Different Approaches of Drugs Interactions Studied using Affinity Capillary Electrophoresis

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List of abbreviations

ACE	affinity capillary electrophoresis
BS	bile salt
CD	cyclodextrin
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
DSC	differential scanning calorimetry
EOF	electroosmotic flow
FTIR	fourier transform infrared
GLB	glycopyrronium bromide
HPLC	high performance liquid chromatography
IBU	ibuprofen
IPA	ion pair agent
IPF	ion pair formation
NMB	neuromuscular blocking agent
NMR	nuclear magnetic resonance
NaGC	sodium glycocholate
NaGDC	sodium glycodeoxycholate
NaTC	sodium taurocholate
NaTDC	sodium taurodeoxycholate
PC	phosphatidylcholine
Pro	propranolol
PS	phosphatidylserine
SC	stratum corneum
SDS	sodium dodecyl sulfate
UV	ultraviolet

List of symbols

V_{app}	apparent velocity
μ_{ep}	electrophoretic mobility
μ_{eof}	electroosmotic mobility
μ_{eff}	effective mobility
E	electric field strength
U	applied voltage
Q	charge
R	radius
H	viscosity
ξ	zeta potential
ε	dielectric constant
T	migration time
L	length
K_a	association constant
k'	capacity factor
X	molar fraction
α	alpha
β	beta
γ	gamma
Log P	partition coefficient
δ	chemical shift

1 Introduction and Aims

In biopharmaceutics, research work is focused on studies of different types of drugs' interactions with specific receptors or ligands: complex formation, ion pairing, etc. These interactions achieve different pharmaceutical purposes which could safely enhance the drug bioavailability. To investigate and characterize such interactions, many effective methods using different analytical techniques were developed.

Cyclodextrin (CD) complexation is one of the most interesting interactions with drugs. Because of CDs' structure of the lipophilic cavity and the hydrophilic external periphery, CDs can interact with different drug molecules forming host-guest inclusion complexes. This complexation could modify the physicochemical properties of the guest drug, and as a consequence could be utilized for specific pharmaceutical purposes. CDs may be used as drug carriers at different administration routes. Because of the complex formation properties, they could enhance the bioavailability of some problematic drugs at target tissues, that are obtained by enhancing the drugs' solubility or stability. In literature, many methods have been described using different analytical techniques to investigate and characterize CD inclusion complexes, either in solid or in liquid form. Furthermore, affinity capillary electrophoresis (ACE) has been defined as a versatile analytical tool for analysis and determination of CDs and their derivatives concerning complex formation with relevant drugs.

In all previous studies, complex interactions were characterized for CDs as excipients in pharmaceutical formulations. In this work, complex interactions were characterized for a new modified γ-CD (sugammadex) as a drug itself. The extended lipophilic cavity depth of sugammadex encapsulates the molecule of steroid drug; rocuronium bromide, the neuromuscular blocking agent (NMB), to reverse its action after administration in post-surgery treatment. Whereas the eight negatively charged extensions in sugammadex structure enhance the electrostatic interactions with the positively charged neuromuscular agent to optimize the complex strength. From the other side, these adaptations could enhance the formation of chemical encapsulation with co-administrated drugs in physiological fluids, that as a consequence could affect the pharmacological actions for both sugammadex and co-administrated drugs.

Ion pair formation (IPF) is defined as interaction of oppositely charged molecules forming the neutral complex of the ionized molecules. The importance of such interactions to be used in pharmaceutical preparations comes mainly from their studied effectiveness in enhancing the

transport rate of the hydrophilic ionized drug across lipid membranes. The highly polar quaternary ammonium group of glycopyrronium bromide (GLB) limits its passage across lipid membranes such as the lipophilic stratum corneum (SC). In this work, IPF of the cationic drug (GLB) with different relevant anionic molecules was studied as a suggestion to enhance the drug penetration across the SC to reach the target site, the muscarinic receptor of sweat ducts.
Furthermore, the mechanism as well as the optimization of the drug release from CD complexes is most interesting in pharmaceutics. At lipid membrane level, only the free form of the drug which is in equilibrium with the complexed form could penetrate. Many mechanisms contribute to drugs' release from the inclusion cavity of CDs leading to improvement of their absorption. These mechanisms include simple dilution of complex, competitive displacement of drug from CD's cavity by exogenous and endogenous substances, drug binding to the protein, drug uptake into the tissue or CD elimination and changes in pH and temperature. This work is focused on the displacement of the drug from especially ß-CD cavity by cholesterol, which is the native biological membrane component. Liposomal vesicles were used to structurally mimic the native cell membranes, the phospholipid bilayer.
As mentioned previously, many studies developed analytical methods using different techniques like nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), etc. to investigate such kinds of interactions. Over other analytical techniques, capillary electrophoresis (CE) offers advantages of simplicity with high speed and great efficiency, saving time and low consumption of sample and buffer volumes. CE is defined as a separation technique to identify different analytes in one mixture by detecting their migration times in a fused silica capillary, and is related to the pH of the buffer, buffer type, voltage and temperature. ACE is one of the most interesting separation modes that depends on the change in the migration behaviour of an analyte to estimate its interaction with ligand or receptor.

In this work, efforts were aimed at investigating and characterizing different interactions of drugs with ligands using the technique ACE. The main objectives of this work can be defined as the following:

- To investigate the possibility of the NMB agent being replaced from the sugammadex cavity by co-administrated drugs like penicillins. And how that could affect the major pharmacological action of sugammadex in reversing the action of the NMB agent. This study was used for investigation of the interactions between sugammadex and penicillins using ACE, and evaluating their affinity strength by calculating the

association constant (K_a) using a non-linear curve equation. The significance of this study is that ACE, a fast and reproducible method for the assessment of complex formation of the new modified gamma CD has been used for the first time for this purpose. (Chapter 3)

- To evaluate the ability of a cationic drug to form ion pairs with anionic molecules and to investigate the possibility of IPF in improving the lipophilic properties of hydrophilic drugs by enhancing its partition behaviour. This study aimed at investigation of IPF between the cationic drug (GLB) and different anionic molecules and evaluation of their formation strength (K_f) using ACE. Studying the effect of the formed ion pair on the partitioning behavior of the drug, from the aqueous phase into the lipophilic n-octanol phase. Therefore, the partition coefficient of the drug in the absence and presence of the ion pair agents (IPAs) at different concentrations was measured using the shake-flask method. Determination of the relationship between the affinity strengths and the partition coefficients to optimize the IPF for GLB with the suitable IPA. (Chapter 4)

- To develop a simple method that could illustrate the release of lipophilic drugs from the CD cavity at the target site and the transfer into the lipophilic membrane enhanced by replacement of the drug from the CD by cholesterol. This study aimed at investigation of the interaction between liposomes as a model membrane and a CD complex of the drug using ACE. Development of an ACE method to study the release of a lipophilic drug from ß-CD complexes and its subsequent transfer into liposomal vesicles and to investigate the influence of cholesterol as a constituent of the liposomes by comparing buffer and sample systems with pure phospholipid liposomes and liposomes with incorporated cholesterol. The drug release from the ß-CD complex and the transfer of the drug into liposomal membrane, would be followed by evaluation of the migration data. (Chapter 5)

2 Theoretical Background

2.1 Capillary Electrophoresis

CE is defined as a high-performance analysis technique and separation method developed from traditional gel electrophoresis. In 1980s, electrophoretic separation efficiency has been improved in CE by the introduction of micrometers diameter fused silica capillary as a separation compartment where solutes in a buffer solution migrate under high voltage up to 30 kV. The reductions in analysis time and in broadening the zone of separation were advantages over the traditional methods. In the late 1980s, CE as an instrument was on the market. Much literature documented the applications of CE in different fields such as pharmaceutics, biopharmaceutics, medicine, biochemistry and chemistry.

2.1.1 Instrumentation

CE has a simple instrumental structure as seen in figure 1. CE apparatus is composed of the following: a high voltage power supplier, two electrodes, a capillary as separation compartment, two buffer reservoirs (electrode jars) where the ends of the capillary are immersed and a detector. Two injection systems are used, which are working in hydrodynamic mode and in electrokinetic mode [1].

The voltage source should supply voltages up to 30 kV and currents up to 200 – 300 μA. A fused silica capillary is the one most widely used due to its chemical and physical resistance and high thermal conductance. An external polyimide layer protects the capillary with an internal uncoated silica surface. Some internal surface could be coated by polymers. Fused silica capillaries are produced with a narrow internal diameter (between 20 and 100 μm) and with length between 20 and 100 cm. It is somewhat cheap in cost.

The detector is placed a few centimeters before the end of the capillary immersed in an output buffer jar. Ultraviolet (UV) radiation is passed through the transparency window in a fused capillary. The window is manually made by burning. Capillaries with windows at different sizes and shapes are also manufactured. In addition to UV detection that mostly applies in CE, a variety of other detection techniques are used such as: laser induced fluorescence, conductimetric, electrochemical amperometric and mass spectrometric for forensic applications.

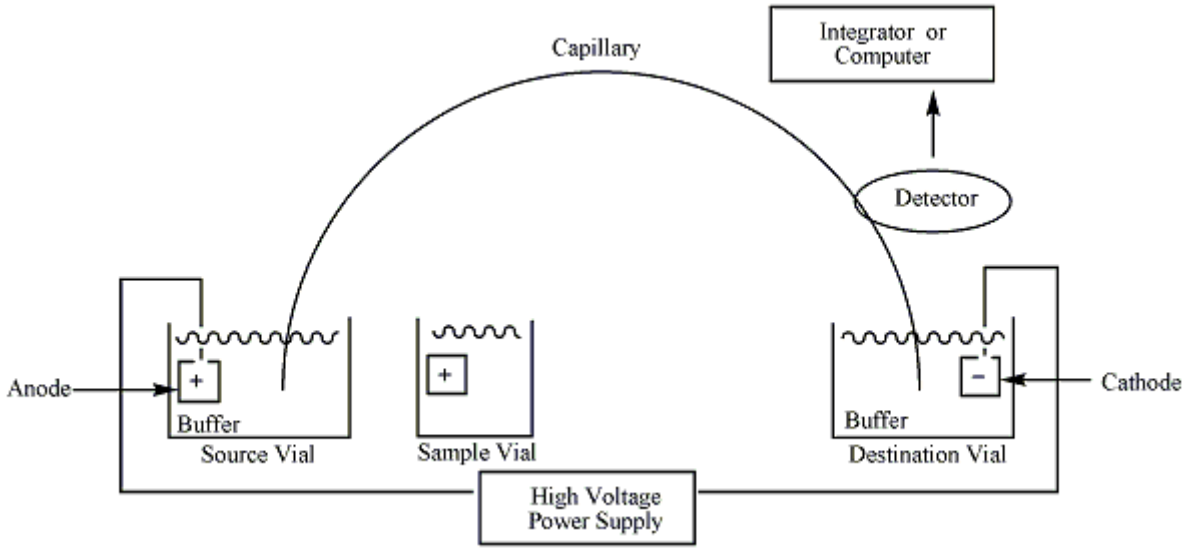

Figure 1 Capillary electrophoresis instrumentation

2.1.2 Separation Modes

Based on the type of used buffer and/or the capillary composition, the separation modes for the same hardware system of CE are classified into: capillary zone electrophoresis (CZE), capillary isotachophoresis, micellar electrokinetic chromatography, capillary electrochromatography, capillary gel electrophoresis, capillary isoelectric focusing and affinity capillary electrophoresis (ACE) [2].

2.1.2.1 Separation Principle

Charged solutes move through the buffer containing the capillary under the force of an electric field. This motion is expressed by the electrophoretic velocity term. The electrophoretic velocity of the charged solute is produced from its electrophoretic migration and the electroosmotic flow (EOF). So, the apparent velocity (V_{app}) is the sum of the vector of the electrophoretic mobility (μ_{ep}) and the electroosmotic mobility (μ_{eof}) in an applied electric field (E):

$$V_{app} = E\,(\mu_{ep} + \mu_{eof}) \qquad \textbf{Equation 1}$$

The electrophoretic migration velocity of the solute (V_{ep}) is represented as the following:

$$V_{ep} = E\,\mu_{ep} = \frac{q.E}{6\pi.\eta.r} \qquad \textbf{Equation 2}$$

The electrophoretic mobility of the ionic solute as shown in equation (2) depends on the ratio of charge to radius of the solute (q/r) and the viscosity of the solution (η). Moreover, the

mobility is affected by the dissociation degree of the ionizable group and therefore depends on the pH of the solution and the pK_a of the group.

The mobility of the solute in free zone electrophoresis is influenced also by the electroosmotic flow. Due to electrostatic forces, the positively charged ions (cations) in the buffer are attracted to the ionized silanol groups (at pH > 2) attached to the inner surface of the fused silica capillary and form an electrical double layer; one rigid and one movable diffuse layers. The electroosmotic flow is generated from the movement of the cations in diffuse layers towards the cathode. So, the electroosmotic velocity is the mobility of the buffer through the capillary under an applied electric field and is expressed by the following equation:

$$V_{eof} = E\,\mu_{eof} = \frac{\xi.\varepsilon.E}{4.\pi.\eta} \qquad \textbf{Equation 3}$$

Where (ξ) is the zeta potential at the capillary-buffer interface and (ε) is the dielectric constant of the electrolyte solution.

The velocity is defined as a rate of change of position with respect to time. In a capillary, the apparent migration time of a solute or drug (t_d) which is required for the solute to move from the beginning of capillary to the detection window, could be calculated from the distance between the inlet and the detection window (L_D) and the apparent velocity, so:

$$\mu_{app} = \frac{L_D}{t_d.E} \qquad \textbf{Equation 4}$$

E is calculated from ($E = U/L_T$); L_T is the total length of capillary and U is the applied voltage. Then the effective mobility of solute (μ_{eff}) obtained from equation 1 is the following:

$$\mu_{eff} = \mu_{app} - \mu_{eof} = \frac{L_D L_T}{U}\left(\frac{1}{t_d} - \frac{1}{t_{eof}}\right) \qquad \textbf{Equation 5}$$

2.1.2.2 Affinity Capillary Electrophoresis

ACE is an electrophoretic mode that is dedicated to investigate and determine the molecular interactions of the solute or drug with specific receptors or ligands. ACE is a widely applicable technique for estimating the binding strength of the molecular interactions such as drug CD complexes, micellar and liposomal drug interactions, drug anion interactions, etc. The ACE method depends mainly on the change in the migration pattern of interacting molecules to identify and quantify specific binding and estimate binding constants.

Principle: Because of the increase of the ligand (L) concentration, complexes (SL) are formed, which in turn leads to changes in the electrophoretic mobility of the injected substrate (S) into a capillary.

The equilibrium between complex and free molecules was achieved at a 1:1 ratio of ligand to substrate [3]:

$$S + L \rightleftharpoons SL \qquad \textbf{Equation 6}$$

By the law of mass action, the association constant (K_a) of the complex in solution is determined by the following equation:

$$K_a = \frac{[SL]}{[S].[L]} \qquad \textbf{Equation 7}$$

It is assumed that, first, there is a fast adjustment of equilibrium between free and bound substrate and second, there are no interactions between the substrate or ligand and the capillary wall. The net effective mobility of (S) is the sum of effective motilities of free (S) and bounded (SL) forms and (x) is the molar fraction:

$$\mu_{eff} = x_S \,.\, \mu_S + x_{SL} \,.\, \mu_{SL} \qquad \textbf{Equation 8}$$

The molar fractions are defined as:

$$x_S = \frac{[S]}{[S]+[SL]} \text{ and } x_{SL} = \frac{[SL]}{[S]+[SL]} \qquad \textbf{Equation 9}$$

The molar ratio of bound to free substrate is defined as the capacity factor (k') since the molecules dissolved in the same volume, then:

$$k' = \frac{[SL]}{[S]} \qquad \textbf{Equation 10}$$

and combining equations (7) & (10):

$$k' = K_a \,.\, [L] \qquad \textbf{Equation 11}$$

and combining equations (9) & (10):

$$x_S = \frac{1}{1+k'} \qquad \textbf{Equation 12}$$

$$x_{SL} = \frac{k'}{1+k'} \qquad \textbf{Equation 13}$$

When the molar fractions as present in equations (12) & (13) are replaced in equation (8), the effective mobility of (S) would be:

$$\mu_{eff} = \frac{\mu_S + K_a.[L].\mu_{SL}}{1+K_a.[L]} \qquad \textbf{Equation 14}$$

As seen in equation 14, the association constant K_a could be obtained from a nonlinear regression analysis of a plot of μ_{eff} against [L]. μ_{eff} is calculated from equation 5 at the corresponding L concentration.

2.2 Cyclodextrins

2.2.1 Definition

CDs are cyclic oligosaccharides, obtained from enzymatic degradation of starch [4]. The natural α-, β- and γ – CDs are the most common, which consist of 6, 7 and 8 (α-1, 4) linked α-D-glucopyranose units, respectively. The glucopyranose units of the chair formation are arranged together to form the cone shape of the CD molecule with both wide and narrow edges, which are substituted to secondary and primary hydroxyl groups (-OH), respectively. The structures of CDs with a central lipophilic cavity and an outer hydrophilic surface are shown in figure 2. The structure of CD, mainly the steric parameters of ligands (geometry and size), makes them capable of interacting with different drug molecules forming host-guest inclusion complexes. This complexation could alter the physicochemical and the biological properties of the guest drug, and as a consequence it could be utilized for pharmaceutical purposes.

γ – CDs have the highest solubility in water compared to α- and β – CDs which are lower in solubility. In general, the aqueous solubility of the natural CDs is limited, due to the strong intramolecular hydrogen bonds. Many scientific papers described the structural modifications of the natural CDs. In addition to improving the solubility of CD derivatives, specified modifications have been aimed pharmaceutically at improving the guest drug association to the CD derivative molecule [5].

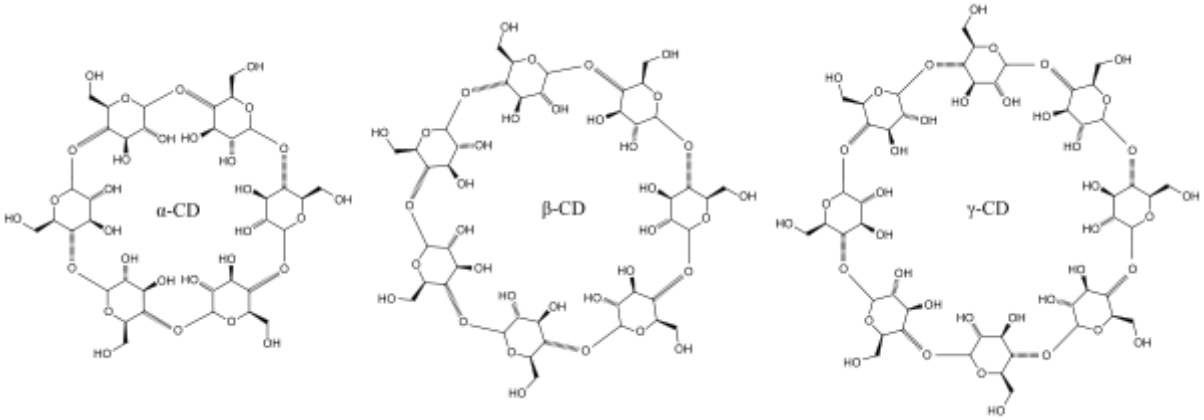

Figure 2 The chemical structures of natural cyclodextrin

2.2.2 Pharmaceutical Applications of CDs

The use of CDs as **drug carriers** at different administration routes is the most common pharmaceutical application. CDs are added as excipients to the pharmaceutical formulations containing drugs. At these formulations, the complexes of drugs to the host molecule could modify their bioavailability by enhancing the solubility of poorly water soluble drugs, as well as the stability for some problematic drugs, and control the drug delivery.

The naturally occurring CDs are generally safe when administrated orally and topically. In oral application, the hydrophilic CDs improve the dissolution rate and its extent for poorly water soluble drugs through complex formation [6]. For example, the hydroxylpropyl-β-CD acts as a powerful solubilizer agent in the oral tablet of ursodeoxycholic acid and hydroxypropyl-ß-CD (HP-ß-CD) complex [7]. Also, ß-CD enhances the absorption of an oral antifungal drug (ketoconazole) into the lipophilic membrane by increasing the dissolution rate of the drug which was encapsulated into the CD cavity. These applications achieve their aims only when the rate-limiting step in the absorption process is a dissolution step, not the ability of the drug to be absorbed into the biological membrane. Moreover, CDs improve the stability of drugs that are liable to hydrolysis, dehydration and oxidation and as a consequence increase the shelf life of drugs. γ–CD enhances the bioavailability of digoxin formulated in sublingual tablets by forming a complex that protects the drug from acid hydrolysis [8]. Other examples of CD complexes which have an enhancement effect on the solubility and the stability of various orally administrated drugs; are listed in [6, 9]. Ocular preparations of HP-ß-CD/ dexamethasone acetate complexes are useful to enhance the corneal bioavailability of drugs [10]. By changing the nasal mucosa permeability, heptikas-(2, 6-di- O-methyl)-ß-CD enhances the bioavailability of morphine at the nasal absorption site. For rectal delivery, the release of an anti-inflammatory drug (flurbiprufen) from a suppository base is enhanced by forming a complex with the hydrophilic CD [8]. At parenteral administration routes, the low aqueous solubility and toxicity of applied molecules should be taken into account. Therefore, because of the nephrotoxicity and low solubility of β-CD and the aggregation formation of γ-CD in aqueous solution, they are not used parenterally. The hydrophilic derivatives of CDs have been mainly used to improve the solubilization and stability of drugs and reduce drug irritation at the site of administration; e.g. hydroxypropyl-β-CD [11, 12].

Modified CDs were also designed and synthesized to be used as **medications**. They serve as host molecules for NMB agents to reverse their actions [13]. To encapsulate the steroidal drug,

the modifications were derived to achieve the needed influence of the hydrophobic cavity. The NMB agents have a positively charged quaternary ammonium group. Negatively charged groups were substituted to the CD's outer surface, to enhance the affinity of drug complexation to CD by strong electrostatic interactions. The complexation induced by these modifications should appropriately enhance the reversal action of NMB agents after surgery. The sugammadex-rocuronium complex is one of the strongest among CDs and their guest drugs.

A modified γ-CD, sugammadex, is a novel compound used in anaesthesia in post-surgery treatment to reverse the muscle relaxant effect of rocuronium [14]. Sugammadex is derived from γ-CD by placing eight carboxyl thio ether groups at the sixth carbon positions as shown in figure 3. These structural modifications extend the lipophilic cavity depth of the CD leading to greater encapsulation of rocuronium. The negatively charged extensions enhance the aqueous solubility of CD and the electrostatic binding to positively charged rocuronium. At the neuromuscular junction, sugammadex, the host molecule, encapsulates the free rocuronium molecules and as a consequence induces the drug's dissociation from the nicotinic acetylcholine receptor to be replaced by acetylcholine neurotransmitter. Sugammadex works as a reversal NMB agent without inhibition of the acetylcholinesterase avoiding undesirable side effects [15].

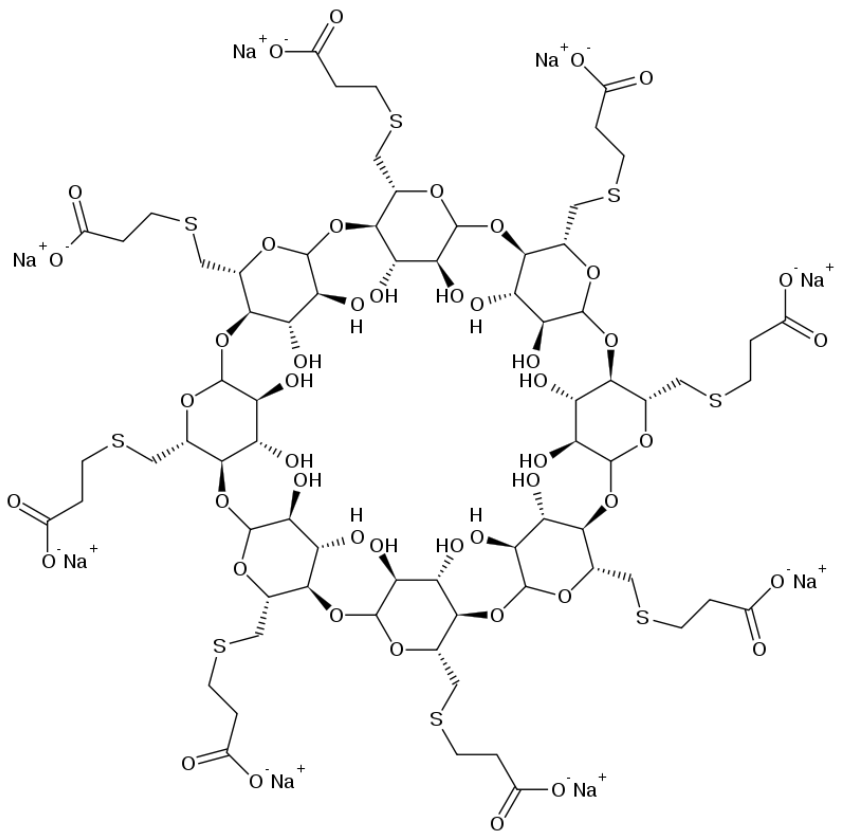

Figure 3 The chemical structure of Sugammadex

2.2.3 Characterization of Cyclodextrins Complexes

Various analytical methods have been used to investigate and characterize CDs' inclusion in complexes, either in solid form or in solution [16]. The complex interactions between drug and CD are accompanied by changes in the physicochemical properties of the guest drug based on the host concentration. By detecting such changes, the complex formation could be investigated and characterized.

The thermo-analytical methods are common techniques in characterization of the multicomponent systems like inclusion complexes of CD in solid state. As an example, differential scanning calorimetry (DSC) evaluated the complex formation by detecting the changes in certain thermal peaks of the drugs upon complexation. Furthermore, the complex formation between CD and drugs in solid state samples could be investigated by comparing the IR spectra of the free drugs and the complex forms using Fourier Transform Infrared (FTIR). The shifts in the absorbance bands, which are due to atom group vibrations, could also indicate the involved groups in the complexation of the binary systems drug-CD [16].

In solution, the equilibrium between the free form of the drug and the complexed one is expressed by the association constant (K_a), which is a quantitative parameter that evaluates the affinity strength of host-guest complex. Many techniques were used to measure the K_a of such complexes. Besides solubility studies, spectroscopic ones are commonly used. One of the most effective spectroscopic methods used to investigate the complex formation between the CD and the drug is *Nuclear Magnetic Resonance* (NMR) [17-19]; ^{1}H NMR. A COSY spectrum has been used for assignment of protons, while a ROSY spectrum has been used for representation of the complex interaction between the drug and the CD. The complexation could be determined by the changes in the chemical shifts of the CD protons, mainly H3 & H5, in position and generally all guest drug protons. The change in chemical shift between free and complexed form expressed by $\Delta\delta$. K_a of the complex interaction could be detected by different methods [20], such as plotting of the change in the chemical shift for drug proton or CD proton versus the corresponding CD or guest drug concentration, respectively. By NMR titration data, the stoichiometry of the drug CD complex should be first determined. A fluorescence spectroscopy was also used to determine the association constants of ß-CD and γ-CD complexation with some drugs [21].

Moreover, the changes in the retention time of the drug in the absence and presence of the CD were detected by HPLC indicating the complex formation. By plotting the changes versus the corresponding CD concentrations, the association constants could be calculated [22].

ACE has been defined as a versatile analytical tool for analysis and determination of CDs and their derivatives' complexations [23-25]. Plätzer et al. [25] described ACE as a simple handling method to study the interactions between five types of CDs and different drugs. In principle, ACE is identifying and quantifying K_a depending on the migration behavior of the interacting molecules, as described extensively in [3]. The analyte or drug (D), which is injected into a capillary filled with a normal buffer, migrates toward the cathode in proportional to its electrical charge and in inverse proportion to its hydrodynamic radius, which is related to the molecular mass. The migration of the drug is expressed by its electrophoretic mobility (equation 5). In the presence of the receptor (CD), dissolved at varied concentrations of defined range in the running buffer, the changes in the electrophoretic mobility could occur as a consequence of the complex formation. These changes are based on the size and charge of complexation partners and on the affinity strength of the complex. Per equation (5), the electrophoretic mobility of the free drug in the buffer solution through capillary is determined by its migration time obtained from the electropherogram. As the concentration of neutral CD increases in the electrolyte buffer, the drug peak shifts towards the EOF peak due to the complex formation and the mobility decreases. The complexation degree increases as the CD concentration increases. At the concentration corresponding to the maximum drug peak shift, the complexation is the optimum (DCD) and the equilibrium is achieved. By plotting the effective mobility of the free drug in CD containing the buffer versus the corresponding CD concentration, the K_a and effective mobility of complex could be determined from a nonlinear least squares curve-fitting equation:

$$\mu_{eff} = \frac{\mu_D + K_a.[CD].\mu_{CDC}}{1 + K_a.[CD]} \qquad \textbf{Equation 15}$$

Several hypotheses described in [26]; assume that the non covalent bonds are involved in CD complexations. The hydrophobic interactions and hydrogen bonds are the major driving forces. In addition, electrostatic interactions take place in complex formation for appropriately substituted CDs such as sugammadex.

2.2.4 Mechanisms of Drug Release from CD

CDs as drug carriers should deliver the drugs into the target tissues. This function could be completely achieved by releasing the drug from the CD cavity at the lipid membrane and by transferring the drug into the membrane. The high molecular weight and the low partition coefficient of CDs prevent their penetration through a lipid membrane. Only the free form of the drug, which is in equilibrium with the complexed one, could penetrate. So the absorption

enhancement effect of the CD for the guest drug could be improved by the mechanisms that increase the dissociation rate of the CD-complex leading to release of the drug from the CD's cavity. Some studies have discussed the possible mechanisms that affect the drug's delivery from CD complexes into biological membranes. The **dilution** enhances the release of the drug from the CD cavity depending on the affinity strength of the complex and the route of administration. Simulations confirm that the dilution factor is an effective and attainable contributor to the drug's release from the weak complex, especially after injection or oral administration [27]. For topical administration, such as the ophthalmic one, the dilution is minimal. For strong complexation, the dilution alone will not have a significant releasing effect, so the contribution of other mechanisms will play a role in a significant release of drugs from the CD cavity. The **competitive displacement** of the drug from the CD cavity by exogenous or endogenous substances enhances the drug's availability at the absorption site. In cases of relatively strong complexes, exogenous substances added to the formulations will compete the drug for the CD cavity, leading to acceleration of the dissociation rate of complexed drug and increasing the presence of the free form of the drug at the absorption site; as a consequence, this enhances the drug's bioavailability through the lipid membrane. Also, some endogenous substances like cholesterol, present in the biological membrane, could play the same role. In addition, the interaction of free CD and the membrane components could alter the transport properties of the membrane and as a consequence enhance the drug's absorption. Many studies have tried to investigate and explain such mechanisms [27-32]. Also, the ability of the drug to **bind protein** reduces the complexed fraction of drug to cyclodextrin. In simulations, the affinity strength and protein concentration increased the dissociation rate of drug-CD interaction, which is accelerated. In cases of the drug's administration where the dilution and competitive interactions are limited, the drug's **uptake into tissue** could enhance the release of the drug from the CD cavity. Other mechanisms and their effects on the release of the drug from the CD cavity at the absorption site, and the consequent drug bioavailability, were also investigated: **CD elimination** and **change in ion state** and **temperature**.

2.3 Ion Pair Formation (IPF)

An ion pair is generally defined as a pair of oppositely charged ions (cation; positively charged ion and anion; negatively charged ion) which associate together by coulomb forces. IPF could be described as a mechanism that enhances the absorption or penetration of charged hydrophilic drugs through biological membranes, i.e. gastrointestinal tract, skin, etc. Per pH-partition hypothesis, the highly polar group of some drugs limits their passage across lipid membranes.

At the pH of the biological membrane, the drug is present in ionized and unionized forms depending on its pK_a. The absorption rate of the drug through the lipid membrane is proportional to the concentration of the unionized form and the partition coefficient. Since some drugs remain ionized over a wide range of pH, their partition coefficients are close to zero and their absorption behaviour is almost negligible. Addition of a lipophilic counter ion to the charged drug forms an ion pair which is electrically neutral and has lipophilic properties. As a consequence, the bioavailability of these problematic drugs could be improved. This improvement in the bioavailability of such drugs is due to the change in their physicochemical properties upon the ion pair interactions. In case of using counter ion molecules whose structures contain a lipophilic group (or tail), the lipophilicity of the drug was increased and consequently, the partitioning of the drug throughout of the lipid membrane into their action sites was facilitated [33, 34]. So to optimize the effect of IPF, a suitable counter ion should be identified. The lipophilicity, sufficient solubility, physiological compatibility and metabolic stability are the needed properties of the counter ion for IPF.

Many studies were aimed at improving the lipophilicity of *the hydrophilic ionizable drugs* by IPF. *In vitro partition studies* investigated the absorption and the partition behaviour of the ionized drug from an *aqueous phase* to a *non-aqueous* one in the absence and presence of a suitable counter ion at different concentrations for several cationic and anionic drugs. To evaluate any change in the lipophilicity of ionizable drugs by IPF, *the partition coefficient* log P is determined as the ratio of concentrations of drug (or solute) in a mixture of two immiscible phases at equilibrium. Most commonly, the non-aqueous solvent is a hydrophobic one such as n-octanol. A phosphate buffer as aqueous phase was recommended for use in the investigation of the ion pair partition behaviour. Many methods of measuring log P were developed, such as the shake-flask, reverse phase HPLC, and pH-metric techniques. *The shake flask method* is the classical and the most reliable method, used commonly [35]. Factors like temperature, pH, etc. could affect the determined value of log P, and should be take into consideration.

The partition behaviour of isopromide iodide, a quaternary ammonium drug from a physiological phosphate buffer into n-octanol, is increased in the presence of the bile salt, sodium glycocholate, in concentration below CMC. Isopromide iodide has no partition in absence of the bile salt. The absorption enhancement returned to the possible situation of IPF between the drug cation and the bile salt anion [36]. It was also reported that the relation between IPF and the enhanced drug absorption especially through the GI tract, occured for other quaternary ammonium drugs [37].

The influence of IPF on the transport of hydrophilic drugs was studied in vitro using different artificial membranes [33]. IPF with fatty acids increased the lipophilicity of cationic drugs and facilitated their transport across isopropyl myristate membranes and their in vitro permeation through excised human skin [34]. The penetration of erythromycin (ERY), the topical treatment of acne vulgaris, into a modified multilayer membrane system (MMS) is enhanced when ion pairing between ERY and octadecansulfonate was used in comparison with the penetration of the ERY base alone [38]. The influence of an ion pair on ex vivo penetration of ERY was also investigated [39].

IPF is an equilibrium reaction between the ion pair complex (C) and the two other component ions, the drug (D) and the counter ion (CI), which is expressed as the following at 1:1 molar ratio:

$$\mathbf{D + CI \rightleftharpoons C} \qquad \textbf{Equation 16}$$

K_a is the association constant that determines the formation strength of the ion pair. This parameter K_a could evaluate the absorption behaviour of the drug as an ion pair. As the potential of K_a increases, the absorption potential increases.

To characterize the ion pair interaction and its strength, K_a is determined. As previously described in section 2.2.3, many different methods were used to characterize molecules' interactions by calculating K_a. Depending on the migration behaviour of the interacting molecules, the K_a of the ion pair interaction is quantified using ACE as described previously in 2.2.3. At the concentration corresponding to the maximum drug peak shift, the ion pair is completely formed and the equilibrium is achieved. The ion pair complex has a neutral charge and as a consequence its migration time would be like the EOF migration time. Thus, the effective mobility of the ion pair complex (μ_c) would be zero and the effective mobility of the drug is defined as [40]:

$$\mu_{eff} = \frac{\mu_D}{1 + K_a \cdot [CI]} \qquad \textbf{Equation 17}$$

3 Study of Interactions between Sugammadex and Penicillins using Affinity Capillary Electrophoresis *(Article in Chromatographia 76(23-24)2013)*

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Abstract

Sugammadex, a new modified gamma cyclodextrin, reverses the neuromuscular blockage induced by rocuronium by forming a strong complex with this muscle relaxant. To evaluate possible interactions with potentially co-administered drugs, interactions between sugammadex and penicillins were investigated using the affinity capillary electrophoresis method for the first time. Capillary electrophoresis coupled to an ultra violet detector was used as an analytical device for the analysis and detection of cyclodextrin inclusion complexes. Changes in the effective mobility of guest drugs (amoxicillin, ampicillin, oxacillin, dicloxacillin and azlocillin) were correlated with the increasing concentration of host molecules in background electrolyte, and successfully fitted into a non-linear curve equation; assuming 1:1 stoichiometric interaction. The calculated association constants (K_a) were: 383.44, 184.54, 265.34 and 95.06 M^{-1} for amoxicillin, ampicillin, oxacillin and dicloxacillin, respectively. No complex formation with sugammadex could be detected for both penicillin G and piperacillin. The difference in the chemical structure of the penicillins, especially in the (R) side chains, is suggested to be responsible for the variety of binding strength between penicillins and sugammadex.The displacement study demonstrated that interactions between sugammadex and co-administered penicillins could reduce the pharmacological effects of both.

3.1 Introduction

Sugammadex is a new modified γ-cyclodextrin (CD) used in post-surgery treatment, reversing the neuromuscular blockage (NMB) induced by rocuronium bromide [41]. The host molecules with their extended lipophilic cavity depth encapsulate the molecules of the steroid drug, whereas their eight negatively charged groups enhance the electrostatic interactions with the positively charged guest molecules. Those adaptations increase the chemical encapsulation of rocuronium leading to the reversal of the neuromuscular blocking effect [42]. Former studies have characterized the complex of rocuronium bromide and sugammadex in solution by nuclear magnetic resonance (NMR) experiments and have determined the association constant by proton NMR chemical shift titration [19]. Isothermal titration microcalorimetry (ITC) data have confirmed the complexation strength [43]. The lipophilic cavity of CDs and in particular the previously described structural modifications of sugammadex could enhance the formation of inclusion complexes with other co-administered drugs in physiological fluids. These potential interactions may reduce the pharmacological effects of both sugammadex and the co-administered drugs. Penicillins (ß-lactam antibiotics) are widely used as antibacterial agents. Amoxicillin and ampicillin belong to the broad-spectrum aminopenicillin family. Penicillin G is an example of natural penicillins, which are preferred to treat many infectious diseases. Oxacillin and dicloxacillin are pencillinase-resistant penicillins. Piperacillin and azlocillin are included in the extended-spectrum of penicillin groups [44, 45]. In general, the structure of all penicillins is based on the same core penam ring, which is attached to the (R) side chain. The different functional groups are responsible for the variety of bindings between receptor or drug and different penicillins [46]. Various analytical methods have been used for the quantitative and/or qualitative analysis and the characterization of CDs inclusion complexes either in solid form or solution [17]. Affinity capillary electrophoresis (ACE) has been defined as versatile analytical tool for analysis and determination of CDs and their derivatives complexations [23-25]. The association constant Ka is a quantitative parameter that expresses the affinity strength of host–guest complex. In principle, ACE is identifying and quantifying K_a depending on the migration behavior of the interacting molecules, as described extensively in [3]. Plaetzer et al. [25]described ACE as a simple handling method to study the interactions between five types of CDs and different drugs. This study investigated the interactions between sugammadex and penicillins using ACE, and evaluated their affinity strength by calculating K_a using non-linear curve equation. Non-linear fitting has been used for estimating K_a, since it allows direct comparison of experimental and theoretical values and is therefore more accurate and precise

than linear one [47]. The significance of this study lies also in the fact that ACE a fast and reproducible method for the assessment of complex formation of the new modified gamma CD has been used for the first time.

3.2 Experimental

3.2.1 Materials

Sugammadex, Bridion_ 100 mg/ml, i.v. (intravenous) was purchased from N.V. Organon (Oss, Netherlands). Amoxicillin, ampicillin, pencillin G, azlocillin, oxacillin, dicloxacillin and piperacillin were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Potassium hydrogen-phosphate and Potassium di-hydrogen-phosphate were purchased from Merck (Darmstadt, Germany). Dimethylsulfoxid was purchased from Fluka Chemie AG (Buchs, Switzerland).

3.2.2 Methods

CE experiments were performed using a diode array detector (190–600 nm) coupled to a Hewlett Packard model G1600AX (Waldbronn, Germany) 3D CE system. The detection wavelength was 200 nm. The fused silica capillary had an internal diameter of 50 μm and a total length of 64.5 cm. The distance to the detector was 56 cm. Analysis conditions: a new capillary was rinsed first with 1 N NaOH at 40°C for 15 min followed by distilled water for 15 min. For each run, the capillary was rinsed with 0.1 N NaOH for 3 min followed by distilled water for 2 min. In the final step, the capillary was rinsed with the buffer solution for 5 min. The temperature was kept at 25°C and the separation voltage was 30 kV. The samples were injected at 50 mbar for 9 s. For each sample, the run was repeated three times.

Principle: As a consequence of the increase of the receptor (CD) concentration, complexes (DCD) are formed, which in turn leads to changes in the electrophoretic mobility of the injected drug (D). Assumptions first, fast adjustment of equilibrium between free and complexed drug; and second, no interactions between the drug or CD and the capillary wall.

The association constant of the inclusion complex in solution (Table 1) was obtained using the origin (7.0) program. The equilibrium between complex and free molecules was achieved at a 1:1 ratio of guest molecule to host molecule:

$$\mathbf{D + CD \rightleftharpoons DCD} \qquad \textbf{Equation 18}$$

When K_a is determined according to the following equation,

$$K_a = \frac{[DCD]}{[D]\,[CD]} \qquad \textbf{Equation 19}$$

where the net effective mobility of (D) is the sum of effective motilities of free and bounded forms and x is the molar fraction,

$$\mu_{eff} = x_D \cdot \mu_D + x_{DCD} \cdot \mu_{DCD} \qquad \textbf{Equation 20}$$

then:

$$\mu_{eff} = \frac{\mu_D + K_a.[CD].\mu_{DCD}}{1 + K_a.[CD]} \qquad \textbf{Equation 15}$$

where μ_{eff} is calculated from,

$$\mu_{eff} = \frac{L_D \cdot L_T}{U}\left(\frac{1}{t_m} - \frac{1}{t_{EOF}}\right) \qquad \textbf{Equation 5}$$

L_T is the total length of the capillary, while L_D is the distance from the capillary inlet to the detector. t_m and t_{EOF} are the migration times of the drug peak and the electroosmotic flow (EOF) peak, respectively. EOF is detected by neutral substance marker.

Table 1. Association constants calculated using nonlinear least squares curve-fitting equations

	K_a ± SD (M^{-1})	μ_{DCD} ± SD ($cm^2.s^{-1}.v^{-1}$)
Amoxicillin	383.44 ± 32.2	-2.93E-04 ± 2E-06
Ampicillin	184.54 ± 26.5	-3.23E-04 ± 2.2E-06
Oxacillin	95.06 ± 17.7	-6.24E-04 ± 3.1E-06
Dicloxacillin	265.34 ± 17.3	-5.08E-04 ± 1.4E-05
Piperacillin	0	-
Azlocillin	13.6 ± 43.4	-6.76E-04 ± 2.4E-06
Penicillin G	0	-

3.2.3 Buffer Preparation

For CE, 10 mM of phosphate buffer was prepared by dissolving 1.05 g potassium hydrogen-phosphate and 0.53 g potassium di-hydrogen-phosphate in 1 L distilled water with pH 7.2. The buffer solution was filtered using a 0.45 µm syringe filter. For ACE, buffer solutions were constituted using increasing concentrations of sugammadex (0, 1.88×10^{-4}, 3.75×10^{-4}, 7.5×10^{-4}, 1.5×10^{-3} and 3×10^{-3} M). All buffer solutions were degassed for 15 min using ultrasound.

3.2.4 Sample Preparation

100 µg/mL of each penicillin sample was prepared in distilled water and filtered through a 0.45 µm syringe filter. Dimethylsulfoxide (DMSO) was added to the samples as neutral marker.

3.3 Results and Discussion

The interactions between sugammadex and penicillins (amoxicillin, ampicillin, penicillin G, oxacillin, dicloxacillin, azlocillin and piperacillin) under the previously described conditions were investigated using ACE. The penicillins had negative electrophoretic mobilities. As shown in figure 4, the drug peak shifted away from EOF and the electrophoretic mobility (inversely related to the migration time) decreased as sugammadex concentrations increased in the running buffer. This change can be attributed to complex formation and is based on the increase of the total negative charge and the mass of inclusion complexes compared with the free drug [2]. The shifting in t_{EOF} could be attributed to any unexpected interaction between the neutral marker and the negatively charged CD. The interaction strength was estimated by calculating the association constant Ka using a non-linear curve fitting method (assuming 1:1 stoichiometric ratio). The relative standard deviation (%RSD) of the penicillins' effective mobilities ranged between 0.02 and 0.78, indicating the precision of the method.

For amoxicillin, ampicillin, oxacillin, dicloxacillin and azlocillin, the effective mobilities as a function of the increasing concentrations of sugammadex were correlated (Figure 5) and successfully fitted into Eq. (15). K_a values and complex effective mobilities were plotted in Table 1. No changes were observed for penicillin G and piperacillin, which allows the conclusion that no complexes were formed.

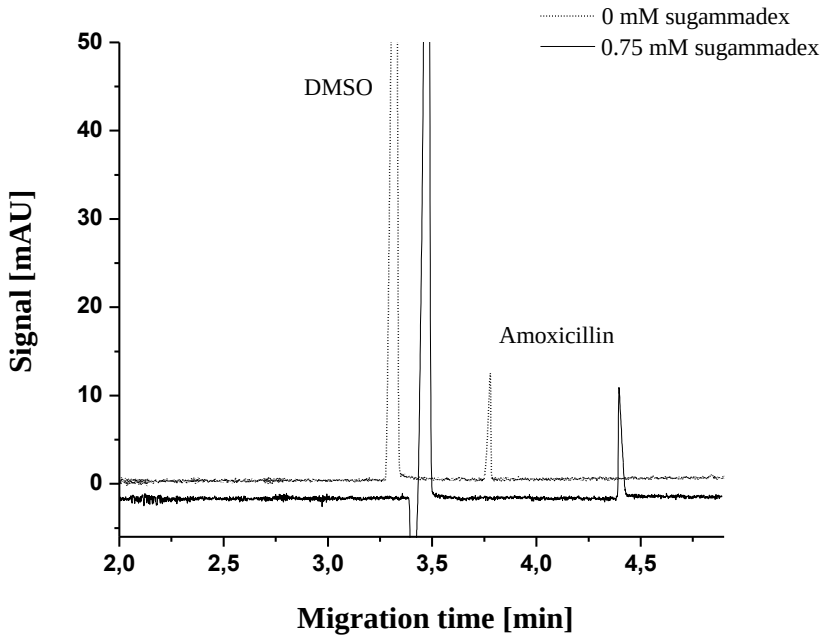

Figure 4 Electropherogram of amoxicillin for two different concentrations of sugammadex 0 mM (dash line) and 0.75 mM (straight line) in phosphate buffer 10 mM, pH 7.2

The affinity strength of the penicillins to sugammadex was arranged as following: Amoxicillin > dicloxacillin > ampicillin > oxacillin; the difference in (R) side chain groups is suggested to play a major role in explaining the differences in the binding activities between various penicillins and sugammadex. A hypothesis, described in [23], assumes that the non-covalent bonds are involved in the interaction between CDs and drugs [4]. Considering the chemical structures of sugammadex and the penicillins, hydrophobic interactions and hydrogen bonds are assumed to have occurred in the complex formation process. The absence of these functional groups responsible for the occurrence of the previously mentioned interactions in penicillin G is assumed to be the reason for the fact that penicillin G does not form complexes with sugammadex. For piperacillin, the steric effect is assumed to play a role. Since the complex concentration [DCD] could be calculated from Eq. (2), it was possible to estimate the amount (%) of penicillins needed to form complexes when coadministered with sugammadex. The calculated amount ranged between 2 and 10 % for those investigated penicillins that interacted with sugammadex. It is a well-established fact that any antibiotic treatment should ensure a preferably constant drug level in the bloodstream. The amount of penicillins that is consumed by complexation would lower their blood level and consequently reduce their pharmacological effect. Therefore, the clinical relevance of the potential interactions investigated in this study should be taken into consideration. Further studies may be necessary.

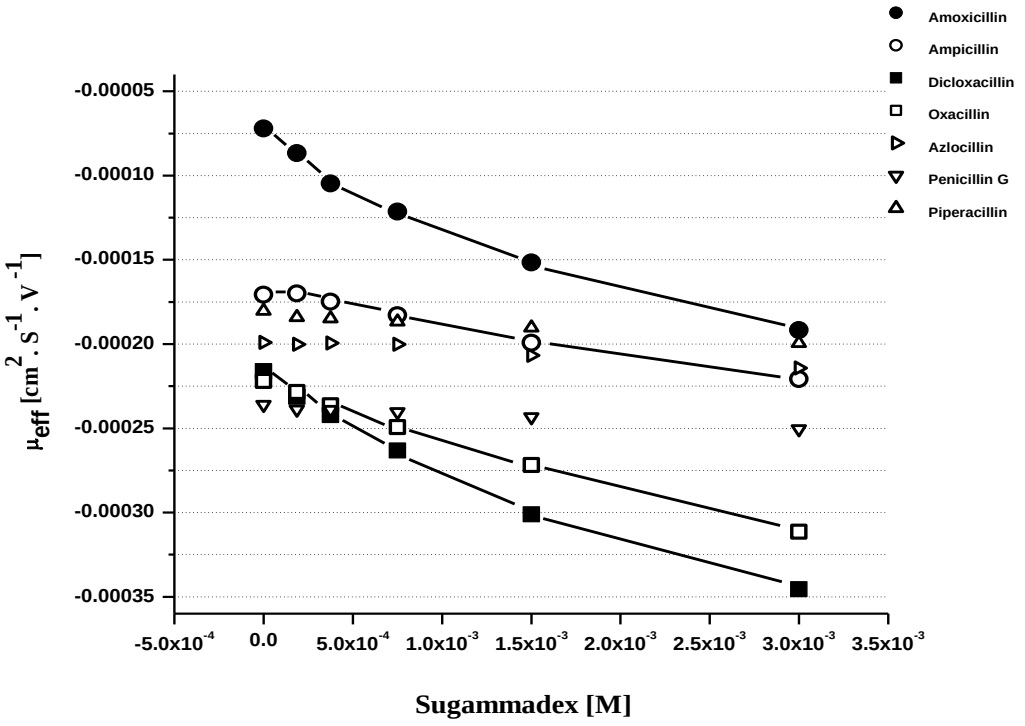

Figure 5 Interactions of sugammadex with penicillins; non-linear curve fitting of the effective mobility changes in corresponding to different sugammadex concentrations

Displacement Study

Penicillins are widely used as antibacterial agents; they can be injected or otherwise administered during or after surgery in case of infections. Thus, it is very likely that displacement interactions occur especially in the case of sugammadex being used for reversing rocuronium induced NMB. The possibility of the formation of penicillins-sugammadex inclusion complexes does exist, as shown in our study. The average electrophoretic mobility of amoxicillin (0.274 mM) was calculated (Eq. 5) after it had been injected into BGE that contained a mixture of sugammadex and rocuronium 1:1 (0.375 mM). The phenomenon of the decreasing electrophoretic mobility of amoxicillin after it had been injected into the sugammadex containing buffer was not present in this case: no change in mobility could be detected. This could be attributed to the strong affinity of rocuronium to sugammadex. The same could be observed in the case of a higher concentration (3 mM). This study confirmed that the administration of penicillins ($K_a \leq 383.44\ M^{-1}$) will not affect the complex formation between sugammadex and rocurronium.

3.4 Conclusions

For the first time, inclusion complexes between sugammadex and penicillin were successfully characterized using a simple and effective method. The difference in the chemical structure, especially (R) side chains of the studied penicillins is assumed to be responsible for the different affinities to sugammadex. On the basis of the 1:1 sugammadex-rocuronium inclusion complex formation, the displacement study was carried out using the ACE method.

Furthermore, this study confirms the potential of ACE for detecting complex formations of sugammadex with other potentially co-administered drugs.

4 Optimization of Ion-Pair Formation between Glycopyrronium Bromide and Different Ion Pair Agents Using Affinity Capillary Electrophoresis

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Abstract

Glycopyrronium bromide (GLB) is an anticholinergic drug. Its highly polar quaternary ammonium group could limit the skin permeation of the topical hydrophilic drug. The ion pair formation with suitable anionic molecules was suggested to play a role in enhancing the cationic drug transport to the target site. The interactions between GLB and different ion-pair agents (IPAs) were investigated using ACE. The changes in the effective mobility of 0.05 mM GLB were correlated with the increasing concentrations of IPAs in 20 mM BGE of pH 6.2, and successfully fitted into a nonlinear binding isotherm equation assuming 1:1 stoichiometric interaction. The formation constants (K_f) were 74.33, 28.5, 18.17, 8.2, 7.6, 5.69, 4.76, and 3.96 M^{-1} for sodium salts of dodecyl sulfate, taurodeoxycholate, taurocholate, glycodeoxycholate, glycocholate, salicylate, quinolone sulfonate, and p-toluene sulfonate; respectively. Surfactant's and bile salts' concentrations were below CMCs. The partition coefficient of GLB between buffer phase and n-octanol was determined successfully in the absence and presence of different IPAs. The study confirmed the linear correlation (R^2 = 0.907) between the affinity strength of ion pair and the partition behavior of GLB in the presence of anionic molecules at 1:1 molar ratio.

4.1 Introduction

Glycopyrronium bromide (GLB) is a **synthetic** quaternary ammonium compound [48]. According to its anticholinergic (antimuscarinic) action [49], it has different pharmacological effects. In anesthesia, it is used preoperatively to reduce gastric acid, salivary, tracheobronchial, and pharyngeal secretions. At the end of anesthesia, it is given in a mixture with anticholinesterase drug for reversing neuromuscular blockage [50, 51]. It is used as a bronchodilator in treating asthma and chronic obstructive pulmonary disease [52-54]. Some recent studies showed the effectiveness of GLB in treatment of hyperhidrosis [55, 56]. The highly polar quaternary ammonium group of GLB limits its passage across lipid membranes. That is an advantage over other anticholinergic drugs such as atropine that passes the blood–brain barrier causing the central nervous side effects. From another side, this property could limit the skin permeation of topically applied GLB. The hydrophilic drug should permeate through the lipophilic stratum corneum; the rate limiting step in the transdermal absorption, especially for hydrophilic substances and macromolecules [57], and reach the muscarinic receptors (example: sweat ducts) located in the dermis layer.

As GLB is a cationic drug, it is suggested that the ion pair formation (IPF) with *suitable* anionic reactant could play a role in enhancing the drug transport to the target site mentioned previously. Studies showed the effectiveness of IPF in increasing the transport rate of the hydrophilic ionized drugs across lipid membranes. This improvement in the bioavailability of such drugs is due to the change in their physicochemical properties upon the ion-pair interactions. In case of using anionic molecules whose structures contain lipophilic group (or tail), the lipophilicity of the drug was increased and as a consequence the partitioning of the drug throughout of the lipid membrane into their action sites was facilitated [33, 34].

ACE has been used to study different interactions (examples: CD–drug complex, drug–anion complex) and to quantify their formation constants (K_f) depending on the migration behavior of the interacting molecules [23, 25, 40, 58-60]. So *first*, this study developed an ACE technique to investigate the IPF between the cationic drug (GLB) and different anionic molecules and to evaluate their formation strength (K_f). The chosen ion-pair agents (IPAs) were as the following: SDS is a hydrophilic anionic surfactant; it has enhanced penetration properties in transdermal drug delivery [61, 62]. Sodium salts of taurocholate, taurodeoxycholate, glycocholate, and glycodeoxycholate are anionic bile salts (BSs), easily dissolved in water. Different studies have shown the bile acids effect on the absorption of different drugs through various biological membranes [63]. The partitioning behavior of the cationic drug into lipophilic phase was enhanced by the effect of BSs (below CMC) [36]. In order to investigate the ion pair interaction

between the cationic drug and the anionic monomer, SDS and BSs were used in an amount below their CMCs [36, 40]. Salicylic acid is a keratolytic agent in topical formulation [64]. Quinoline derivatives have antimicrobial effect [65], so they could play an additive (antiseptic) effective in the GLB topical formulation. The anionic derivative of quinoline (quinoline-8-sulfonic; sodium salt) was chosen. Sulfonic acids (example: p-toluene-sulfonate) are used as active pharmaceutical ingredients' counter ions to improve the physicochemical properties of active pharmaceutical ingredients and so enhance their solubility, absorption, bioavailability, and pharmacological effects.

Second, the effect of the formed ion pair on the partitioning behavior of drug from aqueous phase into the lipophilic n-octanol phase was determined by measuring the partition coefficient of drug in the absence and presence of the IPAs at different concentrations. The lipophilicity of drug and drug–anion complexes was described by measuring the logarithmic value of the n-octanol/buffer partition coefficient [66]. Therefore, the shake-flask method was used as the most common procedure to determine log*P*.

According to the previous details, in brief, the aim of the work was to determine the formation constants for GLB–anions complexes and estimate the effect of the ion pairing on the lipophilicity of GLB by measuring its partition coefficient in presence of different anionic agents. As consequence, the relation between the affinity strengths and the partition coefficients was studied in order to optimize the IPF for GLB with the suitable IPA.

4.2 Materials and methods

4.2.1 Materials

GLB was a gift from RIEMSER Pharma (Greifswald, Germany). SDS, sodium glycocholate hydrate (NaGC), sodium glycodeoxycholate (NaGDC), sodium taurocholate hydrate (NaTC), sodium taurodeoxycholate (NaTDC), sodium ptoluenesulfonate, and sodium salicylate were purchased from Sigma-Aldrich Chemie (Schnelldorf, Germany). Quinolone-8-sulfonic acid sodium salt was purchased from ABCR KG (Karlsruhe, Germany). Sodium dihydrogen phosphate (NaH2PO4), disodium hydrogen phosphate (Na2HPO4), and sodium hydroxide (NaOH) were purchased from Carl Roth KG. DMSO was purchased from Fluka Chemie AG (Buchs, Switzerland).

4.2.2 ACE

Equipment conditions

CE experiments were performed using a DAD (190–600 nm) coupled to a Hewlett Packard model G1600AX (Waldbronn, Germany) 3D CE system. A CE ChemStation equipped with a HP Vectra 486/66U workstation was used for instrument control, data acquisition, and data analysis. The system was controlled by Windows software that was modified to the HP system. The detection wavelength was 195 nm. The fused silica capillary had an internal diameter of 50 µm and a total length of 64.5 cm. The distance to the detector was 56 cm. A new capillary was rinsed first with 1 N NaOH at 40°C for 15 min followed by distilled water for 15 min. For each run, the capillary was rinsed with 0.1 N NaOH for 3 min followed by distilled water for 2 min. In the final step, the capillary was rinsed with the buffer solution for 5 min. The temperature was kept at 25°C and the separation voltage was 20 kV. The sample was injected at 50 mbar for 9 s. The measurements for each sample were done in triplicate in case of the SDS containing buffer solution and in duplicate for the rest.

Principle

As a consequence of the increase of the IPA concentration, complexes (*C*s) are formed, which in turn leads to changes in the electrophoretic mobility of the injected drug (*D*).

Assumptions

First, fast adjustment of equilibrium between free and complexed drug; and second, no interactions between the drug or IPA and the capillary wall. The formation constant of the complex in the running buffer solution was obtained using the OriginPro (8) program, assuming (1:1) drug to IPA stoichiometric interaction.

$$\mathbf{D + IPA} \rightleftharpoons \mathbf{C} \qquad \textbf{Equation 21}$$

When K_f is determined according to the following equation [3]:

$$K_f = \frac{[C]}{[D]\,[IPA]} \qquad \textbf{Equation 22}$$

Where the net effective mobility of (*D*) is the sum of effective motilities of free and complexed form and *x* is the molar fraction,

$$\mu_{eff} = x_D \cdot \mu_D + x_C \cdot \mu_C \qquad \textbf{Equation 23}$$

Then:

$$\mu_{eff} = \frac{\mu_D + K_f.[IPA].\mu_C}{1 + K_f.[IPA]} \qquad \textbf{Equation 24}$$

Third assumption, the effective mobility of the ion-pair complex (μ_c) is equal to zero, that simplified the Eq. (24):

$$\mu_{eff} = \frac{\mu_D}{1 + K_f \cdot [IPA]} \qquad \textbf{Equation 25}$$

Where μ_{eff} is calculated from,

$$\mu_{eff} = \frac{L_D \cdot L_T}{U} \left(\frac{1}{t_m} - \frac{1}{t_{EOF}} \right) \qquad \textbf{Equation 5}$$

L_T is the total length of the capillary, while L_D is the distance from the capillary inlet to the detector. t_m and t_{EOF} are the migration times of the drug peak and the EOF peak, respectively. EOF is detected by a neutral substance marker (DMSO).

Buffer preparation

The buffer is 20mMsodium phosphate buffer solution; composed of 2.6459 g (19.1746 mM) sodium dihydrogen phosphate (MWT = 137.99 g/mole) and 0.2216 g (1.561 mM) disodium hydrogen phosphate (MWT = 141.96 g/mole) dissolved in 1 L distilled water with pH 6.2. The IPAs containing buffer solutions were prepared from the stock solutions (100 mL of 50 mM for each sodium p-toluenesulfonate and sodium quinoline-8-sulfonate, 50 mL of 20 mM for each sodium BS and sodium salicylate, and 25 mL of 4 mM SDS); appropriate volumes of the stock solutions were diluted by buffer to reach the desired concentration ranges: (0.05–1mM) for SDS and NaTDC, (0.1–2 mM) for NaGDC, (0.05–3 mM) for NaTC, (0.05–4 mM) for NaGC and p-toluenesulfonate, (0.5–10mM)for sodium salicylate, and (1–25mM)for sodium quinolone-8-sulfonate. All buffer solutions were degassed by sonication for 10 min and filtered using 0.45 µm syringe filter before use.

Sample preparation

GLB sample was prepared at 0.05 mM concentration (= 20 µg/ml) from 500 µg/ml stock solution in 1 ml distilled water. GLB is white powder, soluble in water and has molecular weight of 398.33 g/mol.

4.2.3 CMC determination

The major methodological approaches based on CE technique to determine CMC of surfactants were described extensively in the review [67]. In this work, the electrophoretic mobility of GLB (0.05 mM) as a function of surfactant or BS concentration was plotted at the experimental conditions mentioned previously. The concentration of SDS or BS at the observed sharp change

in slope was detected as CMC value. The studied analyte (GLB) was used as the marker compound [68].

4.2.4 Partition experiment

Saturated solutions: aqueous phase (sodium phosphate buffer, 20 mM, pH 6.2) saturated by n-octanol and n-octanol phase saturated with buffer solution were prepared in large quantity in 1000 mL volumetric flask and mechanically shacked overnight. Two phases were separated using separatory funnel. Two microliters of saturated buffer contained the cationic drug and the anionic molecule was filled into glass transparent ampule with 2 mL saturated n-octanol. Samples were prepared of drug to IPA at different molar ratios 1:0, 1:1, 1:5, 1:10, 1:20, and 1:40. The concentration of GLB was 0.05 mM (= 20 µg/mL). The ampules were closed well and placed in a water bed overnight. The vibration was applied at the water bed. The temperature was adjusted to 25°C using thermostat. The phases were separated and the aqueous phase was measured by CE couples to UV detector as mentioned previously. Serial concentrations of GLB (2, 5, 10, 20, 40 µg/mL) dissolved in saturated buffer were prepared for calibration curve. The plot of drug concentrations versus area under curve showed good linearity of 0999. And the LOD was detected as 1 µg/mL.

4.3 Results and discussion

4.3.1 IPF between GLB and anionic molecule

The interaction between GLB and IPA (BSs, SDS, sodium quinolone-8-sulfonate, sodium p-toluene sulfonate, and sodium salicylate) under the previously described conditions was investigated using ACE. The RSD (%RSD) of GLB's effective mobilities in running buffer was 0.75 for n = 16, indicating the reproducibility and precision of the method. The changes in electrophoretic mobility were determined for GLB in presence of IPA in running buffer. These changes could be attributed to the IPF and is based on the decrease of the total positive charge and increase in the mass of ion pair compared to free drug. The formation strength was estimated by calculating the formation constant K_f using a nonlinear curve fitting method (assuming 1:1 stoichiometric ratio, μ_c is zero). For BSs and the surfactant (SDS), the concentrations below CMC were used in order to avoid the micelles formation. The maximum concentration was not to exceed CMC (Table 2). CMC values were determined by CE method based on the mobility. Under the previously described experimental conditions, CMC value was the concentration of SDS or BS at which a dramatic change in effective mobility of GLB

occurred. In general, the decreased effective mobilities for the drug as a function of the increasing concentrations of the anionic agent were correlated and successfully fitted into the Eq. (25) (Figures 6 & 7). K_f values were shown in Table 2. In literatures, the formation constants were recorded for 1:1 ion-pair complexation of different cationic drugs with anions. The formation constant values of this work were to some extent in the same range of those determined measurements. By a solvent extraction technique, tetrabutylammonium and isopropamide have formation constants of 50 and 12 M^{-1}, respectively, with salicylate, and 31.4 and 5 M^{-1}, respectively, with p-toluenesulfonate. Otherwise, it was impossible to calculate the formation constants for both drugs with NaTDC below their CMC [66]. The propranolol-taurocholate formation constant (37 M^{-1}) was determined using ACE at BS concentration below CMC with assumption that 1:1 molar ratio of ion-pair complex [16]. The affinity strength of GLB to SDS is the most high, followed by BSs (NaTDC > NaTC > NaGDC > NaGC), sodium salicylate, sodium quinoline-8-sulfonate, and sodium p-toluene sulfonate. The ion pair consists of positive ion and negative ion temporarily bonded together by electrostatic forces, which assumed to be the driving forces between positive quaternary ammonium ion and negative sulfate, sulfonate, and carboxylate ions. The differences between sulfate and sulfonate groups in their attractive molecular interactions with quaternary ammonium group could explain the higher formation constant of GLB to SDS than one to taurine basis BSs [69]. Otherwise sulfonic acids were stronger than carboxylic acids, which could be the reason for more affinity of GLB to taurine-conjugated BSs than to glycine-conjugated ones in the selected pH buffer. In addition steric hindrance could affect the ion-pair association [70]. The presence of IPAs in the running buffer could affect the determined results, so the implications of their added concentrations on the BGE were investigated. ANOVA showed no significant ($p > 0.05$) differences in migration time of EOF betweenBGEwith andwithout IPAs. The ionic strength of the BGE was calculated to 0.024 M. Almost no changes to small changes in the ionic strength (1 mM for SDS, NaTDC containing buffers, 2 mM for NaGDC containing buffer, 3 mM for NaTC containing buffer, 4 mM for NaGC, Na p-toluene sulfonate containing buffers, and 10 mM for Na salicylate containing buffer) were recorded. These changes were not suggested to affect the obtained results.

Table 2 Values for formation constants of ion pairs between GLB and IPAs (n = 2), for SDS (n = 3)

IPA	CMC [mM]	K_f [M^{-1}] ± sd
SDS	1	74.33 ± 9.5
NaTDC	2	28.5 ± 3.11
NaTC	8	18.17 ± 0.8
NaGDC	3	8.2 ± 2.4
NaGC	6	7.6 ± 0.65
Na Salicylate	-	5.69 ± 0.33
Na Quinoline Sulfonate	-	4.76 ± 0.1
Na p-toluene sulfonate	-	3.96 ± 0.51

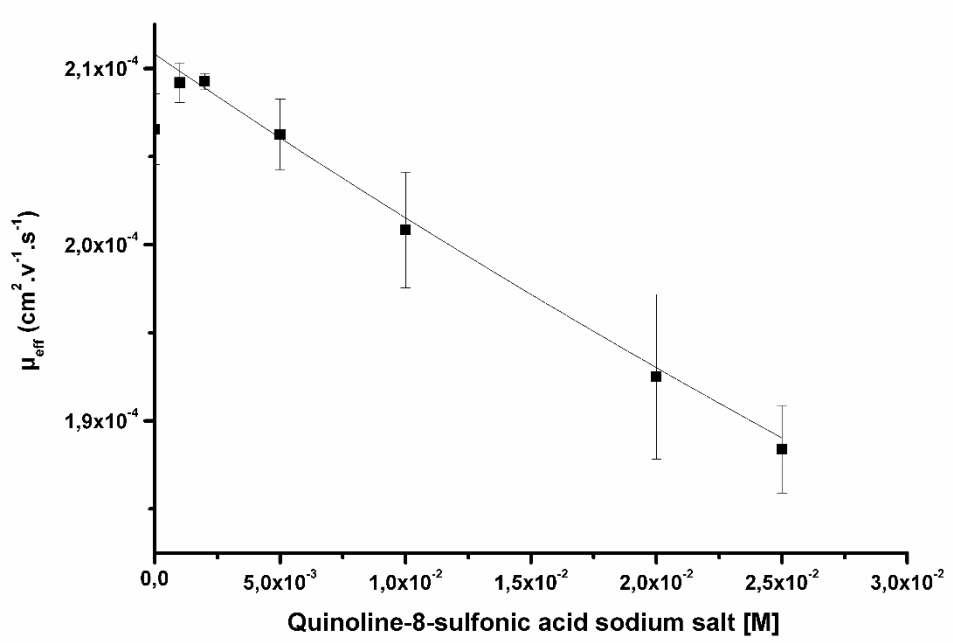

Figure 6 Interaction of GLB with quinoline-8-sulfonic acid sodium salt; nonlinear curve fitting of the effective mobility changes in corresponding to different IPA concentrations (n = 2)

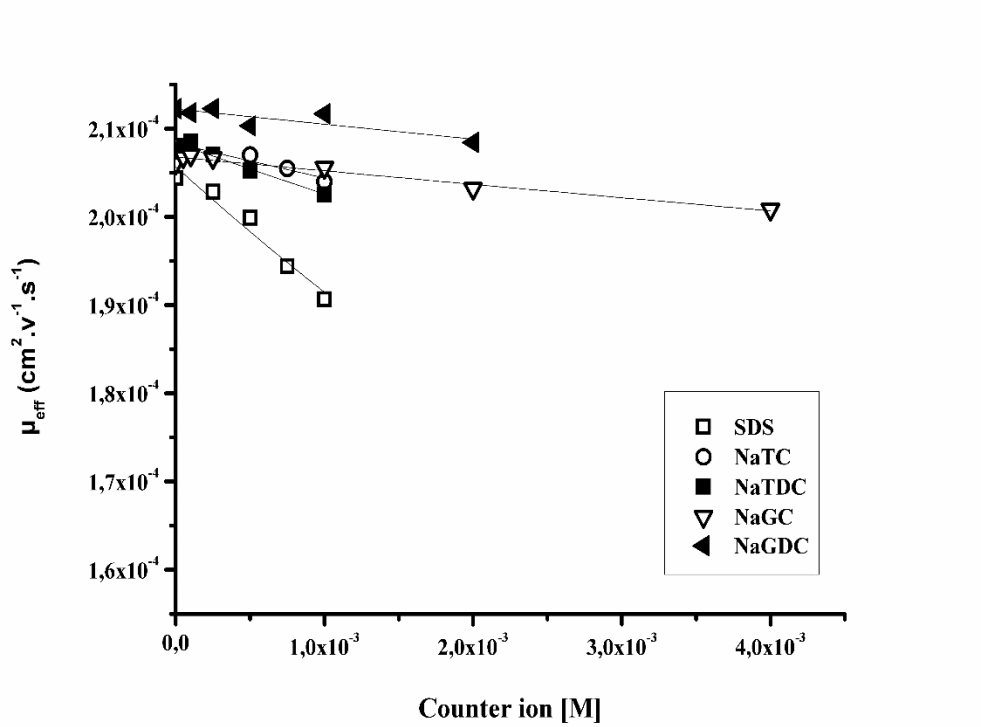

Figure 7 Interaction of GLB with SDS and BSs; nonlinear curve fitting of the effective mobility changes in corresponding to different IPA concentrations below CMC

4.3.2 Partition studies

The partitioning of GLB from aqueous buffer of pH 6.2 into octanol phase was studied in the absence and presence of IPAs at different concentrations. GLB concentrations in the buffer phase were calculated by using their measured peak areas in the equation of the calibration curve, which was linear with a correlation coefficient $R2 = 0.999$. The detection sensitivity of this analysis method was 1 µg/ml. In each case, the calculated concentration in aqueous phase was subtracted from the starting (or total) concentration of GLB to obtain one in octanol phase. And so the partitioning coefficient for GLB was calculated (Table 3). The quaternary ammonium drug alone exhibited a partitioning into n-octanol phase in a very small quantity. The addition of NaTDC (the BS in concentrations below CMC) to the drug containing aqueous buffer showed a progressive increase in the partitioning behavior of drug into the organic phase as the concentration of NaTDC increased. With increasing concentrations, NaTC, NaGDC, and NaGC enhanced the partitioning of the drug into n-octanol phase from aqueous one and partition coefficient ($P_{O/W}$) values were lesser than ones in case of NaTDC. At the studied concentrations of IPA, a very small enhancement in partitioning of drug to organic phase is noticed in case of increasing concentration for Na salicylate, sodium quinolone- and sodium ptoluene- (sulfonate). This small increase in the partitioning of drug could be correlated to the relatively low complexation constants determined. The greatest effect on the partitioning

behavior of drug into the organic phase was accompanied by adding the anionic surfactant ([SDS] < CMC) to the buffer phase. In this study, for the IPF in aqueous phase the following equation might be assumed [36] :

$$D^{+}_{aq} + IPA^{-}_{aq} \xleftrightarrow{K_f} C_{aq} \xleftrightarrow{P_{O/W}} C_{oct} \qquad \textbf{Equation 26}$$

where K_f is the formation constant of ion pair between the cationic (GLB) and anionic (IPA) in the buffer phase (aqueous phase) of pH which is in the skin pH range. $P_{O/W}$ is the partition coefficient of the neutral ion-pair complex (*C*) into the organic phase. The enhanced partitioning of cationic drug into the lipophilic n-octanol phase was suggested most likely to be correlated to the strength of ion-pair complex. That confirmed by linear correlation (R^2 = 0.907) of the partition coefficient values at 1:1 molar ratio with the corresponding values of formation constants (Figure 8).

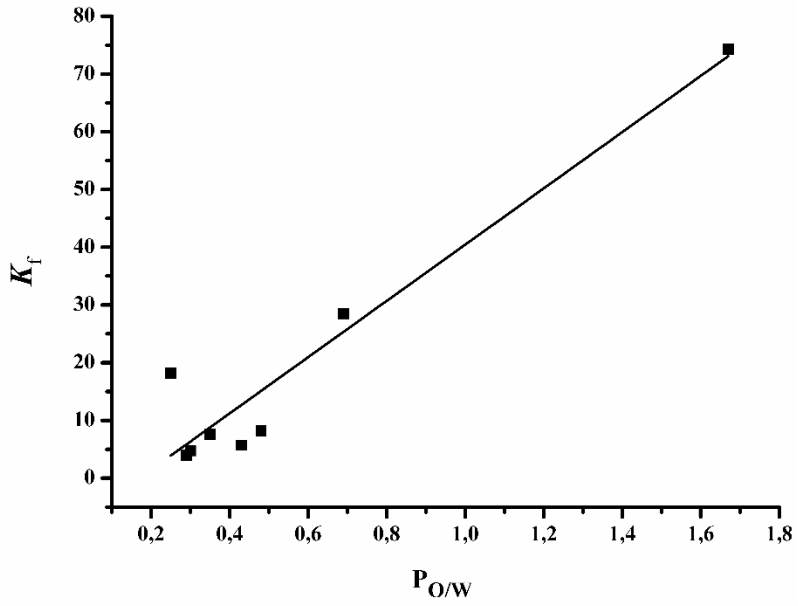

Figure 8 Plot of the GLB partition coefficient values (PO/W) at 1:1 molar ratio (mentioned in Table 2) versus the corresponding values of formation constant (K_f) (mentioned in Table 2)

4.4 Concluding remarks

The IPF between a quaternary ammonium compound (GLB) and different IPAs was successfully characterized using a simple and effective technique. This study confirmed the linear correlation between the affinity strength of ion-pair complex and the partition behavior of the cationic drug, GLB, in the presence of anionic molecules at 1:1 molar ratio. The partition behavior of the cationic drug from the aqueous phase into organic phase was determined

successfully in the absence and presence of different anionicmolecules at different molar ratios. As conclusion, the partitioning of drug into hydrophobic phase is directly increased as its affinity strength to the anion by ion-pair interaction increased. In order to optimize the IPF and its effect, SDS would be the most recommended IPA for this study. SDS has significant effect on K_f and the partitioning behavior of GLB. Furthermore, its properties like availability, low cost, and its friendly impact on human are the contributions for SDS to be preferable chosen.

Table 3 Partitioning coefficient of GLB in presence of different IPAs at different molar ratios

IPA	Molar Ratio [D]:[IPA]	$P_{O/W}$	logP	Increase Factor[a)]
n = 6				
	1:0	0.054 ± 0.01	-1.27 ± 0.11	-
NaTDC	1:1	0.69 ± 0.07	-0.16 ± 0.04	12.7
	1:10	6.62 ± 0.37	0.82 ± 0.02	122.5
n = 4				
NaTC	1:1	0.25 ± 0.02	-0.6 ± 0.04	4.6
	1:10	0.57 ± 0.01	-0.24 ± 0.008	10.5
NaGDC	1:1	0.48 ± 0.04	-0,31 ± 0.04	8.9
	1:10	1.03 ± 0.02	0.016 ± 0.007	19
NaGC	1:1	0.35 ± 0.01	-0.45 ± 0.01	6.5
	1:10	0.8 ± 0.04	-0.1 ± 0.006	14.8
Na salicylate	1:1	0.43 ± 0.06	-0.37 ± 0.06	7.9
	1:10	0.5 ± 0.07	-0.29 ± 0.06	9.3
Na quinoline	1:1	0.3 ± 0.04	-0.52 ± 0.06	5.5
sulfonate	1:10	0.29 ± 0.02	-0.53 ± 0.03	5.4
Na p-toluene	1:1	0.29 ± 0.03	-0.53 ± 0.04	5.4
sulfonate	1:10	0.39 ± 0.06	-0.4 ± 0.07	7.2
SDS	1:1	1.67 ± 0.04	0.22 ± 0.01	31
	1:10	-	-	

a) Ratio of $P_{O/W}$ in presence of IPA to $P_{O/W}$ in absence of IPA.

5 Drug Release from ß-Cyclodextrin Complexes and Drug Transfer into Model Membranes Studied by Affinity Capillary Electrophoresis *(Article in Pharmaceutical Research 33(1175-1181)2016)*

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Abstract

Purpose Is to characterize the drug release from the ß-cyclodextrin (ß-CD) cavity and the drug transfer into model membranes by affinity capillary electrophoresis. Phospholipid liposomes with and without cholesterol were used to mimic the natural biological membrane.

Methods The interaction of cationic and anionic drugs with ß-CD and the interaction of the drugs with liposomes were detected separately by measuring the drug mobility in ß-CD containing buffer and liposome containing buffer; respectively. Moreover, the kinetics of drug release from ß-CD and its transfer into liposomes with or without cholesterol was studied by investigation of changes in the migration behaviours of the drugs in samples, contained drug, ß-CD and liposome, at 1:1:1 molar ratio at different time intervals; zero time, 30 min, 1, 2, 4, 6, 8, 10 and 24 h. Lipophilic drugs such as propranolol and ibuprofen were chosen for this study, because they form complexes with ß-CD.

Results The mobility of the both drug liposome mixtures changed with time to a final state. For samples of liposomal membranes with cholesterol the final state was faster reached than without cholesterol.

Conclusions The study confirmed that the drug release from the CD cavity and its transfer into the model membrane was more enhanced by the competitive displacement of the drug from the ß-CD cavity by cholesterol, the membrane component. The ACE method here developed can be used to optimize the drug release from CD complexes and the drug transfer into model membranes.

5.1 Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides. The natural α -, β - and γ – CDs are the most common. They consist of 6, 7 and 8 α-D-glucopyranose units, respectively, linked together by α-(1,4) glucosidic bonds in ring structures with lipophilic cavity and hydrophilic outer surfaces. The structure of CDs makes them capable of interacting with different drug molecules forming host-guest inclusion complexes. This complexation could alter the physicochemical and the biological properties of the guest drug and as consequence it could be utilized for pharmaceutical purposes. Many reviews [11, 12, 71] report the use of CDs as drug carriers at different administration routes and how that could improve the solubility of poor water soluble drugs as well as the stability for some problematic drugs. The inclusion could eventually enhance their bioavailability under certain conditions depending on CD concentrations. In oral application, CD complexes improve the drugs' bioavailability mainly by improving their dissolution rate; e.g., tablet of ursodeoxycholic acid and hydroxypropyl-ß-cyclodextrin (HP-ß-CD) [7]. ß-CD enhances even the absorption of an oral antifungal drug (ketoconazole). γ – CD enhances the bioavailability of digoxin formulated in sublingual tablets by forming a complex that protect the drug from acid hydrolysis [8]. Other examples of CD complexes, which have an enhancement effect on the solubility and dissolution of various orally administrated drugs, are listed in [9]. Ocular preparations of HP-ß-CD/ dexamethasone acetate complexes are useful to enhance the corneal bioavailability of drug [10]. By changing in the nasal mucosa permeability, heptikas-(2, 6-di-O-methyl)-ß-CD enhances the bioavailability of morphine at the nasal absorption site. For rectal delivery, the release of anti-inflammatory drug (flurbiprufen) from suppository' base is enhanced by forming complex with the hydrophilic CD [8]. At the lipid membrane level, only the free drug which is in equilibrium with the complexed form could penetrate. The high molecular weight and the low partition coefficient (octanol / water) of CDs prevent their penetration into the lipid membrane. So the absorption enhancement effect of CD for the guest drug could be improved by the mechanisms that increase the dissociation rate of the CD-complex leading to release the drug from the CD's cavity. Furthermore, the effect of CD on the drug absorption depends on the nature of drug itself; the enhancement effect is obtained for the BC II drugs (ex: ibuprofen) of high permeability property and low water solubility. The contributions of some mechanisms in drug release from complex comprise simple dilution of complex, competitive displacement of drug from CD's cavity, drug binding to protein, drug uptake into tissue or CD elimination and changes in pH and temperature [27]. Some studies discussed the possible mechanisms that affect the drug delivery through biological membrane from CD complexes [72]. For competitive mechanisms, the drug availability at the

absorption site is enhanced by displacement from CD's cavity by exogenous and endogenous substances [8, 28]. Several investigations tested the ability of ß-CD to extract cholesterol from biological membrane and tried to explain the possible mechanisms for that [29-32]. Furthermore, the stability constants for ß-CD complexes of endogenous cholesterol were determined [73]. Phospholipid bilayer is the most common general structure of biological membranes; other major lipids like cholesterol are integrated. Structurally, liposomal vesicles are mimicking the natural cell membranes. For this reason, liposomes are often used as models of cell membranes in pharmaceutical researches [74]. Partitioning of a drug into biological membranes could be predicted by the study of the interaction between the drug and liposomes, which resemble the natural bilayer phospholipid membranes. Immobilized-liposome chromatography [75, 76] or capillary electrophoresis techniques where the liposomes present in buffer solution, act as a pseudostationary phase are common methods to quantify the interaction by calculating the retention factor in order to determine the predicting partitioning of solutes between liposome and buffer system [77-79]. Affinity capillary electrophoresis (ACE) is a technique which depends on the change in the migration pattern of an analyte to estimate its interaction with ligand or receptor [3]. Upon interaction, the change in the total charge or and change in molecular size of the complexed drug compared to the free form could be reflected as change in migration time. As consequence, the change in electrophoretic mobility (inversely related to migration time) of analyte was determined. The objective of this paper is the application of ACE to study the interaction between liposomes as a model membrane and a CD complex of a drug. Therefore, an ACE method was developed to study the drug release from ß-CD complexes and its subsequent transfer into liposomal vesicles. The influence of cholesterol as a constituent of the liposomes was also investigated comparing buffer and sample systems with pure phospholipid liposomes and liposomes with incorporated cholesterol. The drug release from the ß-CD complex and the transfer of the drug into liposomal membrane could be followed by evaluation of the migration data. These studies were carried out for lipophilic drugs such as propranolol and ibuprofen with logP of 3.48 and 3.97, respectively.

5.2 Materials and methods

5.2.1 Materials

(±)-Propranolol hydrochloride, Ibuprofen sodium salt, L-α-Phosphatidylcholine (PC), 1, 2-Diacyl-sn-glycero-3-phospho-L-serine (PS) and Cholesterol (CHOL) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). ß-CD and Dimethylsulfoxid (DMSO) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Potassium hydrogen phosphate (K2HPO4) and Potassium di-hydrogen phosphate (KH2PO4) were purchased from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH) was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

5.2.2 Methods

Preparation and characterization of liposome

Phospholipid Film Preparation. Two liposomal solutions were prepared from phospholipids at a starting concentration of 5.625 mM (PC; 80%) / (PS; 20%) one with 3.7 mM cholesterol while the other without cholesterol. First, the phospholipid film was prepared by weighing and dissolving the compositions in chloroform in a round bottomed flask, and then the chloroform was evaporated using a rotary evaporator at 30°C. The rotation speed was adjusted to 150 rpm. Finally, the phospholipid film containing flask is kept in a vacuum overnight to ensure the complete dryness of the film.

Phospholipid Film Hydration. 4 ml of filtered potassium phosphate buffer solution was added to the flask and the rotation for 30 min was applied. The hydrated liposomes were 6 times freeze-thawed using liquid nitrogen, and sonicated for 2 h in a water bath sonicator. All liposomal solutions were filtered using 0.22 µm syringe filter. The size and zeta potential measurements were determined using a Zetasizer Nano ZS (Malvern, Worcestershire, United Kingdom).

Buffer and Samples Preparation

For CE, 10 mM of phosphate buffer was prepared by dissolving 1.24 g potassium hydrogen-phosphate and 0.59 g potassium di-hydrogen-phosphate in 1 L distilled water. The pH adjusted to 7.4, using a pH meter. The buffer solution was filtered using a 0.22 µm syringe filter. Stock solutions of studied drugs (20 mM Pro, 20 mM IBU) and of ß-CD (20 mM) were prepared with the filtrated buffer. All the samples and the buffer systems (Table 4) for the entire study were

prepared from the stock solutions of drugs, ß-CD and liposomes by appropriate diluting with the filtrated buffer. The samples to be injected into capillary were: 1 mM Pro (P1), 1 mM IBU (I1) and a mixture of drug and ß-CD at 1:1 molar ratio (1 mM); (P2) for Pro and (I2) for IBU. For the mechanistic study, the sample was composed of drug, ß-CD and liposome with or without cholesterol at 1:1:1 molar ratio of 1 mM concentration for each content; Pro:ß-CD:liposome without cholesterol (S1), Pro:ß-CD:liposome with cholesterol (C1), IBU:ß-CD:liposome without cholesterol (S2) and IBU:ß-CD:liposome with cholesterol (C2). The buffer systems running in the capillary as background electrolyte were normal buffer (B1), 1 mM ß-CD containing buffer (B2) 10 mM ß- CD containing buffer (B3), 1 mM PC/PS liposome containing buffer (B4) and 1 mM PC/PS/cholesterol containing buffer (B5). For the mechanistic study and in order to avoid the dilution effect on the complex formation, the buffer system was composed of ß-CD and liposome without (B6) or with (B7) cholesterol at 1:1 molar ratio of 1 mM concentration for each content. Propranolol and ibuprofen were categorized as soluble drugs in water. 1 mM of propranolol (295.8 g/mole) and 1 mM of ibuprofen (228.3 g/mole) were (295.8 μg/ml) and (228.3 μg/ml); respectively, which were concentrations of drugs dissolve in buffer solution.

Table 4 Buffers and samples

Buffer systems	
B1	Phosphate buffer
B2	1 mM ß-CD containing phosphate buffer
B3	10 mM ß-CD containing phosphate buffer
B4	1 mM PC/PS liposome containing phosphate buffer
B5	1 mM PC/PS/cholesterol liposome containing phosphate buffer
B6	1 mM ß-CD & 1 mM PC/PS liposome containing phosphate buffer
B7	1 mM ß-CD & 1 mM PC/PS/cholesterol liposome containing phosphate buffer
Samples	
P1	1 mM propranolol
P2	1 mM propranolol & 1 mM ß-CD
I1	1 mM ibuprofen
I2	1 mM ibuprofen & 1 mM ß-CD
S1	1 mM propranolol & 1 mM ß-CD & 1 mM PC/PS liposome
C1	1 mM & 1 mM ß-CD & 1 mM PC/PS/cholesterol liposome
S2	1 mM ibuprofen & 1 mM ß-CD & 1 mM PC/PS liposome
C2	1 mM ibuprofen & 1 mM ß-CD & 1 mM PC/PS/cholesterol liposome

CE Equipment

Propranolol. CE experiments were performed using a diode array detector (190 to 600 nm) coupled to a Hewlett Packard model G1600AX (Waldbronn, Germany) 3D CE system. A CE ChemStation equipped with a HP Vectra 486/66U workstation was used for instrument control, data acquisition, and data analysis. The system was controlled by Windows software, which was modified to theHP system. The detection wavelength was 215 nm. The fused silica capillary had an internal diameter of 50 μm and a total length of 64.5 cm. The distance to the detector was 56 cm. The sample was injected at 50 mbar for 9 s.

Ibuprofen. CE experiments were performed using a diode array detector (190 to 600 nm) coupled to a Beckman Coulter P/ACE™ MDQ system with 23 Karat Software. The detection wavelength was 200 nm. The fused silica capillary had an internal diameter of 50 μm and a total length of 60 cm. The distance to the detector was 50 cm. The sample was injected at 1 psi for 9 s.

A new capillary was rinsed first with 1 N NaOH at 40°C for 15 min followed by distilled water for 15 min. For each run, the capillary was rinsed with 0.1 N NaOH for 3 min followed by distilled water for 2 min. In the final step, the capillary was rinsed with the buffer solution for 5 min. The temperature was kept at 25°C and the experimental voltage was 20 kV.

Study of the Exchange Kinetics

Reaching the equilibrium is a slow process, whose kinetic is affected by diffusion of the drug into liposome. In case of cholesterol containing liposome, the presence of an extra factor is suggested that affect the equilibrium and the velocity of the exchange. The cholesterol as a liposomal constituent will enhance the dissociation rate of the drug-CD complexes by competing for the cavity of the CD against the drug. The dilution in the capillary is prevented in this study as the concentrations of the liposome and the CD in the sample and the buffer were the same. The mechanism is illustrated in Figure 9. This hypothesis was investigated using ACE by detecting the migration time of the drug at different buffer systems. The free form of the drug in all studied samples will migrate through the buffer systems under the influence of its net equilibrium resulted from complex formation with CD and/or distribution into liposome. Prior to study the mechanism, the drug migration through B1, B2, B3, B4, B5 buffer systems were detected in order to investigate drug-CD and drug-liposome interactions separately. Those interactions occurred and were investigated at buffer systems in the capillary compartment. The mechanism of drug release from CD and transfer into liposome was studied by detecting the migration time of the drug in samples (S1&2 and C1&2) through

B6 and B7 buffer systems, respectively, at interval times ranged between 0 and 24 h after preparing the samples. Here, the time interval represents the contact time of the drug-CD complex to the liposomal membrane in sample. In contrast to previous interactions, the mechanism (release from and transfer into) interactions occurred in samples (S & C) compartments or vials (offline) and then were investigated in the analytical compartment; the capillary filled with buffer systems. As consequence, the effective mobility which is inversely related to migration time of the free form of the drug changed. The effective mobility of drug was calculated using the following Eq. (5):

$$\mu_{\mathbf{eff}} = \frac{\mathbf{L_D \cdot L_T}}{\mathbf{U}} \left(\frac{\mathbf{1}}{t_{\mathbf{m}}} - \frac{\mathbf{1}}{t_{\mathbf{EOF}}} \right)$$

L_T is the total length of the capillary, while L_D is the distance from the capillary inlet to the detector. tm and t_{EOF} are the migration times of the drug peak and the electroosmotic flow (EOF) peak, respectively. EOF is detected by a neutral substance marker (DMSO).

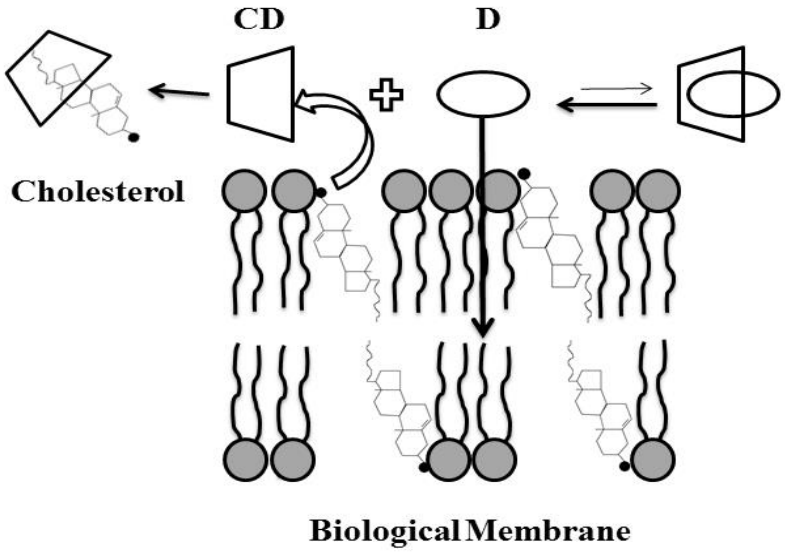

Figure 9 Mechanism for drug (D) release from cyclodextrin (CD) cavity and transfer into biological membrane. The dissociation rate of drug-cyclodextrin complex was increased by cholesterol competitive displacement of drug for its site on CD cavity leading to enhance the absorption of free drug into membrane.

5.3 Results and discussion

Under previously described conditions, first, the interaction of drug complexation with ß-CD, second, the interaction of drug distribution into liposomal membrane and third, the interaction of drug release from ß-CD complexes and transfer into liposomes with and without cholesterol were investigated by detecting the migration behaviour of the drug through appropriate buffer system. The drug migrated through the buffer system under the influence of the change in the total charge or/and size of interacted drug in comparison to the free form and that is a result of its complex formation with CD and/or its partition into liposome. These changes in the migration behaviour of the drug through the buffer system were expressed mathematically in CE method by calculating the value of the drug effective mobility (inversely related to the migration time).

In order to mimic biological membrane, liposome was prepared by using PC as a major phospholipid component. Furthermore, PS was used to obtain the total negative charge of liposome. The negatively charged liposome was more suitable in the case of using uncoated capillary than neutral or positively charged one. It is repelled from the negatively charged capillary wall. So the total charge of liposomes was investigated by measuring zeta potentials in duplicate for each preparation; −103±2.12 mV for PC/PS and −80.8±0.212 mV for PC/PS/CHOL. The average size of each liposome preparation measured five times was 80.96±0.25 nm and 60.34±0.44 nm at 25°C for PC/PS and PC/PS/CHOL; respectively. The polydispersity index was 0.24 for both. Those size and dispersity measurements were repeated for the same liposome preparations after 24 h in order to investigate the stability of the formulation during the incubation time and they showed similar results.

The lipophilic drugs used in this study were propranolol as an example for positively charged drug and ibuprofen for a negatively charged one. The complex formation between propranolol and different CDs (e.g., ß-CD) and their affinity strength were determined [80, 81]. Ibuprofen forms a complex with ß-CD and this complexation found a pharmaceutical application due to enhancing the dissolution of drug formulation containing CD [82].

Interaction Between Propranolol and ß-CD

Propranolol in sample P1 migrates as a cationic drug through the normal buffer (B1) (Table 5). In the ß-CD containing buffer system (B3), the complexation of the drug (P1) is indicated as a decreased electrophoretic mobility due to the increased size of the CD-complex compared to the free propranolol (Table 5). The net charge does not change, since the neutral CD with no electrical charge in aqueous solution below pH 12 was used.

Table 5 The Effective Mobility of Propranolol (Pro) and Ibuprofen (IBU) in Different Buffer Solutions

Buffer System	**Effective mobility ($cm^2.s^{-1}.v^{-1}$)*10^{-4}**		
	Mean **(n = 2)**	± SD	RSD (%)
	Sample P1		
B1	2.34	0.0218	0.93
B3	1.99	0.0087	0.44
B4	-3.10	0.025	0.8
B5	-1.57	0.011	0.7
	Sample I1		
B1	-2.16	0.0064	0.3
B2	-1.30	0.0047	0.36
B4	-2.18	0.0027	0.12
B5	-2.16	0.001	0.04
	Sample I2		
B6	-1.19	0.0027	0.22
B7	-1.22	0.0055	0.45

Interaction Between Propranolol and Liposomal Membrane

In the liposomal buffer system (B4 & B5), propranolol behaves as an anionic molecule (Figure 10). The change in the migration behaviour of the drug attributes to the transfer of lipophilic drug into the negatively charged liposome without or with cholesterol contained in the buffer systems B4 and B5, respectively, and is based on the increase of the total negative charge and size of the interacted molecules in comparison to the free drug. The B4 and B5 buffer systems are without ß-CD, so there is no competition from complexation. The transfer of propranolol into liposomal membrane in these systems represents the optimum transfer or distribution of the drug into these liposomes. In other words, as the reaction came to an end, the equilibrium state between all constituents is reached, so no further changes take place (the final transfer state).

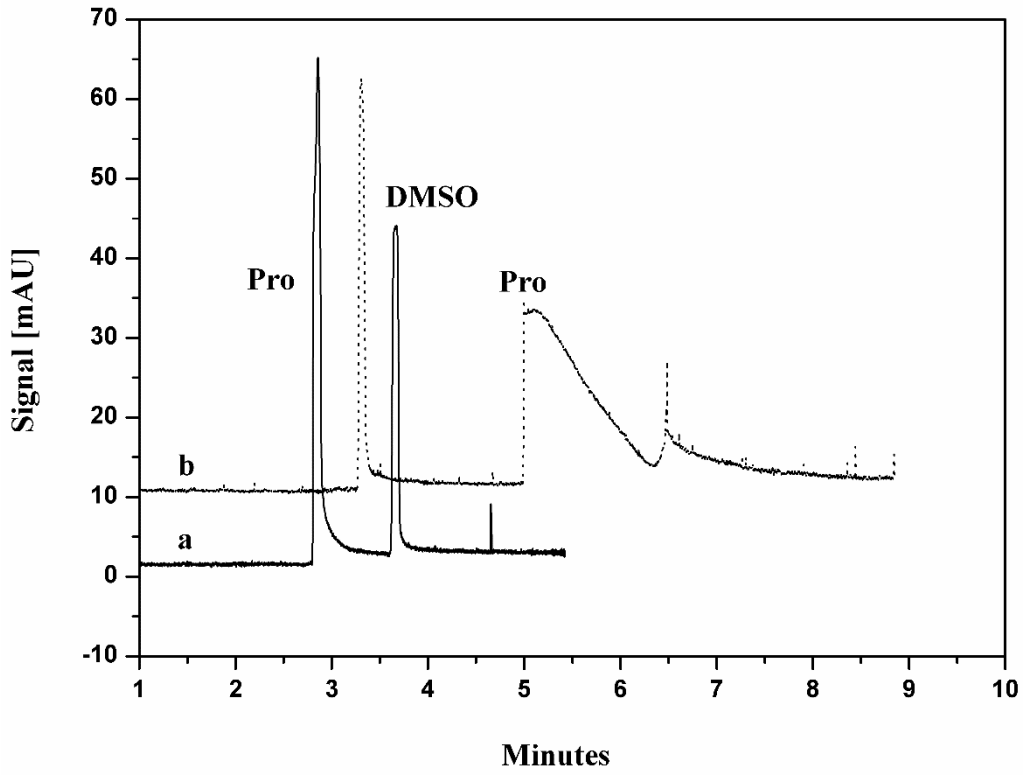

Figure 10 Migration time of 1 mM propranolol (Pro) alone (a) and migration time of 1 mM Pro in liposome (b); experimental conditions: in phosphate buffer 10 mM, pH 7.4 (a: solid line) and in 1 mM PC/PS liposome containing phosphate buffer system B4 (b: dot line)

Study of Exchange Kinetics

Prior to this study, the mobility for propranolol in sample P2 through the buffer systems B6 and B7 was detected as $-2.33*10^{-4}$cm2.s^{-1}. v^{-1} and $-1.24*10^{-4}$cm2.s^{-1}. v^{-1}; respectively. The change in the migration behaviour shows the ability of the drug to release from ß-CD complex and distribute into liposomal membrane already existed in the capillary. The kinetics of propranolol release from ß-CD complex and drug transfer into liposome with and without cholesterol was studied over 24 h. The experiment was carried out using a freshly prepared buffer for each run.

Transfer into PC/PS Liposome Contained in Sample S1

The drug, propranolol, in sample S1 moves through the buffer system B6 under the influence of the net interaction resulted from complex formation with CD and distribution into liposome. In this study, the drug shows an increasing negative mobility (Figure 11a), as the contact time of the complex to the liposomal membrane increased. This change we assign to the enhanced

release of propranolol from ß-CD cavity and transfer into liposome by time, as shown at zero, 30 min, 2 and 4 h (Figure 11a), before it reaches the final state of equilibrium. At 6 h release time the final transfer state is reached and the optimum drug release from complex and transfer into liposome is achieved.

Transfer into PC/PS/Cholesterol Liposome Contained in Sample C1

The influence of cholesterol integrated in the vesicular membrane on the drug release from CD complexes and transfer into liposome was also investigated, recording the migration behaviour of propranolol in sample C1 through the buffer system B7 at different time intervals over 24 h. As shown in Figure 11b, only a minor variation of mobility over the time takes place and an equilibrium of the partition between drug-CD complex and the cholesterol containing liposomes is reached after a short time. The interaction of ibuprofen with ß-CD forming a complex and the interaction of the drug distribution into liposome without and with cholesterol were also investigated. That was by detecting the change in migration behaviour (the mobility) of ibuprofen (sample I1) through the buffer systems B2, B4 and B5; respectively (Table 5). The kinetics of ibuprofen release from ß-CD complex and drug transfer into liposome with and without cholesterol was studied over 24 h.

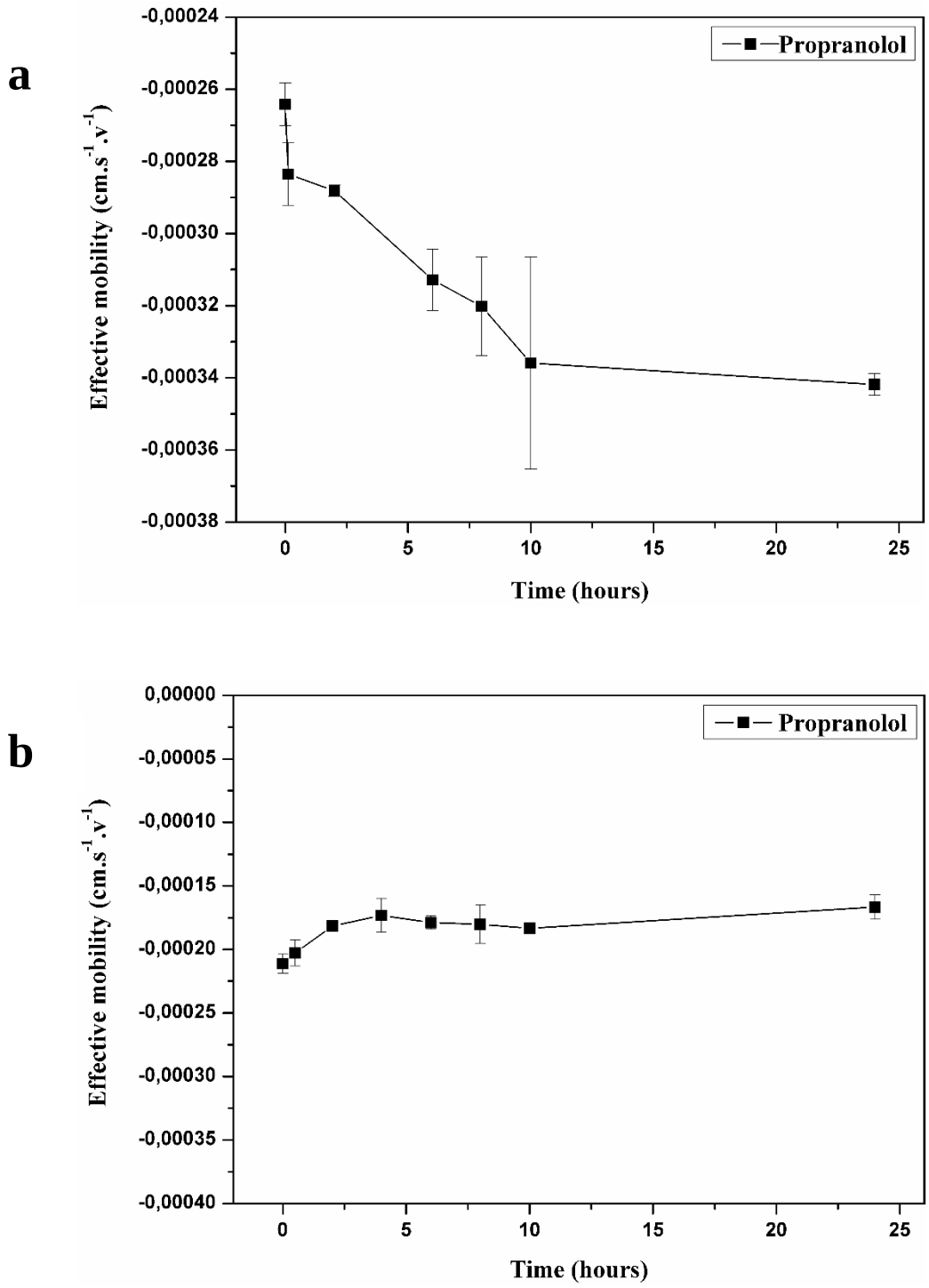

*Figure 11 Release of propranolol (Pro) from ß-CD complex and transfer into liposome without cholesterol **(a)** and with cholesterol **(b)** determined by measuring the effective mobility of 1mMPro contained in S1 sample **(a)** inC1 sample **(b)** versus time (ΔX±SD, n=2); experimental conditions: in 1mMß-CD and 1mMPC/PS liposome containing phosphate buffer system B6 **(a)** in 1 mM ß-CD and 1 mM PC/PS/Cholesterol liposome containing phosphate buffer system B7 **(b)***

Transfer into PC/PS Liposome Contained in Sample S2

As shown in Figure 12a, the mobility of ibuprofen (sample S2) through the buffer system B6 starts to change after the 2 h contact time of complex to the liposomal membrane. That indicates the starting of the drug release from ß-CD and transfer into liposome after 2 h. At 8 h release time, the maximum change in the mobility of ibuprofen is reached and this change remains constant at longer release time. At 8 h, the maximum drug release from ß-CD and transfer into liposome, which is before the final transfer state, can be achieved.

Transfer into PC/PS/Cholesterol Liposome Contained in Sample C2

As shown in Figure 12b, the migration behaviour of ibuprofen in sample C2 through the buffer system B7 reaches the maximum at 2 h. The fast maximum release of drug from ß- CD and transfer into liposome with cholesterol can be achieved at 2 h. In contrast to propranolol, the maximum transfer state for ibuprofen is not the optimum. The study of mechanism for both drugs confirms that, the drug release from ß-CD and transfer into liposome is faster in case of the cholesterol containing samples. Zero and 2 h were recorded as the maximum drug release times for propranolol and ibuprofen, respectively, for cholesterol containing samples (C1 & C2). While the same mechanism is time relating for samples (S1 & S2). As conclusion, the integrated cholesterol in liposome enhances the lipophilic drug release from ß-CD cavity and transfer to biological membrane.

5.4 Conclusions

It could be shown that ACE is a powerful method in order to study the release of drugs from CD complexes and the transfer of drugs into model membranes. The lipophilic drugs propranolol and ibuprofen which form complexes with ß-CD were used as model drugs. Furthermore, it could be demonstrated that ß-CD is able to extract cholesterol from biological membranes and since the affinity of cholesterol to ß-CD is greater than that of chosen drugs to ß-CD the drug release from its cavity and drug transfer into the liposomal membrane is accelerated when cholesterol is present in the model membrane. The method confirms that the lipophilic drugs were more easily released from its CD complex and transferred into liposomal membranes. The method described in this paper can be used to optimize drug release from CD complexes and the drug transfer into model membranes.

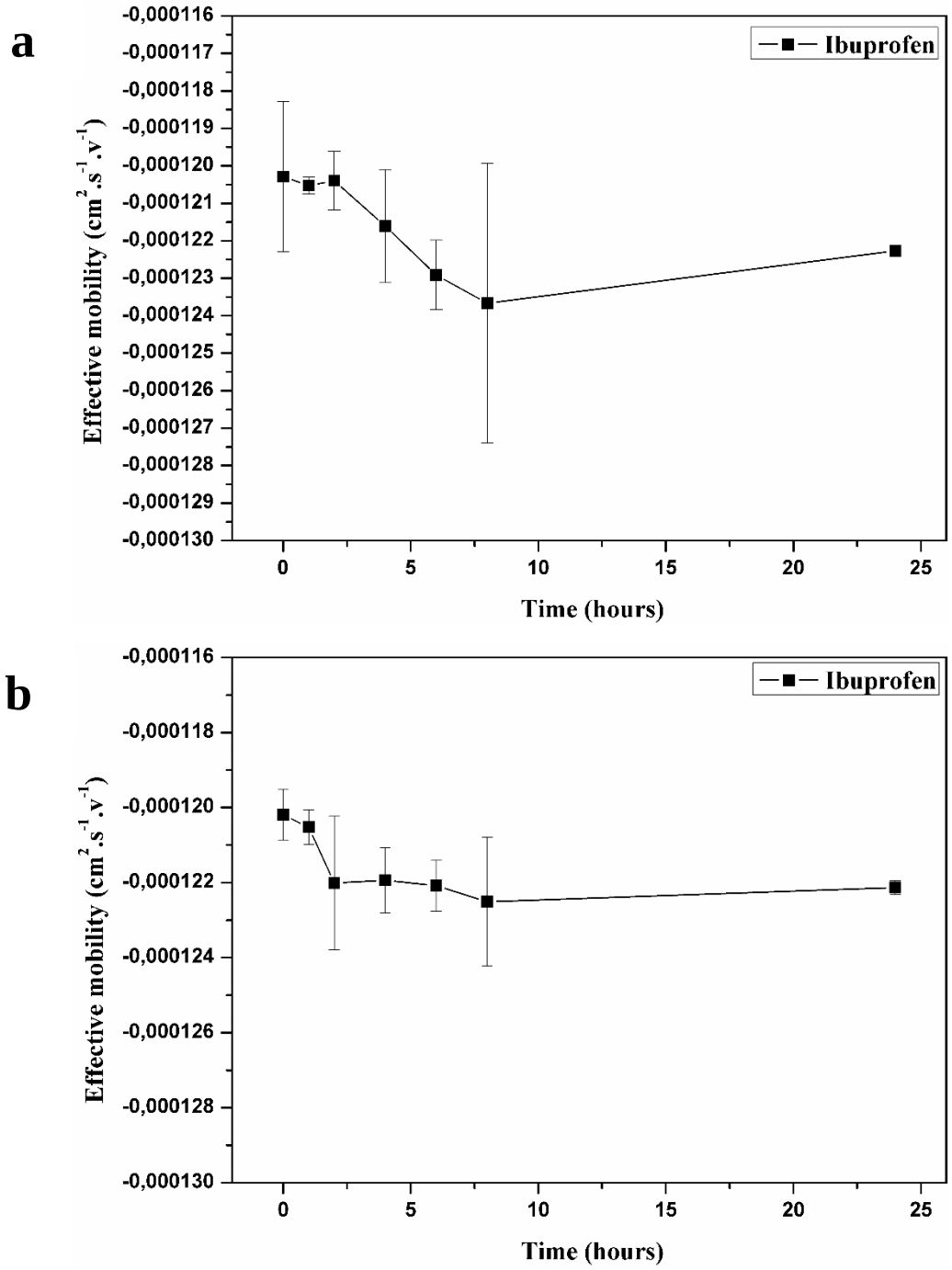

*Figure 12 Release of ibuprofen (IBU) from ß-CD complex and transfer into liposome without cholesterol **(a)** with cholesterol **(b)** determined by measuring the effective mobility of 1 mM IBU contained in S2 sample **(a)** in C2 sample **(b)** versus time (ΔX ± SD, n=2); experimental conditions: in 1 mM ß-CD and 1 mM PC/PS liposome containing phosphate buffer system B6 **(a)** in 1 mM ß-CD and 1 mM PC/PS/Cholesterol liposome containing phosphate buffer system B7 **(b)***

6 Appendix: Study of interactions between sugammadex and penicillins using H-NMR titration

6.1 Introduction

Proton nuclear magnetic resonance (H-NMR) is defined as an application of NMR spectroscopy with respect to the hydrogen-1 nuclei within the molecules of a substance, to determine the structure of its molecules. H-NMR spectra of most compounds are characterized by the chemical shift (δ) of each analyte proton. The protons of the dissolving solvents used should not interfere, so it is preferred to use deuterated ones. Internal standard (δ = 0) such as (TMS or DSS) is used for calibrating the chemical shift for H-NMR spectroscopy in organic or aqueous solutions; respectively. Depending on the change in the chemical shift between free and complexed form (Δδ), the association constant of interacted molecules could be investigated and characterized as mentioned previously in 2.2.3. For the purpose of confirming the interactions between sugammadex and penicillins, an additional NMR titration method has been used to investigate and determine the affinity strength (K_a) between sugammadex (Receptor) and penicillins (Ligands) in water. Amoxicillin and ampicillin have been chosen for this study.

6.2 Materials and methods

6.2.1 Materials

As mentioned previously in 3.2.1.

6.2.2 Methods

Job plot

The equation for calculating the complex association constant Ka only applies to 1: 1 complexes. For this reason, a Job plot is prepared before NMR titration. A Job plot is used to determine the stoichiometry of a binding event. In this method, the total molar concentration of receptor and ligand are held constant, but their mole fractions are varied. A measurable parameter that is proportional to complex formation (here is the observed chemical shift) is plotted against the mole fractions (x) of these two components. The maximum on the plot corresponds to the stoichiometry of the two species as shown in figure 13. This method is named

after P. Job, who first introduced this methodology in 1928 [83]. Stock solutions (5mM) of each of the receptor, sugammadex, and the ligands, amoxicillin or ampicillin, were prepared by dissolving weighted amounts of each in 10 ml volume of deuterated water (D2O). From these stock solutions, the equimolar solutions of the receptor and ligand were prepared and kept overnight before the measurement to ensure the occurrence of interaction.

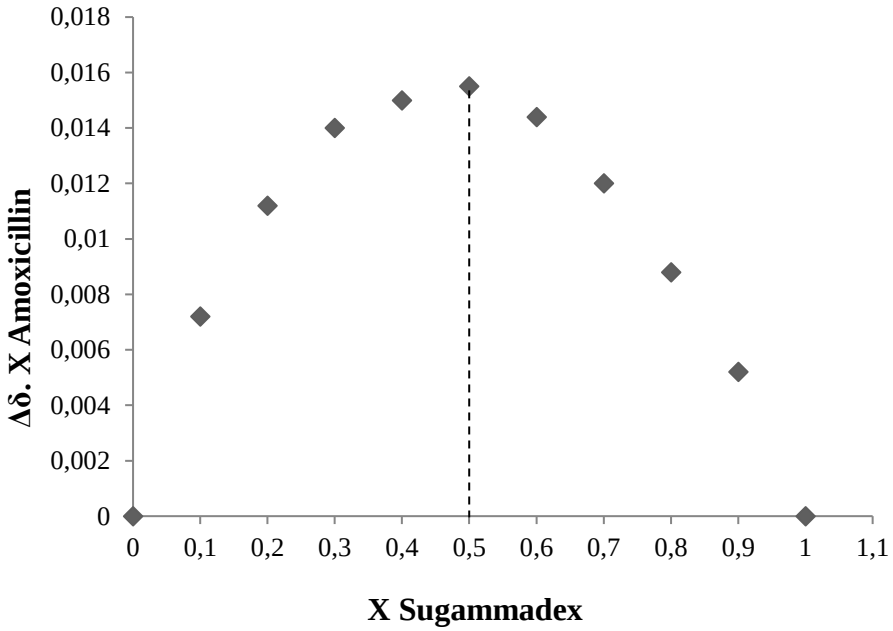

Figure 13 Job plot for titration of amoxicillin with sugammadex in D2O

NMR titration

A series of solution mixtures (ligand: receptor) were prepared at different molar ratios (1:0, 1:1, 1:2, 1:3, 1:5) in D2O. The solutions of samples were homogeneously distributed using a vortex vibrator. The samples were kept overnight to ensure the complex formation. The ^{1}H-NMR spectra were recorded with Agilent Technologies devices 400 MHz VNMRS and 500 MHz DD2. The observed chemical shift of the proton, present in both guest molecules as referred to by the circle in figure 14, is determined for both guest drugs.

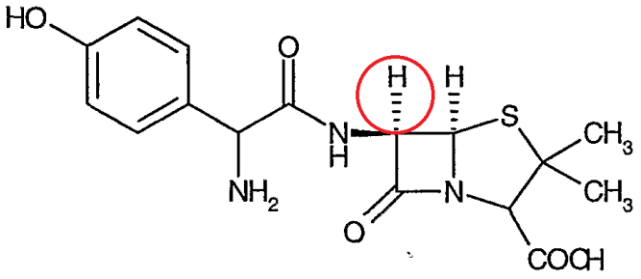

Figure 14 Chemical structure of amoxicillin

All the values of the chemical shift changes (Δδ) were plotted in correspondence to the concentration of the receptor, sugammadex [M] (Figure 15). The fitting curve was represented by an equation, which is applied to conclude the association constant of complex interaction from the value of the dissociation constant of the same interaction and discussed in [84]. From this, the value of K_a was calculated.

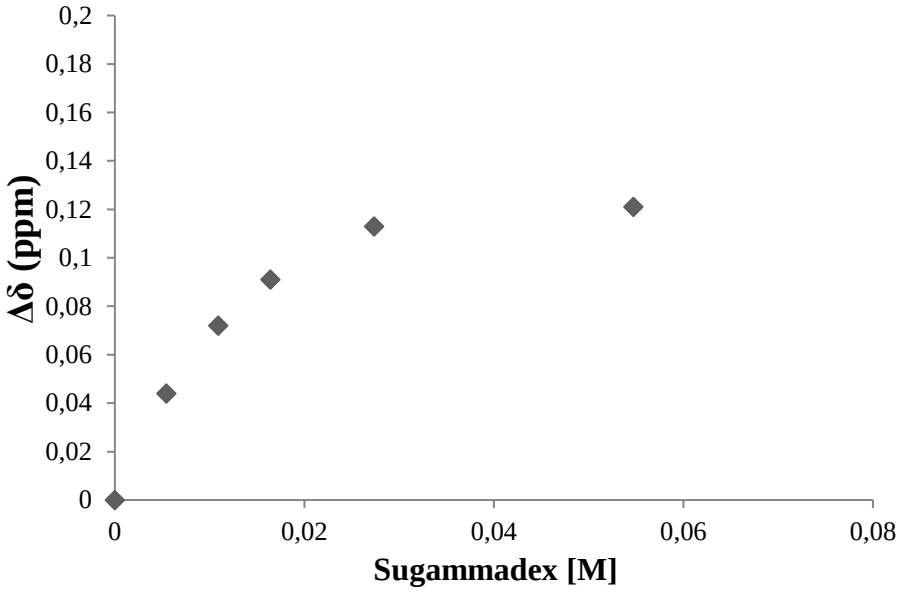

Figure 15 Titration curve represented by a plot of changes in chemical shift of defined guest proton versus the corresponding receptor concentration

Results and discussion

As shown in figure 13, the maximum change in chemical shift for amoxicillin proton (x mole fraction) was at 0.5 mole fraction of the receptor, sugammadex. That means the complex formation occurs at 1:1 molar ratio. The same result was obtained for ampicillin. The stoichiometry of each complex interaction was detected, then the value of K_a could be calculated. Regarding the recorded values of the changes in the chemical shift for the guest proton and the corresponding receptor concentrations, all these data were compensated for in the representative equation of the fitting curve using an Excel file. And as a consequence, the values of the affinity strength of pencillins with sugammadex were calculated as: 257.8 and 133.3 M^{-1} for amoxicillin and ampicillin; respectively. In conclusion, the H-NMR results of K_a values for both amoxicillin and ampicillin are in good agreement with the values of ACE (Table 1) and that confirmed again the complex formation between studied drugs and sugammadex. And from another side, this confirms the suitability of ACE as the simplest, not complicated, non-consuming sample amounts and time-saving analytical method for determination of CD complex formation.

7 Summary

7.1 English version

This study has confirmed the potential of ACE as a simple and effective analytical method for investigation and characterization of some interested interactions in the field of pharmaceutics and biopharmaceutics. The importance of these interactions came from their possibility of influencing the pharmacodynamics and/or the pharmacokinetics of the drug and, as a consequence, influencing the drug's bioavailability and its pharmacological action.

For the first time, inclusion complexes between sugammadex (a modified γ-CD) and penicillin were successfully characterized using ACE. The difference in the chemical structure, especially (R) side chains of the studied penicillins, is assumed to be responsible for the different affinities to sugammadex. Furthermore, this study confirms the potential of ACE for detecting complex formations of sugammadex with other potentially co-administered drugs. So in the future the investigation of sugammadex interactions using ACE could be continued for other different prospective co-administrated drugs.

The IPF between a quaternary ammonium compound (GLB) and different IPAs was successfully characterized using ACE. This study confirmed the linear correlation between the affinity strength of the ion-pair complex and the partition behavior of the cationic drug, GLB, in the presence of anionic molecules at 1:1 molar ratio. The partition behavior of the cationic drug from the aqueous phase into the organic phase was determined successfully in the absence and presence of different anionic molecules at different molar ratios. In conclusion, the partitioning of the drug into the hydrophobic phase is directly increased as its affinity strength to the anion by ion-pair interaction increases. To optimize the IPF and its effect, SDS would be the most recommended IPA for this study. SDS has a significant effect on K_f and the partitioning behavior of GLB. Furthermore, its properties, such as availability and low cost, are the contributions for SDS to be preferably chosen.

It could be shown that ACE is a powerful method to study the release of drugs from CD complexes and the transfer of drugs into model membranes. The lipophilic drugs propranolol and ibuprofen which form complexes with ß-CD were used as model drugs. Furthermore, it could be demonstrated that ß-CD can extract cholesterol from biological membranes and, since the affinity of cholesterol to ß-CD is greater than that of chosen drugs to ß-CD, the drug's release from its cavity and its transfer into the liposomal membrane is accelerated when cholesterol is present in the model membrane. The method confirms that the lipophilic drugs

were more easily released from their CD complex and transferred into liposomal membranes. The method described in this work could be used to optimize drug release from CD complexes and the drug's transfer into model membranes. This method could be used in future for investigation and demonstration of the release from different CDs, and transfer into biological membranes of other lipophilic drugs. On the basis of this study, the method could be developed to be adapted for similar interactions for drugs with different physicochemical characterization.

7.2 Zusammenfassung

In dieser Studie wurde bestätigt, dass die ACE als eine einfache und wirksame analytische Methode für die Untersuchung und Charakterisierung von einigen interessierenden Interaktionen auf dem Gebiet der Pharmazie und Biopharmazie ein großes Potenzial besitzt. Die Bedeutung dieser Wechselwirkungen stammt von ihrer Möglichkeit, die Pharmakodynamik und / oder die Pharmakokinetik des Arzneimittels zu beeinflussen und als Folge die Bioverfügbarkeit des Arzneimittels und dessen pharmakologische Wirkung zu beeinflussen.

Zum ersten Mal wurden Einschlusskomplexe zwischen Sugammadex (einem modifizierten γ-CD) und Penicillinen erfolgreich unter Verwendung der ACE charakterisiert. Der Unterschied in der chemischen Struktur, vor allem (R) -Seitenketten der untersuchten Penicilline wird als verantwortlich für die verschiedenen Affinitäten zu Sugammadex angenommen. Darüber hinaus bestätigt diese Studie das Potenzial der ACE für die Erkennung der Komplexierung des Sugammadex mit anderen potenziell zusammen verabreichten Medikamenten. So könnten in Zukunft die Untersuchungen mit der ACE hinsichtlich von Sugammadex-Wechselwirkungen bei andere koadministrierten Medikamente fortgesetzt werden.

Die Ionenpaarbildung (IPF) zwischen einer quaternären Ammoniumverbindung (GLB) und verschiedenen IPAs wurde erfolgreich unter Verwendung der ACE charakterisiert. Diese Studie bestätigte die lineare Korrelation zwischen der Affinitätsstärke des Ionenpaarkomplexes und dem Verteilungsverhalten des kationischen Arzneistoffs GLB in Gegenwart verschiedener Gegenionen im molaren Verhältnis von 1: 1. Das Verteilungsverhalten des kationischen Arzneistoffes aus der wässrigen Phase in die organische Phase wurde erfolgreich in Abwesenheit und Gegenwart verschiedener anionischer Moleküle bei verschiedenen molaren Verhältnissen bestimmt. Als Abschluss wird die Aufnahme des Arzneistoffes in die hydrophobe Phase direkt als die Affinität des Anions zum GLP bestimmt. Um die IPF und ihre

Wirkung zu optimieren, wäre SDS das beste Gegenion für das GLP. SDS hat signifikante Auswirkungen auf K_f und das Partitionierungsverhalten von GLB.

Es konnte weiterhin gezeigt werden, dass ACE eine leistungsfähige Methode ist, um die Freisetzung von Wirkstoffen aus CD-Komplexen und den Transfer von Wirkstoffen in Modellmembranen zu studieren. Die lipophilen Wirkstoffe Propranolol und Ibuprofen, die mit ß-CD Komplexe bilden, wurden als Modellarzneistoffe verwendet. Darüber hinaus konnte gezeigt werden, dass ß-CD Cholesterol aus biologischen Membranen extrahiert werden kann, da die Affinität von Cholesterin zu ß-CD größer ist als die der ausgewählten Arzneistoffe. Die Arzneistofffreisetzung aus dem Hohlraum des β-CD und der Transfer des Wirkstoffes in die liposomale Membran wird beschleunigt, wenn Cholesterol in der Modellmembran vorhanden ist.

Das Verfahren bestätigt, dass die lipophilen Arzneistoffe leichter aus ihrem CD-Komplex freigesetzt und in liposomale Membranen übertragen wurden. Das in dieser Arbeit beschriebene Verfahren könnte verwendet werden, um die Arzneimittelfreisetzung von CD-Komplexen und den Arzneimitteltransfer in Modellmembranen zu optimieren. Diese Methode könnte in Zukunft für die Untersuchung und Demonstration der Freisetzung von anderen Wirkstoffen aus CD-Komplexen und des Transfers in biologische Membranen verwendet werden. Auf der Grundlage dieser Studie könnte die Methode weiterentwickelt werden, um ähnliche Interaktionen von Arzneistoffen mit relevanten Liganden/Hilfsstoffen zu charakterisieren.

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List of Publications

Research articles

- Kinda A. Darwish, Yahya Mrestani, Reinhard H. H. Neubert, Study of Interactions Between Sugammadex and Penicillins Using Affinity Capillary Electrophoresis, *Chromatographia*, Volume 76, Issue 23 (2013), 1767-1771

- Kinda A. Darwish, Yahya Mrestani, Reinhard H. H. Neubert, Optimization of Ion-Pair Formation between Glycopyrronium Bromide and Different Ion Pair Agents Using Affinity Capillary Electrophoresis, *Electrophoresis*, Volume 36, Issue 21-22 (2015), 2805–2810

- Kinda A. Darwish, Yahya Mrestani, Hans-Hermann Rüttinger, Reinhard H.H. Neubert, Drug Release from ß-Cyclodextrin Complexes and Drug Transfer into Model Membranes Studied by Affinity Capillary Electrophoresis, *Pharmaceutical Research*, Volume 33, Issue 5 (2016), 1175-1181

Poster presentations

- Kinda A. Darwish, Yahya Mrestani, R.H.H. Neubert, Characterization of sugammadex inclusion complexes with penicillins using affinity capillary electrophoresis, *7th polish-German symposium on pharmaceutical science "Interdisciplinary research for pharmacy"*, 24-25 May 2013, Gdansk, Poland.

- Kinda A. Darwish, Yahya Mrestani, R.H.H. Neubert, Study of Sugammadex and Penicillins Inclusion Complexes Using Affinity Capillary Electrophoresis, *CE–Forum*, 16-17 September 2013, Jena, Germany.

- Kinda A. Darwish, Yahya Mrestani, R.H.H. Neubert, Determination of drugs in aqueous media and biological fluids using capillary electrophoresis coupled to different detectors, *CE-Forum*, 29-30 September 2014, Marburg, Germany.

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Curriculum vitae

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Eidesstattliche Erklärung

Hiermit erkläre ich gemäß § 5 Absatz 2b der Promotionsordnung der Naturwissenschaftlichen Fakultät I (Biowissenschaften) der Martin-Luther-Universität Halle-Wittenberg, dass ich die Ergebnisse der vorliegenden Dissertation

Different Approaches of Drugs Interactions Studied using Affinity Capillary Electrophoresis

am Institut für Pharmazie der Martin-Luther-Universität Halle-Wittenberg selbständig und ohne fremde Hilfe erarbeitet und verfasst habe. Ferner habe ich nur die in der Dissertation angegebenen Literaturstellen und Hilfsmittel verwendet und die entnommenen und benutzten Literaturstellen auch als solche kenntlich gemacht. Weiterhin habe ich die vorliegende Arbeit bisher keiner anderen Prüfungsbehörde vorgelegt.

Halle (Saale), im Januar 2018 Kinda Darwish