Development of chromatographic and electrophoretic analytical methods

PhD Thesis

Ida Fejős

Doctoral School of Pharmaceutical Sciences
Semmelweis University

Supervisor:	Dr. Szabolcs Béni, Ph.D.
Official Reviewers:	Dr. Tamás Tábi, Ph.D.
	Dr. Attila Gáspár, Ph.D.
Head of the Final Examination Committee:	Dr. Tamás Török, D.Sc.
Members of the Final Examination Committee:	Dr. Pál Perjési, C.Sc.
	Dr. Krisztina Ludányi, Ph.D.

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Introduction

In the last 40 years the popularity of alternative therapies are increased, as well as the number of dietary supplements and herbal remedies are raising year after year. There is a widespread belief that natural products offer a safe, side effect free and healthy alternative to medicinal products containing synthetic ingredients. However, these herbal products are not harmless, despite their advantages as the easy, anonym sale, the possible risks are endless. Their production and their distribution is much less regulated and controlled, so their safety and toxicity is questionable: contaminants (heavy metals, pesticides), adulteration (to increase efficiency manufacturers illegally add synthetic compounds) often occur.

The number of dietary supplements and herbal medicines used for the treatment of erectile dysfunction and at the same time the adulteration of these products are increasing. In order to effectively increase sexual performance manufacturers add approved phosphodiesterase-5 enzyme (PDE-5) inhibitors to the formulations, such as sildenafil (Viagra®), vardenafil (Levitra®) and tadalafil (Cialis®), or unapproved designer analogs with subtle changes in their structures. Adulteration with PDE-5 inhibitors and their analogs possess serious health risks. Further health issues are also evoked by the lack of safety and toxicity data of the numerous unapproved analogs. Furthermore, these alternative medicines are herbal medicines, with complex composition, complex pharmacological effects, thus require complex analytical methods for monitoring suspicious products. Due to the popularity of PDE-5 inhibitor drugs, their counterfeit pharmaceutical preparations have appeared in various websites offering an easy and anonymous sale. Therefore, official control laboratories need simple, easy-to-use, inexpensive routine methods to screen and determine the illicit adulterants in herbal dietary supplements and in suspicious pharmaceutical preparations.

The aim of the thesis is to develop an easy, low-cost, MS compatible HPLC-UV method for qualitative and quantitative screening of PDE-5 inhibitors and their designer analogs in illegal erectile dysfunction products.

The second part of the thesis deals with the enantioseparation of chiral molecules. The stereoisomers (enantiomers) may interact in different ways with biological structures therefore, despite their similar structures may exhibit widely different pharmacokinetic and pharmacodynamic properties. Since the introduction of enantiopure drugs the characterization of chiral molecules, the separations of the enantiomers and the determination of enantiomer ratio are essential. Pharmacopoeias and regulatory authorities require the determination of contamination over 0.1 % - including enantiomeric impurities.

For the determination of chiral purity besides the well-known chromatographic techniques (high-performance liquid chromatography, gas chromatography, supercritical fluid chromatography) capillary electrophoresis (CE) offers an alternative method. The advantages of CE are the outstanding efficiency, short analysis time, easy sample preparation, fast and easy method development and the high throughput thus from minute sample amount trace enantiomer impurities below 0.1% can be determined, even for more enantiomer pairs, in complex matrices. The successful separation of enantiomers in capillary zone electrophoresis necessitates the application of chiral selectors. The most frequently used chiral additives are the family of cyclodextrins (CDs) thanks to their low price and low UV cut-off, furthermore, due to their wide variety of neutral and charged derivatives easy and fast method development could be achieved.

Applying cyclodextrin-modified capillary electrophoresis the development of a routine, validated method is feasible and could serve as methods of Pharmacopoeias. The main goals of my PhD work have been the development of cyclodextrin-modified capillary electrophoretic methods for the separation of 3 chiral drugs: the enantiomers of the antidiabetic alogliptin, the stereoisomers of the analgesic tapentadol and the stereoisomers of tadalafil used for the treatment of erectile dysfunction. During the method development, the ability of enantioseparation of various CDs, the apparent stability of the analyte-CD inclusion complexes and the enantiomer migration order were studied. Based on the results, the most promising CDs were chosen and the enantioseparation methods were further optimized, moreover, the separations of alogliptin enantiomers and tapentadol stereoisomers were validated.

Objectives

The aims of my PhD work have been the development of an easy, fast, MS compatible HPLC-UV method for qualitative and quantitative screening of the PDE-5 inhibitors sildenafil, vardenafil, tadalafil and their 11 designer analogs in illegal products against erectile dysfunction. The validation of the method and the application to the analysis of dietary supplements and herbal remedies with an indication for enhanced male sexual potency were among our primary objectives as well.

The second aim of my PhD work has been the development of various easy-to-use cyclodextrin-modified capillary electrophoretic methods for the enantioseparation of alogliptin, tapentadol and tadalafil. As the physico-chemical properties of the compounds

affect the electrophoretic behavior and the affinity towards the cyclodextrins, therefore the acid-base profiling of the molecules were completed first. As the next step of the method development, we aimed at studying the enantioseparation ability of various CDs, the stability of the analyte-CD inclusion complexes and the stereoisomer migration order in the case of alogliptin, tapentadol and tadalafil stereoisomers, respectively. The CDs with high enantioselectivity were chosen the methods were optimized and also validated in the case of alogliptin and tapentadol according to the International Conference on Harmonization (ICH) guidelines.

Methods

For the separation of PDE-5 inhibitors an MS-compatible HPLC-UV method was developed. The optimized method was validated in accordance with the appropriate ICH guideline in terms of specificity, selectivity, precision, linearity, limit of quantitation, limit of detection, accuracy, robustness and stability. Sildenafil served as a single external standard for both identification and quantification of all analytes. Relative retentions and reference UV spectra were used for qualitative, whereas correction factors, concentration of sildenafil standard and sample dilution for quantitative analyses, respectively.

For the determination of the protonation macroconstants in the case of alogliptin CE-pH titration and pH-potentiometry while in the case of tapentadol ^{1}H NMR-pH titration and pH-potentiometry were carried out.

In the case of the 3 chiral molecules various neutral, positively and negatively charged α-β- and γ-CD derivatives were studied for chiral separation. In the case of enantioresolution, the Rs values were calculated with the usual formula:

$$R_S = \frac{2(t_2 - t_1)}{w_2 + w_1} \tag{1}$$

where t_1 and t_2 are the migration times of the enantiomers, w_1 and w_2 stand for the extrapolated peak widths at the baseline.

In order to successfully separate the isomers, there should be a mobility difference between the enantiomers.

$$\Delta\mu = \mu_2 - \mu_1$$

$$= \frac{\mu_{free} + \mu_{cplx\,2}\ K_{stab\,2}[CD]}{1 + K_{stab\,2}[CD]} - \frac{\mu_{free} + \mu_{cplx\,1}\ K_{stab\,1}[CD]}{1 + K_{stab\,1}[CD]} \tag{2}$$

where μ_1 and μ_2 are the effective mobilities of the enantiomers, K_{stab1} and K_{stab2} are the selector-enantiomer averaged (apparent) complex stability constants (M^{-1}); μ_{free} and μ_{cplx} are the effective mobilities of the free and the complexed enantiomers; [CD] is the concentration of the chiral selector (M). The $\Delta\mu$ mobility difference was modeled according to Eq. (2) as a function of CD concentration and plotted for various CD systems in case of alogliptin and tadalafil to predict the optimal CD concentration.

In chiral separations, different interactions between the enantiomers and the chiral selector are fundamental, the calculated complex stability constants refers to the strength of the interaction. For the calculation of the analyte-CD apparent binding constants, the measured μ_{eff} - [CD] was fitted by the following equation using Microcal Origin (OriginLabs):

$$\mu_{eff} = \frac{\mu_{free} + \mu_{cplx}\ K_{stab}\ [CD]}{1 + K_{stab}\ [CD]}$$

$$(3)$$

where the fitting parameters are μ_{cplx} and K_{stab}. In the cases of chiral resolution, the μ_{eff1}, μ_{eff2}, μ_{cplx1}, μ_{cplx2}, K_{stab1} and K_{stab2} parameters were evaluated separately for the first and second-migrating enantiomer.

Results

HPLC separation of PDE-5 inhibitor compounds used for the treatment of erectile dysfunction

As the first step of method development, various methanol:acetonitrile mixtures were tested for the extraction of PDE-5 inhibitors from herbal products. As a compromise, a 1:1 (v/v) mixture of methanol and acetonitrile was found to be optimal. Based on their chemical structures and UV spectra, the compounds were classified into 5 groups. 290 nm was found to be the best compromise at which all the compounds have significant absorbance close to an absorption maximum.

The choice of the stationary phase represents a milestone during method development, a core–shell C18 reversed phase column was preferred in order to achieve appropriate retention and peak shape. Because all the examined compounds (apart from tadalafil) contain one or more basic nitrogen centers, the pH of the mobile phase had to be controlled, so a pH 7.0 (200 mM ammonium acetate) system was found to be optimal. The poor separation with both 100% acetonitrile and 100% methanol suggested the use of their mixtures as mobile phase B. The most suitable system contained a 1:1 (v/v) methanol:acetonitrile mixture as mobile phase

B, and a 200 mM ammonium acetate solution as mobile phase A using a gradient elution programs described in Table 1. Figure 1. shows the optimized chromatogram applying 25°C column temperature, 5 µl injection volume and 0.5 ml/min flow rate.

Table 1.: The gradient elution program

Time (min)	B%
0–9	40 → 50
9–17	50 → 80
17–20	80
20–20.5	80 → 40
20.5–25	40
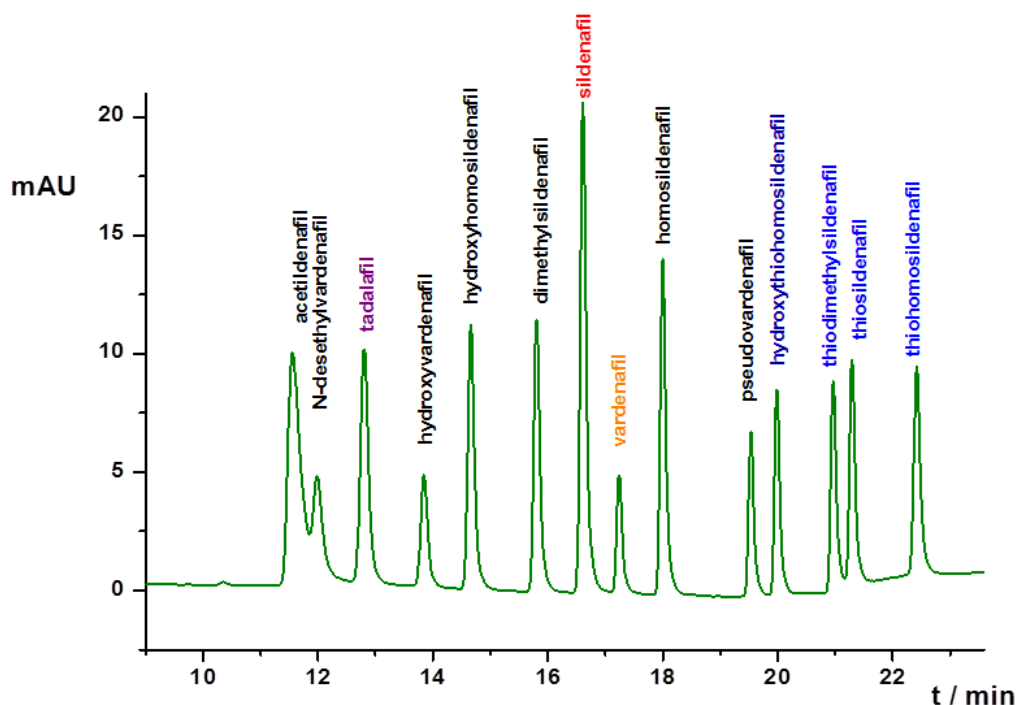
Figure 1.: The chromatogram of the optimized system (Kinetex C18 2.5 µm·100 mm·3 mm column, A-eluent: 200 mM ammonium acetate (pH~7), B-eluent: methanol:acetonitrile 1:1 (v/v), gradient elution (Table 1.), 0.5 ml/min, 25°C, 5 µl inj., 290 nm)

Sildenafil eluted in the middle of the separation window, which was advantageous for qualitative and quantitative analysis, as it was used as the external standard. Sildenafil and vardenafil eluted consecutively, so the system suitability criterion was based on the resolution of these two peaks.

The method was validated in accordance with the corresponding ICH guideline in terms of specificity, selectivity, precision, linearity, limit of detection, limit of quantitation, accuracy, robustness and stability.

In our system, the relative retention of any of the chosen compounds to sildenafil and comparison of the UV spectrum of the same compound to a reference spectrum provide sufficient information to identify the analyte.

In HPLC assays, the lack of reference substance for a compound to be determined implies that its amount can be estimated by the use of a different external standard. This is a common practice in the European Pharmacopeia especially in tests for related substances. In this case, the peak area of the analyte is compared to that of the external standard. To correct for any significant differences in response factors, the peak area of the substance to be determined is multiplied by a correction factor. For each compound, the correction factors used in this method were based on the slope of the regression lines obtained in the linearity study divided by the slope obtained for sildenafil. The PDE-5 inhibitor content (referring to a single dose) could be calculated using the concentration of the external standard, the sample dilution and the correction factors.

In order to prove the applicability of the developed method, the described procedure has been applied to "real samples" including dietary supplements and herbal remedies for the treatment of erectile dysfunction. As a result of the screening, various samples were found to contain sildenafil, thiosildenafil-type analogs, vardenafil analogs and tadalafil (Figure 2.).

Sample E contained 18 mg HV and a PDE-5 analog not included in our sample set. The retention of this compound could not be matched with any of the compounds examined, however the UV data suggested a vardenafil analog. Using mass spectrometric detection in conjunction with our chromatographic system, this compound was identified as hydroxythiovardenafil. Using the response factor of the parent compound vardenafil an approximate amount of 20 mg hydroxythiovardenafil was found in the adulterated sample. The latter case clearly shows the advantage of our method, namely the fast and robust identification and approximate quantitation of PDE-5 inhibitors in adulterated herbal dietary supplements.
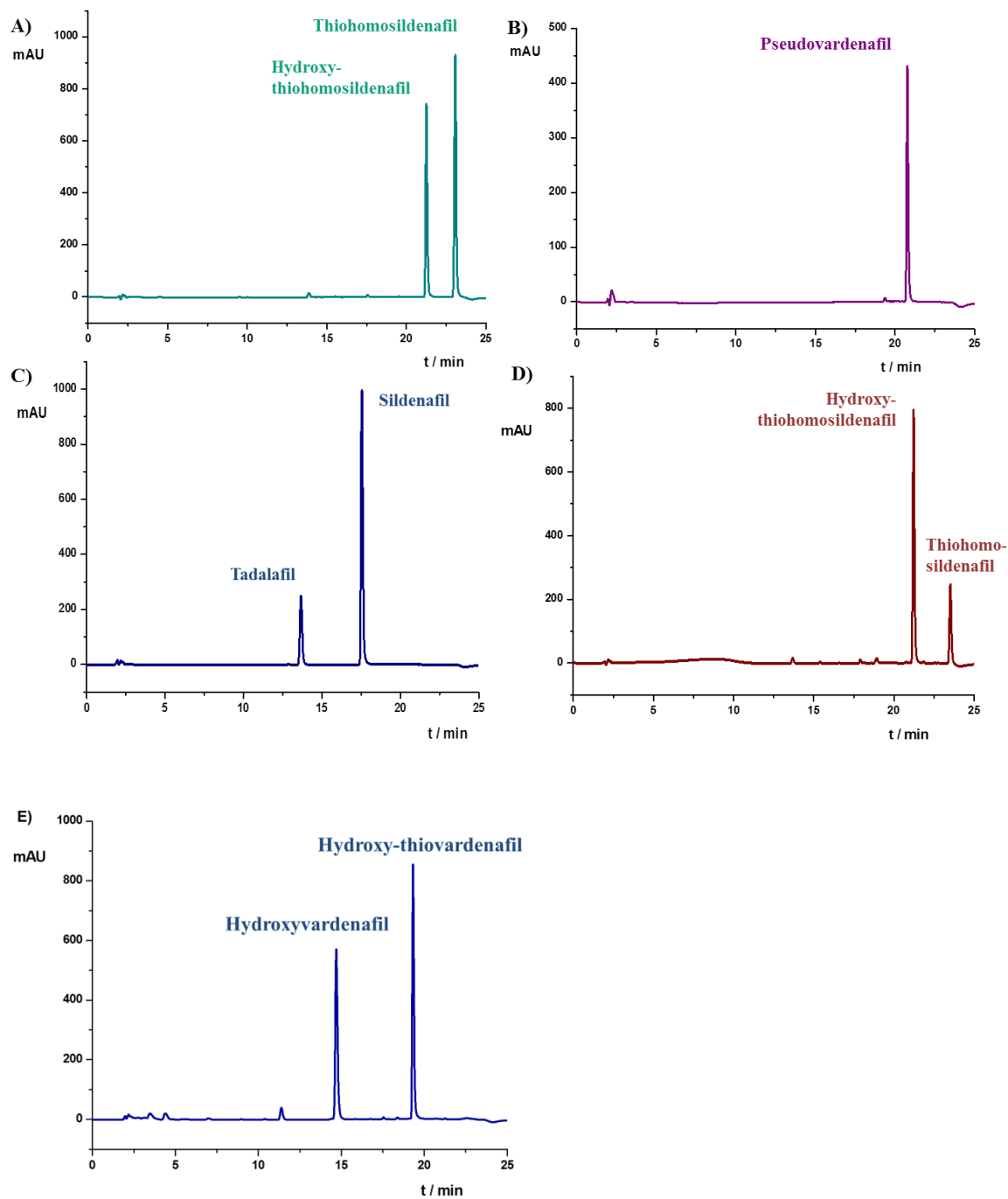
Figure 2.: Adulterated dietary supplements: sample A 60 mg hydroxythiohomosildenafil and 67 mg thiohomosildenafil per capsule; sample B 9 mg pseudovardenafil per capsule; sample C 9 mg tadalafil and 33 mg sildenafil per capsule, sample D 65 mg hydroxythiohomosildenafil and 20 mg thiohomosildenafil per capsule; sample E 18 mg hydroxyvardenafil and 20 mg hydroxythiovardenafil per capsule

Cyclodextrin-modified capillary electrophoretic separation of chiral compounds

As the first step of method development the physico-chemical properties were studied. In order to characterize the complexation of the compounds also with neutral cyclodextrins, the molecules should be in ionized form. The $\log K = 8.34 \pm 0.02$ value was obtained in the case of the CE-pH titration for alogliptin, the results were confirmed with potentiometry ($\log K = 8.50 \pm 0.04$). Therefore, the pH values of all background electrolytes were set at least one pH unit below the $\log K$ of alogliptin, where predominantly the monocationic form exists. Tapentadol is a diprotic compound bearing an acidic phenolic hydroxyl and a basic tertiary amine function, the protonation macroconstants were found to be $\log K_1 = 10.59 \pm 0.01$ and $\log K_2 = 9.44 \pm 0.01$ applying pH-potentiometry, confirmed by ^{1}H NMR-pH titration ($\log K_1 = 10.62 \pm 0.03$ and $\log K_2 = 9.48 \pm 0.03$). Due to the protonation of the amino group, tapentadol is positively charged under acidic conditions (e.g. at pH 2.5) and partially positive under basic conditions (at pH 9.5), thus in CE measurements neutral CDs could also be studied. Since tadalafil lacks basic or acidic functional groups separation of its four uncharged stereoisomers (both migrating with the EOF) requires charged CDs as carrier hosts to afford velocity difference during migration. Furthermore, tadalafil is practically insoluble in aqueous buffers, therefore the sample preparation should be optimized.

The chiral separation was studied using various cyclodextrins: three native, five neutral, and five negatively charged CD derivatives were screened for the separation of alogliptin enantiomers at various CD concentrations in the range of 0.25 – 80 mM in 30 mM (pH 4.75) acetate buffer. Six of the applied CD derivatives resulted in partial or baseline enantioseparation. The sulfopropyl-γ-CD yielded the highest resolution (Rs = 5.16) followed by the sulfobuthyl-ether-γ-CD (Rs = 4.75). Among the β-CD derivatives, the best resolution was achieved with the sulfobuthyl-ether-β-CD (Rs = 4.64).

The chiral separation of R,R- and S,S-tapentadol was determined in the case of two native, five neutral and twelve negatively charged cyclodextrin derivatives in the 0.125-30 mM concentration range in 50 mM Tris-acetic acid buffer at pH 4.75. From the neutral CDs one α- one β-CD derivative was able to separate the enantiomers, while all of the studied charged CDs resulted in enantioseparation, with outstanding enantioresolution in the case of sulfated-α-CD ($R_S = 16.18$). Regarding the chiral separation of all four stereoisomers, under acidic conditions (pH 2.50 phosphate buffer) the hydroxypropyl-CD derivatives separated certain enantiomer pairs: the hydroxypropyl-β-CD separated S,R- and R,S-tapentadol ($R_S = 1.63$),while the hydroxypropyl-γ-CD resulted in baseline separation of

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R,R- and *S,S*-tapentadol ($R_S = 4.50$). Applying the two neutral hydroxypropyl-CD derivatives in one system at appropriate concentration ratio resulted in a dual CD system (4 mM hydroxypropyl-β-CD and 35 mM hydroxypropyl-γ-CD) capable of chiral recognition for all four stereoisomers. Using sulfated-α-CD baseline separation could be achieved under basic (pH 9.5 borate buffer) conditions (with the *S,S, S,R, R,S, R,R*-tapentadol migration order).

The chiral separation of tadalafil isomers was studied in 50 mM Tris–acetic acid buffer (pH 4.75) applying 22 charged CD derivatives in the 0.1–20 mM concentration range: four resulted in partial and eleven in baseline separation. Focusing on the separation of the therapeutically applied *R,R*-tadalafil and its *S,S* enantiomer, three negatively charged β-CD derivatives showed R_S values higher than 7.00. From the α-CD derivatives the sulfated-α-CD demonstrated the highest enantioresolution ($R_S = 5.19$) followed by the sulfobuthyl-ether-α-CD ($R_S = 4.26$).

The different interactions between the analyte enantiomers and the chiral selector are fundamental in chiral separation. The calculated apparent averaged host–guest complex stability constants serve as a mean to quantify the strength of interaction, and help to find the most promising CD for the enantioseparation, which is the crucial point of method optimization.

In cases of chiral resolution, the μ_{cplx}, μ_{cplx2}, K_{stab1} and K_{stab2} parameters were evaluated separately for the first and second-migrating enantiomer.

In the case of alogliptin, the complex stability analysis was performed at various CD concentration levels in the range of 0.25–80 mM (depending on the CD solubility and enantioseparation) in 30 mM (pH 4.75) acetate buffer (Table 2.).

Table 2.: The alogliptin-CD apparent complex stability constants, the mobility of the complexes and the enantiomer migration order (EMO) (a: no reliable estimate could be calculated)

CD	DS	K_{stab1}	K_{stab2}	μ_{klpx1} (10^{-5} cm^{2}V^{-1}s^{-1})	μ_{kplx2} (10^{-5} cm^{2}V^{-1}s^{-1})	EMO
γ-CD	-	7 ± 0,8	11 ± 0,7	a	a	*R; S*
SP-β-CD	~2	194 ± 14	206 ± 6	-7,9 ± 0,3	-8,0 ± 0,2	*S; R*
SP-γ-CD	~2	65 ± 2	84 ± 3	-2,1 ± 0,2	-2,4 ± 0,3	*R; S*
SBE-β-CD	~4	226 ± 6	240 ± 14	-7,2 ± 0,2	-6,4 ± 0,3	*S; R*
	~6,3	388 ± 13	411 ± 20	-17,3 ± 0,5	-18,4 ± 0,5	*S; R*
	~10,4	874 ± 86	911 ± 88	-25,5 ± 1,5	-26,7 ± 1,5	*S; R*
SBE-γ-CD	~4	167 ± 9	187 ± 15	-4,8 ± 0,4	-6,2 ± 0,5	*R; S*

The affinity of alogliptin toward native and neutral CDs was rather poor. In the pH 4.75 acetate buffer, alogliptin carries a positive charge therefore the negatively charged CDs may enhance the complex stability by electrostatic interactions. Moreover, the increased mobility difference achievable with anionic complexes may also contribute to resolution improvement. Comparing the γ-CD and β-CD derivatives, it can be concluded that the averaged stabilities for β-CD derivatives are greater than those with γ-CDs.

The stabilities of tapentadol–cyclodextrin complexes were determined in the case of *R,R*- and *S,S*-tapentadol applying seven α- and twelve β-CD derivatives in the concentration range 0.125-30 mM at 50 mM Tris-acetic acid buffer (pH 4.75, Table 3.). The non-charged hosts show low affinity toward the cationic tapentadol, while outstanding stabilities were detected with carboxymethylated-β and phosphated-β-CD. Despite the high affinity of β-cyclodextrins, the observed enantioresolution was significantly higher in the case of α-CDs. The outstanding enantioresolution in the case of sulfated-α-CD (R_S = 16.18) originates from the complex stability differences and the mobility differences. In the case of other selectors the complex stability differences are dominant, as the mobilities are in the same regime.

Table 3.: The tapentadol-CD apparent complex stability constants, the mobility of the complexes and the enantiomer migration order (EMO) (a: no reliable estimate could be calculated)

CD	DS	K_{stab1}	K_{stab2}	μ_{klpx1} $(10^{-5} cm^2V^{-1}s^{-1})$	μ_{klpx2} $(10^{-5} cm^2V^{-1}s^{-1})$	EMO
α		35 ± 9	40 ± 9	a	a	*R,R ; S,S*
TRIME-α-CD	~18	<10		a		
RAME-α-CD	~11	60 ± 8	75 ± 8	6,4 ± 0,6	6,5 ± 0,5	*R,R ; S,S*
SBE-α-CD	~4	370 ± 10	440 ± 10	-14 ± 0,2	-15 ± 0,2	*R,R ; S,S*
SP-α-CD	~2	210 ± 20	240 ± 20	-4,7 ± 0,9	-5,0 ± 0,8	*R,R ; S,S*
S-α-CD	~12	550 ± 45	620 ± 55	-29 ± 0,9	-32 ± 1	*S,S; R,R*
CM-α-CD	~3,5	480 ± 20	570 ± 20	-13 ± 0,2	-14 ± 0,1	*R,R ; S,S*
β		355 ± 9		7,7 ± 0,08		
TRIME-β-CD	~21	<10	<10	a	a	*R,R ; S,S*
RAME-β-CD	~12	350 ± 60		7,3 ± 0,4		
HP-β-CD	~4,6	246 ± 17		6,4 ± 0,2		
SBE-β-CD	~3,9	1750 ± 150	1720 ± 110	-15 ± 0,3	-15 ± 0,2	*R,R ; S,S*
SBE-β-CD	~6,5	2470 ± 140	2710 ± 160	-28 ± 0,5	-29 ± 0,5	*R,R ; S,S*
SP-β-CD	~4	3940 ± 320	4360 ± 350	-22 ± 0,2	-22 ± 0,2	*R,R ; S,S*
SP-β-CD	~2	2330 ± 70	2490 ± 60	-20 ± 0,1	-20 ± 0,1	*R,R ; S,S*
SHP-β-CD	~2,5	1210 ± 20	1350 ± 70	-11 ± 0,08	-11 ± 0,1	*S,S; R,R*
CM-β-CD	~3	7510 ± 460	8140 ± 480	-17 ± 0,1	-17 ± 0,1	*S,S; R,R*
CE-β-CD	~3	3810 ± 210	3830 ± 220	-3,6 ± 0,1	-3,8 ± 0,1	*S,S; R,R*
Phos-β-CD	~4	6480 ± 200	6740 ± 85	-23 ± 0,1	-24 ± 0,1	*S,S; R,R*

The complex stability of tadalafil isomers was studied in 50 mM Tris–acetic acid buffer (pH 4.75) applying 22 charged CD derivatives in the 0.1–20 mM concentration range (Table 4.)

Table 4.: The tadalafil-CD apparent complex stability constants and the enantiomer migration order (EMO)

CD	K_{stab1}	K_{stab2}	K_{stab3}	K_{stab4}	EMO
S-α-CD	210 ± 80	110 ± 60	70 ± 35	105 ± 45	***R,R*** *; S,S; R,S; S,R*
SP-α-CD	130 ± 30	115 ± 20	120 ± 30	110 ± 25	*R,S; S,R;* ***R,R*** *; S,S*
SBE-α-CD	190 ± 15	200 ± 15	275 ± 15	325 ± 10	*R,S; S,R; S,S;* ***R,R***
CM-β-CD	145 ± 10	265 ± 10	290 ± 25	510 ± 25	*S,S;* ***R,R*** *; S,R; R,S*
CE-β-CD	340 ± 100	580 ± 145	1050 ± 200	1350 ± 150	*S,S;* ***R,R*** *; S,R; R,S*
Succ-β-CD	**630** ± 40	**1090** ± 175	**1650** ± 400	**2300** ± 600	*S,S;* ***R,R*** *; S,R; R,S*
S-β-CD	110 ± 45	125 ± 45	140 ± 40	150 ± 55	*S,S; S,R;* ***R,R*** *; R,S*
SP-β-CD	195 ± 10	340 ± 30	890 ± 50	1250 ± 50	*S,S;* ***R,R*** *; S,R; R,S*
SHP-β-CD	250 ± 30	400 ± 40	675 ± 55	1000 ± 75	*S,S;* ***R,R*** *; S,R; R,S*
MA-β-CD	535 ± 30	365 ± 35	280 ± 45	210 ± 55	*R,S; S,R;* ***R,R*** *; S,S*
PA-β-CD	530 ± 55	375 ± 45	255 ± 10	235 ± 65	*R,S; S,R;* ***R,R*** *; S,S*
IPA-β-CD	310 ± 70	225 ± 60	150 ± 40	110 ± 25	*R,S; S,R;* ***R,R*** *; S,S*

The β-CD derivatives formed less stable inclusion complexes than α-CD derivatives. Consequently, the β-CD derivatives possess the most suitable cavity size for the inclusion of tadalafil stereoisomers. Succinyl-β-CD formed the most stable complex under the investigated conditions (K_{stab4} = 2300) with the *R,S* isomer. The positively charged β-CD derivatives showed slightly lower affinity towards the tadalafil stereoisomers than the negatively charged CD derivatives.

Besides the enantioresolution, the enantiomer migration order (EMO) holds a great significance in chiral analysis since the main component can disturb the analysis of the minor component via system overloading or because of heading/tailing peaks.

In the case of alogliptin cavity size dependent EMO reversal was observed: the *R* enantiomer migrates faster with β-CD derivatives, while the *S* enantiomer migrates first with γ-CD derivatives as a consequence of the different binding affinity toward the enantiomers. Among the β-CD derivatives, sulfobuthyl-ether-β-CD (DS~6.3) was chosen for the further method optimization.

Regarding the EMO of tapentadol stereoisomers the migration order reversal in the case of CM-α- and CM-β-CD may originate from the different cavity size of the CDs (Figure 3.).

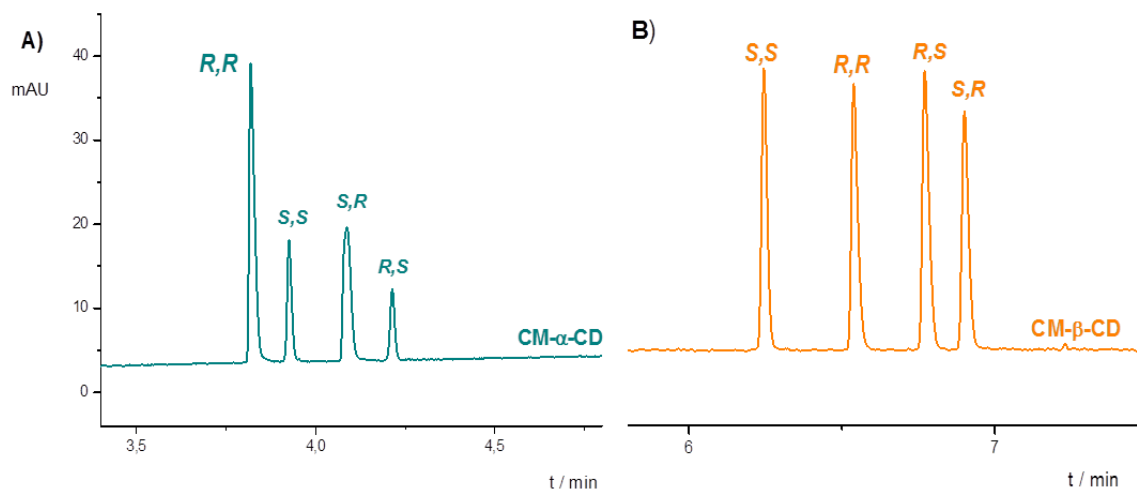*Figure 3.:* Cavity size dependent EMO reversal of tapentadol stereoisomers in the case of A) 15 mM carboxymethyl-α-CD and B) 0.7 mM carboxymethyl-β-CD

Among the α-CD derivatives substituent dependent migration order reversal could be observed in the case of sulfobuthyl-ether-α-CD (*R,R*-, *S,S*-, *S,R*-, *R,S*- tapentadol) and sulfated-α-CD (*S,S*-, *S,R*-, *R,S*-, *R,R*-tapentadol). To separate the stereoisomers and to detect trace chiral impurities the preferred systems are those where the eutomer migrates last: this criteria has been fulfilled only in the case of sulfated-α-CD, thus this cyclodextrin was chosen for the further method optimization.

In the case of tadalafil cavity size dependent EMO change was observed in the presence of sulfopropyl-α-CD and sulfopropyl-β-CD. To investigate potential structural aspects of the cavity size dependent EMO reversal ^{1}H NMR experiments were carried out. Substituent-dependent EMO reversal could be observed in the case of sulfated-α-CD and sulfobuthyl-ether-α-CD. In the case of tadalafil the optimal system to detect trace stereoisomer impurities is the one where the *R,R*-tadalafil migrates as last, thus the sulfobuthyl-ether-α-CD system was chosen for further method optimization.

In order to improve enantioseparation, further parameters such as CD concentration, buffer components and concentration, pH, applied voltage, temperature, and injection parameters were subject to optimization in case of the 3 studied chiral molecules.

Figure 4. shows the electropherogram of the optimized system for the alogliptin enantiomers (25 mM acetate buffer pH 4.75, 5 mM sulfobuthyl-ether-β-CD (DS~6.3), 18 kV, 20 mbar·4s, 16°C, where the resolution is R_S = 8.39), and the electropherogram of the detection of 0.1% chiral impurity.
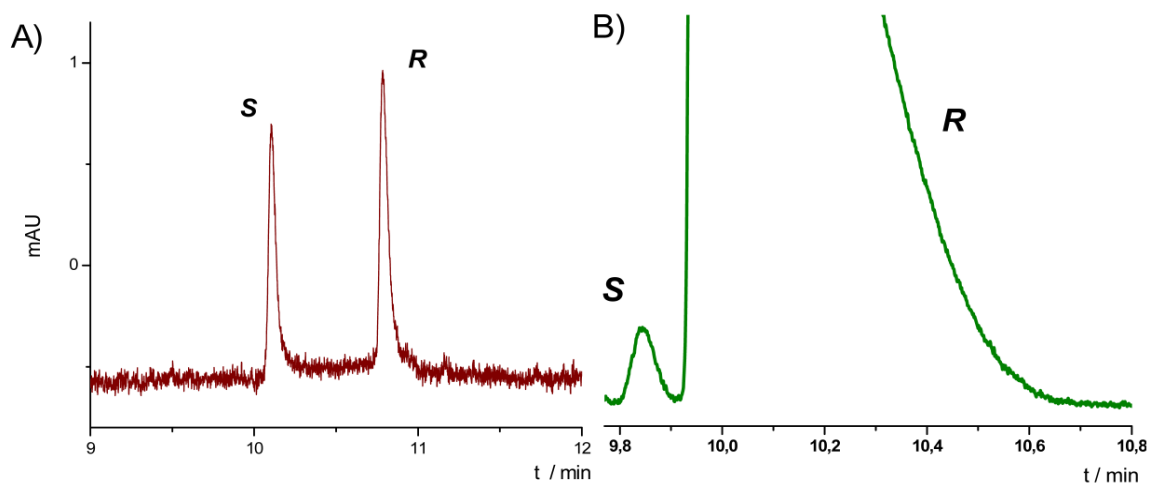
Figure 4.: A) The electropherogram of the optimized system ($R_S = 8{,}39$); B) the detection of 0.1% chiral impurity of *R*-alogliptin

The optimized chiral CE method for the enantioseparation of alogliptin enantiomers was validated in terms of precision, detection limit, quantitation limit, linearity, and robustness.

The system containing 5 mM sulfated-α-CD in 100 mM sodium borate pH 9.5 buffer could be able to determine 0.15 % chiral impurities of *R,R*-tapentadol (Figure 5.). The optimized system was validated according to ICH guidelines.
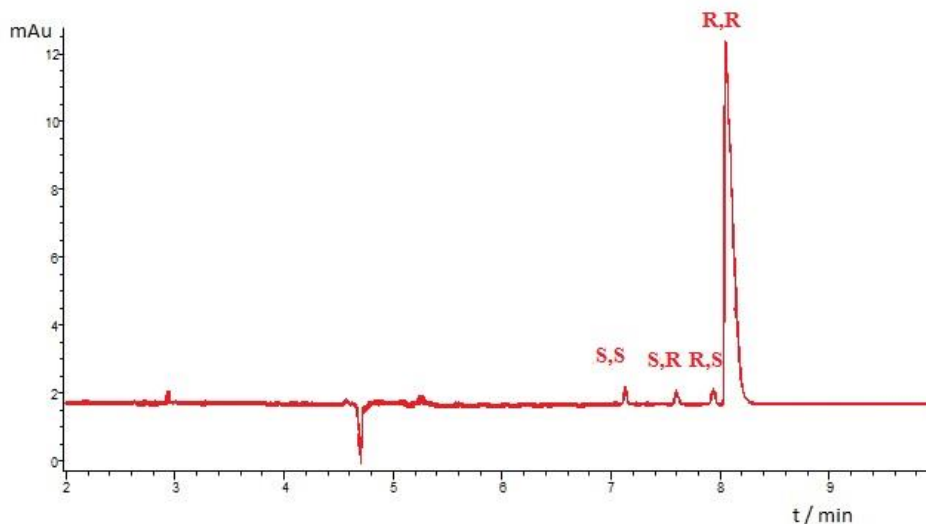
Figure 5.: Determination of 0.15 % chiral impurities of *R,R*-tapentadol with the optimized system

To separate the 4 tadalafil stereoisomers the system containing 7 mM sulfobuthyl-ether-α-CD in 75 mM Tris – acetic acid pH 4,75 buffer found to be optimal applying 25 kV voltage and 25°C capillary temperature. The effect of the method optimization is shown in Figure 6.
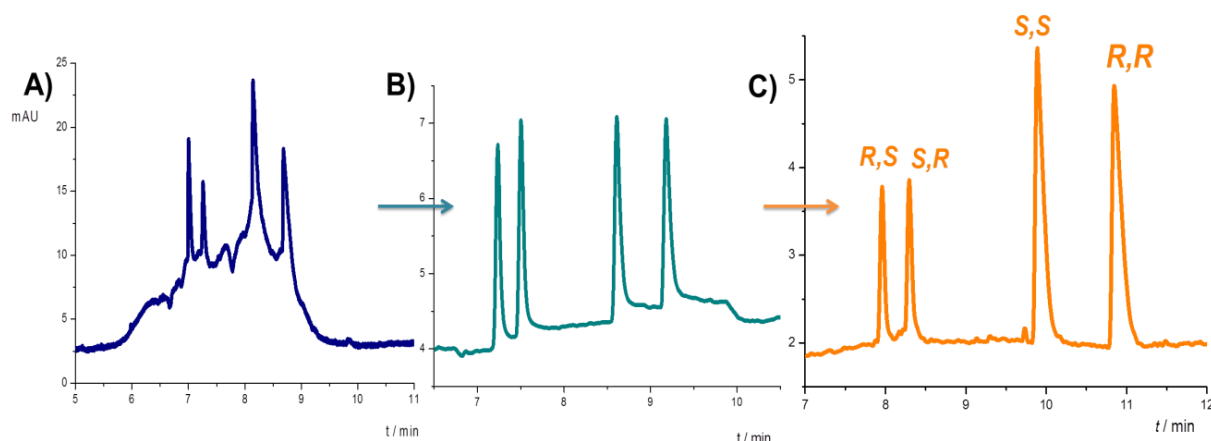
Figure 6.: The electropherograms of tadalafil isomers at different stages of method development using sulfobuthyl-ether-α-CD: A) the electropherogram before optimization B) after optimization of the sample preparation and C) after optimization of the critical parameters

Conclusion

Due to their popularity, medicinal products containing PDE-5 inhibitors sildenafil, vardenafil and tadalafil are often subject to counterfeiting. In addition, illicit herbal dietary supplements adulterated with these substances or their analogs have appeared on the market offering an easy and anonymous sale. As a result of our work a validated RP-HPLC-UV method has been developed for the separation of sildenafil, vardenafil, tadalafil and 11 of their designer analogs. There is no need for complicated sample preparation, furthermore the optimized method allows a rapid screening and determination of PDE-5inhibitor content of illegal erectile dysfunction products utilizing only sildenafil as a widely available external standard both for the qualitative and quantitative analysis of all analytes, for the first time. The identification is based on relative retentions and UV spectra; furthermore, it is possible to perform a tentative preliminary identification of undeclared substances in complex herbal matrices with the developed method. For the confirmation of the analysis MS-compatibility could be utilized. The quantification is easily achievable applying the correction factors, the sildenafil external standard concentration and sample dilution. The optimized and validated method has been successfully applied to the analysis of natural dietary supplements and herbal remedies. Due to the easy use and the low costs of the developed method, it could serve as a routine procedure in control laboratories for fast screening of the suspicious products.

All of the molecules studied with capillary electrophoresis contain one (alogliptin) or more (tapentadol, tadalafil) chiral centers, thus exist in several stereoisomeric forms, which

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possess different biological activity. Comparing the stereoisomers, the *R*-alogliptin, the *R,R*-tapentadol and the *R,R*-tadalafil are the most potent, the pharmaceutical preparations must contain these isomers in enantiopure forms, as the distribution of single-isomer drugs and the analysis of the chiral purity is an essential criteria during authorization. In chiral analysis and in the study of inclusion complex stability capillary electrophoresis is an easy, inexpensive, fast and widely used technique. Cyclodextrin-based chiral capillary electrophoretic methods have been developed for the separation of the antidiabetic alogliptin enantiomers, the analgesic tapentadol stereoisomers, and the tadalafil stereoisomers used for the treatment of erectile dysfunction. Various cyclodextrin derivatives were studied for chiral separation and complex stability in the case of the 3 chiral components. In general, applying charged cyclodextrins instead of neutral cyclodextrin derivatives the enantioselectivity and the complex stability have been increased. Comparing the 3 chiral components, the highest enantioresolution has been achieved in the case of the tapentadol/sulfated-α-CD system, while the most stable complex has been formed in the case of the tapentadol/carboxymethyl-β-CD system. Furthermore, for the inclusion of all 3 examined molecules, the β-CD derivatives possessed the most suitable cavity size.

In the case of alogliptin cavity size dependent EMO reversal was observed: the preferred systems are those containing β-CDs, among which sulfobuthyl-ether-β-CD was chosen for the further method optimization. After the optimization of the critical parameters the chiral CE method was validated according to ICH guidelines. The developed method could be able to detect 0.1% chiral impurity *S*-alogliptin, thus the method could be a routine procedure in Pharmacopoeias.

In the case of tapentadol and tadalafil stereoisomers both cavity size dependent and substituent dependent migration order reversals could be observed. In the case of tapentadol the sulfated-α-CD found to be optimal due to the outstanding enantioselectivity and the ideal migration order, thus this system was further optimized and partially validated. Applying the developed method 0.1% chiral impurity *S,S-, S,R-, R,S*-tapentadol could be detected, it could be served as a routine chiral analytical method.

The most promising system containing sulfobuthyl-ether-α-CD (with an optimal EMO of *R,S; S,R; S,S; R,R* and outstanding enantioselectivity) may serve as a routine CE enantioseparation method for the stereoisomeric impurity profiling of tadalafil.

The study of the cyclodextrin complexation of alogliptin enantiomers, and the development of an optimized, validated method for the separation of alogliptin enantiomers was performed for the first time. All tapentadol stereoisomers were also investigated by cyclodextrin-modified capillary electrophoresis for the first time. To separate all 4 neutral

stereoisomers of tadalafil is challenging in capillary electrophoresis, where the separation is based on the different charge/size ratio. The separation of the tadalafil stereoisomers by CE was accomplished for the first time.

Our measurements enabled a deeper insight into the mechanism of the selector-analyte interactions, the separations of the stereoisomers and the enantiomer migration order reversals. The developed methods could be used to determine trace enantiomeric impurities; furthermore, these methods could serve in the near future as new chiral analytical tools for the Pharmacopoeias or routine methods for control laboratories.

Bibliography of the candidate's publications

Publications associated with the thesis

[S1] Fejős I, Urbancsok Zs, Zhou W, Sohajda T, Hu WH, Szente L. Béni Sz. (2014) Separation of alogliptin enantiomers in cyclodextrin-modified capillary electrophoresis: a validated method, Electrophoresis, 35: 2885-2891.

[S2] Fejős I, Kazsoki A, Sohajda T, Márványos E, Volk B, Szente L, Béni Sz. (2014) Interactions of non-charged tadalafil stereoisomers with cyclodextrins: Capillary electrophoresis and nuclear magnetic resonance studies. J Chromatogr A, 1363: 348-355.

[S3] Fejős I, He Y, Völgyi G, Kazsoki A, Sun J, Chen W, Sohajda T, Szente L, Jiang X, Béni Sz. (2014) Tapentadol enantiomers: Synthesis, physico-chemical characterization and cyclodextrin interactions. J Pharm Biomed Anal, 88: 594–601.

[S4] Znaleziona J, Fejős I, Ševčík J, Douša M, Béni Sz, Maier V. (2014) Enantiomeric separation of tapentadol by capillary electrophoresis—Study of chiral selectivity manipulation by various types of cyclodextrins. J Pharm Biomed Anal, 105: 10–16.

[S5] Fejős I, Neumajer G, Béni Sz, Jankovics P. (2014) Qualitative and quantitative analysis of PDE-5 inhibitors in counterfeit medicines and dietary supplements by HPLC–UV using sildenafil as a sole reference, J. Pharm. Biomed. Anal, 98: 327-333.

Further publication

[S6] Sohajda T, Varga E, Iványi R, Fejős I, Szente L, Noszál B, Béni Sz. (2010) Separation of vinca alkaloid enantiomers by capillary electrophoresis applying cyclodextrin derivatives and characterization of cyclodextrin complexes by nuclear magnetic resonance spectroscopy, J Pharm Biomed Anal, 53: 1258-1266.

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