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COMMUNICATION

Examination of ^{19}F-NMR as a Tool for Investigation of Drug-Cyclodextrin Complexes

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ABSTRACT

Fluorine nuclear magnetic-resonance spectroscopy (^{19}F-NMR) was used to measure complexation of three fluorine-containing drugs—dexamthasone, fluoxetine hydrochloride, and diflunisal sodium—with 2-hydroxypropyl-β-cyclodextrin (HPβCD). Poor aqueous solubility inhibited investigation of dexamethasone complexes with this method. Complexation caused separation of the fluorine peaks that could be assigned to the two enantiomers of fluoxetine hydrochloride. The trifluoromethyl group of the drug was not included, or only partially included, in the cyclodextrin cavity and the shift changes resulting from complexation were small (0.04 and −0.05 ppm). The NMR method, therefore, could not be used to determine complex stoichiometry and complex stability constants, as chemical-shift changes were influenced by changes in the composition of the solvent medium. The difluorophenyl group of diflunisal sodium was fully included in the cyclodextrin cavity and the chemical-shift changes were large, 2.0 and 1.4 ppm, for C2′ and C4′ fluorine atoms, respectively. Using the continuous variation method, a 1:1 stoichiometry was determined for the complex. The chemical shift changes could also be used to determine the stability constant (K_c) for complex formation. The value obtained for the fluorine that enters deeper into the cavity was 2000 M^{-1}. The data shows that, given that the drug has sufficient solubility, one-dimensional ^{19}F-NMR can be a fast and convenient method to investigate drug-cyclodextrin complexes. However, when the results are interpreted it must be taken into account that the solvent medium can affect the chemical shifts of the fluorine peaks.

Key Words: Cyclodextrin; Complexation; Drugs; Fluorine; Nuclear magnetic resonance.

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INTRODUCTION

The prediction of the behavior of guests (i.e., drugs, dyes, flavoring agents, etc.) in cyclodextrin (CD) solution is, among other physicochemical properties, dependent on the complexation constant (also called the stability or association constant, K_C) for the CD-guest complex, and the stoichiometry of complexation. Measurements of complexation induced chemical-shift displacements (up- or downfield) in nuclear magnetic-resonance spectroscopy as a function of concentrations (NMR titrations) can be used to determine the complexation constant and changes in chemical shifts ($\Delta\delta$) resulting from inclusion of the guest molecule in the CD cavity. These methods have, compared to most other methods of equilibrium determinations, the advantage of providing several independent signals for the evaluation of stability constants.[1] In addition, NMR studies will give information about the structure of the complex, i.e., which group is included in the cavity and how it will be oriented. Inclusion of guests into the CD cavity will affect protons inside the CD cavity, as well as the complementary protons on the included guest. Changes in chemical shift for both CD and guest molecules can be used to determine stoichiometry and stability constants of the complexation.[1] More often, NMR titrations are designed to investigate the influence of the included guest on the CD protons. However, in the case of pharmaceutically interesting substituted CDs, random introduction of substituents onto the CD hydroxyls changes the chemical and magnetic environment of the protons inside the CD cavity, making their NMR signals broad and difficult or even impossible to interpret. Chemical-shift variations in the guest molecule can then provide a simpler probe. Fluorine (^{19}F) NMR has been used to demonstrate the complexation of natural βCD with perfluorinated ends of telomeric polymers[2–4] and fluorinated amino acid derivatives.[5] Fluorine as a group is rare in natural compounds, but is a relatively common substituent in drugs. However, there is no systematic study on the value of ^{19}F-NMR as a tool to investigate the complexation between fluorine-containing drugs and CD derivatives. Naturally, this method is limited by the fact that a majority of drugs do not contain fluorine, but when applicable, ^{19}F-NMR should have certain advantages over the more commonly applied proton (^{1}H) and carbon (^{13}C) NMR methods. In comparison to ^{1}H-NMR, the absolute chemical-shift variation is an order of magnitude larger for ^{19}F-NMR, and peak broadening, which often makes CD complexation-induced chemical-shift determinations in ^{1}H-NMR difficult, is less observed. While the absolute chemical-shift variation may be similar in ^{13}C- and ^{19}F-NMR, the latter method is more sensitive since the natural abundance of the measured isotope is 100%, compared to 1.1% for ^{13}C, allowing less time-consuming collection of data. Overlapping of CD and guest signals, which is frequently encountered in ^{1}H- and ^{13}C-NMR, is also eliminated. The work presented here was aimed at investigating whether ^{19}F-NMR is a suitable method for determination of complex stoichiometry and complexation constants of the 2-hydroxypropyl-β-cyclodextrin derivative (HPβCD) and fluorinated drugs.

EXPERIMENTAL

Reagents

Deuterium oxide (99.5 mol% D) and diflunisal D-3281 (5-[2,4-difluorophenyl] salicylic acid, DIF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dexamethasone [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione, DEX] was purchased from Unikem (Copenhagen, Denmark) and fluoxetine hydrochloride [(±)-*N*-methyl-γ-[4-(trifluoromethyl)phenoxy]benzenepropanamine hydrochloride, FXT] was a gift from Omega-Pharma (Reykjavík, Iceland). 2-Hydroxypropyl-β-cyclodextrin (HPβCD, DS 0.6, $MW_{calc} = 1379$) was obtained from Wacker-Chemie (Munich, Germany). All other chemicals were commercially available chemicals of reagent or analytical grade.

Preparation of Diflunisal Sodium

The drug was weighed into a beaker and the exact amount of dilute sodium-hydroxide solution required to reach equimolar concentrations of diflunisal and sodium was added. The suspension was sonicated for 2 hr and then stirred overnight with a magnetic stirrer. Water was eliminated by freeze drying (~0.1 mbar without temperature control) overnight [Snijders Scientific LY-3-TT table-top mini freeze dryer (Tilburg, Netherlands) with a Vacuubrand RD4 rotary vane vacuum pump

(Wertheim, Germany)]. Finally, the material was sieved (500 μm).

High-Performance Liquid Chromatography

The high-performance liquid chromatography (HPLC) equipment consisted of a L-6200A Intelligent Pump operated at 1.50 mL/min, a L-4250 UV-VIS Detector, a AS-2000A autosampler with the injection volume set to 20 μL, and a D-2500 Chromato-Integrator, all from Merck-Hitachi (Berlin, Germany). Reversed-phase chromatography was conducted at ambient temperature using a Phenomenex Luna(2) C_{18} column, 5 μm, 150 × 4.6-mm column (Cheshire, England). The mobile phase for diflunisal sodium contained acetonitrile and acetic acid in water (65:1:34), the retention time was 2.5 min and detection was made at 254 nm. For fluoxetine hydrochloride, the mobile phase consisted of acetonitrile and aqueous 4 mM phosphate buffer containing 0.1% (v/v) triethylamine (adjusted to pH 7.2) (50:50). The retention time was 3.0 min and detection was made at 227 nm. The dexamethasone mobile phase was composed of acetonitrile, tetrahydrofuran, and water (54:1:45). The retention time was 2.2 min and detection was made at 254 nm. Sample concentrations were calculated using the external standardization method.

Sample Preparation

Cyclodextrins and drugs were dried to constant weight in a vacuum oven (~10 torr) at 40°C and stored in desiccators. A stock solution of cyclodextrin was prepared in 30% (v/v) D_2O in water for ^{19}F-NMR measurements. Sample solutions were prepared by diluting the stock solution with 30% (v/v) D_2O in water to obtain predetermined concentrations of cyclodextrin in the samples. An exact amount of the drug was weighed into a glass vial and the sample solution pipetted into the vial. The vials were sealed tightly and shaken before heating to dissolve all the drug. The samples were allowed to equilibrate in darkness at room temperature (22°C–24°C) for 4 to 5 days before the NMR measurement was performed. The sample concentration for K_C determination by ^{19}F-NMR was 0.0075 M for DIF. For continuous variation plots,[6] the total concentration of DIF and HPβCD was kept at 0.05 M for ^{19}F-NMR and 5×10^{-5} M for UV experiments, but the ratio (HPβCD)/([HPβCD] + [DIF]) was varied from 0.1 to 0.9. NMR samples of fluoxetine hydrochloride were 0.022 M, and dexamethasone samples were 0.00027 M in water and 0.0196 M in 10% (w/v) HPβCD. The drug concentration in all samples was measured by HPLC.

NMR and UV Measurements

The NMR spectra were recorded at 297K in 30% (v/v) D_2O in water on a Bruker AC 250 P 250 MHz spectrometer. The instrument was calibrated for ^{19}F-NMR with an external standard of trifluorotoluene. The trifluorotoluene signal was fixed at −64 ppm, relative to $CFCl_3$ at 0 ppm. Spectra were generally obtained in 30 to 80 scans, depending on the sample concentration. The UV absorbance was measured at 254 nm on a Perkin-Elmer Lambda 3A Spectrophotometer (Wellesley, MA, USA).

Calculations

NMR data was plotted as $\Delta\delta$ ($\delta_{\text{withHP}\beta\text{CD}} - \delta_{\text{in water}}$) against $[CD]_T/[D]_T$ and fitted to the following equation using KaleidaGraph 3.0 (Synergy Software, Essex Junction, VT, USA) to obtain K_C (Eq. (1))[6]:

$$\Delta\delta_{obs} = \Delta\delta_{max}\left(\frac{1}{2}\left(\frac{[CD]_T}{[D]_T} + \frac{1}{K_{1:1}[D]_T} + 1\right) - \left\{\frac{1}{4}\left(\frac{[CD]_T}{[D]_T} + \frac{1}{K_{1:1}[D]_T} + 1\right)^2 - \frac{[CD]_T}{[D]_T}\right\}^{1/2}\right)$$

where $\Delta\delta_{obs}$ is the change in chemical shift of the reporter group in response to an addition of a certain CD concentration, $\Delta\delta_{max}$ is the limiting change in chemical shift at infinite CD concentration, $[D]_T$ and $[CD]_T$ are total concentrations in the sample of drug and CD, respectively, and $K_{1:1}$ is the apparent K_C for a 1:1 drug:CD complex.

RESULTS AND DISCUSSION

Fluorine NMR spectra were recorded for dexamethasone (DEX, Fig. 1a) in saturated solutions containing 0% and 10% (w/v) HPβCD. The chemical-shift variation between the two solutions was less

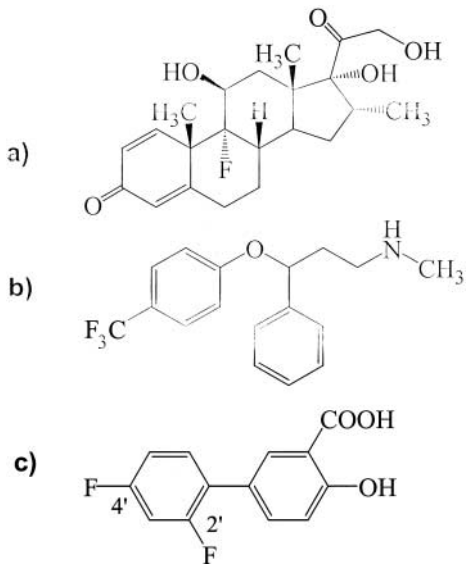

Figure 1. Structures of a) dexamethasone b) fluoxetine and c) diflunisal.

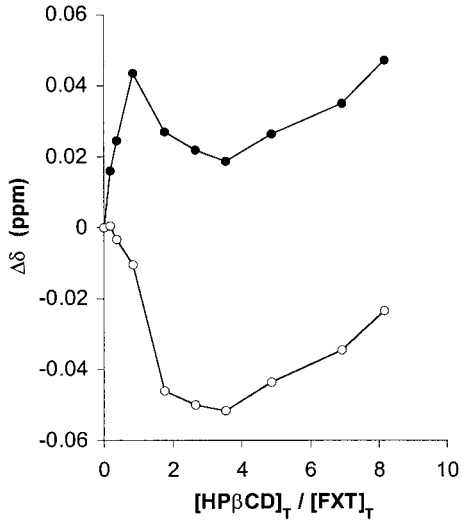

Figure 2. Fluorine NMR chemical-shift variations of each of the two fluoxetine hydrochloride enantiomers with increasing concentration of HPβCD, 0%–25% (w/v) (0–0.181 M HPβCD). Each point represents the average of two experiments. The fluoxetine concentration was 0.021 ± 0.001 M.

than 0.1 ppm. DEX has a very poor aqueous solubility (0.11 mg/mL or 2.7×10^{-4} M), which leads to a poor signal-to-noise ratio in water, making the accurate chemical-shift determination difficult. This, together with the fact that collecting spectra for low-concentration samples is time consuming, makes the ^{19}F-NMR method for K_C determination less suitable for DEX than other more soluble compounds such as fluoxetine hydrochloride (FXT, Fig. 1b) or diflunisal sodium (DIF, Fig. 1c). FXT forms a 1:1 complex with HPβCD, according to a continuous variation plot based on UV measurements (not shown). Complexation can theoretically take place at either the phenyl group or the *p*-trifluoromethylphenyl group. A 2:1 complex, where two CD molecules embed the separate aromatic groups, would be sterically hindered. In the ^{19}F-NMR spectra, FXT has one strong singlet at −61.38 ppm. FXT is a racemic mixture and addition of HPβCD resulted in separation of the enantiomers in the ^{19}F-NMR spectra, with different chemical-shift variations for either enantiomer. Thus, the presence of two enantiomers can be demonstrated simply by the addition of CD, and equal size of the peaks confirmed that the enatiomers were present in equimolar quantities. The changes in shift were, however, less than 0.05 ppm, indicating that the complexation takes place at the phenyl group to a much greater extent and there is no great involvement of the *p*-trifluoromethylphenyl group. One shortcoming of the FXT measurements was the observed medium effects. As seen in Figs. 2 and 3a, complexation induces shielding of one enantiomer and deshielding of the other at lower CD concentrations resulting from complexation with the chiral CD molecule. The separation reaches maximum at 0.05 M HPβCD concentration when the [CD]/[FXT] ratio has reached 1.5. At higher CD concentrations there are changes in the shift, which are nondiscriminating for the enantiomers, e.g., medium effects. Conventional methods such as NMR titrations and Job's plot could therefore not be applied to the FXT ^{19}F-NMR data.

Two signals are observed in the fluorine spectra of DIF: a quartet at −115.06 ppm and a quintet at −112.77 ppm (assigned to C2′ and C4′ fluorines, respectively) (Fig. 3b). A continuous variation plot of DIF in the presence of HPβCD (Fig. 4) shows a maximum value at a 0.5 mol fraction for HPβCD, which is characteristic for formation of a 1:1 complex. No difference is observed whether measured by UV or ^{19}F-NMR. As seen in Figs. 3b and 5, the C2′ signal is shifted more dramatically upfield upon addition of HPβCD than the C4′ signal. This is consistent with the C2′ fluorine penetrating deeper into the cavity when the complex is formed; the upfield shift is a result of the shielding exerted by the CD skeleton.[1] The $\Delta\delta_{max}$ values were 2.092 and 1.378 ppm for C2′ and C4′, respectively. The K_C

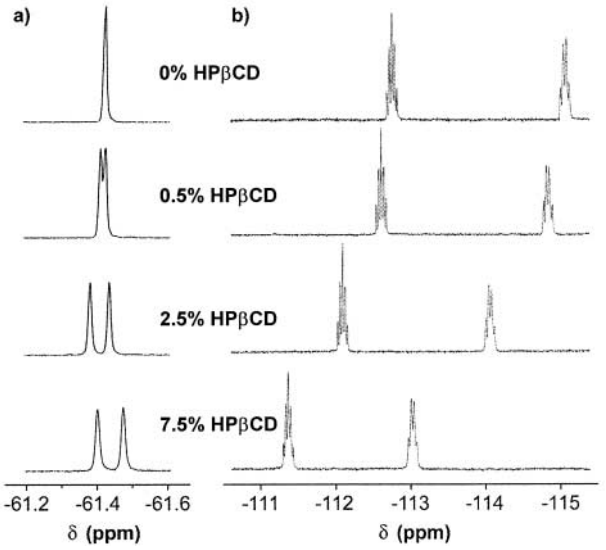

Figure 3. Changes in the ^{19}F-NMR spectra of a) fluoxetine hydrochloride and b) diflunisal sodium upon addition of HPβCD. The HPβCD concentrations (w/v) are shown for each spectra.

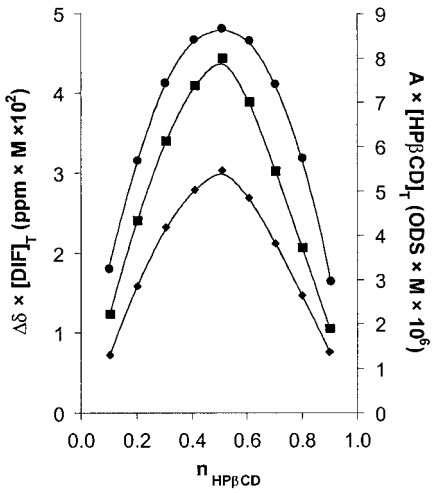

Figure 4. Continuous variation plot of diflunisal sodium with HPβCD, measured either by UV (●, max 0.50) or ^{19}F-NMR (■, C4′ peak, max 0.51; ◆, C2′ peak, max 0.50).

value is always derived from determination of the ratio between free and complexed drug. Accurate determination of the K_C value, which is a measure for the binding affinity, thus requires that significant amounts of the complexing species are found both in free and complexed form. The K_C values were obtained from nonlinear fits of NMR chemical-shift data for HPβCD concentrations between 0.0015 M and 0.010 M. Under these conditions, the drug and

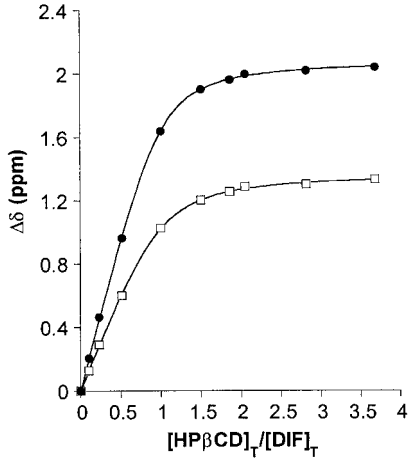

Figure 5. Changes in the ^{19}F-NMR chemical shift of diflunisal sodium with addition of HPβCD, 0%–20% (w/v) (0–0.145 M HPβCD). Solid lines are nonlinear least squares fitting of the data, according to Eq. (1). The diflunisal concentration was 0.0075 ± 0.0007 M. C2′ (●); C4′ (□).

cyclodextrin were between 10% and 90% complexed. The K_C values calculated for each of the fluorine peaks were 2034 ± 403 M^{-1} and 1473 ± 426 M^{-1} for C2′ and C4′ signals, respectively. The K_C value obtained for a UV titration was somewhat higher, or 5069 M^{-1} (data not shown). Previously reported values, which have been obtained by methods such as equilibrium dialysis (3892 ± 360 M^{-1}),[7] titration microcalorimetry (3394 M^{-1}),[7] and potentiometry [5570 ± 40 M^{-1}[8] and 5560 M^{-1}[9]], are closer to the value obtained by UV titration.

Low K_C estimates with the ^{19}F-NMR titration could be caused by solvent effects. When the titration was done for 0.037 M DIF concentrations (data not shown), the $\Delta\delta_{max}$ values were 2.203 and 1.569 ppm for C2′ and C4′, respectively. These values are significantly different from those obtained for 0.0075 M concentrations, which is also an indication of a solvent-medium effect. The medium effects could be caused by fluorine-hydrogen bonds[10] or by self-association of the cyclodextrin complexes.[11]

CONCLUSION

Fluorine NMR titrations and continuous variation methods are easy to perform and fast. These methods can be used to determine complexation stoichiometry or stability constants between fluorine-containing drugs and CD derivatives, provided

that the fluorine is embedded into the CD cavity when the complex is formed. However, care must be taken in interpreting the results as fluorine is also very sensitive to solvent effects. For convenient use of this method, the drug must have a relatively high intrinsic solubility ($>1 \times 10^{-3}$ M) so that the acquisition time for each sample is within few minutes. This method could be used to measure enantiomeric composition of chiral compounds, since only very small additions of CD cause separation of peaks in the spectra.

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