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Inclusion Complexation of Warfarin with β-Cyclodextrins and Its Influence on Absorption Kinetics of Warfarin in Rat

O. KARADAĞ, E. GÖK and I. SERDAR ATEŞ*
Department of Chemistry, Faculty of Science, Hacettepe University, Beytepe, Ankara, Turkey

and

Ö. KIRAN and A. BOZKURT
Department of Pharmacology, Faculty of Medicine, Hacettepe University, Sihhiye, Ankara

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Abstract. The inclusion complexes of warfarin with β-cyclodextrin, 2-hydroxypropyl-β-CD and methyl-β-CD have been investigated in aqueous solution. The apparent binding constants of warfarin are found to be 542 ± 19, 442 ± 18 and 112 ± 6 M^{-1} respectively, calculated from the increments in fluorescence emission of the drug. The influence of the β-CDs on the absorption rate of the drug is investigated with *in situ* experiments in a chronically isolated internal loop, in the small intestine of the rat. The first-order disappearance (absorption) rate constant decreases to 3.6×10^{-4} min^{-1} in β-CD, to 5.0×10^{-4} min^{-1} in 2-hydroxypropyl-β-CD and to 1.4×10^{-3} min^{-1} in methyl-β-CD compared to 3.2×10^{-3} min^{-1} in isotonic phosphate buffer (pH=7.4) solution, all of them showing a good agreement with the percentage of free warfarin in their complexed solutions: 16%, 18% and 47% calculated, respectively.

Key words: Inclusion complexation of warfarin with β-CDs, apparent binding constants, bioavailability of warfarin, β-CD complexes, fluorescence spectroscopy, β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, methyl-β-cyclodextrin.

1. Introduction

The ability of cyclodextrins (CDs) to act as molecular hosts for a variety of hydrophobic organics species is well known. α-, β- and γ-CDs are composed of 6, 7 and 8 α-(1-4) linked units of glucopyranose, forming rings with internal diameters of 5.7, 7.8 and 9.5 Å. In spite of being the least soluble in water, β-CD is the one most frequently employed for pharmaceutical and analytical applications. It seems to be the host of choice when dealing with a variety of compounds that have a size compatible with the interior cavity of β-CD. The extent of inclusion is quantified by the association constant of both the host and guest molecules. Geometric factors rather than chemical factors and the polarity of molecules are decisive in determining the type of guests which can penetrate into the CD cavity.

* Author for correspondence.

CDs are used extensively in the pharmaceutical industry in some oral and parenteral formulations and suppositories [1–3]. They often improve drug stability, increase solubility, promote faster absorption, reduce local irritation and result in improved bioavailability. Stability is conferred on the guest molecule by protecting it from degradation due to heat or light, from sublimation or oxidation. For a poorly water-soluble compound, in particular, its inclusion as a guest in the CD cavity increases the low rate of dissolution or its low solubility and may enhance its bioavailability.

Warfarin (W), a compound which has poor solubility in water, has been selected as a guest molecule for this study. It is a hydroxycoumarin derivative that is used both as an oral anticoagulant in the treatment of thromboembolic disorders or as a rodenticide to help control the rat population. Methods based on spectrophotometry [4, 5], fluorimetry [6, 7], phosphorimetry [8, 9] or HPLC [10–12] are prefered for the routine analysis of this compound, as in the cases of suspected poisoning. Some recent papers have mentioned warfarin complexation with β-CD in connection with its spectrofluorimetric determination in irrigation water [13], its simultaneous determination in bromadiolone mixtures (also a rodenticide) by derivative synchronous fluorescence spectrometry [14], and its binding properties to β-CD by HPLC [15]. They all indicate the formation of only 1 : 1 inclusion complexes, as is usually the case for inclusion of guests in β-CD which are stable in neutral solutions. No work has been published on the interaction of W with β-CD derivatives or on its bioavailability after complexation.

Here we report on the influence of α-, β-, 2-hydroxypropyl-β- and methyl-β-CD on the apparent solubility of W under strictly controlled pH conditions. Their apparent binding constants are studied *in vitro*, the influence of β-CDs on the absorption of W from the small intestine of the rats is studied *in situ* in a chronically isolated internal loop in the rat.

2. Experimental

2.1. Reagents

Stock solutions of warfarin (Aldrich, Milwaukee, MI, USA) were prepared in ethanol at concentrations of 1.0 mg mL^{-1}; α-, β-, 2-hydroxypropyl-β- and methyl-β-cyclodextrin (Sigma, St Louis, MO, USA) were recrystallised once from boiling water, 1×10^{-2} M aqueous solutions being used. A buffer solution (pH=9.0) was prepared from 0.1 M boric acid and 0.1 M sodium hydroxide solution. Isotonic phosphate buffer (pH=7.4) was preferred for *in situ* studies.

2.2. Study of inclusion phenomena

Increasing volumes of 1×10^{-2} M α-, β-, 2-hydroxypropyl-β- or methyl-β-cyclodextrin solutions, 2 mL of buffer solution (pH=9.0) and de-ionised water were added to aliquots of ethanolic warfarin solution and gently evaporated to dry-

TABLE I. The linear regression of $([W]_0/\Delta I_F)$ against $[\beta\text{-CD}]^{-1}$ with correlation constants (R^2) for $[W]_0 = 3.24 \times 10^{-6}$ M and [β-CDs] up to 1×10^{-2} M, calculated apparent binding constants (K_{app}), maximum solubility of warfarin in 4.0 mL 8×10^{-3} M β-CDs and the relative increase in warfarin solubility compared to the pH=7.4 phosphate buffer solution.

	Linear regression* y against z	(R^2)	K_{app}** (M^{-1})	Max. Solubility as $\mu g\ ml^{-1}$	Molar	Rel. sol. increase
Buffer	$y = 2.13 + 22.82z$	(0.995)	-	18	5.84×10^{-5}	1.0
β-CD	$y = 8.29 \times 10^{-5} + 1.53 \times 10^{-7}z$	(0.997)	542 ± 19	1193	3.87×10^{-3}	66.3
2HP-β-CD	$y = 9.72 \times 10^{-8} + 2.20 \times 10^{-10}z$	(0.995)	442 ± 18	1559	5.06×10^{-3}	86.6
Met-β-CD	$y = 6.48 \times 10^{-8} + 5.78 \times 10^{-10}z$	(0.990)	112 ± 6	183	5.94 ± 10^{-4}	10.2

* y is I_F values measured against warfarin concentration (z=[W]) in pH=7.4 phosphate buffer solutions (calibration curve). y is $([W]_0/\Delta I_F)$ values measured against $z = [\beta\text{-CDs}]^{-1}$ as given by Equation (1) for solutions having β-CD, 2HP-β-CD and Met-β-CD.
** The definition of apparent binding constant (K_{app}) is given by Equation (2) and it is calculated experimentally from Equation (1).

ness on a hot-plate, to give a final volume of 10 mL. These samples were sonicated for 15 min and their fluorescence spectra measured.

All fluorimetric measurements were made on a Jasco Model FP-550 spectrofluorimeter using 1 cm^2 quartz cells, the emission spectra being corrected for the spectral response of the system. Monochromatic readings were taken from the digital display with a 0.25 s time constant and with a 3 nm bandwidth on the excitation side, 5 nm on the emission side. The fluorescence intensity of W was measured at 390 nm with excitation at 310 nm, against a reagent blank. All pH adjustments were made by a microprocessor pH-meter (Model HI-8521, Hanna Inst.) with an accuracy of ± 0.05 pH unit.

2.3. Solubility of Warfarin Sodium

The solubility of W was measured in an isotonic phosphate buffer (pH=7.4) and borate buffer (pH=9.0) containing 8×10^{-3} M β-CDs by shaking 4 mL of each solution with 3.083 mg of crystalline W for 24 h at 37° C. After centrifuging at 4000 rpm for 15 min, the fluorescence intensity of the supernatant was measured at $\lambda_{em} = 390$ nm with excitation at 310 nm, against a reagent blank (see Table I).

2.4. Perfusion of W/CD mixtures *in situ*

Male Wistar albino rats were used (230–290 g). An ileal segment of approximately 8 cm and about 10 cm proximal to the ileocaecal junction with intact blood supply was chronically isolated inside the peritoneal cavity of the rat. The two open ends of the loop were connected to the perfusion system by two Delrin cannulas (Figure 1). The perfusion solution was pumped through a heat exchange device to bring the solution to body temperature just before it entered the rat. The details of this procedure are given elsewhere [16].

The rats were perfused in the isolated internal loop with a 60 mL solution of W (5 mg L^{-1}) in isotonic phosphate buffer (pH=7.4) both with and without

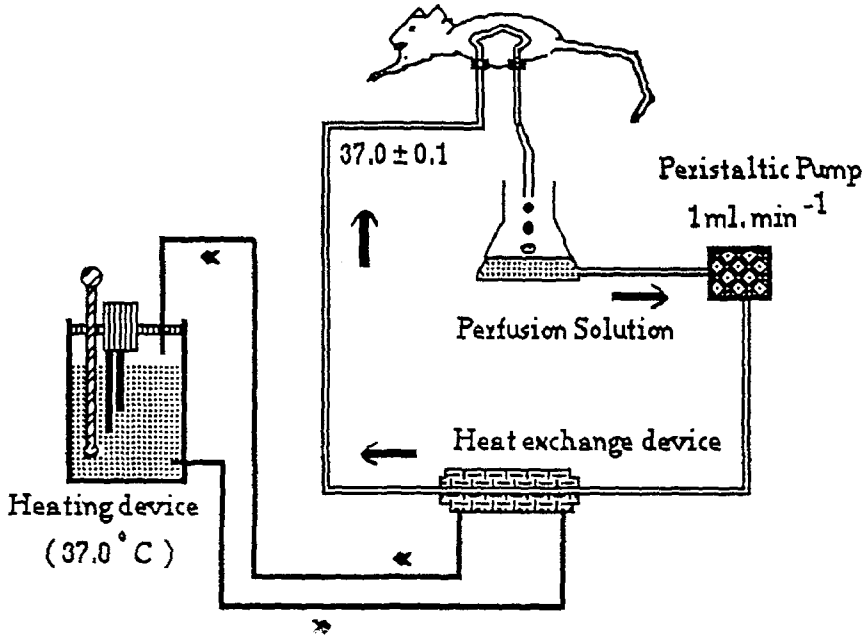

Fig. 1. The chronically isolated internal-loop model in the rat.

1 mM cyclodextrins. All perfusions were performed in the recirculating mode. The absorption of W was determined as the disappearance from the perfusate. A 5 mL sample of perfusate was collected for each time period up to 140 min and relative fluorescence intensities were recorded. Since the W was passively absorbed, a first-order decay in concentration in the perfusate was observed: the absorption was characterized by the first-order disappearance rate constant k_{dis}, which equalled the slope of the plot of ln [remaining W] against time.

3. Results and Discussion

Quantitative data can be obtained from the enhanced fluorescence emission that occurs as the β-CD concentration increases (Figure 2). Similar enhancements can be seen for 2-hydroxypropyl-β-CD and methyl-β-CD but not as much as for α-CD (Figure 3). This enhancement has been attributed to a diminution in rotational freedom in the medium of the hydrophobic cavity, where one part of the warfarin molecule is protected from collisions, upon inclusion complexation of the guest with host [13]. Thus the α-CD cavity is too small for warfarin and no reasonable binding constant can be evaluated.

A contour 3D emission spectrum of the warfarin-β-CD complex is given in Figure 4 for eight different pH values between 3.0 and 10.0. The maximum fluorescence intensity at 390 nm emission is nearly constant for pH values between 6.0 and 10.0. Warfarin, being an acidic drug with a pK_a value of 5.1, is negative-

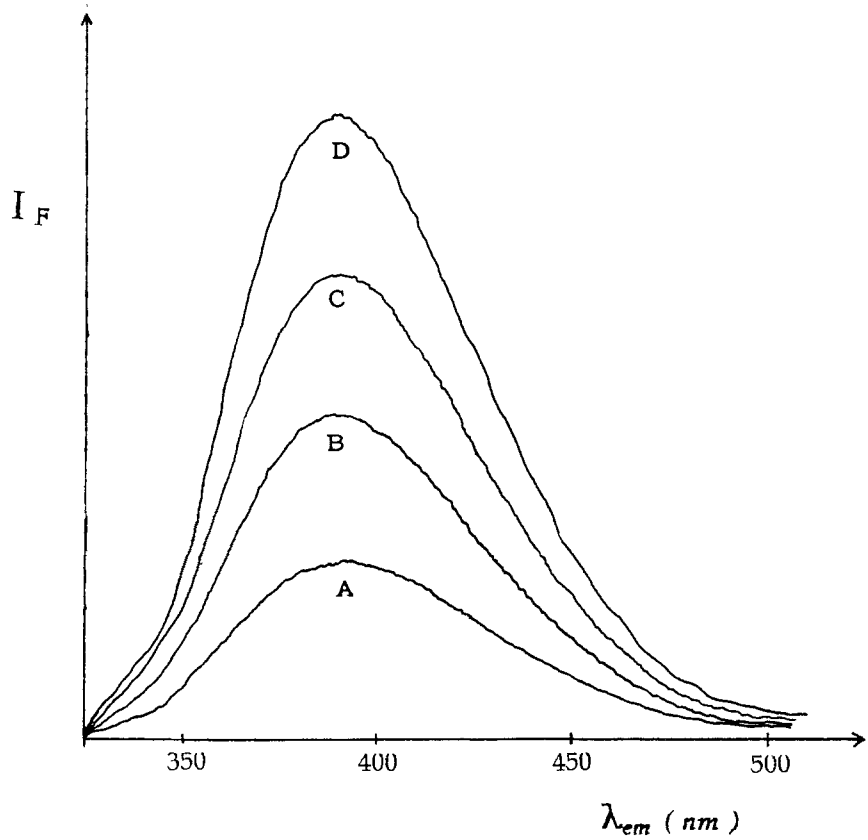

Fig. 2. Influence of the β-CD concentration on the formation of the inclusion complex. $[W]_0 = 3.24 \times 10^{-6}$ M; with A, no β-CD added; B, $[\beta\text{-CD}] = 6.25 \times 10^{-4}$ M; C, 1.88×10^{-3} M and D, 6.25×10^{-3} M. pH=7.4, $\lambda_{ex} = 310$ nm.

ly charged under these conditions. Thus pH values > 6.0 increase the attractive electrostatic interactions between β-CDs and the charged W, causing more complexation. The complex is stable for at least 2 h and the order of addition of reagents is not important.

The apparent binding constants (K_{app}) of warfarin with β-CDs are obtained by the expression described previously [13, 17].

$$(\Delta I_F)^{-1} = (\alpha K_{app}[W]_0\,[\beta\text{-CD}])^{-1} + (\alpha[W]_0)^{-1} \quad (1)$$

$$K_{app} = [W_{bound}] / [W_{free}] \times [\beta\text{-CD}] \quad (2)$$

ΔI_F is the increase in fluorescence intensity after addition of β-CDs to the warfarin solutions ($I_{F\ complexed} - I_{F\ W_0}$), $[W]_0$ is the initial formal concentration of warfarin and α is the proportionality constant that contains all other experimental and instrumental parameters. The linear relationship in Equation (1) between

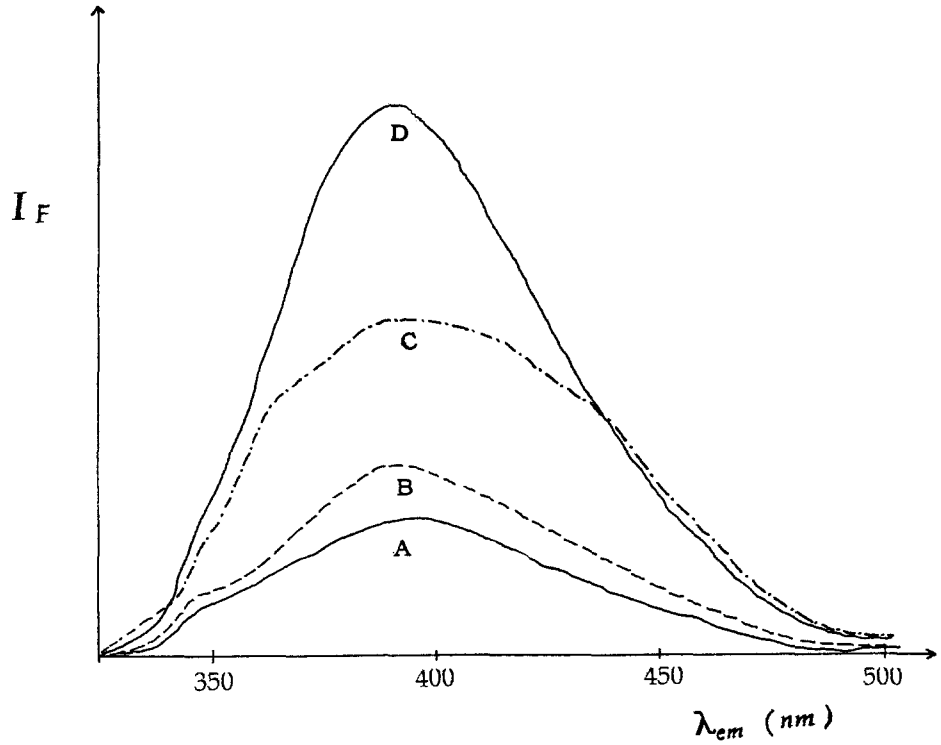

Fig. 3. Fluorescence emission spectra of warfarin in A, buffer solution; B, α-CD; C, methyl-β-CD; D, 2-hydroxypropyl-β-CD. $[W]_0 = 3.24 \times 10^{-6}$ M and all [CDs] = 5.00×10^{-3} M, pH=7.4, $\lambda_{ex} = 310$ nm.

($[W]_0/\Delta I_F$) and $1/[\beta\text{-CD}]$ and the ratio of intersect/slope gives the host–guest association constant, K_{app}. The linear regressions with correlation constants (R^2) given in Table I also show the experimental evidence for the warfarin forming 1 : 1 inclusion complexes with all β-CDs, not only for W-βCD [13–15].

The K_{app} value is found to be 542 ± 19 M^{-1} for the warfarin-β-CD complex. This value is nearly the same as the value of 520 ± 30 M^{-1} reported by Thuaud [15] but higher than the 160 M^{-1} or 149 M^{-1} reported by Marquez [13] and Lin [18]. There are no results in the literature concerning the binding of warfarin to Met-β-CD or 2HP-β-CD. An average of 6.0 to 7.3 β-CD sites being chemically modified to methyl groups decreases the apparent binding constant to 112 ± 6 M^{-1} and an average of 7.6 β-CD sites being chemically modified to hydroxypropyl groups decreases the apparent binding constant to 442 ± 18 M^{-1}. Both methyl or hydroxypropyl substitutions on the β-CD molecule either affects the electrostatic interactions between them and the negatively charged warfarin or introduces a steric hindrance.

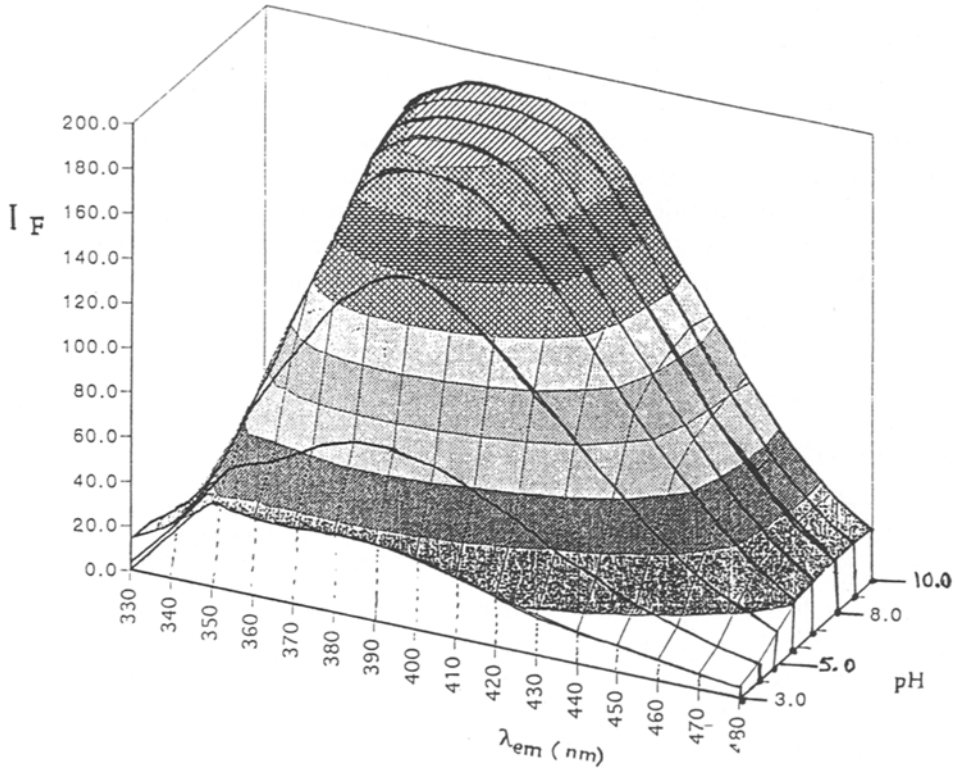

Fig. 4. Contour 3D emission spectra of 3.24×10^{-6} M W in 8.00×10^{-3} M β-CD for pH values 3.00; 4.00; 4.75; 5.60; 6.75; 8.00; 9.00; 10.00. $\lambda_{ex} = 310$ nm. All bold lines correspond to a given pH above with a (• –) sign at their starting points.

The solubilities of crystalline β-CDs in pure water at 25°C increase in the order of 1.8 g/100 mL for β-CD $<\sim$ 50 g/100 mL for Met-β-CD $<\sim$ 60 g/100 mL for 2HP-β-CD. The maximum solubility of the drug increases from 5.84×10^{-5} M to 3.87×10^{-3} M (66.3-fold) when 4.0 mL pH=7.4 phosphate buffer solution with or without 8×10^{-3} M β-CD is treated with excess crystalline warfarin. The experimental data do not match with the expected value of the relative solubility increase of the drug calculated from $K_{app} = 542\ M^{-1}$ when the total solubility is expressed as ($[W]_{free} + [W_{bound}]$). This is because of the high drug concentration used in solubility tests (higher ionic strength of solution, deviation in calibration, experimentally longer saturation time) and/or a change in geometric factors besides chemical factors determining the penetration of warfarin into the CD cavity.

The increment in the drug's relative solubility is 86.6 times for 2HP-β-CD and 10.2 times for Met-β-CD complexation compared to the buffer solution. Although hydroxypropyl or methyl substitution on the β-CD molecule lowers the association constant of warfarin, the experimental data show that the former substitution typically increases the maximum solubility. The increase in concentration of negatively

charged guest molecule probably interacts with the hydroxyl groups of the host molecule besides normal inclusion complexation. 2HP-β-CD seems to be the best choice for warfarin with the highest solubility in water, 1.56 mg mL^{-1}.

According to the phase separation model, the ratio of $[W_{free}]/[W_{bound}]$ is assumed to be constant throughout the concentration range of warfarin used in *in situ* absorption experiments. This means that in the perfusate the thermodynamically active concentration of warfarin is 16% of its formal concentration when β-CD is added to the solution. Table II shows the percentages of free or bound warfarin calculated from the K_{app} values given by Equations 3 and 4.

$$\% \text{ free} = [W_{free}] / [W_{total}]) \times 100 \tag{3}$$

and for $[\beta\text{-CD}] \gg [W_{total}] = [W_{bound}] + [W_{free}]$

$$\% \text{ free} = (1/1 + K_{app} \cdot [\beta\text{-CD}]) \times 100 \tag{4}$$

The formal concentration of warfarin is free (100%) when no β-CDs are added but drop to 47 ± 3%; 18 ± 2% and 16 ± 2% when 1×10^{-2} M of Met β-CD; 2HP β-CD and β-CD are added, respectively.

The residual concentration of warfarin remaining in solution versus time during an *in situ* absorption experiment is measured for different $[W]_0$ and $[\beta\text{-CD}]$ values. The disappearance of warfarin from the solution is found to be first order, as can be seen from the straight lines fitted through the logarithmic data points ($\ln I_{F_t}/I_{FW_0}$) versus time (t) and from the correlation constants given in Table II. The disappearance rate constants from the perfusate, k_{dis}, are calculated from the slope of these lines and their mean ratio, $r(k_{dis+}/k_{dis-})$, from rate constants with and without β-CDs. Thus the percentage of free warfarin (100%, 47%, 18% and 16% corresponding to buffer pH=7.4; Met-β-CD; 2HP-β-CD; β-CD) is well correlated with the reduction in the absorption rates (expressed as $r = 1.00$; 0.43; 0.15 and 0.11, respectively). The mean half-life of the drug's absorption from the perfusate $t_{1/2} = 0.693/k_{dis}$, is also calculated for each β-CD to show their correlation with the percentage of bound warfarin in complex solutions (Table II). *In situ* absorption experiments suggest that only the $[W_{free}]$ is thermodynamically active, giving the driving force for passive diffusion of warfarin over the intestinal wall and the CDs do not have an influence on the absorption step itself.

4. Conclusion

When methyl-β-CD, 2-hydroxypropyl-β-CD and β-CD are added to a solution of warfarin, with which they form inclusion complexes in aqueous solutions, they decrease the active (free) concentration of the drug in the media, the bound form having no active role in the absorption step and preventing the diffusion of the drug through the intestinal wall. Thus the absorption rate of warfarin in the perfused intestine of the rat is accordingly decreased. Despite the fact that β-CDs apparently do not interfere in the membrane passage of the drug, they still play a role in

TABLE II

Apparent binding constants (K_{app}), % free and % bound warfarin in 1×10^{-2} M β-CD solutions calculated from Equation (4), the linear regression of $\ln (I_{F_t}/I_{FW_0})$ against time (t) with correlation constants (R^2). The first-order disappearance (absorption) rate constants (k_{dis}), the mean ratio of rate constants with and without β-CDs given as $r = k_{dis+}/k_{dis-}$ and the warfarin absorption half-life from the perfusate, $t_{1/2}$ for $[W]_0 = 3.24 \times 10^{-6}$ M in pH=7.4 isotonic phosphate buffer.

	K_{app} (M^{-1})	% Free	% Bound	Linear regression* y against t	(R^2)	k_{dis} (min^{-1})	r	$t_{1/2}$** (min)
Buffer	–	100	0	$y = 0.01 - 3.21 \times 10^{-3}t$	(0.917)	$(3.2 \pm 0.8) \times 10^{-3}$	1.00	215 ± 10
Met-β-CD	112 ± 6	47 ± 3	53 ± 3	$y = 0.02 - 1.40 \times 10^{-3}t$	(0.894)	$(1.4 \pm 0.8) \times 10^{-3}$	0.43	506 ± 16
2HP-β-CD	442 ± 18	18 ± 2	82 ± 2	$y = 0.02 - 4.98 \times 10^{-4}t$	(0.881)	$(5.0 \pm 1.0) \times 10^{-4}$	0.15	1400 ± 25
β-CD	542 ± 19	16 ± 2	84 ± 2	$y = 0.01 - 3.62 \times 10^{-4}t$	(0.998)	$(3.6 \pm 1.0) \times 10^{-4}$	0.11	1927 ± 30

* y is $\ln (I_{F_t}/I_{FW_0})$ values measured against time (t), I_{F_t} and I_{FW_0} being the fluorescence intensity of warfarin at time t and $t = 0$ min. Data is given for the 5 mL samples taken from the perfusate *in situ* absorption experiments.

** Calculated from the first-order disappearance rate constant as $0.693/k_{dis}$.

the overall process of drug absorption by enhancing the dissolution rate and the apparent solubility of warfarin.

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