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DFTMP, an NMR Reagent for Assessing the Near-Neutral pH of Biological Samples

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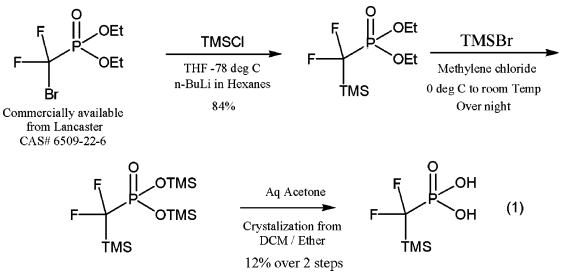
NMR has played a central role in our understanding of biological systems. In particular, high-resolution NMR is quite useful at elucidating atomic-level dynamic information and quantitative concentration information on complex systems such as drug-target interactions, enzymatic and nonenzymatic kinetics, or biological fluids analysis. One important factor in such biochemical systems is sample pH, which affects both biological function and specific NMR parameters. Slight perturbations in the electron distribution around an NMR-active atomic nucleus result in measurable changes in its resonant frequency. Hence it is possible to evaluate events such as ionization and protonation state through changes in the chemical shift ($\Delta\delta$) of nonexchangeable nuclei proximal to the locus of the perturbation. The power of NMR to probe site specific acid−base properties was recognized early, and advancements over the last few decades have positioned NMR as the most powerful tool to carry out such investigations.[1]

In practice, pH measurements are typically made using conventional pH electrodes or by the addition of internal standards that have chemical shifts which are sensitive to pH in and around the pH of interest (such as imidazole for near neutral pH[2]). The former approach has the advantage of direct pH measurement with a tool of determinable accuracy but can be tedious and requires sample manipulation and direct contact with the electrode which can result in sample loss and contamination. The latter avoids these risks but can introduce extraneous signals that may overlap analyte signals of interest in the NMR spectrum.

This situation has stimulated us to seek a novel chemical shift-sensitive reagent for in situ pH measurement in NMR experiments conducted at near-neutral pH. Such a compound must be highly stable and have chemical shifts that (1) do not overlap with analyte components, (2) are sensitive to pH (>0.005 ppm/pH unit) in the pH 6−8 range, and (3) are insensitive to Ca^{2+} and Mg^{2+} concentrations often found in biological samples. We have synthesized and characterized a new chemical entity that meets all of these requirements, 1,1-difluoro-1-trimethylsilanyl methylphosphonic acid (DFTMP) **1**, (Scheme 1). The nine equivalent protons of the trimethylsilyl group offers a single strong, sharp resonance at ∼0.2 ppm. An additional characteristic of this compound is that it contains proton, fluorine, and phosphorus nuclei and can thus serve as a chemical shift reference point and pH indicator for multinuclear studies.

One NMR application that has recently garnered a great deal of interest is the quantitative analysis of endogenous metabolites in biological fluids and the subsequent correlation of changes in biomolecular composition with physiological or genetic perturbation.[3] A common problem with this approach is the pH and metal ion dependence of the chemical shift of individual components,[4] which confounds the identification of endogenous analytes in complex matrixes that often contain hundreds of molecules. This is especially true in urine, which can have rather wide ranges in pH and divalent metal ion content. An accurate knowledge of the urinary pH can both identify abnormal conditions and provide a context upon which to make peak assignments for species whose chemical shifts are pH sensitive.

Scheme 1



To evaluate the utility of **1** for in situ pH measurements using NMR, ^{1}H NMR spectra were recorded on a human urine sample containing **1** and DSS-d_6[5] over a range of pH. Individual aliquots of a single urine sample were mixed with measured amounts of HCl or NaOH then diluted with distilled water. A 550 μL aliquot of the pH-adjusted urine sample was mixed with 65 μL of 3.0 mM DSS-d_6 in D_2O and 35 μL of 1.8 mM **1** in H_2O. Proton spectra were immediately recorded on a Bruker AV-600 NMR spectrometer at 19.0 °C. The pH of each sample was measured by a Tecan robotic system at 19.0 °C immediately after each NMR spectrum was acquired using a WTW inoLab pH level 2 m in conjunction with a Schott N 5900 A-2m (Ag/AgCl) electrode that was calibrated prior to the first sample measurement. The pH of the calibration buffers was remeasured after the last sample and found to be within the acceptable range.

The chemical shift versus pH data for **1** relative to DSS can be adequately modeled using a simple 3-term thermodynamic relationship (eq 1), where pK_a is the pK_a of first deprotonation of **1**, d_{obs}, d_{min}, d_{max} are the observed, fully protonated and fully (mono) deprotonated chemical shift of **1**, respectively.

$$\mathrm{pH} = \mathrm{pK_a} + \log\left[\frac{\delta_{\min} - \delta_{\mathrm{obs}}}{\delta_{\mathrm{obs}} - \delta_{\max}}\right] = 6.246 + \log\left[\frac{0.224 - \delta}{\delta - 0.193}\right] \quad (1)$$

The experimental data and predictive model are shown in Figure 1. The chemical shift of the single resonance from the nine equivalent trimethylsilyl protons ranges from 0.195 and 0.225 ppm, with excellent agreement between calculated and observed pH between pH 4.3 and 8.2. Outside these ranges, the $\Delta\delta$ with pH becomes small and the model loses accuracy. Between pH 5 and 8 the RMSE error of pH prediction is 0.02 pH units, which is comparable to the accepted accuracy of conventional glass electrodes.[1] The overall titration shift between extremes (pH ∼4 to 9) is ∼0.03 ppm with a maximum slope of ∼0.015 ppm/pH unit between pH 5.7 and 7.4 and is sufficiently large for accurate

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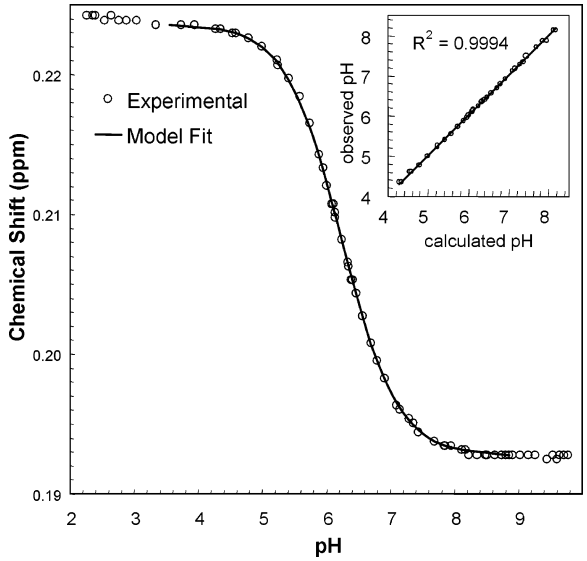

Figure 1. Experimental and modeled chemical shift data for DFTMP in urine. Inset shows correlations between observed and calculated pH between 4.3 and 8.2.

chemical shift determination on modern spectrometers with high homogeneity. Similar calibrations plots will need to be generated to match individual study conditions.

The chemical shifts of the ^{31}P and ^{19}F nuclei in DFTMP also exhibit a smooth sigmoidal transition over a similar pH range with a total $\Delta\delta$ of 0.24 and 3.0 ppm, respectively.

Calcium and magnesium are the most predominant divalent metal ions present in biological fluids, with normal levels ranging approximately between 0.1 and 10 mM.[6] Therefore, it is important that interactions of these ions with any prospective pH reagent be sufficiently small so as to not influence the accuracy of derived pH. The effect of $[Ca^{2+}]$ and $[Mg^{2+}]$ on the $\Delta\delta$ of **1** in aqueous solution is shown in Figure 2. The complexation shifts in the metal concentration range 1−10 mM is <0.001 ppm for both of these metals, suggesting that interference with these two common metal ions is negligible.

To assess stability of **1** in solution, samples prepared in aqueous solution at various pH values from 3 to 11 were analyzed by proton NMR, allowed to sit at room temperature for approximately 3 months, and then were then reanalyzed, with no degradation of the **1** observed at any pH.

Unlike commonly used aqueous chemical shift standards, a sharp resonance from free DFTMP appears in samples of serum and plasma at concentrations as low as of 0.1 mM. Thus, when referenced relative to a pH-invariant peak such as glucose H1α, the reagent will be useful for pH determinations and possibly as a internal standard in proteinaceous biofluids.

Addition of a reasonable working concentration of 0.1 mM DFTMP to a typical rat urine sample showed no measurable changes in the chemical shifts of any endogenous component.

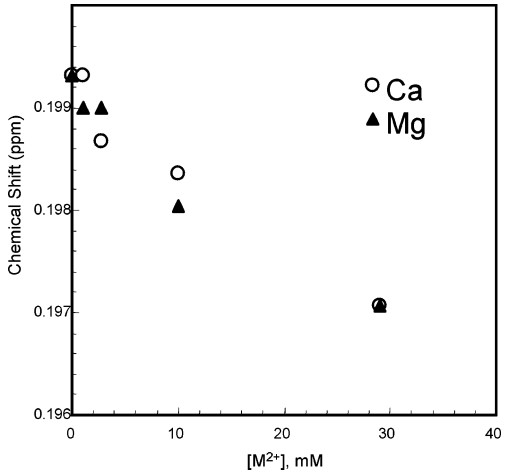

Figure 2. Chemical shift dependence on $CaCl_2$ and $MgCl_2$ for DFTMP in 50 mM TRIS buffer, pH 7.2. Chemical shifts relative to internal DSS.

Using human urine as an example, we have shown that the use of DFTMP, in conjunction with a pH-invariant chemical shift reference standard[7] makes in situ pH measurement trivial in NMR experiments conducted in aqueous solution. We have demonstrated DFTMP to be valuable for NMR analysis of urine and can expect it to have broader application in biological NMR. It is proposed that this reagent will have general utility when NMR studies are carried out wherein pH and/or metal ion concentration either are not known or are subject to change over time.

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Supporting Information Available: Tabular proton NMR chemical shifts of **1** as a function of pH and metal ion concentration, experimental preparations for **1**, commercial source for **1**, detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) We have used DSS in the present study, but any internal component whose chemical shift is independent of pH would be suitable (e.g., glucose).

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