMR is unique in its ability to permit the analysis of the metabolism of both endogenous and xenobiotic compounds such as drugs.

This article will focus on quantitative in vitro metabolic studies of fluorinated or phosphorylated drugs using fluorine-19 NMR (^{19}F NMR) or phosphorus-31 NMR (^{31}P NMR) as the analytical technique. The first part presents an overview of the advantages and limitations of NMR spectroscopy for in vitro analyses of complex biological matrices, with emphasis on the ^{19}F and ^{31}P nuclei. The second part deals with the problems encountered in the quantification procedure and the criteria to be considered for successful quantification by NMR spectroscopy. The third and fourth parts report some examples of quantification of fluorinated drugs and their metabolites in biofluids, tissue extracts, packed intact cells and excised tissues using ^{19}F NMR, and phosphorylated drugs and their metabolites in biofluids using ^{31}P NMR.

2. Advantages and limitations of NMR for in vitro drug metabolism studies

NMR is non-destructive so that the sample is available for analysis by a subsequent alternative technique. NMR enables the direct study of intact biofluids, cells or tissues, without resort to treatment. Problems of extraction recovery and chemical derivatisation and those that may be encountered with pH-sensitive metabolites are consequently avoided. NMR spectroscopy is thus particularly suited to the study of delicate samples.

The method is non-selective and unexpected substances are not overlooked during the investigation, since all low molecular weight molecules in solution (provided they bear the nucleus under investigation and are present at sufficient concentrations) are detected simultaneously in a single analysis. This contrasts with chromatography that usually requires some prior knowledge of the structure of metabolites in order to optimise sample preparation and/or detection; novel metabolites may therefore be missed. NMR also avoids the use of a number of different chromatographic techniques, which is sometimes necessary when metabolites have different chemical structures. This is an important attribute of NMR in the search for novel metabolites when, often, the analyst will have no idea of the type of molecule to look for.

NMR provides uniquely rich structural information. Indeed chemical shifts, multiplicity, integrals, as well as homo- and heteronuclear two-dimensional (2D) experiments make up a powerful toolkit to probe the molecular structure of novel metabolites. Even though metabolites must still be extracted and purified for unequivocal elucidation of the structure, the NMR "behaviour" nevertheless can give a good estimate of the structures of unknown compounds.

Although optimisation of the quantitation of drugs and metabolites in in vitro studies is somewhat tedious, it is nevertheless fairly easy to obtain quantitative data and NMR can be used routinely in the same way as HPLC. Moreover given a good signal-to-noise ratio (S/N), NMR can quantify substances accurately and reproducibly. Quantification may be however affected by the binding of drugs or metabolites to macromolecules in plasma, by micellar substructures in bile or by inhomogeneities and magnetic susceptibility effects in intact cells or excised tissues. This results in significant signal broadening that leads to reduced S/N or even to NMR invisibility of the signal. Some sample pre-treatment may be therefore required; for example, ultrafiltration of plasma that removes lipoproteins and macromolecules. Yet, the diffusion-

sensitised proton NMR technique that completely separates serum macromolecules and low molecular weight serum metabolites by utilising the large difference in their translational diffusion coefficients can also be used. The excellent correlation between the absolute metabolite concentrations obtained by diffusion NMR and ultrafiltration demonstrates the quantitative nature of spectral simplification by diffusion NMR [2].

There are several magnetically active nuclei that can be routinely used for drug metabolism studies (hydrogen, carbon-13, fluorine-19, phosphorus-31, lithium-7, boron-10 and -11 and, to a much lower extent, deuterium, oxygen-17, nitrogen-14 and -15, platinum-195).

The studies reported here focus on fluorine-19 and phosphorus-31 nuclei because of their favourable NMR characteristics: nuclear spin of $\frac{1}{2}$, relatively narrow lines, 100% natural abundance, high sensitivity for fluorine-19 nucleus (83% that of proton) and correct sensitivity for phosphorus-31 nucleus (6.6% that of proton), large chemical shift range (about 500 and 800 ppm for fluorine-19 and phosphorus-31, respectively), which minimises signal overlap.

An important advantage, specific to ^{19}F NMR, is that resonances of fluorine nuclei can be observed without problems of interfering background signals since the level of endogenous fluorine-containing compounds is very low.

Concerning the phosphorus nucleus, the presence of endogenous phosphate and derivatives (e.g. phosphomonoesters, phosphodiesters, etc.) may interfere with signals from phosphorylated drugs and their metabolites. In practice, this is not a large obstacle since these endogenous compounds are relatively few, at least in biofluids, to produce detectable signals.

Moreover, ^{19}F and ^{31}P NMR of biological matrices are not hampered by dynamic range problems as encountered in proton NMR due to the intense signal of water protons that must be suppressed.

Set against these advantages, however, are a number of disadvantages that need to be taken into account. NMR spectrometers are expensive instruments with the cost rising steeply with the field strength of the magnet used. Higher field strengths provide greater chemical shift dispersion and increase the sensitivity. However, presently, the price of a routine NMR spectrometer (7 T corresponding to a proton resonance frequency of 300 MHz) does not exceed that of a sophisticated liquid chromatography–mass spectrometry (LC–MS) machine. Moreover, the magnets have long useful lifetimes (≈15–20 years) and the advances in computer technology may be incorporated continuously in NMR spectrometers at reasonable cost.

The total volume of sample required for NMR analysis ranges between 0.3–0.7 and 2.0–3.0 mL with 5 and 10 mm tubes, respectively, depending if a coaxial capillary is or not employed. This can be a hindrance for pharmacokinetic studies that require numerous plasma samples or for difficult-to-obtain biofluids such as bile, cerebrospinal fluid or those from neonates. But the volume needed for an experiment may be reduced without having to redesign the radio-frequency (RF) coil, using several approaches: utilisation of smaller tubes (for example 3 mm microtubes requiring 130–150 μL) or tubes fitted with plastic plugs matched to the susceptibility of the solvent or spherical microcells. In the two last cases, the volume sample (60–80 μL under favourable circumstances, or 18 μL, respectively) is placed within the active region of the RF coil [3,4].

Moreover, NMR probe manufacturers now offer probes with “saddle-type” coils smaller than those used in standard high-resolution NMR spectrometers, as well as solenoidal coils oriented at the magic angle (54.7°) with respect to the static magnetic field. These probes accommodate total sample volume in the range 140–25 μL for the “saddle-shaped” coils and from 6 μL to a maximum of 40 μL for the solenoidal coils rapidly spinning at the magic angle [3,5,6].

Submillimeter coils have been also designed for static analyses. They require 0.4–1.4 [7] or 200 nL sample volumes [5] with solenoidal microcoils and 30, 120 or 470 nL with planar microcoils [8].

Compared with other techniques in terms of the minimum sample amounts for an analysis, NMR is significantly insensitive, which represents the principal drawback of the technique. Indeed the limit of detection (LOD) of NMR ranges between 10^{-9} and 10^{-11} mol whereas UV–vis absorbance reaches 10^{-13}–10^{-16} mol, radiochemical 10^{-14}–10^{-19} mol, mass spectrometry 10^{-13}–10^{-21} mol and fluorescence 10^{-18}–10^{-23} mol [3].

With the development of microliter- and nanoliter-volume static probes, it is possible to achieve NMR structural elucidation of mass-limited products. Indeed, LOD (defined as the analyte amount that yields a S/N of 3) of 44 pmol was calculated from a proton NMR spectrum of an 82.6 mM menthol solution acquired in 9 min in an observed volume (V_{obs}) of 31 nL, the total sample volume used being 200 nL [5]. Similarly, from 10 min acquisition time of ^{1}H NMR spectra of three sucrose solutions (5 mM for the two first and 500 mM for the third solution) in V_{obs} of 131, 88 and 5 nL (sample volume considered as infinite since a flow probe configuration was used), LODs of 17.6, 34.1 and 18.8 pmol were found, respectively [7,9].

Despite possessing high mass sensitivity, NMR microcoils have poorer concentration sensitivity than conventional-scale NMR probes due to the large difference in probe observation volumes.

This is illustrated by the performance indicators of several NMR probes for hydrogen analysis of sucrose solutions in deuterated water (Table 1). The decrease in observed volume leads to a higher mass sensitivity and a poorer concentration sensitivity.

Microcoil probes offer obvious advantages for structural determination of mass-limited compounds but it must be taken into account that realistic sample volumes that can be handled practically are typically greater than 200 nL when the solvents possess a high viscosity and/or a high surface tension such as water or dimethylsulfoxyde, or 1–2 μL with

Table 1
Performance comparisons of several NMR probes

Probes	Varian 5 mm	Nalorac 1.7 mm	Solenoïdal microcoil 1	Solenoïdal microcoil 2
Volume observed by the coil	222 μL	23 μL	620 nL	5 nL
LOD_m (pmol)[a]	82	36	7.5	2.0
LOD_c (μM)[a]	0.4	2.6	11.9	410

[a] Mass sensitivity and concentration sensitivity limits of detection (LOD_m and LOD_c) are calculated for a recording time of 20 h from the data normalised to 600 MHz reported by Lacey et al. [3].

the organic solvents of low surface tension and/or viscosity (methylene chloride or acetone for example).

Considerations of probe mass sensitivity are weighed against the amount of sample available for analysis and the acceptable concentration range for the particular experiment.

Clearly, quantitative analysis of complex biological matrices for which the goal is to reach the highest concentration sensitivity can be performed with classical RF coils accommodating 3 mm diameter NMR microtubes, 5 mm or even 10 mm diameter NMR tube in which the number of spins observed by the RF coil is four times greater than in a 5 mm tube, leading to a 4-fold increase in concentration sensitivity.

Fifteen years ago, Nicholson and Wilson [10] considered that the detection limit for ^{1}H NMR in biological matrices was theoretically about 10 μM for 500 MHz (or above) spectrometers using 5 mm probe. Even after suppression of the intense signal from water with adequate strategies, the "chemical noise" of the sample caused by the presence of hundreds of compounds near the limit of detection whose signals sum to give an irregular baseline, as well as the line broadening due to macromolecules binding and chemical-exchange phenomena, especially severe in plasma and bile, make that it was often difficult to quantify accurately compounds present at concentrations < 100 μM.

Today, the sensitivity limit for low molecular weight (≤500 Da) metabolites in body fluids is the low micromolar range depending of the number of hydrogens that contribute to a signal, the multiplicity of the resonance and the difficulty to quantitate the metabolites of interest due to chemical noise caused by considerable signal overlaps in some regions of the spectrum especially between 3.5 and 4.1 ppm [11,12].

The detection limit is estimated at 10 μM and the quantification limit at 30 μM for the singlet resonance of the methyl group of creatine in human urine after ≈20 min recording with a 300 MHz spectrometer [13]. A more complete study by the group of Wevers reports the following detection limits after ≈20–30 min acquisition time in human urine using 500 or 600 MHz spectrometers: 5 μM for a singlet resonance and 10 μM for a doublet resonance of a methyl group, 15 μM for a singlet resonance of a methine group and for a triplet resonance of a methylene group, 30 μM for a doublet resonance of a methylene group [14,15].

The limits of sensitivity for ^{19}F and ^{31}P NMR in biological matrices will be presented in the third and fourth parts of the article.

In recent years, the NMR detection limits have been lowered by a factor of 4 with the introduction of the so-called cryoprobes. In these NMR probes, the electronic components are cryogenically cooled to ≈20 K, while the sample remains at ambient temperature. This greatly reduces the electronic noise leading to a 4-fold increase in sensitivity, which for a given amount of sample reduces the experiment time by a factor of 16 over that of a conventional probe.

3. Measurement of absolute concentrations by in vitro NMR

The area of a NMR signal is directly proportional to the number of nuclei giving rise to it. This is only strictly true under well-defined experimental conditions. Indeed for quantitative measurements, a number of NMR criteria have to be considered [16–18].

1. The entire spectral width (SW) must be uniformly excited by the RF pulse. The problem is often overlooked but can be very serious at high fields for ^{13}C and ^{19}F nuclei, for example, for which the resonances extend over a wide range. The RF pulse is applied for a time period (tp) (typical times 10^{-5}–10^{-6} s) also called pulse width (PW) with a very high power (100 W) and a fixed frequency ν_1 corresponding to that of the resonance transition of the nucleus considered, i.e. $\nu_1 = \gamma B_0/2\pi$, B_0 being the flux density of the spectrometer magnetic field and γ the gyromagnetic ratio of the nucleus. The RF pulse contains not only the frequency ν_1, but also a continuous band of frequencies symmetrical about the centre frequency ν_1 over a range of ±1/tp Hz. The intensity distribution of the RF pulse follows a sinc curve so that it falls to zero at ±1/tp Hz. Nevertheless, it is reasonably constant within ±1/4 tp Hz.

   This can be experimentally checked by comparing the signal integrals of several compounds of known concentrations whose resonances spread on the entire SW considered or by acquiring, into one free induction decay (FID), a reference signal using a selection of transmitter offsets (O_1) than span the desired SW and comparing signal integrals obtained in each spectrum [16].
2. Concerning data collection, there are three features to take into account: the avoidance of differential saturation effects and that of differential nuclear Overhauser effect (NOE) enhancements as well as the need to characterise the resonance signal properly.

   The maximum possible signal intensity per FID or transient is produced by rotating the bulk equilibrium static

magnetisation (M_0) aligned along the direction of the spectrometer magnetic field (*z*-direction) into the *xy* plane. This 90° angle of tipping is called the flip angle and the pulse width causing this flip is called a $\pi/2$ or 90° pulse ($PW_{90°}$). After a 90° pulse, a spin system needs an amount of time equal to 5-fold the spin–lattice relaxation time (T_1) to undergo nearly complete relaxation (99.3% of the initial magnetisation is realigned along *z*-direction after $5T_1$). To avoid perturbation of the relative signal intensities in the spectrum, it is essential to wait for the spins to fully relax between pulses, demanding recycle time of at least $5T_1$ (after a 90° pulse) of the slowest relaxing nuclei. Clearly, one requires some knowledge of the T_1 values for the compounds present in the sample of interest or, at least, for compounds with similar structure.

While recycling times of $5T_1$ are usually bearable for proton work, they can be too much long in the study of heteronuclei. Here, fully relaxed spectra are obtained in more reasonable time using small flip angles (10–45°), which allows to shorten the recycle times since, with such angle values, magnetisation decays back to the equilibrium more rapidly.

Moreover, the invasive technique of adding paramagnetic relaxation agents, such as chromium(III) acetylacetonate ($Cr(acac)_3$), shortens the T_1 relaxation times and therefore the recycle times, but a too high concentration must be avoided as it would broaden the lines.

A further source of intensity distortions in heteronuclear spectra recorded with broadband proton decoupling arises from the NOE produced by proton saturation. For quantitative measurement, it becomes worthwhile to suppress the NOE, yet it is still desirable to collect proton-decoupled spectrum for optimum S/N and minimal resonance overlap. This is carried out by the inverse-gated decoupling technique that consists to apply proton decoupling during acquisition time and gate it off during the recovery delay. With a decoupler on/off duty cycle of usually <20%, one can provide a proton-decoupled spectrum without NOE enhancements.

It should be noted that the addition of paramagnetic relaxation agents aids to the suppression of the NOE as it eliminates the dipolar relaxation responsible for this enhancement.

The third fundamental requirement is an adequate digitisation of the signals. To minimise intensity errors, it is necessary to have at least four acquired data points (but many more are preferable) covering the resonance line width. Therefore the spectra of heteronuclei must be recorded with 32K data points or more if a large SW needs to be used.

3. The data processing must be optimised to enhance results:
   - Provided the FID has fallen to zero (or near to zero) when acquisition stops, the definition of the lineshape is enhanced by the use of zero-filling. This process increases artificially the digital resolution by interpolating data points in the frequency-domain. No new information is obtained and the improvement is purely cosmetic. Zero-filling must not be used as a substitute for correct digitisation of the acquired FID.
   - The acquired data can be manipulated prior to Fourier transformation (FT) by mathematical weighting functions known as window functions to reduce the noise amplitude and so increase sensitivity or to narrow the resonances and so enhance resolution. Due to the specific insensitivity of NMR, it is clear that sensitivity must be enhanced. The most common procedure to achieve this is to apply an exponential function to the FID (process called exponential multiplication) that causes broadening of the lines. This helps also to ensure the data are sufficiently well digitised and is particularly benefit for the spectra of heteronuclei.
   - A careful correction of the phase as well as of the baseline distortions of the spectrum is essential for an accurate quantification of the resonances.
   - Peak areas are integrated using manufacturer's standard software (1D WinNMR for Bruker spectrometers). For a Lorentzian line, the integral should, ideally, cover 20 times the linewidth each side of the peak for ensuring that 99% of its intensity have been measured. In practice, it may not be always possible to extend the integral over such distances before various other signals are met. This is particularly true for proton spectra, but also for heteronuclei spectra. Moreover it must be kept in mind that the integration accuracy is S/N dependent: for example, a S/N of 250 is needed to yield an integral value with ±1% precision, even if computer-calculated peaks areas are accurate with S/N > 20 [19]. Typically, spectra are integrated five times sequentially and the mean integral value is considered. With lower S/N values or when the resonance signals are not well resolved, the best results are obtained by the reliable, if somewhat time-consuming, method of cutting out the peaks of interest and weighing [19,20].
4. A reference signal of known concentration must be detected in the SW considered to provide a calibration standard. In biofluids and cell or tissue extracts, the concentrations of the analytes are calculated by comparing the analyte integrals with the reference integral, corrected from the number of nuclei in the resonances.

One can use an internal reference or an external reference contained in a coaxial capillary put into the larger NMR tube. Both of them must have some common specifications i.e. high purity, chemical inertness, low volatility and a resonance signal well resolved from the analyte signals. Moreover an internal reference must present an easy solubility in aqueous media and its resonance should not be broadened by protein binding or chemical-exchange phenomena, problems that can be especially severe in plasma and bile.

If for quantitative proton NMR analysis, 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid sodium salt (TSP) is the quasi-exclusively standard used [14,15,21],

several standards are employed for quantitative ^{31}P NMR (dimethyl-methylphosphonate [22], methylphosphonic acid (MPA) [23,24], phenylphosphinic acid (PPA) [24], triphenylmethylphosphonium bromide [25], glyphosate [26] or 3-aminopropylphosphonic acid which is also used as proton standard [27]) or ^{19}F NMR analysis (*para*-fluorobenzoic acid sodium salt (FBEN) [28–30], sodium fluoroacetate [28,29], trifluoroacetic acid (TFA) [31], *para*-fluoro-D-phenylalanine [32]).

However to provide a field/frequency lock for the spectrometer, deuterium nuclei must be present in the sample analysed. For this purpose, usually 10% (sometimes 5%) of deuterated water (D_2O) are added to the biofluid analysed, or the dried cell or tissue extract samples are re-dissolved in D_2O. These procedures cause a selective deuteriation of acidic protons.

The external reference contained in a coaxial capillary needs to be previously calibrated against solutions of known concentration compounds but has several advantages: (i) one single calibrated capillary can serve for any number of samples; (ii) the external reference is dissolved in a deuterated solvent, which can be not only water, but any organic solvent. The deuterated solvent provides the field/frequency lock for the spectrometer and the addition of paramagnetic relaxation agent shortens the T_1 of the reference. There is no contamination of the sample, which is available for analysis by a subsequent alternative technique; (iii) the external reference can be used for analysis of any biofluid without problems of protein-binding or chemical-exchange phenomena; (iv) one capillary can serve for concentration measurements expressed for example in M in bulk solutions (biofluids or extracts) or in moles in heterogeneous media (packed cells or excised tissues), provided that the cell pellet or the small pieces of tissues are positioned within the sensitive volume of the RF coil. This implies that the external reference has been previously calibrated in number of moles observed by the RF coil against a known number of moles of standard contained in the RF coil sensitive volume.

External reference capillaries are used for some quantitative hydrogen analysis (TSP [33]) and for almost all the quantitative metabolism studies of fluorinated and phosphorylated drugs. For ^{19}F NMR analyses, FBEN in D_2O is employed by Martino's group [34–37] and Kamm et al. [38,39], C_6F_6 and, since 1990, 1,2-difluorotetrachloroethane (DFTCE) in benzene-d6 by Hull's group [40–45], NaF in D_2O by Wyrwicz and coworkers [46]. Hexachlorocyclotriphosphazene (HCCP) in toluene-d8 [23,24] then MPA in D_2O [20] are utilised by Martino's group for ^{31}P NMR analyses.

In some cases, quantification is enabled by the use of external calibration. For each substance to be quantified a calibration curve is established by linear regression analysis using peak integral of the substance and its nominal concentration [47], or the ratio [peak height of the substance/peak height of the internal standard] and the concentration of the pure substance [25]. This procedure avoids the problems of total relaxation between consecutive scans and differential NOE enhancements, but when the calibration is done without an internal standard, the same number of transients must be acquired for each spectrum. This approach of NMR quantification is similar to that used with chromatographic methods but with a lesser sensitivity and, above all, it does not take advantage of the unique property of NMR to quantify in a single analysis all the compounds whatever their structure provided that their concentrations reach the LOD of the method.

The validation of the quantification procedures for ^{19}F and ^{31}P NMR measurements will be discussed in the third and fourth parts of the article.

4. Examples of in vitro ^{19}F NMR quantification of fluorinated drugs

As a number of fluorinated drugs are currently in clinical use and because of the favourable NMR characteristics of the fluorine nucleus, the majority of NMR drug metabolism studies concern ^{19}F NMR. We discuss here of the main results dealing with the commonly prescribed anticancer agent, 5-fluorouracil (FU) and one of its recent prodrug, capecitabine (CAP, Xeloda^{TM}).

4.1. Overview of the metabolism of FU and its prodrugs

FU has been in clinical use for the treatment of malignant disease for over 45 years. It is the mainstay of antimetabolite treatment for solid tumours, especially gastrointestinal tract, breast, and head and neck cancers [48]. FU is inactive until it undergoes complex metabolic pathways leading to fluoronucleosides (FNUCs) and then to fluoronucleotides (FNUCt), the antitumour agents.

As several reviews have been devoted to the biochemistry, metabolism, mechanism of action and pharmacology of FU [48–50], the sequence of its biochemical activation (anabolism) and degradation (catabolism) will be presented briefly.

4.1.1. Anabolism

The anabolism of FU to FNUCt is illustrated in Fig. 1. It occurs through several pathways identical to the de novo pathways followed by uracil.

The conversion of FU into 5-fluorouridine-5′-monophosphate (FUMP) can be (i) a sequential, two-step reaction involving the addition of a ribose by uridine phosphorylase to yield 5-fluorouridine (FUrd), followed by phosphorylation to FUMP catalysed by uridine kinase; (ii) a direct reaction catalysed by pyrimidine phosphoribosyl transferase that transfers a ribose phosphate from phosphoribosylpyrophosphate.

The pathway for conversion of FU to 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) is quantitatively less important. It consists in the addition of deoxyribose-1-phosphate to FU by thymidine phosphorylase leading to 5-fluoro-2′-deoxyuridine (FdUrd), which is phosphorylated to FdUMP by thymidine kinase.

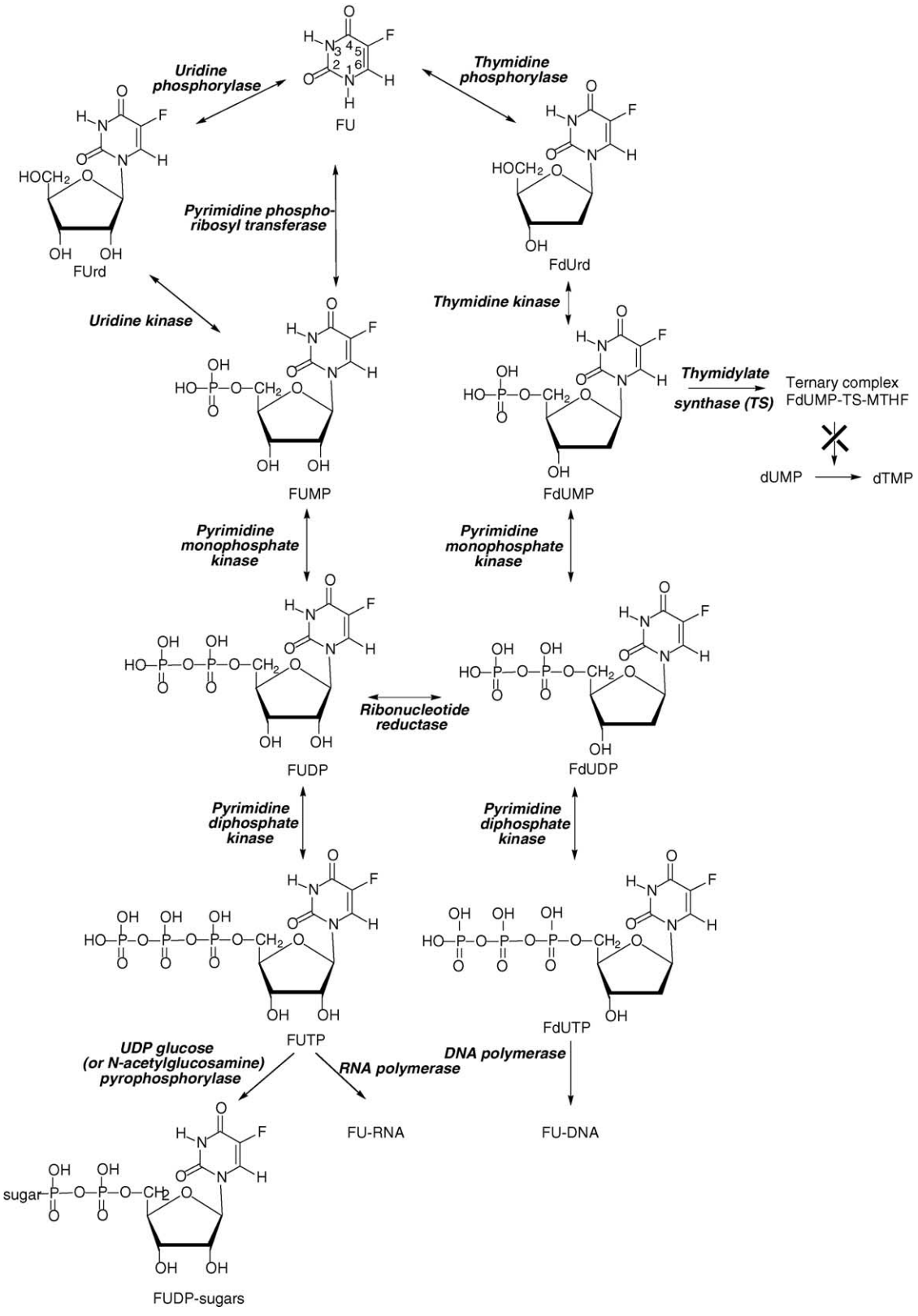

Fig. 1. Intracellular anabolism of 5-fluorouracil. All the compounds are represented in neutral form. FU, 5-fluorouracil; FUrd, 5-fluorouridine; FUMP, 5-fluorouridine-5′-monophosphate; FUDP, 5-fluorouridine-5′-diphosphate; FUTP, 5-fluorouridine-5′-triphosphate; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FdUDP, 5-fluoro-2′-deoxyuridine-5′-diphosphate; FdUTP, 5-fluoro-2′-deoxyuridine-5′-triphosphate; MTHF, N^5,N^{10}-methylene tetrahydrofolate; dUMP, 2′-deoxyuridine-5′-monophosphate; dTMP, thymidine-5′-monophosphate.

FUMP can be metabolised by pyrimidine monophosphate kinase to 5-fluorouridine-5′-diphosphate (FUDP) that can be converted either to 5-fluorouridine-5′-triphosphate (FUTP) by pyrimidine diphosphate kinase, or to the deoxyribosyl nucleotide, 5-fluoro-2′-deoxyuridine-5′-diphosphate (FdUDP) by ribonucleotide reductase. FdUDP is in equilibrium with the deoxyphosphates FdUMP and 5-fluoro-2′-deoxyuridine-5′-triphosphate (FdUTP) through pyrimidine monophosphate kinase and pyrimidine diphosphate kinase, respectively.

The anticancer effect of FU is mainly the result of two mechanisms: (i) the inhibition of thymidylate synthase (TS), and consequently DNA synthesis, by FdUMP that forms a slowly reversible ternary covalent complex with TS and a folate cofactor. The in vivo antitumour action depends on the extent and duration of TS inhibition; (ii) the incorporation of FUTP in place of uridine triphosphate into both nuclear and cytoplasmic RNA fractions. This incorporation alters RNA synthesis as well as RNA processing and function.

Two other mechanisms can be partly responsible for the cytotoxic effect of FU. The first one is the incorporation of FdUTP into DNA, resulting in damage in newly synthesised DNA. The second is the alteration of the membrane function by incorporation of FU-nucleotide-sugars (FUDP-sugars) formed from FUTP [49,50].

4.1.2. Catabolism

The catabolic pathway of FU is illustrated in Fig. 2. It is identical to the reductive pathway of naturally occurring pyrimidines, uracil and thymine.

The first step in the degradation of FU is the reversible reduction of the C5=C6 double bond of the pyrimidine ring. In the presence of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), FU is converted to 5,6-dihydro-5-fluorouracil (FUH_2), the reaction being catalysed by the enzyme dihydropyrimidine dehydrogenase (DPD).

At this stage, a second enzyme, dihydropyrimidinase catalyses the reversible hydrolytic cleavage of the C3–C4 bond leading to α-fluoro-β-ureidopropionic acid (FUPA).

β-Alanine synthase or β-ureidopropionase catalyses the next step. It consists of irreversible conversion of FUPA into FBAL with the concomitant release of ammonia from the N3 position and carbon dioxide from the C2 position of the former pyrimidine ring. Urea is formed by the urea cycle from CO_2 and NH_4^+.

Since FBAL is the major metabolite, the description of the catabolic pathway of FU is most of the time limited to the three steps described above.

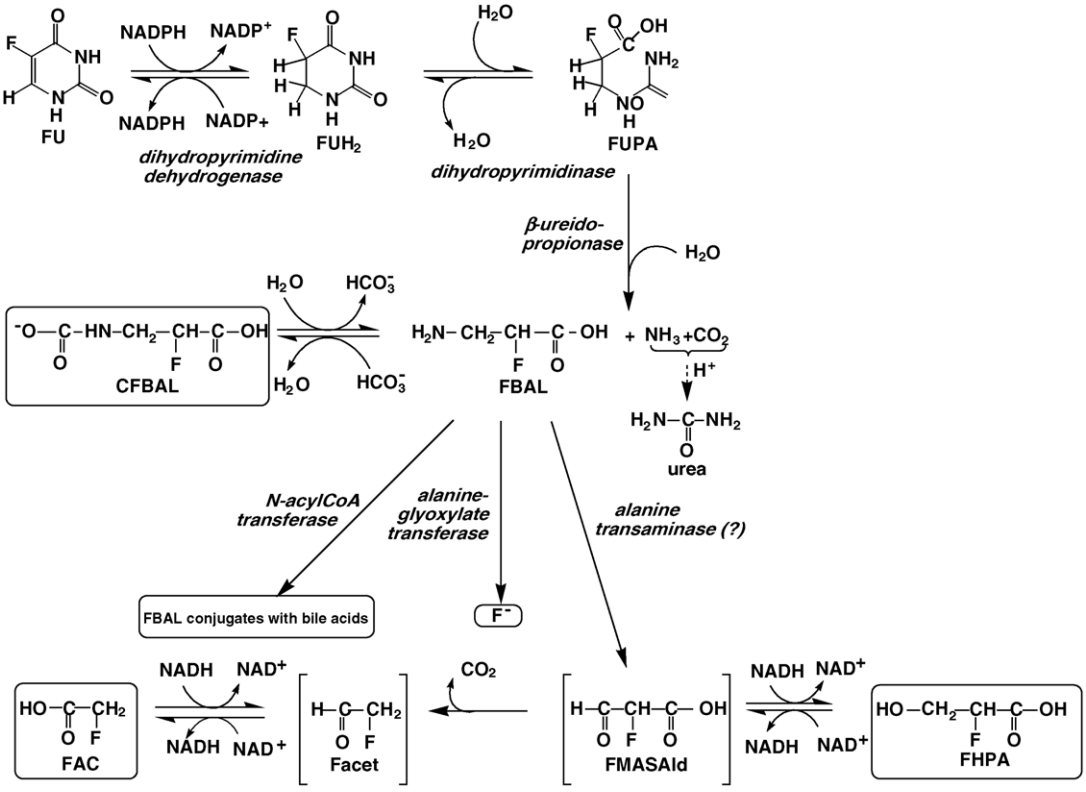

Fig. 2. Catabolic pathway of 5-fluorouracil. All the compounds (except CFBAL) are represented in neutral form. FU, 5-fluorouracil; FUH_2, 5,6-dihydro-5-fluorouracil; FUPA, α-fluoro-β-ureidopropionic acid; FBAL, α-fluoro-β-alanine; CFBAL, *N*-carboxy-α-fluoro-β-alanine; F^-, fluoride ion; FMASAld, fluoromalonic acid semi-aldehyde; FHPA, 2-fluoro-3-hydroxypropanoic acid; Facet, fluoroacetaldehyde; FAC, fluoroacetic acid. Fluorinated metabolites identified for the first time with ^{19}F NMR are represented in boxes. Non-detected fluorinated intermediates are represented in brackets.

4.2. ^{19}F NMR studies of body fluids: a contribution to the understanding of the degradative pathway (catabolism) of FU and CAP

4.2.1. FU

^{19}F NMR studies of biological fluids of patients treated with anticancer FU have furthered understanding of its catabolism. FU, FUH_2, FUPA and FBAL are easily detected in one run analysis in biofluids (plasma, urine) together with two other metabolites identified as fluoride ion (F^-) and *N*-carboxy-α-fluoro-β-alanine (CFBAL) in patients treated with FU.

The assumption that F^- results from the metabolic cleavage of the C–F bond of FBAL is strongly supported by the detection of elevated levels of F^- in acidic urine of rats treated with FBAL. Indeed, FBAL is defluorinated chemically in basic medium only [51]. More recently, it has been shown that L-alanine-glyoxylate aminotransferase catalyses the elimination of F^- from (*S*)-(*R*)-FBAL [52].

CFBAL results from *N*-carboxylation of FBAL in presence of hydrogen carbonate ion (HCO_3^-). This reaction corresponds to the well-known equilibrium between compounds with an amino group (as FBAL) and their corresponding carbamate (*N*-carboxy) derivatives in weakly alkaline aqueous carbonate solution. Owing to its acid lability, CFBAL easily escapes detection. It is thus detectable in plasma and urine at pH superior to 7.3–7.5 [40,53].

Biliary excretion of fluoropyrimidine drugs in humans is low: 2–3% for radiolabelled FU as measured by scintillation counting [54] and 0.8% for the FU prodrug, 5′-deoxy-5-fluorouridine (5′dFUR) as determined by ^{19}F NMR [55]. The excreted metabolites are FBAL and F^- (for about 10%) and conjugates of FBAL (for about 90%) with cholic acid (choloFBAL) and chenodeoxycholic acid (chenoFBAL) in a 74/26% ratio in patients with external bile drainage [55–57]. In a bile sample obtained at surgery from a patient treated with intrahepatic FU, three conjugates of FBAL were detected, choloFBAL, chenoFBAL, and the conjugate of FBAL with the third major bile acid in humans, deoxycholic acid, in a 54/17/29% ratio [55].

Owing to the close resemblance between the structures of the natural β-aminoacid, β-alanine, and its fluorinated analogue FBAL, it was suggested several decades ago that FBAL could be further metabolised into fluoroacetate (FAC) by analogy with the conversion of β-alanine into acetate [58]. This assumption had never been demonstrated until recently. Low levels of two compounds that are fluorinated analogues of β-alanine metabolites are detected with ^{19}F NMR in urine of patients treated with FU. They are 2-fluoro-3-hydroxypropanoic acid (FHPA) and FAC [35,59]. As FU commercial formulations are not pure, FAC and FHPA arise from both the metabolism of impurities and that of FU itself [35,59]. Hull et al. [40] also detected FHPA in urine of patients treated with FU but did not identify this compound. A typical ^{19}F NMR spectrum of a urine sample from a patient treated with FU is presented in Fig. 3.

4.2.2. Capecitabine

CAP is a prodrug of 5′dFUR, another FU prodrug, designed to be used as an oral formulation to overcome the substantial gastrointestinal toxicity (diarrhoea) of 5′dFUR without compromising its antitumour efficacy [60]. CAP is converted to FU through a cascade of three enzymes. Oral CAP passes intact through the intestinal tract and is converted first to 5′-deoxy-5-fluorocytidine (5′dFCR) by carboxylesterase, almost exclusively located in the liver. Cytidine deaminase present in high concentrations in the liver and various types of solid tumours then transforms 5′dFCR into 5′dFUR. Finally, thymidine phosphorylase that is more active in malignant tissue than in normal tissue generates FU selectively within tumours (Fig. 4). Thus, higher amounts of FU are present in the tumour and exposure of healthy tissues to FU is minimized. As CAP and its two first metabolites do not show intrinsic cytotoxicity, the activation pathway is expected to combine high antitumour efficacy with improved clinical safety [61].

Clinical pharmacological studies show that oral CAP and its metabolites are rapidly and extensively excreted in urine. Indeed about 98% of a single 2 g oral dose of radiolabelled CAP is recovered over a period of 6 days based on radioactivity measurements (95.5% in urine and 2.6% in faeces) with a very large proportion (84%) excreted in urine in the first 12 h post-dosing [62].

The mean value for the urinary recovery of drug and its metabolites up to 48 h post-dosing, measured by ^{19}F NMR (84.2%), is in agreement (within 10%) with the 92.3% of the radioactivity recovered at that time, demonstrating the reliability of the ^{19}F NMR spectroscopic quantification [62]. Moreover, all the metabolites of the catabolic pathway: CAP → 5′dFCR → 5′dFUR → FU → FUH_2 → FUPA → FBAL (Fig. 4) are quantified [62,63]. As expected, the major metabolite by far is FBAL, with 5′dFCR, 5′dFUR and FUPA the other significant forms, FU and FUH_2 representing a small percentage of the excretion (Table 2).

In patients treated orally with CAP at a dose of 1000–1250 mg/m^2/day administered twice daily at 12 h interval, the ^{19}F NMR analysis of urine collected 12 h after the first CAP dose shows a total recovery of fluorine-containing compounds of 71% [37] not very different from the 84 and 86% recoveries reported by Judson et al. [62] and Cassidy et al. [63] after 48 and 24 h urine collection, respectively. Moreover, the recovery percentages of all the pre-cited metabolites are similar in the three studies except that of FBAL that is lower in the Desmoulin's study (Table 2) due to its long $t_{1/2}$ value [54]. Some other classical FU catabolites are quantified by Desmoulin et al. [37]: F^-, FHPA and FAC. Since CAP formulation is pure, this demonstrates that FU can be metabolised into FHPA and FAC via FBAL [64]. Moreover, a novel very minor degradative pathway is identified: the transformation of 5′dFCR into 5-fluorocytosine (FC) and then 5-fluoro-6-hydroxycytosine (OHFC) (Table 2). The complete degradative pathway of CAP is depicted in Fig. 4, and a characteristic ^{19}F NMR spectrum is illustrated in Fig. 5.

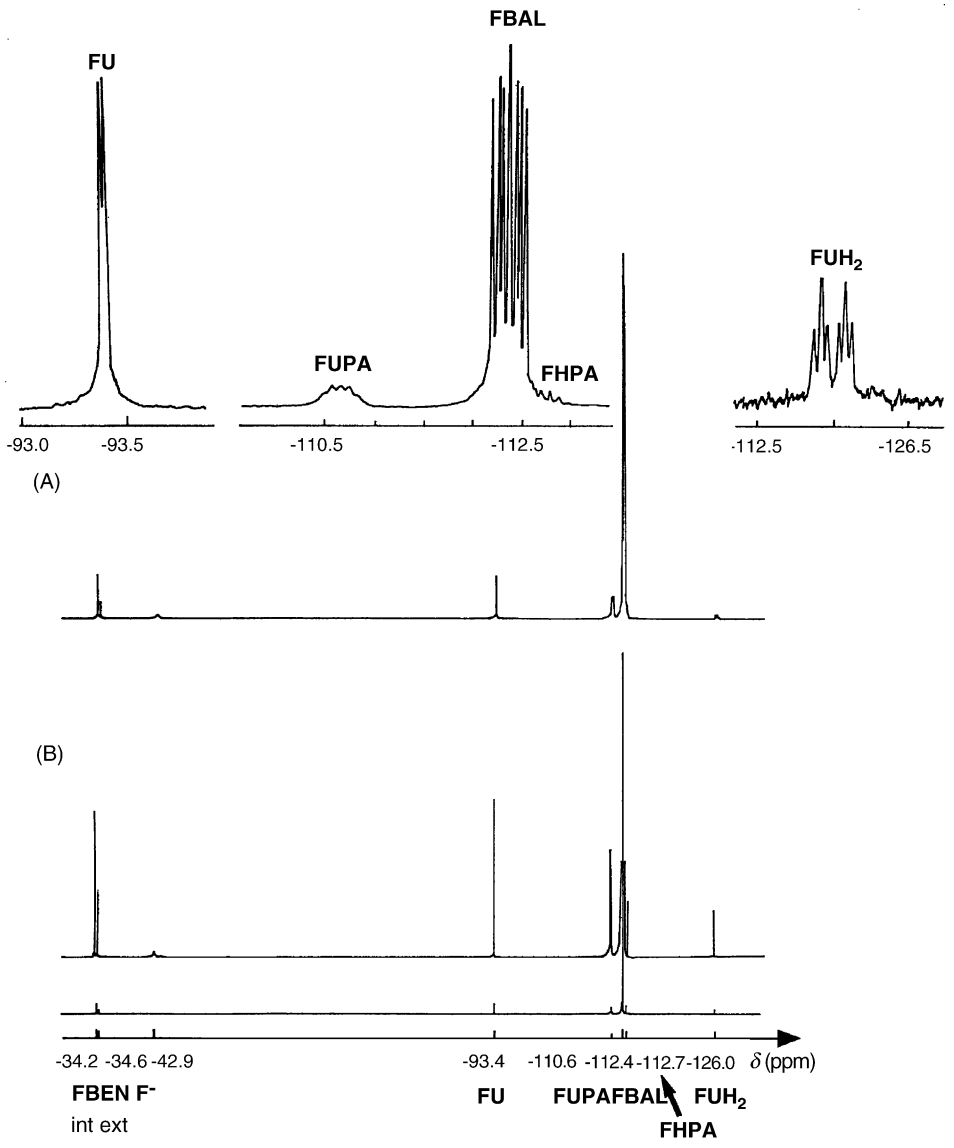

Fig. 3. ^{19}F NMR spectra at 282 MHz without (A) and with proton decoupling (B) of a urine sample from a patient treated with a continuous perfusion of 5-fluorouracil for 4 days at a dose of 0.6 g/m^2/day and folinic acid at a dose of 0.5 g/m^2/day. The sample was recovered at the end of the third day of treatment; pH of the sample: 5.5. The chemical shifts are expressed relative to the resonance peak of TFA (5% (w/v) aqueous solution) used as an external reference. Int and ext FBEN, sodium monofluorobenzoate as internal and external references for quantitation; F^-, fluoride ion; FU, 5-fluorouracil; FUPA, α-fluoro-β-ureidopropionic acid; FBAL, α-fluoro-β-alanine; FHPA, 2-fluoro-3-hydroxypropanoic acid; FUH_2, 5,6-dihydro-5-fluorouracil. The signal of fluoroacetic acid (FAC) is too low to be detected in non-concentrated urine samples. The upper part shows the enlarged signals of FU, FUH_2, and the area FUPA-FBAL-FHPA to display their multiplicity.

4.3. ^{19}F NMR studies of tissues and cells: contribution to the knowledge of the metabolic profile of FU

The cytotoxic activity of FU requires its anabolic conversion into FNUCs and FNUCt. FNUCt are trapped within the cells since the presence of the charged phosphate group prevents their diffusion out of the cell. Consequently, plasma levels of FU and its diffusible metabolites do not correlate well with the response of patients to the treatment. The determination of the fluorinated content of cells is needed for the knowledge of the metabolic profile of FU, especially that of FNUCt involved in the cytotoxic mechanisms (FUTP, FdUTP, FdUMP and FUDP-sugars).

Even if ^{19}F NMR analysis can be carried out in situ in tissues of animals or humans and in perfused living cells or isolated organs, we will only consider here the studies dealing with packed cells or excised tissues and their extracts for which the best resolution of ^{19}F NMR signals can be achieved. During the last 15 years, several attempts have been done to assign individual FNUCs and FNUCt signals in tissue extracts. Since the range of δ for these species at a given pH is small (0.04 ppm for the two nucleosides FUrd and FdUrd [65], and 0.3–0.6 ppm for all the FNUCt [66–68]),

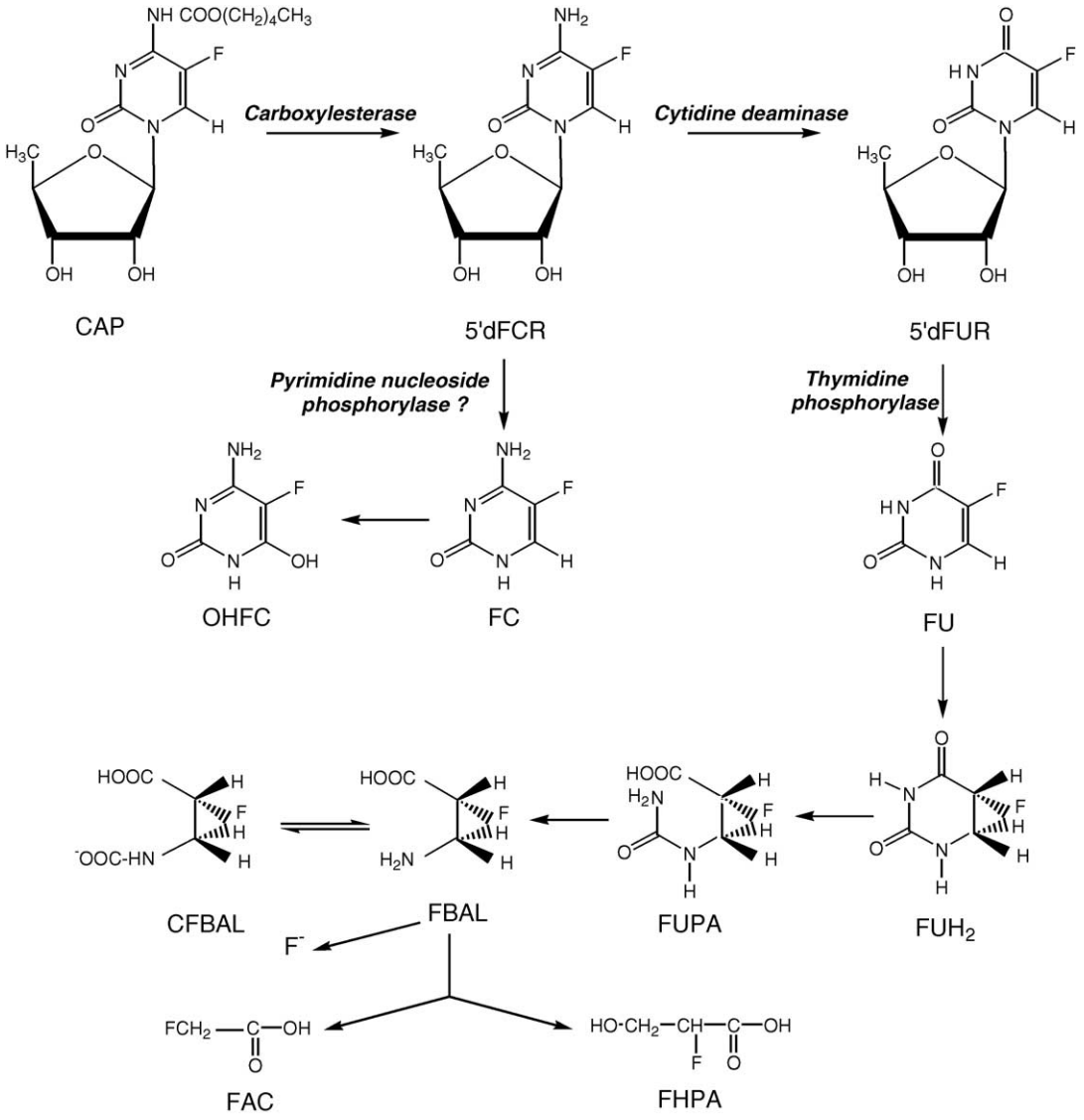

Fig. 4. Catabolic pathway of capecitabine. All the compounds (except CFBAL) are represented in neutral form. CAP, capecitabine; 5′dFCR, 5′-deoxy-5-fluorocytidine; 5′dFUR, 5′-deoxy-5-fluorouridine; FU, 5-fluorouracil; FUH_2, 5,6-dihydro-5-fluorouracil; FUPA, α-fluoro-β-ureidopropionic acid; FBAL, α-fluoro-β-alanine; CFBAL, *N*-carboxy-α-fluoro-β-alanine; F^-, fluoride ion; FHPA, 2-fluoro-3-hydroxypropanoic acid; FAC, fluoroacetic acid; FC, 5-fluorocytosine; OHFC, 6-hydroxy-5-fluorocytosine.

unambiguous a priori assignments are difficult. Using authentic references, Cabanac et al. [65] find that FdUrd resonates 0.04 ppm upfield than FUrd at 25 °C and pH 5–6 in PCA extracts of liver. However, a spectral discrimination between FUrd and FdUrd resonances is not always effective. For example, the sequential addition of FUrd and FdUrd in PCA extracts of murine tumours from mice treated with FU results in an increase of the same peak without any apparent resolution at pH 6.5 and 7 °C [38].

The assignments of the FNUCt resonances are usually accomplished by spiking samples at a given pH with authentic references. Knop and coworkers [67,69] identify the signals of FUMP, FUDP, FUTP and several FUDP-hexoses (FUDP-glucose (FUDP-Glc), FUDP-*N*-acetylglucosamine (FUDP-GlcNAC), and FUDP-*N*-acetylgalactosamine) in chloroform–methanol extracts of tumour cells treated with FUrd or FdUrd at pH 8.0–9.2 and 18.5–22 °C on a 11.75 T spectrometer. In the acid-soluble (AS) fractions of PCA extracts or soluble fractions of acetonitrile extracts of rat liver treated with FU, Arellano et al. [68] detect seven ^{19}F signals at pH 5.4 and 25 °C. Five of them are identified and attributed to FUMP, FUDP, FUTP, FUDP-Glc, and FUDP-GlcNAC (Fig. 6a). From a systematic study of the pH dependence of ^{19}F NMR δ of five nucleotides (FUMP, FdUMP, FUDP, FUDP-Glc, and FUTP) in model solutions, Lutz and Hull [66] recommend that, for a secure assignment of the signals, NMR measurements be done at pH 5 and 7 in order to utilise the differences in titration behaviour. At pH 5, the signal of FdUMP

Table 2
Percentages of capecitabine (CAP) and its fluorinated metabolites recovered in human urine as measured by ^{19}F NMR

Metabolite	% of excreted dose (mean ± S.D. or mean and (% CV))		
	Judson et al. [62] (n = 6)[a]	Cassidy et al. [63] (n = 23)[a]	Desmoulin et al. [37] (n = 14)[b]
CAP	2.9 ± 1.7	2.7 (39%)	3 ± 1
5′dFCR	7.2 ± 2.0	7.4 (42%)	10 ± 3
5′dFUR	11.1 ± 3.0	9.8 (44%)	7 ± 2
FU	0.5 ± 0.1	0.7 (63%)	0.6 ± 0.2
FUH_2	0.4 ± 0.1	0.3 (89%)	0.3 ± 0.2
FUPA	4.9 ± 0.8	3.9 (40%)	4 ± 1
FBAL	57.3 ± 4.5	61.2 (28%)	46 ± 4
F^-			0.2 ± 0.3
FHPA			0.3 ± 0.1
FAC			0.004 ± 0.002[c]
FC			0.01 ± 0.002[c]
OHFC			0.02 ± 0.002
Total	84.2 ± 6.0	86.0 (28%)	71 ± 17

[a] The patients receive a single oral dose of 200 mg of CAP. Six patients are enrolled in the Judson et al. study [62] with radiolabelled CAP and 23 in that of Cassidy et al. [63]. The urines are collected over 48 [62] and 24 h [63] post-dosing, respectively. The samples are concentrated 5-fold before ^{19}F NMR analysis.
[b] The patients (11) are treated orally with CAP at 1000–1250 mg/m^2/day twice daily at 12 h interval. Among these patients, three receive a second treatment 3 months later. The urine samples (14) are collected over 12 h after the first CAP dose and concentrated ≈ 10-fold (8–14) before ^{19}F NMR analysis.
[c] Metabolites observed only in 4 over 14 urine samples.

can be clearly recognized as it resonates upfield from all other species [34,66], whereas FUDP and FUTP signals having very close δ are not resolved or resolved with difficulty [68]. *A contrario*, at neutral pH, it becomes difficult to distinguish FdUMP from FUDP-hexoses and FUTP even if spiking with authentic substances [66]. In a recent study, Knop's group [69] show that at pH ≈ 9, the signals of all the ribosylFNUCt aforementioned and that of FdUMP can be resolved.

Usually, the incorporation of FU into RNA is evaluated either by measuring the radioactivity in isolated RNA using radiolabelled FU or by quantification of the fluorinated compounds resulting from RNA degradation,

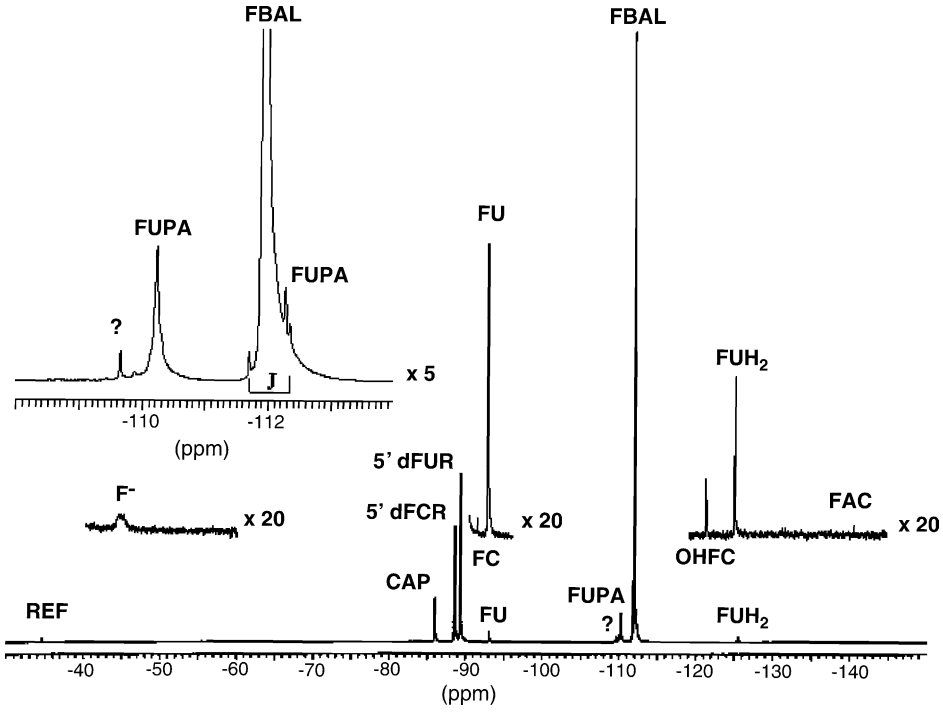

Fig. 5. ^{19}F NMR spectrum at 282 MHz with proton decoupling of a urine sample from a patient treated orally with capecitabine at a dose of 2900 mg/day administered twice daily at 12 h interval. Urine fraction 0–12 h collected after the first dose of 1450 mg and 14-fold concentrated, pH of the sample: 5.5. The chemical shifts are expressed relative to the resonance peak of TFA (5% (w/v) aqueous solution) used as external reference. REF, external reference; F^-, fluoride ion; CAP, capecitabine; 5′dFCR, 5′-deoxy-5-fluorocytidine; 5′dFUR, 5′-deoxy-5-fluorouridine; FC, 5-fluorocytosine; FU, 5-fluorouracil; ?, unknown; FUPA, α-fluoro-β-ureidopropionic acid; FBAL, α-fluoro-β-alanine; FHPA, 2-fluoro-3-hydroxypropanoic acid; OHFC, 6-hydroxy-5-fluorocytosine; FUH_2, 5,6-dihydro-5-fluorouracil; FAC, fluoroacetic acid. J, $^1J_{^{13}C—F}$ coupling constant.

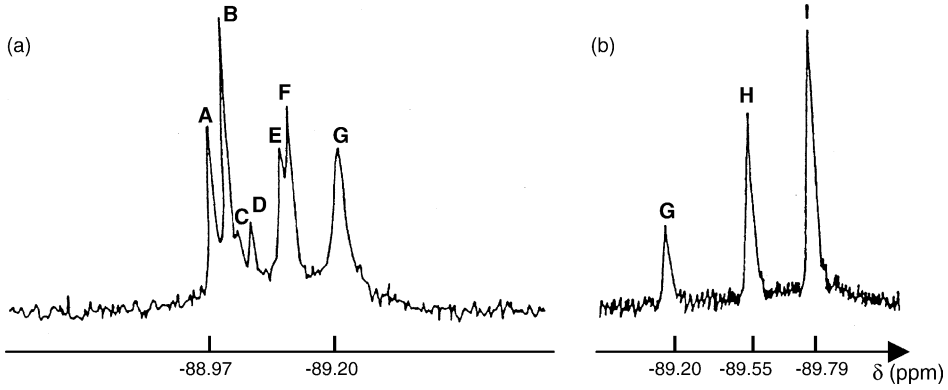

Fig. 6. ^{19}F NMR spectra at 282 MHz of the region of fluoronucleotide and 5-fluorouridine-5′-diphosphate-sugar (FUDP-sugar) resonances at pH 5.4 in acid-soluble (a) and acid-insoluble (b) fractions of a perchloric acid liver extract from a rat treated with 5-fluorouracil. The chemical shifts are relative to the resonance peak of TFA (5% (w/v) aqueous solution) used as an external reference. A: FUDP-*N*-acetylglucosamine (−88.97 ppm); B: FUDP-glucose (−89.00 ppm); C: unknown (−89.02 ppm); D: unknown (−89.04 ppm); E: FUDP (−89.10 ppm); F: 5-fluorouridine-5′-triphosphate (FUTP, −89.11 ppm); G: 5-fluorouridine-5′-monophosphate (FUMP, −89.20 ppm); H: 5-fluorouridine-2′-monophosphate (2′FUMP, −89.55 ppm)*; I: 5-fluorouridine-3′-monophosphate (3′FUMP, −89.79 ppm)*. *: The assignments of H and I resonances are erroneously reversed in reference [68] with respect to the original assignments (3′FUMP appearing upfield relative to 2′FUMP, on the basis of enzymatic and chemical treatment procedures [34]).

namely 5-fluorouridine-2′-monophosphate (2′FUMP) and 5-fluorouridine-3′-monophosphate (3′FUMP) that can be further degraded into FU [70]. ^{19}F NMR cannot be used to measure directly FU incorporated into RNA. The restricted motion of macromolecules results in a substantial signal broadening (≈9 ppm for RNA-incorporated FUTP signal [71,72]), and, consequently, a loss of sensitivity, which precludes the detection of such a signal in tissues after treatment with FU at therapeutic doses. On the other hand, ^{19}F NMR is a valuable method to determine FNUCt resulting from the acid hydrolysis at 70 °C of the acid-insoluble (AI) fraction of PCA extracts. The ^{19}F NMR analysis of the hydrolysate shows three resonances. The enzymatic and chemical treatments demonstrate that they correspond to ribosylFNUCt and not deoxyribosylFNUCt [34,68]. Since the fluorinated content of the AI fraction only concerns fluorine incorporated into macromolecules (FUTP incorporated into RNA, FdUTP incorporated into DNA or the ternary complex FdUMP-TS-folate cofactor), the ribosylFNUCt reflect the incorporation of FU into RNA. The two major resonances are identified as 2′- and 3′FUMP with specific hydrolysis reactions on isolated RNA, and the minor one to FUMP by spiking with the authentic standard [34]. The ^{19}F NMR spectrum of the FNUCt resonance area of the AI fraction of a PCA liver extract is illustrated in Fig. 6b.

An excellent correlation was observed between the fluorinated content of AI fractions of cultures of the fungus *Nectria haematococca* supplemented with unlabelled or [2-^{14}C]-labelled FU measured by ^{19}F NMR (4.4 ± 0.3% of the initial FU) or by scintillation counting (4.0 ± 0.1% of the initial FU), respectively [34]. This demonstrates the reliability of the NMR quantification.

Despite the potentiality of the ^{19}F NMR technique for the resolution of FNUCt and FNUCs components, the recent metabolic studies on FU in extracts of murine colon tumours grown in mice or in mice livers monitor the time-dependence variation of the whole anabolite pool (FNUCt and FNUCs) [38] or that of total FNUCt and FNUCs independently [73,74].

4.4. Spectra recording conditions for quantitative analysis of bulk solutions

Hull's group determines concentrations of fluorine-containing compounds in urine, plasma and tissue extracts using Bruker AM-500 or AM-300 SWB spectrometers (^{19}F resonance frequencies 470.6 and 282.4 MHz, respectively) with 5 or 10 mm diameter tubes without ^{1}H decoupling, which avoids complications owing to NOE signal enhancements [40,41,43].

A coaxial capillary (2 or 3 mm diameter) containing a solution of C_6F_6 [40] or DFTCE [41,43] in benzene-d6 doped with the relaxation agent $Cr(acac)_3$ is employed for field/frequency lock and for chemical shift and quantitation reference. The concentration of the reference is calibrated against solutions of FU or FU and TFA or FC and TFA of known concentrations [40,41,43].

Using the following parameters, fully relaxed spectra are obtained and peak areas are directly proportional to concentrations:

- With the AM-500 spectrometer and for urine sample analysis: probe temperature 28 °C; SW 71,428 Hz; 65,536 data points zero-filled to 131,072; PW 3 μs (22° flip angle); repetition time (RT) 1.5 s; exponential line broadening (LB) 1 Hz. For plasma sample analysis, the same conditions are used except PW 5 μs (11° flip angle), RT 1 s, LB 20 Hz and 65,536 data points with no zero-feeling. For mouse liver extract analysis, the same conditions as in urine are used except SW 83,333 Hz, 65,536 data points with no zero-feeling, PW 3 μs (20° flip angle), RT 2 s [40,41].

- With the AM-300 SWB spectrometer: probe temperature 25 °C; SW 55,555 Hz; 64,000 data points; PW corresponding to 9° flip angle; RT 0.5 s for plasma sample analysis [43].

Martino's group using a Bruker WB-AM 300 spectrometer with the proton decoupling inverse-gated technique and 10 mm diameter tubes measure concentrations of FU or its prodrugs and their metabolites in biofluids, tissue extracts, perfusates and culture media. The magnetic field is shimmed from the ^{1}H NMR resonance of water. The external standard for quantification, namely a solution of FBEN in D_2O doped at saturation with $Cr(acac)_3$, is placed in a coaxial 4 mm diameter capillary. Its apparent concentration is previously calibrated against solutions of FC and fluoroacetamide of known concentrations. $Cr(acac)_3$ (≈2.5 mg) is added to the 2.5–3 mL sample analysed except for concentrated perfusates, culture media and PCA extracts.

The recording conditions are: probe temperature 25 or 4 °C; SW 41,667 or 29,411 Hz; 32,768 data points zero-filled to 65,536; PW 7 μs (40° flip angle in urine, bile and non-concentrated perfusates, ≈30° in PCA extracts and ≈20° in concentrated perfusates at 25 °C); acquisition time 0.39 or 0.56 s; RT 3.4 or 3.6 s for quantification of biofluids, perfusates and culture media, 1.4 or 1.6 s for that of PCA extracts and concentrated perfusates; LB 1–6 Hz, except for culture media, 15 Hz [34–37]. Fully relaxed spectra are obtained for all media analysed. This is demonstrated from experiment in which the repetition time is set to (i) 10 or 10.4 s with $Cr(acac)_3$ instead of 3.4 s with $Cr(acac)_3$ in biofluids or perfusates; or (ii) 3.4 s with $Cr(acac)_3$ instead of 1.4 s without $Cr(acac)_3$ in PCA extracts or concentrated perfusates [35,36,64] but in which all other parameters were left unchanged. The difference between concentration values thus determined change no more than a few percent (5–10%). The high ionic strength of concentrated perfusates and PCA extracts and the high viscosity of the first medium induce a decrease of the flip angle for a given value of PW (see above) and probably of the T_1, leading to an accurate quantification even with a short RT without $Cr(acac)_3$ and a value of 0.4 for the time ratio decoupler on/off duty cycle (instead of a required value < 0.2) in the inverse-gated sequence [16].

The detection threshold is approximately (i) 2–2.5 μM after 20–24 h of recording at 25 °C [37,43], 2 μM after 10 h recording at 7 °C [38] in 10 mm diameter tubes with 300 MHz spectrometers; (ii) 3 μM and 1–2 μM after ≈12 h recording at 25 °C in 5 mm diameter tubes with a 400 MHz spectrometer and in 10 mm diameter tubes with a 500 MHz spectrometer, respectively [39,40].

The detection sensitivity may be increased by concentration of the biofluid of interest or by extraction of a higher amount of tissue, but also by using the NOE enhancement of the signal induced by continuous ^{1}H decoupling application. In the last case, a detection threshold of 0.3 μM in plasma is reached but large quantitation errors are expected since the signal integrals will not be strictly proportional to concentration [43].

The accuracy and precision of the NMR concentration determinations are generally on the order of 5–10% for concentrations > 50 μM and approximately ±1 μM for a concentration of a few μM near the limit of detection [40]. These values seem more realistic than that of ±2% reported by Rengelshausen et al. [43] since the use of a coaxial insert tube reference introduces a level of error of ≈5% [75] (between ≈3 [43] and 7%, personal unpublished data).

4.5. Spectra recording conditions for quantitative analysis of excised tissues and packed cells

4.5.1. Excised tissues

Hull and coworkers are the sole to develop a ^{19}F NMR methodology for the quantification of fluoropyrimidine metabolites in intact human or animal excised tissues at 4 °C [42,45,76]. Their previous study on FdUrd metabolism in Novikoff hepatoma in the rat [42] is completed by an important study of FU metabolism in a rat model with DS sarcoma transplanted in the thigh for a wide range of therapy protocols [45]. Quantitative FU metabolite profiles in tumour, liver and kidney tissue on one rat cohort receiving FU either by intravenous or locoregional intraarterial route at different doses and over different infusion times are compared and correlated with therapy response determined from a second cohort of rats.

The ^{19}F NMR spectra are recorded at 470.6 MHz on a Bruker AM-500 spectrometer in 10 mm diameter tubes at 4 °C without spinning and proton decoupling. Owing to the large linewidths observed for the tissue spectra, proton decoupling would have provided little improvement in resolution or sensitivity at least for FU and anabolites.

Tissue excision is performed as quickly as possible since at room temperature the F-nucleoside triphosphates decrease by ≈13%/min while monophosphates and nucleosides (but not FU) increase in a corresponding manner [77]. This procedure takes about 2 min and tissue samples are immediately frozen in liquid nitrogen and stored at −25 °C until NMR analysis.

Each frozen tissue (0.5–1.2 g) is cut into several small pieces that are placed in a chilled tube. Ice-cold HEPES buffer at pH 7.4 is then added to give a volume of ≈1.3 mL. A sealed 3 mm reference capillary containing a solution of DFTCE in benzene-d6 is inserted in the 10 mm sample tube. The bottom of the tube is adjusted via the sample spinner at a constant position so that the same absolute quantities (nmol) of DFTCE and the entire volume (1.3 mL) containing the small pieces of tissue are within the sensitive volume of the RF coil.

In a separate experiment, the DFTCE signal integral is calibrated against a FU standard solution whose volume (1.2 mL) is within the RF coil sensitive volume. It has been found equivalent to 130 ± 10 nmol F.

The data acquisition parameters used are: SW 83,300 Hz; 16 K time domain data points; PW 3 μs (10° flip angle, α); RT 0.3 s; LB 50 Hz. With these recording conditions,

the saturation factor (F_{sat}) (which represents the fraction of the signal intensity detected relative to that obtained under fully relaxed conditions) calculated using the relation $F_{sat} = (1 - E_1)/(1 - E_1 \cos\alpha)$ with $E_1 = e^{-RT/T_1}$ and $T_1 = 2.3$ s (that of FU in rat tumours at 37 °C [78]) is 0.90. Since the T_1 of FU metabolites in vivo at 37 °C are lower than that of FU (1.3 s for FNUCt in rat tumours [78] and 1.6 s for FBAL in human liver [79]), the T_1 values of FU and metabolites at 4 °C are certainly less than 2.3 s. Therefore, the error in metabolite concentrations due to differences in T_1 relaxation times is less than 10%.

The FIDs acquired over successive 1 h periods are stored separately and used to check for changes in metabolite concentrations over time. The FU catabolites content is stable up to 12 h, whereas FNUCt concentrations decrease by ≈20–40% over 3 h while FNUCs or FU or both increase in a compensate manner. Thus quantitative analysis is performed only with NMR integration data for 1 h or almost 2 h of data acquisition.

The precision of the concentrations from integrals is estimated to be ±10%. For data analysis, ^{19}F signals or overlapping signals quantified are: FU, its major catabolite FBAL, F^-, FNUCs, FNUCt (even if with resolution enhancement, at least two components attributed to FUMP and FUMP-hexoses (≈−88.7 and −88.9 ppm, respectively) are observed) and the sum of FUPA, CFBAL and other FBAL adducts. FUH_2, the first FU catabolite is not detected in any tumour, liver or kidney tissue. The absolute number of moles of fluorine is expressed as concentration in nmol/g wet weight.

The detection threshold for quantification in 1 h measurement is ≈5 nmol/g from 0.5–1.2 g samples.

Comments relative to the saturation factor and the recording conditions used for quantitative analysis in bulk solutions.

With the recording parameters used by Hull's group [40,41,43] for the quantitative analysis of bulk solutions and a T_1 of 3.2 s (T_1 value chosen as an average of FU and FBAL T_1 values found in literature (1.2–4.6 s) in aqueous solutions, human plasma or blood [45,53,73,79,80]), the calculated saturation factor is in the range of 0.89–0.95. The error in concentrations due to differences in T_1 relaxation times does not exceed the precision of the method (5–10%, see above).

The saturation factors calculated from the same T_1 value and the acquisition parameters used by Martino's group are 0.89–0.90 for biofluids and concentrated perfusates and 0.81 for PCA extracts. Since T_1 values are shortened by addition of $Cr(acac)_3$ in biofluids and by the high viscosity and/or ionic strength in concentrated perfusates and PCA extracts, it is expected that fully relaxed spectra are obtained in all media analysed. This is demonstrated by the non-significant modification of the signal intensities measured when RT is increased by a 2.5–3-fold factor [35–37].

4.5.2. Cell pellet

The role of the bystander effect of cytosine deaminase/5-fluorocytosine (CD/FC) gene therapy in a Dunning rat prostate tumour model is studied with ^{19}F NMR [44]. Cultures of a rat prostate cell line transfected with a gene encoding for CD are incubated with a high dose of FC (387 or 774 μM) for various times. Washed cell pellets ((0.3–1) × 10^8 cells) and culture medium are analysed by ^{19}F NMR in separate measurements. The spectra of intact cells show the presence of three broad resonances corresponding to FC (−92.5 ppm relative to TFA as external reference), FU (−93.6 ppm) and a broad composite signal (−88.7 to −89.3 ppm), which may contain any or all FNUCt. A strong signal of FC and a weak resonance of FU are observed in the culture medium. Neither cells nor culture medium exhibit signals for FNUCt or any FU catabolites.

The spectra are recorded using a Bruker AM 500 spectrometer at 470.6 MHz and 4 °C without proton decoupling using the following parameters: SW 25,000 Hz; PW 12 μs (36° flip angle); RT 1.0 s. Successive 1 h acquisition periods are stored separately. The comparison of spectra shows no change in FC and metabolite concentrations over 4 h in cell suspensions. For culture medium, data acquisition is performed up to 10 h. The cell pellet resuspended in a minimum volume (0.3–0.5 mL) of buffer is placed in an 8 mm diameter tube inserted into a 10 mm diameter tube containing deuterated water for field/frequency lock. The vertical position of the 8 mm tube is adjusted within the 10 mm tube so that the cell pellet, settled to the bottom of the tube, is always centred within the RF coil. For NMR measurements of the culture medium, the 8 mm tube is filled to a constant height of 30 mm overcoming the 28 mm height of the RF coil.

The ^{19}F NMR spectra are processed using a constant absolute intensity scale and the signals integrated using the Bruker software routines. The concentrations of fluorine-containing species in the culture medium are calculated by comparing their signal integrals with that of FC in the incubation medium that contains FC in known concentration. For the analysis of the cell pellet, it is necessary to perform a calibration experiment to determine the proportionality constant for signal integral versus an absolute quantity of fluorine nuclei. Increasing volumes of incubation medium (0.2–1.0 mL) with known FC concentrations are put in the 8 mm tube and positioned in the manner employed for cell pellet analysis. The FC signal integral intensity is measured for each solution after recording of N transients (FIDs). A plot of FC signal integral per 1000 transients ($I_{1000} = I(1000/N)$) as a function of mL of medium or FC mole quantities in the medium analysed, displays an initial linear behaviour followed by a plateau region when the sample volume exceeds the observed RF coil volume (0.70 mL in the 8 mm tube) or when the FC number of moles reaches 31.2 nmol. Therefore, the calibration constant per unit I_{1000} is 31.2 nmol and the I_{1000} data obtained from cell pellets can be converted into absolute nanomole quantities for FC and metabolites by simply multiplying I_{1000} by 31.2. Since the total number of cells in each NMR sample is known from the cell count performed after harvesting, the absolute nanomole quantities can be expressed in terms of fmol/cell.

This quantification procedure assumes that the signal integrals are proportional to the molar concentration or to the absolute mole quantities of fluorine-containing compounds within the sensitive volume of the RF coil. The quantitative calibration procedure performed for FC avoids the problem of non-total FC relaxation between scans ($F_{sat} = 0.81$ for FC with the acquisition data used). Since the T_1 of FU and metabolites are not expected to be markedly different from that of FC (1.71 s at 4 °C in incubation medium), no significant error might result from differences in T_1 relaxation times.

A detection threshold of 1 μM is achieved over 10 h recording in incubation medium and ≈2 nmol fluorine in cell pellet for 4 h acquisition periods.

A ^{19}F NMR study of FC metabolism in *Candida* strains with quantification of culture medium and cell pellet was performed with a Cameca 250 spectrometer at 250 MHz in 5 mm diameter tube without proton decoupling, nearly 20 years ago [81]. Fully relaxed spectra are obtained with the recording conditions used for analysis of culture media at 25 °C as well as cell pellets at 4 °C [81]. The concentrations of FC, FU and metabolites are measured by comparing areas of their respective signals with that of the external FBEN reference placed in a coaxial capillary and previously calibrated against known volumes of FU solutions of known concentrations in molar and absolute mole quantities within the sensitive RF coil volume (expressed in pmol/10^6 cells in cell pellet knowing the number of cells in each NMR sample). Using the conversion factor proposed by Corban-Wilhelm et al. [44] of 1 fmol/cell = 696 μM based on an average cell volume of 1.437 pL, a minimal concentration of ≈17 μM is detected in cell pellet [81], whereas the detection threshold in bulk solution is 10 μM [29].

5. Examples of in vitro ^{31}P NMR quantification of phosphorylated drugs

As there are few phosphorylated drugs in current use, only a few ^{31}P NMR drug studies have so far been carried out. Mention can be made here of the metabolism studies of the anticancer oxazaphosphorine drugs, cyclophosphamide (CP) and ifosfamide (IF).

5.1. Overview of the metabolism of CP and IF

CP and its structural isomer IF are among the most widely therapeutically used alkylating anticancer agents. Both compounds are prodrugs that are bioactivated by cytochrome P-450 (CYP-450) enzymes to exert their toxic activity [82–84].

The initial CYP-450-catalysed metabolic step leads to the formation of 4-hydroxymetabolites, 4-hydroxycyclophosphamide (OHCP) or 4-hydroxyifosfamide (OHIF), that equilibrate with their ring-opened aldo tautomers, aldocyclophosphamide (AldoCP) or aldoifosfamide (AldoIF) (Figs. 7 and 8). These aldo intermediates are believed to play a pivotal role in antitumour efficacy of these drugs by partitioning between pathways giving either the cytotoxic agent or a biologically inactive compound. Indeed, the aldo derivatives undergo a non-enzymic β-elimination of urotoxic and nephrotoxic acrolein to yield the ultimate alkylating species, phosphoramide mustard (PM) or isophosphoramide mustard (IPM). Alternatively it may be oxidized to inactive carboxycyclophosphamide (CXCP) or carboxyifosfamide (CXIF) by aldehyde dehydrogenase (ALDH) or reduced to alcocyclophosphamide (AlcoCP) or alcoifosfamide (AlcoIF) by an aldehyde reductase. OHCP and OHIF may also be partially deactivated to 4-ketocyclophosphamide (KetoCP) or 4-ketoifosfamide (KetoIF) [82].

In addition to this ring oxidation, *N*-dechloroethylation of CP or IF can occur, leading to the formation of *N*-dechloroethylcyclophosphamide (DCCP) from CP or 2-dechloroethylifosfamide (2-DECLIF) and DCCP from IF, and to the elimination of chloroacetaldehyde, a compound that may be responsible for the oxazaphosphorine-induced neurotoxicity, urotoxicity and cardiotoxicity [85–87].

^{31}P NMR is used to analyse urine samples from patients treated with CP [20,25,88] or IF [23,24,89,90] and also plasma and cerebrospinal (CSF) samples from a few patients treated with IF [23].

5.2. ^{31}P NMR studies of body fluids: a contribution to a better understanding of the metabolic pathways of CP and IF

Busse et al. [25,88] use ^{31}P NMR to determine the 24 h urinary excretion of CP and its inactive metabolites CXCP, DCCP and KetoCP whereas plasmatic concentrations of CP are measured by HPLC. The aim of their studies is to investigate the influence of dose escalation on CP pharmacokinetics and relative contribution of activating and inactivating elimination pathways. Overall pharmacokinetics of CP is apparently not affected during 8-fold dose escalation, but there is a shift in the relative contribution of different clearances to systemic CP clearance in favour of inactivation pathways. Moreover, dividing the administration of high dose of CP (100 mg/kg over 1 h) over 2 consecutive days (50 mg/kg over 1 h) increases the metabolism of CP [88].

Autoinduction of CP metabolism was first reported by Bagley et al. in 1973 [91] and confirmed by numerous authors (see for example Fasola et al. [92]).

In another ^{31}P NMR study of urine samples from patients treated with CP on 2 consecutive days, Joqueviel et al. [20] show that the 24 h urinary excretion of unmetabolised CP is not significantly different on the first and second days of treatment, but that of its phosphorylated metabolites is much higher after the second CP dose (37% of the daily administered dose) than after the first (20%), also suggesting autoinduction of CP metabolism.

Moreover, the concentrations of CP and all its known phosphorylated metabolites, except the highly unstable tautomeric pair OHCP/AldoCP, i.e. CXCP, DCCP, AlcoCP,

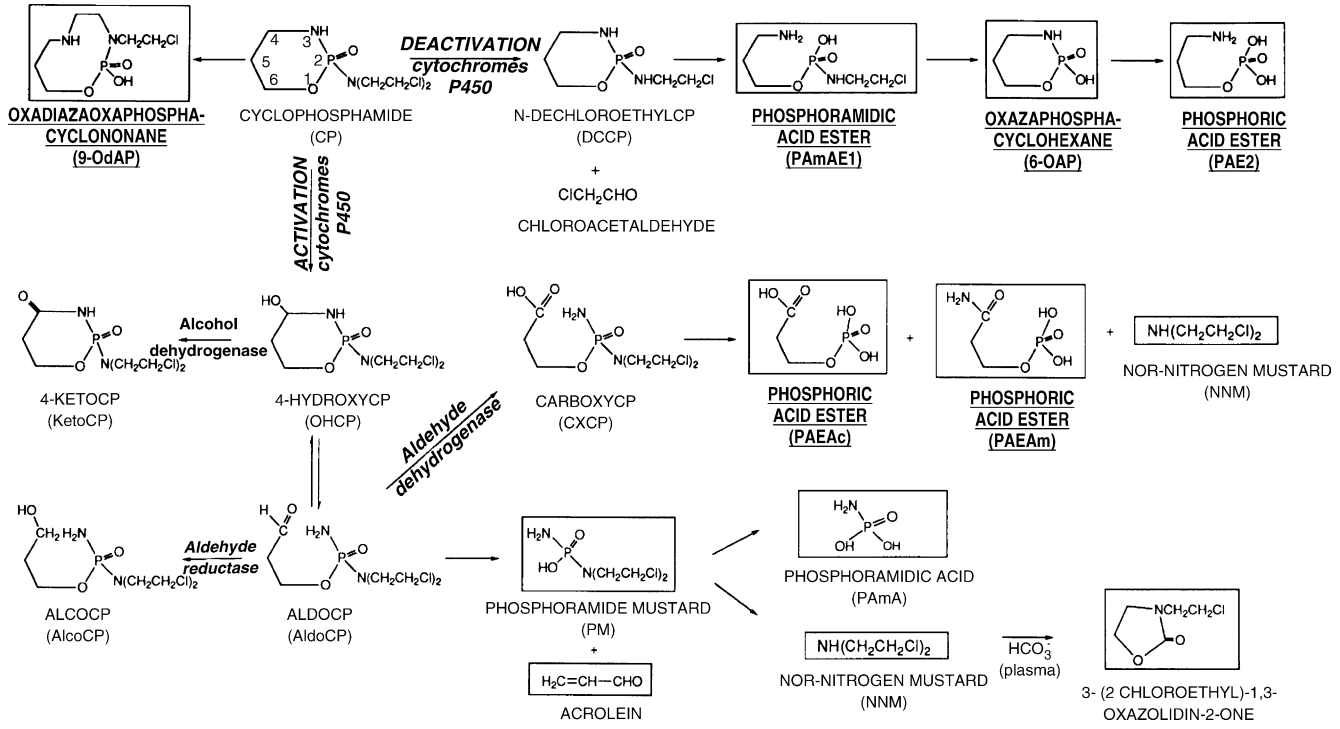

Fig. 7. Metabolism of cyclophosphamide incorporating the new phosphorylated compounds found in urine using ^{31}P NMR. Metabolites that are also degradation products, i.e. spontaneously formed, are represented in boxes. The names of the new metabolites identified for the first time with NMR are underlined.

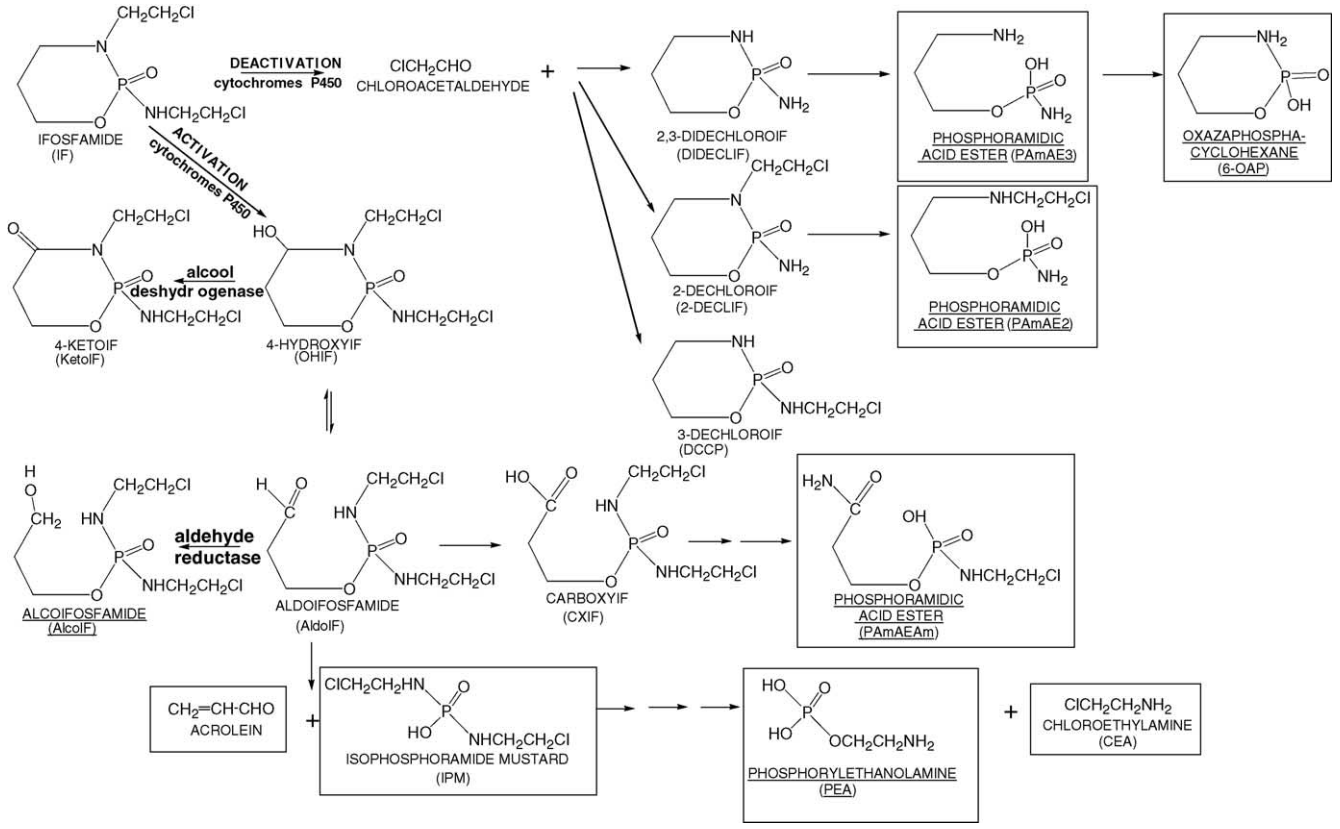

Fig. 8. Metabolism of ifosfamide incorporating the new phosphorylated compounds found in urine using ^{31}P NMR. Metabolites that are also degradation products, i.e. spontaneously formed, are represented in boxes. The names of the new metabolites identified for the first time with NMR are underlined.

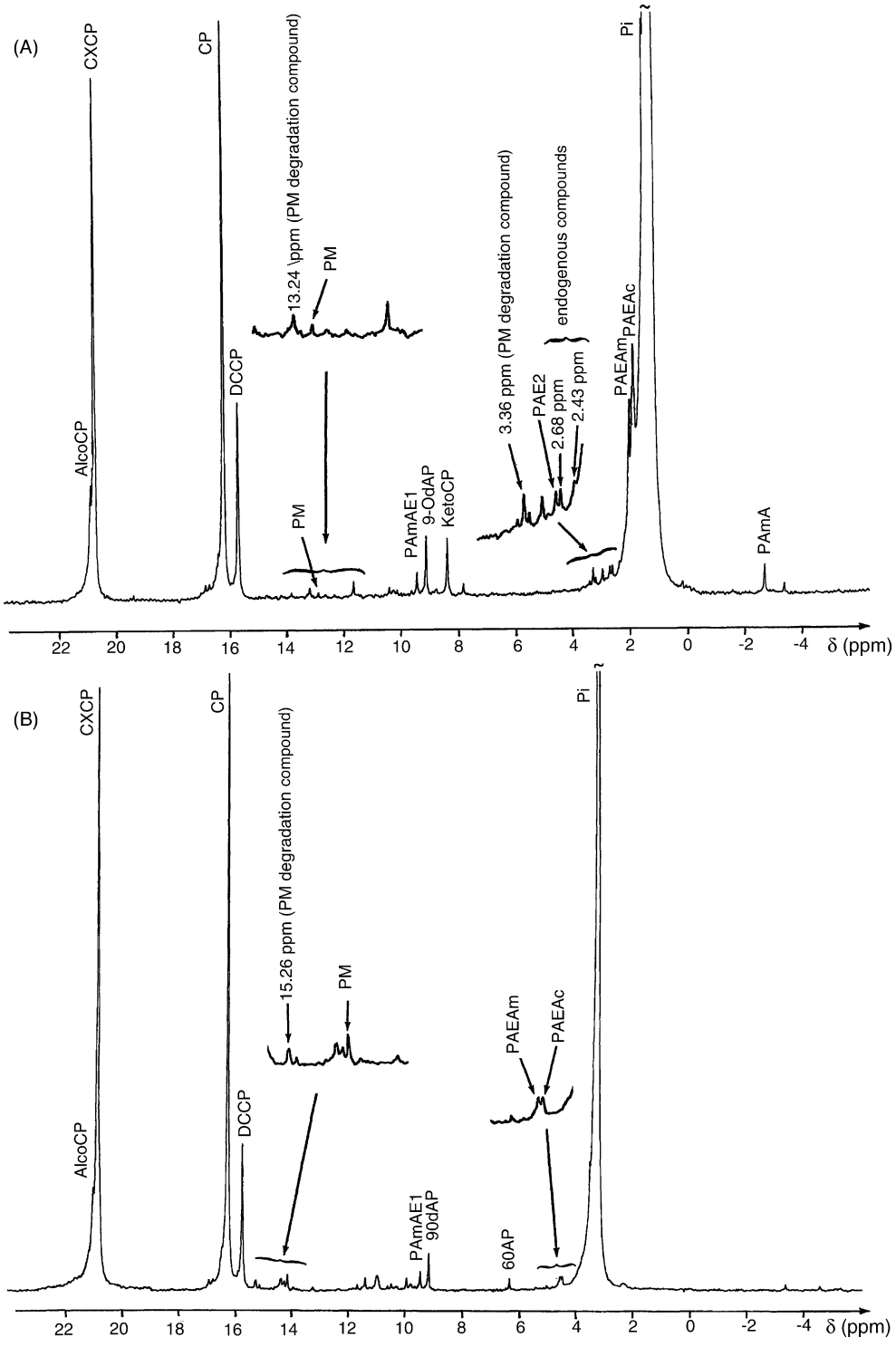

Fig. 9. ^{31}P NMR spectra at 121.5 MHz with proton decoupling of urine samples from patients treated with cyclophosphamide (CP) at a dose of 60 mg/kg/day. (A) Fraction collected 18–24 h after the start of the infusion on the first day and concentrated 3.4-fold (pH 5.8). The signals at 13.24 and 3.36 ppm are derived from the degradation of phosphoramide mustard (PM) but are still unidentified. The signals at 2.68 and 2.43 ppm correspond to endogenous urinary compounds. (B) Fraction collected 0–6 h after the start of the infusion on the second day and concentrated 3.6-fold (pH 7.8). The signal at 15.26 ppm is derived from the degradation of PM. Chemical shifts (δ) are related to external 85% H_3PO_4.

KetoCP and PM are measured. Several other signals corresponding to unknown CP-related compounds are observed. Seven of them are identified. All are hydrolysis products of CP or its metabolites: one from CP (oxadiazaphosphacyclononane, 9-OdAP), two from CXCP (phosphoric acid esters called PAEAc and PAEAm), three from DCCP (phosphoramidic acid ester PAmAE1, oxazaphosphacyclohexane 6-OAP, and phosphoric acid ester called PAE2) and one from PM (phosphoramidic acid $H_2N{-}P(O)(OH)_2$, PAmA) (Figs. 7 and 9). Overall the degradation products of CP and its metabolites newly identified in this study account for ≈3% of administered CP and make up approximately 15 and 10% of the excreted metabolites of CP on days 1 and 2, respectively.

In a pioneering study, Misiura et al. [89] use ^{31}P NMR to quantify the urinary excretion of IF and its metabolites CXIF, DCCP, 2-DECLIF and KetoIF whose attribution is questionable with a poor-performing 60 MHz spectrometer (24.3 MHz ^{31}P resonance frequency).

More recently, Martino's group using a 300 MHz spectrometer analyse urine samples of nine patients treated with IF at a dose of 3 g/m^2 administered as a 3 h intravenous perfusion [23,24]. The 24 h urinary excretion of IF and its classical metabolites, CXIF, DCCP, 2-DECLIF, IPM, KetoIF, is determined. Several signals corresponding to unknown compounds are observed. Six of them are identified: AlcoIF that has not been detected at that time in human biofluids; two compounds resulting from the degradation of 2,3 didechloroethylifosfamide (DIDECLIF) whose resonance is never found, phosphoramidic acid ester called PAmAE3 and 6-OAP; one compound coming from the hydrolysis of the endocyclic P–N bond of 2-DECLIF, phosphoramidic acid ester called PAmAE2; one coming from the degradation of CXIF, identified as the phosphoramidic acid ester PAmAEAm from recent studies on the hydrolytic behaviour of CXIF in urine; and one as a degradation compound of IPM, phosphorylethanolamine (PEA) (Fig. 8). At present time, some of the other unknown compounds can be identified from recent studies on the degradative pathways of *N*-dechloroethylated IF metabolites (DIDECLIF, 2-DECLIF, DCCP) [93], IPM [94] and CXIF. The degradation compounds (identified and unknown) represent ≈14% of the administered IF and ≈43% of the excreted metabolites.

Quantitative ^{31}P NMR analysis of plasma samples, even after deproteinisation, results in the detection of the sole resonance of IF, whereas that of an unique CSF sample shows the signals of IF and DCCP [23].

5.3. Spectra recording conditions for quantitative analysis of biofluids

Busse et al. [25] determine the concentrations of CP and its metabolites with a Bruker ARX 500 spectrometer at 202.5 MHz using 10 mm diameter tubes with proton decoupling to gain by the NOE signal enhancements.

The recording conditions are: probe temperature 25 °C; SW 10,000 Hz; 16 K data points zero-filled to 32 K; PW 12 μs (flip angle ≈ 45°); RT 2.32 s; LB 5 Hz. To the crude urine (2.85 mL) was added 0.15 mL of a pH 7 buffer containing deuterated water for field/frequency lock and the internal standard triphenylmethylphosphonium bromide. Calibration standards are prepared by adding known amounts of the pure reference substance (CP, DCCP, CXCP or KetoCP) to crude urine. Calibration curves are obtained by linear regression analysis using relative peak heights (height pure substance/height internal standard) and the amount of the pure reference substance. As already shown, this methodology avoids the problems of T_1 and NOE and leads to a significant reduction in the acquisition time. For example, a concentration of 10 μM is detected in 2 h. The accuracy (±15%) and reproducibility (±5%) are good even at low concentrations. For a S/N > 3, the detection limit is 7.5 μM. On the other hand, it is necessary to have the pure reference standard and establish a calibration curve for each standard.

Martino's group [20,23,24] with a Bruker WB-AM 300 spectrometer (^{31}P resonance at 121.5 MHz) using the proton decoupling inverse-gated technique acquire quantitative spectra of biofluids (urine, plasma, CSF) in 10 mm diameter tubes. The magnetic field is shimmed on the FID from H_2O in the sample. The following instrumental conditions are: probe temperature 4 °C; SW 15,151 Hz; 32,768 data points zero-filled to 65,536; PW 5 μs (flip angle ≈ 35°); RT 6.08 s; LB 3 Hz.

The urine samples are doped at saturation (about 3 mM) with the paramagnetic agent $Cr(acac)_3$ to shorten the T_1 relaxation times of the phosphorylated compounds. The concentration of all the compounds detected are measured by comparing the areas or their ^{31}P NMR signals with that of MPA, the standard for quantification, which is placed in a sealed coaxial insert. The external standard [MPA in deuterated water that has also been doped at saturation with $Cr(acac)_3$ to shorten its T_1 relaxation time, with the deuterated solvent providing the field/frequency lock for the spectrometer] is calibrated against IF solutions of known concentrations with recording conditions (PW 5 μs (flip angle ≈ 35°); RT 6.08 s) set to produce fully relaxed spectra.

This method is validated for quantification of IF and its metabolites. Indeed the T_1 of 10^{-2} M solutions of IF, KetoIF, CXIF and MPA in blank urine doped with $Cr(acac)_3$ and determined at 25 °C by the inversion-recovery method are 2.2, 2.0, 2.0 and 2.9 s, respectively. The saturation factor calculated with the relation reported above and the highest T_1 value (2.9 s) is 0.98. The decoupler on/off cycle is 0.22, very close to the 0.20 value for which NOE is suppressed [16]. Therefore fully relaxed spectra should be obtained with peak areas directly proportional to concentrations. This assumption is verified for all these compounds as well as for CP and CXCP whose T_1 are considered as representative of cyclic and linear structures of phosphorylated metabolites related. Indeed, recording the spectra of solutions of all these compounds under the conditions described with an RT as long

as 9.08 or 10.08 s, with all other parameters left unchanged, does not alter their signal intensities.

The accuracy and precision of the ^{31}P NMR assay are determined for CP, AlcoCP and DCCP in human urine doped with $Cr(acac)_3$ at concentrations $\approx 10^{-3}$, 5×10^{-4}, 10^{-4}, 5×10^{-5} and 10^{-5} M as well as for aqueous solutions of IF doped with $Cr(acac)_3$ at the same concentrations. The results obtained show that accuracy and precision are less than ±10% for concentrations $\geq 5 \times 10^{-5}$ M and approximately ±20% for concentrations between 5×10^{-5} and 10^{-5} M. At the limit of detection (5 μM after 24 h recording, S/N between 2 and 4), the accuracy and precision are approximately ±30–35% [20].

Because of the length of time required for quantitating CP and its metabolites (9–14 h), the NMR data are acquired in ≈2.5 h data blocks. These blocks are then compared to check the stability of the phosphorylated compounds detected during the period of NMR recording. There are no significant differences with time even for the most acidic urine samples in which degradation is most rapid. Quantitation is therefore carried out using spectra resulting from the sum of all blocks. Since at room temperature, CXCP and CXIF slightly degrade with time in acidic urine, it is essential to perform NMR recording at 4 °C [24,25].

6. Conclusion

Although optimisation procedures for a successful quantification by NMR are somewhat tedious, it is nevertheless fairly easy to use NMR routinely to obtain quantitative data. Especially, in vitro ^{19}F or ^{31}P NMR is a high potential analytical technique for absolute quantification, in a single run, of all the fluorine- or phosphorus-containing species in complex biological matrices. The limit of quantification with conventional probes for ^{19}F NMR is estimated at 1–3 μM in bulk solutions (biofluids, tissue extracts, perfusates or culture media), 2–5 nmol in excised tissues and 2 nmol in cell pellets after 1 and 4 h measurements, respectively, whereas for ^{31}P NMR this limit is approximately 10 μM in biofluids.

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