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Review

Determination of minute enzymatic activities by means of capillary electrophoretic techniques

James J. Bao[a,*], James M. Fujima[b], Neil D. Danielson[b]

[a]*Procter & Gamble Pharmaceuticals, 11530 Reed Hartman Highway, Cincinnati, OH 45241, USA*
[b]*Department of Chemistry, Miami University, Oxford, OH 45056, USA*

Abstract

Capillary electrophoretic analysis of enzymes, co-enzymes, substrates and other chemical species that can be linked to an enzymatic reaction is reviewed with 80 references. Both off-line and on-line assays of minute enzymatic activities are discussed. In addition to heterogeneous on-line enzyme assays, a special emphasis is given to a newly established on-line technique called electrophoretically mediated microanalysis (EMMA). The basic principle, procedure, and various detection modes of EMMA are discussed. The recent developments in on-line determination of various enzyme substrates as well as on-line enzyme kinetic studies are also summarized. Some potential future developments in the determination of enzymatic activities by means of CE are also presented. © 1997 Elsevier Science B.V.

Keywords: Reviews; Electrophoretically mediated microanalysis; Enzymes

Contents

*Corresponding author.

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1. Introduction

The combination of enzymes with separation techniques has been a theme of research for many years. As early as 1970, Brown initiated the use of enzymes in free solution for pretreatment of samples before separation [1]. Since then, there has been significant progress in using enzymes to solve many separation-related problems by HPLC [2].

Capillary electrophoresis (CE) is an important separation technique. In CE, the migration of any charged species depends primarily on the charge-to-mass ratio and, to a certain extent on the shape, size and conformation (for large molecules). When the analytes have similar charge-to-mass ratios and other parameters are very close, CE can become an inadequate technique for their separation. However, the identification of a given species in a mixture is often of importance, such as in the case when there are no authentic standards available. Therefore, other means are required to confirm the presence or absence of a specific species. Enzymes, with their high specificity and unique amplification power are well suited to this purpose. They not only selectively bind to substrates, but they also catalyze some specific reactions and generate sufficient products for detection with common detectors. The combination of these characteristics makes it practical to analyze small amounts of either enzyme or substrate without significant modification of the common CE instrument.

2. Enzyme assays off-line

At first, CE was only used as a separation tool in the determination of enzyme activities. Banke et al. [3] first separated the crude fermentation broth containing *Aspergillus oryzae* by CE in an alkaline buffer and then collected the fractions for analysis of alkaline protease activity. After incubation with substrate, the reaction mixtures were separated by CE again. As little as 0.3 ng of enzyme was detected by this method. The direct monitoring of enzyme activities without enzyme separation and fraction collection steps can be exemplified by the work of Landers et. al [4]. CE was used to rapidly and reproducibly analyze the activity of bacterial chloramphenicol acetyltransferase (CAT) which converts the substrates acetyl coenzyme A (CoA) and chloramphenicol to acetyl chloramphenicol and CoA. In this work, CE is shown to yield similar quantitative results with a non-radiolabelled substrate in a fraction of the time required for other standard analytical methods. Other off-line studies of enzyme activity using CE are summarized in Table 1. A general review of enzyme and antibody/antigen reactions used in conjunction with HPLC and CE is available [27].

Off-line heterogeneous enzyme assays have been coupled with CE. In 1989, Cobb and Novotny combined a narrow-bore enzyme reactor packed with trypsin immobilized on agarose gel with both micro-column HPLC and CE for the characterization of phosphorylated and dephosphorelated β-casein [28]. This miniaturization allowed enzymatic digestion of very small (ng) amounts of proteins. Instead of packing the capillaries, Kuhr et. al reported another way of preparing the trypsin containing micro-reactors. They immobilized trypsin onto the inner surface of an aminoalkylsilane-treated fused-silica capillary via biotin-avidin-biotin coupling [29]. The enzyme-modified capillary was used to digest minute amounts of β-casein simply by flowing a solution of the denatured protein through the capillary at a rate of 40 nl/min and collecting the effluent. The tryptic peptides in the collected digest were separated by CE.

In summary, CE is an excellent tool for studying enzyme activities since it has several advantages over standard single parameter assays, such as: (a) the ability to monitor both loss of substrate and the appearance of products simultaneously; (b) high speed of analysis; (c) no need for radiolabelled substrate; (d) small sample volumes; (e) high sensitivity/resolution; and (f) excellent quantitation capabilities. However, an off-line enzyme assay has its limitations. First of all, there is a time delay from the time of reaction to the time of sample analysis. The reaction must be completely stopped, especially for some kinetics studies, during this delay period, by either adding a reagent or changing the solution conditions. Secondly, even though only a nl-scale sample is needed for CE analysis, significantly larger amounts of starting materials are needed to carry out the reaction. To have a real nl-scale reaction, it is necessary to conduct the reaction directly inside the capillary.

Table 1
Enzyme assays by HPCE off-line

Classification	Enzyme	Substrate	Detectable species	Detection method	Notes
Transferase	chloramphenicol acetyltransferase	chloramphenicol and acetyl coenzyme A	acetyl chloramphenicol	UV-200 nm	no need for radioactive substrate [4]
	acyl Co-A synthetase	octanoic acid and other carboxylic acids	Octanoyl Co-A and others	UV-260 nm	activation of xenobiotic carboxylic acids, MEKC used [5]
	adenine phosphoribosyl-transferase	adenine	adenine and AMP	UV-254 nm	purine-related enzyme [6]
	ADP ribosyltransferase	L-arginine	ADP-ribosylarginine	UV-254 nm	LLD: 0.3 pmole, 100× lower than RPLC [7]
	α-(1→3)-fucosyltransferase	GDP-fucose donors	Lewis X determinant	LIF	biosyntehsis in 3 strains of *Heliobacter pylori* [8]
	β-1,4-galactosyltransferase	UDP-galactose donors	Lewis X determinant	LIF	biosynthesis in 3 strains of *Heliobacter pylori* [8]
	γ-glutamyltranspeptidase	*p*-N-ethyl-N-hydroxy-ethylaminoaniline	p-N-ethyl-N-hydroxy-ethylaminoanilide	UV-200 nm	A blotting membrane micropreparation is an improved microtest plate [9]
	glycosyl transferase	β-galactosyl/GDP-fucose/fluorescent sugar conjugate	fluorescent products	fluorescence, λ_{em}=420 nm	LLD: PAGE, 200 pmole CE, 80 fmole [10]
	protein kinase	kemptide	phosphorylated kemptide	UV	better accuracy and sensitivity than isotope method [11]
Oxidoreductase	dimethylsulfoxide reductase	dimethylsulfoxide	dimethyl sulfide dimethylsulfoxide	UV-200 nm	MEKC detect sub-ng level [12]
	glutathione peroxidase	reduced glutathione	glutathione disulfide	UV-214 nm	MEKC assay agreed well with RPLC and spectrophotometry [13]
	plant phenolic oxidation enzymes	apigenin, isoorientin, or quercetin	chlorogenic acid	UV-325 nm	monitor rate of phenolic oxidation [14]

(Cont.)

Table 1 (Cont.)

Classification	Enzyme	Substrate	Detectable species	Detection method	Notes
Lyase	adenylosuccinase	adenylosuccinate	adenosine monophosphate	UV-254 nm	cell extracts assayed [15]
	adenylosuccinate lyase	succinate-AMP	AMP and S-AMP	UV-254 nm	human erythrocytes, mixed blood lymphocytes and venouos blood [6]
Hydrolase	α-amylase	9-aminopyrene-1, 4, 6-tri-sulfonate (APTS) maltotetraose (G4)	APTS-G2 product	LIF-488	specificity of enzyme action studied, APTS G4 not hydrolyzed [16]
	carboxypeptidase A	methotrexate amino acid prodrugs	methotrexate	UV-204 nm	11 different substrates tested [17]
	carboxypeptidase Y	glucagon-like peptide (GLP-1, R-Ala-OH) and Arg-NH_2	GLP-1 and R-Arg-NH2	UV-200 nm	90% conversion of R-Ala-OH [18]
	chymosin	α- and β-casein	peptides	UV-214 nm	method for monitoring the identification of other peptides [19]
	endoproteinase Arg C(I)	ACTH peptides cleavalbe at Arg-X and other amino acid linkages	cleaved peptide fragments	UV-200 nm	faster results than RPLC [20]
	feruloyl/*p*-coumaroyl	methyl esters of ferulic and *p*-coumaric acid	ferulic and *p*-coumaric acids and esters	UV-scan, 225–345 nm	a feruloyl-containing sugar ester from wheat bran was also used [21]
	α-glucosidase	tetramethyl rhodamine trisaccharide derivative	fluorescently labeled oligosaccharides	LIF-λ_{ex}=543.5 nm	LLD: 50 oligosaccharide molecules [22]
	porcine pepsin	chromogenic peptide substrate	peptide cleavage products	UV-200 nm	kinetic parameters determined [23]
	prorelaxin processing enzymes	peptides representing prorelaxin processing sites	peptide fragments	UV-214 nm	serine protease enzyme identified [24]
	protease (alkaline)	suc-Ala-Ala-Pro-Phe-*p*-nitroanilide or casein	*p*-nitroaniline	UV-200 nm	LLD: 3 ng of enzyme in fermentation broths [3]
	sulfoesterase	chondroitin sulfate and heparin derived dissacharides	sulfated dissacharide products	UV-232 nm	MEKC assay, no need for radiolabelled substrates [25]
	tirpeptidase	Gly-Gly-Phe	peptide	UV-200 nm	enzyme kinetics in crude extract studied [26]

*LLD: Lower limit of detection.

3. Enzyme assays on-line

For an on-line enzyme assay in CE, it is necessary to have conditions that can (a) mix the necessary reagents together, (b) carry out the enzymatic reaction, (c) separate the product(s) from the substrate(s) and/or the enzyme, and (d) detect the results of the reaction. For homogeneous enzyme assays, all of these steps have to be carefully selected, while in heterogeneous enzyme assays, steps (a) and (c) are relatively easy to optimize.

3.1. Heterogeneous enzyme assays by CE

In a heterogeneous enzyme assay, one of the reactants, most often the enzyme, is immobilized onto the capillary. The mixing and reaction occur between the immobilized enzyme and the substrate(s) in the buffer. The resulting product(s) can be directly transported and detected by UV–Vis absorbance, fluorescence or mass spectrometry. Earlier work [28,29] had demonstrated the advantages of using immobilized enzyme capillary microreactors, such as (a) pl–nl volume requirement, (b) longer enzyme lifetimes, (c) higher stability, (d) the ability to reuse enzymes conveniently, (e) extremely efficient sample handling modes, and (f) easy separation of the reaction products from the enzyme reagents. However, all this work required a fraction collection step, which can be omitted if the assay is on-line. In general, the on-line technique is especially well suited to the characterization of minute quantities of species, such as proteins, because it transfers pl to nl volumes of reaction products without excessive sample handling which could lead to losses or contamination [29]. Table 2 summarizes the most recent developments in on-line heterogeneous enzyme assays.

El Rassi and co-workers developed an on-line enzyme assay system called enzymaphoresis, in which a heterogeneous capillary enzyme reactor is coupled in tandem with a CE column [30,31]. RNA-modifying enzymes were immobilized on the capillaries to selectively modify the solutes (or substrates)

Table 2
On-line hetereogeous enzyme assays by CE

Immobilized enzyme(s)	Immobilization method	Applications
RNA-modifying	-immobilized on the inner walls	-qualitative and quantiative analysis of different nucleic acids with good accuracy;
enzymes, e.g., RNase T1, RNase U2,	-either singly enzymeor mixed enzymes	-on-line digestiona nd mappingof minute amount of tRNAs -simultaneous synthesis and separation of nanogram quantities of oligonucleotides
hexokinase & adenosine deaminase [30,31]	-through glutaraldehyde linkage	-identifying minute amounts of dinculeotides as well as the finger-printing of tRNAs -peak locator on the electropherogram with improved system selectivity
trypsin [32,34]	-immobilized on the inner walls	-on-line with CE/ion spray MS for fast peptide mapping (<1 h) -digestion of the oxidized insulin B-chain,
tyrosine phosphatase [33]	-immobilizing tyrosine phosphatase on ther inner walls, or	on-line with CE/LC and electrospray ionization MS -rapid enzymic dephosphorylation and separation of the reaction products
	-immobilizing the phosphatase onto 40-90-m avidin-activated resins	-simple, fast, sensitive, and nonisotopic technique for the detection of tyrosine-phosphorylated peptides and the determination of sites of protein
	-avidin-biotin interaction	tyrosine phosphorylation
aminopeptidase [35]	-Baker's yease cells trapped on a	-profiling cells with different amino acid containing substrates using a on-line
	midcapillary frit containing 1 mm diameter 'through pores'	enzyme assay -required much less number of yeast cells (500 as compared with >500 000 in other methods)

before entering the separation capillaries. This system was used primarily for the analysis of nucleic acids (Table 2). These open-tubular capillary enzyme reactors were chemically and thermally stable for a prolonged period of use under normal CE conditions and were reusable for several RNA samples.

A trypsin microreactor integrated on-line with CE/ion spray mass spectrometry (MS) allowed fast peptide mapping. On-line digestion of the oxidized insulin B-chain by the enzyme reactor, CE separation, and MS identification of the products allowed the entire peptide mapping procedure to be completed in <1 h [32]. A protein tyrosine phosphatase microreactor coupled on-line to either CE or LC and electrospray ionization MS was used for rapid enzymatic dephosphorylation [33]. The microreactor was constructed by immobilizing genetically engineered, metabolically biotinylated human protein tyrosine phosphatase onto the inner surface of a capillary or by immobilization of the phosphatase onto 40–90 μm avidin-activated resins. This is a simple fast sensitive non-isotopic bioanalytical technique for the detection of tyrosine-phosphorylated peptides and the determination of sites of tyrosine phosphorylation in protein. On-line peptide mapping by CE using a trypsin-modified fused-silica microreactor coupled to the separation capillary can be done for pmole levels of proteins in less than 3 h [34].

A novel design of constructing an on-line heterogeneous capillary enzyme reactor by immobilizing Baker's yeast cells on a capillary using a mid-capillary frit was reported [35]. This frit has approximately 1 μm diameter "through pores", which allow the liquid flow to pass the capillary and retain large particles like cells. The cells trapped in the capillary by this frit were profiled with different amino acid-containing substrates using an on-line enzyme assay method developed by Bao and Regnier [36]. This method required a much smaller number of yeast cells (500) than other methods (>500 000).

3.2. Homogeneous enzyme assays by CE

Heterogeneous capillary enzyme reactors have some limitations: (1) Even though the immobilized enzymes generally displayed their usual enzymatic activities toward their substrates, certain other properties of the enzymes, such as the activity-pH dependency were altered when immobilized on the column. This was believed to be attributed to change in the micro-environment due to the close proximity of the immobilized enzymes [31]. (2) These enzyme reactors have only limited use. Enzymes are specific to only one or a few reactions except for a limited number of general functional enzymes, such as trypsin. (3) It is labor intensive and time consuming to prepare the enzyme microreactors. Multiple reaction steps are usually required before an enzyme reactor can be prepared. (4) Only limited conditions can be explored without affecting the immobilized enzymes. It is always desirable to have the freedom of choosing specific conditions, such as pH, to optimize the activities of the enzymes. (5) Quantitation and reproducibility is a major problem in a heterogeneous enzyme reactor. It is very hard to quantitate enzymes actually immobilized on the capillary and to reproduce the same capillary reactor. (6) The lifetime of the enzyme reactor is a concern if the enzyme is not very stable and/or is expensive. (7) Pure enzyme is not always available for immobilization.

Since Bao and Regnier reported an on-line homogeneous enzyme assay of glucose-6-phosphate dehydrogenase (G-6-PDH), a technique called electrophoretic mediated microanalysis (EMMA) has been established [36]. EMMA has become a general approach for ultra-micro enzyme assays in CE [36–54]. EMMA took advantage of CE being able to perform each of the following tasks required in a reaction-based micro-chemical analysis: (a) transferring different reagents together by utilizing mobility differences; (b) mixing the reactants together for reaction without disturbing the mixing zones; (c) allowing sufficient time to carry out the reaction for quantitation by manipulating the electrical potential; (d) transporting the detectable species to the on-line detection window; and (e) having sufficient detection sensitivity. The detection sensitivity can be significantly improved if certain amplification effects, such as the catalytic characteristics of the enzyme, can be employed. In EMMA, the analyte and reagent(s) are introduced into the electrophoretic capillary as distinct zones. These distinct zones tend to merge together during the electrophoresis process because they have different mobilities. The reaction proceeds

during the mixing process in the presence or absence of the electrical field. The products are then transported to the detector by the electrical field. Therefore, EMMA allows the reaction-based chemical analysis to be performed entirely on-column.

In practice, EMMA is most commonly performed in the "continuous supply of reagent(s)" mode, in which enzyme-saturating concentrations of substrate, coenzyme when appropriate, and running buffer were mixed and used to fill the capillary and reservoirs first. The enzyme, such as G-6-PDH is injected by either electrophoresis or siphoning and mixed with the reagents in the capillary immediately. When potential is applied, the enzyme moves inside the capillary and continuously mixes and reacts with new substrates during the electrophoresis process. The originally filled capillary and reservoir vials with the substrate ensure the "continued supply" of substrate to the enzyme. Enzyme activity is assayed by detecting one of the products, NADPH, which is electrophoretically transported to the detection window. The whole process of mixing, enzymatic reaction, product separation and detection can be controlled by a programmed potential. When a high potential is applied, the product is separated from the enzyme after it is formed. Because the rate of product formation is much higher than that of enzyme–product separation, product accumulates. The amount of accumulated product is related to the reaction time, which is inversely related to the operating potential [36]. The same amount of product will be generated at different sites along the capillary during a constant-potential process. Therefore, product formed under constant potential appears as a flat plateau. Reducing the electrical potential will allow a longer reaction time, and, thus, cause more product(s) to be accumulated. The extreme case is the zero-potential mode. The zero-potential assays are generally carried out by electrophoresing the enzyme partially through the capillary and then switching to zero potential before the enzyme reaches the detection window. The potential is resumed after a short break to transport the accumulated product to the detector, and a peak will be observed on top of the plateau. Quantitation of the enzyme activity and concentration can be accomplished by using either the height of the plateau or the area/height of the peak. The lower limit of detection for G-6-PDH (10^{-8} *M*) with simple UV detection was at least two orders of magnitude lower

Table 3
On-line homogeneous enzyme assays by CE

Enzyme	Substrate	Detectable Species	Detection Method/Sensitivity	Note
G-6-PDH	G-6-P	NADPH/NADH	UV-340 nm/10^{-8} M	[36,38,41], post column detection [44,45]
LDH	pyruvate/lactate	NADH	UV-340 nm or LIF/10^{-17} M	UV: horse and pig [38], human [52], PEG stabilized [54], LIF: single RBC (10^{-12} *M*) [42], single molecule [43]
AcP	*p*-nitrophenol phosphate	*p*-nitrophenol	Vis-405 nm/10^{-12} M	post-column reaction for detection [38]
LAP	L-leucine *p*-nitroanalide	*p*-nitroanaline	Vis-405 nm	[38,58]
	L-leucine-4-methoxy-β-naphthylamide	4-methoxy-β-naphthylamine	LIF/10^{-13} M	45 min reaction time [40]
APD	amino acid β-naphthyl-amide	β-naphthylamine	LIF/500 cells	profiling immobilized cell [35]
ALP	*p*-nitrophenylphosphate	*p*-nitrophenol	UV-Vis/10^{-11} M	gel media [47,49]
	p-aminophenylphosphate	*p*-aminophenol	ECD/10^{-7} mg/ml	[49]
β-galactosidase	*o*-nitrophenyl-β-galactopyranoside	*o*-nitrophenol	Vis-405 nm/10^{-5} mg/ml	gel media [47]
ADH	ethanol/acetaldehyde	NADH	UV-340 nm	[41]
6-PGDH	6-phosphogluconate	NADH	UV-340 nm/10^{-8} M	assay with two power supplies [45]
CK	phosphocreatine	ATP	UV-260 nm/0.5 units/mL	[54]
pepsin	β-lactoglobulin	peptide fragments	LIF/fmol	[55]

G-6-P, glucose 6-phosphate; LAP, leucine aminopeptidase; 6-PGDH, 6-phosphogluconate dehydrogenase; ALP, alkaline phosphatase; ADH, alcohol dehydrogenase, APD, aminopeptidase, CK, creatine kinase; ECD: electrochemical detection.

than the conventional spectroscopy method with less than 50 amole samples required.

Much continued on-line analysis research covering various analytes with different detection methods was done after Bao and Regnier's work. For example, Chung and Yeung used pepsin for on-column digestion of proteins for peptide mapping using CE with laser-induced fluorescence (LIF) [55]. Fluorescence of the natural aromatic amino acids was monitored and as little as 10 fmole of β-lactoglobulin could be digested and mapped. Table 3 summarizes these results and lists their characteristics. The scope of EMMA techniques is continuing to increase [56,57].

3.3. Isoenzyme assays

Clinically, it is often necessary to know the activities of more than one enzyme before a definite diagnosis can be accomplished. Isoenzymes are classes of enzymes that catalyze the same reactions. Separation of these isoenzymes is important in cases when either the crucial information can only be drawn from the relative levels of the isoenzymes or only one specific form of the isoenzymes is related to specific diagnosis. The profiling of lactate dehydrogenase (LDH) isoenzymes belongs to the first category. There are five different LDH isoenzymes, LDH-1 to LDH-5. Each of these isoenzymes has four sub-units consisting of the various combination of two different forms, H and M. The relative activities of LDH-1 and LDH-2 can be used to evaluate myocardial infarction. Several research groups have pursued the assay of LDH isoenzymes. Hsieh et al. reported the analysis of human LDH isoenzymes from human serum samples by CE in 1992 [52]. The profiling of human LDH isoenzymes in single human erythrocytes was achieved by Xue and Yeung in 1994 [42]. Fig. 1 shows that LDH-1 (30–40%) and LDH-2 (40–45%) are the major forms of LDH in red blood cells, and LDH-3 (14–16%) is present at a relatively low amount. For 3 of the total 36 cells examined, an extra peak is observed (Fig. 1d,e). This indicates the occasional presence of a high LDH-4 or LDH-5 content compared to normal cells, which typically contain these isoenzymes at 3–5% and 2%, respectively. Similar assays with horse and pig LDH isoenzymes as models under different conditions

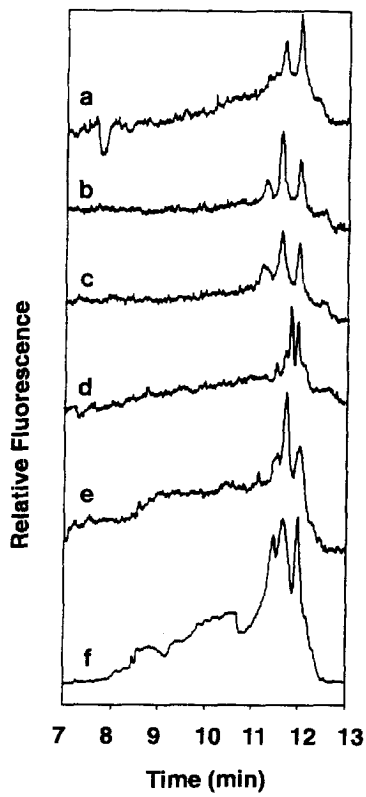

Fig. 1. Electropherograms of LDHs in several individual human erythrocytes: (a–e) – 2 min incubation and (f) 5 min incubation, plotted with a 0.4 scale factor. The migration times have been normalized with respect to that of LDH-1 in each case. Reproduced by permission from Analytical Chemistry [42].

were also studied by Bao and Regnier [37,38]. All of the above studies were based on the EMMA technology described earlier.

The analysis of human prostatic acid phosphatase (HPAcP) belongs to the second category. Serum AcP is derived from a number of tissues, principally the prostate, liver, and spleen, as well as erythrocytes, leukocytes and platelets. However, only the HPAcP isoenzyme has clinical value in the diagnosis of carcinoma of the prostate with metastasis. All of the current analytical methods have to assay the acid phosphatase (AcP) activities twice: one for the total AcP activity of the sample and the other for the remaining AcP activity after adding inhibitors specific to HPAcP. The difference of these two measurements gives the activity of HPAcP. The accuracy of these methods depends on the efficiency of the

inhibitors, such as tartrate, in inhibiting the activity of HPAcP. It is very hard to find perfect inhibitors that only inhibit HPAcP without affecting other AcPs. The on-line enzyme assay technique uses CE to separate HPAcP from other AcP isoenzymes and makes it impossible to assay the activities of individual enzymes [38]. Fig. 2 shows the separation of HPAcP from another AcP isoenzyme having about the same concentration (Fig. 2a) and the enzyme assay result (Fig. 2b). It can be seen that HPAcP can easily be assayed in the absence of inhibitors without the interference from other AcP isoenzymes.

In addition to the above isoenzymes, there are numerous enzymes that generate a common product or different products but with similar detection characteristics. Through the use of either natural or derivatized substrates, it is possible to generate the same product from totally different enzymatic reactions. NADH was the detectable species in the above LDH isoenzyme assays. NADH can also be generated by many other NAD^+ related oxidoreductases, such as G-6-PDH and alcohol dehydrogenase (ADH). Roeraade et al. reported an assay based on the separation of G-6-PDH and 6-phosphogluconate dehydrogenase (6-PDH) by CE [44]. The enzyme activities were monitored after reaction with NAD^+ and glucose 6-phosphate (G-6-P). Similarly, the assay of AcP was based on the production of *p*-nitrophenol, which can be generated by numerous other enzymes, such as alkaline phosphatase (ALP) on nitrophenol-releasing substrates. β-Galactosidase may generate *o*-nitrophenol, which has similar detection characteristics as those of *p*-nitrophenol. Another common product is *p*-nitroaniline, which can be generated by various aminopeptidases [40].

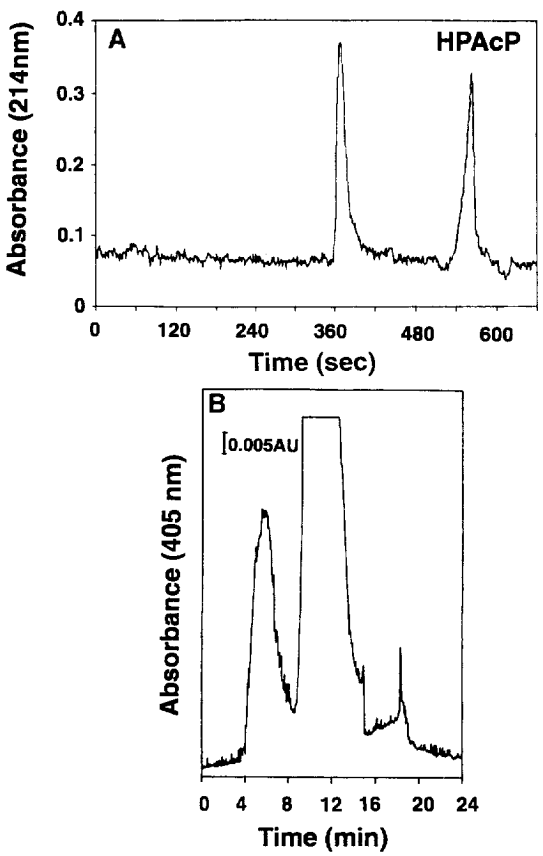

Fig. 2. Separation and simultaneous assay of human prostatic acid phosphatase (HPAcP) and another acid phosphatase with changing conditions. (A) Separation of the enzymes is detected at 200 nm in a 100 m*M* NaAc buffer at pH 5 in the analytical capillary. (B) The product of the enzymatic reaction, *p*-nitrophenol, is detected at 405 nm in a basic buffer at pH>9 in the detection capillary (see Section 3.4.2).

3.4. Detection

Most of the CE detection modes such as absorbance, fluorescence, MS, electrochemistry, chemiluminescence, and charge-coupled device (CCD) have been used for enzyme assays in CE (Table 3). Some of these detection modes may involve various ancillary techniques including chemical derivatization.

3.4.1. Detection modes

It was the amplification power of the enzyme that made it possible to assay G-6-PDH at a concentration of 10^{-8} *M* with a simple UV absorbance detector [36]. A more powerful detection mode, such as LIF, enabled the detection of 10^{-13} *M* leucine aminopeptidase (LAP) [40]. Even single LDH molecules (10^{-17} *M* solution) were detected with this technique [43]. The only limitation of LIF detection is that a suitable fluorophore, either native or derivative, must exist.

Mass spectrometry (MS) has recently been used for on-line enzyme assays with CE. An ion-spray MS was used for the identification of peptide fragments generated by a trypsin microreactor and

separated by CE [32]. An electrospray ionization MS was used for identifying low pmole amounts of tyrosine-phosphorylated peptides in a complex peptide mixture generated by proteolysis of a protein [33].

Chemiluminescence (CL) detection and CCD were also used for on-line enzyme assays [50,51]. Using CL, enzymes in nl volumes of biological samples at zeptomole level were assayed. Oxidases and catalase at levels of 9000 molecules were detected with an inexpensive PMT and amplifier. With horseradish peroxidase and firefly luciferase, the former converts hydrogen peroxide to light and the latter converts adenosine triphosphate (ATP) to light as the end reaction. A series of enzymes that can generate either hydrogen peroxide or ATP can be assayed through these two reactions (Table 4).

The sensitivity of detection in enzyme assays also depends on the biological properties of the enzymes and the physical properties of the environment. The specific activity and the turn-over number of the enzyme will determine how fast an enzyme can amplify a reaction, and, thus, how much gain there is in the detection sensitivity. The reaction conditions such as pH, temperature, presence of competitors and/or inhibitors also have significant effects on the sensitivity of the assays. Extraordinarily high sensitivity can be obtained under optimized conditions. For example, as low as 10^{-12} *M* of HPAcP was detected with a UV detector [38]. Higher sensitivity can also be obtained with a longer reaction time. A 45 min incubation allowed the detection of 10^{-13} *M* LAP [40]. The diffusion problem can be eliminated by increasing the viscosity of the running buffer with added soluble polymers or gel.

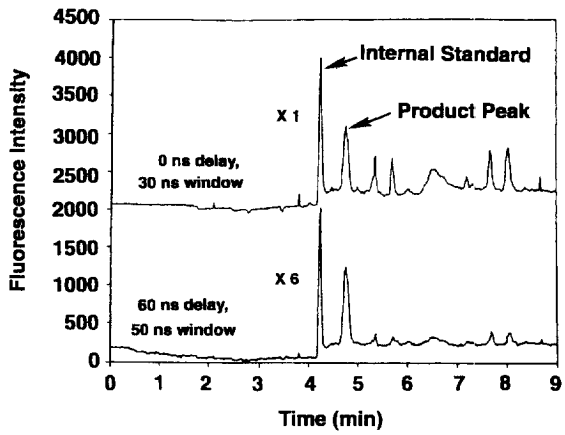

Fig. 3. Dual electropherograms for dialyzed urine samples assayed for LAP. Sample was incubated for 10 min. Data were offset for clarity. The 60 ns time delay in data acquisition improved the appearance of the electropherograms. The product peaks can be easily identified. Reproduced by permission from Analytical Chemistry [40].

3.4.2. Selectivity

In addition to sensitivity, there have been several reports on selective detection of specific compounds [38,40,48]. Selective detection is especially important in the analysis of enzymes in biological ma-

Table 4
Oxidases/catalase and substrates analyzed by on-line enzyme assay with chemiluminescence detection [50,51]

Enzyme	Substrate	Coupled reaction	Detection	Limit of detection
horseradish peroxidase	H_2O_2	direct	post-column CL	-39 fmol/380 fmol* for H_2O_2 assay -120 amole for HRP assay
firefly luciferase	ATP	direct	post-column CL	540 amol
	D-proline	HRP	coupled enzyme CL	1.3 pmol D-proline
D-amino acid oxidase	D-alanine	HRP	coupled enzyme CL	1.5 pmol D-alanine
	D-proline	HRP	EMMA-CL	36 amol D-proline
kinase creatine	ADP	FL	coupled enzyme CL	180 fmol ADP/170 fmol ATP
uricase	uric acid	HRP	EMMA-CL	1.5 fmol (1.3 nU)
D-galactose oxidase	D-galactose	HRP	EMMA-CL	7.7 amol (190 nU)
glucose oxidase	D-glucose	HRP	EMMA-CL	120 zmol (4.0 nU)
catalase	water and oxygen	HRP	EMMA-CL	15 zmol (9300 molecules, 150 nU)

CL: chemiluminescence, HRP: horseradish peroxidase, ATP: adenosine triphosphate, ADP: adenosine diphosphate, FL: firefly luciferase.
*: two different configurations.

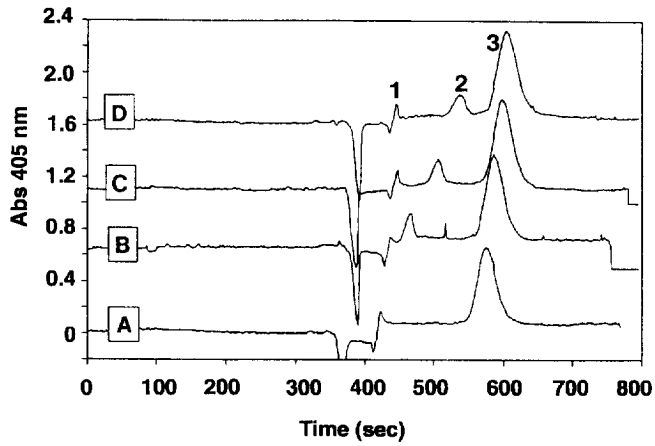

Fig. 4. Electropherograms obtained with zero potential applied at different running times before the enzyme passes the detector. Zero potential was applied after (A) 0, (B) 1, (C) 2, and (D) 3 min from the beginning of the electrophoresis process. Peaks 1 and 3 are the background peaks. Peak 2 is the product peak generated from the reaction [38].

trices. First, it is possible to differentiate the product peak from other peaks by allowing the detection to occur in a different time period. The specific peak with a different height proportional to the reaction time is usually related to the enzyme activity. In the assay of enzymes in complex biological samples, the product peak is often hidden inside many other interfering peaks resulting from the matrix. By repeating the same reaction with a little longer time, the product peak can be identified because it appears significantly higher as compared with the other peaks.

The second way of selective detection is to use the detector to discriminate the product signal from other interferences. By using the time-resolved LIF detector, data can be collected with a certain time, e.g. 60 ns delay. Most of the short-lived fluorescence peaks resulting from the matrix (Fig. 3, top) were eliminated and only the relatively longer-lived product fluorescence was detected (Fig. 3, bottom) [40]. However, this selectivity was gained at the price of a certain sensitivity loss because the product peak was also reduced simultaneously.

Selectivity can also be gained by manipulating the mobilities of different species. The time to detect a product is the total migration time of (a) the substrate

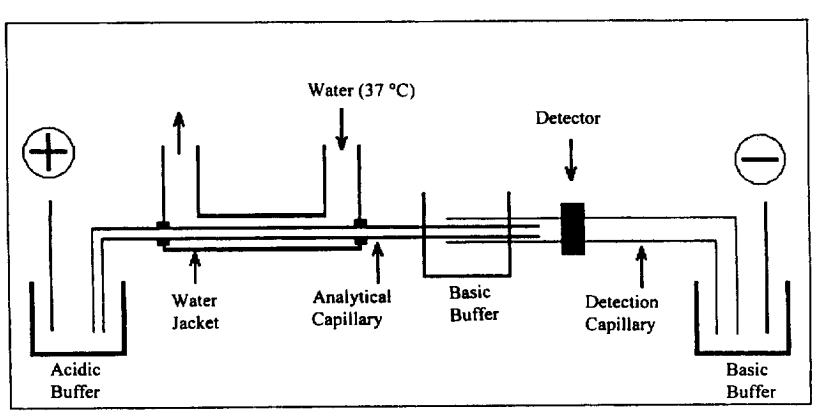

Fig. 5. Capillary electrophoresis system used for the analysis of AcP. The analytical capillary is inserted in plastic tubing. Water controlled at a constant temperature (37°C) is pumped through the tubing to keep the capillary at 37°C [38].

before the enzymatic reaction, (b) the migration time of the enzyme–substrate complex during the reaction, and (c) the migration time of the product to the detection window after it is dissociated from the enzyme. Therefore, the mobilities of both the substrate and the product as well as the mobility of the enzyme–substrate complex can affect the detection. The enzyme reaction is usually accomplished at zero potential when there is no movement but the substrate and product move at significantly different speeds because of their different mobilities. Consequently, by controlling when the analytical reaction is to occur, it is possible to change the time for the product to be detected [38,48,58]. The result of this manipulation is that the product peak can be "moved" from one place to another. In the analysis of LAP, this technique was used to place the product peak in a relatively "clean" area for quantitation. Fig. 4 shows the different positions of the product peak generated at different times [38,48].

Finally, selective detection can be achieved using either post-column addition or reagent gradient in the case where direct detection is not possible under specific conditions. Both of these strategies were used in the analysis of AcP (38). AcP reacts best at acidic pH while the product *p*-nitrophenol is a chromophore only at basic pH. This means that a changing condition has to be provided in order to carry out the enzymatic reaction and detect the product. Fig. 5 shows an instrument designed specifically for this type of application [38]. A regular 50/185 μm I.D./O.D. capillary (analytical capillary) filled with acidic buffer was inserted into a second capillary (detection capillary) with a larger I.D. (>250 μm). The opening in the junction of these two capillaries allows the introduction of different reagents, such as different pH buffers into the system. With this design, the AcP enzymatic reaction was carried out in the analytical column at pH 5. The resulting product, *p*-nitrophenol was transported to the detection capillary by electro-osmotic flow (EOF). When *p*-nitrophenol met the basic buffer (pH>9), it ionized to form a chromophore detectable at 405 nm. Fig. 2 shows the separation of two AcP isoenzymes detected at 200 nm (Fig. 2a) and the corresponding products detected at 40.5 nm (Fig. 2b). Selective detection was achieved by changing the pH conditions with a post-column addition of a basic buffer. Emmer et al. reported a similar post-column system with two detectors [44,45]. One detector is on-column to monitor the CE separation and the other is post-column to monitor the reaction products. In this case, the enzymatic reaction is also carried out in the second capillary, not the first capillary. The first capillary was used only for separation of multiple enzymes and the product was formed by adding a flow of substrate in the post-column section. In the assay of AcP, a reagent gradient was also used to achieve selective detection. A reagent gradient can be created in a single capillary with the two ends having different pH

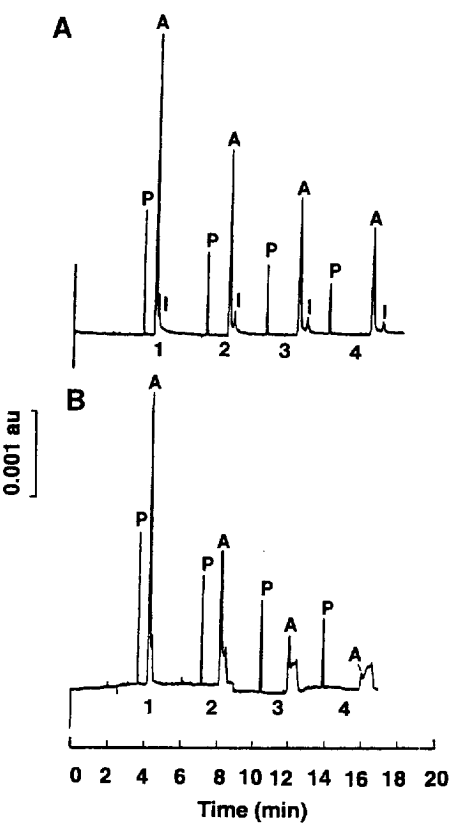

Fig. 6. Electropherograms of adenosine (A), inosine (I) and 3-methylphenol (P) mixture obtained from four consecutive detections. Capillary: 75 μm I.D.×360 μm O.D. bare capillary with 150 cm total length. The four effective window lengths were 35, 66.5, 97.5 and 129 cm. Electric field: 167 V/cm. Injection: 6 cm×10 s. Temperature: 25°C. Run buffer: (a) 0.025 *M* borate at pH 8.01; (b) 0.025 *M* borate with 0.04 units/ml adenosine deaminase at pH 8.01 [63].

buffers. A long-enough capillary was used to ensure the acidic pH for reaction and the basic pH for detection [38].

4. Enzyme kinetics

Lowther and Dunn reported a CE-based method for monitoring the rate of cleavage of basic oligopeptides in 1994 [23]. This method was particularly applicable to monitoring the cleavage rate of peptides where the specificity of the proteinase dictates against the presence of bulky chromophores. The kinetic parameters determined for the cleavage of a related chromophoric substrate were directly comparable. The kinetic analysis of intrinsically or mutationally induced inefficient enzymes can be facilitated by this approach. Affinity capillary electrophoresis (ACE) has been used to determine binding stoichiometries of protein–ligand interactions [59]. Examples studied are carbonic anhydrase inhibition by 4-alkylbenzenesulfonamide and binding of streptavidin to biotin derivatives [60]. ACE was also applied to determine kinetic and equilibrium constants for binding of arylsulfonamides to bovine carbonic anhydrase [61]. The Michaelis-Menton constant for an enzymatic reaction was also recently evaluated by ACE [62]. An enzyme reactor immobilized with β-galactosidase was used to reproducibly determine the Michaelis–Menton constant of the enzymatic reaction with *o*- and *p*-isomers of nitrophenyl-β-galactoside. Sun and Hartwick performed on-line kinetic monitoring for biochemical reactions with multi-point detection in CE [63]. Four detection windows were opened about every 30 cm throughout the entire capillary length of 150 cm. The capillary was bent into loops so the windows fitted in a slit and lens assembly in front of the detector. Fig. 6 shows the on-line monitoring of the deamination reaction of adenosine to inosine. The deamination speed of adenosine to inosine increases with adenosine deaminase concentration. In addition the degradation of the heme protein myoglobin was found to follow first-order kinetics under certain electrophoretic conditions.

5. Enzyme substrate assays

In addition to the direct assay of enzyme activities, there are many other species that can be determined using the amplification power and selectivity of an enzyme. A variety of homogeneous off-line substrate assays are summarized in Table 5. EMMA has been used as an on-line analytical technique for the determination of ethanol using ADH [72]. The formation of NADH was monitored at 340 nm and a linear range of 0.5–6 mg/ml ethanol was determined. The limit of detection was 4 μg/ml or 300 fmole injected. A correlation coefficient of 0.995

Table 5
Assay of enzyme substrates by off-line CE

Substrate	Enzyme(s)	Detection	Note
N-acetyllactosamine derivatives	galactosidase hexisamidase	LIF-543.5 nm	substrates for enzymes present in HT-29 cells [64]
heparin/heparin sulfate dissacharides	heparinase, heparin lyases II, heparinitase	UV-232 nm/50 fmole	dissacharide composition of glycosaminoglycan studied [65]
hypoxanthine IMP inosine	xanthine oxidase catalase nucleoside phosphorylase nucleostidase	UV-250 nm	fish freshness assessed [66]
hyduronan	hyaluronidase	UV	human synovial fluid analyzed [67]
NAD^+ *p*-methylbenzyl hydroperoxide	alcohol dehydrogenase	UV-260 and 300 nm	NADX tautomer products formed [68]
oligosaccharides (fluorescenc conjugates)	chitinase	LIF	kinetics of chitinase also studied [69]
paralytic poisoning shellfish toxins	hydrolytic enzyme from little neck clams	tandem MS	stacking procedure developed [70]
tetramethylrhodamine labelled oligosaccharide	fucosyltransferase	LIF	100 molecule detected [71]

between EMMA and the standard spectrophotometric assay was found. Lactate and pyruvate using LDH has been determined by EMMA by Fujima and Danielson [73]. Fig. 7 shows rapid monitoring of either the NAD^+ peak for pyruvate or the NADH peak for lactate is possible when a short 15×75 cm capillary is used. Linearity of pyruvate from 0.02 to 0.3 m*M* at 30°C was established by monitoring the NAD^+ product peak at 280 nm. Most LDH assays for pyruvate look at the disappearance of the NADH signal which requires two measurements to be made. Linearity of lactate from 2 to 8 m*M* was found by monitoring the NADH product peak at 340 nm. Better lactate detection limits (0.015 m*M*) were possible using hydrazine as a trapping agent. Phosphocreatine using creatine kinase has also been determined by EMMA [74]. Linearity of phosphocreatine from 10 to 40 m*M* was generated by following the ATP peak. A detection limit of 0.025 m*M* phosphocreatine was possible. For both the lactate/pyruvate and phosphocreatine studies, the enzyme activity was stabilized using 1.5% poly(ethylene glycol) (PEG) in solution to ensure reproducibility of the assay for 120 h, as compared to only 2 h without PEG.

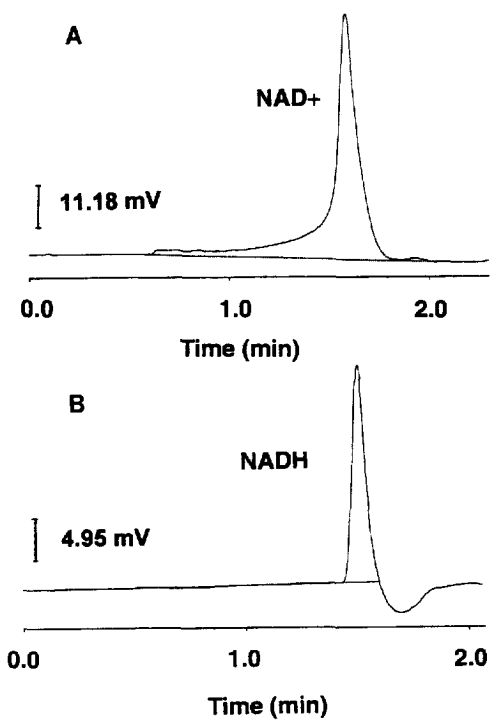

Fig. 7. Pyruvate and lactate assays using EMMA technology. (a) NAD^+ peak (280 nm) from LDH (500 units/ml) enzyme reaction involving 0.1 m*M* pyruvate and 3 m*M* NADH (pH=7.5). (b) NADH peak (340 nm) from LDH (500 units/ml) enzyme reaction involving 4 m*M* lactate and 3 m*M* NAD^+ (pH=10). Both reactions were carried out in a 100 m*M* Tris buffer with 1.5% polyethylene glycol in a 15 cm×75 μm I.D. fused-silica capillary under 25 kV.

6. Future

CE, in conjunction with capillary enzymatic reactors and highly sensitive detectors, has been recognized as an effective tool for the analysis of minute enzyme quantities and enzyme-related substances. It has the potential to carry out microanalytical reactions and separations with chemical assays at the zeptomole level in biological samples. Based on the current trends, we envision that there will be significant progress in (a) single-cell and single-molecule analysis, (b) capillary immunoassay, and (c) automation.

6.1. Single-cell and single-molecule analysis

The profiling of cells [35], the quantitation of LDH within single human erythrocytes [42] and of even a single LDH molecule [43] have demonstrated the potential of CE as an efficient tool for exploring the secrets of nature. The efficient separation of different LDH forms by CE, in combination with on-line reaction and LIF detection, opens up the possibility of multiple-enzyme assays with a single mammalian cell. Yeung points out that the concept of utilizing enzyme amplification to rapidly assay extremely low concentrations of molecules (10^{-17} *M*) can be extended to any species that can catalyze the production of a suitable fluorophore [43]. With the appropriate linkage of LDH to a hybridization tag or an antibody, practically any molecule can be counted in this way. Each molecule can be characterized, not just counted.

With proper design of multi-step reactions, the amplification power of enzymes can be cascaded when the reactions are linked together properly [44,75]. It is feasible to conduct single-cell and single-molecule assays with even less sensitive detectors, such as UV–Vis. It is further expected that

this method will have great potential for DNA analysis in single-cell and single-molecule analysis when the amplification power of the polymerase chain reaction (PCR) is properly utilized.

6.2. Capillary immunoassay

Immunoassay has high specificity. By combining the specificity of the antibody–antigen interaction with the amplification power of enzymatic reactions, various immunoassay techniques, such as enzyme-linked immunosorbent assay (ELISA) and enzyme-multiplied immunoassay (EMIT), have been well accepted by the life science community. The addition of the separation power of CE to immunoassay will definitely enhance the power of these techniques.

A homogeneous enzyme immunoassay using CE to determine drugs in hemolyzed, lipemic or icteric serum samples was reported [76]. An EMIT assay kit for digoxin in human serum was evaluated. CE was used to separate the enzymatic reaction product (NADH), remaining substrate (NAD^+), and the internal standard (*p*-nitrophenol) after the enzyme immunoassay. Both an internal calibration plot for NADH and a dose-response curve for digoxin in serum were constructed. Calibration serum, patients' sera with hemolyzed, lipemic, and icteric interference factors, and other pigmented blood components (e.g., serum albumin, bilirubin, Hb, uric acid, coproporphyrin, melanin, protoporphyrin IX, and uroprophyrin) demonstrated no interference. This method was believed to be useful for analysing digoxin in hemolyzed, lipemic and icteric blood samples that are known to create problems in conventional EMIT assays and may be applicable to other EMIT-based assays for monitoring drugs in complex biological matrixes. Chen et al. reported an on-line capillary immunoassay with a combination of homogeneous immunoassays and enzyme assays [53,75]. Recently, immunoassay for triiodothyronine (T3) employing enzyme amplification and EMMA has been presented [77] to achieve low detection limits.

6.3. Automation

Automation of enzyme analysis is progressing rapidly. Multi-array capillaries have been utilized to increase the throughput of CE methods [78]. The fabrication of CE on glass chips has made it more feasible to create instruments that can handle thousands of samples simultaneously [79,80]. A great demand from pharmaceutical and biotechnology industries for high-throughput screening methods to identify potential drug candidates from combinatorial libraries has inspired the rapid development of automation in CE. It is expected that multi-array enzyme assays will play an important role in searching potential hits in future pharmaceutical and biotechnology developments.

7. List of abbreviations

ACE	affinity capillary electrophoresis
AcP	acid phosphatase
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
ALP	alkaline phosphatase
ATP	adenosine triphosphate
CCD	charge-coupled device
CE	capillary electrophoresis
CK	creatine kinase
CL	chemiluminescence
ECD	electrochemical detection
ELISA	enzyme-linked immunosorbent assay
EMIT	enzyme enzyme-multiplied immunoassay
EMMA	electrophoretically mediated microanalysis
FL	firefly luciferase
G-6-P	glucose 6-phosphate
G-6-PDH	glucose 6-phosphate dehydrogenase
HPAcP	human prostatic acid phosphatase
HRP	horseradish peroxidase
LAP	leucine aminopeptidase
LDH	lactate dehydrogenase
LIF	laser-induced fluorescence
MS	mass spectrometry
NAD^+	nicotinamide adenine dinucleotide
NADH	the reduced form of NAD^+
NADPH	the reduced form of nicotinamide adenine dinucleotide phosphate
PCR	polymerase chain reaction
6-PGDH	6-phosphogluconate dehydrogenase
PEG	poly(ethylene glycol)
T3	triiodothyronine

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