Enantiomeric separation on cyclodextrin-based chiral selectors

Ph.D. THESIS

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1. Introduction

In the 1980s the interest for the chiral drugs significantly increased, due to the different physiological effect of the enantiomers, which was proven in many drug products for instance in the case of Thalidomide (Contergan). Thalidomide was marketed at the end of the 1950s but in the early 1960s it was withdrawn after being found to be responsible for many different forms of birth defects. In the 1980s it was proven that in the case of Thalidomide, manufactured in racemic form, only one enantiomer causes abnormalities. After the incident the U.S. food and medicines supervisory authority, the FDA ordered pharmaceutical companies to study the pharmacological effects of both enantiomers of all chiral substances before the pharmaceutical marketing. However, the FDA also recommended that racemic drugs should not be banned from sale, but the final authorization must be based on a complex set of information, including the pharmacokinetic and pharmacodynamic characterization of both enantiomers and the mixtures as well as the analytical methods for their determination.

In physical and chemical processes appearing in the human body drug molecules have interactions with asymmetric biological macromolecules (proteins, polynucleotid, glycopeptides). This chiral environment is capable of distinguishing the enantiomers, in other words chiral drug molecules in biological systems are recognized stereospecifically, i.e. the stereoisomers may posses various physiological effects.

For instance:

- same pharmacology effect, but higher toxicity,
- other pharmacology effect or
- antagonism.

As a consequence, the discrimination and separation of the enantiomers of drug substances is an indispensable task, to meet these requirements in addition to liquid chromatography, capillary electrophoresis is one of the most frequently used analytical methods nowdays.

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2. Aims

The primary aim of this work was to develop new chiral capillary electrophoretic (CE) and liquid chromatographic methods for the separation of different biologically and pharmaceutically important molecules.

The objectives to be studied were the followings:

a) separation of *ß*-methyl substituted amino acid enantiomers by CE technique (using different cyclodextrin based chiral selectors),
b) enantiomer separation of *ß*-substituted tryptophan analogs by CE (using different cyclodextrin based chiral selectors),
c) enantiomer separation of *ß*-lactams in reversed phase conditions by novel cyclodextrin based stationary phases, and
d) separation of structurally divergent molecules by novel cyclodextrin based stationary phases.

Further objective was to investigate the effect of changes in the separation conditions during CE and liquid chromatographic measurements. Depending on the applied technique the effect of different parameters such as the nature and composition of the mobile phases, the buffer concentration, pH, the nature and structure of investigated compounds and chiral selectors on the chiral separation process were studied to be able to draw conclusions for the chiral discrimination processes.

3. Experimental

3.1. Apparatus

The pH measurements were performed on Thermo Orion 420 pH meter (Orion, USA).

The measurements of capillary electrophoresis were performed by HP^3CE instrument (Agilent Technologies, Palo Alto, CA, USA), applying diode array detector for the detection, and Chemstation softver for the data evaluation.

Two kinds of HPLC system was applied for the measurements of liquid chromatography

1. DIONEX Ultimate 3000 and
2. Agilent 1100 type liquid chromatograph.

(Quaternary pump, autosampler, and diode array detector were applied for both systems).

3.2. Applied cyclodextrins

During the CE measurements we used the following cyclodextrins: native α-CD, sulfopropyl-α-CD (SP2-α-CD: degree of substitution=2), β-CD-sulphate (degree of substitution=12), sulfopropyl-β-CD (SP2-β-CD: degree of substitution=2 and SP4-β-CD: degree of substitution=4), as well as sulfopropyl-γ-CD (SP2-γ-CD: degree of substitution=2) (Cyclolab R&D Ltd. Budapest, Hungary).

3.3. Applied columns

The applied chiral stationary phases were the following: **PMBCD** (permetyl-β-CD, **Quest 1**), **HPBCD** (hydroxypropyl-β-CD, **Quest 2**), **BCD** (β-CD, **Quest 3**) 250x4.0 mm, 5 μm particle size (Chiroquest, Budapest, Hungary).

3.4. Investigated analytes

- β-methyl-substituted amino acids
- β-substituted tryptophan analogs
- β-lactams
- structurally divergent molecules

4. Results

New chiral CE and liquid chromatographic methods were developed for the separation of different biologically and pharmaceutically important molecules. During the capillary zone electrophoresis and direct chiral HPLC enantioseparations the effect of different separation conditions such as the pH, nature and concentration of chiral additives in CE, nature and concentration of mobile phase compositions, structure of analytes were examined on the chiral discrimination processes.

1. *β*-Methyl-substituted amino acid enantiomers were separated with CE technique. *α*-CD, SP2-*α*-CD, SP2-*β*-CD, SP4-*β*-CD and *β*-CD-sulfate were used as chiral selectors. The pH dependence of enantioseparation with application of SP2-*α*-CD showed that the migration time first decreased with increasing pH than increased. Hovewer, the selectivity and resolution continuously decreased with incresing pH (exceptions were *threo-β*-MeTyr and *threo-β*-MeTic in borate buffer). This can be explained that the protonation and complexation of the amino acids strongly depend on the pH. Besides the pH of background electrolyte (BGE) the separations strongly depend on the structure of analytes. The retention of molecules possessing two ring systems was much longer than the molecules having one ring system. Molecules possessing two rings probably fit better in the cavity of SP2-*α*-CD, however, despite the higher migration time the $\boldsymbol{\alpha}$ and $\boldsymbol{R_s}$ values of the *β*-MeTic were much lower than those for the *β*-MePhe, *β*-MeTyr or *β*-MeTrp. The enantiomers of both *erythro*- and *threo-β*-MeTic are conformationally very constrained; the complex stabilities of the two enantiomers of *threo-β*-MeTic may be very similar, resulting in no or very poor separation.

In the presence of SP2-*β*-CD and SP4-*β*-CD the migration times measured, in most cases increased when SP4-*β*-CD was applied (exceptions were *erythro-β*-MeTyr and *erythro-β*-MeTrp in borate buffer, *erythro-β*-MeTic in acetate buffer, and *threo-β*-MeTrp in acetate and phosphate buffers). Comparing the data with the SP2-*β*-CD and SP4-*β*-CD selectors, the $\boldsymbol{\alpha}$ and $\boldsymbol{R_s}$ values in most cases were larger on SP4-*β*-CD, and especially high resolutions were obtained for the *β*-MeTic analogs. However, these higher resolutions did not reach the $\boldsymbol{R_s}$ values obtained with the SP2-*α*-CD selector, indicating the better fit of these amino acids in the SP2-*α*-CD cavity (exceptions were the *threo-β*-MeTyr and *erythro*- and *threo-β*-MeTic enantiomers). The special behavior of

conformationally very constrained *ß*-MeTic enantiomers during the CE separation with the application of CDs indicates the importance of steric effects in chiral discrimination. In the case of a mixture of *erythro*- and *threo*-*ß*-MePhe, *ß*-MeTyr and *ß*-MeTrp, all four stereoisomers were baseline resolved with the application of SP4-*ß*-CD in acetate or borate buffers, while the *erythro*- and *threo*-*ß*-MeTic enantiomers could be separated by the application of *ß*-CD-sulfate in the presence of acetate buffer.

2. New CE methods were developed for the direct enantioseparation of various *ß*-alkyl or aryl-substituted Trp analogues. Comparing with *ß*-Me-substituted Trp analogues where sulfated *ß*-CDs such as SP2-*ß*-CD és SP4-*ß*-CD were the most useful selectors for the enantioseparation of *ß*-MePhe, *ß*-MeTyr and *ß*-MeTrp while for *ß*-MeTic the *ß*-CD-sulfate seemed to be most applicable, however for *ß*-alkyl or aryl-substituted Trp analogues (*ß*-2-PrTrp, *ß*-3-PentTrp, *ß*-PhTrp and *ß*-diMeOPhTrp) the SP2-*α*-CD was the most useful selector. For optimization of migration time, selectivity and resolution the nature of BGE, pH and applied voltage were varied.

Migration time, selectivity and resolution of the enantiomers of *ß*-substituted Trp analogues applying SP2-*α*-CD as chiral selector strongly depend on the pH of BGE and the structures of the analytes. Amino acids with aromatic side-chain in *ß*-position usually are retained more strongly than those with aliphatic side-chain (except *treo*-*ß*-2-PrTrp). It was also observed that migration times increased for the amino acids with the aliphatic side chains possessing higher carbon number. However, in spite of the longer migration times, in some cases lower selectivity and resolution were observed (*ß*-3-PentTrp, *ß*-MeTrp).

As concerns the separation efficiency, separation factor and resolution in all three buffer systems were much lower applying SP2-*ß*-CD selector than for SP2-*α*-CD. Probably this was due to the worse fitting and the lower stability of the complex applying SP2-*ß*-CD selector. The migration order of *ß*-substituted Trp analogues was checked by spiking with authentic enantiomers obtained from enzymatic digestion. It is interesting to note that the sequence of elution of the *erythro*- and *threo*-diastereomers depends on the nature of amino acids and background electrolytes. The change of BGE in most cases resulted in the change of the order of migration of the diastereomers, while the migration

order of the enantiomers remained the same. For the elution sequence no general rule could be established.

3. New HPLC methods were developed for the separation of *ß*-lactam enantiomers on three different „novel" columns (prepared by new bonding technique) such as **BCD**, **HPBCD** and **PMBCD**. It was established that the structure of *ß*-lactam molecules, the size of the aliphatic and aromatic groups attached to the *ß*-lactam ring, the presence and position of double bonds in the attached rings and the nature (polarity) and position of substituents on the attached aromatic rings all influenced the formation of the inclusion complexes with substituted *ß*-CDs thereby significantly affect the chiral recognition.

On the three CSPs the effect of structure of *ß*-lactams showed that an increase in size of the attached ring resulted in longer retention times and enhanced enantioselectivity on the **PMBCD** CSP, while **BCD** and **HPBCD** CSPs proved to be less effective. On the **PMBCD** column the presence of a double bond in the attached ring of *ß*-lactams resulted in all cases in lower retention time and in higher resolution. The beneficial effect of the double bond on the **BCD** and the **HPBCD** CSPs was not detected.

On the **PMBCD**-bonded column excellent chiral separation was obtained for the unsubstituted phenylazetidinone (**22**), while the enantiomers of analytes containing methyl or halogen substituent in *„para"* position on aromatic ring were not separated [only *p*-fluorophenylazetidinone (**27**) was partially resolved on this CSP]. The unresolved compounds (**23**,**26** and **28**) however, were at least partially separated on the **HPBCD** and on the **BCD** CSPs. The **PMBCD** column showed different interaction for „*para*" substituted molecules than the **HPBCD** and **BCD** CSPs, however this interaction depends on the size of the substituent. For the halogen-substituted analogs possessing halogen atoms in „*para*" position the retention times increased in the sequence F, Cl, Br on each of the tested cyclodextrin-bonded CSPs. In the sequence of F, Cl, Br the size of the molecule is increasing, the polarity is decreasing and the analyte containing F, Cl and Br atoms are getting more and more apolar.

Enantioseparation was achieved for 17 of the 19 *ß*-lactams on three novel cyclodextrin-based CSPs. The **PMBCD** selector proved to be the most effective among

the three CSPs. In summary, it can be stated that the investigated three novel stationary phases have complementary capabilities to separate *ß*-lactam enantiomers.

4. Methods were developed for the enantioseparation of structurally very divergent analytes on three different „novel" columns (native **BCD**, **HPBCD** and **PMBCD** as mentioned above). The 14 structurally different molecules provide a good benchmark for the characterization of the efficiency of the three different CSPs.

In the case of three coumarin analogs no obvious relationships were found between retention (***k***), selectivity (***α***) and resolution (***R*** $_S$). For example on **PMBCD** column in case of component (**31)** the best separation was achieved at the shortest, while in case of component (**29**) the highest resolution was obtained at the longest retention time. This phenomenon shows the complexity of the enantiomeric separation on CD-bonded stationary phases, *i.e.* strong inclusion interaction is not always sufficient for the desired enantioselectivity.

For *Dns*-amino acids it seems that the *Dns*-core of analytes determines the separation. The best resolutions were achieved on the **PMBCD** CSP for *Dns*-Phe and on the **BCD** CSP for *Dns*-Leu while *Dns*-Met exhibited partial resolution only on **BCD** and **PMBCD** CSPs.

The effect of the nature of the substituent on the phenyl ring of the propionic acid analogues was observed. The retention factors of all three propionic acid analogues were largest on **BCD** and **HPBCD** columns, inspite of the fact that **PMBCD** possesses the largest cavity for inclusion complexation. Despite of the large retentions observed on the **BCD** and **HPBCD** columns, the chiral recognition was less effective on these selectors. It was observed that the methyl group of the methyl-substituted propionic acid analog may interact with the methyl group of permethylated-*ß*-CD hereby improving the selectivity. The large ***R*** $_S$ value obtained for the three-chloro substituted analog probably was due to the better fit of the molecule in the cavity of permethylated-*ß*-CD.

Tropic acid and terbutaline possess an extra aliphatic or aromatic hydroxyl groups which may induce H-bond interaction between the analyte and the free hydroxyl group on the rim of the *ß*-CD. At optimized chromatographic conditions for both analytes

HPBCD CSP proved to be the best due to the possible H-bond interaction between the hydroxyl groups of the rim and analytes.

The lack of the aromatic ring in permetrine acid shed light to the different separation mechanism on *ß*-CD-based CSPs. The cyclopropyl-ring is too small to fit inside the cavity of *ß*-CD, however large $\boldsymbol{R_s}$ values for *cis* and *trans* enantiomers of permetrine acid on **PMBCD** was achieved. These results support the importance of interactions on the rim of substituted *ß*-CD with the groups of chiral analytes, as it was stated in the case of separation of propionic acid analogues.

Of the 14 tested modelcompounds two on **BCD** column, four on **HPBCD** column and nine on **PMBCD** column could be baseline separated. In most cases the retention was largest on **BCD** and on **HPBCD** column.

We found that the molecular and chemical structure of the stationary phase and analytes determined the main possible interactions hereby helping the choice of the suitable selector. The three different CSPs were complement to each other, *e.g.* if one of the stationary phase ensured at least partial separation, it is likely that the separation on one of the two other stationary phases may be successful. The separation mechanism on the three-stationary phases probably are different, which was supported by the different elution sequences determined for some analytes.

5. Publications

5.1. The thesis is based on the following publications

1. István Ilisz, **Gábor Fodor**, Róbert Iványi, Lajos Szente, Géza Tóth, Antal Péter
   Enantioseparation of ß-methyl substituted amino acids with cyclodextrins by capillary zone electrophoresis
   J. Chromatogr. B, 875 (**2008)** 273-279. **Impact factor: 2.500**

2. István Ilisz, **Gábor Fodor**, Róbert Berkecz, Róbert Iványi, Lajos Szente, Antal Péter
   Enantioseparation of ß-substituted tryptophan analogues with modified cyclodextrins by capillary zone electrophoresis
   J. Chromatogr. A, 1216 (**2009**) 3360-3365. **Impact factor: 4.101**

3. **Gábor Fodor**, István Ilisz, Júlianna Szemán, Róbert Iványi, Lajos Szente, Gábor Varga, Enikő Forró, Ferenc Fülöp, Antal Péter
   HPLC Enantioseparation of ß-Lactam Stereoisomers Using ß-Cyclodextrin-Based Chiral Stationary Phases
   Chromatographia 71 (**2010**) S29-S34. **Impact factor: 1.075**

4. Gábor Varga, **Gábor Fodor**, István Ilisz, Júlianna Szemán, Júlia Visy, Lajos Szente, Antal Péter
   Comparison of Separation Performances of Novel ß-Cyclodextrin-Based Chiral Stationary Phases in High-Performance Liquid Chromatographic Enantioseparation
   J. Pharm. Biomed. Anal. 70 (**2012**) 71-76. **Impact factor: 2.976**

Total impact factor: 10.652

5.2 Posters

2007 István Ilisz, **Gábor Fodor**, Róbert Iványi, Géza Tóth, Antal Péter
Enantioseparation of *ß*-methyl substituted amino acids with cyclodextrins by capillary zone electrophoresis
31st International Symposium on High Performance Liquid Phase separations and related techniques, June 17-21, Gent, Belgium, 2007

2009 **Gábor Fodor**, István Ilisz, Júlianna Szemán, Róbert Iványi, Lajos Szente, Gábor Varga, Enikő Forró, Ferenc Fülöp, Antal Péter
HPLC Enantioseparation of *ß*-Lactam Stereoisomers Using Cyclodextrins-Based Chiral Stationary Phase
8th Balaton Symposium on High Performance Separation Methods, 2-4. September, Siófok, Hungary, 2009

2011 István Ilisz**, Gábor Fodor,** Zoltán Pataj, István Szatmári, Ferenc Fülöp, Lajos Szente, Antal Péter
Capillary Electrophoretic Enantioseparation of Aminonaphthol Analogs
36th International Symposium on High performance Liquid Phase Separations and Related Techniques, 19-23 June, Budapest, Hungary, 2011

2011 Julianna Szemán, Júlia Visy, Éva Jámbor, **Gábor Fodor,** Róbert Ohmacht, Gábor Varga
Cyclodextrin Based Cation-Exchanger Chiral Columns
36th International Symposium on High performance Liquid Phase Separations and Related Techniques, 19-23 June, Budapest, Hungary, 2011