Evaluation of three neutral capillary coatings for the determination of analyte-cyclodextrin binding constants by affinity capillary electrophoresis. Application to N,N'-disubstituted piperazine derivatives.

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Highlights

Three neutral coatings are evaluated to avoid the analyte and CD adsorption

CD adsorption on the HPC and PEO coatings is proved

PDMA coating allows to determine polycationic analyte-CD binding constants

Complex structure is elucidated through structure-binding constant relationships

Abstract

The performances of three neutral static coatings (hydroxypropyl cellulose, polyethylene oxide and poly(N,N-dimethylacrylamide) have been evaluated in order to determine the binding constants of the complexes formed between four polycationic compounds (piperazine derivatives) and four cyclodextrins of pharmaceutical interest (β-CD, HP-β-CD, Me-β-CD and sulfobutyl ether-β-CD) by affinity capillary electrophoresis. The physically-adsorbed poly(N,N-dimethylacrylamide) coating proves to be the more efficient to mask the silanol groups of the capillary wall since the lowest electroosmotic flow was measured for this coating. Moreover, it drastically reduces the adsorption of the compounds since it allows a correct repeatability of their migration time, higher efficiencies of the peaks and no baseline shift. Then, it was verified for four complexes that this coating allows a correct determination of the binding constants avoiding the CD adsorption which is responsible of an undervaluation of binding constants. The highest binding constants are obtained using the anionic sulfobutyl ether-β-CD (SBE-β-CD). The structure of the complex formed between the tacrine derivative and the SBE-β-CD was further investigated through 2D ROESY NMR experiments and structure-binding constant relationships. Results suggest that the inclusion in the SBE-β-CD cavity occurs through the aliphatic ring portion of the tacrine moiety.

1. Introduction

Cyclodextrins (CDs) are macrolytic compounds with several D-glucopyranose units linked by α-1,4-glycosidic bonds. The common α-CD, β-CD and γ–CD are composed of 6, 7 and 8 glucose units, respectively. The shape of CDs is a truncated cone with a central cavity due to the chair conformation of the glucopyranose units. Their exterior surface is hydrophilic thanks to the presence of hydroxyl groups whereas the central cavity is lined by skeletal carbons and ethereal oxygens of the glucose residues which give it a relatively lipophilic character [1, 2]. These properties make them first interest complexing agents since they are able to form inclusion complexes with a great variety of molecules of appropriate polarity and size [3]. In pharmaceutical formulations, CDs are generally used to enhance the solubility, bioavailability and stability of drugs [4-6]. CDs can be found in at least 35 pharmaceutical products: the β-CD, the hydroxypropylated HP-β-CD, the methylated Me-β-CD and the sulfobutyl ether SBE-β-CD are the most commonly used CDs [7].

Quantification of the interactions between the drug and the cyclodextrins based systems is a recurrent question in the literature. Many techniques may be used to assess the apparent stability constants of drug-CD complexes. Among them, the capillary electrophoresis has been widely used [8] because of its automatisation, miniaturization, efficiency, rapidity and small amount of sample required. Affinity capillary electrophoresis (ACE) is especially adapted to investigate the ligand-cyclodextrin interactions. It consists in studying the electrophoretic mobility variations of the ligand according to the various CD concentrations in the background electrolyte (BGE) [8].

Alzheimer's disease (AD) is the most prevalent cause of dementia. It combines two pathophysiological mechanisms: amyloid pathology and Tau pathology [9]. Itis characterized by a progressive decrease in acetylcholinesterase (AChE) enzymatic activity accompanied by a cholinergic neuron degenerescence. Only symptomatic treatments are currently available. Designing multitarget drugs is a recent and promising approach against multifactorial illnesses as neurodegenerative diseases such as Alzheimer's disease. We have designed and developed a novel family of N,N'-disubstituted piperazine [10, 11] that modulate APP metabolism by reducing the release of selective species of Aβ peptides, especially the long and toxic Aβ species, and by increasing several APP metabolites including the gene regulatory fragment of APP that is the amyloid intracellular domain (compounds **2**, **3** and **4-** Figure 1) [11]. We recently designed multifunctional compounds derived from N,N'-disubstitued piperazine and available AChE inhibitors. Compound **1** provided high potential as a

drug candidate for AD as it inhibited hAChE very efficiently (IC_{50} < 1nM), and was able to decrease both amyloid and Tau pathology *in vivo* by oral route [12, 13].

Since the compound **1** possesses a tacrine moiety, particular attention must be paid to its eventual metabolites. Pool et al. [14] have described the metabolism of tacrine by single or multiple hydroxylations of its aliphatic ring. These metabolites have been described to be responsible for hepatotoxicity. In order to protect its aliphatic ring against this metabolism, we have chosen to investigate the interest of CDs in the formulation of compound **1**. Before envisaging a microsomal study of the metabolism of **1** under free and complexed forms, a study of the **1**-CDs complexes must be achieved in order to select the more appropriate CD with a sufficient binding constant and a favourable structure.

Early CE analysis at pH 7.4 of compound **1**, which contains a N,N'-disubstituted piperazine moiety (polycationic character), have shown its adsorption on the naked capillary. This adsorption may be relied to electrostatic interactions with the anionic surface of the capillary due to the deprotonation of the silanol groups at pH 7.4. We used coated capillaries in order to avoid this problem and to allow an accurate determination of the binding constants of the **1**-CDs complexes.. To the best of our knowledge, no study regarding the determination of analyte-CD binding constants by ACE using coated capillaries is reported in literature. Only some chiral separation methods using such CDs are developed using coated capillaries to improve the enantioresolution and to reduce peak tailing resulting from solute interactions with silanol groups of capillary wall (for methods developed at neutral or basic pH) [15-17]. As described in a recent review [18], the coating of the inner wall of the capillary may be achieved in dynamic or static fashions. The dynamically coated capillaries require a regular regeneration and the addition of the coating agent into the BGE which is necessary to maintain the coating. Static coatings are the most popular capillary coatings. Two kinds of static coatings exist: the static-covalent coatings whose preparation are quite laborious but present a long-term stability and the static-adsorbed coatings which can be more easily regenerated.

In this paper, the performances of three static coatings obtained using hydroxypropylcellulose (HPC), polyethyleneoxide (PEO) or poly(N,N-dimethylacrylamide (PDMA) are evaluated: ability to cover the inner surface of the capillary and ability to strictly reduce the adsorption of the polycationic compounds **1**-**4** while avoiding the adsorption of the CDs. Then, the binding constants of the

complexes formed between **1** and four CDs of pharmaceutical interest (β-CD, HP-β-CD, Me-β-CD and SBE-β-CD) is determined by ACE to select the more appropriate CD. Last, the structure of the complex displaying the greater binding constant is investigated through a 2D ROESY NMR study and structure-binding constant relationships.

2. Material and methods

2.1 Chemicals

The original derivatives **1**-**4** (Figure 1) were designed and synthesized by some of us according to previously described protocols [11-13, 19]. Tacrine, ibuprofen, carbamazepine and poly(ethylene oxide) (PEO, average Mw 200,000) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Hydroxypropylcellulose (HPC, average Mw 80,000) was purchased from Ashland Aqualon (Wilmington, SA). Poly(N,N-dimethylacrylamide) (PDMA, average Mw 2,400) was purchased from Polymer Source (Dorval, Quebec, Canada). The structure of these polymers are displayed in Figure 1. Risperidone was a gift from Janssen-Cilag (Issy-les-Moulineaux, France). Sodium hydroxide, chlorhydric acid 37%, sodium dihydrogenophosphate monohydrate and di-sodium hydrogenophosphate were purchased from Merck (Nogent-sur-Marne, France). Deionized water was obtained from Milli-Q system (Millipore, Saint-en-Yvelines, France). Deuterium oxide (100%) was purchased from Euriso-top (Gif sur Yvette, France).

2.2 Cyclodextrins

β-CD, HP-β-CD and Me-β-CD were kindly supplied by Roquette Laboratories (Lestrem, France). The HP-β-CD and Me-β-CD represent multicomponent mixtures with molar substitution (MS) of 0.61 and 0.57 per glucose unit, respectively. Sulfobutylether-β-CD (SBE-β-CD, Captisol®, average degree of substitution: 6.2-6.9) was kindly supplied by Cydex Pharmaceutical (La Jolla, California, USA). Averaged molecular weight was taken into account to calculate the molar concentration of the substituted CD.

2.3 Nuclear Magnetic Resonance

The NMR experiments were realized on a Bruker AVANCE 500 with a TXI probe operating at 500.13 MHz. To study the structure of the complexes by 2D ROESY experiments (homonuclear dipolar correlation ^{1}H-^{1}H), experiment spectra were recorded with a mixing time of 500 ms. Five hundred µL of solutions were introduced into standard 5 mm NMR tubes and the experiments were performed at 298 K. The 67 mM phosphate buffer pH 7.4 was prepared by mixing appropriate amounts of both sodium salts in D_2O. The concentrations of **1** and the CD (β-CD or SBE-β-CD) results from a compromise between a relative high analyte concentration (for better signal/noise ratios) and a good separation of analyte resonance signals (if possible). The optimal condition was obtained for an equimolar mixture (4 mM for **1** and 4 mM for the CD).

2.4 Capillary electrophoresis

CE experiments were performed on a Beckman P/ACE MDQ Capillary Electrophoresis system, including an on-column diode-array UV-detector driven by the 32 Karat software (Beckman Coulter, Villepinte, France) package for system control, data collection and analysis. It was equipped with 30.2 cm (20 or 10 cm effective length) x 50 µm I.D. capillaries (Composite Metal Services, Ilkley, UK). Each new fused-silica capillary was conditioned before use by successive flushes with NaOH 1 N (20 psi, 5 min), NaOH 0.1 N (20 psi, 5 min) and deionized water (20 psi, 5 min). For use of an uncoated capillary, the rinses between each run consist of flushes with deionized water (1 min, 20 psi) and BGE (3 min, 20 psi).

For the complexation study, the capillary was mounted on a cartridge thermostated at 298 K ± 0.1 K. The 67 mM phosphate buffer pH 7.4 (BGE) was prepared by mixing appropriate amounts of NaH_2PO_4 and Na_2HPO_4. The applied voltage was 15 kV, the highest voltage that prevents significant Joule heating in our conditions. The concentration of the analytes was 0.1 mM (or 0.01 mM for ibuprofen) in water with 0.02% DMSO as EOF marker. To make the model valid, the analyte concentration must be at least ten times lower than the CD concentrations used [20]. Hydrodynamic injections were made with a 5 s injection time at 1.0 psi pressure. Analytes were detected using the diode-array detector between 190 and 290 nm.

2.4.1 HPC coating

The capillary was coated with hydroxypropylcellulose (HPC) as described by Shen et al. [21].

The fused-silica capillary was filled with a 5% w/v hydroxypropyl cellulose solution (in water) using a syringe pump (Harvard apparatus, Holliston, MA, USA). An amount of 20 µL was percolated at a flow rate of 0.5 µL/min and incubated for 2 h at room temperature. The capillary was purged under nitrogen pressure (20 psi) for 10 min, then heated from 60 to 140°C for 30 min, in a GC oven (Varian, Les Ulis, France).

2.4.2 PEO coating

The coating based on poly(ethylene oxide) (PEO) was prepared as described by Thuy Tran et al. [22]. The solution of PEO at 0.2% in 0.1 M HCl was prepared as follows : 20 mg of PEO were dissolved in 9 mL of water at 85°C and stored at room temperature. Then 1 mL of 1 M HCl was added to the PEO solution just before use. The coating procedure consists in successive flushes with water (20 psi, 2 min), HCl 1 M (20 psi, 5 min), PEO 0.2% (20 psi, 5 min) and BGE (20 psi, 2 min). It was necessary to regenerate the capillary daily and between each run with this rinse procedure to maintain the EOF constant.

2.4.3 PDMA coating

The solution of poly(N,N-dimethylacrylamide) (PDMA) was prepared in HCl 0.1 M at a concentration of 0.1% [17]. The coating procedure consists in successive flushes with water (20 psi, 2 min), HCl 1 M (20 psi, 5 min), PDMA 0.1% (20 psi, 5 min) and BGE (20 psi, 2 min). It was necessary to regenerate the capillary daily and between each run with this rinse procedure to maintain the EOF constant.

2.4.4 EOF measurements

For uncoated capillaries, the EOF was measured using the migration time of a neutral marker (DMSO 0.02%). For neutral coated capillaries, for which EOF is too low to be classically evaluated, the residual EOF was measured according to the method developed by Williams and Vigh [24].

Briefly, a neutral marker (DMSO 0,02%) was injected (1 psi, 5 s) in the capillary previously rinsed with the BGE (band N_1). A pressure (0.5 psi, 30 s) was applied to shift the band into the capillary. A similar second band (N_2) of DMSO was injected and a pressure (0.5 psi, 30 s) was applied to shift both bands N_1 and N_2 into the capillary. A voltage (V) of 15 kV was applied during 5 minutes (t voltage). During this time, both bands migrated towards with a mobility equal to EOF. A third band of DMSO was

injected (N_3) and a final pressure of 0.5 psi was applied during 5 minutes to allow the passage of the three bands past the UV detector. The times required for the bands N_1, N_2 and N_3 to be pushed past the detector permit the calculation of the EOF.

2.4.5 Mesurement of the viscosity

The determination of analyte-CD binding constants by ACE requires correction to negate the changes in mobility caused by variation of the buffer viscosity induced by the addition of CD. The viscosities of the BGE without CD (η_0) or with CD from 1 to 10 mM (η) were evaluated using the Hagen–Poiseuille law as described by François et al. [25]. All mobilities were corrected by the ratio η_0/η. These ratios varied from 0.99 to 0.97 for the β-CD, from 0.99 to 0.96 for the Me-β-CD, from 0.99 to 0.95 for the HP-β-CD and from 0.99 to 0.93 for the SBE-β-CD.

3. Results and discussion

3.1 Preliminary experiments

The first analyses of **1** performed using a fused-silica capillary and a phosphate buffer pH 7.4 display an awful repeatability of the migration time and EOF (Fig. 3). .Seven successive analyses of **1** were performed and the migration times progressively increase as the EOF decreases which supposes a retention mechanism through an adsorption phenomenon. The severe peak broadening observed and the lack of efficiency is a second indication of the adsorption of **1** onto the negatively charged capillary surface. Similar observations were made with compounds **2**, **3** and **4** (Figure A, Supplementary material). As the four compounds present a similar bis-aminoalkylpiperazine chain, their adsorption may be attributed to their polycationic character. Then, our strategy to analyze by CE these polycationic compounds was based on the use of coated capillaries. Numerous static coatings exist and are described in various reviews; most of them are developed to reduce the protein adsorption [18, 26, 27]. Additionally, the selected coatings must be based both on the the analyte and the cyclodextrin electrical charges: the compounds of pharmaceutical interest are polycationic, then the CDs selected will be neutral or anionic. Therefore, we chose to study the interactions between both partners potentially presenting opposite charges by focusing on neutral coatings. The compound **1** was used as

a model to investigate and to reduce this adsorption phenomenon. It is worth mentioning that neutral coatings drastically reduce or eliminate the EOF [26].

3.2 Evaluation of neutral coatings

Two kinds of static neutral coating capillaries are intensively used: the covalently-linked and the physically-adsorbed neutral polymers. Among the neutral polymers, four have proven their high efficiency to coat the capillary and then to avoid protein adsorption: the cellulose derivatives, the polyvinylalcohol (PVA), the polyethylene oxide (PEO) and the acrylamide-based polymers [27, 28] or copolymers [29]. We chose to study the performance of a covalently-linked HPC capillary, previously used in our laboratory [30], and two physically adsorbed coated capillaries using PEO and PDMA. These polymers were selected since they possess a high affinity for the capillary wall and then form stable coating. Moreover, even if these coatings are not recommended for proteins (proteins possess hydrophobic patches that interact with these more hydrophobic polymers) [31], it seems to be good candidates for the study of interaction between a cationic compound and various CD. Effectively, a moderately hydrophobic capillary coating must limit the adsorption of the CDs which present hydrophilic surfaces.

Four factors were considered to evaluate the coatings: (i) the measurement of the residual EOF, (ii) the repeatability of the migration time of the analytes, (iii) the separation efficiency N (plates per meter) and (iv) the presence (or absence) of a baseline shift which is an indicator of an irreversible adsorption [26].

According to the EOF values displayed in Table 1 and as expected the three coatings appear to be effective to drastically reduce the EOF. EOF mobilities using the PEO, HPC or PDMA coatings are around 350, 650 or 2700 times smaller than the EOF mobilities measured using the uncoated capillary, which prove the ability of these neutral polymers to mask the silanol groups of the capillary wall responsible of the analyte absorption. However, the RSD of the EOF appears to be higher than the one without coating (around 20-30%) but this result must be carefully discussed. Indeed, the EOF is so low that a variation of 0.01 minute on the migration times of the neutral marker, according to the Williams and Vigh's method, greatly impacts the very low EOF value. This result does not question the reliability of the coatings. The PDMA coating displays the lowest EOF (inferior to 1.10^{-5} $cm^2.V^{-1}.min^{-1}$) which is in accordance with the results published by Chiari et al. [31]. Due to its more hydrophobic

character, it is able to displace the solvent water molecules from the surface and then forms more stable coatings through the formation of a higher number of hydrogen bonds with the absorptive sites of the silica-capillary. Using the PDMA coating procedure, the silanol groups are more effectively shielded which provides the slowest EOF. Table 1 displays the three factors allowing to evaluate the performance of the coatings to avoid the adsorption of **1**. The RSD of its migration time is very satisfactory whatever the nature of the coating (≤ 0.3%). These results are associated to higher peak efficiencies, the theoretical plates are around two times higher even if a slight tailing is still observed. Additionally, no baseline shift is observed with the use of the three coated capillaries. Similar results are obtained for the compounds **2**-**4** (Fig.B, supplementary material). Therefore, these results show that, whatever the nature of the polymer, the neutral coatings are efficient to strictly limit the adsorption of the compounds **1**-**4** on the capillary wall.

3.3 Determination of apparent binding constants

For analyte/CD complexes of assumed 1:1 stoichiometry, Benesi-Hildebrand's method [32] and Scott's modified equation [33] allow the calculation of apparent binding constants from the three following linear equations obtained after mathematical rearrangements (y-reciprocal (1), double-reciprocal (2) and x-reciprocal (3)):

$$\frac{[CD]}{\mu_i - \mu_f} = \frac{1}{\mu_c - \mu_f}[CD] + \frac{1}{(\mu_c - \mu_f)K} \quad (1)$$

$$\frac{1}{\mu_i - \mu_f} = \frac{1}{(\mu_c - \mu_f)K}\frac{1}{[CD]} + \frac{1}{(\mu_c - \mu_f)} \quad (2)$$

$$\frac{(\mu_i - \mu_f)}{[CD]} = -K(\mu_i - \mu_f) + K(\mu_c - \mu_f) \quad (3)$$

where K is the apparent binding constant, [CD] is the free CD concentration in the capillary, μ_i is the electrophoretic mobility observed and μ_f and μ_c are the electrophoretic mobilities of the analyte in their free and complexed forms, respectively. It is noteworthy that the free CD concentration is considered to be the total CD concentration since the complexed CD concentration is insignificant (the

concentration of the injected analyte is at less ten times lower than the CD one). The electrophoretic mobilities are corrected for viscosity changes by multiplying the mobilities by the ratio η_0/η. The three linear equations are used since differences due to relative uncertainties of the variables before and after transformation for plotting can occur [34, 35]. Moreover, deviations from linearity can be more easily observed using x- or double-reciprocal plot than the y-reciprocal plot [36]. However, no significant difference was obtained; all the reported results in this study are determined using the y-reciprocal equation. The binding constants are apparent because the concentrations are considered (instead of activities) and averaged when the complexation occurred with modified CDs of averaged degree of substitution. Since coated capillaries are employed, it is necessary to study the possible adsorption of the CD on the surface capillary. Indeed, if CD adsorption occurs, the CD concentration free to interact with the analyte is lower and this results to an undervaluation of the binding constant. Then, the determination of analyte-CD binding constants will be performed after proving the absence of CD adsorption.

3.3.1. Study of the CD adsorption on capillary wall:

The method classically used to study the adsorption on capillary wall consists in the evaluation of the recovery through the measurement of the peak areas, using a CE instrument with two on-capillary detectors [37] or only one detector [38] (these methods were developed to study the protein adsorption). In the case of non UV-absorbing substances such as CDs, these methods cannot be employed. If adsorption of CD onto the capillary wall occurs, the EOF may be altered. Thus, monitoring the EOF in absence and presence of CD should be informative. However, the addition of CDs in the BGE can generate other modifications such as the viscosity and the ionic strength of the BGE. Moreover, the interaction between the neutral marker and the CD can alter the measurement of the EOF. As previously described, the influence of the viscosity can be easily removed. The influence of ionic strength (which changes the ζ–potential) is more problematic and can be significant using anionic SBE-β-CD. Müllerova et al. [39] have demonstrated that DMSO is a suitable neutral marker to determine the EOF in presence of CD in the BGE : the interaction between DMSO and CD is sufficiently weak to neglect the effect of their complexation on the mobility measurements. The EOF was evaluated for each coating in absence or presence of the neutral β-CD at various concentrations in the BGE. Results are displayed in Figure 3. As illustrated, the errors associated to the electroosmotic mobilities are large and the results must be carefully discussed. For the PDMA coating,

the EOF values are very low whatever the β-CD concentration and no significant trend may be found. With the PEO and HPC coatings, a decrease of the EOF values using higher β-CD concentration in the BGE is suggested. This may be in accordance with a CD adsorption on the capillary wall which may mask more efficiently the silanol groups. However, the lack of accurate conclusions about the adsorption of the CD by monitoring the EOF lead us to propose another methodology.

To the best of our knowledge, in literature, all the determinations of analyte-CD binding constants for neutral or anionic CD are evaluated using fused-silica capillaries and any adsorption phenomenon of these CD is reported. Moreover, various studies point out the good agreement between the binding constants obtained by ACE using uncoated capillaries and other static techniques as isothermal titration calorimetry (ITC) recognized as the reference method [40]. We have determined the binding constants using fused-silica capillary for the complexes ibuprofen-β-CD (K = 9 332 M^{-1}) and ibuprofen-HP-β-CD (K = 5 178 M^{-1}). Our results are in total agreement with the published results obtained by ITC (K = 9502 $M^{-1}$and K = 5146 M^{-1}, respectively) [41]. So, we have chosen to evaluate the possible adsorption of CDs by comparison of the binding constants determined for various analyte-CD complexes with the uncoated and coated capillaries (the reference being the result obtained with the uncoated capillary).The complexes must be carefully chosen, after will be assured that the analytes do not adsorb on the capillary wall.Several complexes were studied in order to generalize the study of the CD adsorption. Due to the neutral character of the capillaries, at least one partner of the interaction must be ionized to allow the migration of the analyte. We excluded cationic CD because the CD of pharmaceutical interest are neutral or anionic. Complexes with identical charge for both partners (CD and analyte) were also excluded for reasons of electrical repulsion. The β-CD and SBE-β-CD have been selected to study CDs presenting different charge and substitution. Four analytes, the carbamazepine, tacrine, risperidone and ibuprofen (which are neutral, cationic, cationic or anionic at pH 7.4, respectively) were selected after checking that they do not adsorb on the four capillary surfaces. by evaluation of their migration time repeatability (RSD under 1%), efficiency and absence of baseline shift (Table A, Supplementary material) Note that tacrine displays an excellent repeatability of its migration time on the fused-silica capillary (RSD = 0.5%) contrary to the compounds **1** derived from tacrine (32.5% RSD for tm of compound **1**, Table 1). This result confirms that their adsorption may be due to the polycationic character of their piperazine chain. Lastly, four complexes (Table B, Supplementary material) were studied using the fused silica capillary and the three coatings in order to

select the best one to avoid CD adsorption.

3.3.2 Choice of the coating

The apparent and averaged binding constants determined for the four complexes using fused-silica capillary or the three coated capillaries are displayed in Table 2. Relative standard deviation and bias calculated taking as reference the result obtained with the uncoated capillary are enclosed. First, it appears that the binding constants determined using the PEO coating display poorer repeatabilities. RSD values are higher than 5%, the generally admitted limit for binding constant determination by ACE [40, 42]. Moreover, the binding constants measured with both PEO and HPC coatings are systematically lower than the reference value obtained using the uncoated capillary (bias around -10 to -20%). The undervaluation of the constants obtained with both more hydrophilic polymers can be explained by an adsorption of the CD through surface hydrophilic interactions, which reduce the CD concentration free to interact with the analyte. Only the PDMA coating permit to obtain binding constants values in agreement with those obtained using the uncoated capillary (bias around ± 5%).. Moreover, for the complex risperidone-β-CD, the apparent binding constant determined with the PDMA coating is 234 M^{-1}, which is in accordance with our previously published results (K = 221 M^{-1}) [42]. According to all these results, the PDMA coating is the best coating able to strictly limit the adsorption of the CDs and the adsorption of the compounds **1**-**4**. Then, this coating was selected to investigate the complexation of the compound **1** with the four CDs of pharmaceutical interest.

3.3.3 Study of the (**1**)-CD complexes using the PDMA coating

First, the complexation of **1** with neutral CDs (β-CD, HP-β-CD and Me-β-CD) has been investigated. Whereas the migration time shifts of **1** observed with additions of β-CD and HP-β-CD in the BGE are too low to permit the binding constant determination, the additions of Me-β-CD involved sufficient migration time shifts to quantify the binding constant of the **1**-Me-β-CD complex around 50 M^{-1}. Due to the cationic character of **1** and so as to enhance the complexation, the complexing ability of the anionic SBE-β-CD, the only anionic CD used in marketed pharmaceutical products [7], was evaluated. It is worth mentioning than a previous work of Luppi et al. [43] about nanoparticles carrying cyclodextrins in order to load tacrine, has shown that SBE-β-CD significantly increases the drug loading whereas β-CD and HP-β-CD did not affect it. Here, the apparent and averaged binding

constant determined for the analyte 1-SBE-β-CD complex is more than 20 times greater than with the neutral Me-β-CD, around 1210 M^{-1}. This result can suggest a high contribution of electrostatic interactions in the complexation process between the sulfobutylether group of the CD and the cationic piperazine chain of **1** but this contribution would be only partially responsible of the interaction taking into account the results of Luppi et al [43]. Among the four CD, the SBE-β-CD appears to be the more appropriate CD to complex the compound **1**.

3.4 Study of the structure of the 1-SBE-β-CD complex :

Before envisaging investigating the influence of the SBE-β-CD on the metabolism of **1**, it is essential to study the structure of the complex. The ROESY is a powerful tool to investigate intra- and inter-molecular interactions. Presence of cross-peaks between the protons of two different species, which are generated by Nuclear Overhauser Effects (NOE) indicates that they are in a spatial contact within 3-5 Å [44]. The partial ^{1}H NMR 2D ROESY spectrum obtained for the complex formed between **1** and SBE-β-CD is displayed in Figure 4. One intramolecular interaction is observed between the protons a and e. Three cross-peaks may be attributed to intermolecular interactions between the analyte and the CD. However, the attribution of these interactions with the interior or exterior of the cavity is impossible due to overlapping of the various signals of the modified CD, The complex **1**-β-CD was further studied since the ^{1}H signals of the β-CD are well resolved and assigned, compared to the modified SBE-β-CD, and could provide more detailed information about the geometry of the complex. However, no cross peak was observed for intermolecular interactions and no information could be obtained due to low interactions between both partners even with an important accumulation of scans. A previous study on the complexation between tacrine and β-CD by NMR and molecular dynamics [45] gave evidence of absolute lack of internal or external interaction between them. This result points that the structural modification of tacrine by substitution with the piperazine chain does not favour the interaction with the native β-CD.

Since NMR does not allow to obtain information on the structure of the **1**-SBE-β-CD complex, we have chosen to investigate structure-binding constant relationships by studying the complexes formed between this CD and the compounds presenting structural modifications on the tacrine moiety or on the piperazine chain. The apparent and averaged binding constants are displayed in Table 3.

First, the comparison of the binding constants determined for tacrine and **1**-**4** points out that the interaction between the piperazine chain and the SBE-β-CD provide a high contribution to the binding constant. Indeed, the binding constant K obtained for tacrine is 3 to 9 times lower than those obtained for the substituted compounds **1**-**4**.

Second, the comparison of the binding constants obtained for the various compounds **1**-**4** presenting structural modification on the tacrine moiety shows that the interaction is dependent of the nature of the ring. Only the replacement of the tacrine moiety by the benzimidazole moiety has low influence on the binding constant (1210 M^{-1} for **1** and 1066 M^{-1} for **2**). On the contrary, the replacement by a quinoleine moiety in compound **4**, has a great influence. The enhancement of the binding constant observed for **3** (1954 M^{-1}) may be related to its chloro substitution and then its higher hydrophobicity which may favour its inclusion in the hydrophobic cavity of the CD. The comparison of the analogues **1** and **4** which present the tetrahydro-acridine ring or a quinoleine ring, respectively, without any other structural modification, is more interesting. Indeed, it appears that the aliphatic ring portion of the tacrine moiety greatly contributes to the interaction with the CD since the binding constant determined for the compound **1** is two times greater than for **4**. This last result suggests that an inclusion of **1** in the SBE-β-CD cavity could occur through this ring.

4. Conclusions

Among the three neutral coatings, the best performances were obtained for the PDMA coating. It proves to be more efficient to mask the silanol groups of the capillary wall and then to strictly reduce the adsorption of the polycationic compounds **1**-**4**. Moreover, it permits to correctly determine the analyte-CD binding constants avoiding the CD adsorption. The results obtained by ACE using the PDMA coating and isothermal titration calorimetry (recognized as the reference method) are in good agreement. This coating would be further used for analysis of various polycationic molecules in presence of CD in the BGE (for chiral analysis or determination of binding constants). The SBE-β-CD appears to be the best pharmaceutical CD to complex our compounds. The investigation of the structure of the **1**-SBE-β-CD complex shows that the inclusion of **1** in the CD cavity seems to occur through the aliphatic ring portion of the tacrine moiety. This structure may be favorable to a modification of the metabolism since it has been demonstrated by Pool [14] that metabolism of tacrine

occurs by single or multiple hydroxylation of this aliphatic ring. Further study will consist to study the metabolism of **1** in the uncomplexed form or in association with the SBE-β-CD.

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Table 1. Evaluation of the EOF mobilities using the fused-silica capillary and the three coatings.Repeatability of the migration time, separation efficiency and presence of a baseline shift obtained for the compound **1** using the various capillaries

	EOF ($cm^2.min^{-1}.V^{-1}$) (RSD)		RSD tm	N	Baseline shift
Fused-silica capillary	**$1.2\ 10^{-2}$**	**(2%)**	**19%**	**1793**	**Yes**
HPC coating	$1.8\ 10^{-5}$	(32%)	0.3%	3251	No
PEO coating	$3.4\ 10^{-5}$	(22%)	0.3%	3802	No
PDMA coating	$4.4\ 10^{-6}$	(18%)	0.2%	3666	No

mean values calculated from seven determinations

Table 2. Apparent and averaged binding constants determined for the four complexes using the three coatings

	K (M^{-1}) (%RSD) ; bias (%)			
Complex	**Fused-silica capillary**	HPC coating	PEO coating	PDMA coating
Ibuprofen - β-CD	**9332 (2.7%)**	8034 (2.0%) ; - 14%	7225 (7.2 %) ; -22%	8975 (1.3%) ; -4%
Risperidone - β-CD	**224 (2.9%)**	199 (4.6%) ; -11%	199 (7.8%) ; -11%	234 (2.7%) ; +4%
Carbamazepine - SBE-β-CD	**1063 (3.8%)**	806 (0.1%) ; -24%	934 (5.9%) ; -12%	1117 (2.4%) ; +5%
Tacrine - SBE-β-CD	**234 (2.4%)**	185 (5.2%) ; -21%	198 (6.7%) ; -15%	217 (4.7%) ; -5%

mean values calculated from three determinations
bias calculated taking as reference the binding constant obtained with the fused-silica capillary

Table 3. Apparent and averaged binding constants K determined by ACE for the complexes formed between Tacrine or compounds **1**-**4** and the SBE-β-CD.

	K (M^{-1})	%RSD
Tacrine	215	5%
1	1210	3%
2	1066	1%
3	1954	5%
4	673	4%

Mean values calculated from three experiments.

Figure captions

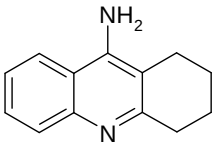
Figure 1. Structure of the studied compounds and structure of the polymers used for the coatings

Figure 2. Electropherograms of seven successive analyses of **1** with a fused-silica capillary. Conditions : capillary 30.2 cm (effective length, 20 cm) 50 mm id ; BGE, 67 mM phosphate buffer, pH 7.4; anodic injection (1 psi, 5 s); temperature, 298 K; voltage, 15 kV (* : EOF signal)

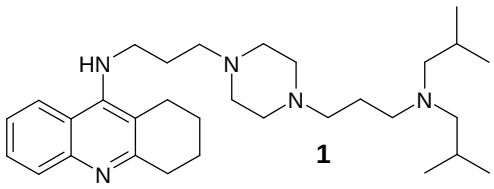
Figure 3 : Effect of increasing β-CD concentration on EOF mobilities using the HPC, PEO or PDMA coatings. Conditions : capillary 30.2 cm (effective length, 20 cm) 50 mm id ; BGE, 67 mM phosphate buffer, pH 7.4; anodic injection (1 psi, 5 s); temperature, 298 K; voltage, 15 kV ; neutral marker, DMSO (the EOF mobilities are corrected by the ratio η_0/η, mean values from 3 experiments)

Figure 4. Extract of the 2D-ROESY spectrum of the **1** - SBE-β-CD complex ([**1**] = [SBE-β-CD] = 4 mM).

Figure 1



Tacrine



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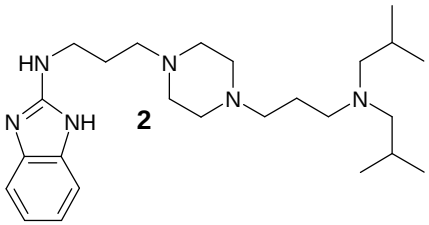

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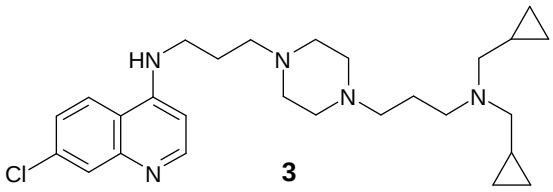

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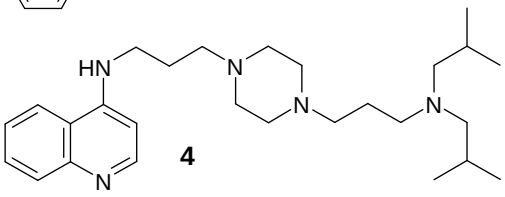

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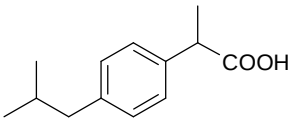
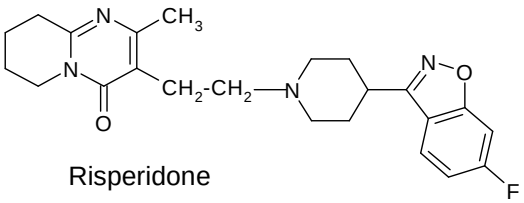
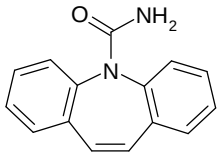


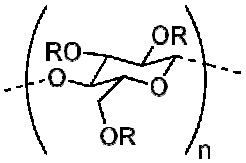
Ibuprofen



Risperidone

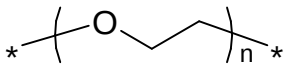


Carbamazepine

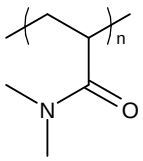


R = H or $CH_2CH(OH)CH_3$

Hydroxypropyl cellulose, HPC



Polyethylene oxide, PEO



Poly(N,N-dimethylacrylamide), PDMA

Figure 2

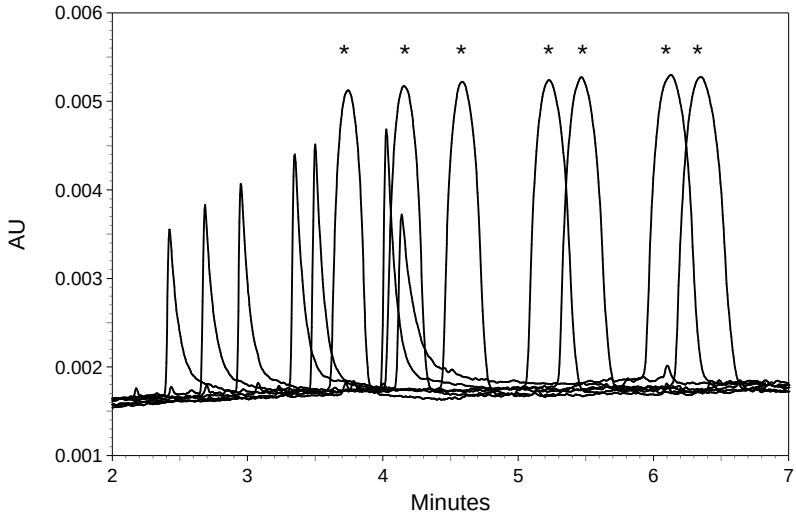


Figure 3

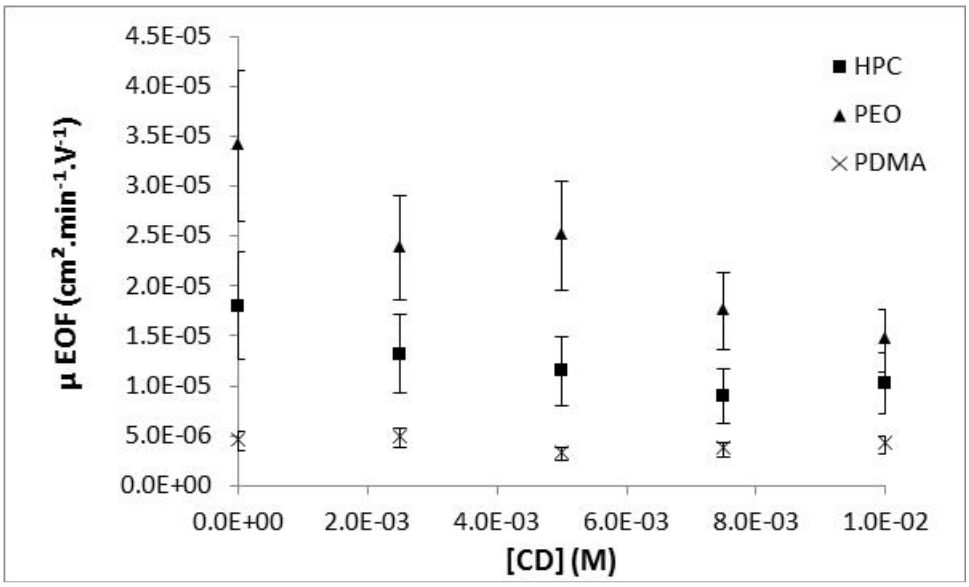
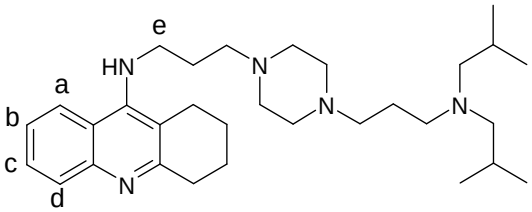

4.5E-05
4.0E-05
3.5E-05
3.0E-05
2.5E-05
2.0E-05
1.5E-05
1.0E-05
5.0E-06
0.0E+00
μ EOF (cm².min⁻¹.V⁻¹)
0.0E+00
2.0E-03
4.0E-03
6.0E-03
8.0E-03
1.0E-02
[CD] (M)
HPC
PEO
PDMA

Figure 4


e
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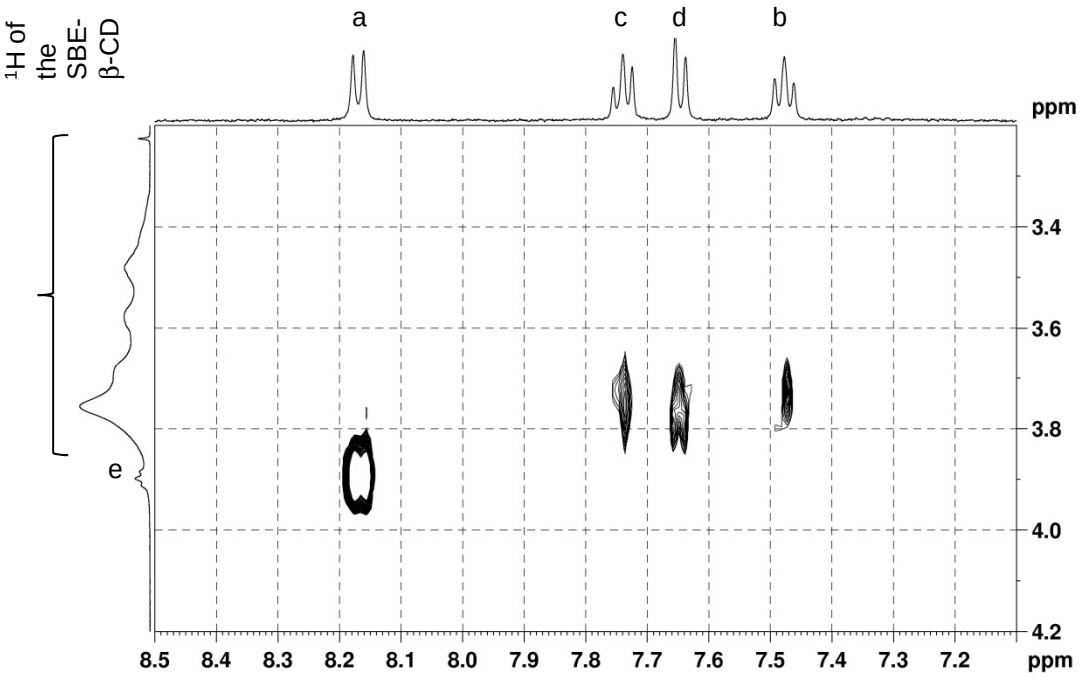
^{1}H of the SBE-β-CD
a
c
d
b
ppm
e
3.4
3.6
3.8
4.0
4.2
8.5
8.4
8.3
8.2
8.1
8.0
7.9
7.8
7.7
7.6
7.5
7.4
7.3
7.2
ppm

Supplementary material

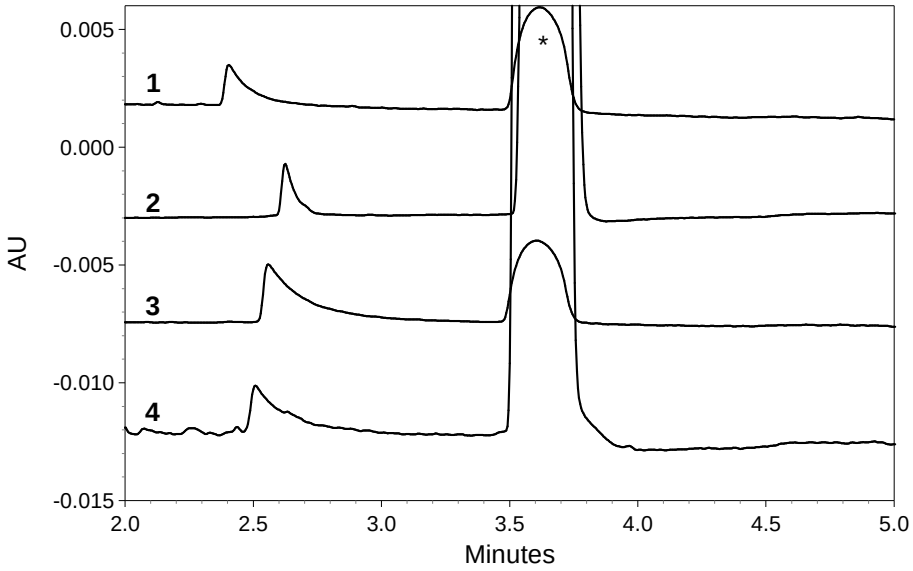

Figure A. Severe peak broadening observed on the electropherograms of **1**, **2**, **3** and **4** using a fused-silica capillary. Conditions : capillary 30.2 cm (effective length, 20 cm) 50 mm i.d. ; BGE, 67 mM phosphate buffer, pH 7.4; anodic injection (1 psi, 5 s); temperature, 298 K; voltage, 15 kV (* : EOF signal).

Results obtained rinsing the capillary with NaOH (20 psi, 10 min), H_2O (20 psi, 10 min) and BGE (20 psi, 5 min) between each run to find the correct EOF again.

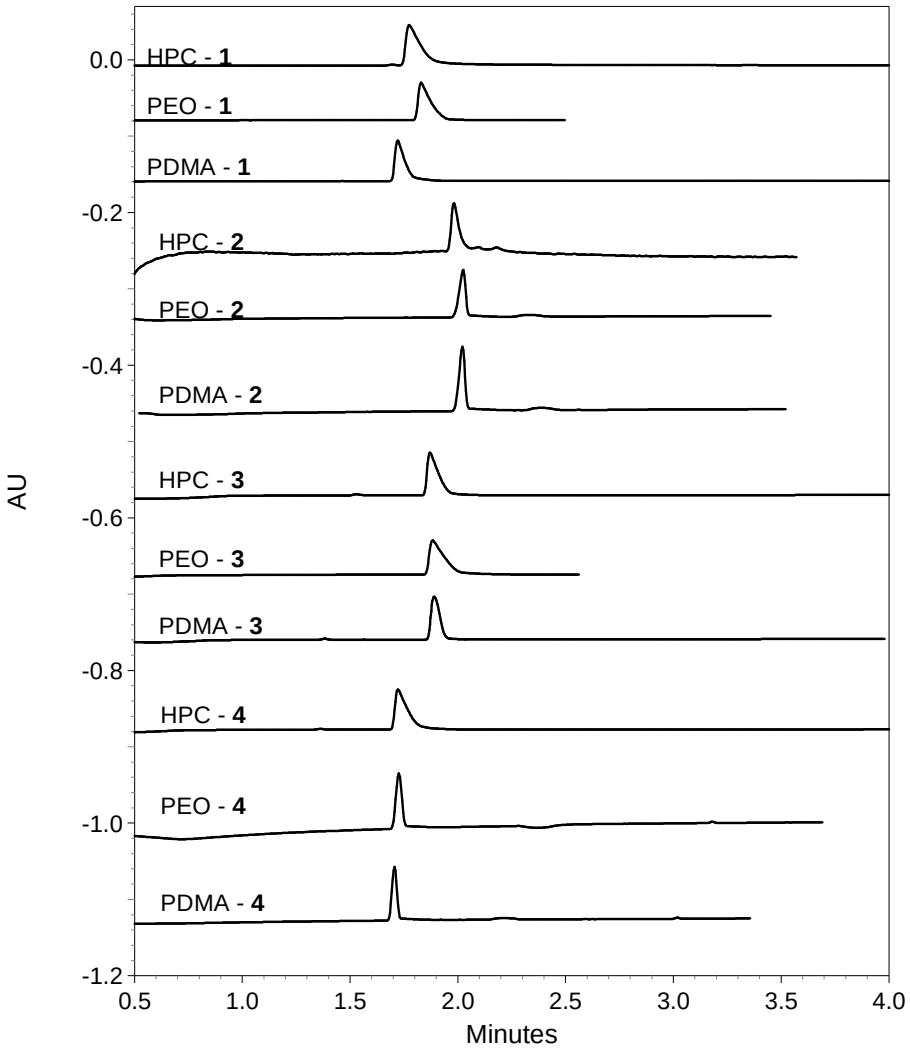

Figure B. Electropherograms of **1- 4** with the HPC, PEO and PDMA coatings

Conditions : capillary 30.2 cm (effective length, 10 cm) 50 mm id ; BGE, 67 mM phosphate buffer, pH 7.4; anodic injection (1 psi, 5 s); temperature, 298 K; voltage, 15 kV

Table A. RSD of the migration times and efficiencies measured for the four model analytes using uncoated and coated capillaries

	%RSD tm ; N			
Analyte	**Fused-silica capillary**	HPC coating	PEO coating	PDMA coating
Ibuprofen	**0.2% ; 42 542**	1.1% ; 14 438	0.9% ; 17 098	0.7 % ; 23 587
Risperidone	**0.4 % ; 24 929**	3.1% ; 4 810	2.4% ; 9 964	0.3% ; 10 474
Carbamazepine	**0.7% ; 5 513***	n.d.	n.d	n.d
Tacrine	**0.5% ; 21 256**	0.9% ; 5 604	2.4% ; 6 983	1.1% ; 10 478

mean values calculated from three determination
*: lower efficiencies obtained for the neutral carbamazepine was expected since its signal is joined with the DMSO one.
n.d. : not determined since the neutral carbamazepine does not migrate without charged CD in the BGE with these three neutral capillaries
Conditions : capillary 30.2 cm (effective length, 20 cm) 50 mm i.d. ; BGE, 67 mM phosphate buffer, pH 7.4; anodic injection (1 psi, 5 s); temperature, 298 K; voltage, 15 kV

Table B. The four complexes studied for the evaluation of the CD adsorption

Complex	Charge of the compound	Charge of the CD
Ibuprofen - β-CD	Anionic	Neutral
Risperidone - β-CD	Cationic	Neutral
Carbamazepine - SBE-β-CD	Neutral	Anionic
Tacrine - SBE-β-CD	Cationic	Anionic