Review Article

Capillary Electrochromatography: A Versatile Tool for Biochemical Analysis

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Received: 12-05-2020; **Revised:** 24-07-2020; **Accepted:** 30-07-2020.

ABSTRACT

Capillary Electro chromatography (CEC) is a small scale separation technique which involves principle of both chromatography and capillary electrophoresis. Separation of analyte is done on the basis of differences in ratio of partition between stationary phase and mobile phase or due to differences in their electrophoretic mobility. Capillary Electro chromatography (CEC) combines the principles used in HPLC and CE. The mobile phases are driven across the chromatographic bed using electro osmosis instead of pressure (as in HPLC). This review describes the general introduction to CEC, the various advantages of CEC over HPLC and Electophoresis, instrumentation, history, various stationary phase and mobile phases, column technology and vast applications of CEC in a variety of fields.

Keywords: Capillary Electro chromatography, Instrumentation, Biochemical analysis.

INTRODUCTION

The Capillary electro chromatography is a modern hybrid analytical tool that combines the Principle of both high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) Hence it is important to first know what actually is HPLC and CE.

High Performance Liquid Chromatography is a type of column chromatography which uses columns in which packing material (stationary phase), pump which moves mobile phase through the column, along with the detector which shows the retention time of the analyte are present. HPLC is generally used in the separation, identification and quantification of active compounds. [1] It is very famous as well as popular analytical tool used to separate each constituents of mixture. The pressurized fluid and sample mixture both are passed with the help of pumps, thus both the mobile phase and sample enter into the column which is filled with solid adsorbent material[5]. Different modes of operation or types of HPLC are normal phase chromatography, reversed phase chromatography, size exclusion chromatography, ion exchange chromatography, affinity chromatography[1]. HPLC uses simple chromatographic mechanisms which are as follows: adsorption, partition, size exclusion and ion exchange.

Partition chromatography is said to occur when liquid stationary phase is not miscible with the eluent and that is coated on an inert support)[2]. When an ionically charged surface that is different from charge of sample is used it is termed as ion exchanged chromatography. When samples are separated by virtue of their molecular size it is termed as size exclusion chromatography. [6]

Electrophoresis can be considered as a method of separation based on the differential rate of migration of charged species in an applied electric field. An electrophoretic separation can be done by injection of a small band of sample into aqueous buffer solution, a high voltage is then applied along the length of buffer by means of electrodes. Two types of Electrophoresis are characterized i.e slab electrophoresis and capillary electrophoresis. Capillary electrophoresis is carried out with the help of phenomenon known as electro-osmotic flow. Electro-osmotic flow is generated when a high voltage is applied across a fused silica capillary tube containing buffer solution, and the bulk liquid migrates towards the cathode.[7] Thus in capillary electrophoresis the separation is performed in capillary column using electro-osmotic flow (EOF) as the driving force.[8] Capillary gel electrophoresis is miniature version of typical gel electrophoresis. Basic system of capillary gel electrophoresis consists of two buffer reservoirs, a capillary, a high voltage power supply, sample introduction device, detector. Electrophoresis is used to separate a wide range of biological molecules including amino acids, peptides, proteins, nucleic acids. Capillary electrophoresis has found its application in pharmaceutical analysis of basic drugs and related substances.[9] A capillary gel electrophoresis method has also been developed for fast and selective characterization and quantification of viral proteins. [10]

When electrophoretic and chromatographic adsorption combines it gives a more versatile tool for analysis that is capillary electro chromatography. Capillary electro chromatography can be defined as a separation technique wherein the flow of mobile phase is driven through a chromatographic column with the help of an electric field rather than pressure. Due to this the technique is a

combination of separation and selectivity potential of HPLC and high efficiency capillary electrophoresis.[11] This modern liquid chromatography uses electro-drive to improve chromatographic performance. Here the EOF drives the liquid through the column, which is movement of bulk of liquid except surface layers. This is unlike capillary electrophoresis wherein separation is mainly based upon the differential migration of charged species under an applied electric field.[12] Based on the interactions between solutes and the stationary, the uncharged and charged substances can be separated. There are different separation modes of CEC which can be classified as: ligand affinity, chiral recognition, ion exchange, hydrophilic partitioning and reversed phase. The main component of CEC are the columns. There are three types of columns characterized namely: packed, monolithic and open tubular. As compared to packed columns, monolithic and open tubular are mostly used due to its inherent advantages of unambiguous preparation and frit less design. Open tubular columns are very much suitable for introduction of innovative material as stationary phase. Monolithic columns offer higher efficiencies and resolution.[13] There are many advantages of CEC, these include: analysis of both neutral and charged solutes is possible, sample requirements are quite low, the amount of organic solvents used is also quite low, the separation efficiencies are also higher, shorter analysis time[8]. The band broadening in column chromatography is affected by a number of processes such as eddy diffusion, molecular diffusion, resistance to mass transfer, system effects. CEC is not much affected by eddy diffusion and resistance to mass transfer.[12] Other advantages of CEC include separation of neutral compound occurs by chromatographic mechanism(partition) and charged compounds get separated by both chromatographic as well as electrophoretic mobility, the plug like flow in EOF yields in reduced band dispersion, back pressure generation in the column is absent in CEC this results in extremely high efficiency.[14] To increase column efficiency and resolving power, smaller particles and longer column are utilized in CEC because velocity of EOF is independent of particle size in packed bed.[15]

There are a vast number of applications of CEC which include: drug analysis, sample analysis, chiral separation, enantiomeric separation, biological sample analysis, separation of drugs (such as NSAIDS, sulphonamides, cephalosporins etc). [13]

The review discusses about the history of CEC, basic theory or principle involved behind CEC, its instrumentation, its various stationary and mobile phases, columns and specific applications of CEC.

HISTORY

Strain in 1939 first reported the use of electro-osmotic flow in chromatography. Recognition of difference between electrophoresis and electro chromatography on one hand and partition of analyte between mobile phase and stationary phase on the other hand was also done by Strain. EOF is important in electrically driven chromatography was confirmed by the early work done on separation of large biological entities which utilized electro drive to aid movement of analytes through separative medium. It is possible to analyze basic, neutral, acid molecule using electro migration; this was reviewed and demonstrated by Strain and Sullvian. Separation of polysaccharides using EOF through colloidal membrane was reported by Mould and Synge. Theoretical foundation of CEC was laid in 1980s as well as significant progress began. CEC is possible to be performed using open tubular columns was reported by Stud and co-workers. [12]

1. THEORY AND PRINCIPLE

In CEC instead of pressure, electro osmosis is used to move the mobile phase across chromatographic bed [16]. Electro osmosis is the main principle or phenomenon behind CEC. Electro osmosis can be defined as a process which occurs when a liquid containing electrolyte moves to a stationary charged surface by the virtue of an applied electric field. The surface charge is usually due to ionization. [15] The generation of EOF in CEC is similar to that in CE. It occurs as follows: the stationary phase and the surface of capillary wall both possess charge, when a voltage is applied beyond the column due to accumulation of counter ions an electric double layer is formed, thus bulk fluid flow results, and hence formation of EOF. The value of EOF generated μ_{eo} is directly proportional to the zeta potential ζ which is due to the double layer. This is explained mathematically by following equation: [8]

$$\mu_{eo} = K_o K \zeta / \eta \quad [8]$$

Where, K_o = Permittivity of vacuum

K= Dielectric constant

η= viscosity of solvent

There are various factors that affect electro osmotic flow [15]. These are:

a. **Column Packing**- EOF generation is mainly dependent upon the packing of column. EOF velocity gets decreased in open tube packing. [15]

b. **Voltage-** According to the equation velocity and electric field strength μ_{eof} is directly proportional E or voltage V, considering the length of column (L) is constant. [15]

$$E = V/L$$

c. **pH of mobile phase-** With reduction in pH there is also reduction in μ_{eof} [15]

d. **Diameter of packing material-** The mobile phase velocity is independent on diameter of packing material. This is explained by Kozeny- Carman equation. [15]

e. **Organic groups attached to packing material-** Silica particles that are bonded to alkyl, octadecylsilyl groups are most commonly used stationary phase. [15]

f. **Composition of mobile phase**- EOF mobility gets influenced due to type and composition of mobile phase.[15]

INSTRUMENTATION

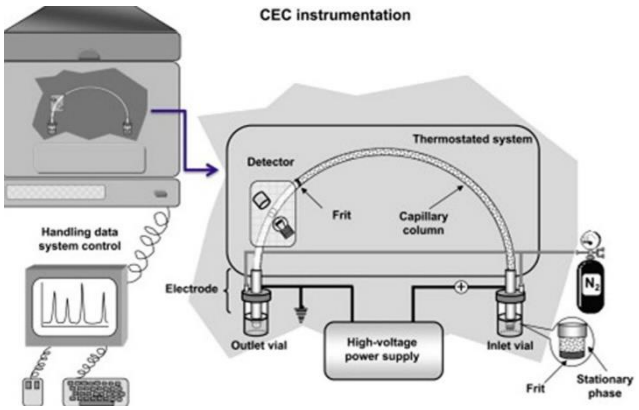

Figure 1: Instrumentation of CEC [3]

Figure 1 depicts the instrumentation of CEC. The CEC consist of some basic components i.e a system for electro kinetic injection, column, a high voltage power supply, and a detector.[12] The instrument also contain some additional components such as vials for solvents, inlet for column, waste outlet, a temperature control system, signal collection workstation.[14] There are two main types of CEC: Isocratic and Gradient. Gradient CEC can be further divided into 2 types: a. the one in which conventional liquid chromatography pump is present which drives mobile phase along the column. b. Two high voltage supply are used to control the EOF velocity and two different mobile phases are also used in this case. [12]

STATIONARY PHASES AND MOBILE PHASES USED IN CEC

Stationary Phases

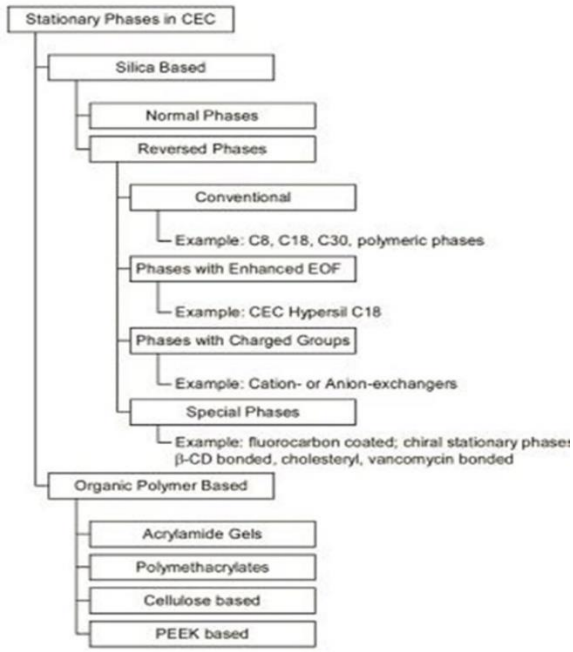

Figure 2: Stationary Phases used in CEC with their examples [3]

Figure 2 depicts the various stationary phases which are presently extensively in use in CEC. At first conventional HPLC stationary phases were used in CEC too. But this imposed certain problems such as these HPLC stationary phases were unable to develop stable EOF, this led to development of novel generations of stationary phase wherein there is advanced silanization. Thus, this advanced salinization reduces the number of free silanols which in turn generates the required EOF. [11]

a. Normal Phases

Normal phases are a type of stationary phases which are more polar in comparison to non polar stationary phases. Normal phase CEC was used by Lai and Dabek-Zlotorzynska to separate caffeine, theophylline, theobromine on silica. They used mobile phases such as isopropanol, hexane, ammonium acetate, acetonitrile which gave them efficiency of up to 63,000 plates/m. Maruska et al utilized columns that were packed with polygosil 100-10 silica as the stationary phase to separate non polar to very polar compounds. Maruska et al had used pure acetonitrile, methanol, ethanol, hexane as mobile phase. For the size exclusion system in CEC Stol et al., used LiChrosorb Si Silica and Nucleosil silica as stationary phase along with dimethyl formamide and lithium chloride as the mobile phase. Another successful separation was achieved by Ye et al; bare silica was utilized in the separation, which was coated with CTAB (cetyltrimethyl ammonium bromide). With the help of CTAB coating Ye et al was successful in separation of anilines and peptides.

One more remarkable and successful stationary phase that is hydroxypropyl-β-cyclodextrin was found out which showed cation exchange mechanism. Thus hydroxypropyl-β-CD was coated on bare silica and was used by Wei et al.[11]

b. Reversed Phase

There are 4 types of reversed phase stationary phase characterized.

Conventional reversed phase- Table 1 gives a list of commonly used conventional reversed phases. These are generally HPLC reversed phase stationary phases. These possess high silanol activity; these are mostly used because they provide a stable EOF. [11]

Phases with enhanced EOF- These are derived from conventional types itself. They provide a much stable EOF owning to its presence of very high silanol content. Hypersil C18 is an example of such type of stationary phase. [11]

Phases with charged groups- *This* type of stationary phase possess charges on silica surface. [11]

Chiral and special type of stationary phase- Examples of chiral stationary phase include, vancomycin bonded type, β-cyclodextrin bonded type, amino acid bonded type, naproxen bonded type, cellulose derivative type. Special purpose stationary phase includes antibody type,

fluorinated type, polymer coated type, ionenene coated type. [11]

Table 1: Complete list of examples of Reversed Phase-Stationary phase along with their manufacturers [4]

Reversed Phases	Manufacturers
Hypersil C18 Hypersil C8 Hypersil Phenyl	Hypersil ThermoQuest
Spherisorb ODS (type I, II) Symmetry Shield	Waters
Nucleosil (C18 and C8)	Macherey-Nagel
NPS (non-porous) Synchropack	Micra Scientific
LiChrosper, Purosper, Monospher, Chromspher	Merck
GromSil ODS	Grom
Alltech, Exsil	AllTech

C. Sol- gel Stationary Phase

Sol-gel technology for development of stationary phase is very versatile approach. It has a number of advantages. Sol-gel technology provide better homogeneity from raw material, low temperature of preparation, mixing is good for multi component system, control of particle size, shape and properties is possible, process occur in very mild conditions. Cortes et al was the first one to report that sol-gel technology can be used to create monolithic ceramic beds inside small capillaries. He used these capillaries as columns in liquid chromatography. The sol-gel technology was then successfully used to bind chromatographic stationary phase to inner surface of column. Reagents for preparing sol-gel stationary phase normally include one metal alkoxide (precursor), solvent in which the precursor disperses, a catalyst, water. Irradiation can also be another method for formation of sol-gel stationary phase. In irradiation method, precursor used is methacryoxypropyl trimethoxy silage, this precursor is irradiated with UV light. Reaction that takes place in formation of sol-gel stationary phase is that first the hydrolysis of precursors occurs, sol-gel active species in the solution undergo water or alcohol condensation, condensation continues to form a 3-D sol-gel network. General procedure involved in preparation of sol-gel stationary phase: Pre-treatment of capillary, fabrication of sol-gel stationary phase, post gelation treatment, characterization (using SEM, TEM, NMR, XRD etc).[17]

Mobile Phase

Figure 3 gives an overview of various mobile phases used in CEC. Generally mobile phases used in CEC comprise of a mixture if aqueous buffer, along with one or more modifiers (eg: acetonitrile). Sometimes non aqueous buffers are also used. Using non aqueous buffers have shown to decrease the stability of EOF. Acetonitrile is the of choice of modifier in the mobile phase because it has most optimal K/ η ratio.

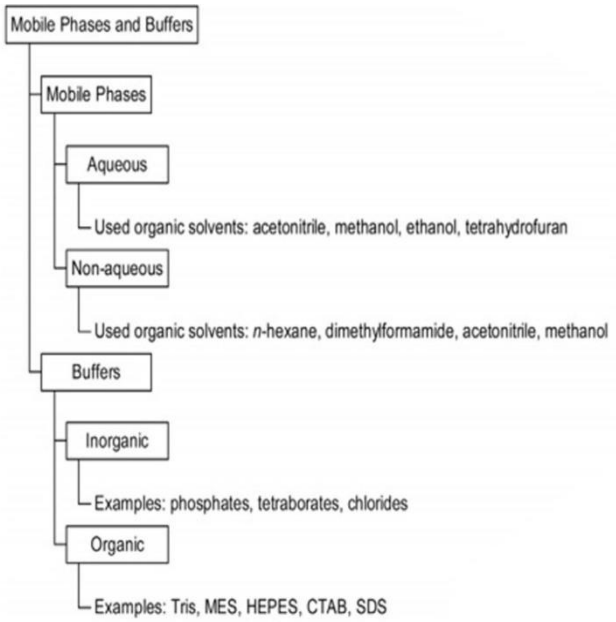

Figure 3: Various mobile phases used in CEC [4]

The values of EOF using acetonitrile systems are twice higher than for methanol and about thrice higher than THF. It has also been found out that EOF depend on the percentage of organic modifier in mobile phase. Among the non aqueous mobile phases commonly used are lithium and ammonium salts. Basically, non aqueous mobile phase is used because of samples possessing high retention properties as in fats or fullerenes. [11]

COLUMN TECHNOLOGY

There are 3 main types of columns used in CEC. *Packed bed columns*, *Continuous bed or Monolithic columns* and *Open tubular columns.*

Table 2: Success rate of packing of capillaries by different methods [20]

Packing method	Percentage of capillaries successfully packed and tested	Percentage of packed and tested column and gave satisfactory CEC
Super critical CO_2	70	75
Super critical CO_2 + ultrasonic probe	70	80
Liquid Slurry	75	75
Liquid Slurry + ultrasonic probe	70	80

Packed bed Columns

These types of columns are commonly used because a large variety of stationary phases are used. In this type of columns the stationary phase remains in the capillary. Columns are packed in a variety of methods such as slurry packing which is conventional type, electro kinetic packing, ultrasonic packing, and packing by super critical fluid, and using centripetal forces. Frits are the major disadvantages of using packed bed columns. Unlike HPLC, in CEC frits are required. In CEC stationary phases that are charged migrate under the effect of applied electric field and come out through the capillary, therefore frits at both the extremes (inlet and outlet) are required. The method by which the frits are made removes the protective polyimide coating, thus resulting in susceptibility of the column to breakage. [8] There are 3 main basic procedures for manufacture of frits. First by using a mixture of potassium silicate and formamide, then tapping capillary into the slurry of silica gel and potassium silicate, and lastly sintering the pure silica in the column post packing. After the frits are formed bubble formation within the packing of column or frits is the real problem. There are 2 theories which implicate the bubble formation, ohmic heating can result into bubble formation or other reason might be change in the EOF velocity. To prevent bubble formation low systems such as TRIS and MES are used, since these do not contribute to ohmic heating. Lelievre et al showed an alternative approach to decrease the bubble formation, this included use of high percentage of organic mobile phase.[12] The packing material that has to be used in CEC should be having a narrow size, should support the EOF for neutral as well as charged compounds. Most commonly used packing materials are CEC Hypersil C18 and Spherisorb ODS I.[18]

Continuous bed or Monolithic columns

With large column capacity, high efficiency and high selectivity continuous bed or monolithic column are much more convenient. According to fabrication method we can classify monolithic columns into two types: Direct copolymerization and incorporation strategy[13]. Monolithic column has been found to offer large advantage because no frits are necessary to hold the stationary phase in place. Thus, monolithic column is more robust. Some other types of monolithic column are also categorized into 3 types such as organic porous polymer, immobilized particles and silica sol-gel.[8] The organic polymer columns are prepared using polymerization of monomers in situ, by using UV. Acrylamide based polymers have been developed for CEC applications with the help of soft gel columns. These polymers have advantages such as providing stability over a wide pH range. Compounds such as peptides, proteins, aromatic compound have been separated. [8]

Open tubular columns (OT)

The walls of open tubular columns have a ligand attached to them, here the separation is achieved using chromatographic interaction and electrophoretic mobility. These types of column when used give smaller retention and they also have low sample capacities because of less surface area. Apart from these drawbacks, OT columns are easier to fabricate because of absence of frits, making it advantageous to use. When etched capillaries were used it showed improvements in resolution and high retention.[8] There are various methods by which open tubular columns are prepared these are adsorption (using cationic surfactant, polymeric surfactant, charged polymers), covalent bonding/ cross-linking, porous layers, chemical bonding after etching, sol-gel techniques, columns for chiral separation[19].

APPLICATIONS

Applications of CEC are vast and are applied to many fields such as: pharmaceutical analysis, biotechnological application, food and agricultural analysis, environmental analysis. [7] Other applications include enantiomeric separations, application in biochemical analysis (amino acid, peptides, proteins), and industrial applications. [21]

Specific applications of CEC are as follows:

To determine Sulfonyl urea herbicides using capillary electrochromatography

Sulfonyl ureas a newer class of herbicides which have been determined by the use of HPLC and GC. Earlier Dinelli et al used capillary zone electrophoresis (CZE) to detect sulfonyl urea in tap water. Krynitsky recently reported liquid chromatography-MS method for 12 sulfonyl urea herbicides. CEC serves as a connecting link between high efficiency of CZE and high selectivity of micro HPLC. Thirteen sulfonyl urea herbicide compounds were investigated using CEC. The 13 compounds are as follows: Sulfometuron methyl, Benzsulfuron methyl, Thifensulfuron methyl, Trisulfuron methyl, Chlorimuron ethyl, Methsulfuron methyl, Chlorsulsuron, Triasulsuron, Nicosulfuron, Prosulfuron, Sulfosulfuran, Primisulfuron, Halosulfuron methyl. These 13 sufonyl urea herbicides were successfully determined using Electropak column and 70% acetonitrile-30%ammonium acetate. [22]

To determine inorganic anions using capillary electro chromatography

As mentioned earlier CEC is very effective separation for charged species also. Separation of charged species is advantageous by using CEC because 2 mechanisms are combined: chromatography and electrophoresis. Li et al was the first one to report the separation of inorganic anions using CEC, I^-, IO_3, ReO_4^- were the entities whose separation was compared by the use of silica based strong anion exchanger. It has been reported that OT-columns have also shown successful separation of inorganic anions. This was shown by Nutku et al. Krohkin and co-workers studied the separation of inorganic anions and anionic metal complexes by the use of cationic polymers. [23]

Analysis of Phenols in mainstream and side stream tobacco smoke

CEC can be used to rapidly analyze quantitatively mono and dihydroxy phenols found in tobacco smoke. It is very much important to accurately determine the phenols produced during combustion of tobacco smoke. Many phenols and their derivatives have been identified in the smoke of tobacco, these are hydroxyquinone, resorcinol, catecol, phenol, o-, m-, p- cresols. These can be found both in the inhaled (mainstream) and exhaled (side stream). If gas chromatography is used in case of analysis of phenols in tobacco smoke, then it is disadvantageous. Due to high boiling points and polar characteristics of phenols require derivatisation using agents such as bis-N, O-trimethylsilyl-trifluoroacetamide. Thus, procedure is very time consuming hence a better option is CEC. Using CEC gives high resolution, good separation, short analysis time, minimal sample preparation, reduced operation cost. [24]

Analysis of Ketorolac and its impurities using capillary electro chromatography

Ketorolac (KT) is a chiral NSAID which is structurally similar to indomethacin. It has high efficiency and usually employed to treatment of short term pain. Its main mechanism of action is inhibition of COX (cyclo-oxygenase) enzyme. Ketorolac causes many serious side effects such as gastro intestinal disturbances. In its pharmaceutical dosage ketorolac has been reported to possess 3 impurities such as 1-hydroxy analog of KT, 1-keto analog of KT, decarboxylated KT. CEC has been found to be a very versatile method of identifying and separating the impurities of ketorolac even at very minute quantity. Thus easy quantitative analysis of ketorolac and its related impurities can be done using CEC. [25]

Analysis of Triglycerides using capillary electro chromatography

Analysis of triglycerides has been extensively done by liquid chromatography, super critical fluid chromatography. Nowadays CEC using reversed phase stationary phases have been employed for separation of triglycerides. In comparison to liquid chromatography, CEC offers much better resolution for triglycerides in vegetable oils. The analysis of triglycerides and determination of fatty acid have open new perspective for elucidation of unknown triglycerides as well as fatty acids determination.[26]

Separation of NSAIDS by using capillary electro chromatography

Successful separation of 10 non steroidal anti inflammatory drugs was carried out using packed capillary with RP-18 silica particles as stationary phases. The mobile phase comprised of a mixture of ammonium formate buffer at pH 2.5 and acetonitrile. Ten analytes that were analyzed includes: carprofen, cicloprofen, fenoprofen, flurbiprofen, indoprofen, ketoprofen, naproxen, suprofen and tiaprofen. Resolution, retention factor and efficiency were the parameters taken into consideration. All the ten NSAIDS can be obtained as baseline separation. Thus efficient and accurate separation of NSAIDs was possible using CEC. [27]

Enantioseparation of ten basic drugs using capillary electro chromatography

Separation of 10 drugs, as been possible with the help of CEC. β-cyclodextrin based open tubular CEC columns have been used in the separation. Drugs which were successfully separated based on enantioseparation are: zopiclone, chlorpheniramine maleate, brompheniramine maleate, dioxopromethazine hydrochloride, carvedilol, homatropine hydrobromide, homatropine methyl bromide, venlafaxine, sibutramine hydrochloride, terbutaline sulphate. 25mM Tris- phosphoric acid buffer of pH 2-3 was used as the mobile phase. Due to increase in EOF, it was found that increase in pH, migration time of all the analytes was decreased. [28]

Separation of Tetracyclines by capillary electro chromatography

Separation of various tetracyclines and their degradation products have been done both by HPCE (high performance capillary electrophoresis) and CEC (capillary electro chromatography). Minocycline, tetracycline, chlorotetracycline, oxytetracycline, doxycycline, methacycline, meclocycline are different types of tetracyclines which were separated using both HPCE and CEC. But HPCE showed many limitations as compared to that separation done by CEC. [29]

Separation of basic solutes by capillary electro chromatography

At low pH basic solutes were separated by CEC. Parameters such as solvent composition, pH, and temperature have effect on retention behavior of basic solutes. Thus basic drugs been successfully separated and investigated using reversed phase capillary electro chromatography. [30]

CONCLUSION

Capillary Electro chromatography is much more advantageous than HPLC and Capillary Electrophoresis along with many other different instrumental methods of analysis. Its applications are diverse which include enantiomeric separation, separation of amino acids, proteins peptides, carbohydrates. It also extends its application to pharma sectors which include identifying acidic drugs, basic drugs, along with vitamins, in industrial sector also it used in case of polymer science. Thus, CEC has very vast applications in various fields.

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Source of Support: None declared.

Conflict of Interest: None declared.