



Pre-Concentration Approaches Combined with Capillary Electrophoresis in Bioanalysis of Chiral Cardiovascular Drugs

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ABSTRACT

Currently, there is a remarkable interest in the separation and quantification of enantiomeric compounds because of their importance in biochemistry, therapeutic drug monitoring (TDM), pharmaceutical, forensic, etc. In past decades the pharmacopoeia was governed by racemates, but nowadays the U.S. Food and Drug Administration (FDA) exclusively has been issued a statement that racemic new drugs could not be marketed unless they are thoroughly investigated of the pharmacological and toxicological properties of pure enantiomers. Demands for accurate and precise analytical method for separation and determination of drugs enantiomers and their metabolites in biological fluids are highlighted. Capillary zone electrophoresis (CZE) is an interesting technique that shows promise for the resolution of enantiomers. This is carried out by adding suitable chiral selector to the background electrolyte results to enantioseparation. According to WHO organization report, cardiovascular diseases (CVDs) rank the leading cause of death, globally. Therefore the related risk factors addressing CVDs should be considered and diagnose to prevent the disease. This article provides a brief review on applying off-line and on-line sample preconcentration methods for enhancing the detection limit of cardiovascular drugs.

Introduction

Chirality

Stereochemistry is a field of chemistry involving the study of geometric arrangement of atoms in molecules. Chirality is described as the optical property of a rigid

materials such as drugs. Chiral compounds have identical formula but they are distinguished in spatial arrangement which shows non-superimposable mirror images with each other (optical isomers).

Table 1. Definitions of important terms in stereochemistry.

Term	Definition
Stereoisomers	Stereoisomers have similar molecular formula but differ in spatial orientation of their atoms
Enantiomers	Enantiomers are two stereoisomers with non-superimposable mirror images
Distomer	Enantiomer with lower biological activity
Eutomer	Enantiomer with higher biological activity
Racemate	A mixture of equal amounts of enantiomers
Chiral switch	A racemate switch to single enantiomer
Epimerization	Bioinversion of enantiomers
(+) Optical isomer	Rotates the light plane clockwise (dextrorotary)
(-) Optical isomer	Rotates the light plane counter-clockwise (levorotary).
(R) and (S)	According to enantiomer configurations which are right- or left-handed.

Chiral molecules can rotate plane of polarized light in opposite directions and according to their optical rotation of plane-polarized light, they can be classified as (+) and (-) enantiomers. On the basis of another

classification, amino acids and sugars are distinguished as (d) and (l) system. For example one enantiomer rotates the light to the left direction (levorotary or (-)-enantiomer or R-form), therefore the other will rotate

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the light to the right (dextrorotatory or (+)-enantiomer or S-form).¹ It is concluded that each enantiomer may behave very distinguished in its biological effects. There are several examples that the prefixes have been used to mark active pharmaceutical ingredients (API) e.g. es- (esomeprazole), ar- (arbupivacaine), lev- (levibuprofen) and dex- (dexmethylphenidate). More defined terms related to stereochemistry are shown in Table 1.

Chirality in biological system

Basic building blocks in biological system such as amino acids, carbohydrates, and lipids show chirality. Furthermore, biological matrices or drug targets such as receptors, enzymes, proteins and membranes (e.g. phospholipids) display three-dimensional structure. Enantiomers of a chiral molecule show identical properties in an isotropic environment but in anisotropic environments they exert different properties. Biochemical interactions are stereoselective, therefore the drugs interactions with corresponding targets should be stereoselective.² Easson and Stedman in 1993 suggested the "three point fit" model between the active enantiomer and its receptor.³ As is illustrated in Figure 1, the biological response of drugs depends on their binding behavior. Therefore, it is reasonable that a chiral drug molecule may show different therapeutic properties as its enantiomer counterpart has the different binding mode with biological targets.

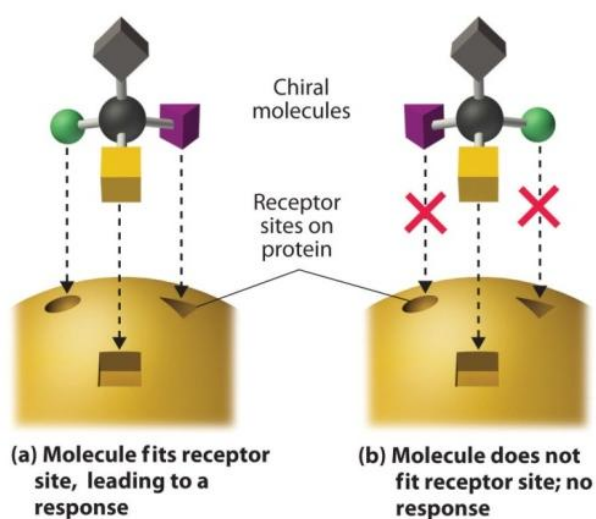


Figure 1. Interaction between chiral molecule and receptor.⁴

Drugs with chiral center

Drugs with chiral centers show a remarkable attention in the pharmaceutical field. About 70% of drugs in the pharmaceutical market are chiral compounds, and it is growing very fast.^{2,5} In better words, almost 1 in 4 of marketed drugs is in racemic form rather than their enantiomers.⁶ The fact that 9 of top 10 drugs have chiral active ingredients, may emphasize the important aspect of chiral drugs in pharmaceutical investigations. In general, administration of more active isomer of

racemic drugs causes to following advantages: diminishing probable side effects due to the antipode (counterpart) isomer, decrease in producing of unwanted isomer, halved dosage for a patient and new commercial opportunities for racemic switching.⁷

Drugs with chiral center can be categorized in three major groups;

1. Racemic drugs with one major bioactive enantiomer
2. Racemic drugs with equally bioactive enantiomers
3. Racemic drugs with chiral inversion

Most of the attention was focused on drugs with one enantiomer having major activity. Another enantiomer can be therapeutically present inactive, with toxic effects or even with reducing effect on active form. Enantiomers may differ in both their pharmacodynamic and pharmacokinetic profiles. Thalidomide is a famous example that elucidates the importance of chirality in human body. It was synthesized in 1953 as a racemat and prescribed for morning sickness in pregnant women. Unfortunately, it leads to born almost 10,000 babies with defects in Europe. Although the first estimation was indicated the undesirable role of (S)-enantiomer in teratogenicity, but subsequent researches proved that both enantiomers have equal teratogenic potency in the rabbit. Another hypothesis discussed on the bioinversion of thalidomide in acidic media.⁸ Although the chirality story about thalidomide is a disaster but the great impact of thalidomide in development of chiral drugs is not deniable.

Despite the dark past of thalidomide, it is transformed gradually into a pretty useful drug. It is also noteworthy to mention that thalidomide currently has shown hopes to treat leprosy, acquired immunodeficiency syndrome (AIDS), behcet disorder, lupus, sjogren syndrome, rheumatoid arthritis, inflammatory bowel disease, molecular degeneration and some cancers.⁹ Recent studies confirm that (S)-thalidomide slows down the growth of tumors as well as angiogenesis thus it contributes in revolutionized treatment of myeloma.¹⁰ Contrary to the fact that thalidomide shows very toxic side effects such as peripheral neuropathy, somnolence, constipation, rash, deep vein thrombosis and teratogenicity, but the statistics show that till 2001 it has survived the hundreds of years of life in myeloma treatment.

Importance of enantioseparation

There is a high demand for enantioseparation at analytical scale for therapeutic drug monitoring (TDM), optimization of pharmacotherapy and for research in pharmacokinetic and pharmacodynamic investigations. Furthermore, as certain enantiomers represent illicit or banned substances, chiral discrimination shows an important challenge in forensic analysis and doping control in biological fluids (typically plasma, serum or urine).^{7,11}

Nowadays, TDM is widely accepted as an applied method to improve the effectiveness and safety of therapeutic agents. TDM is based on measurement of

drug concentrations in biological fluids to assess useful information to personalized dosage, keep concentrations within a target range, increase efficacy or decrease toxicity of drugs.¹²

Because of above mentioned rationals, since 1992 the Food and Drug Administration (FDA) and the European Committee for Proprietary Medicinal Products (CPMP) have been made a statement in order to investigate the enantiomers of chiral drugs with respect to their individual properties before taken to market as a racemic form or a pure enantiomer.¹¹ Inactive enantiomers are going to be administrated less, but they provide information to design safe generation of useful analogues.

Electromigration techniques in chiral separation

There are various enantioseparation methods such as high-performance liquid chromatography (HPLC),¹³ gas chromatography (GC),¹⁴ supercritical-fluid chromatography (SFC),¹⁵ and capillary electrophoresis (CE).¹⁶⁻²¹ Stereospecific drugs resolution with liquid chromatography (LC) methods are accompanied with rather expensive stationary phases and consumption a large amount of organic solvents. In the last 20 years, chiral separation techniques based on capillary electromigration methods have been intensively studied.²²

CE has been accepted as a powerful separation tool in chiral and achiral compounds. The use of CE to discriminate between enantiomers dated back to the work of Gassmann et al. in 1985.²³ One of the important features of CE is its versatility in the kinds of separation modes allowing the separation of a wide range of analytes from macromolecules to small ions for many different applications such as food, pharmaceuticals, chiral, etc. Capillary zone electrophoresis (CZE) is the simplest version of CE and modification on this basic form creates other modes such as micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), capillary isotachopheresis (CITP), non aqueous capillary electrophoresis (NACE) and capillary electrochromatography (CEC).

CE has been proven as a powerful instrument in the chiral analysis area due to its low samples and solvents consumption, high separation efficiency, rapid method development, fast migration times, versatility, possibility of adding various selectors to the background electrolyte (BGE), and simple instrumentation. Chiral separation by CE is performed either indirectly by construction a diastereomeric pair with chiral compound that can be resolved under achiral condition or directly which separation is performed with addition of the chiral selector/selectors into BGE.²⁴

The frequently used CE modes in enantioseparation include; CZE, MEKC, NACE and CEC.²⁵ Gübitz and Schmid documented the recent progresses in chiral separation by various modes of CE.^{21,26,27}

MEKC is performed by solubilization of

pseudostationary phase such as surfactants into BGE. CEC is a hybrid form of chromatographic and electrophoretic principles, extends the utilization of chromatographic principles to CE-based enantioseparation. Selectors can be both bond on stationary phase or added into BGE. It can be useful for aqueous or non-aqueous buffers and those selectors with UV absorption or risk of mass-spectrometer (MS) detector contamination.²⁸

Among variety of CE modes, most of them utilize aqueous BGEs and this creates a constraint for the use of partially soluble selectors, therefore NACE is providing for this purpose. Additionally, non-aqueous solvents show good compatibility with MS detection. Also, NACE is beneficial for those sample preparation methods that analytes extract into apolar and viscose solvent. So, the need for solvent evaporation, back-extraction and/or sample reconstruction is eliminated and sample injection can be directly applied.

Capillary zone electrophoresis (CZE): Basic principle

The following section will be dedicated to CZE, as the most commonly used version of CE and also this mode is relevance in the present project. CZE is the most widely used mode of CE, developed since the late 1980s, which the mechanism is based on analytes differences in charge to size ratios and therefore differences in electrophoretic mobilities.

In CZE, the separation is performed by differential migration of analytes upon the application of a current through the capillary. Figure 2 shows the schematic view of a typical CE instrument. The apparatus consist of a fused silica capillary (usually around 25 to 100 μm internal diameter (i.d.) while both ends were immersed in buffer solutions (also known as a (BGE)). Electrodes are connected to both end of the capillary with a high voltage power supply. In a regular analysis, the sample is loaded into column at the anodic (positive) end and then with applying the voltage, analytes migrate towards the cathodic (negative) end, where the detector is located. The applied voltage also causes to make a difference across the capillary and results a migration of a bulk portion of liquid called electro-osmotic flow (EOF).

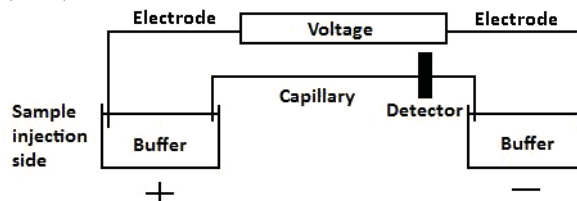


Figure 2. Schematic of a capillary electrophoresis unit.

Mechanism of enantioseparation by CZE

In direct separation mode in CZE, chiral resolution is performed by adding chiral selector into BGE. Among the various chiral selectors, i.e. chiral micelles, crown ethers, chiral ligands, proteins, oligo- and poly-saccharides, and macrocyclic antibiotics, cyclodextrins

(CDs) and CD derivatives exhibit acceptable results in chiral resolution for a wide range of analytes. More details about the mechanism can be found in references.^{29,30} CDs are cyclic oligosaccharides that become the most popular selectors in chiral separation due to their availability, UV-transparent, presence in charged and neutral forms and almost low cost. Commercial CDs are α -CD, β -CD and γ -CD which differ in the number of glucose units (i.e., α -CD contains 6 glucose units, β -CD has 7 glucose units (Figure 3) and γ -CD has 8 glucose units).

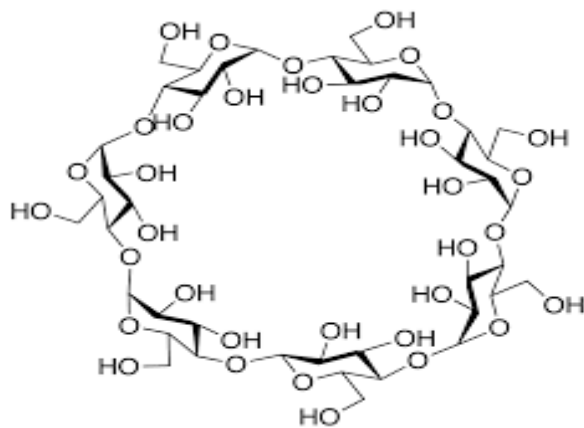
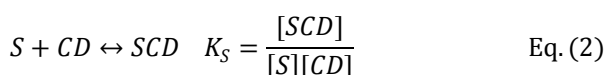


Figure 3. Chemical structure of β -CD.

Descriptions the theoretical background on how the separation occurred, present in equations 1 and 2 The thermodynamic complexation equilibria between the enantiomers R and S and the CD are defined by the complexation constants K_R and K_S , respectively, assuming the formation of a 1:1 complex between the enantiomers and the chiral selector.



Effective mobility μ_{eff} of an analyte is the sum of its portion traveling in the free form, μ_f and the complexed form, μ_{cplx} . The differential μ_{eff} between enantiomers indicates the separation and the fundamental equation developed by Wren and Rowe in equation 3.³¹

$$\Delta\mu = \mu_{\text{eff}}^R - \mu_{\text{eff}}^S = \frac{\mu_f + \mu_{\text{cplx}}^R K_R [C]}{1 + K_R [C]} - \frac{\mu_f + \mu_{\text{cplx}}^S K_S [C]}{1 + K_S [C]} \quad \text{Eq. (3)}$$

μ_{eff}^R and μ_{eff}^S are the effective mobilities of the R- and S-enantiomers, μ_{cplx}^R and μ_{cplx}^S are the mobilities of the complexed R- and S-enantiomers and [C] is the concentration of the CD.

Two main points can be driven from equation 3. Differences in enantiomers complexation constants cause to enantioseparation. Another important concluded tip from the equation 3 is that the chiral selector concentration plays a crucial role in enantioseparations

and should be in optimum value. In order to have chiral discrimination, enantiomers must show differing chromatographic interactions with the chiral selector whereas they have virtually identical electrophoretic mobilities in achiral media. The enantiomers are inserted into CDs hydrophobic cavity and based on their different interactions they can be resolved. Now these complexes differ in electrophoretic mobility due to their charge-to-size differences and thus resolved. Beside this inclusion phenomenon, the interaction between selector and selectant may included molecular interactions including; ion-ion bonds, dipole-dipole bonds such as hydrogen bonds, van der Waals forces and ion-dipole bonds. CDs are inherently chiral compounds, therefore, binding of enantiomers results in diastereomeric complexes. Several disadvantages are associated with indirect enantioseparation methods including; a) need of a functional group that can be formed a diastereomeric pair b) derivatization reagent should be in high level of purity and c) forming diastereomeric pair add time-consuming process to analyzing steps. The improvement in chiral separation is mostly associated with direct separation methods which are based on the interaction between a chiral selector and optical stereoisomer.

Detection in CE

The most commonly used detection system in CE is a UV absorbance detector, with detection at single wavelength, multiple wavelengths or even over a wide range of wavelengths using a photo diode array detector. There is a limitation in quantification of drugs via CE which is related to poor LOD (limit of detection) of CE in determination of drugs particularly in biological samples. This is caused by the shorter cell path length (typically 50 to 100 μm) and lower volume of sample injected in comparison with HPLC.³² In order to overcome these limitations, some strategies such as off-line/online preconcentration and/or replacement the UV detector with more sensitive one have been emerged. Utilizing more sensitive detection systems (e.g. MS, laser induced fluorescence (LIF) and electrochemical detection (ED)) have their drawbacks. For example, as many compounds have not possesses naturally fluorescent chromophore, a time consuming sample derivatization step is normally needed for LIF detection. Although, if the alternative sensitive detector was replaced, sample pretreatment step is still necessary especially in complex biological media.

Most instruments cannot handle complex sample matrices directly and therefore a sample pre-treatment step is vital in an analytical procedure. Sample pre-treatment step is intensively required for extracting, isolating and concentrating the target analytes from complex matrices.³³ Bringing the analytes to the detectable concentration levels is necessary before analysis. Sample preparation should be fast, compatible with analytical instrument, easy to use and with little solvent consumption.

Another option to improve the detector response is to increase the sample concentration prior to analysis. There are two practical strategies to pre-concentration, which are sample pre-concentration out of the capillary or performing on-line sample enhancing methods.

Off-line pretreatment

Sample preparation step including the extraction of interested analyte from the sample matrix which shows an important role in the case of biology related samples. Off-line pre-concentration methods are performed out of the analytical instrument and traditionally, there are two main techniques, liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Although, these methods are efficient but they are complicated, time- and solvent-consuming and tedious.^{34,35} Loss of sample or even sample contamination have occurred in utilizing SPE cartridges and cause to make errors in quantitative analysis. Utilizing large amounts of extraction solvent in LLE, relatively alters the polarity of an aqueous sample solution so it increases possibility of solubilization of lipophilic analytes in the aqueous phase. Furthermore, the phase separation is difficult in LLE. Therefore, miniaturization of extraction performance seems to significant try to stop this possible waste amount of analyte.

In order to overcome such limitations, simple, inexpensive and microscale extraction methods were emerged in the mid-to-late 1990s. Solid phase microextraction (SPME) is a simple and efficient technique, which reduces the need of using large volume of solvents.³⁶

Analytes adsorb on the fiber which is suitable to complex matrix such as plasma. SPME can be performed in two ways based on the position of the fiber relative to sample solution; direct immersion SPME (DI-SPME) that fiber was immersed within the aqueous sample and headspace (HS-SPME) which fiber was placed above the sample solution. Both DI- and HS-SPME techniques can be coupled with CE. In HS-SPME mode, fiber is not in direct contact with sample solution, therefore the fiber damage is diminished and generally more than 100 samples can be determined by the same device.³⁷ Apart from the easy manual operation and applicability of SPME for complex biological matrix, SPME shows some disadvantages for examples the fiber is quiet expensive and fragile and recommended for single use only and irreproducible results occurred due to the ageing of fiber and/or clogging the pores of cartridge.

In order to diminish the use of organic solvents in LLE performance, miniaturization of LLE was emerged in 1996.³⁸ Liquid-phase microextraction (LPME), as its name suggests, requires only several μL of solvent rather than several mL in conventional methods. The basic theory fall in the extraction of the aqueous analytes into small amount of organic phase. Thus, the equilibrium is attained in the short time than exhaustive

LLE or SPE. As analytes collected into small volumes of acceptor phase, the enrichment factor is high. The more enrichment can be attained with evaporation the solvent and re-generation the analyte in the low amount of suitable carrier. In the case of complex matrix such as biological one, the enrichment factor is highly proportional to recovery of the target analyte or in another word it belongs to clean-up ability of the sample preparation methods.

LPME falls in three categories;

1. Single-drop microextraction (SDME)
2. Hollow-fiber microextraction (HF-LPME)
3. Dispersive liquid-liquid microextraction (DLLME)

In typical SDME, the tiny drop of organic solvent was hanging from the tip of a syringe and immersed into an aqueous sample solution in order to extraction the analytes from aqueous phase into hanging drop.

HF-LPME developed by Pedersen-Bjergaard and Rasmussen in 1999³⁹ after some difficulties associated with SDME such as low stability of the hanging drop and reproducibility. In this method, the acceptor solution was placed on a membrane hollow fiber by dipping the supported membrane into organic solvent, therefore the analytes extract into extracting solvent. The concept of supported liquid membrane (SLM) is the same but differs technically which the aqueous sample and acceptor phase were pumped into a SLM through the syringes creating flow system.³⁹ As mentioned previously, the solvent consumption in LPME methods significantly decreased and in the SLM it is few volumes. SLM was used in limited number of paper maybe it is not affordable for most labs.

Electromembrane extraction (EME), firstly was developed in 2006, is a combination of HF-LPME set-up and voltage-assisted extraction of analytes to an acceptor phase.⁴⁰

DLLME, a novel technique for pre-concentration, was developed in 2006 by Rezaee et al.⁴¹ This technique is attracting much attention because of its simplicity of operation, rapidity, low cost, high recovery, and good enrichment factor.⁴² Briefly, a dispersion was formed after rapid injecting of extracting and dispersing solvents mixture, then sedimentation was separated with a centrifuge and finally was collected for analysis.

On-line enhancing methods

To overcome the relatively poor detection limit associated with commercially poor UV detectors, several reports are available based on electrophoretic or chromatographic principles.

Field Amplified Sample Stacking (FASS)

The principle behind FASS, is shown in Figure 4. sample is prepared in solution of lower concentration than the BGE. Upon application a voltage, sample ions migrate towards BGE region and are concentrated. This accumulation or "stacking" increases sample concentration and results in an increased signal.⁴³

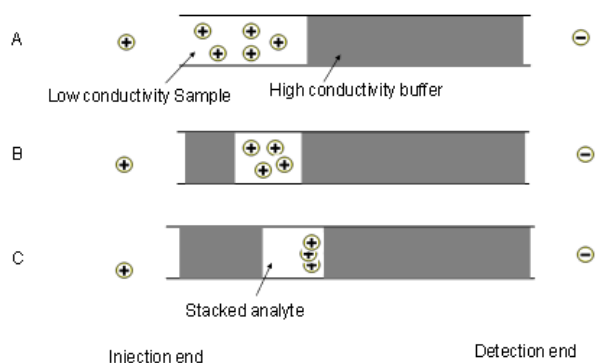


Figure 4. Schematic diagrams of a FASS model. (A) The sample is prepared in low conductivity media than BGE; (B) upon application of an electric field, sample is migrated through the capillary and stacked and (C) the concentrated analytes move toward the detector.

Large volume sample stacking (LVSS)

To improve the sensitivity, in contrary to normal injection, large amount of sample (dissolved in low-conductivity buffer) is loaded into capillary and is called LVSS. The large volume of sample matrix hinders the analyte stacking, therefore the sample matrix should be removed in order to enhancing the sensitivity (Figure 5). Accordingly, LVSS technique subdivided into two versions: matrix removal with or without polarity switching. In polarity switching mode, generally after stacking the analytes, the polarity is inverted to push out the matrix from the capillary. Then the polarity is changed to normal and stacked analyte migrated towards detection end by the aid of EOF.⁴⁴ Inversion the polarity may be not affordable for all of the commercial CE instruments or in some cases polarity cannot be changed instantly and it cause to create irreproducible analysis.

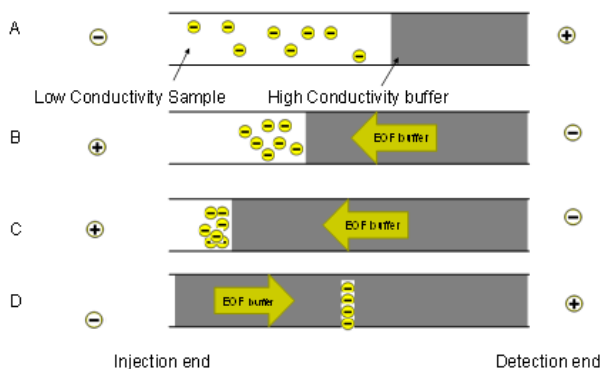


Figure 5. Schematic representation of the operated LVSS-CE method with polarity switching. (A) A large volume of the sample (dissolved in low-conductivity buffer) is injected hydrodynamically; (B, C) polarity is changed for a while and resulting EOF pushes the negatively charged sample matrix away from the column. The negatively charged analytes were focused at the sharp boundary due to a field amplified stacking effect and (D) when the sample matrix has been almost completely removed out of the capillary, the polarity was switched to normal mode and the analytes were separated by CZE.

Sweeping

Sweeping is characterized as a phenomenon which analytes was accumulated with the aid of pseudostationary phase (e.g. micelles, CDs, etc.).⁴⁵ Sample solution conductivity should be as the same as the BGE, thus the conductivity along the capillary is homogenous. Despite of the stacking which concentration was happened when sample zone and BGE have different conductivity, in sweeping mode the sample conductivity is not matter⁴⁵ but biological sample matrix effect should be regarded.⁴⁶ The schematic view of sweeping is shown in Figure 6.

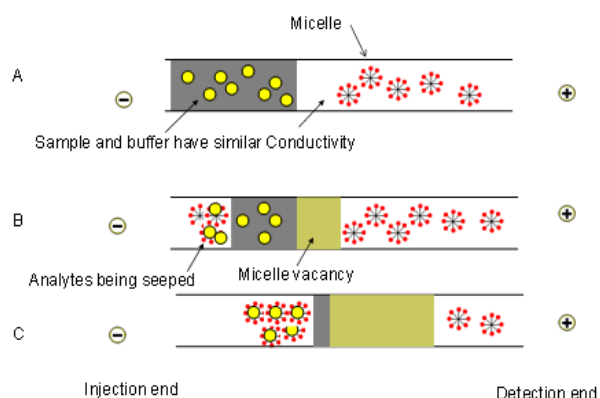


Figure 6. Schematic diagrams of a sweeping model with micelles. (A) The BGE consists of a pseudostationary (for example, assuming neutral surfactant) and electrolytes to form a micelle buffer, but the sample (assuming neutral) is dissolved in a surfactant-free buffer; (B) after the injection of the BGE and the sample solution, separation voltage is turned on and sample swept with surfactants. The mobility of pseudostationary is higher than its complex form, so the vacancy was appeared and (C) the concentrated analytes move toward the detector.

Isotachopheresis (ITP)

ITP is one of the well-known performed modes in CE. In contrary to other sample stacking versions, ITP is performed in the presence of two different conductivity buffers, leading and lagging electrolytes, which sample zone is surrounding by these buffers. After introducing the electrolytes and sample into capillary, voltage is applied (Figure 7). If the sample mobility is intermediate between electrolytes mobility, analytes is sandwiched between buffers and is focused. The principle of ITP can be applied to preconcentration in zone electrophoresis techniques (CZE, EKC), which is termed as transient ITP (tITP).⁴⁷

In transient "pseudo-isotachopheresis" (p-ITP), better known as "acetonitrile stacking" (AS) acetonitrile acts as "pseudo terminating ions" and on the other hand salts act as "leading ions". This method have dual benefits; removing the proteins and compatible with high salt samples. Additionally, AS can be applicable for those CE-based sample preparation methods which analytes were extracted into viscose or low volatile

solvent that needed to evaporate the solvent such as DLLME method.

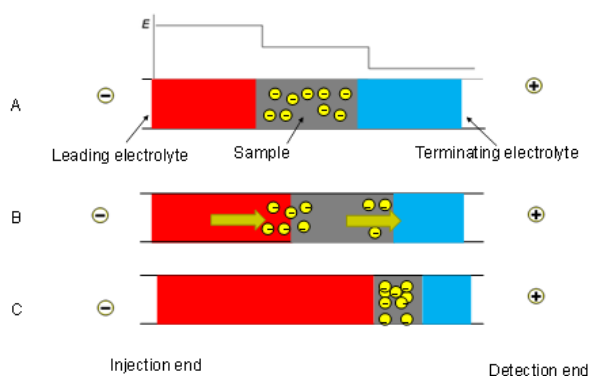


Figure 7. Schematic diagrams of an ITP mode. (A) The sample is introduced between leading and terminating electrolytes; (B) due to a difference in electrical field, ions will move faster when come in the previous zone, and slower when come in the next zone. Therefore, they will return to their "own" zone and (C) the concentrated analytes move toward the detector.

Partial filling technique (PFT)

In PFT, the capillary is filled with BGE without chiral selector. Then, it is filled with a short plug of chiral selector and finally the procedure is followed by introducing the sample solution (Figure 8). After applying the voltage, the separated enantiomers move

along the capillary towards the detector while they left chiral selector zone behind themselves.⁴⁸ In addition to increase the sensitivity, PTF prevents the contamination of ionization source by chiral selector. The summary of different types of on-line pre-concentration methods are listed in Table 2.

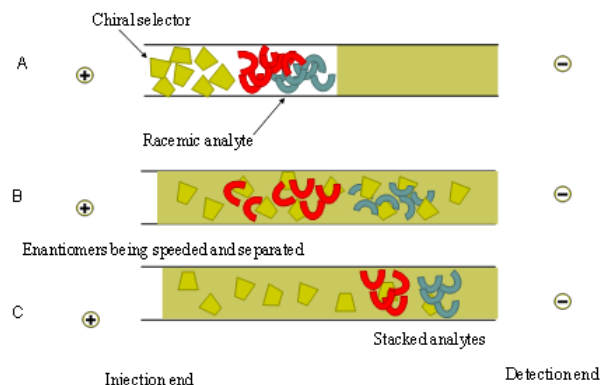


Figure 8. Schematic view of basic PFT performance by CE-MS. (A) Introduction of neutral chiral selector solution and the analyte, sequentially; (B) separation of enantiomers in the chiral selector zone and (C) migration of single enantiomers towards the detector.

Table 2. On-line pre-concentration methods using CE.

Method	Principle	Remarks
FASS LVSS	In stacking modes analyte from a low-conductivity sample migrates towards a high-conductivity zone and is concentrated. In LVSS mode, removing the sample matrix occurred with/without polarity inversion.	Separation only cations or anions in one run. Not applicable for neutral analytes. Easy to perform by simply altering the electrical conductivities of the sample and BGE.
Sweeping	Unlike the stacking methods, sample solution conductivity is close to BGE, and upon applying the electric field into capillary analytes accumulate by pseudostationary phase.	Applicable for neutral and charged analytes. This method concentrates those analytes which interact with stationary phase migrate (sample purification aspect). Application is limited by availability of appropriate pseudostationary phase.
ITP	Analyte migrates between the highest (leading ion) and lowest (terminating ion) electrophoretic mobility zones.	Analysis of neutral compounds is not applicable. ITP technique is applicable to charged analytes and simultaneous analysis of oppositely charged analytes is not possible. On the other hand, this limitation can be understood as an advantage in terms of selective removal of neutral or oppositely charged (sample purification aspect). Applicable to samples with a significant matrix ion (acting as a leader). Applicable for low conductivity samples with the aid of leading ion. Direct analysis of water soluble supernatants of precipitated proteins samples. Precipitation agent (e.g., acetonitrile) serves simultaneously as terminating ion (transient pseudoisotachophoresis).
PFT	Filling a discrete portion of the capillary with BGE containing chiral selector.	Low consumption of selector (vials do not contain selector).

Cardiovascular diseases (CVDs)

According to American Heart Association annual report, more than 50% of mortality is related to CVDs.⁴⁹ 25% of top 200 drugs advocates to cardiovascular drugs. On the other hand, CVDs are mostly associated with hypertension that approximately 1.56 billion people all over the world suffer from hypertension.⁵⁰ Many of cardiovascular drugs and their metabolites possess single or multi chiral center/s which have stereoselective interaction with corresponding targets.² It is reasonable that a chiral drug may show different therapeutic properties from its enantiomers or even racemic counterpart due to the different binding mode with biological targets.

Pharmacotherapy is the main management procedure of hypertension with one or more drugs from following classes: diuretics, β -blockers, β_2 -agonists, calcium-channel blockers (CCB), angiotensin-converting enzyme (ACE)-inhibitors, anticoagulants, sympathomimetic drugs, antiarrhythmic drugs and angiotensin receptor blockers (ARBs).⁵¹

β -blocker drugs are one of the most important class of drugs in the pharmacotherapy management of hypertension. Recently the use of β -blocker drugs for the treatment of cardiac arrhythmias, cardioprotection after myocardial infarction (heart attack), and hypertension has been tremendously increased, therefore enantiomeric analysis is essential to understand their stereoselective biological properties. Although thiazides and diuretics are proposed as the first-line therapy for most patients with hypertension, β -blockers have a successful indication in patients with high-risk conditions such as heart failure, myocardial

infarction (MI), coronary disease, and diabetes.¹⁶ Central inhibition of sympathetic nervous system outflow, inhibition of the renin-angiotensin system by reducing renin release from the juxtaglomerular apparatus, and sensitivity modification of baroreceptors are responsible for antihypertensive action of β -blockers.^{17,19} β -blockers also are used to treat other cardiovascular disorders such as angina, arrhythmias, bleeding esophageal varices, coronary artery disease, heart failure and migraines, and to prevent heart attacks.⁵²

Available β -blockers have one or more chiral centers. For those of β -blockers with one chiral center, the main biological effect is attributed to their (-) enantiomer. Enantioseparation of β -blockers because of therapeutic and forensics reasons has been gain much attentions. Nearly all β -blockers are used as their racemic form.

β -blockers are classified according to their selectivity on β_1 or β_2 adrenergic receptors. The non selective β -blockers including: propranolol, oxprenolol, pindolol, nadolol, timolol and labetalol. Selective β_1 -blockers including metoprolol, atenolol, esmolol, and acebutolol are recommended to whom β_2 -blocker associated with adverse side effects for them. Such patients are suffer from asthma or diabetes, or patients with peripheral vascular disease or Raynaud's disease.⁵³ In addition to therapeutic purposes, most of cardiovascular drugs include in prohibited list according to the World Anti Doping Agency (WADA) regulation. Stereospecific pharmacokinetics of some beta blockers in humans after single oral doses of the racemates are summarized in Table 3.

Table 3. Stereospecific pharmacokinetics of some beta blockers in humans after single oral doses of the racemates.

Drug	Dose (mg)	Isomer	C _{max} (ng/ml)	T _{max} (h)	AUC (ng.h/mL)	CL _R (mL/min)	t _{1/2}	Protein binding (unbound ratio -:+)	Ref.
Acebutolol	200	-	221±106	2.4±1.5	1380±380	91±36	7.6±4	1.0	57
		+	209±91						
Atenolol	50	-	226±136	2.7±1.1	1640±602	129±32	6.13	1.0	58
		+	251±138						
Carvedilol	25	-	34.2±22.5	0.67	125±66				59
		+	73.5±44.3	1.0	288±186				
Metoprolol	200	-			679±388	70±22	2.9±1		60
		+			408±409	75±22	2.8±1		
Pindolol	15	-	33±7	1.7±0.4	209±73	222±66	2.62	1.0	61,62
		+	36±10	1.6±0.5	244±90	170±55	2.85	In human plasma	
Sotalol	160	-	619±164	3.1±0.6	6760±1200	158±38	7.9±1.2	1.0	63,64
		+	615±167						
Propranolol	80	-	81.7±31		329±118		4.5±1.2	0.86	65,66
		+	46.5±25		217±114		5.2±2.4	In human plasma	

Abbreviations: C_{max}: maximum plasma concentration, T_{max}: time to reach C_{max}, AUC: area under the plasma concentration-time curve, CL_R: renal clearance, t_{1/2}: plasma half life.

Therefore, screening the low levels of enantiomers in biological fluids is important. Generally (S)-isomer of all β -blockers is more potent than R isomer in blocking β -adrenoceptors. (S)- Propranolol is a drug that is 100 times more potent than its R form. Complementary study shows that d,l- and d-propranolol can inhibit the conversion of thyroxin to triiodothyronin. This lead to conclusion that single d-propranolol can be contribute to reduce plasma concentration of T_3 in hyperthyroidism patients.^{54,55}

The pharmacological potency of (S)-verapamil, a member of CCB class, is 20 times more than (R)-form. On the other hand, it was discovered later that verapamil exerts application in cancer chemotherapy at high doses following high cardiotoxicity. This problem can be overcome using R-enantiomer in cancer chemotherapy as following studies reveal.⁵⁶ Table 4 provided the CE-based methods for quantification of chiral cardiovascular drugs.

Table 4. CE-based methods for quantification of chiral cardiovascular drugs in biological fluids.

Analyte (s)	Chiral selector	Pre-treatment	Sample/Volume	Organic solvent(s)/volume	CE mode	Linear range For single enantiomer	Ref.
Pindolol, Oxprenolol, Propranolol	DM- β -CD + TM- β -CD	Filtration and dilution	Urine/-	-	CE/FASI	0.12-750 nM	⁶⁷
Metoprolol, O-demethylmetoprolol, Metoprolol acidic metabolite, α -hydroxymetoprolol	CM- β -CD	LLE	Urine/-	Ethyl acetate/5 mL	CE/UV	1-50 μ g/mL	⁶⁸
Carteolol, Atenolol, Sotalol, Metoprolol, Esmolol, Propranolol	CM- β -CD	LLE	Human serum/250 μ L	Ethanol/250 μ L	CZE/UV/FASI	0.05-5 μ g/mL 0.25-25 μ g/mL 2.5-250 μ g/mL	⁶⁹
Labetalol	HDAS- β -CD + ODAS- γ -CD	SPE	Plasma/0.5 mL	CH ₂ Cl ₂ + isopropanol +NH ₄ OH/300 μ L	CE/UV	-	⁷⁰
Warfarin	Methylated β -CD	LLE	plasma/1 mL	CH ₂ Cl ₂ /5 mL	CE/UV	0.2-0.4 mg/mL	⁷¹
Warfarin	tapered capillary packed with 5- μ m (3R,4S)-Whelk-O1 CSP	PP and SPE	plasma/-	-	CEC/ESI-MS	0.1-5 μ g/mL	⁷²
Warfarin, Hydroxywarfarin	Poly-L,L-SULV	SPE	Plasma/250 μ L	-	MEKC/ESI-MS/MS	2-5000 ng/mL 5-1000 ng/mL 10-1000 ng/mL	⁷³
Warfarin	DM- β -CD	Salting out extraction	Urine/25 mL	ACN/25 mL	CE/UV /AS	5-500 ng/mL	⁷⁴
Arotinolol	Sodium taurocholat	SPE	Human serum/1 mL	-	MEKC /UV	50-500 ng/mL	⁷⁵
Clenbuterol	HE- β -CD	LLE	Human urine/2 mL	Hexane <i>tert</i> -butyl methyl ether/10 mL	+ CE/UV	0.5-10 ng/mL	⁷⁶

Table 4 Continued.

Clenbuterol	DM- β -CD	SPE	Plasma/3 mL	-	CE/MS/PFT	50-500 μ g/mL	⁷⁷
Celiprolol	S- β -CD	LLE	Human urine/1 mL	CH ₂ Cl ₂ + propan-2-ol/5 mL	CE/UV	0.06-0.14 mg/mL	⁷⁸
Oxprenolol metabolites	and HP- β -CD	LLE	Urine/5 mL	Etyl acetate /8 mL	CE/UV	0.4-16 μ g/mL	⁷⁹
Amlodipine	HP- β -CD	LLE	Human serum/1 mL	CHCl ₃ /1 mL	CE/UV	2-16 μ g/mL	⁸⁰
Amlodipine	HP- α -CD	SLM	Human urine/1.5 mL	-	CE/UV	10-500 ng/mL	⁸¹
Carvedilol	HP- β -CD	PP	Serum	-	CE/UV	50-4000 ng/mL	⁸²
Carvedilol	Succinyl β -CD + methyl α -CD	LLE	Plasma	Diethylether/1.1 mL	CE/LIF	1.56-50 ng/mL	⁸³
Carvedilol	HP- β -CD	PP and LLE	Serum	Ethyl ether	CE/UV	1000-6200 ng/mL	⁸⁴
Phenprocoumon	α -CD	Direct injection	Urine	-	CE/LIF	0.2-20 μ g/mL	⁸⁵
Propranolol	HP- β -CD	HS-SPME	Urine/5 mL	ACN/10 μ L	CE/UV/FASI	0.05-10 μ g/mL	⁸⁶
Propranolol	Poly (methacrylic acid-co-ethylene glycol dimethacrylate) monolithic capillary	In-tube SPME	Urine/1 mL	MeOH/25 μ L	CEC/UV	20-5000 μ g/mL	⁸⁷
Disopyramide	S- β -CD	PP and LLE	Plasma/0.5 mL	CH ₂ Cl ₂ /800 μ L	CD-EKC/ECL	5×10^{-7} to 2×10^{-5} mol/L	⁸⁸
Clenbuterol	β -CD	Centrifugation and Dilution	Urine	-	CE/UV/FASI-tITP	0.005-5 μ g/mL	⁸⁹
Metaproterenol, Methoxamine, Carvedilol	β -CD	PP	Serum/100 μ L	-	CE/UV/LVSS	05-3.5 μ g/mL	⁹⁰
Carvedilol	CM- β -CD	PP and DLLME	Plasma/0.5 mL	CHCl ₃ /100 μ L	CE/UV/FASI	12.5-100 ng/mL	⁹¹
Verapamil	CM- β -CD	PP and DLLME	Plasma/400 μ L	CHCl ₃ /120 μ L	CE/UV/FASI	25-350 ng/mL	⁹²

Abbreviations: DM- β -CD: heptakis (2,6-di-O-methyl)- β -CD, TM- β -CD: heptakis (2,3,6-tri-O-methyl)- β -CD, CM- β -CD: carboxy methyl- β -cyclodextrine, HDAS- β -CD: heptakis(2,3-diacetyl-6-sulfato)- β -cyclodextrin, ODAS- γ -CD: octakis(2,3-diacetyl-6-sulfato)- γ -cyclodextrin, HP- β -CD: hydroxypropyl- β -cyclodextrin, HE- β -CD: Hydroxyethyl- β -cyclodextrin, β -CD: β -cyclodextrin, poly-L,L-SULV: polysodium N-undecenoyl-L-Leucylvalinate, S- β -CD: sulfated β -cyclodextrin, PP: protein precipitation, LLE: liquid-liquid extraction, DLLME: dispersive liquid-liquid microextraction, SPE: solid phase extraction, SPME: solid phase microextraction, HS-SPME: head space solid phase microextraction, SLM: supported liquid membrane, CH₂Cl₂: dichloromethane, NH₄OH: ammonium hydroxide, CHCl₃: chloroform, ACN: acetonitrile, MeOH: methanol, FASI: field amplified sample injection, CE: capillary electrophoresis, CEC: capillary electrochromatography, ESI-MS: electrospray ionization mass spectrometers, ECL: electrochemiluminescence, LIF: Laser-induced fluorescence, MEKC: micellar electrokinetic chromatography, PFT: partial filling technique, LVSS: large volume sample stacking, AS: acetonitrile stacking, tITP: transient isotachopheresis.

Conclusion

Chirality becomes the major concern in the modern pharmaceutical industry. This importance can be ascribed to a growing awareness that racemic drugs may have different pharmacokinetic and pharmacodynamic profiles owing to the fact that the biological systems is chiral selective. The FDA in 1992 approved a guideline based on investigation of enantiomers of drugs for their pharmacological and metabolic pathways, separately. A complete statement is available at www.fda.gov/cder/guidance/stereo.htm.

Drugs with a chiral center consist of more than half of the currently used drugs. Understanding the contribution of individual enantiomers to follow the drugs action and concentration is necessary in drug development, pharmacokinetic and pharmacodynamic studies, TDM and forensic. Therefore, demands for accurate and precise analytical method for separation and determination of drugs enantiomers and their metabolites in biological fluids are highlighted. The sample pretreatment is vital to attain low matrix noise and enhancing sensitivity.

cardiovascular drug therapy is important in the management of CVDs. Description of *in vivo* behavior of these drugs and their pharmacokinetics (absorption, distribution, metabolism and excretion) have been in circulation for recent decades. As before is mentioned, it is attributed to selectivity of biological environment to different enantiomers. Enantiomers are considered as different compounds, thus it is worthy to follow their pharmacological, pharmacokinetic, and toxicological activities distinctly.

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Conflict of interests

The author claims that there is no conflict of interest

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