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Review

Biomedical Applications of Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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ABSTRACT: Capillary electrophoresis (CE) is a high-efficiency analytical technique that has had a great impact as a tool in biomedical research, clinical and forensic practice in the last ten years. Only in one of the applications, the DNA analysis, it has had an explosive exponential growth in the last few years. This impact is expressed in an enormous amount of CE articles and many reviews. The CE advantages with respect to other analytical techniques: the required very small sample volume, rapid analysis, great resolution power and low costs, have made this technique ideal for the analysis of a numerous endogenous and exogenous substances present in biological fluids. The different modes of CE have been coupled to different detection techniques such as UV-absorbance, electrochemical, mass spectrometry and laser-induced fluorescence detection (LIFD) to detect different nature and molecular size separated analytes. This review focuses mostly on the applications of CE–LIFD, to measure drugs and endogenous neuroactive substances such as amino acids and monoamines, especially in microdialysis samples from experimental animals and humans. CE–LIFD trends are discussed: automated faster analysis with capillary array systems, resolution power improvement, higher detection sensitivity, and CE systems miniaturization for extremely small sample volume, in order to make CE easier and affordable to the lab bench or the clinical bed. Copyright © 2001 John Wiley & Sons, Ltd.

Key words: microdialysis; amino acids; neuroactive substances; drug analysis; biological fluids; brain; human; rat

Introduction

Capillary electrophoresis (CE)

Capillary electrophoresis (CE) is a high efficiency analytical technique with a great impact in the biomedical research, clinical and forensic practice in the last decade. This impact has been expressed in an enormous amount of CE application articles and many review articles [1–5]. Only in one application, DNA sequencing analysis, the explosive growth is evident in the last two years [6–8].

CE development began almost four decades ago. In 1967, Hjertén accomplished the first electrophoretic separations in a 3-mm ID glass tube [9]. Mikkers *et al*. used a 200-μm bore PTFE tube for the separation [10]. In 1981, Jorgenson and Lukacs demonstrated successfully the high resolving power of CE in a fused silica capillary less than 100-μm bore, with high voltage and on-column UV detection [11]. And in 1984, Terabe introduced micellar electrokinetic chromatography (MEKC) to separate neutral compounds by solvophobicity [12].

CE is a technique based on the application of high-electric field on a narrow bore-fused silica capillary to separate molecules contained in

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complex mixtures in small volume samples. The separation in CE is driven by two forces generated by an electrical field: the electrophoretic mobility of the analyte and the electroosmotic mobility due to bulk flow generated by the double layer due to the silanol groups on the wall of a silica capillary filled with buffer. Although many times in the literature CE is referred indistinctly as capillary zone electrophoresis (CZE), the last one is considered the most used mode of CE. CZE is used to describe the method that allows the efficient separation of charged species based on minute differences in their electrophoretic mobilities. But CZE is not useful to separate structurally similar neutral compounds. Instead, MEKC, another CE mode, performs the separation of neutral analytes by adding a detergent above its micellar critical concentration to the running buffer to form a pseudo-stationary phase made of micelles. Neutral solutes are separated based on their differential partitioning between an electroosmotically-pumped aqueous mobile phase and the hydrophobic interior of the micelles, which are charged, and moving at a velocity different than that of the mobile phase due to electrophoretic effects [12]. In addition, CZE can be performed in gel-filled capillaries. In this case, the electroosmotic flow is suppressed and the ionic species are separated by seiving effect.

CE has been coupled to different detection methods including ultraviolet UV- visible absorption [11], conductimetry, mass spectrometry (MS), patch clamp, electrochemical (EC) [4,13] or LIFD [14,15]. This versatility of separation and detection methods has allowed analyzing a wide spectrum of substances ranging from ions to proteins.

CE comparison to other analytical methods. Sensitive analytical techniques such as high-pressure liquid chromatography (HPLC) and gas chromatography (GC) have been extensively used for the analysis of biological active substances. HPLC, one of the most used separation techniques has acceptable limits of concentration detection (LOCD), but requires at least a few microliters sample and liters of solvent. GC has an elevated number of theorical plates, that can be coupled to MS, but is limited to volatile compounds: it is not widely available because of its cost. CE separates different compounds such as inorganic ions, organic molecules and larger molecules, using the same instrument and in most of the cases using the same column changing only the composition of the mobile phase. CE has some other advantages such as the required very small sample volume, rapid analysis, great resolution power, and low cost. Finally, an additional advantage of the CE is the possibility of running several times the same sample, since only a nanoliter or less is injected every time. Besides, the resulting CE waste is safe for the environment, while HPLC produces large amounts of organic solvent waste, and GC volatile compounds, that are sometimes toxic which pollute the environment [3]. All these advantages convert CE as almost ideal for the analysis of numerous endogenous and exogenous substances present in biological fluids [4,16,17].

CE is the adequate analytical technique for applications that require analysis of low nanoliter samples and subfentomole quantities because CE has downscaled the sample volume to less than picoliters and the mass detection limits to the zeptomoles (10^{-21}) range [18,19]. Therefore, CE has been used for chemical analysis at a single cell and even subcellular level.

The present review is focused especially on the applications of CE coupled with laser-induced fluorescence detection (LIFD), to measure neuroactive compounds such as amino acids (AA), amines, and drugs in microdialysis samples from experimental animals and humans. CE trends towards a high throughput technique, concentration limit of detection improvement, and miniaturization are also commented upon.

Detection techniques coupled to CE

One of the most used detection techniques is UV absorbance [11,20]. UV absorption detection, although versatile has poor detection limits due to the extremely small pathlength characteristic of the fused silica capillaries. Besides, many compounds such as AA or monoamines cannot be detected by UV. MS detection gives high sensitivity, it is useful to separate and identify

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proteins, provides structural information but its high cost prevents more spread application as a routine analytical technique [6,21]. EC detection has better mass sensitivity; it does not need sample derivatization, and is tunable because its selectivity is a function of the potential applied [22]. But it still has poor concentration sensitivity and difficult manipulation that makes the technique the domain of the specialist in electrochemistry [4].

CE analysis for small volume samples such as individual cell or microdialysates of short collection time needs to be coupled to a high sensitivity detector. High sensitivity detectors included those based on MS, EC, LIFD. Since fluorescence intensity is dependent on both pathlength and incident power, the use of lasers as an excitation source greatly improves the detection sensitivity. Thus, one way to increase the sensitivity of CE has been LIFD detection [23,24].

Laser-induced fluorescence detection. It is the most sensitive on- and post-column detection mode available for CZE and MEKC. Gassman *et al*. in 1985 separated dansyl AA using for the first time CE coupled to LIFD; the detection was done with a He–Cd at 325-nm [25]. They obtained a limit of concentration detection at 10^{-10} M level. The introduction of post-column detection in a sheat flow cuvette [14] or collinear geometry [26] lowered the limit of concentration detection to 10^{-13} M. These improvements made single molecule detection the ultimate level of precision for CZE analysis [15,27–30]. As a consequence, the CE high resolution power and sensitivity within the zeptomolar range (10^{-21} M) made possible the successful use of CE-LIFD in endogenous neuroactive substances, DNA and illicit drug analysis [5,6,31].

For the LIFD, it is necessary to have fluorescent analytes, but most of them do not have native fluorescence. There are two ways to detect substances that do not have native fluorescence. The first is through the use of indirect detection methods using a fluorescent running buffer in which the analyte displaces or pairs with the fluorescent molecules. Indirect laser-induced fluorescence detection is based upon charge displacement and is not based upon any absorption or emission property of the analyte. Indirect laser-induced fluorescence detection has been used to detect charged species and does not need pre- or post-column derivatization treatment [32–33]. Currently, indirect laser-induced fluorescence detection is used less because its sensitivity is lower than the one obtained with direct laser-induced fluorescence. The second is by derivatization. This means to change the analyte structure with a derivatizing agent to make the analyte fluorescent and detectable. The peaks of radiation absorption of the fluorophore should match the wavelength of the laser in order to reach maximum sensitivity. Thus, LIFD requires a simple sample pre-treatment with a dye to make the analytes fluorescent in order to detect them [14,15,29].

The best reagent for primary amines (amino acids and peptides) would be one with a high quantum yield, rapidity of derivatization, and stability. There are several different derivatizing agents that have been used so far. The *o*-phthaldehyde (OPA) that produces fluorescent isoindoles is detected in the fentomolar range [34]. Naphtalene-2,3-dicarboxaldehyde (NDA), an analog of OPA, with higher quantum yield, and 3-benzoyl-2-quinolinecarboxaldehyde (BQCA) have more stable derivatives than OPA [35,36]. 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CNBQCA) forms highly fluorescent isoindoles, just as OPA and NDA, but with advantages such as detection limits in nanomolar range, fast reaction time and good product stability [37]. OPA, NDA, CNBQCA and BQCA products are detected with He–Cd laser. The agent 3-(2-furoyl)-quinoline-2-carbaldehyde (FQCA) produces fluorescent isoindoles with an excitation maximum near the 488-nm induced by an Ar-ion laser [38]. Fluorescein isothiocyanate (FITC) is the derivatizing agent with higher quantum efficiency compared with the previously mentioned agents. FITC is useful to produce AA derivatives, relatively easy to form, with good electrophoretic properties and strong fluorescent signals induced by the 488-nm line of an Ar-ion laser. But FITC requires longer times of derivatization than the other previously mentioned dyes. It is necessary to have at least 16–18 h of incubation to obtain stable and well detected FITC derivatives [29,30]. CE-LIFD analysis of FITC derivatives gives subattomolar determination of AA mixture,

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which represents few thousands of injected molecules [14,27,29,39,40].

There are three criteria to choose a laser. First, the wavelength of the laser should match the peak of absorbance of the analyte, while the second that of spatial mode quality. The beam should be focused on a small spot to excite the analyte efficiently. Third, laser power above 20 mW is well suited for high sensitivity fluorescence detection for CZE [27]. He–Cd and Ar-ion lasers are relatively inexpensive and easy to operate and feature output radiation in the visible range, 442 nm for the He–Cd, and 457, 488 and 514 nm for the Ar-ion. Therefore, the main inconvenience of LIFD is the limited number of laser wavelengths available. Nevertheless, there are multiwavelength lasers, which are more convenient because of their wider range of detection but they are much more expensive and energy consuming. One way to increase the sensitivity of the LIFD is to use a collinear optical arrangement with high numerical aperture collecting lenses. This geometry improves the signal-to-noise ratio [26].

CE coupling to microdialysis

Part of the success of CE application in the biomedical field, basic and clinical research is due to the coupling to microdialysis. Microdialysis is a valuable *in vivo* tool in basic and clinical research in animals and humans, especially in the neuroscience field [15,31,41–46]. The microdialysis probe monitors continuously the extracellular environment by solute exchange through a porous membrane following chemical gradients. In this way, endogenous substances such as neurotransmitters and exogenous analytes such as drugs can be extracted from tissues or body fluids. Since microdialysis samples are pure ultrafiltrate without protein, deproteinization and ultrafiltration of samples prior to the analysis are not required. This makes possible direct injection of the sample to the analytical apparatus making the analysis easier and faster [22,47].

The temporal resolution of microdialysis depends on the sensitivity of the analytical technique, the sample volume requirements of the analytical system and the recovery of the microdialysis probe [48]. At first, most of the microdialysates were analyzed by HPLC. This technique requires a relatively large sample volume, which increases the microdialysis sampling time, and reduces the microdialysis temporal resolution [49]. Thus, CE with its very small sample volume requirements, high-resolution power, high sensitivity detection method is the alternative to HPLC in order to analyze microdialysates collected in very short times, at very low flow rate to increase the analyte concentration in the sample. In this way, it is possible to improve microdialysis temporal resolution [15,22,29,31] from 20-min sampling period to a few minutes or even seconds [30,50,51]. The on-line coupling of microdialysis with a continuous flow derivatization has been accomplished preserving the high temporal resolution of microdialysis and preventing losses of sample. This technical improvement of the microdialysis and CE coupling requires very short derivatization periods. Therefore, it is critical to use derivatizing agents such as OPA [52–54] or NAD [39,55,56] to produce the fluorescent derivatives in reaction times as short as a minute. This it is not possible using FITC. The on-line derivatization can also be performed in a conscious animal [39].

Summary

CE is a highly efficient analytical technique that separates molecules of different nature and size contained in small sample volume. It is a simple, rapid and not expensive technique. It needs a smaller sample volume than HPLC and its cost is much lower than GC. CE can be coupled to different detection methods: UV, EC, LIFD or MS. LIFD is one of the most popular detection methods because of the detection power, and a simple sample derivatization. This procedure uses different dyes according to the type of the analyte, to make it fluorescent and detectable with the appropriate wavelength laser. Coupling CE with microdialysis, an *in vivo* technique, allows monitoring with high temporal resolution, the biochemical extracellular environment. The pure ultrafiltrate microdialysates make easier and faster the analysis by averting time-consuming sample pre-treatment.

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Biomedical applications of CE

Pharmaceutical, clinical and forensic applications

In the last few years, CE has been extensively used as a separation technique in the pharmaceutical industry [4,17,49,57,58], and in the clinical chemistry modern laboratory [59–63]. This success is due to, its quick analysis time, low costs, small sample volume convenient for microdialysates and capillary blood samples, the versatility to resolve different molecules and high resolution power [16].

At present, CE is considered an established method for the pharmaceuticals analysis. CE applied to the drug analysis in complex biological matrices has replaced HPLC in many cases. There are dozens of recent reviews and hundreds of articles that focus on the CE pharmaceutical and biological applications, some with emphasis in the method development [1,64–66]. Nishi reviews the growth of CE application in the analysis of pharmaceuticals: the main components, natural medicines, besides the measurement of physicochemical properties of drugs [1]. Boon *et al*. present a review of more than 200 bioanalysis of drugs by CE, at this moment it must be larger than the number of drugs analyzed [65]. De Lorenzi reviews the strategies for the analysis of drugs and metabolites in body fluids such as capillary methods of sample concentration and single-step analyses with direct injection of body fluids on-column, and the different modes of CE applied to the drug analysis [66]. CE emerges as a promising, effective and economic approach for the enantioselective determination of drugs in body fluids. Currently, there is a great interest in the separation of enantiomers, which are biologically important compounds. It is important to study the relation between chirality and a drug therapeutical efficacy, drug metabolism and pharmacokinetic investigation [67–69]. Hadwiger *et al*. were the first to employ CE–EC to analyze enatiomers of isoproterenol in microdialysates by adding a chiral agent, to the running buffers [70]. CE coupled to microdialysis has allowed evaluating the pharmacokinetics and pharmacodynamic characteristics of drugs [71,72].

Since the commercial availability of CE in the late 1980s, the CE applications have moved from the laboratory of analytical chemists to the laboratories of the clinical chemists [47,60,73,74]. CE is useful as clinical tool in the diagnosis and course of metabolic disorders such as phenylketonuria [75], homocystinuria or glutathione synthase deficiency [16] or porphyria [47].

Prescription, illicit drugs or other substances analysis. *Prescription drugs and other substances*. CE coupled to EC or LIFD has been successfully employed to the analysis of very diverse types of drugs. The drug companies frequently use CE to monitor the main components in pharmaceuticals preparations [76]. CE has been used for the separation of barbiturates, cardioactive drugs, benzodiazepines, anticonvulsants, diuretics and xantines or antibiotics [77,78], or for single separation of particular drugs [47]. One of the first intents to separate B6 vitamers with native fluorescence in biological fluids was done with MEKC–LIFD [79]. Then, methotrexate, an antineoplasic drug, was analyzed in patients' serum by CE–LIFD. The drug was detected with a He–Cd laser in 10^{-10} M range concentration, two orders of magnitude lower than other techniques at that time [80]. Riboflavin vitamers in human plasma have been determined by MEKC–LIFD far below the physiological concentrations [81]. Drugs used in neurological diseases have been analyzed by CE–LIFD. Gabapentin a new anticonvulsant has been determined in FITC derivatized microdialysates of rat plasma [82]. Valproic acid, another anticonvulsant, has been analyzed in human serum with a detection limit of 0.9 μg/ml [83]. Amantadine was detected in human plasma, with a mass detection limit of 9.5 fmol–115 attomol, previous derivatization with Cy5.29.Osu [84]. Baclofen, a GABA agonist on GABA B receptor, used in spasticity conditions, was detected in nM level with He–Cd laser [85]. The pharmacokinetics of diverse drugs such as, antibiotics, expectorants, anti-inflammatory and analgesics has been quantified and monitored mostly by CE–LIFD [86–91]. CE–LIFD has been used for the other type of screening, such as carbohydrates in urine, cancer screening in urine [92,93], even for selective detection of unusual substances as bacterial enterotoxins [94]. Some

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other prescription drugs such as cloramphenicol, clozapine, and metronidazol, and body proteins, such as myoglobin have been analyzed by CE–EC in human blood or urine [95–98]. The monitoring of plasma levels of antineoplasic drugs in pediatric patients is done by CE analysis of capillary blood samples [99]. The use of microdialysis coupled to CE has allowed access to the previously inaccessible information of drug distribution in humans. Pharmacokinetics of L-DOPA, a drug used in Parkinson disease, was detected by CE–EC in intravenous dialysates after administration. Detection limit was 156 attomol [22]. 5-Fluorouracil, an antineoplasic drug was monitored in tumor and subcutaneous adipose tissue of patients with breast cancer who received the chemotherapy [100]. For more specific information about CE and drug analysis, look for recent reviews [1,3,4,65].

Illicit drugs. CE is also a new powerful tool for the analysis of illicit/controlled drugs in biological samples [5,101]. At present, CE is applied on a routine basis in forensic laboratories, and it is used successfully in the screening procedures for toxicological or doping control [74,102]. Many drugs have been tested for these purposes in biological fluids as urine or plasma. MEKC–UV detection has been used to separate impurities in illicit drugs such as heroin and cocaine [103,104]. Multistep solid-phase extraction of different classes of drugs from a single aliquot of urine followed by sequential MEKC with polychrome UV absorption detection provides a rapid and effective way to confirm multiple drugs of abuse in human urine: barbiturates, opiods, cocaine metabolite, amphetamines, methaqualone and cannabinoids [105]. The combination of CE–MS has also been used for opiods and codeine screening [106]. CE–LIFD is also suitable for screening of illicit drugs in urine or blood. Tramadol [107], metamphetamine [108] and LSD [109] have been determined by CE–LIFD. This combination of techniques was used to detect amphetamine in rat brain microdialysate after its systemic administration. The mass detection limit was 3 attomol [110].

DNA analysis. One of the most successful applications of CE is the analysis of biopolymers (nucleic acids, proteins and peptides, lipids and carbohydrates). Without doubt, DNA analysis is the most prominent application of CE–LIFD in gel-filled capillaries. Millions of theoretical plates per column and high sensitivity have made CE the method of choice for the sequencing of the human genome [3]. In order to apply genetic analyses to large groups of patients or population screening, automation of a sensitive and precise method is highly desirable. CE facilitates the development of methods, which can rapidly process large number of patient samples in an automated fashion [6,8]. The CE instruments in the market for DNA sequencing operate 96 capillaries simultaneously and automatically, and generate about 1000 electropherograms a day which means millions of pairs of bases analyzed per day. This makes this technique the most important analytical tool in use today in the DNA analysis field [6]. There are at least 65 reviews in the last 5 years and 24 reviews in the last two years alone about this CE application. Therefore, this topic is beyond the scope of this paper [6–8,111–113].

CE–LIFD coupled to microdialysis studies. Neuroactive substances analysis

Microdialysates from animal or human's brain, other tissues or body fluids have been used especially to determine basal concentrations and changes of neurochemical compounds such as AA, catecholamines (CA) and other neuroactive substances. Traditionally, the microdialysates were analyzed mostly by HPLC. The microdialysis flow-rates were 1–5 μl/min and the collection times were between 5 and 30 min to be adapted to the minimum requirements of sample volume for HPLC analysis. These flows produce great depletion of the chemicals around the probe. On the other side, the neurotransmitters are released and reuptaken in fractions of a second; therefore, the analytical technique to study the release has to be fast and sensitive enough to allow the analysis of small sample volume collected in very short times. The appropriate technique for this is the CE. Thus, it is possible to reduce flow rate and maintain short collection times. CE requires only nanoliter volumes or less for injection while offering high sensitivity, high separation efficiency and rapid

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separations [29,31]. CE coupled to UV detection is not convenient for endogenous compounds such as neurotransmitters including: AA, amines and peptides, because of the low sensitivity of UV detection. Instead, CZE and MEKC have been used coupled to EC [4,114,115] or LIFD [29,116–120] for those analysis. The most convenient and sensitive detection method is LIFD, for the majority of neuroactive substance analysis. Most of the fluorescent agents react with primary amines while a large number of the neurochemical constituents of brain microdialysis samples could be detected [29,117,118]. The samples obtained through microdialysis are ultrafiltrates. They can be injected directly into the analytical system, making the deproteination procedures unnecessary. To speed up the analysis and prevent losses of sample, the derivatization can be done coupling microdialysis on-line [39,52,53,55,56].

In the last decade, most of the determinations of classical neurotransmitters such as AA and monoamines, peptides and other neuroactive compounds have been done with CE–LIFD [29,30,37,51,117,118,120]. The use of CZE–LIFD in combination with brain microdialysis and CSF analysis has shed some light on central nervous system (CNS) function as well as its disorders.

Amino acids. The AA have been analyzed by CE–LIFD with CZE or MEKC. The different AA possess different chemical properties basic, acidic, polar, hydrophilic, and hydrophobic which complicates the separation by a single method. The combination of CZE high efficient separation and LIFD high sensitivity detection, in a fused silica capillary filled with alcaline carbonate buffer, is especially good for acidic AA such as glutamate (Glu) and aspartate (Asp), and for basic AA such as arginine (Arg) and lysine (Lys). The detection limit reaches the subattomol level [14,15,26,29,30,116]. The applicability of CE may be enhanced if the spectral analysis is incorporated. Simultaneous measurements of CE fluorescence AA peaks and their spectra were obtained by adding to the CE equipment a monochromator and a charge-coupled device (CCD) [121].

The harmonic function of the CNS depends mainly on the balance of excitation and inhibition which depends on the balance of excitatory neurotransmitters such as Glu and Asp, and inhibitory neurotransmitters such as gamma aminobutyric acid (GABA) and glycine (Gly). Glu, the main excitatory transmitter in the brain is related to complex cerebral functions such as memory and learning and also to CNS disorders such as schizophrenia and brain damage by excitotoxicity. Glu and Asp can be analyzed in complex matrices such as brain dialysates, during the emission of different behaviors by CZE–LIFD in freely moving rats [122,123]. Using a collinear LIFD, Glu was detected in the nanomolar range in 25 μm id capillaries. Since the injection volume was 23 pl, the mass detection limit is in the zeptomole (10^{-21}) range. In addition, by reducing the migration time to a minute or less, large throughput can be achieved [15,26,29]. In these conditions, Glu has been measured in 100 nl brain dialysates collected every 6 s from anesthetized rat [30]. Lada *et al*. measured Glu and Asp changes in rat brain microdialysates collected every 5 s after electrical stimulation, or tetrodotoxin [54]. This improvement of time resolution of brain microdialysis might be necessary to enhance the comprehension of the mechanisms of neurotransmitter release when *in vivo* monitoring techniques are used. The fluorescence signals in the LIFD of derivatized Glu and Asp can be increased by the addition of ciclodextrines to the running buffer [124]. Basic AA, such as Arg, the precursor of nitric oxide related to the neurochemistry of pain, can also be analyzed by CE–LIFD [125].

GABA is the main inhibitory AA in the CNS and it has been related to neurological and psychiatric disorders such as epilepsy and anxiety. Gly is also an inhibitory AA, but is also related to glutamatergic function. Both AA are non-polar or neutral and they cannot be separated by CZE. But they are separated by MEKC that involves partitioning the analyte between the electrophoretic buffer and a 'stationary phase' composed of micelles [37,117,120,126]. Bergquist *et al*. by using CE–LIFD, separated GABA and Gly plus eight more AA in normal and pathological human cerebrospinal fluid (CSF); GABA detection limit was in the subnanomolar range, and the detected differences in the AA could be attributed to disease or age.

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Thus, CE–LIFD in CSF analysis provides a rapid, sensitive method for diagnosis and of the mechanisms of CNS diseases [37]. The same technique was applied in the rat brain microdialysates to analyze other AA besides GABA and Gly. Glutamine (Gln), valine (Val), alanine (Ala), Arg, Glu and Asp were detected in the nano and subnanomolar range [117]. We identified and detected neutral AA by MEKC–LIFD in human brain and plasma microdialysates with a better resolution, by modifying the Bergquist buffer conditions and using a smaller one inside the diameter capillary. The brain microdialysis was done in a patient during surgery for treatment of Parkinson's disease. It was possible to monitor GABA changes during the procedure [120]. GABA, Glu and Asp changes in nM concentrations were monitored by CE–LIFD in rat brain microdialysates after peripheral administration of dexfenfluramine [119]. GABA changes were determined by microdialysis and CE–LIFD after the local administration of morphine in rat brain [126]. MEKC coupled to LIFD is the most used combination for GABA analysis [37,117,120], although the detection has been done in some cases with other techniques such as MS [21]. We performed in vitro microdialysis of plasma or CSF before the AA analysis by CE–LIFD. The procedure gave us ultrafiltrates, making the pre-treatment of samples unnecessary and reducing the sample volume that has to be collected. This is very convenient when we need to analyze blood in pediatric patients or collect CSF samples [75,120].

Several other AA with proved or possible role in neurotransmission have been determined by using MEKC–LIFD in microdialysates or biological fluids. Tirosine (Tyr), phenylalanine (Phe), threonine (Thr), isoleucine (Ile), leucine (Leu), serine (Ser), methionine (Met), taurine (Tau), Val, Ala and Gln have been determined simultaneously with Glu, Asp, Arg and Lys in the same sample [37,75,117,120,127]. The simultaneous analysis of AA in biological fluids by CE–LIFD gives characteristic AA profiles. These patterns permit at a glance to recognize errors of metabolism such as phenylketonuria with a high level of Phe and reduced level of Tyr [75].

The thiols have importance in pharmaceutical, clinical and biological fields, and they can be determined by CE–LIFD. The thiols are AA that contain sulfur such as Met, cysteine (Cys), cystine, and the peptide gluthatione. Monitoring in vivo the thiols glutathione and Cys derivatized on line were found in the low micrometer range in rat caudate dialysates [128]. Met, Cys and homocysteine in plasma are analyzed by CE–LIFD of routine determination of clinical studies [129–131].

Catecholamines (CA). The CA including noradrenaline (NA), adrenaline (A) and dopamine (DA) are the most studied neuroactive compounds in the nervous and endocrine systems. They have been extensively analyzed by HPLC with EC in the past two decades. CA were one of the first analytes to be separated by CE. Since CA exhibit nearly ideal electrochemical properties for detection purposes, such as easy oxidation to a low potential, CA are detected by CE coupled to EC amperometric detection [4,13,114]. With the same technique it is possible to resolve the monoamine serotonin from DA [115]. DA was detected in brain microdialysates by CE–EC with low-mass (attomol) detection limit, and low-concentration (nM) detection limits [132]. The CA have been analyzed with CE–EC even in single cells. DA was determined as a part of the cytoplasm or in the whole cell. DA was measured in a large dopaminergic cell in the brain of the pond snail *Planorbis corneus* [133] and in single lymphocytes, which indicates that CA are not the only ones produced in the adrenal glands or nervous tissue [134,135].

CE is also used coupled to LIFD to determine NE, E and DA in microdialysates and single cells. In microdialysates, the CA determination by CZE–LIFD has been performed in low concentration, small volume samples derivatized with NAD to give highly fluorescent compounds. Robert *et al*. [136] and Bert *et al*. [137] have accomplished very reliable and sensitive determinations of CA. NE detection limit was 5×10^{-9} M in rat medial frontal cortex microdialysates [136]. NE detection with derivatization on-line of 2-min microdialysates reached high microdialysis temporal resolution [50]. In dialysates collected in 30-s periods, the detected levels of DA were in attomol and for NE in zeptomol ranges [51]. The performance of the method is

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demonstrated *in vitro* by monitoring rapid fluctuations in the CA concentration in the external microdialysis medium. It was possible to reach the separation of subnanomolar concentrations of DA and NA in less than 2-min run and with high efficiency [137]. The derivatization with NDA has been performed on line [55]. The DA release in rat striatal microdialysates was measured by CZE–LIFD [138]. In single cells, CE and LIFD have also allowed measuring NA and A in individual adrenal medullary cells with high sensitivity down to nanomolar concentration [139]. DA detection in femto to attomol levels in pheochromocytoma cell [140], and the secretion of NA and A released from the adrenal medullary cell induced by acetylcholine were also measured by CE–LIFD [141]. Currently, these methods for biogenic amines have been updated for the single cell analysis and adapted to microchip CE techniques [142].

Other endogenous neuroactive substances. Acetylcholine, one of the most studied classical neurotransmitters, was determined by CE–EC with potentiometric detection down to 10^{-7} M [143]. The nitric oxide end products were measured using CE in brain microdialysates of rats with cerebral trauma [144]. Kinurenine, a tryptophan metabolite, is in very low concentration in brain. It could be detected by CE–EC in microdialysates obtained at a very low flow rate [250 nl/min] to improve the recovery of the analyte [22]. The kinurenic acid synthesized from tryptophan and kinurenine is an excitatory AA receptor antagonist related to neuropsychiatric disorders and was detected by CE–LIFD in microdialysates [145]. Ascorbate and lactate changes in rat brain dialysates were detected by CZE in response to elevated potassium [52]. Glucose has also been determined by CE–LIFD in transdermal human samples with detection limits of 80 nM [146].

CE is also a good separation technique for routine analysis of peptides [147]. CZE and MEKC separate large peptides molecules in a complex mixture. CE has advantages over HPLC in that it only separates the peptides that differ in their charges and has a longer run for each chromatogram [148]. CE has detected substance **P** with post-column derivatization and LIFD in brain microdialysates [149]. Using CE–LIFD, glucagon and insulin can also be detected in the islets of Langerhans [150], and the opiod peptide, enkephaline in the rat serum [151].

CE and single cell analysis

Nanotechnology applied to the study of single cells analysis and even subcellular components such as secretory vesicles makes possible the individual chemical analysis by CE–LIFD in attoliter volumes introduced into the capillaries [19]. Krilov *et al*. showed that single cell analysis with CE–LIFD gives more accurate metabolic information than cellular extract analysis [152]. The CA have been analyzed by CE–EC or CE–LIFD in different single cells [133–135,139–141,153]. Steroids were found in single R2C cell while progesterone secretion was monitored from culture cells and subsequently in single cells. The mass detection limit was 10^{-18} mol of dansylated steroids [154]. In identified neurons of marine slugs, Arg and citrulline, AA related to the synthesis of nitric oxide [155], and insulin in single pancreatic cells [156], a release of insulin from cell line beta TC3 permeabilized by digitonin [157] were all analyzed by CE–LIFD. Gluthatione in single human erythrocytes was analyzed by CZE and amperometric detection with a detection limit of 26 amol [158]. Glutamate content in single cell human erythrocytes and baby rat brain neurons were determined by monitoring NADH by CE–LIFD, and its fluorescence related to the Glu content in each cell [159]. Not only whole individual cells can be analyzed but even subcellular components were analyzed. Thus, secretory vesicles of the mollusk *Aplysia Californica* have been chemically analyzed individually by a combination of optical trapping and CE–LIFD. One vesicle 10^{-18} l volume is injected into the capillary, lysed and derivatized with NDA [160]. All these works show that CE permits quantitative analysis at the single cell and even at subcellular level.

Summary

CE has become a major tool for the chemical analysis in the pharmaceutical industry, clinical and forensic medicine. Thanks to its advantages, CE has successfully replaced other analytical

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techniques in many applications. CE coupled to CE or LIFD has allowed to monitor body fluids (plasma, urine, etc.) or microdialysates, a broad nature of prescription or illicit drugs, toxins or endogenous substances. For DNA sequencing CE is now the appropriate technique. CE coupled to microdialysis helped to understand neuroactive substance roles in the brain or in other extracellular fluids. The study of CA, AA and peptides has contributed to the knowledge of the nervous function in physiological conditions as well as in neuropsychiatric disorders. The microdialysis high temporal resolution together with the CE–LIFD high efficiency and sensitivity, have permitted to study simultaneous changes of several analytes at a very low concentration (nM) and in short collection time samples (less than a minute). This gives us information about the very rapid synaptic chemical changes. Finally, the chemical analysis of single cell and subcellular compartments is possible with CE.

Future developments and applications

In the next future, we will see major developments to speed up, to increase sensitivity and to improve resolution power of CE analysis. The current tendency to make CE easier and affordable for the basic and clinical research will continue. There will also be more emphasis in the chemical analysis of single cells and subcellular compartments. Consequently, miniaturization of CE systems and the further development of CE microchip technology and its applications will accelerate.

Automation and capillary array

Faster analysis will make CE a suitable technique for screening analysis for large number of people, and for running large experiments in animals. Automated CE systems with capillary arrays and autosamplers, to run dozens of samples simultaneously for great diversity of chemical analysis will lead to generate knowledge more rapidly. We have developed a new generation of CE–LIFD system to run samples in 20 narrow capillaries (25 μm inside diameter) simultaneously. The system is equipped with autosampler and is totally computerized (personal communication).

The already high-resolution power of the CE can be enhanced by increasing the high voltage up to ultrahigh-voltage [161], and through the use of radial field control of electroosmotic flow [162]. The recent microchip technology applied to CE can also increase the separation efficiency using inexpensive disposable polyester microchips [163].

The protein analysis will have a great demand in the near future because once the genome project is complete, the interest will be focused on the proteomic research (set of proteins expressed in a cell). CE is a very powerful tool for protein identification; therefore, automatized instrument for protein analysis will be in high demand [6].

Improved detection

Improvement in the detection techniques will be necessary to match the analysis of minute samples with low concentration of analytes. Thus, more sensitive detection techniques coupled to CE will be needed. For LIFD, better lasers are required. The ideal laser would be the one with several wavelengths for matching more fluorescent compounds, but with lower cost and less expenditure of energy than the ones currently available. Increasing the fluorescence signal or reducing the background signal must be accomplished to improve LIFD. CE–LIFD systems make use of visible laser lines so that they can only be applied to analytes that have been chemically derivatized with a suitable fluorescent tag matching the excitation wavelength. But the CE–LIFD can be expanded to include natively fluorescent analytes by employing either improved UV pulse or continuous wave laser systems or multiphoton–excitation [164]. A highly sensitive LIFD detection system based on a 635-nm laser diode and a confocal microscope is used for planar microfluidic CE chip [165]. The near infrared fluorescence also offers an attractive alternative to visible fluorescence because of its very low detection limits [166]. One way to increase detection sensitivity is by increasing the analyte low concentration in the capillary by electrokinetic techniques like sample stacking. This achieves the limits of quantification in the low micrograms per liter

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and offers the most promising results to quantify analytes in biological fluids [24]. Besides, it is possible to enhance the signal in CE by using microchip channels for stacking [167,168]. More and better on-line derivatization systems are desirable for microdialysates analysis to prevent losses of sample and improve the detection [55,56]. There is interest in the future development and potential applications of the coupling of CE with the biosensor detection, which is based on miniaturized electrophoretic chambers, that yields short analysis times, provides automation of the sample handling and for which small sample volumes are required [169].

Miniaturization of CE systems

The tendency in the future will be the miniaturization of the CE systems to analyze progressively smaller sample volumes. At present, there is interest in single cell or subcellular analysis and therefore in developing the appropriate instrumentation and methods [170–172]. CE miniaturization will continue either through reducing column dimensions or placing entire electrophoresis systems on planar microfluidic chips [165,173–179]. The electrophoretic separations in microchips significantly faster than the conventional separations, will have an effect especially in DNA separations [180]. The miniaturization requires smaller volume load with high sensitivity detection. The extremely small sample volumes will be forced to develop appropriate technologies to handle nanoliter volumes, which are very common in individual cells or individual subcellular compartment analysis [18].

Future application of CE as the routine analysis in the pharmaceutical industry and in clinical medicine

CE–LIFD application in the pharmaceutical industry and in clinical medicine will permit a routine analysis of many hundreds or thousands of small volume samples of biological fluids. This can be done by empowerment of CE–LIFD for routine parallel analysis, improving autosampler techniques, adapting advanced detection technology and improving capillary array systems. More emphasis in the coupling of microdialysis *in vivo* or *in vitro* for pharmacokinetics will increase the number of CE applications in the pharmaceutical and clinical field [72]. All of this will show the superiority of CE over classical analytical assays especially in the field of low volume samples [63].

Conclusion

At present, CE a highly sensitive analytical technique, has become one of the most used methods of chemical analysis in the biomedical field. CE has great prospectives in the near future to become a routine analysis technique in the research and clinical laboratories for a large number of endogenous and exogenous substances, toxic or non-toxic compounds, prescription or illicit drugs. The coupling of CE with microdialysis, to monitor the chemical extracellular environment in the human beings or animals, has been very successful especially in neurochemical research and in the clinical environment. This coupling produces high temporal resolution microdialysis that allows monitoring changes in different neuroactive substances in very short periods of time. The coupling also takes advantage of CE benefits that permit to analyze multiple analytes at very low concentration in a few nanoliter samples in the same run. Using CE with more sensitive detection methods such as LIFD or MS, it is possible to measure analytes in attomolar and zeptomolar range. For the near future, there are high expectations for CE with improvements in separation, detection to analyze with higher resolution, better sensitivity, developing faster automatized and miniaturized systems for the analysis of very small volume samples. All of this will make CE even more suitable for the routine chemical analysis and also for research.

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