

**ENANTIOSEPARATION OF KETOCONAZOLE
AND MICONAZOLE BY CAPILLARY
ELECTROPHORESIS USING
HYDROXYPROPYL- β -CYCLODEXTRIN AS A
CHIRAL SELECTOR**

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UNIVERSITI SAINS MALAYSIA

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by

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LIST OF ABBREVIATIONS

^1H	Single proton
2-AHP- β -CD	2-O-acetyl-2-O-hydroxypropyl-beta-cyclodextrin
2D	Two dimension
AGP column	α -1-acid glycoprotein column
BGE	Background electrolyte
CDR	Chiral derivatizing reagent
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CE- β -CD	Carboxyethyl-beta-cyclodextrin
CIEF	Capillary isoelectric focusing
CMC	Critical micelle concentration
CMPA	Chiral mobile phase additive
CM- β -CD	Carboxymethyl-beta-cyclodextrin
CO ₂	Carbon dioxide
CPMP	Committee for Proprietary Medicinal Products
CSA	Chiral solvating agent
CSPs	Chiral stationary phases
Cu	Copper
CZE	Capillary zone electrophoresis
DAD	Diode array detector
DMSO-D ₆	Dimethyl sulfoxide-D ₆
DM- β -CD	Heptakis(2,6-di-O-methyl)-beta-cyclodextrin
DNA	Deoxyribonucleic acid
DTAC	Decyl trimethyl ammonium chloride

EKC	Electrokinetic chromatography
EOF	Electroosmotic flow
FDA	Food and Drug Administration
GC	Gas chromatography
HB- β -CD	Hydroxybutyl-beta-cyclodextrin
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HP- β -CD	Hydroxypropyl-beta-cyclodextrin
HSA column	Human serum albumin column
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MEKC	Micellar electrokinetic chromatography
Min	Minutes
MS	Mass spectrometry
M- β -CD	Heptakis-6-methyl-beta-cyclodextrin
NaOH	Sodium hydroxide
Ni	Nickel
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
pH	Potential for hydrogen
PTA	Paratartaric acid
RSD	Relative standard deviation
SBE- β -CD	Sulfobutylether-beta-cyclodextrin
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEE- β -CD	Sulfoethylether-beta-cyclodextrin

SFC	Supercritical fluid chromatography
TM- β -CD	Heptakis(2,3,6-tri-O-methyl)-beta-cyclodextrin
UV-Vis	Ultraviolet-visible
Zn	Zinc
β -CD	Beta-cyclodextrin

LIST OF SYMBOLS

%	Percent
μL	Microliter
μm	Micrometer
C18	Carbon 18 column
g mol^{-1}	Gram per mole
i.d	Internal diameter
K	Formation constant
kV	Kilovolt
M	Molarity
mbar	Millibar
mg	Milligram
mg L^{-1}	Milligram per liter
mL	Milliliter
mM	Millimolar
nm	Nanometer
$^{\circ}\text{C}$	Degree Celsius
R^2	Coefficient of determination
R_s	Resolution
Sec	Second
α	Alpha
β	Beta
γ	Gamma
π	Pi

**PEMISAHAN ENANTIO BAGI KETOKONAZOL DAN MIKONAZOL
OLEH ELEKTROFORESIS RERAMBUT DENGAN MENGGUNAKAN
HIDROKSIPROPIL- β -SIKLODEKSTRIN SEBAGAI PEMILIH KIRAL**

ABSTRAK

Kaedah elektroforesis rerambut (CE) dengan menggunakan β -siklodekstrin asli (β -CD) dan hidroksipropil- β -siklodekstrin terubahsuai (HP- β -CD) sebagai pemilih kiral telah dikaji dan dibandingkan untuk pemisahan enantio serentak bagi dua agen antikulat imidazol. Dua agen antikulat imidazol iaitu ketokonazol dan mikonazol telah berjaya dipisahkan dengan resolusi yang baik (R_s : masing-masing 1.6 dan 3.7) dalam masa 16 min dengan menggunakan 1.5 mM HP- β -CD dalam latarbelakang elektrolit (BGE) yang mengandungi 35 mM larutan penimbal tris-fosfat (pH 2.5) pada 25 °C dan 25 kV. Sampel disuntik secara hidrodinamik pada 50 mbar bagi 5 saat dan pengesanan telah dijalankan pada 200 nm. Kaedah bagi setiap enantiomer adalah linear bagi kepekatan 0.25 – 50 mg L⁻¹ dengan pekali penentuan yang tinggi ($R^2 > 0.999$) dan had pengesanan dan had kuantifikasi masing-masing ialah 0.075 dan 0.250 mg L⁻¹. Kaedah yang dicadangkan telah diaplikasikan pada sampel krim antikulat terpilih dan perolehan semula yang baik dicapai, antara 82.0 – 105.7%, ($RSD < 7\%$, $n = 3$). Tambahan pula, untuk memahami kemungkinan mekanisma pemisahan kiral dengan lebih mendalam bagi kedua-dua sebatian imidazol dengan β -CD dan HP- β -CD, pembentukan kompleks kemasukan telah dianalisis menggunakan teknik spektroskopi ¹H NMR dan spektrofotometri UV-Vis dan akhir sekali, struktur kompleks kemasukan yang dicadangkan telah dilukis dan dipersembahkan.

**ENANTIOSEPARATION OF KETOCONAZOLE AND MICONAZOLE
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CYCLODEXTRIN AS A CHIRAL SELECTOR**

ABSTRACT

Capillary electrophoresis (CE) method employing native β -cyclodextrin (β -CD) and modified hydroxypropyl- β -cyclodextrin (HP- β -CD) as chiral selectors was studied and compared for simultaneous enantioseparation of two imidazole antifungal agents. The two imidazole antifungals agents which were ketoconazole and miconazole were successfully resolved with good resolution (R_s : 1.6 and 3.7, respectively) within 16 min using 1.5 mM of HP- β -CD in the background electrolyte (BGE) containing 35 mM of tris-phosphate buffer (pH 2.5) at the 25 °C and 25 kV. The samples were injected hydrodynamically at 50 mbar for 5 sec and detection was carried out at 200 nm. The method of each enantiomer was linear over the concentration ranges of 0.25 – 50 mg L⁻¹ with high coefficient of determination ($R^2 > 0.999$) and the limit of determination (LOD) and limit of quantification (LOQ) were 0.075 and 0.250 mg L⁻¹, respectively. The proposed method was also applied to selected antifungal cream samples and good recoveries were found, ranging from 82.0 – 105.7%, (RSD < 7%, n = 3). In addition, in order to understand in depth the possible chiral separation mechanism of both imidazole compounds with β -CD and HP- β -CD, the formation of inclusion complexes were analyzed using ¹H NMR spectroscopy and UV-Vis spectrophotometry techniques and lastly, proposed structure of inclusion complexes were drawn and presented.

CHAPTER 1

INTRODUCTION

1.1 General background of research

Chirality is the study of geometric properties of molecules and ions. Chiral compounds comprise of four different groups attached to at least one carbon atom and the carbon atom is called chiral center or chiral atom. This molecule known as isomer can rotate itself and form its pair that is mirror image which is non-superimposable of each other as shown in **Figure 1.1**. The pair of isomers is known as enantiomer or optical isomers. While, the mixture of enantiomers is called racemic. The enantiomers are identical in terms of configuration number, physical and chemical properties, however, the pharmaceutical, pharmacokinetic, pharmacodynamics, and toxicity properties are might be stereoselective among enantiomers (Kar, 2017; Ribeiro *et al.*, 2018). One enantiomer might be biologically or pharmaceutically active, while the other is inactive or even toxic to the non-target consumers (Zhou *et al.*, 2018). Therefore, the study of chirality becomes one of the most important consideration in pharmaceuticals, drugs, agricultural and food industries.

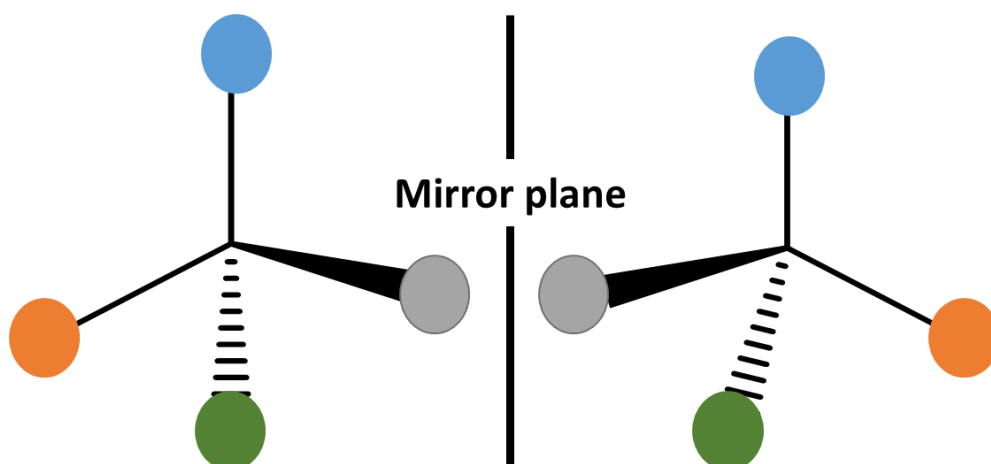


Figure 1.1 Non-superimposable mirror image of chiral compounds.

A lot of studies have been reported about the stereochemistry properties of some enantiomers of pharmaceutical compounds. For example, (S)-propranolol is 100-fold more antagonistic effect on β -adrenergic receptor than (R)-propranolol (Kasprzyk-Hordern, 2010; Stanley *et al.*, 2006), while (S)-citalopram is pharmacologically active as a selective serotonin reuptake inhibitor, whereas (R)-citalopram is inactive and even being inhibitor for (S)-enantiomer (Sánchez *et al.*, 2004). Besides that, the enantiomer can be toxic and harmful such as, (S)-penicillamine is beneficial as anti-arthritic while (R)-penicillamine is extremely toxic (Kasprzyk-Hordern, 2010). Other than that, (R)-thalidomide is used as a sedative treatment for pregnant women and effective for morning sickness, but the (S)-thalidomide is causing birth defect because it inhibits the release of TNF- α from stimulated mononuclear blood cells (Stirling, 2000).

The same phenomenon has occurred to imidazole antifungal agents such as ketoconazole and miconazole, however, there are still scarce reports describing these profiles due to the limited availability of large amounts of enantiomerically pure compounds (Cirilli *et al.*, 2002). To the best of our knowledge, there is a report stated that the cis-(2S,4R) and -(2R,4S) enantiomers of ketoconazole is 40 times more potent as antifungal agent than trans-(2R,4R) and -(2S,4S) diastereoisomers of ketoconazole, therefore the trans-ketoconazole are identified as the impurities of cis-ketoconazole (Anwar-Mohamed *et al.*, 2016). The pharmacokinetic performance of ketoconazole is also different among the enantiomers (Allqvist *et al.*, 2007). Interestingly, a study has reported that the performance of ketoconazole in mixture displayed weaker effect compared to individual enantiomers (Novotna *et al.*, 2014). On the other hands, only two studies had been done to investigate the antifungal activity and the pharmacokinetic activities of the enantiomers of miconazole and (R)-miconazole has been reported to have high antifungal activities against series of yeast (*Candida krusei*,

Cryptococcus neoformans) and filamentous fungi (*Penicillium chrysogenum*, *Aspergillus niger*), while (S)-miconazole shows almost no antifungal activity (Mangas-Sánchez *et al.*, 2011). The pharmacokinetic of each enantiomer in rat plasma is also stereoselective (Du *et al.*, 2017). Moreover, ketoconazole is not free from adverse effect as it can affect the synthesis of ergosterol for non-target consumers because it is a potent inhibitor of the drug metabolizing agents (CYP 3A4). When the antifungal cream is applied to human, it sometimes can affect the inhibition of cell growth and influence the drug-drug interaction. According to the previous studies, this adverse effect is different among the enantiomers (Castro-Puyana *et al.*, 2005; Dilmaghanian *et al.*, 2004).

Since stereochemistry is important for drugs biological effect, in 1992, the US Food and Drug administration (FDA), the European Committee for Proprietary Medicinal Products (CPMP), and other drugs controlling agencies have recommended the industries to develop and market only the optically active isomer instead of mixture (Food & Administration, 1992). Besides that, they have also issued to state the enantiomeric purity for pharmaceutical and drug products at their labels. Nevertheless, most products are sold in mixture of enantiomers without specifying the purity (Calcaterra & D'acuarica, 2018; Gübitz & Schmid, 2008). This is because, the production of individual enantiomer is usually complicated, time consuming and high cost (Blaser, 2002; Federsel, 2009). Therefore, suitable analytical chiral separation techniques are becoming important to verify the enantiomer purity in the drug development, pharmaceutical quality control and to enable further study of individual enantiomer profiles.

The separation of chiral compounds nowadays is easily performed using chromatographic methods such as liquid chromatography (LC), gas chromatography (GC), and supercritical fluid chromatography (SFC). As most of chiral drugs are non-volatile, polar or ionic, the separation method is more favored in liquid phase. Therefore, enantioseparation in GC requires derivatization step in order to increase the volatility. Nevertheless that method is sometimes quite tedious and complicated (Ilisz *et al.*, 2008). Other than that, direct chiral analysis which involves the addition of chiral selector into mobile phase or onto the stationary phase as known as chiral stationary phases (CSPs) is more preferable compared to derivatization technique to preserve the analyte. Hence, liquid chromatography, especially high performance liquid chromatography (HPLC) becomes a common technique for the resolution of chiral drugs using chiral stationary phases (Bounoua *et al.*, 2016; Pérez-Fernández *et al.*, 2011). However, CSPs are relatively expensive, lower efficiency compared to regular HPLC column other than having the ability to separate limited range of chiral compounds only (Patel *et al.*, 2016). Besides that, HPLC separations have problems of low plate counts, which result to band broadening, poor resolution and longer run time (Bernal *et al.*, 2002). Considering to all limitations above, researchers started to develop more interesting innovations, including synthesizing of novel chiral stationary phases, adding various modifiers or additives and also using other instruments in order to improve the separation of enantiomers. In the meanwhile, the enantioseparation by SFC is considered as an alternative to HPLC because it promises high efficiency and higher flow rates (Felletti *et al.*, 2019; Kalíková *et al.*, 2018). Despite of these, the use of SFC is still uncommon as in some cases, the enantiomer is strongly attached to the CSPs when using pure carbon dioxide (CO₂) as eluent. Due to this, high percentage of alcoholic modifier is needed to improve the resolution (Bernal *et al.*, 2000).

On the other hand, electrophoretic method such as capillary electrophoresis (CE) has been widely recognized over the past decades as an important option in separation method because of its powerful technique and fully automated instrumentation (Lin *et al.*, 2004). CE technique possesses its simplicity, high efficiency, extremely low volume of solvent and standard needed and thus economic. This technique also provides rapid analysis with high resolution power and low background noise (Szabó *et al.*, 2019). In the separation of enantiomers, CE has the advantage of having the ability to change or modify the separation media easily compared to HPLC. Besides that, CE has its versatility and flexibility as it is applicable in many separation techniques such as capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), electrokinetic chromatography (EKC), micelles electrokinetic chromatography (MEKC), capillary isoelectric focusing (CIEF) and capillary electrochromatography (CEC). Consequently, capillary electrophoresis becomes popular for separation technique as hundreds of research papers, review articles and book chapters that discuss about this instrument (Bounoua *et al.*, 2016; Gübitz & Schmid, 2000, 2004).

In CE chiral separations, there are two approaches which are direct and indirect methods. Indirect method is done by transforming enantiomers into diastereoisomers using chiral additives before undergo achiral separation (Batra & Bhushan, 2018; Raikar *et al.*, 2018). While, direct method is usually performed by adding chiral selector into the background electrolyte. Each enantiomer interacts with chiral selector differently to form diastereomeric pairs. The stability of diastereomeric complex will possess different electrophoretic mobility and leads to the successful enantioseparation (Chankvetadze, 2018). The examples of chiral selector are proteins, crown ether, polysaccharides, antibiotics, chiral ligands and metal, surfactants and

many more; however cyclodextrins (CDs) is the most common one (Van Eeckhaut & Michotte, 2006).

The uniquenesses of CDs attract the researchers to explore them in diverse fields including analytical chemistry and separation sciences. Basically, CDs are truncated cone-shaped oligosaccharides which consist of six, seven and eight glucose units joined through α -1, 4 linkages and they are classified as α -CD, β -CD, and γ -CD, respectively (Dass & Jessup, 2000). Their structures contain hydrophobic cavity at the inner part and hydrophilic at the outer surface. The inner hydrophobic layer can form inclusion complex with the aromatic compounds, while the hydrophilic outer layer can be bonded through hydrogen bonding, dipole-dipole and $\pi - \pi$ interactions and also possible for functionalization.

Despite of the difference in hydrophobic cavity (cavity size: α -CD < β -CD < γ -CD), β -CD is the most popular one because of low cost, high chemical stability and its capability to interact with wide range of organic and inorganic compounds. However, native β -CD has low solubility, therefore it is reported to possess poor separation of enantiomers (Wang & Khaledi, 2000). Therefore, various innovations are done to modify and develop new β -CD derivatives by adding substituents such as alkyl, acetyl, benzoyl, hydroxypropyl, phenylcarbamoyl, carboxymethyl and many more (Guo *et al.*, 2003) to increase the solubility, vary the complex properties and adding the charged functional groups (De Boer *et al.*, 2000; Karbaum & Jira, 2001; Saz & Marina, 2016).

To the best of our knowledge, only few works had been reported about the enantioseparation of ketoconazole and miconazole using HPLC and CE for past ten years. By using HPLC, the researchers had faced few challenges as various types of

CSPs were applied and different mobile phase compositions were manipulated to obtain the good result, hence the best resolution (R_s) for each compound was 4.99 for ketoconazole (analysis time: 10 min) using chiralpak IA column as CSP, 100% methanol as mobile phase (Andrade *et al.*, 2015), and 3.69 for miconazole (analysis time: 25 min) using chiralpak IC column and n-butanol: n-hexane (15:85 v/v) of mobile phase (Zhu *et al.*, 2018). Meanwhile, two analyses using CE had been reported and in order to get good separation ($R_s > 1.5$), the addition of sodium dodecyl sulphate (SDS), additive such as 1.0% methanol or ionic liquid were required to be added into the BGE (Ibrahim *et al.*, 2015; Zhao *et al.*, 2014). Moreover, separating both compounds simultaneously was also quite challenging due to the similar pK_a values (ketoconazole: 3.96, 4.60 and 6.75, miconazole: 6.64). Therefore, in this study, a simple CE method was proposed and developed to separate the enantiomers of ketoconazole and miconazole using commercial β -CD and HP- β -CD as chiral selectors. The method was also validated and applied into real antifungal cream samples. Meanwhile, the mechanism of enantioseparation was investigated by analysing the inclusion complex formation between the β -CD and HP- β -CD with the ketoconazole and miconazole by using UV-Vis spectrophotometer and NMR spectroscopy. This proposed method was hopefully not only to improve and simplify the previous methods, but also a potential technique to be applied to another chiralazole compounds and basic drugs.

1.2 Scope of research

This research was proposed to investigate the performance of β -CD and HP- β -CD as chiral selectors in the mode of capillary zone electrophoresis (CZE). In the first part (Part I), β -CD and HP- β -CD was being added, respectively into the background

electrolyte (BGE) to resolve the enantiomers of ketoconazole and miconazole. Based on the chiral separation performance, modified HP- β -CD was selected as the best chiral selector as two enantiomers of ketoconazole and miconazole, respectively were successfully separated. After the selection of chiral selector, the method was further optimized, validated and lastly, the developed method had been applied into real sample which was the antifungal creams. The concentration of each enantiomer of ketoconazole and miconazole in the cream were determined from linear calibration curve and they were being compared with the amount stated at the labels. Meanwhile in the second part (Part II), series of investigations were done to compare the formation of inclusion complexes between β -CD and HP- β -CD with ketoconazole and miconazole, respectively. In this study, the inclusion complexes were synthesized using kneading method and they were determined by ^1H nuclear magnetic resonance (^1H NMR) spectroscopy. In addition, the formation of inclusion complexes and formation constant values (K) were also being studied using ultra-violet visible (UV-Vis) spectrophotometer. The mechanism and differences in the formation constant between β -CD and HP- β -CD were subjected to the result of enantioseparation of the analytes.

1.3 Objectives of research

Main objective:

To study and compare the performance of β -CD and HP- β -CD as chiral selectors for the enantioseparation of chiral ketoconazole and miconazole antifungal by capillary electrophoresis.

The following were the sub-objectives of this study:

- 1) To develop, optimize, and validate the chiral separation method of ketoconazole and miconazole using β -CD and HP- β -CD as chiral selectors by capillary electrophoresis.
- 2) To apply the developed method in determining the concentration of the ketoconazole and miconazole in real samples such as antifungal creams.
- 3) To investigate and compare the formation of inclusion complexes occurred between β -CD and HP- β -CD with ketoconazole and miconazole, respectively using UV-Vis spectrophotometer and NMR spectroscopy.

1.4 Outline of thesis

This thesis was separated into five chapters. Chapter 1 consisted of the research background and research objectives. Next, the literature review of this research was presented in Chapter 2. In Chapter 3, the methodology of the research was classified into two parts; Part I and Part II. In part I, the methodology of chiral separation of ketoconazole and miconazole using β -CD and HP- β -CD as chiral selectors in capillary electrophoresis was explained while the formation of inclusion complexes between β -CD and HP- β -CD with ketoconazole and miconazole were explained well in part II. In Chapter 4, data analysis and discussion were tabulated and presented systematically and finally the Chapter 5 was discussed about conclusion and future perspectives.

CHAPTER 2

LITERATURE REVIEW

2.1 Chirality

In 1848, a young French chemist, Louis Pasteur had discovered the study of molecular chirality which at that time he had used the terms “handedness” or “dissymétrie”. He had proposed the chirality of crystal in which some crystal showed “right-handed” while the other showed “left-handed” morphology (Gal, 2013). In order to understand the chirality morphology, Pasteur had examined the characteristic of sodium ammonium (\pm)-tartrate which was also been called as “racemic acid” or paratartaric acid (PTA). These two PTA salts, (+)-TA and (-)-TA showed equal optical activity, but in opposite direction. Based on this phenomenon, he concluded that PTA was 1:1 combination of dextro- and levo-TA and the molecule was dissymmetry (Gal, 2011). Later, the terms of “chiral” and “chirality” that was inspired from Greek word “cheir” which means “hand” were coined by Lord Kelvin in 1884 and being used until today as stated in a literature entitled “A History of Chirality” (Mauskopf, 2006). Finally, the other important terms for chirality (e.g., enantiomer, enantioselective, enantioseparation, etc.,) was introduced by Carl Friedrich Naumann in 1856 (Gal, 2007).

Chirality is referring to a molecule that has at least one carbon attaches to four different groups, therefore it will form two mirror image isomers that are non-superimposable to each other (Eliel & Wilen, 2008). This pair of isomer is known as enantiomers or optical isomers because of their difference in the direction of rotation in plane-polarized light. The optical isomer that rotates plane-polarized light in the clockwise direction is known as dextrorotatory (*D*) or (+)-enantiomer while antipode (opposite enantiomer) is levorotatory (*L*) or (-)-enantiomer. The combination of 1:1 of

two enantiomers is called as racemic. Another important nomenclature for chiral compounds also been applied according to Cahn-Ingold-Prelog priority rules (CIP), which also known as R/S system. The R- and S- labels are defined through the priority of each substituent based on the atomic number (Fujita, 2014). The chirality is also affect the property of peptides, protein, and polysaccharides as they are exist in the form of *D*- and *L*- configuration. For instance, natural sugars are presence in *D*- while amino acids are in *L*- configurations.

The fact that 50% of compounds in nature are chiral attracts the researchers to deeply investigate their properties (Hutt, 2002). This is because, many findings in pharmaceutical, drugs, and agrochemicals analyses indicate that the biological activity of each enantiomer are stereoselective. For pharmaceuticals and drugs, the first possibility is one enantiomer usually shows higher pharmacological activity or more potent while the other is less active, inactive or toxic. The metabolism rates, pharmacodynamic and pharmacokinetic processes are also different among enantiomers (Rentsch, 2002). Secondly, some enantiomers are having the almost equal pharmacological activities (Kim *et al.*, 2002). Thirdly, the composition of enantiomers is also different qualitatively and quantitatively.

In agriculture, chiral pesticides interact enantioselectively with the insects and weeds as they have different bioactivity. The active enantiomer of pesticide might possess desired effect on the target species, however the other might be inactive or harm the non-target species such as humans and non-target animals (Garrison, 2006). As the microbial degradation of pesticides is also dissimilar among the enantiomers, the accumulation of pesticides into the soils and underground water will give a long term effects to the environment and other organisms (de Albuquerque *et al.*, 2018;

Gámiz *et al.*, 2016). Therefore, the authority in the Netherland and Switzerland has issued the registration of optically active pesticides compared to mixture. In Sweden, Norway, Denmark and France, the tax has been implemented to reduce the use of pesticide and to encourage the use of optically active pesticides only (Böcker & Finger, 2016; Ye *et al.*, 2010). Hence, stereochemistry is important to be considered when dealing with drugs, pharmaceutical, agriculture products and optically pure active compounds are recommended to be commercialized at all time.

2.2 Development of chiral separation techniques

Chiral separation of enantiomeric mixture or enantioseparation is practically easy rather than synthesizing pure enantiomers. There are two types of chiral separation or chiral purification techniques such as non-chromatographic and chromatographic (Stalcup, 2010). The non-chromatographic methods consist of membrane separation and crystallization. Using membrane separation, the enantiomers have been transported across the enantiomer-selective liquid or solid membrane and the optical active compounds are collected as product (Hadik *et al.*, 2005). The crystallization method is divided into two types; diastereomeric crystallization and direct crystallization. For diastereomeric crystallization, pure chiral reagent is added to form diastereomers like the following illustration mechanism, $A_{DL} + B_D \rightarrow A_D B_D + A_L B_D$, in which A_{DL} is the racemates, B_D is the pure active compound that is used as chiral resolving agent while $A_D B_D$ and $A_L B_D$ are the diastereomers. As they are not in the form of chiral anymore, these diastereomers are separated according to their different properties such as melting point and molecular or crystal structure (Pham *et al.*, 2009). Meanwhile, the direct crystallization is done by selectively seeding with crystal of preferred enantiomers in supersaturated solution.

The cooling and heating processes are repeated several times to generate the formation of pure crystals. Recently, there is also the report for hybrid method which combine the membrane separation and crystallization (Svang-Ariyaskul *et al.*, 2009, 2012).

The enantioseparation by chromatographic technique can be done by using indirect or direct methods. Through indirect method, chiral derivatizing reagent (CDR) is added to convert the enantiomer into diastereomer before undergo normal achiral separation method (Ilisz *et al.*, 2012). The common CDRs that are commercially available are α -methoxy- α -(trifluoromethyl)phenyl acetic acid, R-(+)- or S-(-)-1-phenylethylisocyanate, L-(-)-N-trifluoroacetyl prolyl chloride and (-)-champhanic acid chloride (Weiß *et al.*, 2015). In order to ensure the diastereomers are successfully formed without any contaminants, few things should be considered such as the purity and concentration of CDRs, temperature, and reaction time. This indirect method is applicable to many chiral compounds such as chiral compounds with functional groups of amines, alkanolamines and hydroxyl compounds.

The chiral separation can also be achieved through direct method by applying chiral selectors throughout the analysis. Chiral selector is the chiral compound that enable to interact with the enantiomers stereoselectively, therefore they can be discriminated by separation system. Before this, chiral selector is just being added into the mobile phase as chiral additive. Even though the method is simple and the result is good, but it is impractical because it cannot be reused and cost-intensive with expensive reagents . Therefore, it is more convenient for the chiral selector to incorporated either in stationary phase as chiral stationary phases (CSPs) in chromatographic instruments such as gas chromatography (GC), supercritical fluid chromatography (SFC) and high performance liquid chromatography (HPLC). In

CSPs, the chiral selector is adsorbed or chemically bonded to the stationary phase. The discrimination happens when the enantiomer interacts selectively with the CSP. The enantiomer that attaches firmly to the CSP will move slowly through the detector compared to the other one. The example of CSPs are polysaccharides-based CSPs, macrocyclic antibiotic-based CSPs, pirkle-type CSPs, molecularly-imprinted, cyclofructans, synthetic polymers, and CD-based CSPs (Teixeira *et al.*, 2019; Xie & Yuan, 2019). Yet, there is no universal CSPs that enable to separate all types of chiral compounds. Therefore, until now, many researches have been developed towards better CSPs. On the other sides, the use of capillary electrophoresis (CE) has become popular among the researchers.

2.3 Capillary electrophoresis as chiral separation technique

CE has achieved great attention among researchers as the alternative to HPLC because it is simple, flexible and versatile. In addition, as compared with the parabolic flow in HPLC, the introduction of “plug flow” in CE, which means the velocity of liquid is nearly uniform within the narrow capillary minimizes the broadening of bands and promises sharp peak (Scriba, 2013). Therefore, it results to high efficiency and high resolution of peaks. The use of CE has been widely used for many application and many review papers have been published about various CE techniques since 90’s (Yu & Quirino, 2019).

The basic principle of separation in CE is based on the difference in mass-to-charge ratio as illustrated in **Figure 2.1**. The molecular weight of compound follows the relationship of force, (F) in which mass (m) is opposed the acceleration (a) of the particles ($F = ma$). Meanwhile, the analyte is a charged ions if it has one or more charged functional groups or it has to covalently formed with any of background

electrolyte components such as chiral selector, additive or micelles (Sanger–van de Griend *et al.*, 2013). The ions are moving across the narrow capillary with the influence of electric charge. Without the presence of electric field, the charged molecules are freely and randomly move in the buffer. When the numbers of positively and negatively charged are the same, they will cancel each other and create the neutral molecules. After applying the electric field, they are starting to move, in which the cations (positively charged ions) move to the cathode (negatively charged electrode) while the anions (negatively charged ions) move to the anode (positively charged electrode) (Whatley, 2001). Besides that, bigger charge is also enhance the velocity of ions compared to smaller charge. This type of movement is known as electrophoretic flow (EPF).

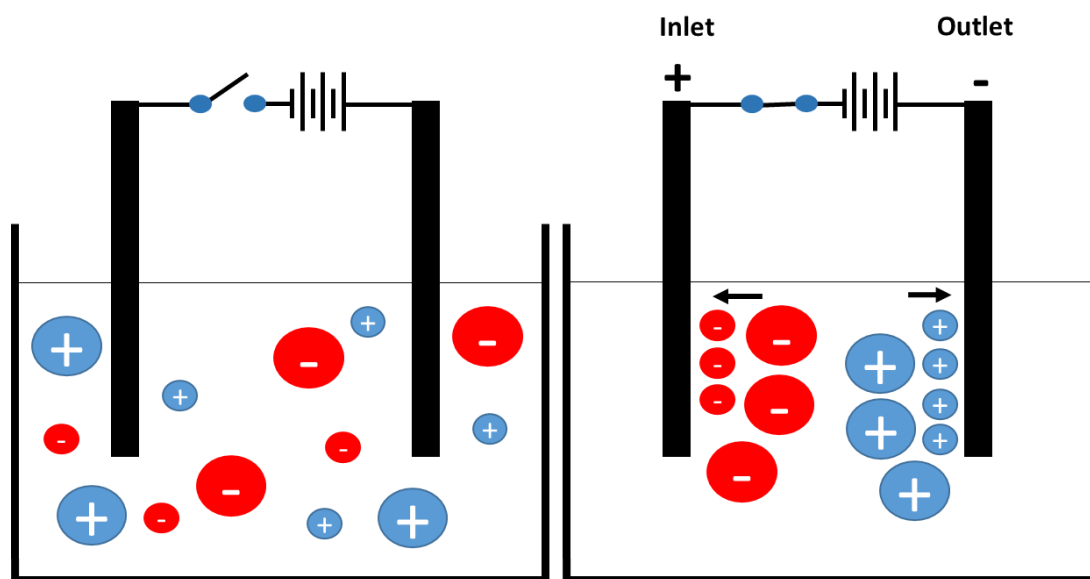


Figure 2.1 Basic principle of capillary electrophoresis method.

Besides EPF, another factor to allow the separation is electroosmotic flow (EOF). EOF means the flow of buffer fluid near the charged surface due to the presence of electrical field. This EOF is consistently flow from anode (+ve electrode) to cathode

(-ve electrode) and the migration velocity is depends on few factors such as concentration of buffer, temperature, voltage, and pH of buffer. However, as the EOF is always greater than EPF, it causes the movement of all compounds, regardless of charge to flow in the same direction but with different velocity. Compared to all, cation tends to migrate the fastest since the EOF and EPF are in the same direction, while neutral compounds are carried at the same velocity as EOF without being separated to each other. The net movement of these charged compounds are illustrated in **Figure 2.2**.

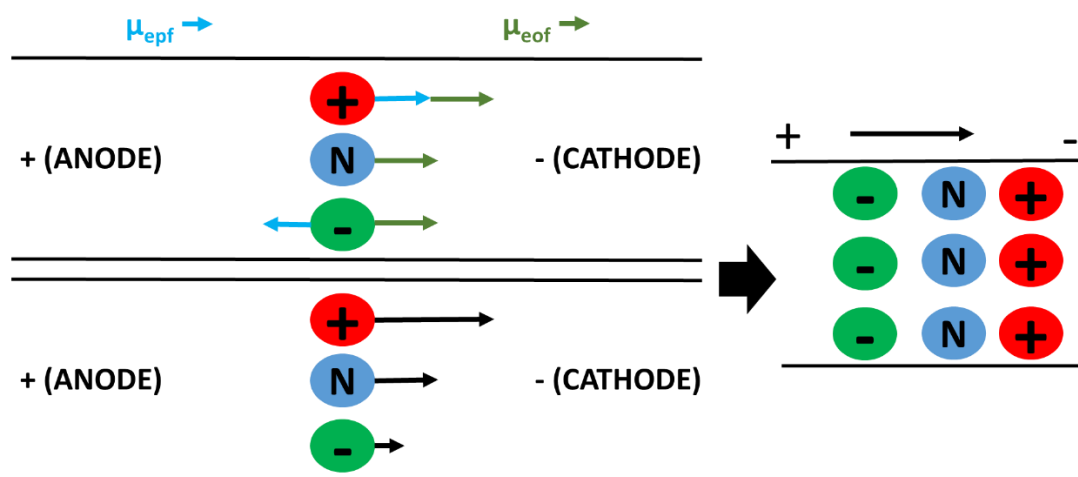


Figure 2.2 Pictorial representation of combined effect of both electrophoretic (EPF) and electroosmotic (EOF) flows on the migration of molecules.

In chiral separation, the simplest mode of CE is CZE as it is based on mass-to-charge ratios of the analytes across the open capillary under low viscosity buffer systems. However, as the enantiomers have the same mass-to-charge ratios to each other, the normal separation is almost impossible. Hence, chiral selector is added into the background electrolyte (BGE) to selectively and rapidly form complex with the enantiomers and carry them to the detector. The apparent mobility is hence depends

on the binding strength of the complex and the mobility of the enantiomer-selector complex (Yu & Quirino, 2019). Besides that, in order to allow the separation, there are three conditions required such as firstly, the enantiomer or the chiral selector needs to be in charged form, secondly the enantiomers must have different affinity for the chiral selector and thirdly, the each enantiomer-selector must have different apparent mobility. Therefore, the selection of chiral selector is important to ensure a success separation. There are many types of chiral selectors have been explored such as crown ethers, carbohydrates, proteins, macro-cyclic antibiotics, ligand-exchange, and chiral ion-pairing reagent. Among all, cyclodextrins (CDs) are the most common one.

Different chiral selectors have different mechanisms towards different types of targeted analytes as reported in some reviews (D'Orazio *et al.*, 2019; Yu & Quirino, 2019). For example, the crown ethers is preferred to compounds containing amino groups such as amino acids and dipeptides as they stereoselectively form inclusion complex with primary amines (Adhikari & Lee, 2018; Lee *et al.*, 2019). Besides that, proteins and carbohydrates are potential to be chiral selectors as they are exhibit as neutral and charged compounds (Chen *et al.*, 2005). Proteins have the ability to negatively and positively charged based on the pH of buffer (Gübitz & Schmid, 2008). In addition, the macrocyclic antibiotics have assymmetrical centres and multiples functional groups to interact with the enantiomers either inclusion complex or ionic interactions such as hydrogen bonds, $\pi - \pi$, and dipole-dipole interactions (Aboul-Enein & Ali, 2000; Ward & Rabai, 2004). Chiral selectors such as ligand exchange is as the chiral selector ligand with metals such as Cu(II), Ni(II), and Zn(II) as central ions interact to form diastereomers mixed with metal complexes (Hu *et al.*, 2019), while chiral ion-pairing reagents are practically applied for non-aqueous medium

(Hödl *et al.*, 2007). Hence, the selection of chiral selector becomes to be one of the important steps towards a successful resolution of chiral compounds.

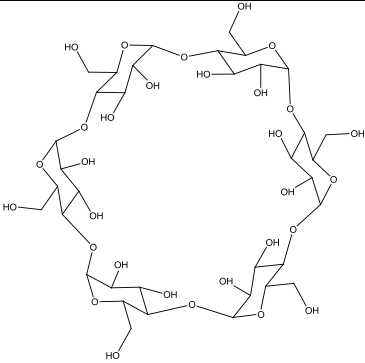
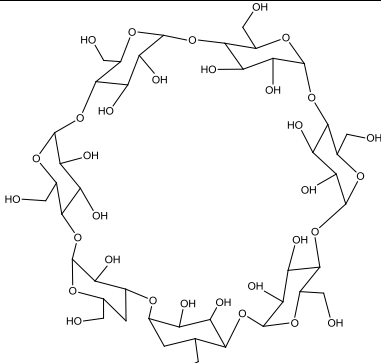
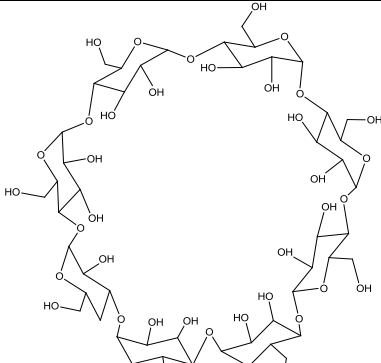
Despite of high resolving power, the CZE method is only available for ionic or charged compounds since the separation is based on the electrophoretic mobility. Therefore, an innovation has been proposed by Terabe and co-workers in 1984 by introducing a method known as electrokinetic chromatography (EKC) method, in which it is the hybrid of CE and chromatographic separation principle (Palmer, 2015). In this method, the ionic pseudo-stationary phase has been introduced into the BGE to separate the neutral analytes. Some pseudostationary phases are microparticles, microemulsions, micelles, and macromolecules (Pyell, 2018; Šlampová *et al.*, 2019). The addition of micellar solutions of ionic surfactants has become the most popular method and it is named as micellar EKC (MEKC). Surfactants are amphiphilic molecules and above the critical micelle concentration (CMC), micelles are formed. The enantioseparation is achieved based on the interaction of enantiomers with micelles at the pseudostationary phase inside the buffer (Ghiasvand *et al.*, 2018). The examples of surfactants are sodium dodecyl sulphate (SDS), bile salts, saponines, polymeric amino acids and dipeptide based surfactants.

2.4 Application of cyclodextrins in chiral separations

The discovery of cyclodextrins (CDs) in science field was recorded by Villiers in 1891. The CDs are produced from the treatment of starch with amylase by bacterial enzymes, *Bacillus macerans* (Kurkov & Loftsson, 2013). CDs are natural cyclic truncated cone-shaped oligosaccharides consisted of at least six glucose unit joined through α -1, 4 linkages (Del Valle, 2004). CDs are commonly known based on the glucose units, in which α -CD, β -CD and γ -CD containing six, seven, and eight glucose

units, respectively with different cavity size (cavity size: α -CD < β -CD < γ -CD) as shown in **Table 2.1**. Besides that, CDs also have hydrophobic inner cavity and hydrophilic outer surface in which the larger and smaller opening of the cavity are lined with secondary and primary hydroxyls, respectively. These allow a number of possible interactions might be present between the inner and outer surfaces with the enantiomers (Loftsson & Duchene, 2007). Other than that, several factors which attract the researchers to use CDs are because of their stability through out the pH, thermally stable and they also do not absorb UV or visible light.

Table 2.1 The physico-chemical description of α -, β -, and γ -cyclodextrins, respectively.

Cyclodextrin types	α	β	γ
Molecular structure			
Number of glucose units	6	7	8
Molecular weight (g mol^{-1})	973	1135	1297
Inner diameter of cavity (nm)	0.47 – 0.52	0.60 – 0.64	0.75 – 0.83
Outer diameter of cavity (nm)	1.46	1.54	1.75
Solubility in water at 25 °C [%] (w/v)	14.5	1.8	23.2

Starting 1970s, the CDs are applied in various fields including food and cosmetic industries (Bilensoy, 2011; Feng *et al.*, 2018). In food industries, CDs was used as stabilizer for flavoring agents and to reduce unpleasant odor and taste (Madene *et al.*, 2006). While in cosmetic industries, CDs acted as stabilizer for chemical labile compounds, to obtain prolong action, to decrease local irritation, and to reduce unpleasant odor (Buschmann & Schollmeyer, 2002). In 1990s, the US based company had launched cyclodextrin-based softener for fabric applications (Chauhan *et al.*, 2018). After that, the application of CDs have been widely explored in pharmaceutical industry as solubilizer and stabilizer (Maazaoui & Abderrahim, 2015; Panda *et al.*, 2016). Apart from that, worldwide, 30 – 40 different drugs had been marketed in the form of cyclodextrin complexes.

In separation sciences, CDs have been popularly used especially in chiral separation (De Boer *et al.*, 2000). The first application of CDs in separation was reported in 1959 as they were introduced as crystallization agents for separation of racemates. Based on their good performance, CDs were used as mobile phase additive in thin-layer chromatography (TLC), chiral selectors in CE (Guo *et al.*, 2003), modifier (Noorashikin *et al.*, 2014), molecular imprinted polymer (MIP) (Surikumaran *et al.*, 2015), act as pseudostationary phase in CD-MEKC and covalently or chemically bonded with stationary phases as CSPs in HPLC, GC, and SFC (De Klerck *et al.*, 2012; Xiao *et al.*, 2012).

Among all CDs, β -CD is the most popular since it is easily used, cheap and can interact with wide range of compounds. Even though γ -CD possesses the largest cavity, the presence of odd number of glucose rings decreases the symmetry of the cavity and hence, increase the resolving power of β -CD. The interesting, unique features of β -CD

is that, its molecule has 35 chiral centers, therefore chiral discrimination can be achieved via the interaction between chiral β -CD and enantiomers. In the inner hydrophobic cavity, the enantiomer can interact well with β -CD via hydrophobic interactions such as host-guest or inclusion complex and Van der Waals force. While, at the exterior of the cavity, β -CD has C_7 symmetry cavity and 14 hydroxyl groups which suitable to attract with the polar substituents of enantiomers via hydrophilic interaction or hydrogen bonding as shown in **Figure 2.3**.

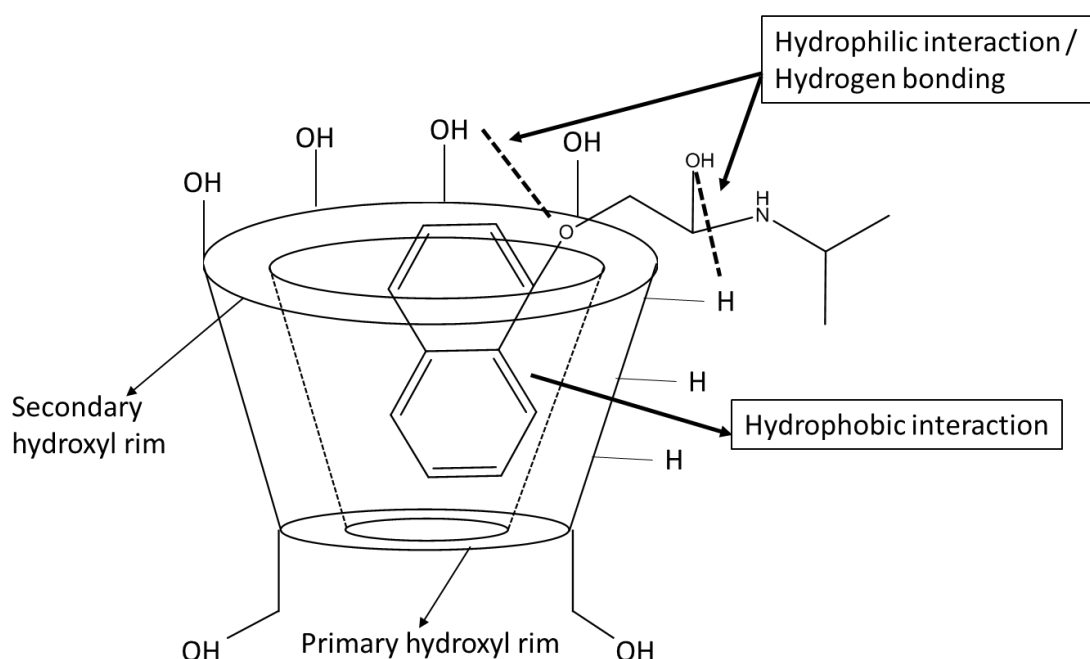


Figure 2.3 Illustration of interactions between CD and enantiomers.

However, the enantioseparation of enantiomers using native β -CD are usually unsatisfied because the binding cavity of native β -CD is found to be too symmetrical to induce large electroselectivities. Moreover, the solubility of native β -CD is also very low compared to other CDs as shown in **Table 2.1**. This solubility issue has been related with the ability of the CDs to interact with the enantiomers through their cavities. Therefore, more works have been developed by adding functional groups

such as alkyl, acetyl benzoyl, hydroxypropyl, phenylcarbamoyl and many more to modify the exterior surface and the depth of cavity of the β -CD (Gübitz & Schmid, 2000; Van Eeckhaut & Michotte, 2006). The functionalized β -CDs are not only improve the solubility and the binding constant of inclusion complex, it also allows the added groups to interact with the analyte via ionic interactions such as hydrogen bonds, $\pi - \pi$, and dipole-dipole interactions (Guo *et al.*, 2016; Zhou *et al.*, 2015).

The use of different substituents vary the enantioselectivity of the modified β -CDs. The modified β -CDs can be formed in neutral, cationic and anionic forms (De Boer *et al.*, 2000). The common substituents for uncharged β -CDs are hydroxybutyl- β -CD (HB- β -CD), 2-O-acetonyl-2-O-hydroxypropyl- β -CD (2-AHP- β -CD), heptakis-O-methyl- β -CD (M- β -CD), heptakis (2,6-di-O-methyl)- β -CD (DM- β -CD), and heptakis (2,3,6-tri-O-methyl)- β -CD (TM- β -CD) (Saz & Marina, 2016). The methylation of the hydroxyl groups has enlarged the size of cavity therefore, it enhances the steric interactions. However, the efficiency of stereoselectivity is also weakened due to the reducing of hydrogen bonding sites. Other than that, the uncharged β -CDs cannot separate uncharged molecule because as they flow as the same as EOF, no separation can be obtained. Hence, the substituents like amino groups based for cationic such as 6A-methylamino- β -CDs, mono (6-amino-6-deoxy)- β -CD, and heptamethylamino- β -CD are suitable for hydroxy acid derivatives (Muderawan *et al.*, 2005; Tang *et al.*, 2005; Tang & Ng, 2008). While, ethyl or phenyl carbamoylated such as carboxyethyl- β -CD (CE- β -CD), carboxymethyl- β -CD (CM- β -CD), sulfobutyl- β -CD (SBE- β -CD) and sulfoethyl- β -CD (SEE- β -CD) for anionic β -CDs are widely used for as various binding sites and the ionic substituents enable them to form many interactions with enantiomers (Zhu & Scriba, 2016). Interestingly, the charged CDs

can act as pseudostationary phases and their enantioseparation is using different electrophoretic mobilities.

Moreover, the study of host-guest complexation or inclusion complex mechanism has achieved great interest among the researchers. This is because, the formation of inclusion complex is one of the factor to improve the resolution of chiral compounds and the solubility of pharmaceutical products. However, it is still a challenge for researchers to really understand this because the interaction between CDs and analytes are uniquely different among the types of CDs, the substituents that being attached to modified CDs, and the medium in which the complexes are formed (Chankvetadze *et al.*, 2012). For example, the affinity patterns of enantiomers of norephedrine to native α - and β -CDs are opposite to each other (Lomsadze *et al.*, 2012), and the same phenomenon occurs for enantiomers of ephedrine (Vega *et al.*, 2011). Therefore, many approaches have been done until now using different instruments such as differential scanning calorimetry (Fernandes *et al.*, 2002), UV-vis spectrophotometer (Wang *et al.*, 2007), fourier transform infrared spectroscopy (FTIR) (Crupi *et al.*, 2007), X-ray diffractometry , scanning electron microscopy (SEM) and nuclear magnetic resonance spectroscopy (NMR) (Rahim *et al.*, 2018).

2.4.1 HP- β -CD as selected chiral selector

Our selected chiral selector for this study is hydroxypropyl- β -CD (HP- β -CD) and its structure is shown in **Figure 2.3**. HP- β -CD is the hydroalkyl based β -CD which is chemically modified by treating the solution of β -CD with propylene oxide and thus, the hydroxyl groups at the exterior of β -CD are replaced with the alkyl groups. By modifying the β -CD with alkyl group not only improve the water solubility (> 600

mg/mL (w/v)), but also change the size and flexibility of the cavity. This modification helps to increase the stability of the complex formed. Besides that, this compound is not only possesses low toxicity, but it also has been applied in pharmaceutical industries as the alternative drug solubilizer compound to the native β -CD.

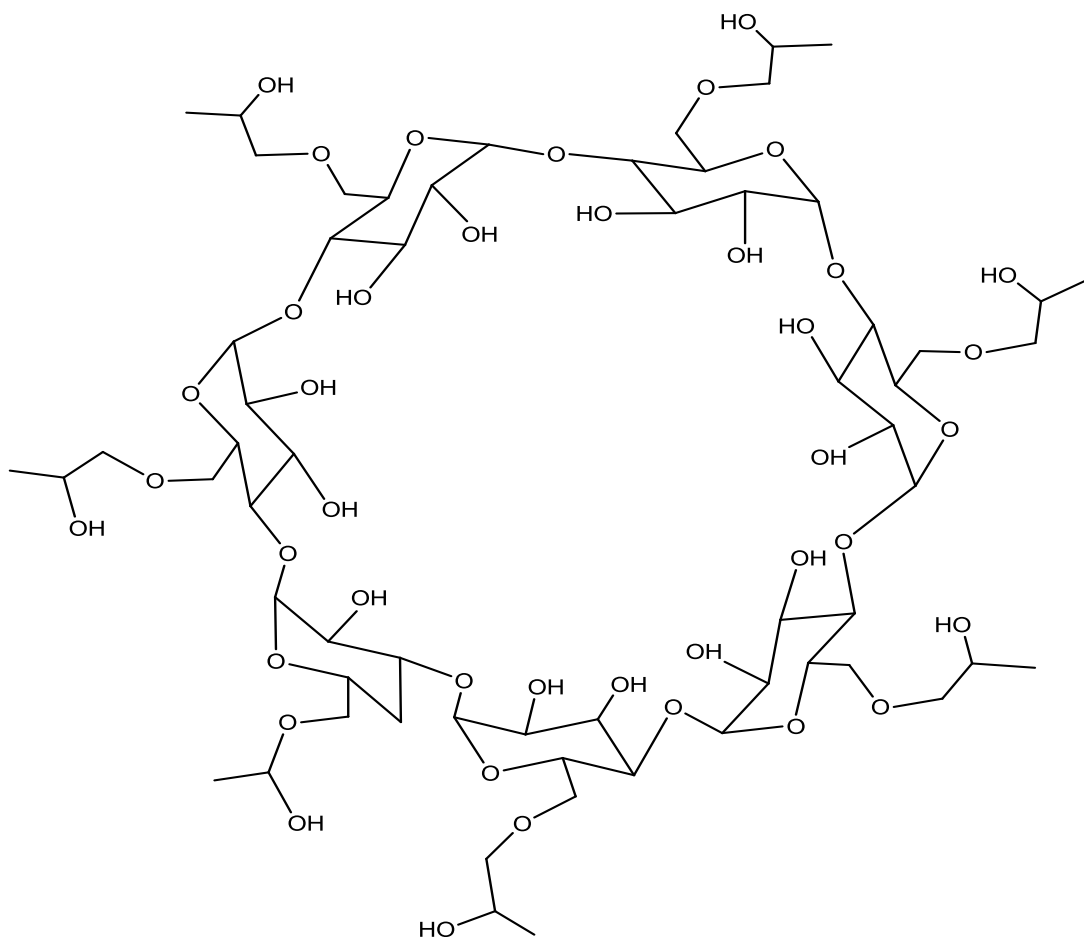


Figure 2.4 The structure of HP- β -CD.

In chiral separation, the HP- β -CD has been used as one of the most common neutral chiral selectors. It is an effective chiral selector in CE because the hydroxyl groups not only increase the depth of inclusion complex, but it also increases the number of hydrogen bonding sites which promote many potential interactions. As a neutral CD, it migrates at the velocity of the electroosmotic flow and the discrimination