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6-Fluoropyridoxol: a novel probe of cellular pH using ^{19}F NMR spectroscopy

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Abstract

6-Fluoropyridoxol was evaluated as an intracellular pH indicator. This molecule exhibits exceptional sensitivity to changes in pH (~10 ppm acid/base shift) and a pK_a ~8.2 appropriate for physiological investigations. Using ^{19}F NMR spectroscopy we determined both intra- and extracellular pH in whole blood and confirmed the measurements using traditional techniques: ion-electrodes and ^{31}P NMR spectroscopy.

Key words: ^{19}F NMR; pH; Fluoropyridoxol; Blood

1. Introduction

Intracellular and interstitial pH are thought to play significant roles in cellular regulation and tissue development. It has been shown that tumor pH influences cell thermosensitivity, radiation sensitivity and proliferation [1]. In addition, pH is reported to influence the efficacy of certain chemotherapeutic drugs for cancer therapy [1,2]. Thus, an accurate non-invasive assessment of tissue pH may provide a prediction of therapeutic success. pH measurement by a variety of techniques including microelectrodes, fluorescence spectrophotometry, partitioning of radio-labelled weak acids and NMR has been extensively reviewed [3,4,5]. NMR offers several potential advantages: continuous non-destructive monitoring of pH in vivo; spatial resolution and simultaneous determination of pH in several compartments [4,5]. NMR has been used to monitor titratable groups of endogenous metabolites on the basis of chemical shift with respect to changing pH; in particular ^{31}P NMR of inorganic phosphate (P_i) [5,6] or phosphomonoesters [7] or ^{1}H NMR of carnosine [8] or histidine [9]. pH measurement using endogenous metabolites is not always successful owing to crowded spectra, broad lines or low metabolite concentration. Thus, various infusable agents have been developed [10,11]. In particular ^{19}F-labelled molecules, which exploit the high visibility of fluorine, the great sensitivity of ^{19}F to the micro-environmental millieu and the lack of background signal [10,12]. Widespread application of such molecules has been hindered by low response to changes in pH [10] or pK_a far from the physiological range [13]. The vitamin B6 analogue 6-FPOL (Fig. 1) has previously been reported to have a pK_a in the physiological range and large chemical shift response [14]. We have now investigated the feasibility of using 6-FPOL to probe cellular pH and demonstrate its application in whole blood.

2. Materials and methods

We have synthesized 2-fluoro-3,4-di(hydroxymethyl)-5-hydroxy-6-methyl-pyridine (6-Fluoropyridoxol or 6-FPOL) using Korytnyk's method [15]. Briefly, this requires a three-step synthesis starting from pyridoxine hydrochloride. Diazotization of pyridoxine with benzene diazonium chloride yielded 6-phenazopyridoxol and dithionite reduction produced 6-aminopyridoxol. In our experience the yield and purity of the product are significantly enhanced by careful repeated extraction at this stage from residual inorganic salts. 6-FPOL was then obtained using a modified Schiemann reaction [15]. NMR (^{1}H, ^{19}F), IR, and melting point confirmed the identity and purity of 6-FPOL. ^{19}F NMR experiments were performed using a 7 Tesla Oxford vertical bore magnet (^{19}F at 282.3 MHz) under control of a Techmag console. ^{19}F chemical shift titration curves were established for 6-FPOL in distilled water and in the presence of dominant physiological ions (300 mM Na^+, 300 mM K^+, 5 mM Mg^{2+}, or 5 mM Ca^{2+}), in blood plasma and at the temperatures 27, 37 and 45°C. Sodium trifluoroacetate (TFA) was used as an external reference (0 ppm).

Fresh whole blood was drawn from the lateral ear vein of New Zeeland White rabbits and stored chilled in the presence of heparin (up to 1 week) prior to NMR observation. Typically, 1–12 mg 6-FPOL were dissolved in ~500 μl water, plasma or whole rabbit blood for NMR studies. The pH of each sample was measured in the NMR tube using a combination pH electrode (Wilmad, Buena, NJ) attached to a pH meter (Corning 220, Sudbury, UK). Shimming was performed on the water signal and ^{19}F NMR spectra were typically obtained in 1 min by averaging 16 transients with 2k data points across ± 5000 Hz. Data were apodized with a 20 Hz exponential line broadening prior to Fourier transformation. ^{31}P NMR spectra were obtained at 121.5 MHz with 512 transients and 4k data points across ± 4000 Hz. In this case a 25 Hz line broadening was applied and methylenediphosphonic acid (MDPA) was used as an external reference. In some cases red blood cells and plasma were separated by mild centrifugation. ^{19}F relaxation measurements were made using a traditional pulse-burst saturation recovery (T1) sequence [16]. In one case the blood was saturated with carbon monoxide, to reduce line broadening associated with deoxyhemoglobin, and ^{19}F and ^{31}P NMR were performed at 37°C.

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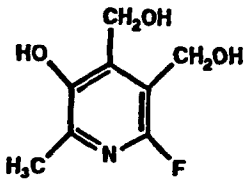

Fig. 1. Structure of 6-Fluoropyridoxol (6-FPOL)

3. Results

3.1. Titration

6-FPOL exhibits a single sharp ^{19}F NMR resonance in aqueous solution or plasma (line-width ~20 Hz). ^{19}F NMR chemical shift of 6-FPOL is very sensitive to pH with a change of 9.76 ppm between acid and base (Fig. 2). From the titration curve in Fig. 3 coefficients of the Henderson-Hasselbach equation were determined as $pK_a = 8.2$; δ(acid) = −9.85 ppm; δ(base) = −19.61 ppm. The pH dependent ^{19}F NMR chemical shift of 6-FPOL was essentially independent of the presence of metal ions (e.g. Na^+, K^+, Ca^{2+}, Mg^{2+}) across the whole pH range (Fig. 3B). A similar titration curve was obtained in blood plasma. The titration curves were found to be independent of temperature in the range 27–45°C.

3.2. Blood

Adding 6-FPOL to whole blood produced two distinct well resolved ^{19}F resonances immediately (Fig. 4a) at −10.72 ± 0.03 ppm (I) and −11.34 ± 0.06 ppm (E) corresponding respectively to pH 7.19 ± 0.02 and 7.45 ± 0.02. A signal-to-noise ratio of 5 was obtained for the larger upfield signal in a single acquisition using 4 mg of 6-FPOL in 2 ml of blood (~10 mM). The resonances were assigned respectively to intracellular (I) and extracellular (E) compartments as follows.

(i) The separation of red blood cells from plasma by centrifugation produced a dominant ^{19}F resonance (I) (Fig. 4b). Addition of saline to red blood cells resulted in increase of the second signal (E) (Fig. 4c). Separated plasma showed a single ^{19}F signal (E) (Fig. 4d).

(ii) The downfield peak had shorter spin-lattice relaxation time: T_1(I) ~0.87 ± 0.12 s, and T_1(E) ~1.21 ± 0.08 s.

(iii) The pH electrode indicated an extra-cellular pH = 7.54 ± 0.05 corresponding with the shift of the upfield signal.

In a separate experiment using blood saturated with carbon monoxide to reduce intracellular paramagnetic line broadening from deoxyhemoglobin ^{31}P NMR indicated intra-cellular pH ~7.14 ± 0.06 from the chemical shift difference of 2,3-DPG (diphosphoglycerate) ($\Delta\delta$ = 0.89 ± 0.02 ppm) [17]. This corresponded with the downfield ^{19}F resonance at δ = −10.80 ppm (pH 7.23), whilst the upfield resonance (δ = −11.53, pH 7.52) corresponded with the electrode determination of pH 7.50.

4. Discussion

We have shown that 6-FPOL is a highly sensitive probe of pH and have exploited this molecule to measure both intra- and extracellular pH in whole blood. The pH dependent ^{19}F NMR chemical shift of 6-FPOL is essentially independent of temperature, the presence of metal ions (e.g. K^+, Mg^{2+}) or plasma proteins and diffuses rapidly into red blood cells.

NMR measurement of pH generally exploits the chemical shift titration of endogenous inorganic phosphate (P_i) or phosphomonoesters. This is entirely non-

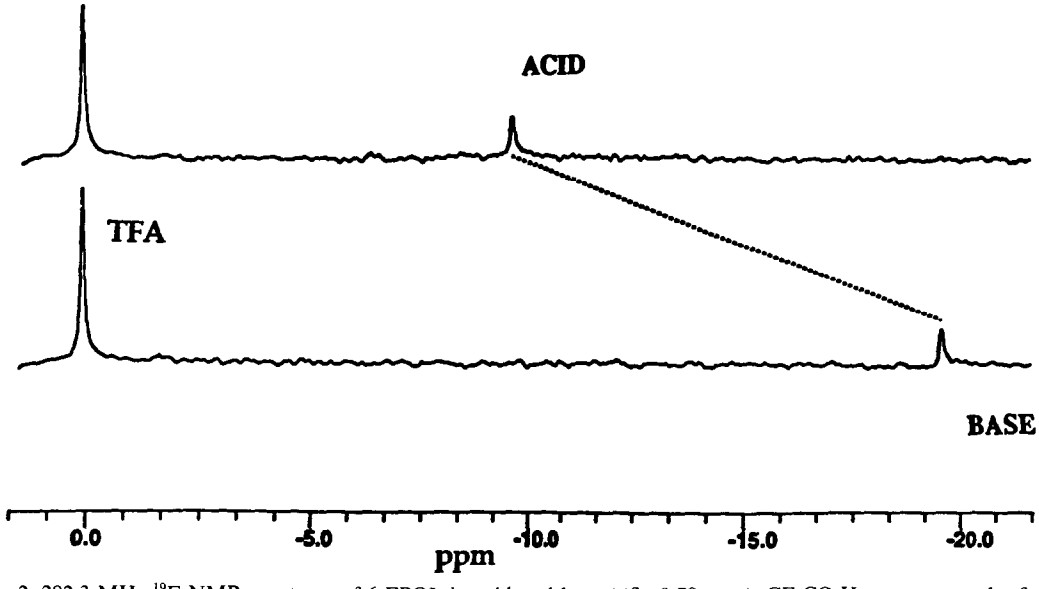

Fig. 2. 282.3 MHz ^{19}F NMR spectrum of 6-FPOL in acid and base ($\Delta\delta$ ~9.78 ppm). CF_3CO_2H as an external reference.

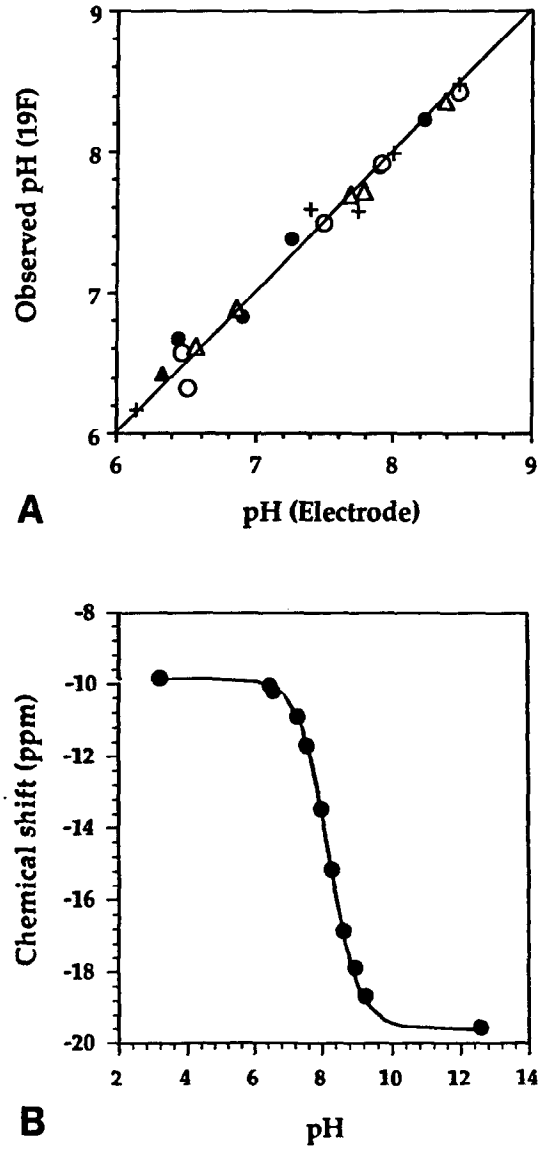

Fig. 3. (A) Plot of ^{19}F chemical shift of 6-FPOL vs. pH in water at 37° C. pK_a ~8.2, δ(acid) = −9.85 ppm, δ(base) = -19.61 ppm. (B) Influence of typical physiological metal ions or blood plasma on chemical shift. Plot of pH predicted from chemical shift in the presence of metal ions using Henderson-Hasselbach equation (+300 mM Na^+, △ 300 mM K^+, ○ 5 mM Ca^{2+}, ▲ 5 mM Mg^{2+}, ● whole plasma) vs. pH determined by electrode.

invasive and is generally reported to provide pH estimates ± 0.02 pH unit close to the pK_a of 6.6 [5], but ± 0.3 may be more typical in vivo [18,19] .The pK_a ~6.6 of P_i is ideally suited to monitor intracellular acidification, but is less suitable for basic compartments. In addition, the pK_a is sensitive to metals ions (e.g. [Mg^{2+}]) and ionic strength and errors as great as 0.5 pH units have been estimated [6,17,20,21]. The chemical shift range of P_i is only 2.4 ppm and ^{31}P NMR spectra are often crowded with broad unresolved resonances and baseline distortion. In blood, P_i is often masked by 2,3 DPG [18,19,22].

Infusable pH-sensitive NMR probes may be designed with a specific pK_a and resonances that do not overlap endogenous signals. ^{19}F is particularly attractive due its high detection sensitivity, large chemical shift ($\Delta\delta$) in response to minor alterations in pH and lack of background signal. Deutsch et al. have pioneered this approach [10] and have successfully designed fluoromethyl alanine derivatives with a range of different pK_a values [10]. The first generation molecules were somewhat insensitive ($\Delta\delta$ acid–base ~1 ppm) [10,23,24], but they have nonetheless been used in isolated cells [10] and perfused organs [25,26]. Second generation molecules (fluoroanilines) have been described, but exhibited inappropriate pK_a [13].

6-FPOL has a large chemical shift range and pK_a appropriate for certain physiological investigations, in particular in the basic range (pH > 6.5). This would complement studies of acidosis using P_i. The variation in chemical shift is smooth and monotonic indicating protonation of a single group. 6-FPOL was originally reported in 1973 [15] and the titratability was exploited to probe the mechanism of glycogen phosphorylase activity [14]. The molecule does not appear to have been used to probe pH in vivo hitherto, although an acute toxicity >100 mg/kg has been reported [15]. There is also some evidence for growth inhibition of tumor cells in vitro [15] and we shall investigate possible cytotoxicity in the future.

As discussed by others a pH probe should ideally meet several criteria: high NMR visibility, narrow lines, stability, no cytotoxicity, no physiological interference, cell penetration, fast exchange between acid and base forms, but slow exchange across cell membranes, large chemical shift range and pK_a in the physiological range [10,18]. We have now established that 6-FPOL penetrates and leaves red blood cells rapidly, but exchange is sufficiently slow to provide separate resonances for intra- and extracellular compartments. Acid–base exchange is in the fast exchange regime so that a single narrow line is observed for each compartment.

Our pH measurements using 6-FPOL are independently confirmed using pH electrodes (extracellular) and the ^{31}P NMR chemical shift differences of the resonances of 2,3 DPG (intracellular). The values also correspond with literature reports for blood derived with various techniques [17,27]. We are currently investigating the general utility of 6-FPOL to measure pH in a variety of cells and tissues and the possibility of targeting specific organs.

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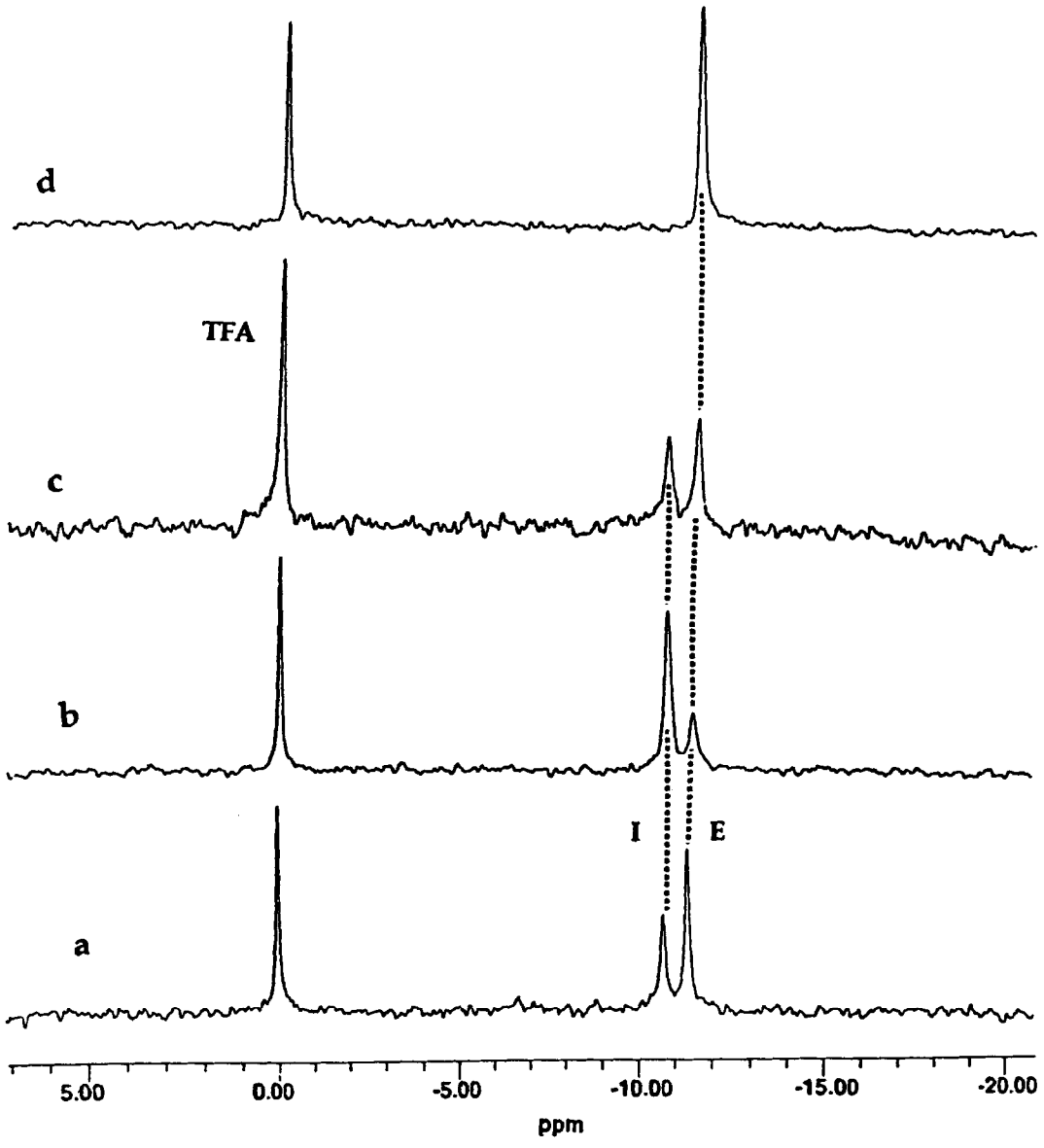

Fig. 4. 6-FPOL (10 mM) was added to whole blood with external TFA standard. The ^{19}F NMR spectrum showed two distinct signals, which were assigned respectively to internal (I = −10.72 ppm; intracellular) and external (E = −11.34 ppm; plasma) compartments. (a) Whole blood; (b) red blood cells obtained by centrifugation of a; (c) saline added to (b); (d) plasma from (a).

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