DEVELOPMENT OF NOVEL COLUMNS FOR DETERMINATION OF VARIOUS ß-BLOCKERS BY CAPILLARY ELECTROCHROMATOGRAPHY

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ABSTRACT

DEVELOPMENT OF NOVEL COLUMNS FOR DETERMINATION OF VARIOUS ß-BLOCKERS BY CAPILLARY ELECTROCHROMATOGRAPHY

ß-blockers are one group of the most widely used drugs to treat heart failure, high blood pressure and abnormal heart rhythms. The purpose of this study was to develop capillary columns of stationary phases from molecular imprinting and sol-gel methodology for the determination of ß-blockers prior to capillary electrochromatography (CEC) analysis. In the first part of the study, molecularly imprinted organic based polymers (MIPs), silica-based molecularly imprinted polymers (SMIPs) and silica-based sorbents were developed, characterized and utilized for sorption of metoprolol, an example of ß-blockers. The three different types of sorbents were studied as stationary phases of capillary column. Critical parameters on sorption of metoprolol were investigated. In the second part, the subsequent studies were concentrated on the preparation new capillary column for separation and determination of metoprolol for CEC analysis.

The performance of three different types of sorbents was compared. Molecularly imprinted organic polymers show higher sorption and selectivity towards metoprolol as compared with the other sorbent types.

The preparation of stationary phases of capillary columns with molecular imprinted organic polymers was proposed for determination of metoprolol prior to CEC analysis. After stationary phase optimization studies, an open tubular column was prepared and its performance was examined.

ÖZET

ÇEŞİTLİ ß-BLOKERLERİN KAPİLER ELEKTROKROMATOGRAFİ İLE TAYİNİ

ß-bloker tipi ilaçlar konjestif kalp yetmezliği, hipertansiyon, anormal kalp ritmi gibi hastalıklarının tedavisinde en yaygın kullanılan ilaç gruplarındandır. Bu projede ß-bloker ilaç grubuna ait olan analitlerin kapiler elektrokromatografi (CEC) ile tayini için moleküler baskılama tekniği ve sol-gel metodu kullanılarak yeni kapiler kolon dolgu maddesi sentezi amaçlanmıştır. Çalışmanın ilk kısmında, moleküler baskılanmış organik bazlı polimer, silika bazlı moleküler baskılanmış polimer ve silika bazlı sorbentler hazırlanmış, karakterize edilmiş ve metoprolol'ün sorpsiyonu için kullanılmıştır. Sentezlenen üç farklı sorbent kapiler kolon dolgu malzemesi olarak çalışılmıştır. Metoprolol'ün sorpsiyonu için kritik parametreler araştırılmıştır. Çalışmanın ikinci kısmında, metopolol'ün kapiler electrokromatografi ile ayırımı ve tayini için yeni kolonlar geliştirilmesi üzerine yoğunlaşılmıştır.

Sentezlenen üç farklı sorbentin sorpsiyon kıyaslanmıştır. Moleküler baskılanmış organik polimerler metoprolol'e yüksek sorpsiyon kapasitesi ve seçicilik göstermiştir.

Metoprolol tayini için moleküler baskılanmış organik polimer durgun faz olarak seçilmiştir. Durgun faz optimizasyon çalışmalarından sonra açık tübümsü kapiler kolon geliştirilmiş ve kolonun performansı incelenmiştir.

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CHAPTER 1

INTRODUCTION

1.1. ß-adrenoceptor Blocking Agents (beta blockers)

ß-adrenoceptor blocking agents (ß-blockers) were reported to be discovered by Sir James Black in 1962 at the Imperial Chemical Industries in the United Kingdom (Wachter and Gilbert 2012). Propranolol was the first applied clinical drug in the family of ß-blockers and become prototype of whole family. Sir James Black won the Nobel Prize in 1988 for synthesizing propranolol (Gullestad et al. 1996). ß-blockers have been commonly used in cardiovascular medicine for reducing morbidity and mortality in patients. They have been used in a variety of cardiovascular diseases such as myocardial infarction, angina, hypertension, cardiac arrhythmia as well as in the control of anxiety, migraine and glaucoma (Cleophas and Kalmansohn 1995).

ß-adrenoceptors correlate the sympathetic nervous system and the cardiovascular system. They have essential role in the cardiac function. Three different types have been distinguished in different tissues, which are $ß_1$, $ß_2$ and $ß_3$ adrenoceptors. $ß_1$ receptors are mostly located in the hearth; $ß_2$ receptors are found in vascular smooth muscle; and, $ß_3$ receptors are located in mainly fat tissue (Ladage, Schwinger, and Brixius 2013). ß-blockers block action the of these receptors. The first generation of ß-blockers is non-selective. They block both $ß_1$ and $ß_2$ receptors. Second generation have higher affinity towards to $ß_1$-adrenoceptors. The most widely used $ß_1$-blockers are atenolol (ATE), metoprolol (MET), esmolol (ESM), nebivolol (NEV) and bisoprolol (BIS) (Tsuchiya and Mizogami 2013).

Norepinephrine and epinephrine are naturally occurring compounds which are found in human body. They are both harmful for cardiac functions (Bristow 2000). Norepinephrine acts like both stress hormone and neurotransmitter. It increases the heart rate and skeletal muscle contraction. Epinephrine, more commonly known as adrenaline, is released into the blood by adrenal medulla under the influence of fear and anger. It causes an increase in hearth rate and blood pressure. ß-blockers bind to $ß_1$-

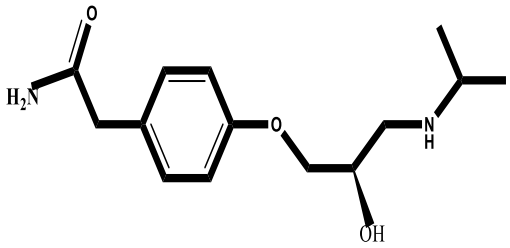
adrenoceptors in hearth and block the binding and action of the epinephrine and norepinephrine. Therefore the rate and force of cardiac contraction is reduced (Baker 2005).

As the blocking agent of norepinephrine and epinephrine, ß$_1$-blockers have been used in several olympic games such as snooker, darts, bowls, archery and shooting. ß$_1$-blockers are prohibited only in certain sports because of their anti-anxiety effect by World Anti-Doping Agency (WADA) (Fitch 2012). Therefore drug control has become crucial part of olympic sports.

1.2. ß$_1$-Cardio Selective Blockers

Metoprolol, atenolol and esmolol are all ß$_1$-adrenoceptor blocking agents of cardiovascular drugs. They inhibit the function of ß$_1$ adrenoceptor at heart. They consist of two structural features: an alkolamide side chain terminating in a secondary amine group and an aromatic group with various substituents.

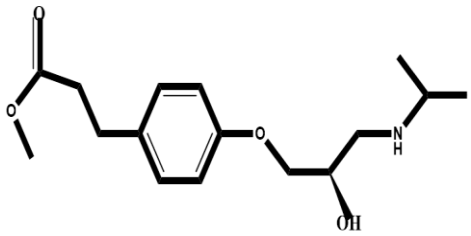
Table 1.1. Structures, molecular weights and pKa values of related ß$_1$ blockers



ATENOLOL

Mw: 266.34 gmol^{-1}

pKa: 9.6



METOPROLOL

Mw: 267.364 gmol^{-1}

pKa:9.5

1.2.1. Mix ß1-blockers in Water

Possible pollution of water from these compounds can occur via household wastes, hospital wastes and wastes from pharmaceutical production industries. Through these ways, drinking waters can be affected if any proper waste water treatment or drinking water treatment is not applied.

1.2.2. Diseases Caused by $ß_1$-Blockers

Hypertension or high blood pressure is a medical disorder in which many pregnant women suffer from its adverse effect. ß-blockers are commonly consumed during the first trimester of pregnancy because of its anti-hypertensive effects. Therefore they can affect adversely for humankind during pregnancy. The most crucial adverse effect is teratogenicity (the capability of producing fetal malformation) (Yakoob et al. 2013).

1.2.3. Determination of ß- Blockers

There are many analytical methods used for the determination and separation of ß-blockers in many samples. In some of these, commercial catridges were used in the preconcentration process prior to chromatographic determination. Gracia et al. (2017) developed an analytical method for the detection of 6 ß-blockers including metoprolol and their metabolites in human plasma. They used Lichrosper RP-4 ADS (Merck) column for pretreatment step before column-switching HPLC determination of ß-blockers. Metoprolol was found in a concentration of 59.3 μgL^{-1} in waste water samples. Another group developed molecularly imprinted extraction method for the enantiomeric separation of propranolol, metoprolol, atenolol and pindolol in natural water prior to HPLC-UV detection. The reported recoveries of ß-blockers were nearly 100.0% (Morante-Zarcero and Sierra 2012b). In another work, HPLC-DAD was used for the enantioselective detection of ß-blockers (propranolol, metoprolol, atenolol and pindolol). For the preconcentration of ß-blockers, molecularly imprinted polymer extraction process was used (Morante-Zarcero and Sierra 2012a). Yılmaz et al. (2009) determined the trace level metoprolol and atenolol in human plasma by GC-MS.

Metoprolol was extracted with a mixture of ethyl acetate and diethyl ether with liquid-liquid extraction. The analytical recovery was found as 91.20%. In another work, Brunelli et al. (2006) investigated two different class of doping agents namely ß-blockers and diuretics prior to GC-MS detection. OV-1701 commercial GC column showed better performance for metoprolol. Limit of detection for metoprolol in human plasma was found 50 μgL^{-1} at GC-MS analysis.

Wang et al. (2011) studied determination and separation of $ß_1$-blockers in biological sample. Capillary electrophoresis and electrochemiluminescence (ECL) detection was used for simultaneous determination of metoprolol, atenolol and esmolol in human urine. Recoveries were obtained in the range of 98.7-105 %. Alnajjar et al. (2013) studied chiral separations of five ß-blockers (propranolol, Esmolol, atenolol, metoprolol and bisoprolol) by capillary electrophoresis using six cyclodextrins as chiral selectors. Carboxymethylated-ß- cyclodextrin (CM-ß-CD) exhibited higher selectivity. (Zhou et al. 2008) described the separation of seven ß-blockers by CE-UV detection. They achieved the separation using titanium dioxide nanoparticles in buffer solution. The detection limit was found as 1.0 μgL^{-1} for both metoprolol and atenolol. This method was applied successfully for pharmaceutical tablets including ß-blockers.

1.3. Capillary Electrophoresis (CE)

Capillary electrophoresis is an analytical method that separates ions based on their electrophoretic mobility the use of applied voltage. The technique is used commonly because it gives faster results and provides high resolution separation.

1.3.1. Historical Background and Development of CE

Electrophoresis is a separation technique which is based on movement of ions by the application of an electric field. This separation technique was introduced by Tiselius in 1937. Tiselius was awarded a Nobel Prize thanks to for his research on electrophoresis of nature of complex serum proteins (Novotny 2017). In 1967, separation of proteins was achieved using a 3 mm-id capillary by Hijerten (Hjertén 1967). Afterwards, Virtanen demonstrated electrophoresis in smaller diameter

capillaries made from glass or Teflon in 1974. In the early 1980s, Jorgenson and Lukacs developed a CE method by using 75- µm id fused silica capillaries. They proved the high separation ability of capillary electrophoresis as an analytical method. (Jorgenson and Lukacs 1981). In that time, CE can be considered a method for using electrophoresis to separate cations, anions, and neutrals in a single analysis (Shamsi and Danielson 1994, Ericson et al. 1997).

1.3.2. Principles of Separation in CE

Capillary electrophoresis is carried out in narrow capillary column, typically 25 to 75 µm inner diameters. Use of narrow capillary column has several advantages. High voltage (10-30 kV) can be applied due to high electrical resistance of the capillary. High separation efficiency and short analysis time are achieved due to the usage of high electrical field. Very small -or even absence of- sample volume is consumed (1-10 nL). It is very effective method for the analysis of large and small molecules in size (Lukacs and Jorgenson 1985). One important feature of CE is the simple instrumentation during analysis. The basic instrumental set-up consist of a high voltage power supply, a fused silica (SiO_2) capillary with a protective layer of polyimide on the outside, two buffer reservoirs, two electrodes, and a detector (Figure 1.1). Briefly, the ends of fused silica capillary are placed in buffer reservoirs which are filled with background electrolyte (BGE). Reservoirs also have electrodes which are used to connect high voltage supply and fused silica capillary. Sample injection is achieved by replacing one of the buffer reservoirs with a sample vial. The analyte is injected hydrodynamically (by a pressure difference) or electrophoretically (by applying voltage) in capillary. Analyte is carried with background electrolyte from inlet to outlet reservoir. High electrical voltage is applied along the capillary column. Optical detection performed on-capillary walls.

Separation by electrophoresis is determined by two factors: electrophoretic mobility and electroosmotic flow (EOF). Electrophoretic mobility is the motion of solute in a capillary column under an electrical field. The velocity (v) of an ion in a capillary column can be given by Equation 1.1.

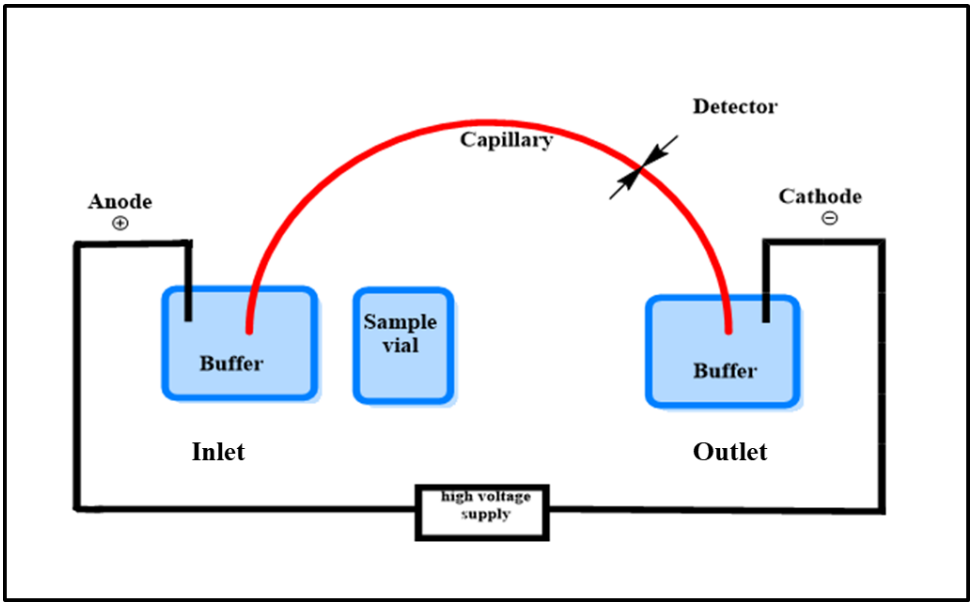


Figure 1.1. Instrumental set up for CE system.

Here v is ion velocity, μ_e is electrophoretic mobility and E is applied electrical field. According to the equation the mobility, for a given ion and medium, is constant which is characteristic of that ion. The electrophoretic mobility is proportional to electric force (F_E) and inversely proportional to frictional force (F_F) in medium, which is shown in Equation 1.2.

$$v = \mu_e \times E \quad (1.1)$$

$$\mu_e \alpha = F_E / F_F \quad (1.2)$$

The electric force and fractional force can be given by Equation 1.3 and 1.4, respectively.

$$F_E = q \times E \quad (1.3)$$

$$F_F = -6\pi\eta rv \quad (1.4)$$

Here q is ion charge, η is solution viscosity, r is radius of ion, v is ion velocity. During electrophoresis, electrical force and frictional force balance to form a steady state. At this point the forces equal but opposite.

$$qE = 6\pi\eta rv \quad (1.5)$$

When the obtained Equation (1.5) is substituted into Equation (1.1), an equation of mobility in terms of physical parameters is obtained as Equation (1.6).

$$\mu_e = q/6\pi\eta r \qquad (1.6)$$

According to final equation, for constant viscosity, separation depends on only q/r ratio. Therefore, small and highly charged particles have high mobililties whereas, large and low charged particles have low mobilities.

1.3.3. Electroosmotic Flow (EOF)

Electroosmotic flow (EOF) is a crucial constituent of CE operation. The EOF is generated by electric field which is applied on the electrolyte solution across the double layer of the capillary wall. The inner surface of the capillary column possesses silanol groups (SiOH) under aqueous conditions. At pHs above 3, these silanol groups are deprotaneted and anionic form (SiO^-) is formed (Slater, Tessier, and Kopecka 2010). The attraction of positive ions from buffer and negatively charged capillary wall form an electrical double layer and create a potential difference very close to the wall (Figure 1.2). This is known as zeta potential. When the voltage is applied across the capillary column, the hydrated cations migrate toward the cathode (Smith and Evans 1994).

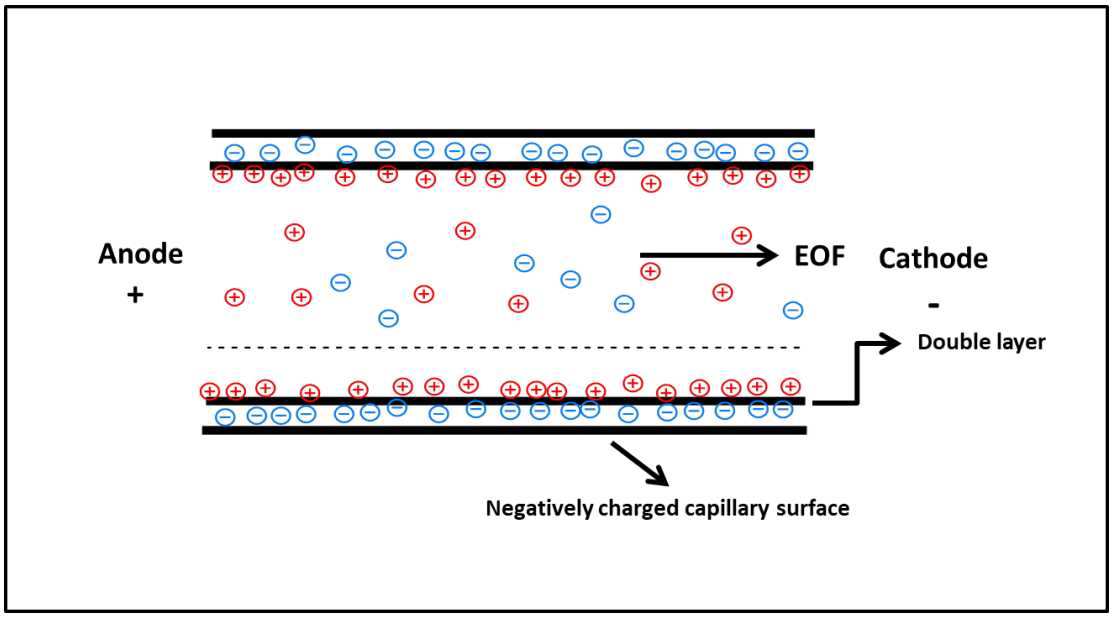


Figure 1.2. Formation of electroosmotic flow from inside the capillary column.

The magnitude of the EOF can by expressed in terms of velocity or mobility by the equations 1.7 and 1.8, respectively.

$$v_{EOF} = (\varepsilon\zeta/\eta)\, E \qquad (1.7)$$

$$\mu_{EOF} = (\varepsilon\zeta/\eta) \qquad (1.8)$$

Here, ε is the dielectric constant of the buffer solution, ζ is the zeta potential, η is the viscosity of buffer solution and E is the electric field over the capillary.

The zeta potential is determined by the surface charge on the capillary wall. The charge of the capillary wall depends on pH of the background electrolyte. Therefore magnitude of EOF varies with pH. At high pHs, the silanol groups are deprotonated and the EOF is greater than that of low pHs where silanol groups are protonated (Rathore 2002).

The most important feature of EOF is the flat profile of the flow in capillary, as depicted in Figure 1.3. There is no pressure drop within the capillary. In contrast to capillary electrophoresis, the flow profile of the mobile phase in is laminar liquid chromatography due to external pump. For this reason EOF does not directly contribute to peak broadening causing an increase in separation efficiency of CE. Therefore narrow band and sharp peaks are obtained (Figure 1.3.(a)). Laminar flow results in increased diffusion and band broadening in liquid chromatography (Figure 1.3.(b)) (Li et al. 1992).

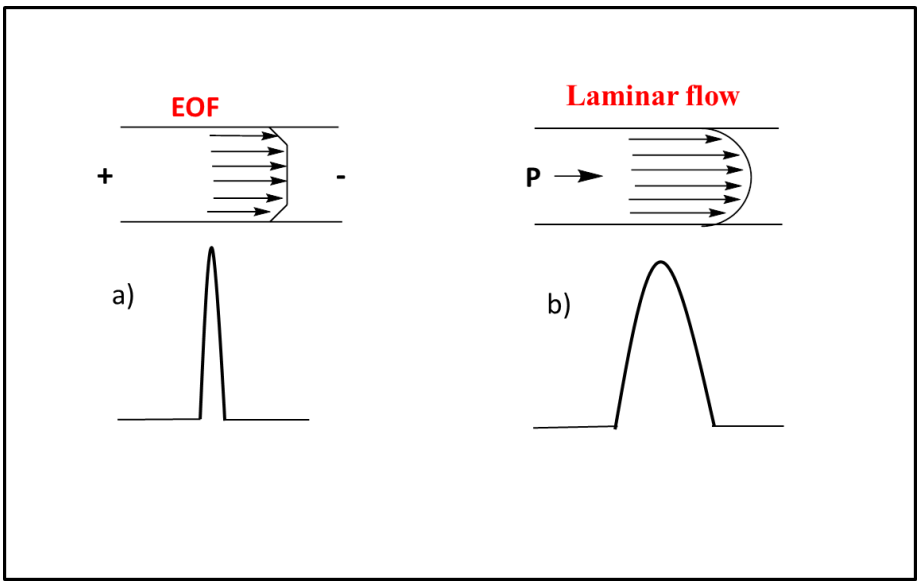


Figure 1.3. A comparison between EOF and laminar flow. (a) narrow and sharp peak obtained in CE, (b) broad (widened) peak obtained in LC

1.3.4. Modes of Capillary Electrophoresis

Capillary electrophoresis include lots of techniques that have quietly different separation characteristics. These modes are summarized briefly Figure 1.4. In this thesis, separation is achieved by using CEC method. Therefore this technique will be explained in more detailed in the proceding section.

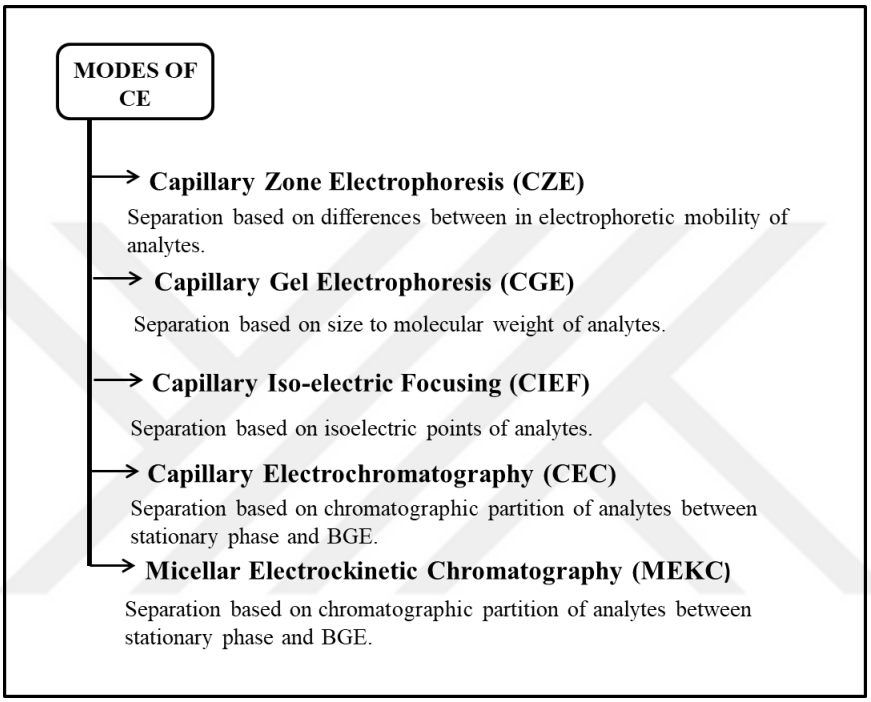


Figure 1.4. Classification of common capillary electrophoresis modes.

1.4. Capillary Electrochromatography (CEC)

Capillary electrochromatography is a hybrid separation technique consisting of combination of CE and HPLC. This technique combines the separation and selectivity potential of HPLC and the high efficiency of CE. In CEC, the capillary column is filled with a stationary phase and the liquid mobile phase is driven by electroosmosis instead of pressure to transport analytes through capillary column (Colon et al. 1997, Altria, Goodall, and Rogan 1994). The capillary columns of stationary phases in CEC technique are similar to other chromatographic techniques. Capillary columns and their

stationary phases are categorized into three main parts: open tubular, packed and monolithic capillary columns. Figure 1.5. represents the types of capillary columns.

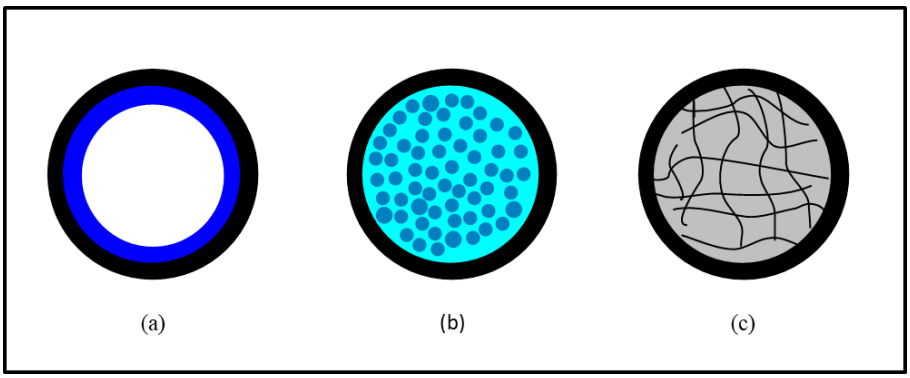


Figure 1.5. Capillary columns (a) open tubular (OT), (b) packed, (c) monolithic.

In open tubular columns (Figure 1.5 (a)), the inner wall of capillary is coated with the stationary phase. The OT capillary column does not need frit which is required in packed columns to hold packed particles inside the column, and it has not tendency to create bubbles. Formation of bubbles may cause an unstable baseline and disruption of the current. The major disadvantage of OT-CEC column is low surface to volume ratio of the coating. Stationary phase is coated only on the interior surface of column. Therefore interaction between analyte and stationary phase originates just on the capillary wall. There are many coating materials used as stationary phases including MIPs, organic inorganic hybrid materials, nanomaterials, natural and synthetic polymers. In some studies, organic-inorganic hybrid MIPs were synthesized as stationary phase of OT-CEC column (Chen et al. 2017). Wei et al. (2011) developed novel capillary column in order to separate racemic propranolol mixture. Under the optimized conditions, the propranolol enantiomers can be separated entirely by using OT-CEC column. In the other study, MIPs coatings were prepared for chiral separation of ketoprofen and naproxen. They prepare MIPs coating materials in capillary column. As a result, the chiral separation of ketoprofen and naproxen was achieved successfully.

Traditionally, packed columns (Figure 1.5 (b)) have commonly been used in CEC process due to the ease of preparation. In CEC, column filling materials contain several functional groups which should be ionic form to support EOF. Otherwise, the column cannot produce EOF sufficiently even if high voltage is applied. Because of this reason, mostly silica based materials are used as the packing material whose size can be

reduced up to 0.5 µm. Packed capillary column provides high sample capacity and reproducibility with high sensitivity. The most critical problem for this kind of capillary column is the formation of bubbles caused by frit which is used to prevent filling material to flow through the column. HPLC pump helps to push solution into capillary to prevent bubble formation.

In last decade, monolithic CEC monolithic (Figure 1.5(c)) columns have become popular in various applications. Monoliths are continuous porous structures as stationary phase throughout the capillary column. Monolithic materials fill the capillary uniformly and bonded chemically on the inner wall. Therefore monolithic columns do not require the use of outlet frits which lead to bubble formation (Tanret, Mangelings, and Heyden 2009). Preparation of monolithic CEC columns is classified into four categories: particle-fixed, silica-based, polymer-based and MIP-based polymers. The major advantage of polymeric monolithic columns is in situ preparation by using one-step polymerization procedure. The polymerization process starts from polymerization mixture and is applied directly in the capillary column. Wide variety of monomers (acrylate/methacrylate, styrene and acrylamide) is used to synthesize of stationary phases (Bedair and El Rassi 2004). Augustin et al. (2008) developed a new polyacrylate-based monolithic column via photo polymerization. The resulting column was employed for the separation of alkyl benzenes, basic drugs, anilines and peptides. This study shows promising result for separation of complex mixtures. Silica-based monolithic columns have wide variety of application areas compared to polymeric columns. In a study, silica based monolithic column was synthesized via sol-gel method. Silica based monoliths have good mechanical strength, high column efficiency, and stability towards organic solvents (Tanret, Mangelings, and Heyden 2009). Xie et al. (2005) examined electrochromatographic separation of 5 ß-blockers (alprenolol, propranolol, pindolol, metoprolol and atenolol) extracted from species used in Traditional Chinese medicine. The five ß-blockers were separated in CEC by using cation-exchange mechanism.

Nowadays, molecular imprinting has shown promising improvement as a method for the preparation of monolithic stationary phase. Molecular imprinting is a synthetic technique to prepare monolithic column where the polymer has specific binding sites against target analyte with high sensitivity (Wu et al. 2008, Hilder, Svec, and Frechet 2004). Imprinting sites are achieved by covalent or non-covalent interaction between monomer and template molecule during polymerization step. Template

molecule is removed from the polymer network. The resulting polymer has cavities that are specific to template (Qin et al. 2006). Xu et al. (2005) prepared an (S)-naproxen imprinted monolith by using thermal initiated polymerization. They investigated several parameters for the chiral selectivity. They concluded that the addition of organic modifiers in buffer solution improve the chiral separation. The purpose of another group was to achieve chiral separation of metoprolol and propranolol using molecularly imprinted monolithic columns prior to CEC analysis. The polymerization was performed under UV source in a capillary column. Chiral separation was achieved in a short analysis time (Schweitz, Andersson, and Nilsson 1997).

In this thesis, molecular imprinting and sol-gel method were used to prepare CEC columns.

1.5. Molecular Imprinting Technology

Receptors can recognize their complementary molecule and make complexes strongly. Large numbers of naturally occurring receptors are available for selective and sensitive recognition of the target analytes. Unfortunately, natural receptors are expensive molecules and decomposed easily. Due to these limitations, researchers have developed artificial receptors inspired by mechanism of enzyme-catalysis, lock and key model (Wackerlig and Schirhagl 2016). These polymeric artificial receptors are able to mimic biological receptors such as antibody and enzymes. Molecular imprinting is a promising approach to create polymeric artificial materials (Vasapollo et al. 2011). Molecularly imprinted polymers are low cost and robust materials which have high stability and activity during variety of conditions. Thanks to lots of advantages compared to natural receptors, MIPs are used in wide range of separation processes. (Chen et al. 2015).

Natural receptors are preferable compounds for treatment of biological samples and waste water. For industries, they are quite expensive and time consuming to fabricate. To date, MIPs are favorable synthetic approach for the separation and purification of ß-blockers from the biological and aquatic matrices. The ability of high affinity towards target analyte in matrix, MIPs have proven to be one of the most versatile materials for determination of ß-blockers.

During synthesis of MIP, firstly an interaction occurs between the analyte (template) and functional monomers (pre-polymerization step). The addition of excess amount of cross linking agent, rigid polymer network can be formed around the resulting template-monomer complexes. After polymerization step, the template is removed with a suitable solvent. Resulting cavities exhibit specific selectivity and affinity toward template molecule (Shahar, Tal, and Mandler 2016).

Figure 1.6 shows three fundamental steps of molecular imprinting process: pre-polymerization, polymerization and removal of template.

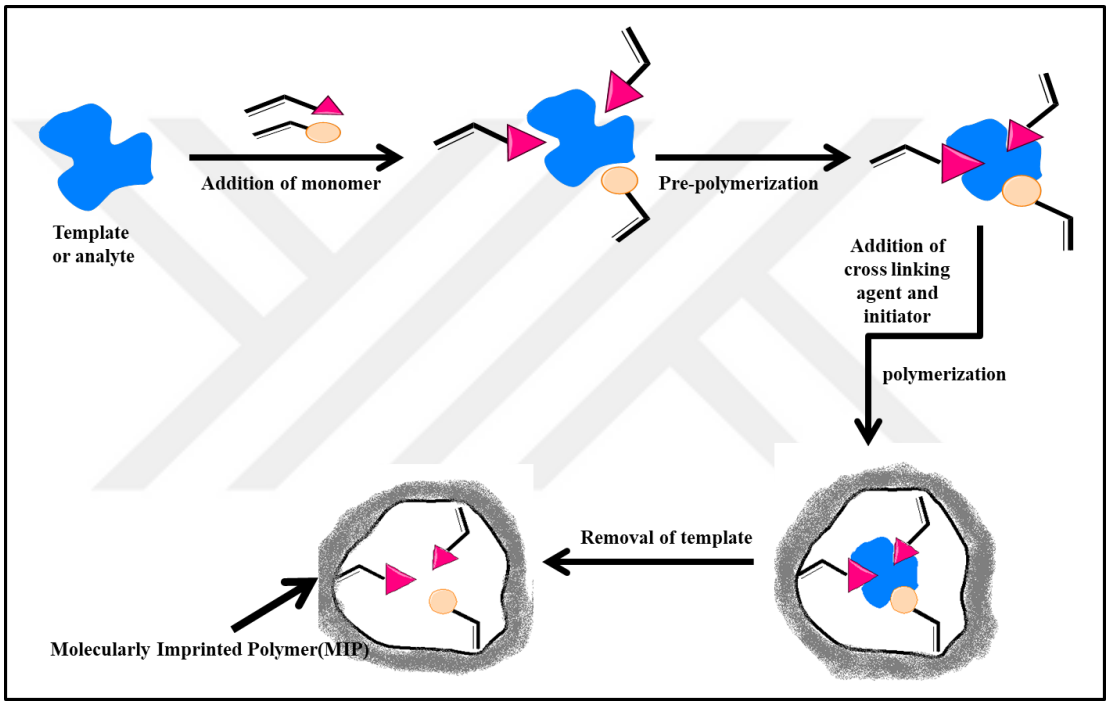


Figure 1.6. Schematic representation of molecular imprinting process.

In pre-polymerization step analyte and template connect each other via non-covalent interactions such as hydrophobic, hydrogen, ionic and wan der Waals interactions or covalent bonding. Non-covalent approach is the most preferably method due to its simplicity. Template is easily removed from non-covalent imprinted polymers (Yan and Row 2006).

Type of solvent is crucial in molecular imprinting process. Two types of solvents are available for polymerization: aprotic solvents (DCM, acetonitrile, and toluene) and polar protic solvents (methanol, ethanol and water). Polar protic solvents lead to form covalent bonding between template and monomer. In contrast, aprotic

solvents promote the possibility of hydrogen bonding during polymerization (Fontanals, Marcé, and Borrull 2010).

In the polymerization step, crosslinking agent is used to form three dimensional network around the template and monomer complexes and supply robustness to the polymer. Furthermore, they can control morphology of polymer matrix. Thus the ratio of crosslinking agent with respect to the total number of monomers and the solvent amount plays crucial role in the physical morphology of MIPs. Low crosslinking ratio produce gel-type polymer. These types of polymers have insufficient specific binding sites and mechanical stability. In contrast to this, when high amount of cross linking agent is used, macroporous type polymer is obtained. Macroporous polymers have more specific cavities and stability compare of gel-type polymer. Several initiators can be used as radical source for initiating radical polymerization. Molecular oxygen should be removed from the polymer matrix in order to prevent retardation of radical polymerization. Removal of free molecular oxygen can be achieved by degassing the polymer mixture with inert gases such as argon or nitrogen. In addition to these, high temperatures have a negative effect on the polymerization stability. Thus lower temperatures are preferred. Under these conditions, photochemically active initiators can be used for polymerization (Yan and Row 2006).

1.5.1. Preparation Methods of MIPs

Large number of methods can be used for preparation of MIPs; for instance, swelling, suspension, grafting, bulk and precipitation polymerization. Precipitation polymerization was preferred in this thesis. Precipitation polymerization is a one-step method to synthesize micro or nano-sized spherical particles. Consequently, in this procedure, crushing, grounding, sieving process is not applied for the resulting polymers (Cormack and Elorza 2004). To prove the specific cavities in MIP, another polymer is synthesized in the absence of template molecule. Therefore formation of specific binding sites is prevented. This polymer is called non-ımprinted polymer (NIP). The binding capacities of two polymers are compared.

1.6. Sol-gel Methodology

A sol is a dispersion of small solid particles (0.1-1µ) in a liquid where Brownian motion suspends the particles. In sol, solid particles may connect each other by Van der Waals forces and hydrogen bonding. A gel is a state where both liquid and solid are dispersed in each other, which presents a solid three dimensional network containing liquid components. The network is formed by agglomeration of solid particles. Gelation is due to the formation of covalent bonds and it is irreversible. Briefly, Sol-gel is a process in which solid particles dispersed in a liquid (a sol) and formed continuous three dimensional networks due to polycondensation reaction (a gel) (Danks, Hall, and Schnepp 2016). Sol-gel process may be utilized to prepare materials with several different shapes including porous structures, thin fibers, dense powders and thin films. Figure 1.7 shows a scheme of the different processing routes leading from the sol to variety of materials.

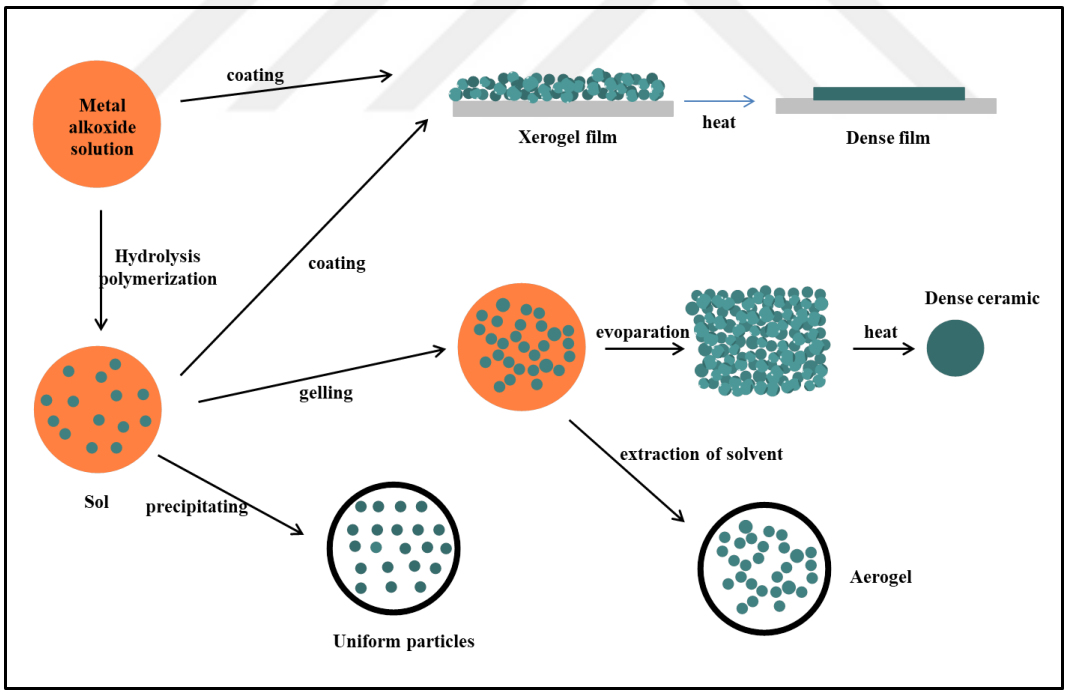


Figure 1.7. Sol-gel processing options.

A sol-gel process occurs in several steps; hydrolysis and condensation of precursors, gelation (sol-gel transformation), aging and drying. The most common

precursors are metal alkoxides. The first step in sol-gel process is hydrolysis. Synthesis of the sol is achieved by hydrolysis reaction of alkoxide ions with water in the presence of alcohol. Hydrolysis reactions can be catalyzed in either acidic or basic medium. The morphology of resulting gel is depending on catalyst type. Acid catalysis causes more extended and straight chain whereas base catalysis causes more compact and highly branched clusters. Immediately, condensation step leads to formation of gel. In drying process, the obtained gel is converted to xerogel or aerogel. Evaporation of the solvent leads to formation of xerogel. Solvent is extracted carefully under supercritical conditions in order to obtain aerogel form.

1.7. The Aim of This Study

The purpose of the study is to develop novel capillary columns of stationary phases for specific recognition of metoprolol prior to Capillary Electrochromatography. For this purpose, molecularly imprinted polymers (MIPs) and silica-based MIPs were prepared and investigated in terms of binding capacity. After preparation of capillary column, optimization parameters were examined such as pH, injection pressure, time and voltage. Prepared column was tried by spiking metoprolol in water samples.

CHAPTER 2

EXPERIMENTAL STUDY

2.1. Optimization of Instrumental Parameters

2.1.1. HPLC-DAD Parameters

For the HPLC-DAD optimization process, firstly 1000.0 mgL^{-1} of metoprolol solution was prepared in methanol. The stock solution was stored in an amber glass bottle at 4.0 °C in refrigerator. Standard samples and solutions were prepared daily with proper dilutions.

All analyses were performed with Agilent 1200 Series with Diode Array Detector. The optimization parameters were shown 2.1. The limit of detection (LOD) and limit of quantification (LOQ) were calculated by analyzing the least concentrated (0.25 mgL^{-1}) standard 20 times with HPLC-DAD.

Table 2.1. HPLC-DAD optimization parameters.

Column	Supelco C18 (Lichrosphere RP 18-5, 25 cm×4.6 mm) column
Mobile phase	85:15 methanol:water (acetic acid, pH:3.0)
Thermostat temperature	30 ºC
Sample injection volume	20.0 µL
Flow rate	0.9 $mLmin^{-1}$
Standard concentration	1.0 mgL^{-1}

2.1.2. CE-DAD Optimization Parameters

All analysis were perfomed with Agilent 7100 CE-DAD system. The studied parameters were given Table 2.2.

Table 2.2 CE-DAD optimization parameters

Electrolyte solution	25.0 mM borate buffer (NaOH pH, 9.5)
Column	Agilent technologies, FS, Undeactivated (75 µm id, 57 cm total capillary length, 50 cm effective length)
Thermostat temperature	25 °C
Injection pressure and time	50 mbar, 5 s
Voltage, Power, Current	20.0 kV, 6.0 W, 300 µA
Data collection rate	2.5 Hz

2.2. Synthesis of Molecularly Imprinted and Non-Imprinted Polymers (MIPs and NIPs)

To prepare stationary phase of capillary column for metoprolol, various solid phase extraction (SPE) sorbent were synthesized before HPLC-DAD CE-DAD and CEC-DAD analyses.

MIPs and NIPs should be synthesized under the same conditions. The synthesis of molecularly imprinted organic based polymers was achieved by precipitation polymerization method. Firstly, 0.5 mmol metoprolol (template), 4.0 mmol methacrylic acid (MAA, monomer) and 200.0 mL acetonitrile (ACN, porogen) were added into 250.0 mL amber reaction vessel and strirred 1.0 hour for pre-polymerization. After that, 20.0 mmol trimethylpropan trimethacrylate (TRIM, cross linker) added. 4,4'-azobis(4-cyanovalericacid) (AIVN, initiator) was used % 2 mole of all reactants except

metoprolol. The mixture was purged with nitrogen gas for 15 min to remove and dissolved oxygen polymerization was performed in oil bath at 60°C overnight. After polymerization process solid and rigid MIPs were obtained. Finally, to remove template molecule metoprolol, two different solvents (methanol and methanol:water (acetic acid pH:3) (85:15) mixture) were applied. After removal of metoprolol, MIPs and NIPs were dried in an oven at 60 °C and the sorbents was ready to experiments. The same experimental strategy was applied for NIP except the addition of metoprolol. The schematic representation is given in Figure 2.1.

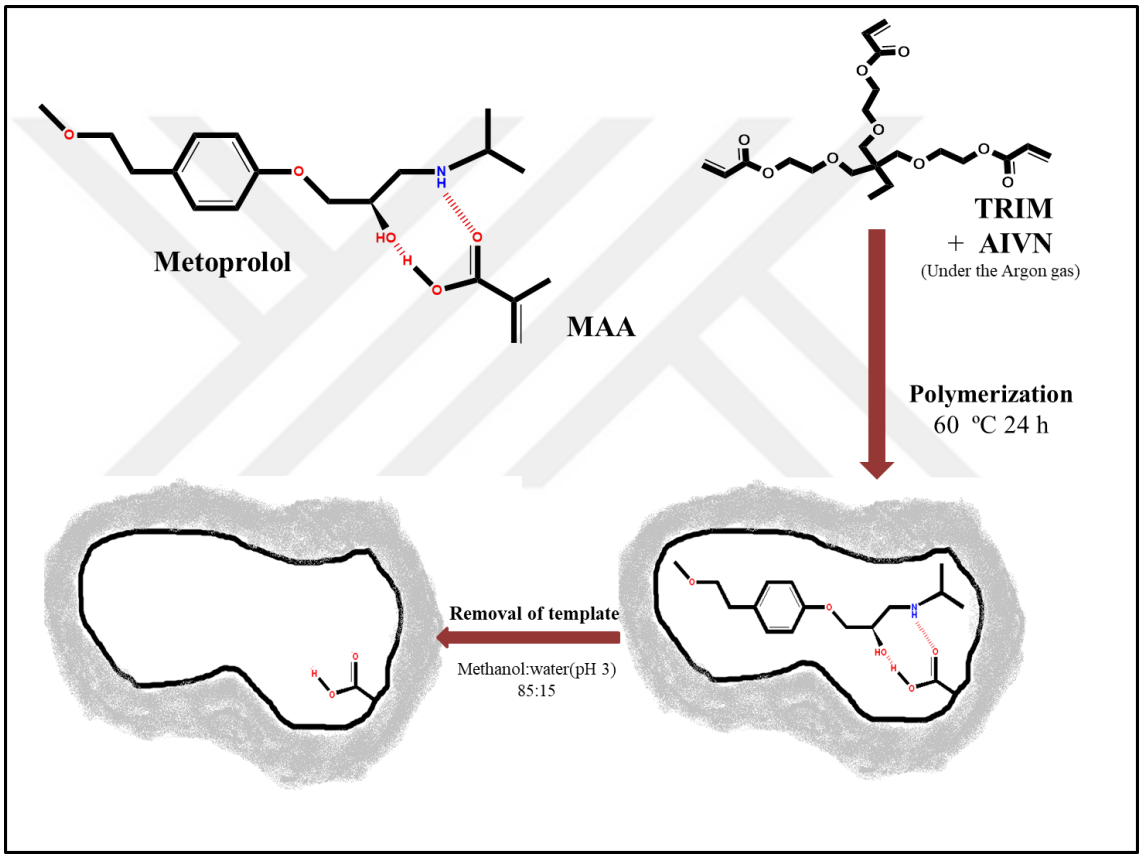


Figure 2.1. Scheme of the synthesis of MIP by copolymerization of MAA and TRIM.

MIP50 was synthesized as MIP200, but this time 50.0 mL acetonitrile was used instead of 200.0 mL. Identity, mole ratios of reagents and particle sizes of all synthesized polymers are given in Table 2.3.

Table 2.3. MIP and NIP compositions.

	identity	Metoprolol (mmol)	MAA (mmol)	TRIM (mmol)	AIVN (mmol)	ACN (mL)	Particle size (μm)
Molecularly imprinted monolith polymers	MIP50	0.5	4.0	20.0	0.48	50.0	NA
Molecularly non-imprinted monolith polymers	NIP50	0.0	4.0	20.0	0.48	50.0	NA
Molecularly imprinted microspherical polymers	MIP200	0.5	4.0	20.0	0.48	200.0	0.400
Molecularly non-imprinted microspherical polymers	NIP200	0.0	4.0	20.0	0.48	200.0	0.402

*NA: not applicable

In the second part of the study, silica based MIPs were synthesized. Firstly, 0.5 mmol metoprolol (template) was dissolved in a 70.0 mL tetrahydrofuran (THF) and water mixture. After, 4.0 mmol triethoxyphenylsilane (TPES) and 3-aminopropyltriethoxysilane (APTES) (functional monomers) were added in reaction medium, followed by the addition of, 16 mmol tetraethyl orthosilicate (TEOS) under constant conditions. After 30 min., 7.0 mL ammonia (25%, w/w) and 10.0 mL ethanol were added and allowed to mix overnight at room temperature. The obtained solid polymers were dried in oven at 60°C overnight. To remove the template molecule from the silica matrix, the polymers were washed with methanol-acetic acid (MeOH: HOAc, 9:1, v/v) mixture. Silica-based non-imprinted polymers (NIPs) were synthesized with the same strategy except addition of template molecule. The mechanism of synthesis of inorganic-based (MIPs) was showed in Figure 2.2.

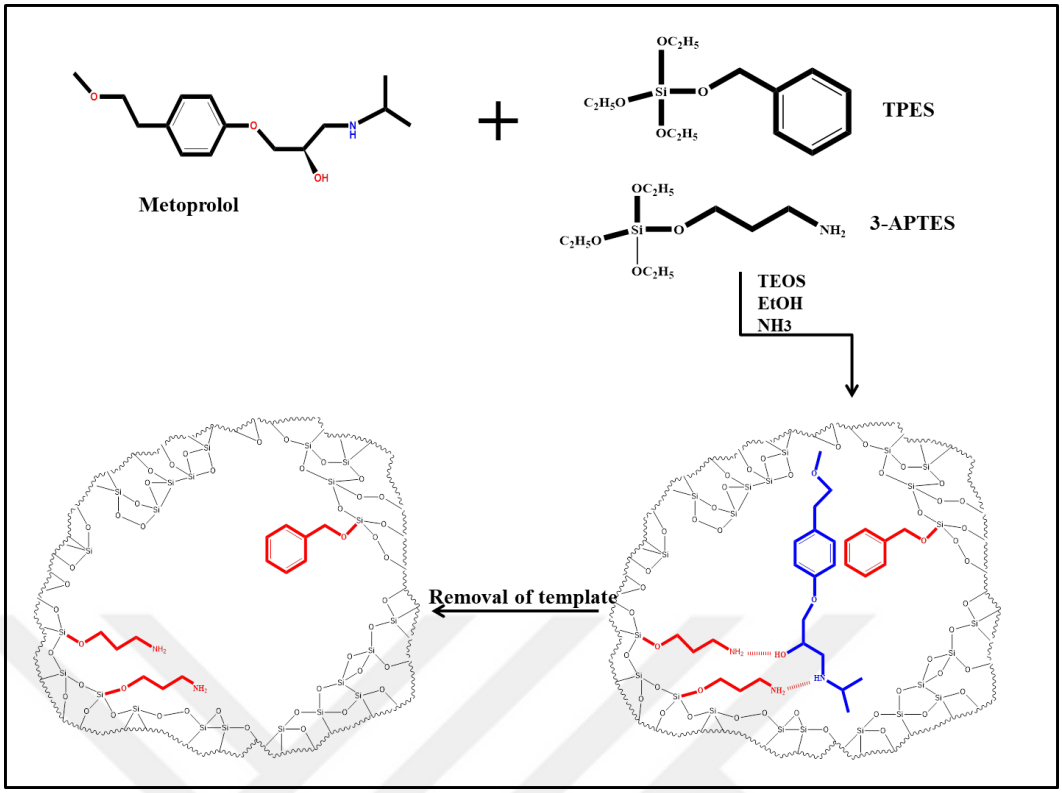


Figure 2.2. Scheme of the synthesis of inorganic-based MIPs.

2.3. Synthesis of Silica Particles

In this part, TEOS was added into 70 mL EtOH/water mixture to form alkoxide solution. After that, ammonia solution was added slowly as a catalyst to obtain gel form. This mixture was stirred overnight at room temperature. After centrifugation, silica sorbent was obtained. The mechanism of synthesis silica via sol-gel method is represented in Figure 2.3.

2.4. Characterization Experiments

Characterization experiments were performed to prove presence of specific cavities in MIPs by comparing sorption capacities of all sorbent types. After that, to understand the affinity of MIPs against metoprolol, the experiment was performed in the presence of NSAIDs.

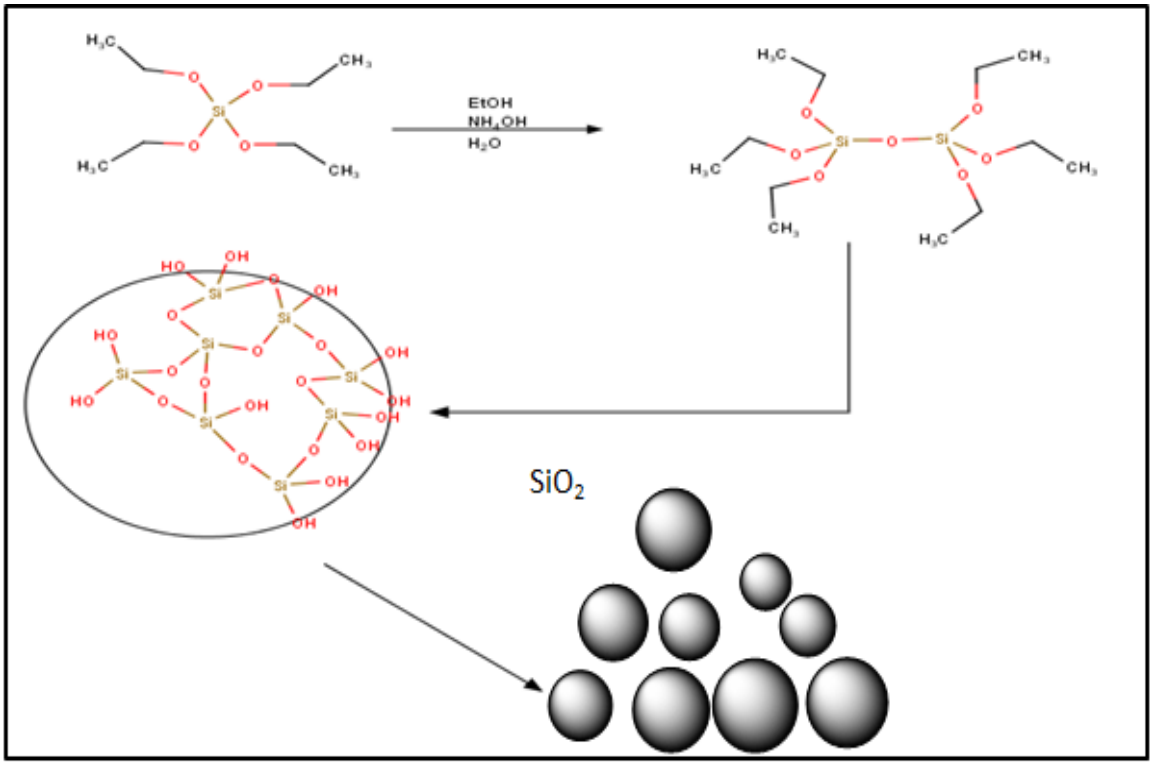


Figure 2.3. Mechanism of silica sol-gel with TEOS and basic catalyst.

2.4.1. Binding Characteristic Assay

The binding capacities and the dissociation constant for molecular imprinted and corresponding non-imprinted polymers were realized by rebinding experiments.

In batch type procedure, metoprolol solutions with different concentrations (10.0 mL) prepared and 10.0 mg of MIP/NIP sorbents were added. The mixtures were shaken at 560 rpm for 24 h. After binding, these mixtures were filtered with sartolon polyamide membranes (0.2 µm pore size) to separate sorbents. Effluents were analyzed with HPLC-DAD and CE-DAD at 220 nm. The same procedure was applied for each type of the sorbents. Parameters are given in Table 2.4. The adsorption capacity Q was calculated according to equation 1.

$$Q = \frac{(C_0 - C_f)\mathbb{V}}{m} \quad (2.1)$$

C_0 and C_f (µmol/L) are initial and final concentration of metoprolol, V (L) is the total volume of sample , m (g) is the mass of MIP/NIP particles Q and Qmax (µmol/g) are amount of metoprolol adsorbed at equilibrium and saturation, respectively.

Table 2.4. Parameters of binding characteristic assay.

Standard concentrations	1.0, 5,0, 10,0, 25.0, 50.0, 100.0, 250.0 mgL^{-1}
Amount of sorbent	10.0 mg
Sample volume	10.0 mL
Sorption time	24 h
Shaking speed	560 rpm
Ambient temperature	25 ℃

2.4.2. Cross Selectivity

Selective recognition studies were performed with metoprolol, ibuprofen and ketoprofen at concentration of 50.0 mgL^{-1}. 10.0 mL of this mixture was added into vials which contain 10.0 mg MIP/NIP solids. Mixtures were shaken at 560 rpm overnight for sorption. Filtration was made with sartolon polyamide filter (0.2 µm pore size). Effluents were analyzed with HPLC-DAD and CE-DAD at 220 nm. The studied parameters for cross selectivity experiments are shown in Table 2.5.

2.4.3. Elemental Analysis

Elemental analysis was used for the determination of nitrogen and carbon contents in polymer matrix.

Table 2.5. Studied parameters for cross selectivity.

Standard concentration	50.0 mgL^{-1}
Amount of sorbent	10.0 mg
Sample volume	10.0 mL
Sorption time	24 h
Shaking speed	560 rpm
Ambient temperature	25 °C

2.5 Preparation of Capillary Columns

In this section, stationary phases of capillary column were prepared for separation of metoprolol in CEC analysis.

2.5.1 Capillary Pre-treatment and Silanization Methods

In order to coat polymers onto the capillary wall, the internal surface of the fused silica capillary needs to be modified. In this study, to increase the amount of available silanol groups, the fused silica capillary (30 cm, 75 µm-id) were activated with 1.0 M NaOH for 30 min fallowed by MeOH solution for 15 min and ultrapure water for 15 min. Afterthat capillary filled with 4.0 µL 3-(trimethoxysilyl) propyl merthacrylate (γ-MPS) solution in 1.0 mL acetic acid for 1.5 h and dried with air.

2.5.2. Polymerization Procedures

In this section, the MIP and silica particles reaction mixtures was filled into the capillary. MIP200 solution was prepared as described by section 2.2. Pre-polymerization mixture was sonicated until the homogenous solution obtained. After that, resulting slurry solution was filled into vial. The slurry solution flushes with for 30 min with inlet of the capillary into a reservoir with gas pressure. The filled capillary was

sealed at both ends and put into oven at 60 ℃ overnight. On the other hand, the same slurry filled with capillary column with a syringe. The CEC-DAD parameters are given Table 2.6.

The same procedure was applied by using silica-based MIP/NIP as described in section 2.2. In addition to this, the slurry was immersed in a capillary with layer by layer. The prepared columns and conditions were given in Table 2.6.

Table 2.6. Novel column synthesis with MIP-NIP and SMIP-SNIP

Identity	Procedure	Reaction start	Filling mode	Layer
SNIP1	Silica-based NIP	in column	with CE	x
NIP1	Polymer-based NIP	in column	with CE	x
NIP1.1	Polymer-based NIP	in column	with CE	1
NIP1.1.1	Polymer-based NIP	in column	with syringe	1
MIP2	Polymer-based MIP	out of column	with syringe	x
NIP2	Polymer-based NIP	out of column	with CE	x
SMIP2	Silica-based MIP	out of column	with CE	x
SNIP2	Silica-based NIP	out of column	with CE	x
MIP3	Polymer-based MIP	out of column	with CE	x
NIP3	Polymer-based NIP	out of column	with CE	x
NIP3.1	Polymer-based NIP	out of column	with CE	5
NIP3.1.1	Polymer-based NIP	out of column	with CE	10

CHAPTER 3

RESULT AND DISCUSSION

3.1 Optimization of Instrumental Analysis

Optimization parameters were determined for both HPLC-DAD and CE-DAD analysis. These parameters were given Table 2.1 and Table 2.2, respectively.

For HPLC-DAD analysis, optimum parameters for mobile phase composition, flow rate and column temperature were determined as 85:15 MeOH:H_2O (acetic acid, pH 3.0), 0.9 $mLmin^{-1}$ and 30ºC, respectively. Under the optimum HPLC conditions, the chromatogram of metoprolol was obtained as (Figure 3.1). Limit of detection (LOD_{3s}) was calculated as 0.036 mgL^{-1} and Limit of quantification (LOQ_{10s}) was found as 0.120 mgL^{-1}. These figures of merits were considered to be sufficient for the purpose of the study; at the initial stages therefore, no attempt was tried to enhance these values. Calibration graph for metoprolol is shown in Figure 3.2.

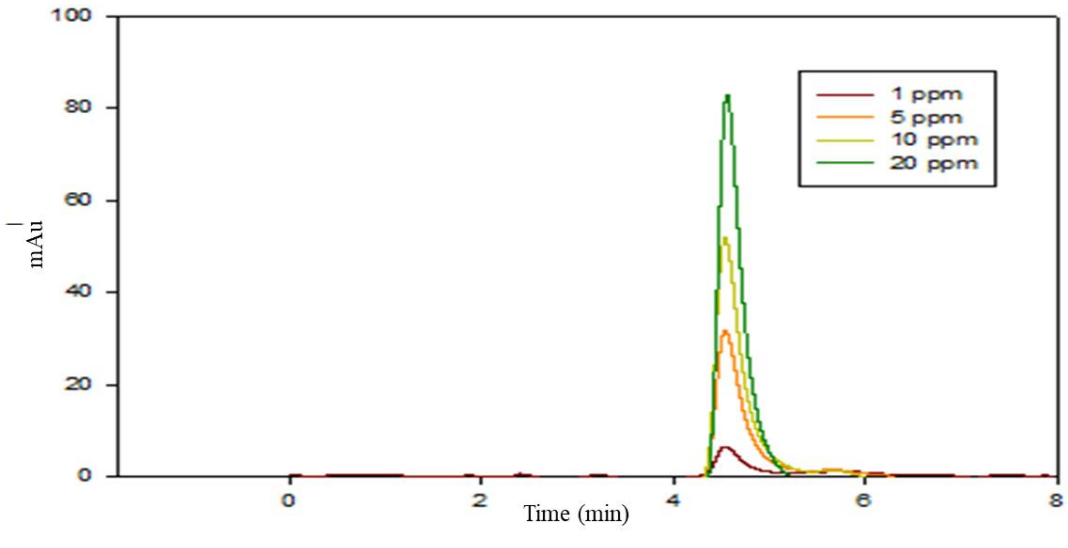


Figure 3.1. Chromatogram of metoprolol. (Agilent 1200 Series HPLC-DAD system, Supelco C18 (Lichrosphere RP 18-5, 25cm×4.6mm) column, 85:15 MeOH:H_2O (pH 3.0) mobile phase, 0.9 $mLmin^{-1}$ flow rate, 220 nm)

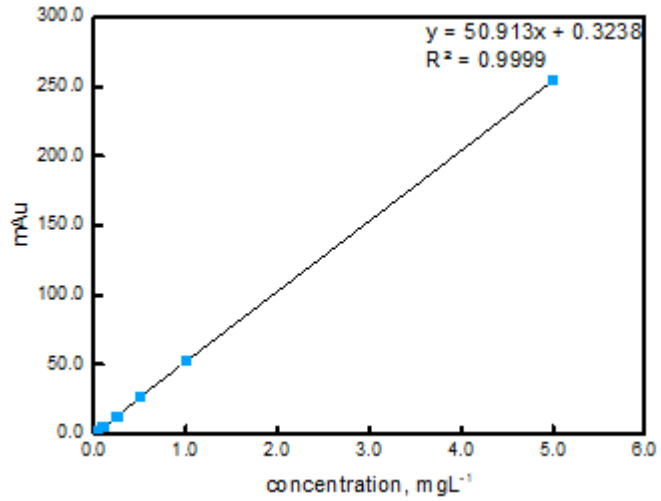


Figure 3.2. Calibration plot for metoprolol. (Agilent 1200 Series HPLC-DAD system, Supelco C18 (Lichrosphere RP 18-5, 25cm×4.6mm) column, 85:15 MeOH:H_2O (pH 3.0) mobile phase, 0.9 $mLmin^{-1}$ flow rate, 220 nm).

For CE-DAD analysis, optimum parameters for background electrolyte, injection pressure and time, voltage were determined as 25.0 mM borate buffer (adtusted with NaOH pH 9.5) 50.0 mbar, 5.0 s, 20.0 kV, respectively. Limit of detection (LOD_{3s}) value was calculated as 0.22 mgL^{-1} and limit of quantification (LOQ_{10s}) was found as 0.73 mgL^{-1}. The electropherogram obtained under optimum conditions of metoprolol is given Figure in 3.3 and calibration graph of metoprolol is shown in Figure 3.4.

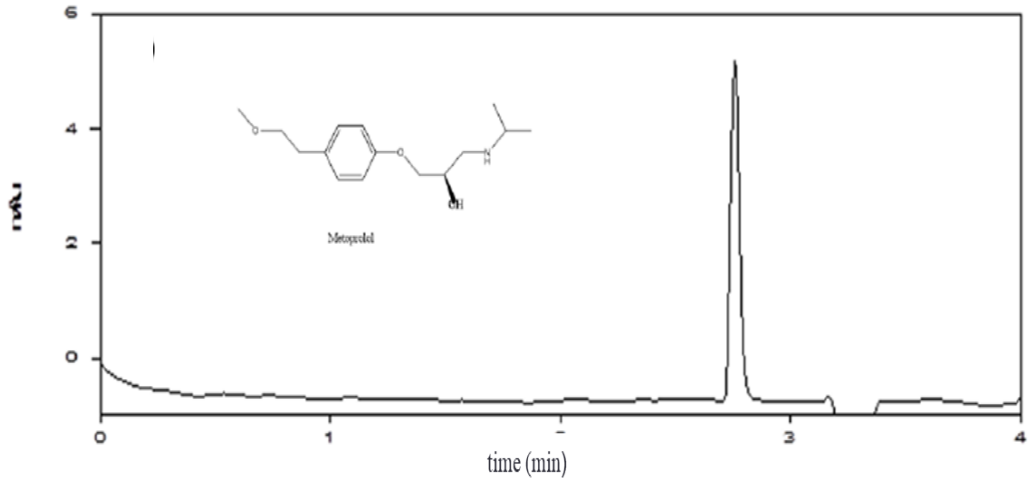


Figure 3.3. Electropherogram of metoprolol. (Agilent 7100 series CE-DAD system, Agilent technologies, FS, undeactivated (75 µm id, 57 cm total capillary length, 50 cm effective length), 25.0 mM borate buffer (pH 9.5), 220 nm).

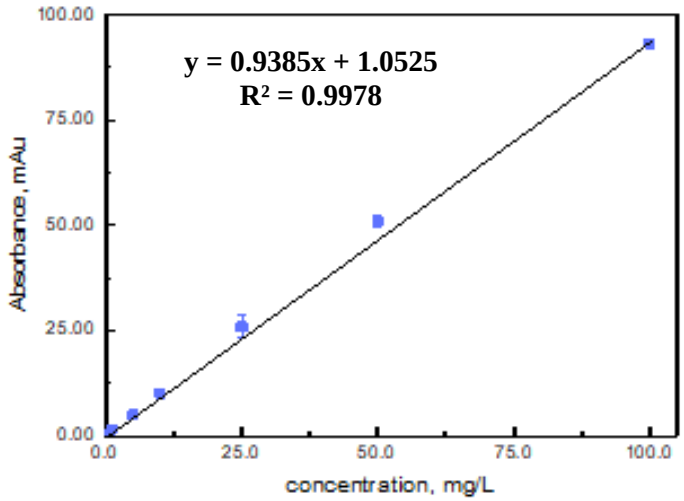


Figure 3.4. Calibration plot for metoprolol. (Agilent 7100 series CE-DAD system, Agilent technologies, FS, undeactivated (75 µm id, 57 cm total capillary length, 50 cm effective length), 25.0 mM borate buffer (pH 9.5), 220 nm).

3.2. Synthesis of Sorbents

Silica particles, molecularly imprinted organic polymers and silica-based polymers were synthesized. Scanning Electron Microscope (SEM) images for all type of the sorbents are given in Figure 3.5, Figure 3.6 and Figure 3.7.

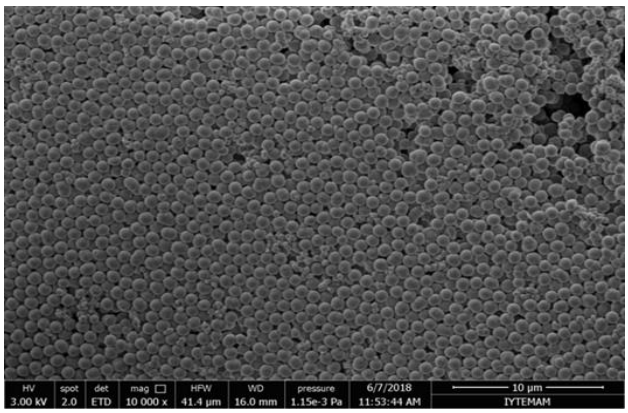


Figure 3.5. SEM image of synthesized silica sorbent.

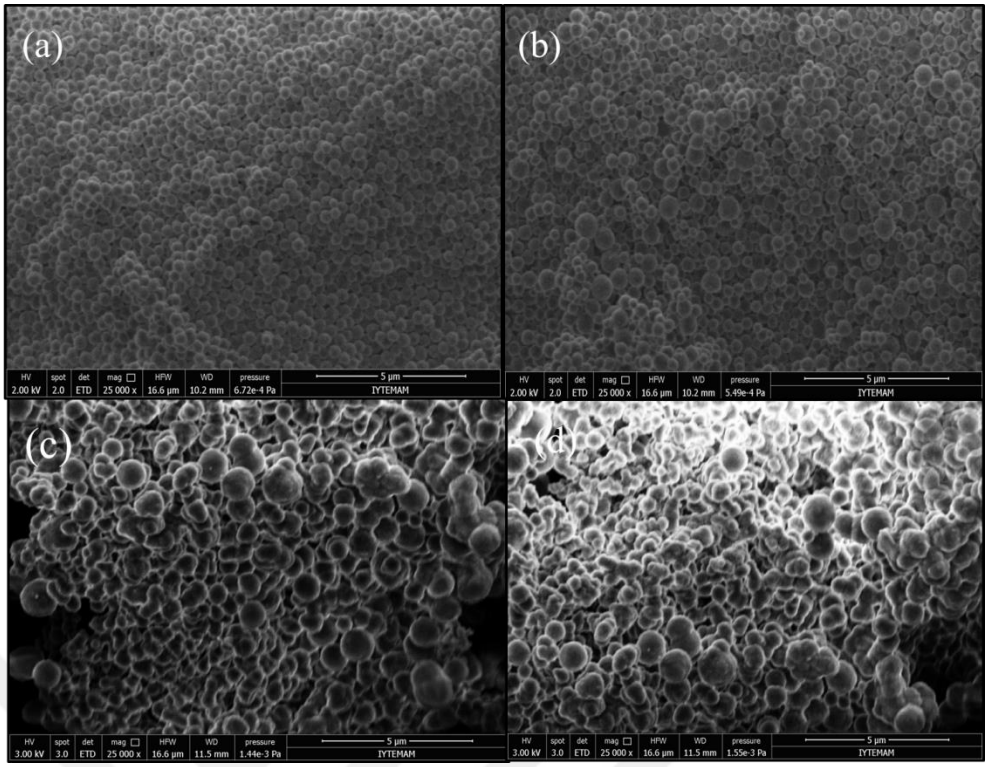


Figure 3.6. SEM images of MIPs: (a) MIP200, (b) NIP200, (c) MIP50, (d) NIP50.

The usage of different amounts of porogen (total monomer/porogen, w/v %) determines the physical morphologies of MIPs/NIPs networks. If this ratio is smaller than 5%, polymers are synthesized with precipitation method, which results in spherical shaped particles. Spherical shaped particles supply homogenous binding site distribution for template. For MIP200 and NIP200 the ratio was calculated as 2.08 %. This ratio proves spherical shaped of particles (Figure 3.6 (a)). If the ratio is bigger than 5%, bulk polymerization occurs. For MIP50 and NIP50, this ratio is 8.32 % (Figure 3.6 (c)). This ratio explains the monolithic morphology. Briefly, when sufficient solvent is provided for precipitation polymerization the particles have spherical shape rather than grow by agglomeration.

According to SEM images, there is no difference between MIP and NIP particles. Specific recognition sites for metoprolol are too small to be seen in SEM images. Transmission electron microscopy (TEM) may be helpful for seeing the cavities.

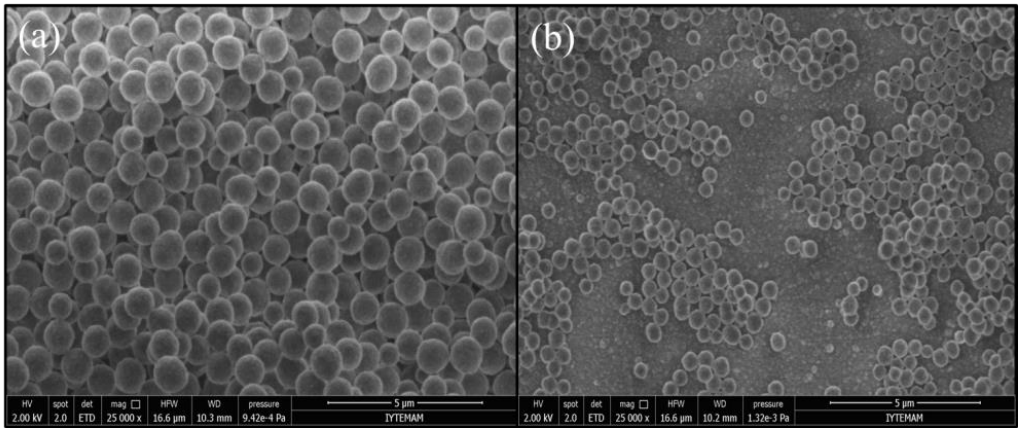


Figure 3.7. SEM images of silica-based MIP, (b) silica-based NIP.

In addition, silica-based MIP/NIP was synthesized. Spherical shape particles were obtained. Aggregation does not occur due to the large amount of solvent, meaning that they have sufficient area to escape aggregation.

After the synthesis of polymers, metoprolol was removed from polymer matrix. The electropherogram of metoprolol in Figure 3.8 explains that, after 20. wash metoprolol is completely removed from MIPs.

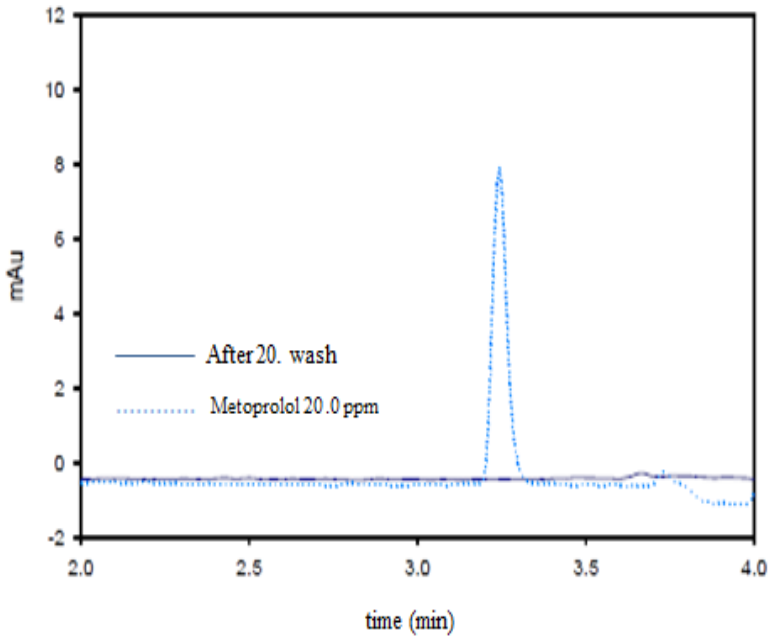


Figure 3.8. Electropherogram of wash solution for metoprolol before and after the washing steps. (Agilent 7100 series CE-DAD system, Agilent technologies, FS, Undeactivated (75 µm id, 57 cm total capillary length, 50 cm effective length), 25.0 mM borate buffer (pH 9.5), 220 nm)

3.3. Characterization Experiments

3.3.1. Binding Characteristic Assay

Sorption capacities of MIP200/NIP200, silica-based MIPs/NIPs and silica particles as a function of metoprolol concentration are shown in Figure 3.9.

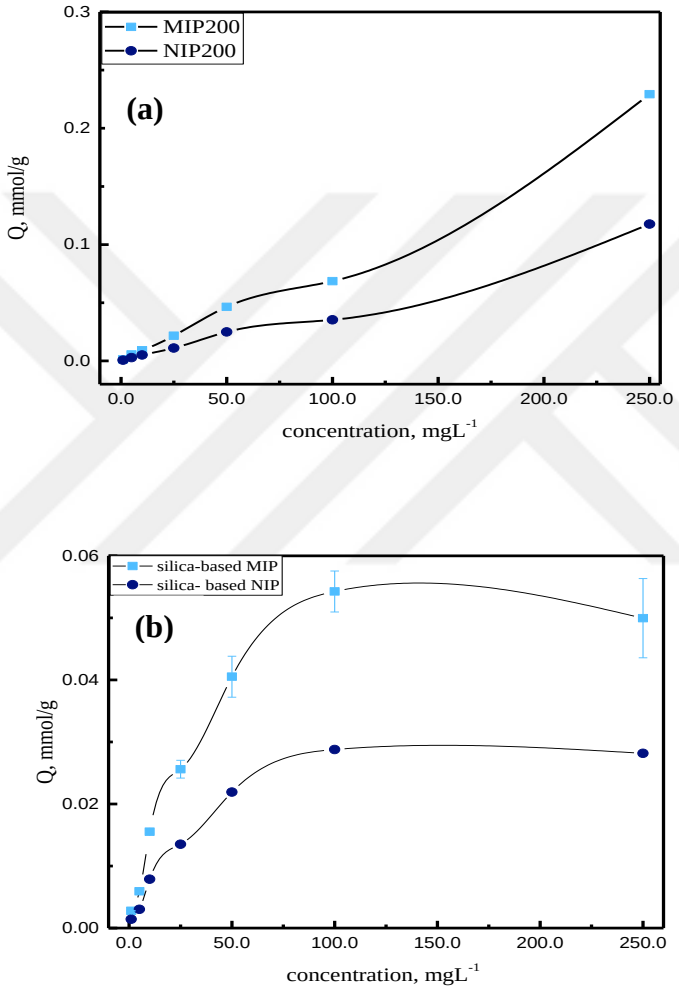


Figure 3.9. Sorption capacities of (a) MIP200/NIP200, (b) silica-based MIP/NIP, (c) silica particles. (Agilent 7100 series CE-DAD system, Agilent technologies, FS, Undeactivated (75 µm id, 57 cm total capillary length, 50 cm effective length), 25.0 mM borate buffer (pH 9.5), 220 nm and n=3**).**

(Continue on next page.)

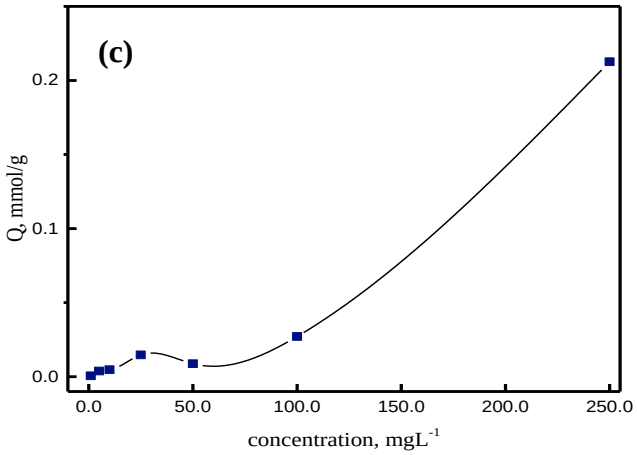


Figure 3.9. **(Cont).**

The ordinate Q shows the ratio of mmol of metoprolol to 1.0 g of MIP or NIP. At low concentrations, there is no noticeable difference between MIP200 and NIP200 (Figure 3.9 (a)). The reason for this appears to be the efficiency of the functional groups even in NIP200 for lower metoprolol concentrations. But, the sorption capacities of both MIP200 and NIP200 increased with increasing concentration. As clearly seen, the MIP200 shows higher sorption capacity for metoprolol than corresponding NIP200 at least up to 250.0 mgL^{-1}.

Silica-based MIP/NIP particles have maximum sorption at 100.0 mgL^{-1} (Figure 3.9 (b)). But, the sorption capacity difference between silica-based MIP and NIP is noticeable even at low concentrations. There is a very small decrease in sorption capacities of silica-based MIP and NIP above 100.0 mgL^{-1} which shows that the sorbents reach saturation point at this concentration.

Silica particles do not have specific binding sites for metoprolol. Template could be adsorbed only onto surface of silica particles. Because of this reason, non-specific interactions exist between metoprolol and silica particles at high concentrations (Figure 3.9 (c)).

The reason of showing higher sorption capacities for MIP200 and silica-based MIP is the specific recognition sites in their structures against metoprolol. Moreover, silica particles can only interact with metoprolol nonspecifically.

3.3.2. Cross Selectivity

The specific binding characteristic of MIP200/NIP200 and silica-based MIP/NIP to metoprolol were investigated in the presence of ketoprofen and ibuprofen.

As shown in Figure 3.10 (a), MIP200 demonstrated higher sorption capacity towards metoprolol than NIP200. For ketoprofen and ibuprofen, NIP200 showed better sorption capacity than MIP200. These compounds can possible be interacting with the surface of MIP200 only since cannot enter the metoprolol-specific cavities in MIP. Besides this, the possible functionality in NIP may have been removed during synthesis.

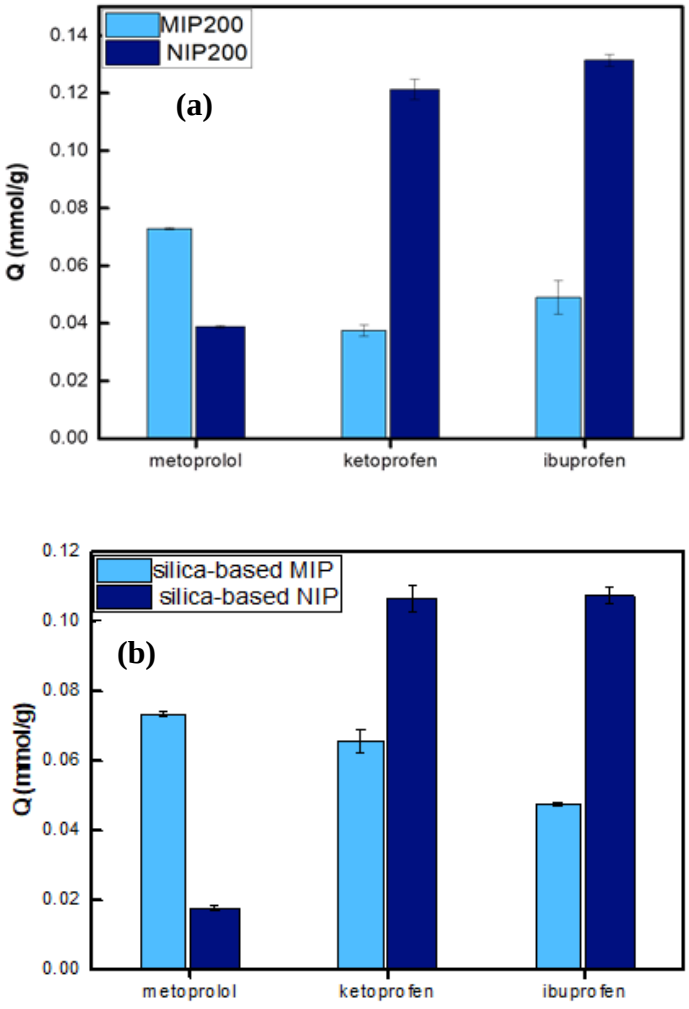


Figure 3.10. Sorption capacities of (a) MIP200/NIP200 and (b) silica-based MIP/NIP in the presence of NSAIDs. (Agilent 7100 series CE-DAD system, Agilent technologies, FS, Undeactivated (75 µm id, 57 cm total capillary length, 50 cm effective length), 25.0 mM borate buffer (pH 9.5), 220 nm and n=3).

In addition to this, similar results were obtained for silica-based MIP/NIP (Figure 3.9 (b)). Silica-based MIP shows higher sorption capacity to metoprolol than silica-based NIP. But, silica-based MIP hold to ketoprofen and ibuprofen higher amount compare to MIP200. These result demonstrated that, organic molecularly imprinted polymer is better sorbent type compare to silica-based MIP.

3.3.3. Elemental Analysis Results

Table: 3.1. Elemental analysis results

Molecular imprinted and non-imprinted organic based polymers	Percentage (w/w)		
	C	H	N
NIP200 (Except metoprolol)	60.0	7.4	0.53
BWMIP200 (before removal of metoprolol)	59.87	7.7	0.90
AWMIP200 (after removal of metoprolol)	58.22	7.7	0.54

Elemental analysis was also used to further show the efficiency of first formation and then removal of specific cavities during MIP synthesis. As given Table 3.1, both MIP and NIP have nitrogen in their structures. The nitrogen percentage 0.53 % in NIP must be originated from the AIVN 4,4'-azobis(4-cyanovalericacid). After the synthesis (before washing), the MIP has 0.90 % nitrogen which is thought to be stemming from amine groups in metoprolol, the template. After washing step for the removal of the template metoprolol from the MIP, the nitrogen content drops to 0.54 %, a very close value to that of NIP. This result, when considered together with the findings explained in section 3.2 (Figure 3.8), demonstrates the success in both the synthesis and the template removal steps in MIP preparation.

3.4. Preparation and Characterization of Capillary Columns

Due to their specific recognition ability for metoprolol MIP200 and silica-based MIP was demonstrated to be good candidates as a SPE sorbent. In this section, interior of capillary columns were prepared for separation of metoprolol in CEC analysis. Experimental procedure was explained section 2.5.2.

Scanning electron microscopy (SEM) was used for the examination of the interior surface of the distal end capillary column; capillary tubing was cut into 1 cm length and placed into SEM planchets. Samples were coated with gold before obtaining images. Figure 3.11. shows a cross sectional view of bare silica capillary.

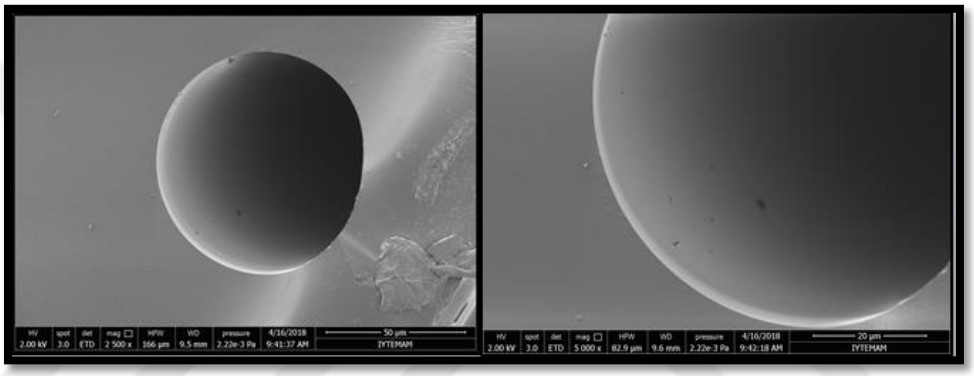


Figure 3.11. The cross sectional view of bare capillary column; magnified (a) 2.500 times, (b) 5.000 times.

Figure 3.12. shows the inside coating at the end of view of NIP1.1.1 capillary column. NIP200 solution was passed through the capillary tubing with a syringe just before the polymerization.

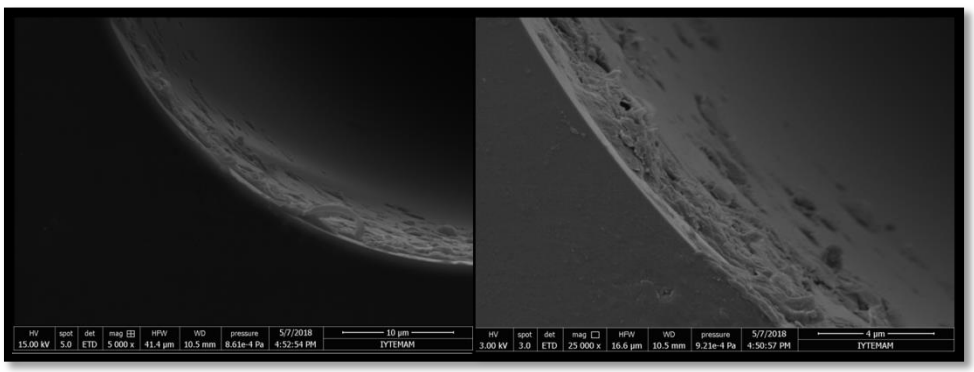


Figure 3.12. The cross sectional view of NIP1.1.1 column; magnified (a) 5.000 times, (b) 25.000 times.

Silica-based NIP was prepared as described in Section 2.2. The polymerization observed the out of column and slurry was filled into capillary column with CE. The SEM images demonstrated in Figure 3.13. Ass seen in figure, there are sections of coated layers, however this layers are not homogenous. This finding suggests that, there is a need for a better coating procedure and homogenous coating will be the aim of the further studies.

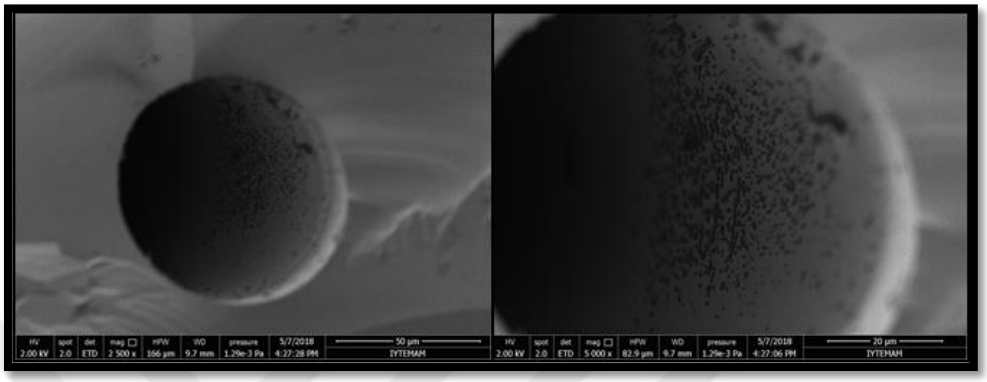


Figure 3.13. The cross sectional view of silica-based NIP column; magnified (a) 2.500 times, (b) 5.000 times.

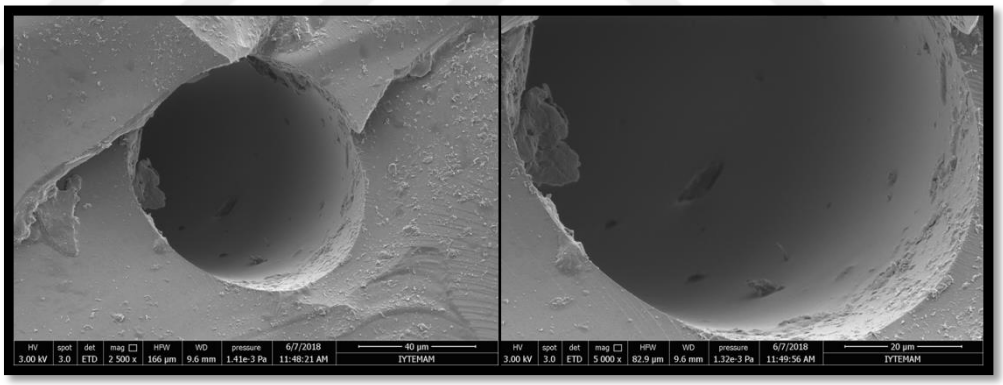


Figure 3.14. The cross sectional view of MIP1 column; magnified (a) 2.500 times, (b) 5.000 times.

3.5. Capillary Electrophoresis Study

In order to investigate whether or not CEC column would be used in the separation of the selected analytes, first, bare silica column was used as a starting point. Within the scope of these studies, for the selection of the appropriate buffer solution

which is the most important variable for CE; buffer concentration, buffer type, working parameters have been systematically changed and optimal conditions have been determined for the separation of selected ß-blockers. Firstly, the electrolyte solution was determined. Since metoprolol and atenolol are basic analytes, buffer solutions, providing basic pH were selected, namely, phosphate buffer and borate buffer. No significant peak was observed with phosphate buffer (pH 7.0) and it was not possible to prepare buffers pHs higher than 8.0. Secondly, borate buffer (pH 9.5) was tried and relatively good signals were observed. The reason is thought to be stemming from the pKa values of atenolol and metoprolol which are close to this pH (9.6 and 9.5 respectively).

The thickness of the electrical double layer increases with increasing buffer concentration. The operating parameters of CE-DAD system were varied and the best optimum results were underlined (Table 3.2).

Table 3.2. CE-DAD optimization parameters.

Column	Agilent technologies, FS, undeactivated (75 µm id, 57 cm total capillary length, 50 cm effective length)
Concentration of background electrolyte solution	Borate buffer 10.0 mM, <u>25.0 mM</u>, 50.0 mM
Injection pressure and time	50 mbar, 5 s
Applied voltage	20.0 kV, <u>25.0 kV</u>, 30.0 kV
Power, Current	6.0 W, 300.0 µA
Data collection parameters	2.5 Hz

The electropherograms obtained with use of the optimized parameters are given in Figure 3.15 and Figure 3.16.

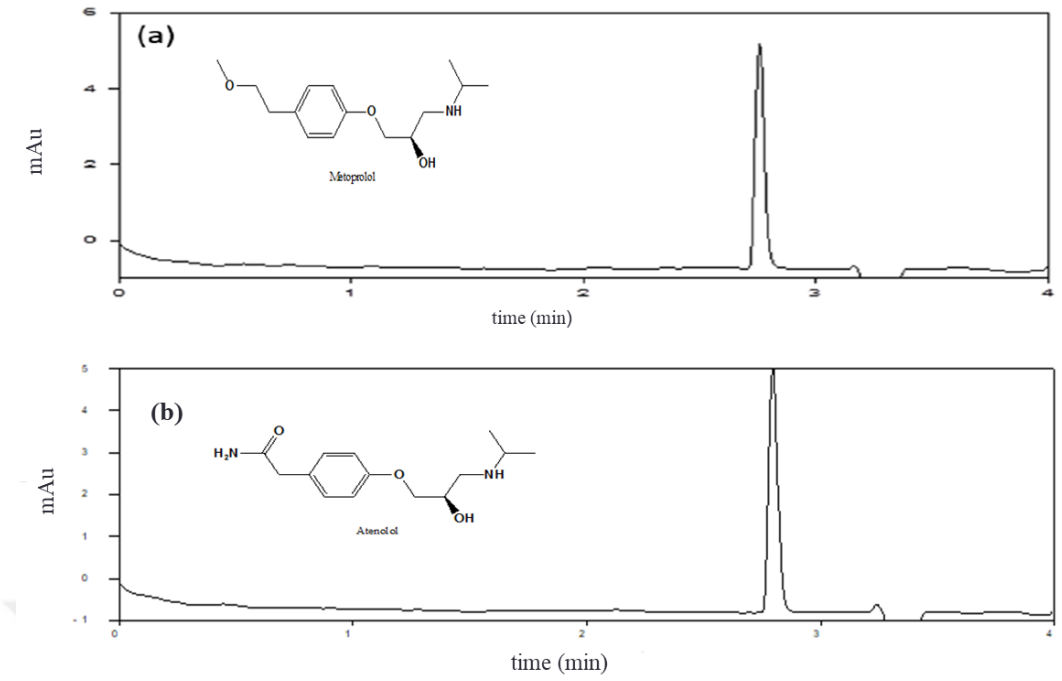


Figure 3.15. Electropherogram of (a) metoprolol, (b) atenolol. (Agilent 7100 series CE-DAD system, Agilent technologies, FS, Undeactivated (75 µm id, 57 cm total capillary length, 50 cm effective length), 25.0 mM borate buffer (pH 9.5), 220 nm).

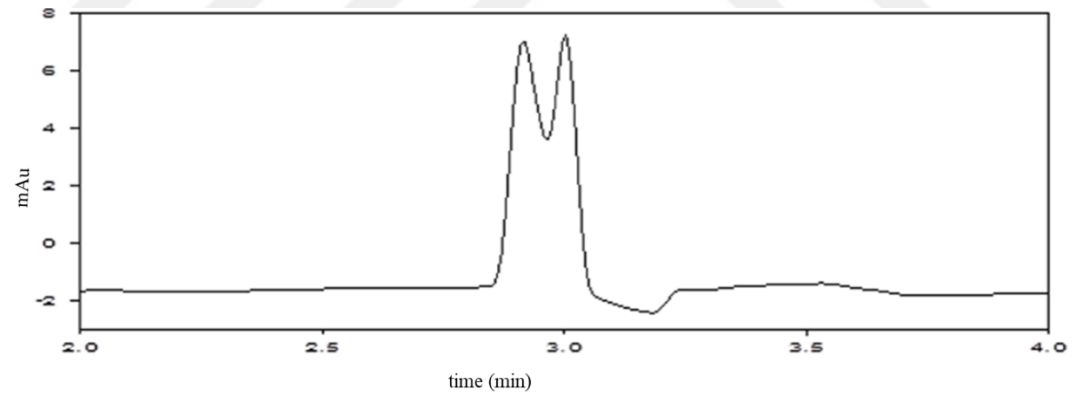


Figure 3.16. Electropherogram for mixture of metoprolol and atenolol. (Agilent 7100 series CE-DAD system, Agilent technologies, FS, undeactivated (75 µm id, 57 cm total capillary length, 50 cm effective length), 25.0 mM borate buffer (pH 9.5), 220 nm) .

Despite all the optimization studies, no separation could have been achieved between metoprolol and atenolol with CE-DAD method due to possibly the very close pKa values. A hydrophobic fused-silica capillary surface causes poor separation efficiency for these analytes. Therefore, the subsequent studies were concentrated on the

preparation of new capillary column for the separation of metoprolol and atenolol with CEC-DAD system. Molecularly imprinted organic polymer was chosen as the stationary phase and capillary column was prepared as explained in Section 2.5. Rest of the experiments was carried out with this capillary column.

3.6. Capillary Electrochromatography Study

In capillary electrochromatography (CEC), the stationary phase has two important roles: first, to provide charged sites to permit the desired EOF for mass transport across the column, and second, to create interactive sites for chromatographic retention. Two routes can be used for making open-tubular stationary phases: sol-gels and organic-inorganic molecularly imprinted polymers. Polymeric method is more preferable because of its recognition ability against the analyte. In this study, molecularly imprinted organic polymers were chosen as a filling material. The procedure consists of four main steps: first, using a promoter solution for activation (3-(trimethoxysilyl) propyl merthacrylate) of the capillary walls to increase sorption sites; second, the polymerization is initiated by heat in oil bath and third, the slurry mixture is passed through the capillary tubing using inlet vial of CE by pushing the polymerization solution under gas pressure (3.0 bar). Lastly, unreacted components are removed with using MeOH: H_2O (pH: 3.0). A detector window must be created for absorbance measurements.

Metoprolol and atenolol mixture were prepared at 50.0 mgL^{-1} and 100.0 mgL^{-1} in ultra-pure water for CEC-DAD analysis. After that, the 25.0 mM borate buffer is prepared as a running buffer at pH 9.5. To minimize any potential disruption of the stationary phase, electrokinetic injection was used mainly. The OT-capillary column was fixed into capillary cassette and cassette temperature was adjusted to at 25 ℃ for analysis. A sample containing metoprolol and atenolol was injected at different voltage and injection times setting. The optimum parameters for CEC-DAD analysis are given in Table 3.2.

Table 3.2. Optimum parameters for CEC-DAD.

Column	Agilent technologies, FS, Undeactivated (75 µm id, 30.0 cm total capillary length, 23.0 cm effective length)
Concentration of buffer	25.0 mM borate buffer (adjusted with NaOH pH, 9.5)
Thermostat temperature	25 °C
Injection pressure and time	50.0 mbar, 5.0 s
Voltage, Power, Current	15.0 kV, 6.0 W, 300.0 µA
Data collection rate	2.5 Hz

As illustrated in Figure 3.17 metoprolol and atenolol were separated with the optimized conditions.

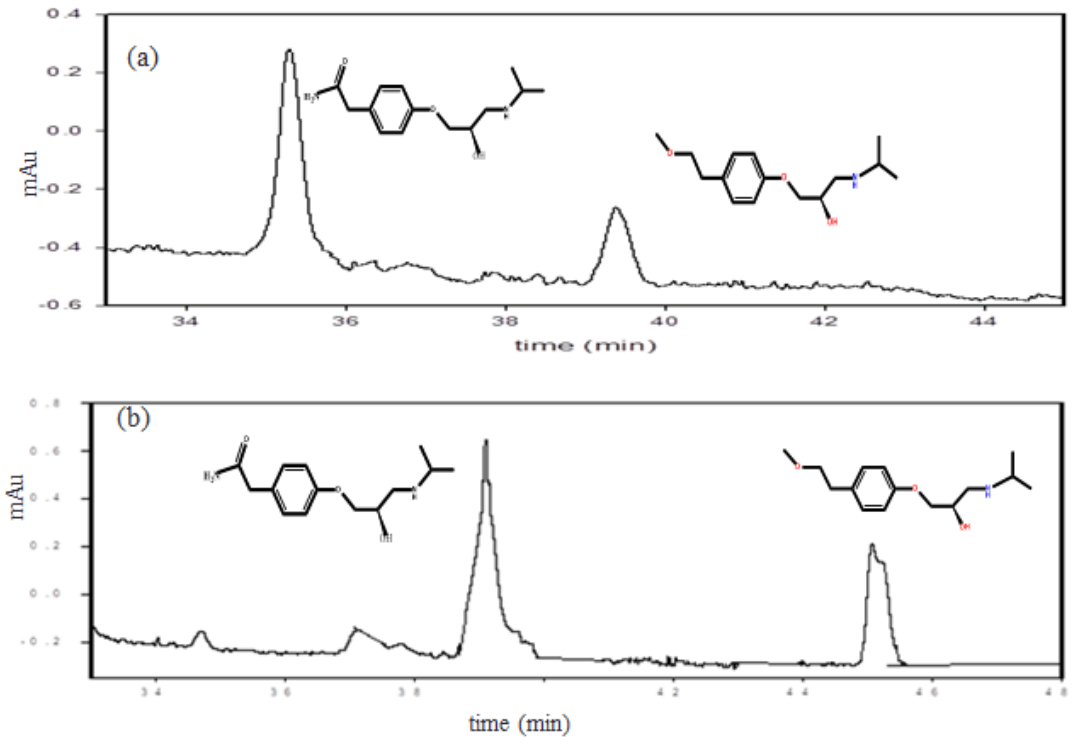


Figure 3.17. Electrochromatogram for mixture at (a) 50.0 mgL, (b) 100.0 mgL^{-1}. (Agilent 7100 series CE-DAD system, (75 µm id, 30 cm total capillary length, 23 cm effective length), 25.0 mM borate buffer (pH 9.5), 220 nm).

It can be said that, open tubular capillary column prepared has specific recognition sites against metoprolol which explains the longer retention time. The more important outcome of this result is that, these two related compounds can be separated using the capillary column prepared. On the other hand, the coating appears to be inhomogeneous and retention times are irreproducible. Although the irreproducibility in the retention times can be overcome with the use of internal standards, the inhomogeneity in the reactive phase in the capillary column cannot be tolerated. Therefore, the study will be continued with a more concentration on the coating homogeneity.

The calibration graphs of metoprolol and atenolol are shown in Figure 3.18. As seen, the graphs are linear at least upto 300.0 mgL^{-1}. Limit of detection (LOD_{3s}) and limit of quantification (LOQ_{10s}) were calculated as 10.0 mgL^{-1} and 30.02 mgL^{-1}, respectively. These numbers appear to be high and not very convenient for a study which aims to determine low analyte concentrations in biological fluids. However, it can be postulated that the combination of the capillary electrochromatography methodology proposed in this study can be connected to a more sensitive detection system such as mass spectrometry and can be used for very low concentrations of metoprolol and atenolol.

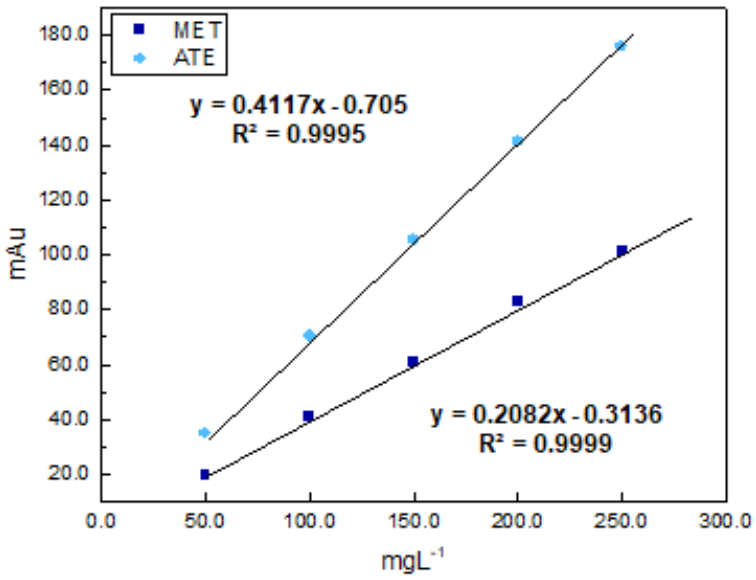


Figure 3.18. Calibration graph of metoprolol and atenolol. (Agilent 7100 series CEC-DAD system, Agilent technologies, FS, Undeactivated (75 µm id, 30 cm total capillary length, 23 cm effective length), 25.0 mM borate buffer (pH 9.5), 220 nm).

CHAPTER 4

CONCLUSION

This study details a method for separation and identification of ß-blockers using a molecularly imprinted organic based polymers OT-capillary column. Capillary electrochromatography coupled with the UV detectors was explored. The proposed CEC-DAD technique provides an effective and efficient method for the separation and identification of analytes.

In the initial stages of the study, three different types of SPE sorbents were synthesized successfully with molecular imprinting technology and sol-gel methodology to have selectivity and specificity for metoprolol. Molecular imprinted organic polymers demonstrated better sorption performance than the other sorbent types and was found to be more specific to metoprolol. The separation of analytes was tried to be achieved with CE-DAD method. Since the selected analytes had very close pKa values and there is no polarity difference between them, they could not have been separated. Still, for metoprolol determination with CE-DAD on aqueous solutions containing no potential interferences, limit of detection (LOD_{3s}) and limit of quantitation (LOQ_{10s}) were calculated as 0.22 mgL^{-1} and 0.73 mgL^{-1}, respectively, under the optimum conditions.

The subsequent studies were concentrated on the preparation of new capillary column for separation of metoprolol and atenolol mixture for CEC-DAD analysis. Molecular imprinted organic polymers were chosen as stationary phase and an open tubular capillary column was prepared for specific recognition of metoprolol. The stationary phase of capillary column provides charged sites to permit the desired EOF for mass transport across the column and to create interactive sites for chromatographic retention. The separation and determination was achieved by the prepared column. Limit of detection (LOD_{3s}) was calculated as 10.0 mgL^{-1} and limit of quantitation (LOQ_{10s}) as 30.02 mgL^{-1} for metoprolol using the optimized parameters in CEC-DAD system. It should be mentioned that the OT-capillary column prepared appears to give irreproducible results. Still, this new methodology

combining MIP stationary phase with CEC-DAD detection has shown to enable promising results for the selective determination of metoprolol in water samples.

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