Effect of pH on binding of agonists and antagonists to rat heart muscarinic receptors

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The pH-dependence of antagonist and agonist binding to rat heart muscarinic receptors was investigated at 25°C, in the absence and in the presence of GTP. The small inhibitory effect observed at the lowest pH investigated (pH 6.0) on [*N*-methyl-³H]-methscopolamine chloride and [*methyl*-³H]oxotremorine-M acetate binding indicated that one or more amino acid residues of the receptor had to be deprotonated for optimal binding affinity. The low p*K* value of these residues (between 5 and 6) prevented their identification. The binding of scopolamine (p*K* 7.6) was favoured by a positive charge in the titratable amine, but binding with a lower affinity remained possible without this charge. GTP did not affect antagonist binding at any pH, but converted more than 90% of agonist binding sites into a low affinity conformation. In the absence of GTP, we observed a time- and pH-dependent conversion of the super-high- and high-affinity receptors to a low-affinity GTP-insensitive state. This conversion was markedly accelerated at high pH (above pH 8.0). In the presence of GTP, a positive charge on the titratable amine of pilocarpine (p*K* 7.05) and oxotremorine (p*K* 8.60) was required for binding. These results support the view that antagonist (e.g. methscopolamine) binding to receptors was largely facilitated by hydrophobic interactions, whereas agonist binding to low-affinity sites was mainly driven by ionic interactions.

Competition curves for rat heart muscarinic cholinergic receptors yield Hill coefficients lower than 1 for agonists (Cavey *et al.*, 1977; Fields *et al.*, 1978; Berrie *et al.*, 1979; Wei & Sulakhe, 1979) but equal to 1 for antagonists. We have previously shown that competition curves obtained with a rat heart membrane preparation are compatible with the presence of only two classes of receptors (Waelbroeck *et al.*, 1982), having respectively 'super-high' (*K_D* 1–10 nM) and 'high' (*K_D* 10–100 nM) affinity for agonists (according to the classification of Birdsall *et al.*, 1978). These two classes of muscarinic receptors display, however, the same low affinity (*K_D* 1–10 μM) for agonists when exposed to GTP (Waelbroeck *et al.*, 1982). Recent data (M. Waelbroeck, P. Robberecht, P. Chatelain, P. De Neef & J. Christophe, unpublished work) indicate that agonists form very few hydrophobic interactions with muscarinic receptors in the presence of GTP (the driving force of the reaction being an ionic

interaction), whereas antagonist binding involves both ionic and hydrophobic interactions.

One of us (Waelbroeck, 1982) has proposed a model to study the effect of pH on a hormone–receptor interaction, assuming that binding is possible only if all the titratable 'active groups' of the ligand and receptor are in the correct ionic state, and unaffected by the charge of all other residues. This model was used in the present study to test the hypothesis that ionic bonds are, indeed, absolutely necessary for binding. To achieve this aim, we compared the effect of pH (6.0–9.0) on binding to rat heart membranes of muscarinic agonists and antagonists that were either titratable in this pH range, i.e. pilocarpine (p*K* 7.1), oxotremorine (p*K* 8.6) and scopolamine (p*K* 7.6), or non-titratable, i.e. atropine (p*K* 9.9) (Jencks & Regenstein, 1976), [³H]NMS, [³H]Oxo-M and carbamoylcholine.

Materials and methods

Chemicals

[³H]NMS (sp. radioactivity 54 Ci/mmol) and [³H]Oxo-M (sp. radioactivity 84 Ci/mmol) were

Abbreviations used: [³H]NMS, [*N*-methyl-³H]methscopolamine chloride; [³H]Oxo-M, [*methyl*-³H]oxotremorine-M acetate.

obtained from New England Nuclear Corporation (Dreieich, Germany). Oxotremorine sesquifumarate was obtained from Aldrich Chemical Co. (Beerse, Belgium), atropine and scopolamine from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and carbamoylcholine from Federa (Brussels, Belgium). GTP was purchased from Boehringer (Mannheim, Germany).

Methods

Preparation of cardiac membranes. Rat cardiac membranes were prepared by the method of Snyder & Drummond (1978) with a few modifications. Wistar albino rats, fed *ad libitum* on standard chow, were decapitated. The hearts were quickly removed, rinsed at room temperature with iso-osmotic NaCl and stored in liquid N₂. All subsequent operations were performed at 4°C. Each heart was homogenized with a glass/Teflon homogenizer in 5 ml of 20 mM-Tris/HCl (pH 7.5) enriched with 2 mM-dithiothreitol and 5 mM-MgCl₂. After a 2-fold dilution in the same buffer, the homogenate was filtered through two layers of medical gauze and centrifuged at 520 g for 10 min. The membrane pellet was washed once with 10 ml of homogenization buffer. The resulting pellet was resuspended in 5 ml of 20 mM-Tris/HCl buffer (pH 7.5) containing 0.25 M-sucrose and 5 mM-MgCl₂. An equal volume of 20 mM-Tris/HCl buffer (pH 7.5) enriched with 0.25 M-sucrose, 2 mM-dithiothreitol and 2.5 M-KCl was added dropwise. The final mixture was left for 1 h in an ice-bath, under continuous stirring, and then centrifuged at 37 000 g for 10 min. The resulting pellet was washed three times in 10 ml of 20 mM-Tris/HCl buffer containing 0.25 M-sucrose and 2 mM-dithiothreitol. The membranes were finally resuspended in the last buffer, at a final concentration of approx. 6 mg of protein/ml and stored in liquid N₂. Proteins were determined by the method of Lowry *et al.* (1951).

Association kinetics. Membrane proteins (0.1 mg/ml) were incubated for the indicated periods of time at pH 6.0, 7.4 or 9.0, in 1.2 ml of 50 mM-sodium phosphate buffer, enriched with 2 mM-MgCl₂ and 1% bovine serum albumin, and 0.08 nM-[³H]NMS or 0.08 nM-[³H]Oxo-M, in the presence or in the absence of 1 μM-atropine.

The samples were then filtered on glass-fibre filters (GF/C; Whatman, Maidstone, Kent, U.K.) and rinsed four times with 2 ml of ice-cold 20 mM-Tris/HCl (pH 7.5) enriched with 0.25 M-sucrose, 2 mM-dithiothreitol and 1% bovine serum albumin. The filters were dried and the radioactivity was counted by liquid scintillation. Non-specific binding (mainly due to interaction with the filters) was defined as tracer binding in the presence of 1 μM-atropine. It represented less than 1% of the total tracer concentration and was not affected by pH. It

was subtracted from total binding, yielding specific binding.

In some experiments, membrane protein or tracer was omitted for a pre-incubation period of 60 min at 25°C, then added at time zero, and the samples were further incubated for the indicated periods of time. The pH of the incubation buffer did not vary during the incubation.

Dissociation kinetics. Membrane proteins (0.1 mg/ml) were pre-incubated for 45 min at pH 6.0, 7.0, 7.4, 8.0 or 9.0, in 1.2 ml of sodium phosphate buffer, enriched with 2 mM-MgCl₂, 1% bovine serum albumin, 0.5 nM-[³H]NMS, in the presence or in the absence of atropine. Atropine (12 μl; 0.1 mM; isotopic dilution) or water (control) were then added to each tube at time zero. Specific binding was measured at appropriate time intervals as indicated above. Results were expressed as percentages of specific binding after addition of water (the control).

Scatchard analysis of [³H]NMS and [³H]Oxo-M binding. Membrane proteins (0.1 mg/ml) were incubated for either 45 min (with [³H]NMS) or 10 min (with [³H]Oxo-M) at pH 6.0, 7.0, 7.4, 8.0 or 9.0, in 1.2 ml of 50 mM-sodium phosphate buffer enriched with 2 mM-MgCl₂, 1% bovine serum albumin, increasing concentrations of [³H]NMS (0.08 to 1.6 nM) or [³H]Oxo-M (0.08 to 1.6 nM), and in the presence or in the absence of 1 μM-atropine. Specific binding (less than 10% of total tracer concentration) was measured as indicated above. The 'free' concentration was calculated as total concentration minus specific binding. The results were analysed by the method of Scatchard (1949). Linear regression analysis of the results yielded regression coefficients ($r^2 > 0.9$ for [³H]NMS and $r^2 > 0.8$ for [³H]Oxo-M).

[³H]NMS competition curves. Membrane proteins (0.1 mg/ml) were incubated in 1.2 ml of 50 mM-sodium phosphate buffer (pH 7.4) enriched with 2 mM-MgCl₂, 1% bovine serum albumin, 0.5 nM-[³H]NMS and various concentrations of agonist or antagonist for 45 min at pH 6.0, 7.0, 7.4, 8.0 or 9.0. Specific binding was measured by filtration, as indicated above. The free tracer concentration did not change significantly with pH.

Results

For the sake of clarity, data obtained at pH 7.0 and 8.0 are not shown in all Figures, but are represented in Figs. 4 and 9, and in Table 1.

Antagonist binding

Association kinetics. The association rate of [³H]NMS was markedly lower at the lower pH values (Fig. 1). Binding was stable at all pH values investigated, suggesting that no degradation of the

Fig. 1. Time course of [^3H]NMS (0.08 nM) binding to rat heart membranes at 25°C

Specific [^3H]NMS binding was measured as a function of time at pH 6.0 (●), 7.4 (○) or 9.0 (■). The experiment shown is representative of three experiments.

tracer or receptors had occurred. An incubation period of 60 min, sufficient to reach steady state at all pH values investigated, was chosen for all equilibrium binding studies (see below).

Dissociation kinetics. The dissociation of bound [^3H]NMS was consistent with a first-order reaction mechanism at all pH values investigated. The dissociation rate constant [$(5.5 \pm 1.9) \times 10^{-3} \text{ s}^{-1}$] was slightly (but not significantly) increased at high pH (Fig. 2; other results not shown).

Equilibrium binding studies. The dissociation constants of atropine and scopolamine were measured by competition curves, and that of [^3H]NMS by Scatchard plot analysis.

As shown on Fig. 3(a), atropine binding (in the presence or in the absence of GTP) was not affected by the pH of the incubation buffer, from pH 6.0 to 9.0. In contrast, scopolamine binding (Fig. 3b) was markedly inhibited at higher pH values. A small but significant decrease of the affinity of [^3H]NMS was found at pH 6.0 (Fig. 4; Table 1).

The experimental results were compared with theoretical curves (continuous lines) obtained by the method of Waelbroeck (1982), assuming that binding was possible only when one group of the receptor (with a pK value of 5.5) was deprotonated, and one group of the drug (with a pK of 9.9 for atropine, and a pK of 7.6 for scopolamine) (Jencks & Regenstein, 1976) was protonated (Fig. 4). Scopolamine binding at pH 9.0 was greater than expected from this model, indicating a significant affinity of the deprotonated form of this antagonist for the receptor. To take this into account we adapted our model by assuming that one protonated

Fig. 2. Time course of dissociation of [^3H]NMS (0.5 nM) from rat heart membranes at 25°C

Membrane proteins (0.1 mg) were pre-incubated at pH 6.0 (●) or 9.0 (■) in the presence of 0.08 nM [^3H]NMS for 45 min. Atropine (1 μM final concentration) was then added, and specific binding was measured at the indicated time intervals after atropine addition. The experiment shown is representative of three others.

group (of unknown pK) increased the affinity of scopolamine for the muscarinic receptor (see the Appendix). Fitting this new model to the experimental results indicated that a titratable residue of $pK 8.2 \pm 0.6$ (not significantly different from the pK of scopolamine) increased the affinity of this

Fig. 3. Effect of pH on dose-effect curves of inhibition of [³H]NMS binding to rat heart membranes by two muscarinic antagonists: atropine (a) and scopolamine (b)

Specific [³H]NMS binding was measured at the indicated pH values, in the absence or presence of 10 μM-GTP, and in the absence or in the presence of the indicated concentrations of atropine (a) or scopolamine (b). The results were expressed as percentages of maximal binding in the absence of unlabelled antagonist. The experiment shown is representative of three experiments.

antagonist for the receptor (6 ± 3)-fold when protonated.

Agonist binding

We have previously shown that [³H]Oxo-M recognizes only the super-high-affinity site under our incubation conditions (Waelbroeck *et al.*, 1982).

Association kinetics. [³H]Oxo-M binding was maximal after 10 min of incubation at all pH values investigated, then decreased with increasing in-

Fig. 4. Effect of pH on the binding of three muscarinic antagonists to rat heart membranes

The logarithm of the K_D values of [³H]NMS (■ and □), atropine (▲ and △) and scopolamine (● and ○) in the absence (filled symbols) or presence (open symbols) of 10 μM-GTP was plotted as a function of pH. The theoretical curves (continuous lines) were drawn as indicated in the text. The standard error of the mean was below 5% of the average K_D values for [³H]NMS (*n* = 5) and 10% of the average K_D values for scopolamine and atropine (*n* = 3).

Table 1. Effect of pH on the K_D values of [3H]NMS and total muscarinic receptor concentration of rat heart membranes, in the presence or in the absence of $10\mu M$ -GTP
Results are means $\pm$ S.E.M. of three to six experiments.

pH	Without GTP		With GTP	
	K_D (nM)	Total receptor concentration (fmol/mg of protein)	K_D (nM)	Total receptor concentration (fmol/mg of protein)
6.0	1.41 ± 0.18	804 ± 113	1.20 ± 0.23	857 ± 152
7.0	1.19 ± 0.15	862 ± 156	1.01 ± 0.08	887 ± 144
7.4	1.16 ± 0.23	865 ± 133	1.03 ± 0.07	856 ± 105
8.0	0.97 ± 0.15	826 ± 153	0.92 ± 0.12	788 ± 181
9.0	0.97 ± 0.18	851 ± 176	0.90 ± 0.08	778 ± 132

Fig. 5. Time course of [3H]Oxo-M (0.08 nM) binding at pH 6.0 or 9.0 to rat heart membranes that were pre-incubated (b) (or not; a) in the absence of ligand but at the same pH and the same temperature ($25^\circ C$)

(a) Specific [3H]Oxo-M binding was measured as a function of time at pH 6.0 (●) or 9.0 (■). The results were not affected by a 30 min pre-incubation of the tracer at the same pH values, i.e. 6.0 (○) and 9.0 (□) respectively. (b) [3H]Oxo-M was added after a 30 min pre-incubation of the membranes, at pH 6.0 (●) or 9.0 (○). Specific binding was then measured as a function of time. The experiment shown is representative of three others.

heart membranes (Fig. 5a). By contrast, pre-incubation of the membranes before addition of tracer led to a markedly lower binding (Fig. 5b), supporting the hypothesis that super-high-affinity receptors were inactivated.

Equilibrium binding studies. This was further documented by comparing [3H]Oxo-M and [3H]NMS Scatchard plots before and after a 30 min pre-incubation period at various pH values. As shown in Tables 1 and 2, the total receptor concentration (measured by [3H]NMS binding) did not vary with pH or after pre-incubation of heart membranes. The concentration of super-high-affinity receptors measured by [3H]Oxo-M binding) was, however, lower at pH 9.0 than at pH 6.0 and decreased markedly after a pre-incubation at pH 9.0 (Table 2).

Competition curves, after a 60 min pre-incubation period at high pH values, indicated that both classes of muscarinic receptors were converted into a low-affinity state, similar to that observed in the presence of GTP (not shown). We did not attempt, therefore, to analyse the effect of pH on the K_D of agonists for super-high- and high-affinity receptors, but limited our studies to the pH-dependence of muscarinic agonist binding to the low-affinity state, in the presence of GTP. The results (Figs. 6–8) were analysed by the method of Waelbroeck (1982) and are summarized in Fig. 9. It appears that the binding of carbamoylcholine, oxotremorine and pilocarpine to rat heart muscarinic receptors was: (1) unaffected by the ionization of receptor groups with pK values varying from 6.0 to 9.0; and (2) strongly dependent on a positively charged group in the agonists oxotremorine (pK 8.6) and pilocarpine (pK 7.1) (Jencks & Regenstein, 1976).

Discussion

The effect of pH on muscarinic antagonist and agonist binding to rat heart membranes was in-

cubation times, suggesting that the super-high-affinity receptors, the tracer, or both, were inactivated under these conditions (Fig. 5). This inactivation was more rapid at high pH values. Pre-incubation of the tracer under identical conditions did not affect its subsequent binding to rat

Table 2. Effects of pH on K_D values of [3 H]NMS and [3 H]Oxo-M and on the corresponding concentrations of total muscarinic receptors (for [3 H]NMS) and 'super-high-affinity' receptors (for [3 H]Oxo-M) in rat heart membranes pre-incubated (or not) for 30 min, at pH 6.0 or pH 9.0
The results are representative of four experiments.

K_D (nM) Receptor concentration (fmol/mg of protein)	Ligand ...		[3 H]NMS				[3 H]Oxo-M			
	Preincubation ...									
	pH ...		—		+		—		+	
			6	9	6	9	6	9	6	9
			1.52	0.90	1.70	0.72	2.3	1.5	2.9	1.7
			503	633	567	604	259	159	217	66

Fig. 6. Influence of pH on dose-effect curves of inhibition of [3 H]NMS binding to rat heart membranes by the agonist carbamoylcholine

Specific [3 H]NMS binding was measured at the indicated pH values, in the absence (top) or in the presence (bottom) of 10 μ M-GTP, and in absence or in the presence of the indicated concentrations of carbamoylcholine. The results were expressed as percentages of maximal binding in the absence of unlabelled agonist. The experiment shown is representative of four experiments.

Fig. 7. Influence of pH on dose-effect curve of inhibition of [3 H]NMS binding to rat heart membranes by the agonist pilocarpine

Specific [3 H]NMS binding was measured at the indicated pH values, in the absence (top) or presence (bottom) of 10 μ M-GTP, and in the absence or in the presence of the indicated concentrations of pilocarpine. The results were expressed as in Fig. 6. The experiment shown is representative of four experiments.

vestigated at 25°C. The total number of receptors (measured by [3 H]NMS binding) did not vary in the pH range studied (Table 1). The affinity of [3 H]NMS for the receptors was slightly, but significantly, decreased at pH 6.0 (Fig. 4), indicating that the presence of one or more deprotonated active residue(s) in the receptor was necessary for binding. This effect was, however, observed at pH values too

low to allow an accurate measurement of the pK value of the active group(s), so that their nature, number and role (ionic interaction with the drug or structural?) remain to be determined.
The K_D of atropine (pK_{9.9}) (Jencks & Regenstein, 1976) measured by competition curves was not affected by pH in the pH range studied (Fig. 4).
For scopolamine (pK_{7.6}; Jencks & Regenstein, 1976), the affinity decreased with increasing pH,

Fig. 8. Influence of pH on dose-effect curve of inhibition of $[^3\text{H}]\text{NMS}$ binding to rat heart membrane by the agonist oxotremorine

Specific $[^3\text{H}]\text{NMS}$ binding was measured at the indicated pH values, in the absence (top) or in the presence (bottom) of $10\ \mu\text{M}$ -GTP, and in the absence or in the presence of the indicated concentrations of oxotremorine. The results were expressed as in Fig. 6. The experiment shown is representative of four experiments.

suggesting that the positively charged drug had a greater affinity than the uncharged, deprotonated molecule. The experimental results deviated significantly, however, from the theoretical curve (Fig. 4), drawn assuming that deprotonated scopolamine was unable to recognize the receptor. This indicates that the affinity of deprotonated scopolamine for the receptor was not negligible. We then calculated the K_D of deprotonated scopolamine (assuming that protonation of the molecule merely increased its affinity for muscarinic receptors) and obtained a value only 6-fold higher than the K_D of protonated scopolamine.

These results support the view that ionic bond formation was not the principal driving force for antagonist binding to the receptors (M. Waelbroeck, P. Robberecht, P. Chatelain, P. De Neef & J. Christophe, unpublished work). It should be borne in mind, however, that the most favourable conformation of deprotonated and protonated scopolamine may be quite different in solution, and markedly affects the relative values of the observed K_D , so that the differences in binding free energy of deprotonated and protonated scopolamine may not reflect the free energy change due to ionic bond formation.

Fig. 9. Effect of pH on the binding of three muscarinic agonists to rat heart membranes in the presence of GTP

The logarithm of the K_D values of the unlabelled agonists for the low-affinity state of the receptors (in the presence of $10\ \mu\text{M}$ -GTP) was plotted as a function of pH. Symbols: $\blacktriangle$, oxotremorine; $\square$, pilocarpine; $\bullet$, carbamoylcholine. The theoretical curves (continuous lines) were drawn as indicated in the text. Results are means of four experiments. The standard error of the mean was below 5% of the average K_D values of the unlabelled agonists at all pH values.

We were surprised to detect a small, but significant, increase of the EC_{50} value (concentration decreasing tracer binding by 50%) of carbamoylcholine at high pH values (Fig. 6). This, and the abnormal binding kinetics of $[^3\text{H}]\text{Oxo-M}$ (Fig. 5), led us to suspect that the super-high- (and possibly high-) affinity receptors were not stable at the highest pH value investigated, but transformed to a lower-affinity state. Pre-incubation of the membranes for 30 or 60 min at pH 9.0 before the binding experiment allowed us to confirm that both classes of agonist receptors were progressively transformed into a low-affinity GTP-insensitive state (Fig. 5; Tables 1

and 2). This 'conversion' was markedly accelerated at the highest pH values investigated. The precise mechanism of this conversion is not known. The fact that a similar conversion is observed in mouse brain (Gurwitz & Sokolovsky, 1980) and rat heart (M. Waelbroeck, unpublished work) after heat treatment (5 min pre-incubation at 50°C) and in rat brain and ileum (Matsumoto *et al.*, 1982) after partial tryptic digestion suggests, however, that inactivation of a GTP-modulated protein, associated with the receptors in the high- and super-high-affinity states, might be responsible for this effect.

In the presence of GTP, carbamoylcholine binding was not affected in the pH range 6.0–9.0 (Fig. 9), suggesting that any effect of pH on pilocarpine and oxotremorine binding to the receptor would be due to protonation or deprotonation of the drug. Under these conditions, the effect of pH on the binding of the two titratable agonists studied to the receptors was compatible with the hypothesis that the uncharged drugs do not bind.

These results support the hypothesis that the high affinity of muscarinic antagonists for the receptors mostly relies on hydrophobic interactions, whereas the binding of agonists in the presence of GTP depends much more upon ionic than on hydrophobic interactions.

APPENDIX

Let us suppose that, in the pH range studied, the affinity constant of the drug for the receptor is not affected by titration of active groups of the receptor and that the affinity of the deprotonated (uncharged) drug is smaller, but not negligible, when compared with the affinity of the protonated drug. The binding reaction may then be written as:

where F^+ is the concentration of free protonated drug, F^0 the concentration of free uncharged drug, R the concentration of receptors, B^+ the concentration of the 'protonated' complex, B^0 the concentration of the 'unprotonated' complex and H^+ the concentration of protons. The equilibrium constants may be written as:

$$K_{D^+} = \frac{R \times F^+}{B^+} \quad (1)$$

$$K_{D^0} = \frac{R \times F^0}{B^0} \quad (2)$$

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$$pK = -\log \left(\frac{F^0 \times H^+}{F^+} \right) \quad (3)$$

$$= -\log \frac{F^0}{F^+} + pH \quad (4)$$

The total concentration of the complex (B_T) is equal to the sum of the protonated and unprotonated complex concentrations:

$$B_T = B^+ + B^0 \quad (5)$$

Using eqns. (1) and (2):

$$B_T = \frac{R \times F^+}{K_{D^+}} + \frac{R \times F^0}{K_{D^0}} \quad (6)$$

Using eqn. (4), the concentration of protonated drug can be written as:

$$F^+ = F^0 (10^{pK - pH}) \quad (7)$$

so that eqn. (6) now becomes:

$$B_T = RF^0 \left\{ \frac{(10^{pK - pH})}{K_{D^+}} + \frac{1}{K_{D^0}} \right\} \quad (8)$$

The total concentration of free drug (F_T) is equal to the sum of the protonated and unprotonated drug concentrations:

$$F_T = F^+ + F^0 \quad (9)$$

and using eqn. (7):

$$F_T = F^0(1 + 10^{pK-pH}) \quad (10)$$

Eqn. (8) then becomes:

$$B_T = R \times F_T \left\{ \frac{(10^{pK-pH})}{K_{D^+}(1 + 10^{pK-pH})} + \frac{1}{K_{D^0}(1 + 10^{pK-pH})} \right\} \quad (11)$$

$$= R \times F_T \left\{ \frac{K_{D^+} + K_{D^0}(10^{pK-pH})}{K_{D^0}K_{D^+}(1 + 10^{pK-pH})} \right\} \quad (12)$$

The apparent K_D of the drug at a given pH is defined as:

$$K_{Dm} = \frac{R \times F_T}{B_T} \quad (13)$$

$$= \left\{ \frac{K_{D^0}K_{D^+}(1 + 10^{pK-pH})}{K_{D^+} + K_{D^0}(10^{pK-pH})} \right\} \quad (14)$$

$$K_{Dm} = \frac{K_{D^+}(1 + 10^{pK-pH})}{\frac{K_{D^+}}{K_{D^0}} + (10^{pK-pH})} \quad (15)$$

so that:

$$\frac{K_{D^+}}{K_{D^0}} = \frac{K_{D^+}}{K_{Dm}} + \frac{K_{D^+}}{K_{Dm}} 10^{pK-pH} - 10^{pK-pH} \quad (16)$$

and:

$$\frac{K_{D^+}}{K_{Dm}} = \frac{K_{D^+}}{K_{D^0}} - 10^{pK} \left\{ \left(\frac{K_{D^+}}{K_{Dm}} - 1 \right) 10^{-pH} \right\} \quad (17)$$

At very low pH values, F_0 is close to zero compared with F^+ , and K_{Dm} is a good estimate of K_{D^+} . This value can then be used to estimate K_{D^+}/K_{Dm} at all pH values.

Plotting K_{D^+}/K_{Dm} as a function of $[(K_{D^+}/K_{Dm}) - 1]10^{-pH}$ yields a straight line, with a slope equal to -10^{pK} and ordinate equal to K_{D^+}/K_{D^0} .