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Determination of phosphate functional group acid dissociation constants of clindamycin 2-phosphate using ^{31}P Fourier transform NMR spectrometry

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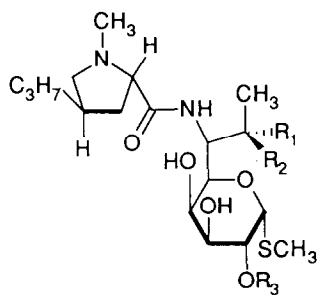
Summary

^{31}P-NMR spectrometric titration was used to determine the apparent first and second acid dissociation constants of the phosphate functional group in clindamycin 2-phosphate at 21°C. Using a standard rapid exchange model, values of 0.964 and 6.06 were determined.

Introduction

As part of an investigation into the mechanism of hydrolysis of clindamycin 2-phosphate (**2**, see Fig. 1), estimates of the acid dissociation constants were desired. This will enable calculation of ionic species distribution as a function of pH. The eventual goal is a more thorough understanding of the mechanism of hydrolysis in relation to pH. A literature search for pK_a values



1: R_1 = Cl; R_2 = H; R_3 = H
2: R_1 = Cl; R_2 = H; R_3 = PO_3H_2
3: R_1 = H; R_2 = OH; R_3 = H
4: R_1 = H; R_2 = OH; R_3 = PO_3H_2

Fig. 1. Structure of clindamycin (**1**), clindamycin 2-phosphate (**2**), lincomycin (**3**) and lincomycin 2-phosphate (**4**).

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afforded information on the dephosphorylated molecule, clindamycin (**1**), and its 7-hydroxyl analog, lincomycin (**3**), but no information on the acid dissociation constants of their phosphate esters, **2** and lincomycin phosphate (**4**). (See Fig. 1 for structures.)

The expected second pK_a of the phosphate group in **2**, which should be approximately 6 (Cori et al., 1937; Ashby et al., 1954; Bunton et al., 1958), lies sufficiently close to the expected pK_a of the pyrrolidinium group in **2** (7.72 reported for clindamycin; Harvey, 1980) that a simple titration would not be expected to resolve this difference. Accordingly, inflection points in a simple acid-base titration were not easily discerned at a drug concentration of 0.023 M. Alternative methods of pK_a determination were therefore sought.

Proton (Zoltewicz and Helmick, 1973; Tanokura et al., 1976), carbon-13 (Van de Weijer et al., 1976; Kimberly and Goldstein, 1981), nitrogen-15 (Paschal and Dorman, 1978) and sulfur-33 (Crumrine et al., 1986) NMR spectrometry, to name a few examples, have been successfully utilized for determining pK_a values. ^{15}N- and ^{13}C-NMR spectrometry were initially considered for the estimation of the pK_a of the pyrrolidine amino group in **2**. The use of these nuclei, however, conflicted with two additional requirements: the need to maintain as low an ionic strength as possible to minimize salt effects, and the need to minimize hydrolysis of the drug at low and high pH values. The natural isotopic abundances of ^{13}C and ^{15}N (1.01 and 0.37%, respectively) and low inherent sensitivity limited their usefulness.

Phosphorus-31 has some useful NMR properties – high isotopic abundance (100%) and a wide range of chemical shifts. Although ^{31}P chemical shifts in phosphate monoesters are relatively insensitive to the local environment (*O* substitution, hydrogen bonding), they are very sensitive to changes in the molecular geometry about the phosphorus atom (Gorenstein, 1975). Thus the chemical shift is affected appreciably by the ionization state of the phosphate group. This property has been exploited to obtain reliable measurements of the degree of ionization (Gorenstein et al., 1976), and pH (DeFronzo and Gillies, 1987). A ^{31}P-NMR spectrum of **2** (0.01 M) with sufficient signal-to-noise ratio was easily obtained after 64 scans (approx. 7 min). In this paper, we wish to report the determination of apparent pK_a values of the phosphate group in clindamycin 2-phosphate by use of ^{31}P FT-NMR spectrometry.

Theoretical

In general, the NMR spectrum generated by molecules in chemical equilibrium is a complex function of both the exchange rates and the chemical shifts of the individual species. When the exchange rate (in Hz) is large relative to the absolute value of the difference in chemical shift ('rapid exchange' limit), a single line spectrum is observed. For a monobasic acid, the observed chemical shift (δ_{obs}) is the average of the chemical shifts of the conjugate acid and base weighted by the respective mole fractions. Similarly, for a dibasic acid, H_2X, consisting of three species, H_2X, HX^- and X^{2-}, in equilibrium:

$$\delta_{obs} = f_{H2X}\delta_{H2X} + f_{HX}\delta_{HX} + f_X\delta_X \quad (1)$$

where f_{H2X}, f_{HX} and f_X are mole fractions. Values δ_{H2X}, δ_{HX} and δ_X are the corresponding chemical shifts. The mole fraction of each species is described by the following equations:

$$f_{H2X} = a_H^2/D \quad (2)$$

$$f_{HX} = a_H K_1/D \quad (3)$$

$$f_X = K_1K_2/D \quad (4)$$

where $D = a_H^2 + K_1a_H + K_1K_2$, and a_H is the hydronium ion activity. K_1 and K_2 are the apparent, not thermodynamic, acid dissociation constants. Apparent, or mixed (Perrin and Dempsey, 1974a) dissociation constants, are dependent on the ionic strength of the medium. Substitution of Eqns 2–4 into Eqn 1 affords:

$$\delta_{obs} = \frac{\beta_2 a_H^2 + \beta_1 a_H + \beta_0}{a_H^2 + \alpha_1 a_H + \alpha_0}, \quad (5)$$

where $\alpha_0 = K_1K_2$, $\alpha_1 = K_1$, $\beta_0 = K_1K_2\delta_X$, $\beta_1 = K_1\delta_{HX}$ and $\beta_2 = \delta_{H2X}$.

Both acid dissociation constants, K_1 and K_2, and the chemical shift of ^{31}P in each ionic species can now be calculated from the coefficients, α_i and β_i, in Eqn 5.

Materials and Methods

Solutions were analyzed by 121.513 MHz ^{31}P FT-NMR spectrometry using a Bruker Instruments Inc. ACP-300 NMR spectrometer. A standard 5 mm NMR sample tube was fitted with an internal 2 mm (o.d.) coaxial capillary. After introduction of an approx. 90 mM solution of sodium pyrophosphate in D_2O, the internal capillary was tightly sealed. The capillary solution provided the spectrometer field/frequency lock as well as an external ^{31}P chemical shift reference standard.

Chemical shifts were determined using a 'peak picking' routine supplied with the instrument. This software (DISNMR^{TM}) estimates chemical shifts by fitting a parabola through the three most intense data points of the resonance in question. The fitted maximum of the parabola is then used as the estimate of the chemical shift. Since the software uses the same number of data points as the number of parameters to be determined (i.e., three), the variance of the estimated chemical shift cannot be determined. However, the maximum error is on the order of the interval between data points in the frequency domain spectrum. The acquisition conditions chosen for the present series of experiments lead to an estimated error in the fitted chemical shift of less than 0.2 Hz.

A fresh drug solution was prepared for each chemical shift measurement. A stock solution of 0.05 M clindamycin-2-phosphate in distilled, deionized water was prepared. For each trial, 20 ml of the stock solution was combined with 60 ml of distilled, deionized water, and the solution adjusted to the desired pH. The pH meter (Beckman, Model Φ71) was calibrated to N.B.S. standards at pH 1.68 and 4.00. In addition, a buffer at pH 1.00 was prepared (Perrin and Dempsey, 1974b) to check for meter accuracy outside the calibration range (values of 0.998, 1.001 and 1.001 were obtained). The drug solution was finally diluted to 100 ml, and the pH redetermined. The freshly prepared drug solution was added to the outside annulus of the sample holder assembly. After each run, the outer 5 mm sample tube and the outside of the capillary insert were thoroughly rinsed with water and dried. An ambient temperature of 21°C was maintained throughout each experiment.

Results and Discussion

The data shown in Table 1 were fitted to Eqn 5 by a nonlinear least-squares regression using the sequential simplex algorithm of Nelder and Mead (Nelder and Mead, 1965). The ^{31}P chemi-

TABLE 1

Results from NMR titration

pH	Chemical shift (ppm) [a,b]	Spin-spin coupling constant (J, Hz) [b]
0.034	5.158	8.384
0.183	5.205	8.397
0.539	5.358	8.324
0.836	5.495	8.433
1.051	5.593	8.336
1.249	5.693	8.445
1.665	5.877	8.506
2.052	5.969	8.470
2.460	6.014	8.494
2.864	6.033	8.457
3.267	6.042	8.482
3.646	6.055	8.530
4.073	6.078	8.530
4.481	6.134	8.518
5.014	6.358	8.494
5.478	6.892	8.494
6.002	7.916	8.530
6.555	9.136	8.664
7.004	9.741	8.688
7.510	10.033	8.725
8.040	10.137	8.725
8.526	10.177	8.737

[a] Relative to external pyrophosphate standard.

[b] Determined by the 'peak picking' routine described in the text.

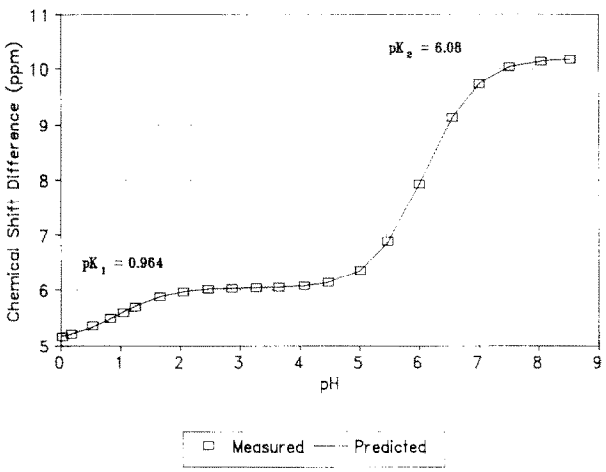

Fig. 2. Phosphorus-31 chemical shift (relative to external pyrophosphate standard) vs pH.

cal shift values (relative to the external pyrophosphate standard) vs pH are plotted in Fig. 2. The fitted function is shown as a solid curve. The standard deviation in each parameter was estimated by the Gauss-Newton method (Gallant, 1975). A computer program, written in PASCAL (Turbo PASCAL™, Version 5.0, Borland International, Inc.) was run on a Compaq 386S™ microcomputer, equipped with an 80387SX math coprocessor. Ten byte reals (extended precision) were used throughout the program. Parameter estimates and their standard deviations are listed in Table 2.

The ^{31}P resonance of the phosphate group was split into a doublet, due to spin-spin coupling between phosphorus and the proton attached to the adjacent carbon, C-2 of the pyranoside ring. J values are listed (in Hz) in Table 1. The coupling constants were fitted to Eqn 5 in which J is substituted for the dependent variable δ. The results, graphically represented in Fig. 3, indicate that although the fit is considerably poorer than regression of chemical shift on pH, reasonable pK_a values ($pK_1 = 1.3$; $pK_2 = 6.5$) could also be obtained. To our knowledge, this is the first instance in which a clear correlation between ^{31}P-^1H spin-spin coupling in a phosphate monoester and its ionization state has been demonstrated.

As shown in Table 3, the estimated pK_1 and pK_2 values of clindamcyin 2-phosphate are significantly lower than those reported for orthophosphoric acid. This is consistent with the behavior of other organic phosphate esters, as shown in the table.

TABLE 2

Parameters estimated by nonlinear regression

Parameter	Estimate	Standard error
By regression of ^{31}P chemical shift on pH		
K_1	0.109 [a]	4.69×10^{-3}
K_2	8.30×10^{-7} [b]	7.73×10^{-9}
δ_X	10.18	6.26×10^{-3}
δ_{HX}	6.04	4.30×10^{-3}
δ_{H2X}	5.07	1.19×10^{-2}
By regression of J (^{31}P–^1H) on pH		
K_1	4.91×10^{-2} [c]	4.22×10^{-2}
K_2	3.41×10^{-7} [d]	1.75×10^{-7}
J_X	8.74	2.26×10^{-2}
J_{HX}	8.50	1.35×10^{-2}
J_{H2X}	8.36	2.74×10^{-2}

[a] $pK_1 = 0.964$.
[b] $pK_2 = 6.081$.
[c] $pK_1 = 1.31$.
[d] $pK_2 = 6.47$.

A study of the effect of ionic strength on the pK_2 of methylphosphonate (DeFronzo and Gillies, 1987) indicated a change of less than -0.12 pH unit over a 213 mM change in ionic strength. Application of the Davies equation af-

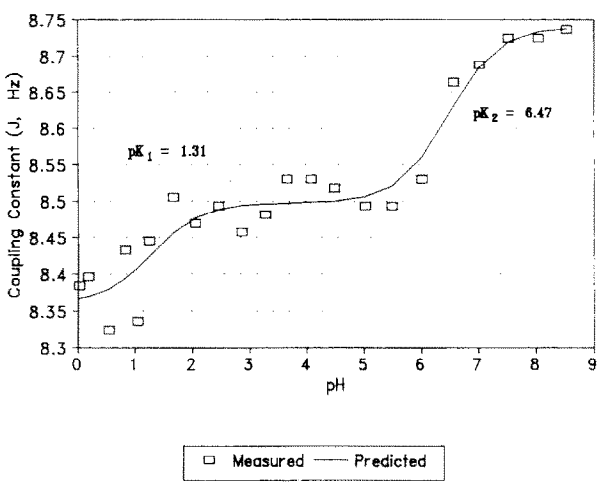

Fig. 3. ^{31}P–^1H coupling constant (J) vs pH.

TABLE 3

Comparison of pK_a values of clindamycin 2-phosphate with those of other organic phosphates

Compound	pK_1	pK_2	Conditions [a]
Orthophosphoric acid [b]	2.15	7.20	25 °C (extrapolated to $I = 0$)
Clindamycin 2-phosphate [c]	0.964	6.081	21 °C ($I \approx 0.11$ for K_1; $I \approx 0.008$ for K_2)
Methyl phosphate [d]	1.52	6.58	22.0 °C (pK_1, $I > 0$) 22.5 °C (pK_2, $I > 0$)
α-D-Glucose 1-phosphoric acid [e]	1.11	6.13	25 °C ($I > 0$)
Hexose 6-phosphoric acid [f]	0.97	6.11	30 °C ($I > 0$)
Glycerol 2-phosphoric acid [g]	1.335	6.650	25 °C (extrapolated to $I = 0$)

[a] 'I' refers to the ionic strength at which pK_a values were either measured or for which an extrapolated value was calculated.
[b] Perrin and Dempsey (1974c).
[c] This paper. Estimation of ionic strength is described in the text.
[d] Bunton et al. (1958).
[e] Cori et al. (1937).
[f] Meyerhof and Lohmann (1927).
[g] Ashby et al. (1954).

forded an estimate of −0.06 pH unit/100 mM change in ionic strength. This is similar to results observed for orthophosphoric acid and 2-D-glucose 6-phosphate (Roberts et al., 1981). At a clindamycin phosphate concentration of 0.01 M, the ionic strength at $pH = pK_2 = 6.06$, is calculated to be less than 10 mM. Thus, the value of pK_2 derived in our investigation should provide a reliable estimate of the thermodynamic value.

The calculated ionic strength at $pH = pK_1 = 0.964$ is approx. 110 mM. At this high ionic strength, due largely to the hydronium ion concentration, the estimate of thermodynamic pK_a for the first acid dissociation is less reliable than that for second proton loss. Examination of Table 1 indicates, however, that the pK_1 estimate is in agreement with the thermodynamic pK_1 determinations of other phosphate monoesters.

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