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Determination by NMR of the binding constant for the molecular complex between alprostadil and α-cyclodextrin Implications for a freeze-dried formulation

Tore Ramstad[*], Chad E. Hadden[1], Gary E. Martin, Stanley M. Speaker, Dirk L. Teagarden, Thomas J. Thamann

Pharmaceutical Sciences, Pfizer Corp., Kalamazoo, MI 49001, USA

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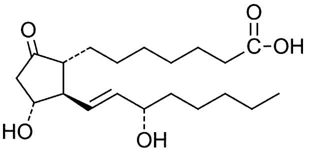
Abstract

A binding constant was determined for the complexation reaction between alprostadil (PGE_1) and α-cyclodextrin (α-CD). This constant was used to calculate the fraction PGE_1 free upon reconstitution of Caverject dual chamber syringe, indicated for the treatment of erectile dysfunction. The determination was based on the measurement of the chemical shift of the C20 methyl protons of PGE_1. The observed chemical shift varies as a linear function of the amount of PGE_1 bound. The binding constant was obtained from the binding isotherm, a curve of the observed chemical shift versus free ligand (α-CD) concentration, through the application of non-linear regression analysis. A value $K_{11} = 966\,M^{-1} \pm 130\,M^{-1}$ ($2s$), measured at 27 °C, was obtained. This value is in good agreement with those reported in the literature. The percent PGE_1 free was subsequently calculated for the reconstituted solution and in the corpora cavernosum after injection. The latter showed PGE_1 to be delivered essentially quantitatively to the targeted site.

Keywords: Binding study; Cyclodextrins; NMR; Stabilization; Freeze-drying

1. Introduction

Caverject^{TM} *sterile powder for injection*, a product of Pfizer, is indicated for the treatment of erectile dysfunction. The active ingredient is a prostaglandin, alprostadil (PGE_1). Caverject is marketed as a lyophilized powder in a vial presentation in strengths of 5, 10, 20, and 40 μg. Each vial is reconstituted with 1.0 ml of bacteriostatic water for injection (BWFI), using a companion syringe.



Alprostadil; PGE_1

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[*] Corresponding author. Tel.: +1 269 833 7432; fax: +1 269 833 0791.
E-mail address: tore.ramstad@pfizer.com (T. Ramstad).

[1] Present address: Eli Lilly, Indianapolis, IN, USA.

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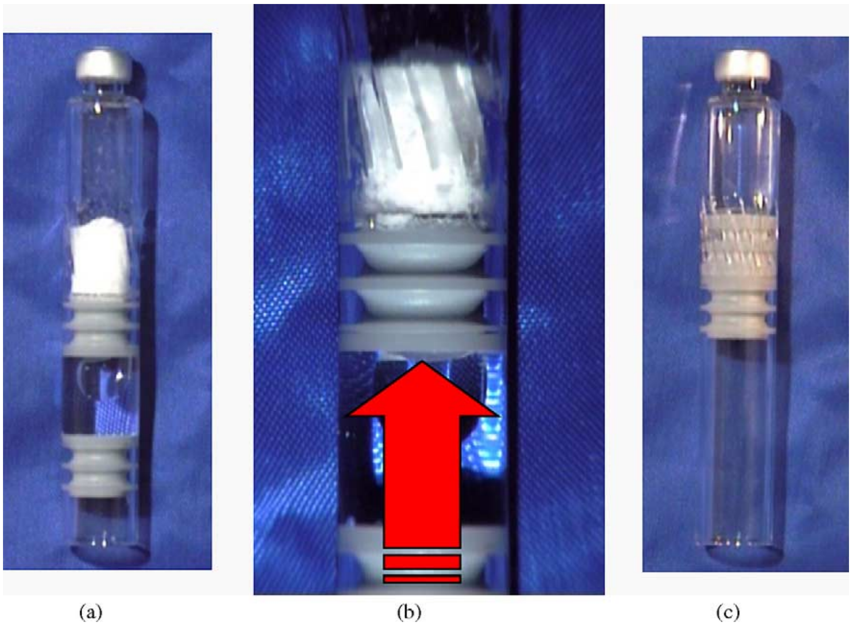

Fig. 1. Action of dual-chamber syringe: (a) prior to reconstitution; the freeze-dried powder is in the forward chamber and the BWFI diluent in the rear; (b) during reconstitution; (c) after reconstitution.

Caverject sterile powder dual chamber (DC) syringe is a recently developed new formulation of Caverject. Its distinguishing feature is a convenience package consisting of a special syringe in which lyophilized powder is contained in a front chamber and diluent (BWFI) in the rear. It is available in two strengths, 10 and 20 μg. Due to a smaller diluent volume compared to the vials, the concentrations after reconstitution are 20 and 40 μg/ml. The delivery volume is 0.5 ml. A disposable administration device is packaged along with the syringe, thereby making for a user-friendly package. This is shown in Fig. 1.

The composition of Caverject DC differs from Caverject principally through inclusion of a complexing agent, α-cyclodextrin (α-CD). A guest–host complex forms between alprostadil and α-CD during the lyophilization process. As the bulk solution containing PGE_1 and α-CD becomes more concentrated during freeze-dry manufacturing, the fraction of PGE_1 that combines with α-CD increases to the point where, if a sufficient excess of α-CD is present, virtually all of the PGE_1 substrate will be complexed in the solid-state. Alprostadil decomposes through an apparent second order reaction mechanism in a solid-state matrix, forming the dehydration product PGA_1 (Teagarden and Baker, 2002). PGA_1 is the only degradation impurity of note for alprostadil. The complex acts to impede the mobility of alprostadil, thereby retarding chemical decomposition. The other constituents of the formulation are lactose, a diluent, and sodium citrate for pH control. A 2-year shelf life at room temperature has been achieved through inclusion of α-CD.

When reconstituted, α-CD is believed to associate with alprostadil in the manner depicted in Fig. 2 (Frömming and Szejtli, 1994a). (By comparison, β- and γ-CD are believed to complex with different portions of the prostaglandin molecule (Frömming and Szejtli, 1994a).) The degree to which PGE_1 and α-CD

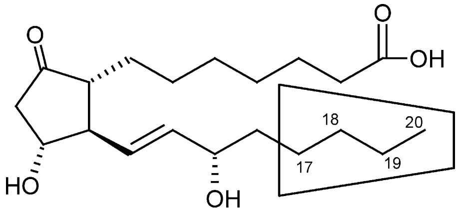

Fig. 2. Schematic representation of the molecular complex between PGE_1 and α-CD (Frömming and Szejtli, 1994a). The trapezoid is a side-view, bucket representation of the ring formed by the six glucopyranose units joined together to form α-cyclodextrin.

interact is reflected in the binding constant. From a formulation standpoint, a balance needs to be struck between inclusion of a sufficient amount of α-CD to impart the desired stability in the lyophilized state, yet not so high that upon reconstitution PGE_1 remains substantially bound. The goal is to free essentially all the PGE_1 for delivery to the patient, since only non-bound PGE_1 has the biological activity. Hence, in order to appropriately design the formulation, the binding constant for the molecular complex between PGE_1 and α-CD needs to be known.

1.1. Background

For an assumed 1:1 stoichiometry (Uekama and Otagiri, 1987; Steffan et al., 1992) the desired binding or equilibrium constant is given by (Connors, 1987)

$$K_{11} = \frac{[\mathrm{SL}]}{[\mathrm{S}][\mathrm{L}]} \qquad (1)$$

where [S] is the concentration of free substrate (PGE_1), [L] is the concentration of free ligand (α-CD), and [SL] the concentration of the complex (PGE_1-α-CD). The subscript 11 signifies the 1:1 stoichiometry. Molecular modeling and NMR measurements utilizing the nuclear overhauser effect (NOE) provide support for the structure depicted in Fig. 2, in aqueous solution (Uekama and Otagiri, 1987; Steffan et al., 1992). Because the process represented by Eq. (1) is a dynamic one, [S] and [SL] or [L] and [SL] are not readily measurable. In lieu of measuring separate concentrations, the nearly universal approach to determine molecular complexation constants is to generate a binding curve and then to extract K via regression analysis.

Using this approach, we have:

$$S_t = [\mathrm{S}] + [\mathrm{SL}] \qquad (2)$$

where S_t is the *total* substrate concentration, which is always known. In addition, we have the expression:

$$f_b = \frac{[\mathrm{SL}]}{S_t} \qquad (3)$$

where f_b is the fraction of S bound. Combining Eqs. (2) and (3) with Eq. (1) leads to the well-known binding isotherm (Connors, 1987)

$$f_b = \frac{K_{11}[\mathrm{L}]}{1 + K_{11}[\mathrm{L}]} \qquad (4)$$

In order to utilize this expression, f_b needs to be described in terms of a measurable system parameter whose magnitude varies as a function of the amount of substrate bound.

Numerous techniques for determining binding constants have been reported in the literature. In principle, any technique in which a measurable parameter changes as a function of ligand concentration may serve as the basis for determining the binding constant. Many studies have been conducted on the binding of cyclodextrins to a wide assortment of compounds, some utilizing NMR (Steffan et al., 1992; Uekama et al., 1975; Yamamoto and Inoue, 1989; Inoue, 1993; Djedaini et al., 1990; Wiese et al., 1991; Fronza et al., 1992; Funasaki et al., 2001, 2002, 2003). The topic of the inclusion complexes with cyclodextrins, including the determination of binding constants has been reviewed by several authors (Thorsteinn and Brewster, 1996; Connors, 1997; Schneider et al., 1998; Liu et al., 2004). Optical absorbance is a preferred technique when applicable. However, in order to use optical absorbance, there must be either a shift in the wavelength of maximum absorbance or a change in A_{max} as a function of ligand concentration. Because PGE_1 possesses only end (short wavelength) absorption, absorption spectroscopy is not possible. NMR is probably the most generally informative of all the techniques, as it affords high specificity and can yield structural information on the nature of the complex (Schneider et al., 1998). Chemical shift, spin coupling, and relaxation times are all sensitive to the short-range interactions encountered in molecular complexes. Applications of NMR to the study of molecular complexes generally, and to cyclodextrin inclusion complexes specifically, have been reviewed (Yamamoto and Inoue, 1989; Inoue, 1993; Liu et al., 2004).

Various NMR techniques, including two-dimensional NMR, can be used to study CD inclusion complexes, both in solution and in the solid-state. Two groups have applied NMR to the study of the inclusion complex between PGE_1 and α-cyclodextrin. Wiese et al. measured the chemical shifts of protons in several different environments (Wiese et al., 1991). They found the greatest shifts for *trans* double bond methine protons and for the methyl protons. Studies were also conducted to try to elucidate the molecular geometry of the complex. Since different protons yielded different values for the dissociation constant, a statistically derived mean value was reported. While it may seem that all protons, regardless of environment, should yield identical values of the binding constant, statistical averaging of the association constants calculated for protons at different sites is viewed as improving the precision of the final result (Djedaini et al., 1990). Steffan et al. measured the chemical shift induced for the methylene protons in PGE_1 with the intent to extract information on the amount of PGE_1 bound in Prostavasin upon reconstitution and dilution (Steffan et al., 1992). They prepared a stock solution of PGE_1 and α-CD, then made dilutions and measured the chemical shift at each dilution. Although they did not report a binding constant, one could be calculated from their data (vide infra). They also concluded through application of NOE that a 1:1 complex was formed, consistent with the structure shown in Fig. 2.

In order to utilize Eq. (4), an expression for f_b involving chemical shift is needed. The requirement imposed on the molecular complex is that the monitored proton's chemical shift differs in the free and bound states. If δ_S is the chemical shift of S, and δ_{SL} the chemical shift of SL, then the observed chemical shift, δ, is given by (Connors, 1987)

$$\delta = f_f\delta_S + f_b\delta_{SL} \tag{5}$$

where f_f is the fraction of S free. The weighted chemical shift represented by Eq. (5) is a consequence of reversible, rapid exchange between the free and bound states. Since $f_f + f_b = 1$, Eq. (5) becomes

$$\delta = f_b(\delta_{SL} - \delta_S) + \delta_S \tag{6}$$

Defining $\Delta = \delta - \delta_S$ and $\Delta_{max} = \delta_{SL} - \delta_S$, and combining these two equations with Eq. (6), one obtains

$$f_b = \frac{\Delta}{\Delta_{max}} \tag{7}$$

Eq. (7) shows that the fraction bound may be determined directly from the measurement of the observed frequency δ. A plot of f_b versus Δ is a straight line with slope $1/\Delta_{max}$. Sustituting Eq. (7) into Eq. (4) yields

$$\Delta = \frac{\Delta_{max}K_{11}[L]}{1 + K_{11}[L]} \rightarrow \Delta = \frac{\Delta_{max}K_{11}[CD]}{1 + K_{11}[CD]} \tag{8}$$

where we have made the substitution CD, referring to α-cyclodextrin, for L.

Eq. (8) is the binding isotherm as it pertains to NMR for a 1:1 molecular stoichiometry (Connors, 1987). It is utilized by measuring δ as a function of [CD]. Δ_{max}, which corresponds to the hyperbolic asymptote, cannot be experimentally determined in the PGE_1/α-CD system, because complete binding is not achievable at attainable concentrations of α-CD. However, Δ_{max}, and ultimately K_{11}, may be extracted from Eq. (8) through application of non-linear curve fitting to the empirical data.

2. Materials and methods

In using the binding isotherm represented by Eq. (8), the concentration of either the substrate or ligand (usually the former) may be kept constant, while varying the concentration of the other over a suitable range. Alternatively, a stock solution containing both substrate and ligand may be prepared at a suitable ratio, then serially diluted. For most of our work we prepared a stock solution and then made dilutions, consistent with the work of Wiese et al. (1991) and Steffan et al. (1992). The chemical shift was then measured at each dilution.

2.1. Chemicals and sample preparation

The alprostadil used was a reference standard of 98.7% purity, synthesized at Pfizer, formerly Pharmacia. The α-cyclodextrin was obtained from Cerestar, formerly American Maize. Deuterium oxide (D_2O) and benzyl alcohol were obtained from Aldrich. A stock solution containing PGE_1 and α-CD in the ratio present in both 10 μg and 20 μg Caverject DC (1:32.5 on a weight basis, 1:11.8 on a mole basis) was prepared in D_2O. Dissolution of PGE_1 was facilitated by the addition of PGE_1 from a concentrated solution of deuterated ethanol. The weight of α-CD was corrected for water. No correction for HPLC impurities was required. These solutions were diluted to cover a concentration range from approximately 200 μg/ml PGE_1/6.49 mg/ml α-CD to 1 μg/ml PGE_1/0.032 mg/ml α-CD. The stock solution was assayed by HPLC to determine the exact concentration of PGE_1. Solutions were refrigerated until used to inhibit decomposition of PGE_1. Decomposition was negligible under the conditions employed.

2.2. NMR measurements

Changes in the ^{1}H chemical shift of the aliphatic methyl protons (C20) of PGE$_1$ were measured as a function of the fraction PGE$_1$ bound (see Fig. 2). A difference in chemical shift between free and complexed PGE$_1$ results from the shielding effects of α-CD protons. As the complexation/decomplexation kinetics are rapid on the NMR timescale, only a single peak (the *observed* chemical shift), i.e., the weighted average of the chemical shifts of free and bound PGE$_1$, is observed. The methyl protons resonated at about 0.7 ppm. The data were acquired on a Bruker AMX-500 spectrometer operating at a proton frequency of 500.13 MHz using a 5-mm Bruker IDTG probe. All spectra were recorded at a temperature of 300 K (27 °C). As noted, most data were collected on solutions of differing absolute concentration but with the same ratio of [PGE$_1$] to [α-CD]. Relatively few transients were required for the highest concentrations of PGE$_1$ (up to 200 μg/ml). The resultant data accumulation time was short (about 5 min). By comparison, at the lowest concentrations (down to 1 μg/ml) many transients were collected and the data acquisition time was long (12 h).

2.3. Data analysis

The data were analyzed according to the Eq. (8) using non-linear regression analysis from which both $\Delta_{\max} = \delta_{SL} - \delta_S$ and K_{11} were extracted. The measured change in chemical shift was plotted versus free α-CD ([α-CD]). The curve fitting feature in SigmaPlot was used to accomplish the regression analysis.

3. Results

The measured chemical shift of the methyl protons (C20) versus CD$_t$ is given in Table 1. The parts per million (ppm) shift is referenced to solvent protons at 4.63 ppm. The methyl resonance shifts downfield (to higher ppm) as the fraction of PGE$_1$ complex increases. When inserted into the apolar cavity of α-CD, the methyl group is in a less polar environment than when free in solution. The resulting complex shields the PGE$_1$ methyl group, thereby inducing a downfield shift of the methyl proton resonance. The methyl protons resonated at ~0.7 ppm, 0.2 ppm less than what Wiese et al. (1991) reported for the same protons . This is most likely explained by the temperature difference in the two studies. Wiese acquired data at ambient temperature, whereas our study was conducted at 300 K (27 °C). Based on the model of Fig. 2, the C17–C19 methylene protons, penetrating farther into the toroidal cavity of α-CD, would be expected to exhibit a greater chemical shift than the C20 protons. However, each of the methylene protons resides in a slightly different chemical environment. Also, their resonances overlap partially with CD protons inside the cavity. Hence, analyzing the methylene protons is not accomplished without difficulty. Therefore, we selected the C20 methyl protons for measurement of chemical shift. The methyl protons were among the several measured by Wiese et al. (1991), whereas Steffan measured the adjacent methylene protons only (Steffan et al., 1992).

The data of Table 1 were applied to the SigmaPlot worksheet and then worked up as shown in Table 2. In order to solve Eq. (8) exactly, an expression for [CD] must be invoked, since only CD$_t$ and not free CD is known to the experimenter. [CD] may be calculated

Table 1
δ_{obs}, Δ, and f_b for serial dilutions of a stock solution of PGE$_1$/α-CD

PGE$_1$ (μg/ml) (=E_t)[a]	α-CD (mg/ml) (=CD$_t$)[b]	δ (Hz)	$\Delta = \delta - \delta_S$	$f_b = \Delta/\Delta_{\max} = \Delta/45.19$
195.7	6.49	407.21	37.89	0.838
97.9	3.25	404.26	34.94	0.773
39.1	1.30	394.58	25.26	0.559
19.6	0.65	386.58	17.26	0.382
9.79	0.32	379.84	10.52	0.233
3.91	0.13	373.95	4.63	0.102
0.979	0.033	369.74	0.42	0.009
0	0	369.32	0	0

[a] E_t, total PGE$_1$ concentration.

[b] CD$_t$, total α-CD concentration.

Table 2
SigmaPlot worksheet used to generate the binding curve of Fig. 3

E_t (μg/ml)	E_t (M)	CD_t (mg/ml)	CD_t (M)	Shift (Hz)	Δ (Hz)
Saturated solution		0.000	0.000	369.32	0.00
0.9785	2.76×10^{-6}	0.03246	3.34×10^{-5}	369.74	0.42
3.9141	1.1×10^{-5}	0.1298	1.33×10^{-4}	373.95	4.63
9.7850	2.76×10^{-5}	0.3246	3.34×10^{-4}	379.84	10.52
19.57	5.52×10^{-5}	0.6492	6.67×10^{-4}	386.58	17.26
39.14	1.1×10^{-4}	1.298	1.334×10^{-3}	394.58	25.26
97.85	2.76×10^{-4}	3.246	3.337×10^{-3}	404.26	34.94
195.7	5.52×10^{-4}	6.492	6.673×10^{-3}	407.21	37.89

Note: (1) col(2) = 1×10^{-3}[col(1)/354.49]; (2) col(4) = col(3)/972.85; (3) col(6) = col(5) − 369.32.

from CD_t through use of the expression

$$[CD] = CD_t - E_t\left(\frac{\Delta}{\Delta_{max}}\right) \tag{9}$$

The binding curve obtained is shown in Fig. 3. The parameter values obtained from the curve fitting analysis are $\Delta_{max} = 45.19$ Hz and $K_{11} = 966 \pm 130\ M^{-1}$ (at 27 °C). The associated error refers to 2σ about the mean.

Through the application of Eq. (7) ($f_b = \Delta/\Delta_{max}$), where Δ_{max} was determined to be 45.19 Hz, we may calculate f_b corresponding to any Δ. Doing this for the values of Δ shown in Table 1, we obtained the values of f_b shown in the last column of the table. The corresponding plot is shown in Fig. 4. The plot in Fig. 4 may be used to define the fraction bound, f_b, corresponding to the measured Δ for any concentration of PGE_1 and α-CD.

3.1. Calculation of percent PGE_1 bound

The ultimate goal of this work was to assure that, upon reconstitution, PGE_1 is quantitatively delivered to the patient. With the binding constant, $K_{11,}$ in hand, the free PGE_1 may be calculated for any combination

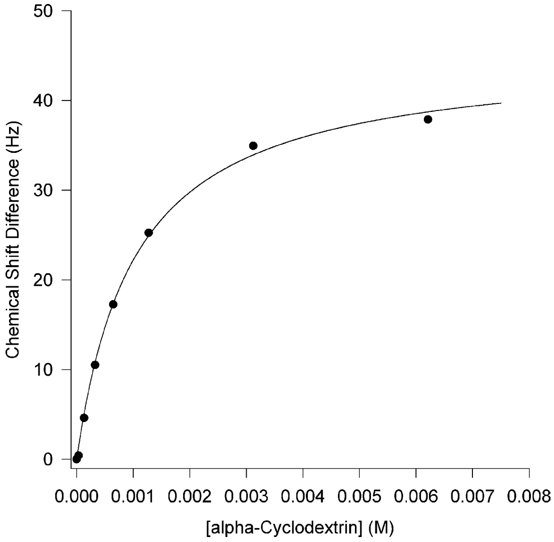

Fig. 3. Binding curve generated from the data in Tables 1 and 2. The filled circles correspond to the experimental data points; the smooth curve is the best (spline-smoothed) fit based on Eqs. (8) and (9).

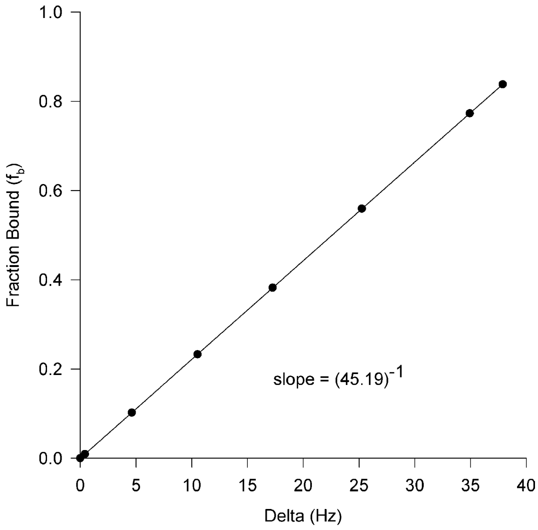

Fig. 4. Plot of fraction bound, f_b vs. Δ for the data of Tables 1 and 2, and the binding curve of Fig. 3.

of $(\mathrm{PGE}_1)_\mathrm{t}$ and α-CD_t using the equation

$$E = \frac{-(K_{11}\mathrm{CD}_\mathrm{t} - K_{11}E_\mathrm{t} + 1) + \sqrt{(K_{11}\mathrm{CD}_\mathrm{t} - K_{11}E_\mathrm{t} + 1)^2 + 4K_{11}E_\mathrm{t}}}{2K_{11}} \tag{10}$$

where E = free PGE_1. This equation can be derived using an analysis similar to that used above to arrive at the binding isotherm, Eq. (8).

The fraction free is then calculated from $f_\mathrm{f} = E/E_\mathrm{t}$. For the 10 μg strength (20 μg/ml) of Caverject DC, with $K_{11} = 966\,\mathrm{M}^{-1}$, 62% of the PGE_1 is calculated to be free in the syringe upon reconstitution. For the 20 μg (40 μg/ml) strength, the corresponding figure is 45%. Assuming an intercavernosal blood volume of 10 ml in the flaccid state (Schwartz et al., 1989), the percent PGE_1 free after injection becomes 97% for the 10 μg strength and 94% for the 20 μg strength. If a blood volume of 50 ml is assumed after tumescence (Schwartz et al., 1989), these values increase to 99.4 and 98.7%, respectively. Hence, the alprostadil is seen to be almost entirely free. Interestingly, over a considerable range for the binding constant (700–1200 M^{-1}), its magnitude has only a slight effect on the amount free immediately after injection, and virtually none after tumescence is achieved.

In practice Caverject DC is reconstituted with BWFI, which contains 0.945% (w/v) benzyl alcohol. The experiment described above did not include benzyl alcohol in the measured solutions. From models, we would expect benzyl alcohol to fit inside the cavity of α-CD (Szejtli, 1988; Frömming and Szejtli, 1994b). The actual amount of PGE_1 free in the presence of benzyl alcohol will then be somewhat higher than that calculated above due to competition for α-CD between PGE_1 and benzyl alcohol. We confirmed this by generating a binding curve for PGE_1 in the presence of 0.945% benzyl alcohol; the curve is shown in Fig. 5. The worksheet, analogous to Table 2 for the aqueous case above, is given in Table 3. A value of $K_{11} = 393 \pm 59\,\mathrm{M}^{-1}$ was extracted by regression, this compared with $K_{11} = 966 \pm 130\,\mathrm{M}^{-1}$ obtained in the absence of benzyl alcohol. In contrast to the experiment above where the ratio of PGE_1 to α-CD was maintained constant through serial dilutions of a stock solution, the binding isotherm with benzyl alcohol was generated with the concentration of PGE_1 maintained constant (at about 26 μg/ml), while the α-CD concentration was varied over the range ∼0–17 mg/ml. Interestingly, although the presence of benzyl alcohol significantly affects the amount free upon reconstitution, its subsequent effect after injection is greatly diminished due to dilution through the penile vasculature. In actuality, the amount free is higher than calculated, as the analysis above only takes into account the effect of dilution. Competitive displacement by endogenous lipophilic materials that are more strongly bound than alprostadil, e.g., lipidic components of the cell membrane (Ohtani et al., 1989), binding to plasma and tissue proteins, and preferential uptake of the drug in tissue may also play a role in release of the drug from the drug–CD complex (Stella et al., 1999). These contributions become increasingly important the stronger the complexation between drug and cyclodextrin. Where complexation is relatively weak, as for binding constants under about 1000 M^{-1}, as here, dilution is thought to play the dominant role in the release of drug (Stella et al., 1999). An additional point, not part of the analysis above, is that

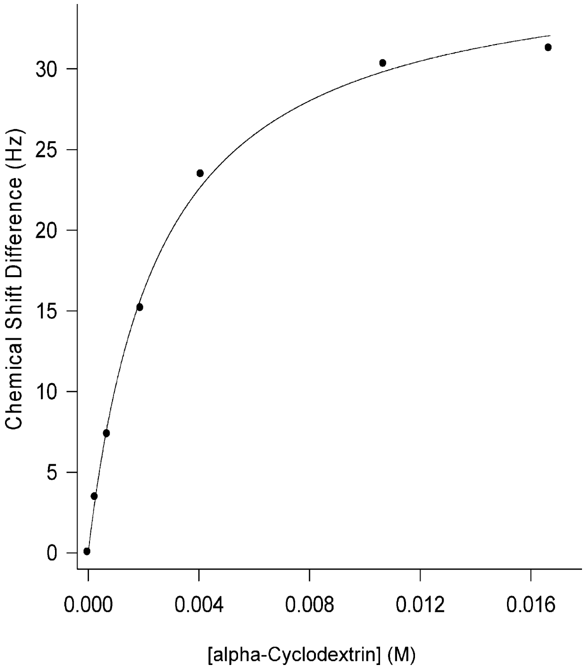

Fig. 5. Binding curve in the presence of 0.945% benzyl alcohol generated from the data in Table 3.

Table 3
SigmaPlot worksheet used to generate the binding curve of Fig. 5 for 0.945% benzyl alcohol

E_t (μg/ml)	E_t (M)	CD_t (mg/ml)	CD_t (M)	Shift (Hz)	Δ (Hz)
26.32	7.414×10^{-5}	0.000	0.000	368.02	0.00
26.32	7.414×10^{-5}	0.2588	2.660×10^{-4}	371.44	3.42
26.32	7.414×10^{-5}	0.6964	7.158×10^{-4}	375.35	7.33
26.32	7.414×10^{-5}	1.884	1.937×10^{-3}	383.16	15.14
26.32	7.414×10^{-5}	4.024	4.136×10^{-3}	391.46	23.44
26.32	7.414×10^{-5}	10.455	1.07×10^{-2}	398.30	30.28
26.32	7.414×10^{-5}	16.279	1.67×10^{-2}	399.27	31.25

Note: (1) col(2) = 1×10^{-3}[col(1)/354.49]; (2) col(4) = col(3)/972.85; (3) col(6) = col(5) − 368.02.

the extent of binding is reduced at elevated (physiological) temperature (37 °C).

4. Discussion

In comparing our regression results to published values, Wiese's work makes for the most relevant comparison since they also measured the chemical shift of the methyl protons. Depending on how the calculation was made, values for K_{11} between 957 and 964 M^{-1} were obtained. Wiese additionally applied the analysis to non-methyl protons. Although one might expect the value of K_{11} to be independent of the proton measured, slightly different values were obtained for different protons. When Wiese combined the results for all protons, he reported an average value of 917 M^{-1}. Steffan, by contrast, measured the chemical shift of methylene protons on the C17–C19 carbon atoms. Although he did not report a value for the binding constant, we were able to calculate K_{11} from his data. Depending upon how the data were processed, values ranging from 805 to 825 M^{-1} were obtained. While Wiese's data exhibited considerable scatter, Steffan obtained a good fit between the experimental data and the regression analysis.

The goodness of the fit in Fig. 3 strongly supports the assumed 1:1 stoichiometry. Generally, a good fit for a curve that sweeps out 75% or more of the full binding range provides strong evidence for the validity of the assumed model (Connors, 1987). In our case, nearly 85% was swept out. An excellent fit was similarly obtained for the binding curve in Fig. 5, generated in the presence of 0.945% benzyl alcohol.

The mathematical analysis made (Eq. (10)) once the binding constant was in hand allowed worst-case conclusions to be made regarding the amount of biologically active (i.e., free) PGE_1 present. It is worst-case, since it does not take into account any physiological processes, which would decrease the amount bound. Having made the calculations, we were able to predict efficacy relative to the earlier developed Caverject sterile powder for injection (vide supra), which contains no α-cyclodextrin. This greatly compressed the time required to bring Caverject sterile powder dual chamber syringe to market, as only an abbreviated clinical program was then necessary.

5. Conclusions

A binding constant was determined by NMR spectroscopy for the molecular complexation reaction between PGE_1 and α-CD. A value of 966 ± 130 M^{-1} (2*s*) was obtained. Our value agrees well with those reported in the literature. With the binding constant known, we were able to estimate the fraction PGE_1 free upon reconstitution and after subsequent injection with its attendant dilution by the cavernosal blood supply. For 10 μg Caverject DC (20 μg/ml), we calculated approximately 97% to be free immediately after injection (i.e., in the flaccid state), and greater than 99% after tumescence. The corresponding values for 20 μg Caverject DC (40 μg/ml) are 94 and nearly 99%. These are conservative estimates, based only on the effect of dilution. They do not take into account the effect of benzyl alcohol preservative, the effect of endogenous constituents competing for the cyclodextrin cavity, of any plasma or tissue protein binding, or of uptake of drug into surrounding tissue. All these factors will increase the percentage of unbound PGE_1. Additionally, physiological temperature (37 °C), as opposed to the

27 °C used here, will contribute to a higher percentage of PGE_1 free.

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References

Connors, K.A., 1987. Binding constants. In: The Measurement of Molecular Complex Stability. John Wiley & Sons.

Connors, K.A., 1997. The stability of cyclodextrin complexes in solution. Chem. Rev. 97, 1325–1357.

Djedaini, F., Lin, S.-Z., Perly, B., Wouessidjewe, D., 1990. High-field nuclear magnetic resonance techniques for the investigation of a β-cyclodextrin:indomethacin inclusion complex. J. Pharm. Sci. 79, 643–646.

Frömming, K.-H., Szejtli, J., 1994a. Cyclodextrins in Pharmacy. Klumer Academic, Dordrecht, The Netherlands, p. 77.

Frömming, K.-H., Szejtli, J., 1994b. Cyclodextrins in Pharmacy. Klumer Academic, Dordrecht, The Netherlands, p. 48.

Fronza, G., Mele, A., Redenti, E., Ventura, P., 1992. Proton nuclear magnetic resonance spectroscopy studies of the inclusion complex of piroxicam and β-cyclodextrin. J. Pharm. Sci. 81, 1162–1165.

Funasaki, N., Nomura, M., Ishikawa, S., Neya, S., 2001. NMR chemical shift references for binding constant determination in aqueous solutions. J. Phys. Chem. B 105, 7361–7365.

Funasaki, N., Ishikawa, S., Neya, S., 2002. NMR chemical shift methods for binding constant determination of an organic anion and α-cyclodextrin. Bull. Chem. Soc. Jpn. 75, 719–723.

Funasaki, N., Ishikawa, S., Neya, S., 2003. Proton NMR study of α-cyclodextrin inclusion of short-chain surfactants. J. Phys. Chem. B 107, 10094–10099.

Inoue, Y., 1993. NMR studies of the structure and properties of cyclodextrins and their inclusion complexes. In: Webb, G.A. (Ed.), Annual Reports on NMR Spectroscopy, vol. 27. Academic Press.

Liu, Y., Han, B.-H., Zhang, H.-Y., 2004. Spectroscopic studies on molecular recognition of modified cyclodextrins. Curr. Org. Chem. 8, 35–46.

Ohtani, Y., Irie, T., Uekama, K., Fukunaga, K., Pitha, J., 1989. Differential effects of α-, β- and γ-cyclodextrins on human erythrocytes. Eur. J. Biochem. 186, 17–22.

Schneider, H.-J., Hacket, F., Rüdiger, V., 1998. NMR studies of cyclodextrins and cyclodextrin complexes. Chem. Rev. 98, 1755–1785.

Schwartz, A.N., Graham, M.M., Ferency, G.F., Miura, R.S., 1989. Radioisotope penile plethysmography: a technique for evaluating corpora cavernosal blood flow during early tumescence. J. Nucl. Med. 30, 466–473.

Steffan, B., Fischer, W., Cordes, G., Habon, I., Müller, R., 1992. ^{1}H-nuclear magnetic resonance (NMR) studies on the inclusion complex of prostaglandin E_1 (PGE_1) with α- cyclodextrin. Pharm. Res. 9, 575–577.

Stella, V.J., Rao, V.M., Zannou, E.A., Zia, V., 1999. Mechanisms of drug release from cyclodextrin complexes. Adv. Drug Deliv. Rev. 36, 3–16.

Szejtli, J., 1988. Cyclodextrin Technology. Kluwer Academic Publishers, Dodrecht, The Netherlands, pp. 287–288.

Teagarden, D.L., Baker, D.S., 2002. Practical aspects of lyophilization using non-aqueous co-solvent systems. Eur. J. Pharm. Sci. 15, 115–133.

Thorsteinn, L., Brewster, M.E., 1996. Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. J. Pharm. Sci. 85, 1017–1025.

Uekama, K., Otagiri, M., 1987. Critical Reviews in Therapeutic Drug Carrier Systems, vol. 3. CRC Press, Boca Raton, FL, p. 7.

Uekama, K., Otagiri, M., Kanie, Y., Tanaka, S., Ikeda, K., 1975. Inclusion complexes of cinnamic acids with cyclodextrins. Mode of inclusion in aqueous solution. Chem. Pharm. Bull. 23, 1421–1430.

Wiese, M., Cordes, H.-P., Chi, H., Seydel, J.K., Bachensfeld, T., Müller, B.W., 1991. Interaction of prostaglandin E_1 with α-cyclodextrin in aqueous systems: stability of the inclusion complex. J. Pharm. Sci. 80, 153–156.

Yamamoto, Y., Inoue, Y., 1989. NMR studies of cyclodextrin inclusion complex. J. Carbohydr. Chem. 8, 29–46.