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β-cyclodextrin complex formation and protonation equilibria of morphine and other opioid compounds of therapeutic interest

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ABSTRACT

The inclusion complex formation of morphine and its 18 opioid derivatives with β-cyclodextrin has been studied using nuclear magnetic resonance spectroscopy. Initially, the protonation equilibria and the acid-base properties of dibasic opioid compounds have been fully characterized. Apparent protonation constants and the relative concentration of the microspecies in cyclodextrin excess were also determined. The 1:1 complex stoichiometry was confirmed by the continuous variation method of Job using UV-VIS spectroscopy. The stability constants of the different protonation forms were determined by ^{1}H NMR titrations. The highest stability was observed in highly alkaline solutions where the amino group is in its unprotonated, neutral state. The structures of the complexes were investigated by two-dimensional ROESY experiments. Based on the stability constants and ROESY experiments, morphine derivatives with longer side chain on the nitrogen atom such as nalbuphine and naltrexone show stronger complexation. The protonation state of the phenolate group, positioned outside the CD cavity, has only a slight influence on the complex stability.

1. Introduction

Morphine, alongside with its semi-synthetic and synthetic derivatives is a valuable family of analgesic agents in the treatment of severe acute and chronic pain. Therefore, the structure and detailed physicochemical and pharmacokinetic properties of these molecules have widely been studied (Mackay and Hodkin, 1955; Mazák et al., 2009, 2012, 2015, 2019). The general structure-activity relationships (SARs) have also been reported (Fürst and Hosztafi, 2008; Devereaux et al., 2018). Some studies have also investigated the effect of cyclodextrins (CDs) on the absorption and metabolism of some opioid analgesics (Bernards, 1994). α- and β-cyclodextrins were found to enhance the rate and extent of bioavailability of morphine (Uekama et al., 1995; Kondo et al., 1996). However, the structure and physicochemical description of these inclusion complexes have not been investigated yet.

Cyclodextrins are water-soluble cyclic oligosaccharides composed by five or more glucose units with a lipophilic central cavity responsible for the host-guest bond formation and a hydrophilic outer surface (Szejtli, 1998). The pharmaceutical industry uses cyclodextrins to improve drug characteristics such as solubility, stability and bioavailability. The use of these oligosaccharides can have other advantages, namely reduced toxicity, masking of undesirable taste and control of the rate of release of several drugs (Buschmann et al., 2001; Loftsson and Duchêne, 2007; Rasheed et al., 2008).

In this study we examined in detail the stability of beta-cyclodextrin complexes of morphine and some of its derivatives using NMR spectroscopy. NMR-titration data can directly give the stability (binding) constant and the stoichiometry of the host-guest complex (Schneider et al., 1998; Fielding, 2000; Orgován et al., 2016). To identify the complex stoichiometry, the classical Job's method of continuous variation was used (Job, 1928; Saha et al., 2017).

In addition, the protonation equilibria of the free and complexed compounds also have been studied. By determining the ionization state of a molecule at a particular pH - using ^{1}H NMR-pH titrations - it was also possible to examine which species is responsible for the stability of the host-guest complexes. The geometry of the inclusion complexes was investigated by 2D rotating frame nuclear Overhauser effect spectroscopy (ROESY) technique since the cross-peaks on the spectra show the space-vicinity of two protons in the host and guest molecules (Neuhaus and Williamson, 2000; Buschmann et al., 2001).

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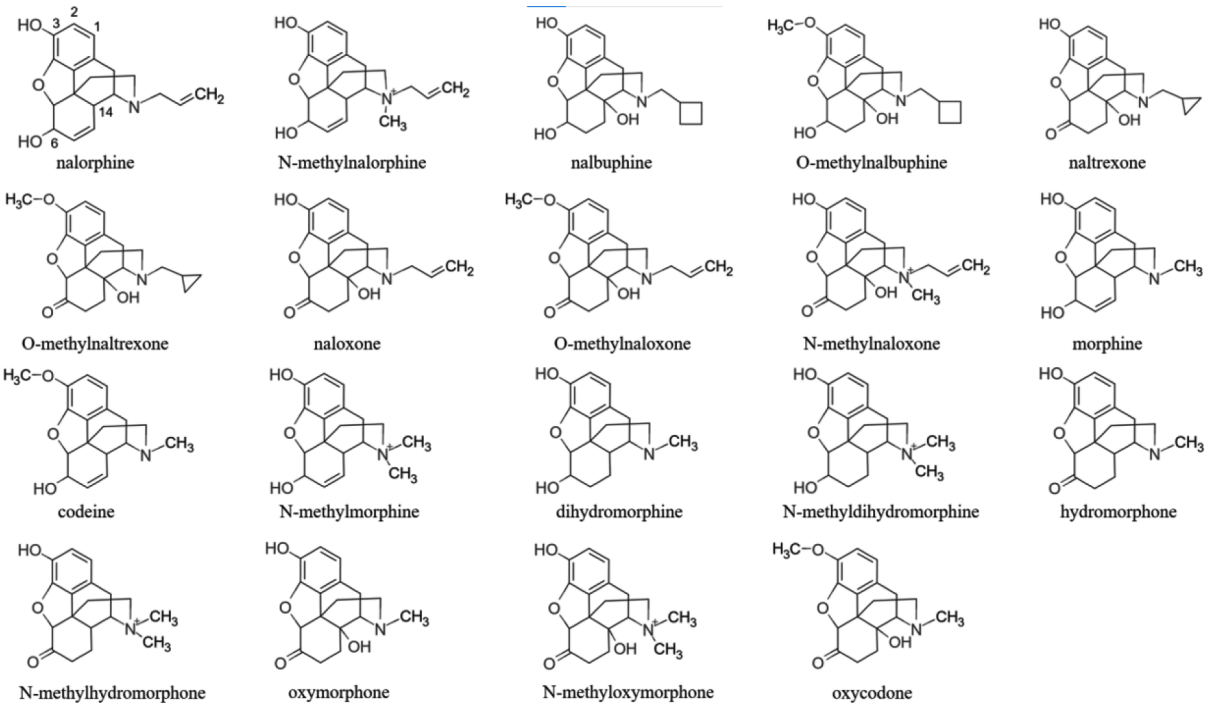

Fig. 1. The structure of the investigated opioid compounds.

2. Materials and methods

2.1. Materials

The opioid compounds (Fig. 1) were prepared by procedures already described in detail (Mazák and Noszál, 2012, Mazák et al. 2015, 2017). The β-CD was from Cyclolab Ltd. (Budapest, Hungary).

2.2. Methods

2.2.1. Determination of complex stoichiometry

UV spectroscopic measurements were carried out on a Jasco V-550 UV/VIS spectrophotometer with 10-mm quartz cuvettes at 25°C. The stoichiometry of the nalorphine - β-CD complex was obtained by the continuous variation method of Job. The stock solutions contained 1 mM of the given compound. The guest/host molar ratio was varied from 0 to 1 and the absorbances at each molar ratio were measured.

2.2.2. Nuclear magnetic resonance measurements

All NMR measurements were performed on a Varian VNMRS spectrometer (599.9 MHz for ^{1}H). Spectra were recorded at 25 °C. Titrations were carried out in solutions containing 90 % (*V/V*) H_2O and 10 % (*V/V*) D_2O. The sample volume was 600 μL. NMR spectra were referenced to the internal standard methanol (3.350 ppm), as methanol is a compound without any significant interaction with cyclodextrins (Tárkányi, 2002). The ionic strength was kept at 0.15 M by the presence of NaCl.

The water signal was suppressed by presaturation (Somlyay et al., 2015). Spectra were processed using VNMRj 3.2a software. Initial pH values were read on a Metrohm 2.780.0010 precision pH meter with a 6.0258.600 Unitrode glass Pt 1000 electrode (Metrohm AG, Herisau, Switzerland), calibrated using National Bureau of Standards (NBS) buffer solutions. For the analysis of NMR titration curves of proton chemical shifts versus pH, the software Origin Pro 8 (OriginLab Corp., Northampton,MA, USA) was used (Jakab et al., 2019).

2.2.3. Determination of protonation constants

To obtain the protonation constants of the compounds ^{1}H NMR-pH titrations were carried out in solutions containing 90 % (*V/V*) H_2O and 10 % (*V/V*) D_2O, with the addition of small amounts of 0.1 M HCl and NaOH. 1 M NaOH was also used to achieve highly basic pH values. The concentration of the investigated opioid compounds was 2 mM. The exact pH in the NMR tube was measured *in situ* by NMR-pH indicator molecules acetic acid, imidazole, TRIS and t-butylamine (Orgován and Noszál, 2011). The concentration of these indicators was 1 mM.

Table 1
The concentration of the samples of the opioid compounds and β-CD during the NMR-titrations with the final [CD]/[L] ratio.

Concentration of the opioid compound (M)	Concentration of the β-CD (M)	[CD]/[L] ratio
0.0005	0	0
0.0005	0.00025	0.5
0.0005	0.0005	1
0.0005	0.001	2
0.0005	0.002	4
0.0005	0.003	6
0.0005	0.004	8
0.0005	0.006	12
0.0005	0.008	16
0.0005	0.01	20
0.0005	0.012	24

The apparent protonation constants in CD excess were also determined using the same method. The solutions contained 1 mM of the opioid compound and 16 mM of the β-cyclodextrin. Due to the possible complexation of the NMR-pH indicator molecules in these CD containing solutions, a calibrated glass electrode was used to show the exact pH values.

2.2.4. Determination of apparent stability constants by NMR titrations

The experiments were carried out in different inorganic buffer solutions, namely phosphate, borate and acetate. The samples were equilibrated for at least 12 h before the experiments. Samples of the opioid compounds and β-CD were prepared with a molar ratio [CD]/[L] ranging from 0 to 24. Concentrations of the stock solutions were set to 6 mM for the guest and 15 mM for the CDs. In this method the concentration of the guest molecule was kept constant throughout the titration.

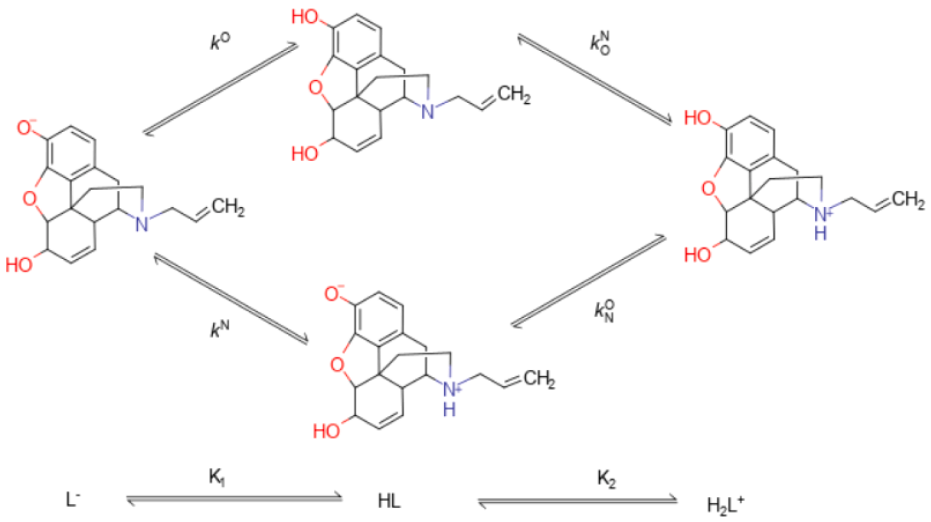

Fig. 2. The protonation scheme of nalorphine.

The exact concentration values are shown in Table 1.

2.2.5. Determination of the structure of the inclusion complexes

Structural information on the inclusion complexes was obtained by 2D ROESY NMR technique. The ROESY spectra were recorded on a 650 μL sample in a phosphate buffer at pH = 12.4 with 10 % (*V/V*) D_2O, containing 5 mM of the opioid in question and 20 mM β-CD with 304 increments using a mixing time of 300 ms.

3. Results and discussion

3.1. Protonation constants

Some of the investigated opioid compounds are amphoteric ones which contain a basic tertiary amino and an acidic phenol group, and consequently exist in solutions in four microscopic protonation forms. Such ligands (abbreviated as L) in highly alkaline solutions carry one negative charge on their phenolate site. By taking up a single proton they will be transformed into either a zwitterionic or an uncharged form. These two forms differ only in the site of protonation and are known as protonation isomers. The uptake of a second proton results in a single cationic species. The protonation scheme of such an amphoteric compound exemplified by nalorphine is depicted in Fig. 2.

K_1 and K_2 are the stepwise macroscopic protonation constants, small case *k* stands for the microscopic or site-specific protonation constants, indices N and O designate the nitrogen and oxygen atoms in the amino and phenolate groups, respectively. Subscript (if any) stands for the group already holding proton during the given microequilibrium protonation process, whereas superscripts of microconstants indicate the group protonating in the process (Mazák and Noszál, 2016).

Some of the relationships between the micro- and macroconstants of dibasic opioid compounds are as follows:

$$\beta_1 = K_1 = k^N + k^O \quad (1)$$

$$\beta_2 = K_1 \cdot K_2 = k^N \cdot k_N^O = k^O \cdot k_O^N \quad (2)$$

3.1.1. Determination of macroscopic protonation constants

To obtain the macroscopic protonation constants of the compounds NMR-pH titrations were carried out using indicator molecules to show the exact pH values. Although these molecules contain up to twenty protons connected to carbon atoms, the chemical shift of only those protons changes significantly, that are located close to the protonating site. We followed as many protons as possible unless they were overlapped by other interfering signals. Typically, the aromatic doublets H1 and H2, the signals of H9, H10 and H16 protons, and those located in the

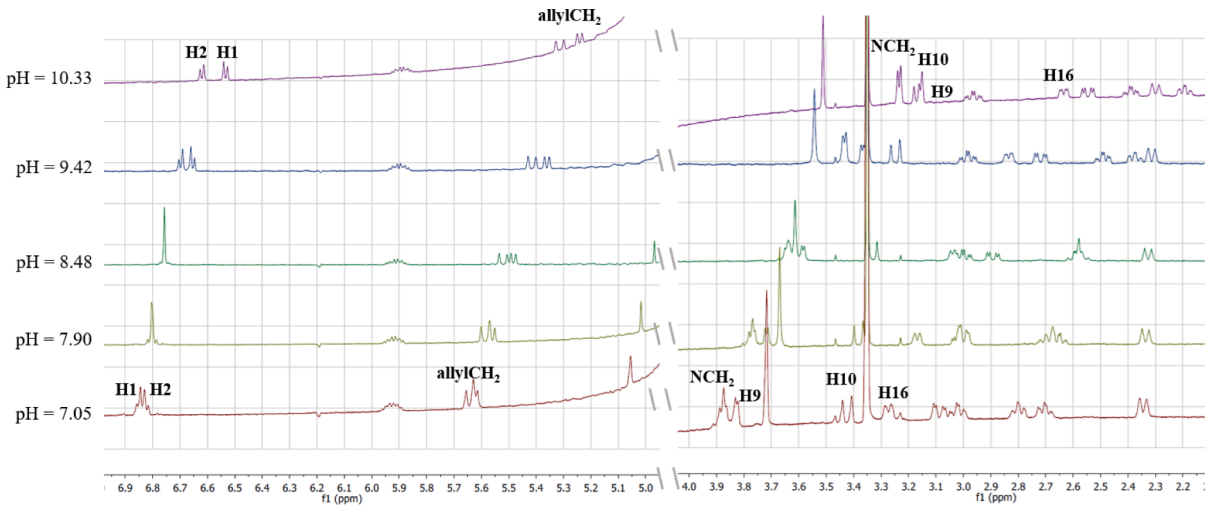

Fig. 3. Representative ^{1}H NMR spectra of the changes of chemical shift of H1 and H2, allyl-CH_2, the nuclei of the side chain NCH_2 and the signals of H9, H10 and H16 protons of naloxone at different pH values.

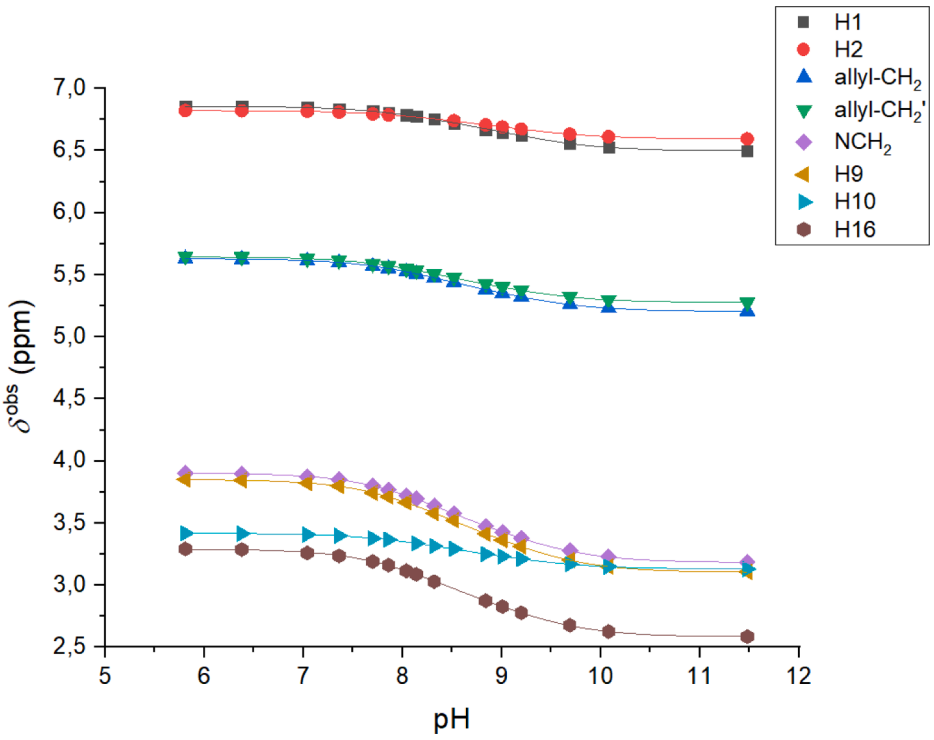

Fig. 4. NMR-pH titration curves for the aromatic doublets H1 and H2, the nuclei of the side chain NCH_2 and allyl-CH_2, and the signals of H9, H10 and H16 protons of naloxone. Computer fits for log K_1 and logK_2 are shown in solid lines.

Table 2
Protonation macroconstants of the investigated compounds (standard error in parentheses).

Compound	logK_1	logK_2
nalbuphine	10.22 (0.01)	8.86 (0.03)
O-methylnalbuphine	9.64 (0.04)	
naltrexone	10.07 (0.01)	8.59 (0.02)
O-methylnaltrexone	9.30 (0.01)	
naloxone	9.29 (0.01)	8.21 (0.01)
O-methylnaloxone	8.38 (0.01)	
nalorphine	9.34 (0.04)	7.89 (0.01)
N-methylnalorphine	8.82 (0.01)	
dihydromorphine	9.90 (0.03)	8.79 (0.03)
N-methyldihydromorphine	8.98 (0.01)	
morphine	9.44 (0.03)	8.27 (0.01)
codeine	8.37 (0.01)	
N-methylmorphine	8.84 (0.01)	
hydromorphone	9.37 (0.03)	8.32 (0.02)
N-methylhydromorphone	8.67 (0.01)	
oxymorphone	9.72 (0.01)	8.53 (0.01)
oxycodone	9.05 (0.01)	
N-methyloxymorphone	8.50 (0.01)	

side chain close to the nitrogen atom were read and evaluated. Since protonation processes are fast in the NMR time scale, only one common peak can be observed with a δ^{obs} chemical shift that is a weighted average of the chemical shifts of the distinct protonation forms (δ_{L^-}, δ_{HL}, $\delta_{H_2L^+}$).

$$\delta^{obs} = \delta_{L^-}\cdot x_{L^-} + \delta_{HL}\cdot x_{HL} + \delta_{H_2L^+}\cdot x_{H_2L^+} = \frac{\delta_{L^-} + \delta_{HL}\cdot K_1\cdot[H^+] + \delta_{H_2L^+}\cdot\beta_2\cdot[H^+]^2}{1 + K_1\cdot[H^+] + \beta_2\cdot[H^+]^2} \quad (3)$$

where x_{L^-}, x_{HL} *and* $x_{H_2L^+}$ are the mole fractions of the anionic, neutral and cationic macrospecies, respectively.

The pH-dependent chemical shifts of the carbon-bound naloxone protons are shown in Fig. 3.

Table 3
Protonation microconstants and pair-interactivity parameters of the investigated dibasic compounds.

Compound	logk^O	logk^N	logk_O^N	logk_N^O	log$E_{N,O}$
nalbuphine	9.44	10.14	9.64	8.94	0.50
naltrexone	9.36	9.98	9.30	8.68	0.68
naloxone	9.12	8.80	8.38	8.70	0.42
nalorphine	9.29	8.41	7.94	8.82	0.47
dihydromorphine	9.45	9.71	9.24	8.98	0.47
morphine	9.30	8.87	8.41	8.84	0.46
hydromorphone	9.11	9.02	8.58	8.67	0.44
oxymorphone	9.20	9.56	9.05	8.69	0.51

The NMR-pH titration curve of naloxone is in Fig. 4. The protonation macroconstants are collected in Table 2.

3.1.2. *Determination of the protonation microconstants*

The dibasic opioid compounds contain an amino and a phenolate group, which are of comparable basicity. We chose therefore the deductive method for the determination of microconstants, which requires an appropriate model compound to mimic the minor species (Mazák and Noszál, 2016).

When the major protonation pathway includes the uncharged microspecies, the macroconstant of the N-methyl derivative of the given opioid compound can be used as the k_N^O microconstant of the dibasic compound. Similarly, when the zwitterionic microspecies lies on the major protonation pathway, the macroconstant of the O-methyl derivative of the given opioid compound can be treated as the k_O^N microconstant. In case of dibasic compounds, knowing one microconstant and the two macroconstants, all other microconstants can be calculated (Table 3). This table also contains the log $E_{N,O}$ pair-interactivity parameter that characterizes how the protonation at one basic site decreases the basicity of the other site.

$$\log E_{N,O} = \log k^N - \log k_O^N = \log k^O - \log k_N^O \quad (4)$$

Table 4
Apparent protonation macroconstants of the investigated opioid compounds in CD excess (standard error in parentheses).

Compound	logK_1	logK_2
nalbuphine	9.48 (0.02)	7.45 (0.03)
O-methylnalbuphine	7.56 (0.01)	
naltrexone	9.22 (0.02)	7.78 (0.01)
O-methylnaltrexone	7.91 (0.01)	
naloxone	9.14 (0.03)	7.23 (0.01)
O-methylnaloxone	7.30 (0.01)	
N-methylnaloxone	8.67 (0.01)	
nalorphine	9.43 (0.03)	6.85 (0.01)
N-methylnalorphine	8.98 (0.01)	
dihydromorphine	9.59 (0.05)	8.19 (0.01)
N-methyldihydromorphine	9.00 (0.01)	
morphine	9.35 (0.03)	7.64 (0.01)
codeine	7.76 (0.01)	
N-methylmorphine	9.00 (0.01)	
hydromorphone	9.26 (0.04)	7.79 (0.01)
N-methylhydromorphone	8.72 (0.01)	
oxymorphone	9.38 (0.02)	8.30 (0.02)
N-methyloxymorphone	8.76 (0.03)	

Table 5
Apparent protonation microconstants and interactivity parameters of the investigated dibasic compounds in CD excess.

Compound	logk^O	logk^N	logk^N_O	logk^O_N	log$E_{N,O}$
naloxone	9.12	7.70	7.25	8.67	0.45
nalorphine	9.43	7.30	6.85	8.98	0.45
dihydromorphine	9.52	8.78	8.26	9.00	0.52
morphine	9.33	7.99	7.66	9.00	0.33
hydromorphone	9.21	8.33	7.84	8.72	0.49
oxymorphone	9.20	8.92	8.48	8.76	0.44

3.1.3. Determination of apparent protonation constants in CD excess

The apparent protonation constants in CD excess were determined using the same method. Table 4 shows the apparent protonation macroconstants of the investigated opioid compounds.

The presence of CD decreases the basicity of the amino group, since it is positioned in the lipophilic central cavity, which preferably accommodates neutral species. Consequently, the k^O microconstant of all these compounds lies always in the major protonation pathway and the N-methyl derivatives can be used to obtain the protonation microconstants. In case of nalbuphine and naltrexone, the N-methyl compounds were not available, thus we did not calculate their microconstants. Table 5 collects the apparent protonation microconstants and interactivity parameters of the investigated dibasic compounds in CD excess.

3.2. Stoichiometry of the inclusion complexes

A potent method for determining the stoichiometry of the host-guest inclusion complexes is the continuous variation method of Job (Job, 1928; Saha et al., 2017). We measured the UV-VIS absorbance of solutions of nalorphine and β-CD, varying the guest/host molar ratio from 0 to 1. Two stock solutions were prepared, one of them containing 1 mM of nalorphine and another containing 1 mM of β-CD. The difference in absorbance of nalorphine with and without CD (ΔA) weighted by the mole fraction of nalorphine (x_{nal}) was plotted against the mole fraction of nalorphine. For all the solutions absorbances were measured at a wavelength of λ_{max} = 284.5 nm at 25 °C. The minimum of the plot belonged to the mole fraction 0.5 (x_{nal} = 0.5), indicating a 1:1 stoichiometry for the nalorphine – β-CD complex (Fig. 5). After proving the 1:1 composition for nalorphine, we assumed that all of the remaining morphine derivatives form complexes of analogous stoichiometry.

3.3. Structure of the complexes

β-CD is a cyclic oligosaccharide composed by seven α-D-glucopyranoside units forming a truncated hollow cone with a lipophilic central cavity and a hydrophilic outer surface. Secondary hydroxyl groups (O(2)-H and O(3)-H) are located on the wider rim, the primary hydroxyl group on the narrower side of the ring. The H-3 and H-5 CH groups and the glycosidic oxygens are located inside the cavity, H-6 protons lie near the narrow opening of the cavity, while the other CH groups, H-1, H-2 and H-4 are positioned on the exterior surface of the CD (Fig. 6) (Béni et al., 2007; Sohajda et al., 2009).

The ^{1}H NMR assignment of β-CD was already known from literature

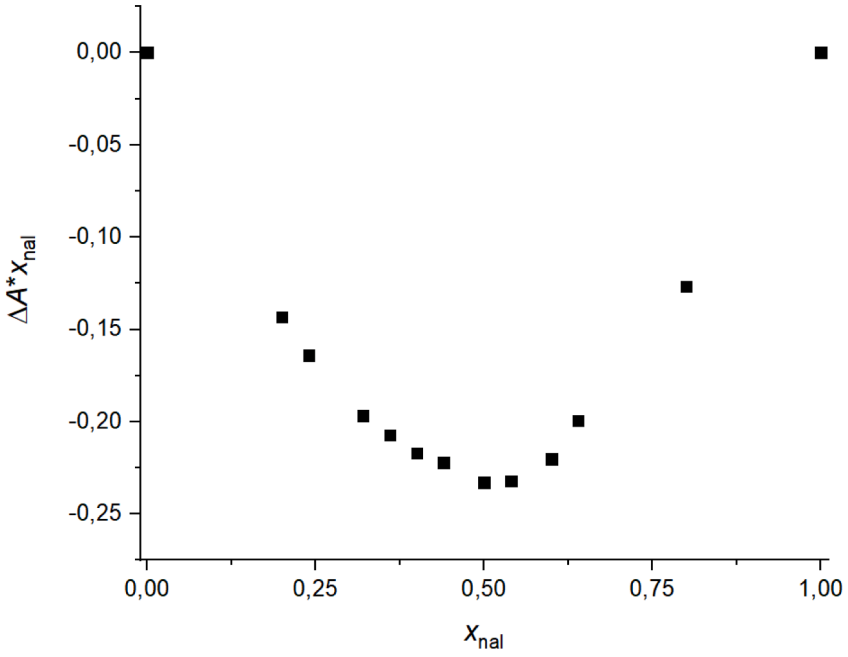

Fig. 5. Job plot of the nalorphine – β-CD system at λ_{max} = 284.5 nm at 25°C, where ΔA is the difference in absorbance of nalorphine with and without CD and x_{nal} is the mole fraction of nalorphine.

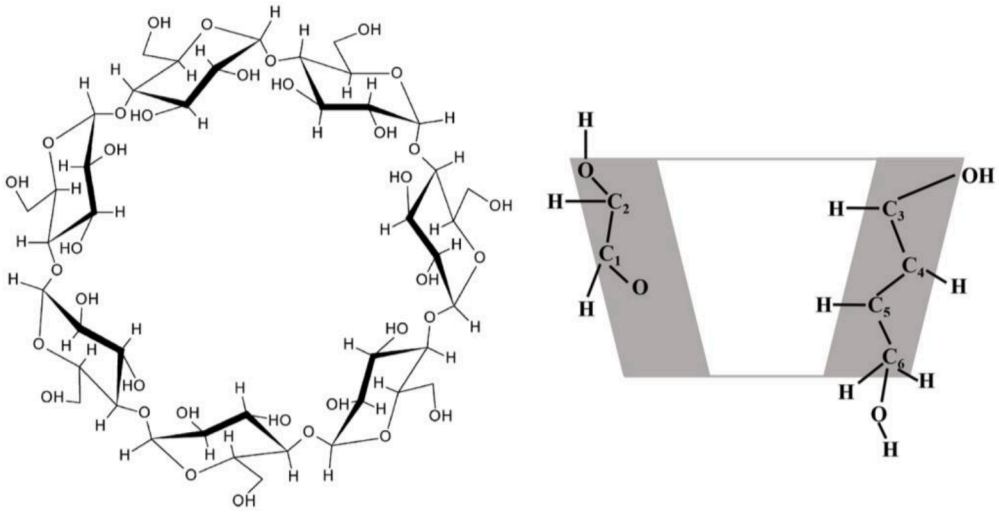

Fig. 6. Chemical structure of the natural β-CD (left) and a schematic view of the cross section of the molecule (right, with numbering) (Béni et al., 2007).

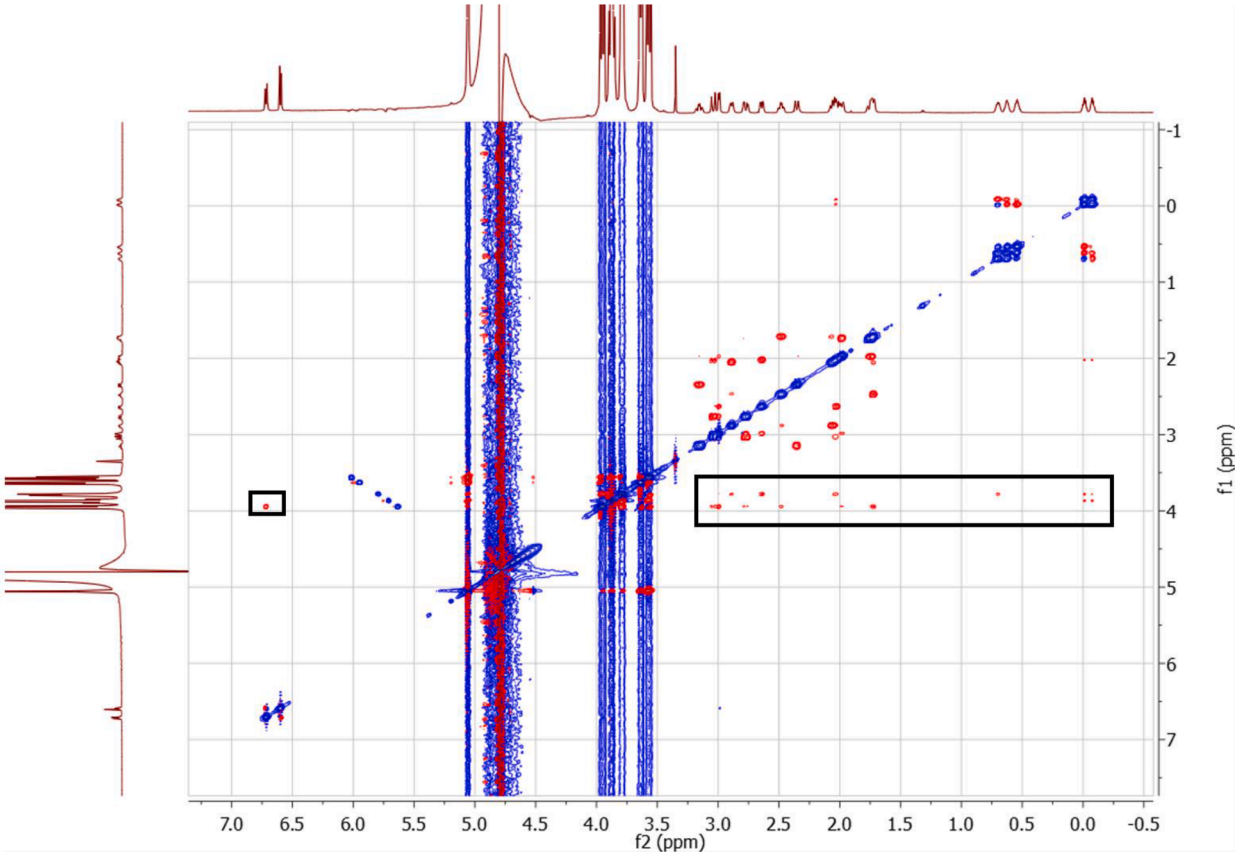

Fig. 7. (a) The ROESY spectrum of naltrexone and β-CD at 1:4 molar ratio. The space-vicinity cross peaks of the inclusion complex are shown in a black frame. (b) Expansion of the ROESY spectrum of naltrexone and β-CD showing the space-vicinity cross-peaks of the complex.

(Zhao et al., 2016). The H-3, H-6 and H-5 CH protons which are important from the point of view of complexation can be seen in Fig. 7. in case of the naltrexone – β-CD complex. The observed chemical shifts are 3.95, 3.88 and 3.79 ppm in the complexed molecules, respectively.

2D ROESY NMR spectra were recorded to obtain further detailed structural information of the complexes. The spectra contained intermolecular cross-peaks between the H3 and H5 protons of the inner cavity of β-CD and certain nuclei of the encapsulated opioid compounds. Based on these spectra, the side chain on the nitrogen atom is responsible for the complexation as cross signals can be found with the side chain and other protons close to the amino group, like H9 and H16. Therefore, inclusion of the investigated molecules proceeds through the wider rim. In case of nalbuphine and naltrexone that contain longer side chains, the H6 proton of the β-CD which is located on the narrow rim also shows space-vicinity that implies a more stable complexation. In some cases, especially with only an N-methyl group in the side chain, the

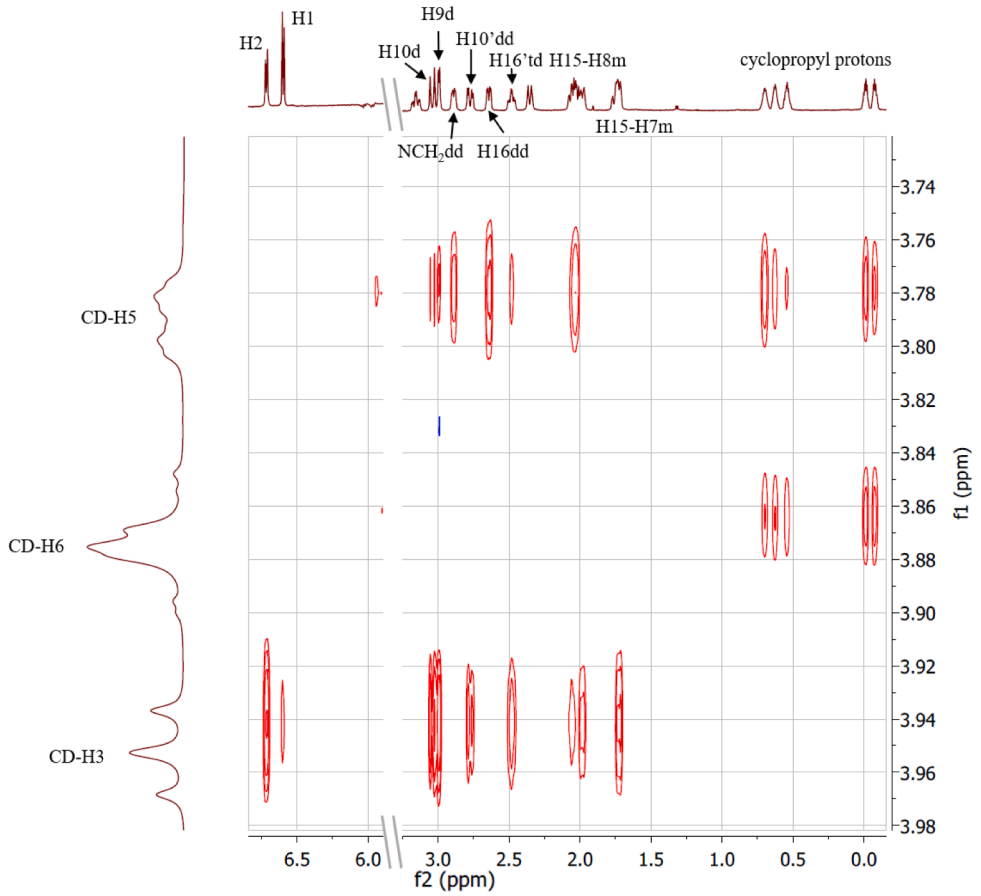

Fig. 7. (*continued*).

Table 6
The observed space-vicinity cross-peaks in the ROESY spectrum of naltrexone and β-CD.

Protons of β-CD	Protons of naltrexone
H3 (3.95 ppm)	H2 d (6.72 ppm)
	H1 d (6.60 ppm)
	H10 d (3.04 ppm)
	H9 d (2.99 ppm)
	H10' dd (2.77 ppm)
	H16' td (2.48 ppm)
	H15-H8 m (2.04 ppm)
	H15'-H7 m (1.74ppm)
	cyclopropyl protons (0.77; 0.58 and -0.04 ppm)
H5 (3.79 ppm)	H10 d (3.04 ppm)
	H9 d (2.99 ppm)
	NCH2 dd (2.89 ppm)
	H16 dd (2.64 ppm)
	H16' td (2.48 ppm)
	H15-H8 m (2.04 ppm)
	cyclopropyl protons (0.77; 0.58 and -0.04 ppm)
H6 (3.88 ppm)	cyclopropyl protons (0.77; 0.58 and -0.04 ppm)

signals are not intense which suggests a weak complexation. The expansion of the 2D spectrum of naltrexone is shown in Fig. 7, while the space-vicinity cross-peaks are listed in Table 6. A proposed structure of the inclusion complex based on the ROESY experiments is in Fig. 8.

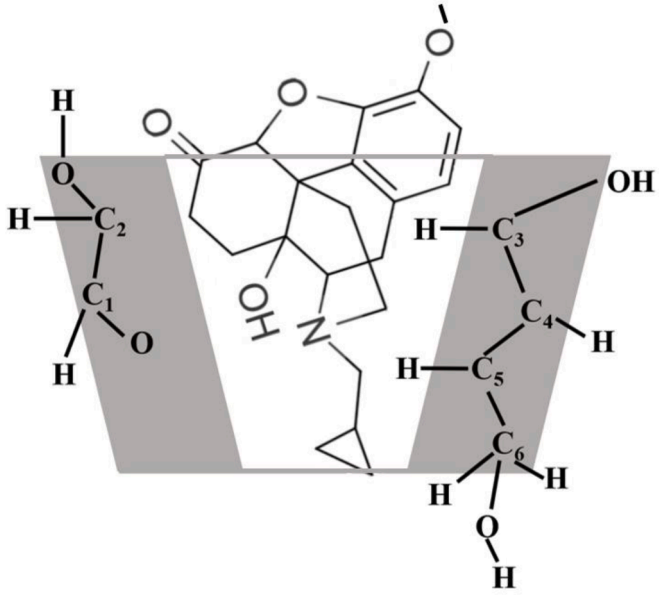

Fig. 8. Proposed structure of the naltrexone – β-CD inclusion complex, based on the ROESY experiment.

3.4. Stability of the complexes

^{1}H NMR spectroscopy is an efficient method for quantification of molecular interactions with regard to stability constants ranging from 10 to 10^4 (Fielding, 2000). ^{1}H NMR chemical shift changes are observable for both the ligand and CD protons as the chemical environment of some nuclei changes upon complexation (Wood et al., 1977; Wilson and Verrall, 1998). Consequently, the chemical shift titration method was used, followed by non-linear curve fitting (Schneider et al., 1998; Fielding, 2000). Robust, unbiased estimates of stability constants

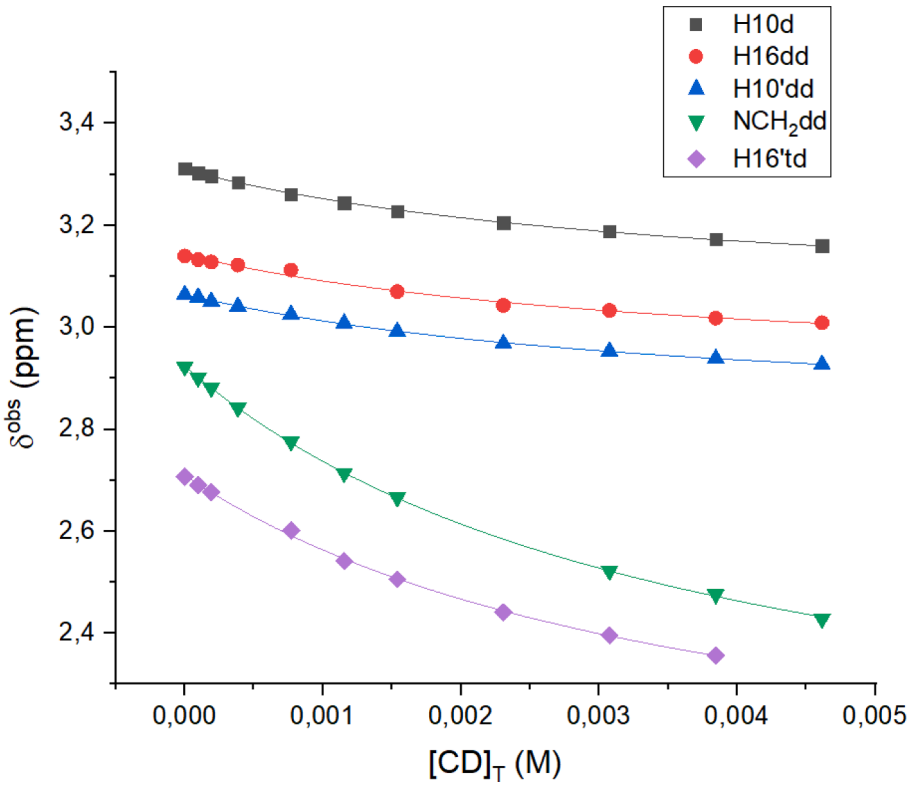

Fig. 9. The ^{1}H NMR titration curves of H10, H16 and NCH_2 protons of naltrexone as functions of the analytical β-CD concentration, together with the fitted curves.

Table 7
Apparent CD stability constants of the investigated opioid compounds at different pH values (standard error in parentheses).

Compound	pH	*K*	log*K*
nalbuphine	5.53	20.67 (6.52)	1.32
	8.64	537.1 (32.0)	2.73
	8.74	925.7 (29.1)	2.97
	8.91	876.5 (29.9)	2.94
	11.98	4441 (132)	3.65
naltrexone	5.53	14.67 (5.41)	1.17
	8.64	220.7 (12.9)	2.34
	8.74	279.3 (7.0)	2.45
	8.91	290.7 (10.1)	2.46
	11.98	1585 (33)	3.20
naloxone	8.24	440.6 (6.3)	2.64
	8.74	690.9 (12.9)	2.84
	12.36	657.0 (16.0)	2.82
nalorphine	8.24	504.1 (7.0)	2.70
	8.74	542.8 (13.1)	2.73
	12.36	499.9 (9.6)	2.70
dihydromorphine	8.91	205.3 (22.8)	2.31
	9.26	259.2 (5.1)	2.41
	12.36	326.1 (8.1)	2.51
morphine	8.74	199.9 (5.3)	2.30
	12.36	226.9 (7.1)	2.36
codeine	12.36	291.5 (5.9)	2.46
hydromorphone	8.69	196.1 (16.9)	2.29
	9.26	185.3 (10.0)	2.27
	12.36	162.4 (4.6)	2.21
oxymorphone	8.69	88.59 (21.12)	1.95
	9.33	70.22 (7.90)	1.85
	12.36	110.9 (2.4)	2.04

and small errors can be achieved by including datasets of several nuclei into the simultaneous evaluation (Béni et al., 2007). Besides obtaining the apparent stability constants at a given pH value, the NMR titration data also provide insight into the structure of complexes (Schneider et al., 1998; Fernandes et al., 2003; Ribeiro et al., 2005). During the titrations, the chemical shift of those nuclei changes the most that take part in the complex formation, as exemplified on naltrexone in Fig. 9. The experiments were carried out in buffer solutions in order to keep the pH constant during the titrations. Otherwise the protonation equilibria could also have some effect on the chemical shift values.

The observed chemical shift (δ^{obs}) of the nucleus in question is the weighted average of the free (L) and the complexed species (LCD):

$$\delta^{obs} = \delta_L \cdot x_L + \delta_{LCD} \cdot x_{LCD} \tag{5}$$

The formula of the stability constant is

$$K = \frac{[LCD]}{[L] \cdot [CD]} \tag{6}$$

where [LCD], [L] and [CD] are the equilibrium concentrations of the complex, the given opioid compound and the CD, respectively. In the knowledge of

$$[L]_T = [L] + [LCD] \tag{7}$$

and

$$[CD]_T = [CD] + [LCD] \tag{8}$$

the concentration of the complex can be expressed in terms of the analytical concentrations of L and CD, since only these quantities can be measured directly in contrast to the equilibrium concentrations. Combining Eqs. (5)., (7). and (8)., we can get the following result:

$$\delta^{obs} = \delta_L \cdot \frac{[L]_T - [LCD]}{[L]_T} + \delta_{LCD} \cdot \frac{[LCD]}{[L]_T} = \delta_L + \Delta\delta \cdot \frac{[LCD]}{[L]_T} \tag{9}$$

Table 8
pH-dependent distribution of opioid compounds in the presence of CD.

Compound	pH	log$K$	Mole fraction of the anionic microspecies	Mole fraction of the uncharged microspecies	Percentage of the unprotonated amino site
naloxone	8.24	2.64	0.1029	0.7805	88.34%
	8.74	2.84	0.2786	0.6683	94.69%
	12.36	2.82	0.9994	0.0006	100.0%
nalorphine	8.24	2.70	0.0584	0.9047	96.31%
	8.74	2.73	0.1678	0.8217	98.95%
	12.36	2.70	0.9988	0.0012	100.0%
dihydromorphine	8.91	2.31	0.1493	0.6082	75.75%
	9.26	2.41	0.3012	0.5481	84.93%
	12.36	2.51	0.9983	0.0014	99.97%
morphine	8.74	2.30	0.1853	0.7208	90.61%
	12.36	2.36	0.9990	0.0009	99.99%
hydromorphone	8.69	2.29	0.1929	0.6389	83.18%
	9.26	2.27	0.4917	0.4382	92.99%
	12.36	2.21	0.9992	0.0007	99.99%
oxymorphone	8.69	1.95	0.1267	0.4100	53.67%
	9.33	1.85	0.4491	0.3329	78.20%
	12.36	2.04	0.9990	0.0007	99.97%

where $\Delta\delta = \delta_{LCD} - \delta_L$.

With the help of Eqs. (6)., (7). and (8)., after rearrangement the equilibrium concentration of LCD can be obtained.

$$[LCD] = \frac{[L]_T + [CD]_T + \frac{1}{K} - \sqrt{\left([L]_T + [CD]_T + \frac{1}{K}\right)^2 - 4\cdot[L]_T\cdot[CD]_T}}{2} \quad (10)$$

Combining Eqs. (9). and (10)., the observed chemical shift of a given nucleus can be expressed using the analytical concentrations of L and CD, and the stability constant.

$$\delta^{obs} = \delta_L + \Delta\delta\cdot\frac{[L]_T + [CD]_T + \frac{1}{K} - \sqrt{\left([L]_T + [CD]_T + \frac{1}{K}\right)^2 - 4\cdot[L]_T\cdot[CD]_T}}{2\cdot[L]_T} \quad (11)$$

From δ^{obs} versus $[CD]_T$ datasets the stability constants (K) can be calculated by non-linear parameter fitting using Equation 11., as $[CD]_T$ was kept constant during the titration (Orgován et al.,2016).

Experiments were carried out in highly alkaline solutions and at a pH value near the isoelectric point of the given morphine derivative. In case of nalbuphine and naltrexone where we expected the largest log$K$ values there were also measurements in acidic solutions with a protonated amino group. The chemical shifts of other opioids in an acidic solution change to a very small extent (less than 0.01 ppm), thus complexes are too weak for accurate determination of the stability constants. The values of stability constants (K and log$K$) in solutions of different pH values are listed in Table 7.

These results are in good agreement with the ROESY spectra, since there were more and stronger cross peaks for compounds that have a longer side chain on the nitrogen atom, such as nalbuphine or naltrexone, where the stability is the highest.

In the knowledge of the apparent microscopic protonation constants in CD excess and the pH of the solutions, the pH-dependent distribution of macro- and microspecies can be calculated. The ratio of the major microspecies – the anionic, the uncharged and the sum of all microspecies with an unprotonated amino group – is presented in Table 8 alongside with the log$K$ stability constant at the given pH value.

As Table 8 shows, the unprotonated amino group always results in more stable inclusion complexes. For most compounds, there is no great difference between the stability of the uncharged and anionic form.

In addition, there is a correlation between the complex stability and the effect of β-CD on the protonation of the amino group. The more stable the inclusion complex, the greater drop occurs in the apparent basicity of the amino group. As the inner cavity represents a lipophilic moiety a positive charge is disadvantageous in terms of inclusion. Due to the presence of the β-CD, the apparent basicity of the amino site decreases, thus K_1 and K_2 protonation macroconstants largely characterize the major protonation pathway, with K_2 practically equal to k_O^N In Table 9 we collected the stability constants in highly alkaline solutions and the change of the value of logk_O^N, where

$$\Delta \log k_O^N = \log k_O^N \text{ of the complexed form} - \log k_O^N \text{ of the free compound} \quad (12)$$

Table 9
The apparent stability constants in highly alkaline solutions and the change of the value of logk_O^N.

Compound	log$K$	Δlogk_O^N
nalbuphine	3.65	-2.08
naltrexone	3.20	-1.39
naloxone	2.82	-1.13
nalorphine	2.70	-1.09
dihydromorphine	2.51	-0.98
morphine	2.36	-0.75
hydromorphone	2.21	-0.74
oxymorphone	2.40	-0.57

In case of nalbuphine and naltrexone we approximated the logk_O^N microconstants with the macroconstants of the O-methyl derivatives.

The correlation between the log$K$ and the Δlogk_O^N values can be seen in Fig. 10, with a correlation coefficient around 0.93, which represents good linearity. pH-independent ionization state-specific complex stability constants can also be calculated in the knowledge of the distribution of the microspecies and the apparent stability constants.

$$K = K_{an}\cdot x_{an} + K_{un}\cdot x_{un} + K_{ca}\cdot x_{ca}, \quad (13)$$

where subscripts stand for the anionic (an), uncharged (un) and cationic (ca) species, respectively. In highly alkaline solutions (in which the pH was approximately 12), morphine derivatives are present predominantly as the anionic species, thus the contribution of the uncharged and cationic form is insignificant to the apparent stability constant. Consequently, the K of the anionic species can be calculated at this pH. In solutions near the isoelectric point of the compounds the K of the

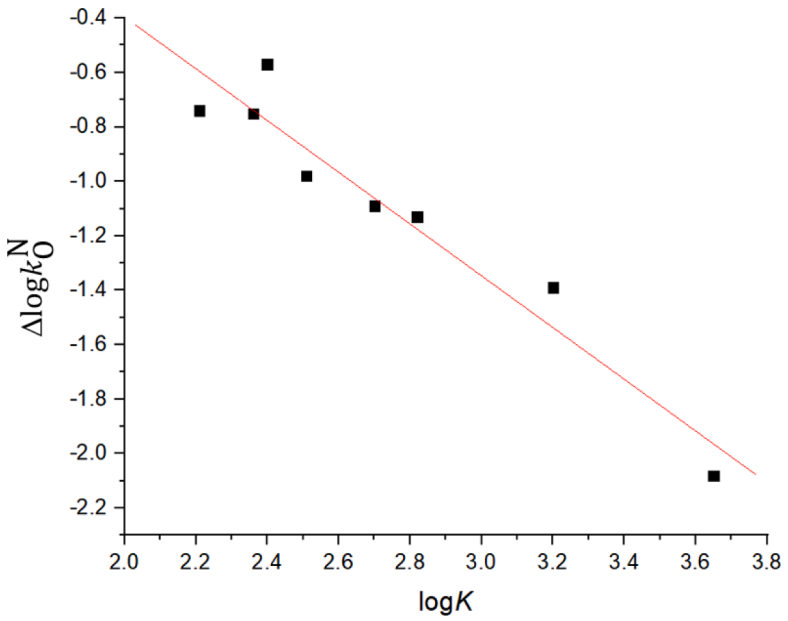

Fig. 10. The correlation between the log*K* and the $\Delta\log k_O^N$ values, computer fit is shown in solid line.

Table 10
Ionization state-specific complex stability constants.

Compound	log*K* of the anionic species	log*K* of the uncharged species
nalbuphine	3.65	not known
naltrexone	3.20	not known
naloxone	2.82	2.78
nalorphine	2.70	2.73
dihydromorphine	2.51	2.44
morphine	2.36	2.34
hydromorphone	2.21	2.40
oxymorphone	2.04	2.02

uncharged species can be obtained by subtracting the contribution of the anionic form. We carried out these calculations at each pH and as a result, the average value of the ionization state-specific stability constants can be seen in Table 10. In the case of nalbuphine and naltrexone not all the microconstants are known, thus log*K* of the uncharged species could not be calculated.

Typically there is only a small difference between the log*K* of the anionic and the uncharged species. The reason is that both the phenol or phenolate groups equally lie outside the CD cavity, therefore this protonation state influences the stability constant to a little extent only. Similarly, the replacement of the phenol with a methoxy group in the morphine-codeine pair hardly changes the stability constant.

4. Conclusions

In this study, the interaction of opioid compounds with β-cyclodextrin was characterized with regard to ionization state-specific complex stability constants. After proving the 1:1 stoichiometry of the inclusion complexes ^{1}H NMR-pH titrations were carried out to obtain the protonation constants of the free and complexed morphine derivatives. By means of the deductive method, using N- and O-methyl derivatives the protonation microequilibria were fully described.

The geometry of the inclusion complexes was elucidated by the 2D ROESY technique. The complex stability NMR measurements confirmed the results of ROESY spectra. Introducing extended side chain on the nitrogen atom results in an enhanced stability of the CD-complex. The unprotonated (-NH_2) form of the amino group, contrary to its protonated –NH_3^+ counterpart, causes a relatively large stability constant value. On the other hand, the protonation state of the phenolate site has slight effect only on the complex stability, since latter is positioned outside the CD cavity.

CRediT authorship contribution statement

Boglárka Tűz: Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. **Béla Noszál:** Writing – review & editing, Supervision. **Sándor Hosztafi:** Resources. **Károly Mazák:** Conceptualization, Writing – review & editing, Supervision.

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