MASARYK UNIVERSITY BRNO
FACULTY OF SCIENCE
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APPLICATION OF ELECTROPHORETICALLY MEDIATED MICROANALYSIS FOR ENZYME ASSAYS

DOCTORAL THESIS

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BRNO 2004

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The process of scientific discovery is, in effect, a continual flight from wonder.

-A. Einstein (*Life*, 9 January 1950)-

Poděkování

V první řadě bych ráda poděkovala své Alma Mater za to, že mi umožnila postgraduální studium na přírodovědecké fakultě v oboru biochemie. Dále děkuji všem zaměstnancům Oddělení pro vědu, výzkum, zahraniční vztahy a doktorské studium, speciálně paní ing. Vérostové a panu ing. Hladíkovi, kteří byli vždy velmi ochotní a pomáhali mi řešit veškerou administrativu spojenou s doktorským studiem.

Mé největší díky patří panu doc. RNDr. Zdeňku Glatzovi, CSc., který mi věnoval v průběhu celého doktorského studia spoustu odborných rad, času, trpělivosti a ochoty. Bez jeho podpory, a to nejen vědecké, by tato práce mohla jen velmi těžko vzniknout.

I would like to thank Catholic University Leuven for an occasion to spend one academic year in the Laboratory for Pharmaceutical Chemistry and Drug Analysis. I wish to thank professor Jos Hoogmartens and professor Ann Van Schepdael for the favour to accept me there and for the kind admission to their laboratory. Professor Ann Van Schepdael, thank you for all you did for me!

Especially, thank you Sigrid, for nice cooperation and for all the laughs and fruitful CE discussions we had during the 'Belgian year' of my PhD studies.

Dále děkuji všem kolegům z katedry biochemie za jejich technickou pomoc a odborné rady. Paní Ludmile Polákové za pomoc při práci v laboratoři a za vytvoření příjemného pracovního prostředí. Dále svým kolegům-studentům Petře, Lence, Magdě, Katce a Jiřímu za veškeré rady, diskuse (více či méně odborné) a za milou společnost v laboratoři.

I want to express my deepest gratitude to the people of the 'Belgian CE lab': Sigrid, Steffi and Pieter form Belgium, Kaale from Tanzania, Ha from Vietnam, Humphrey from Cameroon for the long discussion we had (scientific and others) and for the unforgettable moments together in 'our' little laboratory. And finally, all the other colleagues for being a part of the simulating international atmosphere: Cindy, Ivo, Mieke, Anne and Kris from Belgium, Raja and Raju, Murali, Reddy, Satish and Kumar from India, José from Congo, Dora from Hungary, Wen from China and Yadian from Cuba.

Mé poděkovaní patří též Grantové agentuře ČR a Fondu rozvoje vysokých škol MŠMT za poskytnuté granty a dále Akademické informační agentuře a

Ministerstvu školství mládeže a tělovýchovy za zprostředkování mé belgické stáže.

The acknowledgement also belongs to Ministry of Flemish Community for the financial support of my Belgian stay.

Dále bych ráda poděkovala MUDr. Janu Blatnému i celému kolektivu OKH v Dětské nemocnici FN Brno za psychickou podporu při dokončování mé disertační práce.

V neposlední řadě chci poděkovat své nejbližší rodině za veškerou podporu, které se mi dostalo v průběhu celého mého studia. Nejsrdečnější dík patří mému manželovi za jeho porozumění, láskyplnou trpělivost a ochotu mi vždy pomáhat.

Soňa, September 2004

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LIST OF PUBLICATIONS

The work described in this thesis has been published in international journals:

1. NOVÁKOVÁ S., GLATZ Z. (2002) Determination of Kinetic Parameters of Rhodanese by EMMA in Partially Filled Capillary. **Electrophoresis**, 23, 1063-1069. IF 4.325

2. NOVÁKOVÁ S., TELNAROVÁ M., GLATZ Z. (2003) Inhibition Study of Rhodanese by means of EMMA. **J. Chromatogr. A**, 990, 189-195. IF 2.922

3. VAN DYCK S., NOVÁKOVÁ S., VAN SCHEPDAEL A., HOOGMARTENS J. (2003) Inhibition Study of Angiotensin Converting Enzyme by Capillary Electrophoresis after Enzymatic Reaction at Capillary Inlet. **J. Chromatogr. A**, 1013, 149-156. IF 2.922

4. NOVÁKOVÁ S., VAN DYCK S., VAN SCHEPDAEL A., HOOGMARTENS J., GLATZ Z. (2004) Study of Enzyme Kinetics of Phenol Sulfotransferase using Electrophoretically Mediated Microanalysis. **J. Chromatogr. A**, 1032, 319-326. IF 2.922

The work described in this thesis is part of two review articles published in following international journals:

1. VAN DYCK S., KAALE E., NOVÁKOVÁ S., GLATZ Z., HOOGMARTENS J., VAN SCHEPDAEL A. (2003) Review: Advances in Capillary Electrophoretically Mediated Microanalysis. **Electrophoresis**, 24, 3868-3878. IF 4.040

2. NOVÁKOVÁ S., VAN DYCK S., VAN SCHEPDAEL A., HOOGMARTENS J., GLATZ Z. (2004) Review: Electrophoretically Mediated Microanalysis. **J. Chromatogr. A**, 1032, 173-184. IF 2.922

I am co-author of the following articles that are not part of this thesis and have been published in international journals:

1. GLATZ Z., NOVÁKOVÁ S., ŠTĚRBOVÁ H. (2001) Analysis of thiocyanate in biological fluids by capillary zone electrophoresis. **J. Chromatogr. A,** 916, 273-277. IF 2.793

2. TELNAROVÁ M., VYTISKOVÁ S., GLATZ Z. (2004) Study of Enzymatic Reaction by Mediated Microanalysis in a Partially Filled Capillary and Indirect and Direct Detection. **Electrophoresis**, 25, 290-296. IF 4.040

3. TELNAROVÁ M., VYTISKOVÁ S., GLATZ Z. (2004) Application of Electrophoretically Mediated Microanalysis with Partial Filling Technique and Indirect and Direct Detection for Inhibition Study of Haloalkane dehalogenase. **Electrophoresis**, 25, 1028-1033. IF 4.040

CONGRESSES[♣]

The work described in this thesis has been presented in international symposiums or meetings as an oral presentation:

1. NOVÁKOVÁ S., GLATZ Z. Application of Capillary Zone Electrophoresis for the Study and Assay of Enzymes.
   6th Meeting of Biochemists and Molecular Biologists, 2002 Brno, Czech Republic

2. VYTISKOVÁ S., GLATZ Z. Capillary Electrophoresis as a Tool for the Study of Enzymes.
   15th International Conference: Chromatografic Methods and Human Health, 2003 Piešťany, Slovakia

3. VAN SCHEPDAEL A., VAN DYCK S., HOOGMARTENS J., NOVÁKOVÁ S. In-line Study of Enzymatic Reactions by Capillary Electrophoresis.
   27th Symposium on High Performance Liquid Phase Separations and Related Techniques HPLC 2003, Nice, France

The work described in this thesis has been presented in international symposiums as a poster presentation:

1. NOVÁKOVÁ S., TENAROVÁ M., GLATZ Z. Electrophoretically Mediated Microanalysis of Rhodanese.
   Developments in Chromatography and Electrophoresis & Chiranal 2002, Olomouc, Czech Republic

2. NOVÁKOVÁ S., GLATZ Z. Determination of the Kinetic Parameters of Rhodanese by Electrophoretically Mediated Microanalysis in a Partially Filled Capillary.
   2nd International symposium „Separations in the Biosciencies“ 2001, Prague, Czech Republic

3. NOVÁKOVÁ S., TELNAROVÁ M., GLATZ Z. Inhibition Study of Rhodanese by means of EMMA.
   3th International Symposium on Capillary Electroseparation Techniques ITP 2002, Helsinki, Finland

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4. VAN DYCK S., NOVÁKOVÁ S., VAN SCHEPDAEL A., HOOGMARTENS J. In Capillary Kinetic Study of Angiotensin Converting Enzyme (ACE) combined with the In-line Evaluation of Inhibitors such as Captopril.
   16th International Symposium on Microscale Separations and Analysis HPCE 2003, San Diego, USA

5. NOVÁKOVÁ S., VAN DYCK S., VAN SCHEPDAEL A., HOOGMARTENS J., GLATZ Z. Study of Enzyme Kinetics of Phenol Sulfotransferase using Electrophoretically Mediated Microanalysis.
   27th Symposium on High Performance Liquid Phase Separations and Related Techniques HPLC 2003, Nice, France

6. TELNAROVÁ M., NOVÁKOVÁ S., GLATZ Z. Determination of the Kinetic Parameters of Haloalkane Dehalogenase by Electrophoretically Mediated Microanalysis in a Partially Filled Capillary.
   27th Symposium on High Performance Liquid Phase Separations and Related Techniques HPLC 2003, Nice, France

7. GLATZ Z., NOVÁKOVÁ S., TELNAROVÁ M. Application of Capillary Zone Electrophoresis for the Study of Enzymes.
   27th Symposium on High Performance Liquid Phase Separations and Related Techniques HPLC 2003, Nice, France

8. VYTISKOVÁ S., PAPEŽOVÁ K., GLATZ Z. Application of EMMA for Study of Enzymes – **Awarded by Agilent Technologies**
   17th International Symposium on Microscale Separations and Analysis HPCE 2004, Salzburg, Austria

I was presenting author of the work that is not described in this thesis and has been presented in international symposiums as a poster presentation:

1. NOVÁKOVÁ S., GLATZ Z. Analysis of thiocyanate in biological fluids by capillary zone electrophoresis.
   5th Meeting of Biochemists and Molecular Biologists, 2001 Brno, Czech Republic

2. TELNAROVÁ M., VYTISKOVÁ S., PAPEŽOVÁ K., MONINCOVÁ M., GLATZ Z. EMMA with Partial Filling Technique and Indirect or Direct Detection as a Tool for Inhibition Studies of Enzymatic Reaction.
   17th International Symposium on Microscale Separations and Analysis HPCE 2004, Salzburg, Austria

3. TELNAROVÁ M., PAPEŽOVÁ K., VYTISKOVÁ S., GLATZ Z. Complex study of haloalkane dehalogenase by means of Electrophoretically Mediated MicroAnalysis.
   14th International Symposium on Capillary Electroseparation Techniques ITP 2004, Rome, Italy

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
β-Gal	β-Galactosidase
BGE	Background electrolyte
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CHAPSO	3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CGE	Capillary gel electrophoresis
CMC	Critical micellar concentration
CZE	Capillary zone electrophoresis
CTAB	Cetyltrimethylammonium bromid
DAD	Diode array detection
DMSO	Dimethylsulfoxide
DTAB	Dodecyltrimethylammonium bromid
ES	Enzyme-substrate complex
EMMA	Electrophoretically Mediated Microanalysis
EOF	Electroosmotic flow
G-6-P	Glucose-6-phosphate
G-6-PDH	Glucose-6-phospate dehydrogenase
HA	Hippuric acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid
HHL	Hippuryl-L-histidyl-L-leucine
HL	L-histidyl-L-leucine
HPLC	High performance liquid chromatography
ID	Internal diameter
IS	Internal standard
LIF	Laser induced fluorescence detection
LOD	Limit of detection
LOQ	Limit of quantitation
MEKC	Micellar electrokinetic chromatography
MES	2-(N-morpholino)-ethanesulfonic acid
MS	Mass spectrometry

NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced NADP
PAGE	Polyacrylamide gel electrophoresis
pH	Negative decadic logarithm of the proton concentration
pKa	Negative common logarithm of the ionization constant of an acid
pNP	p-Nitrophenol
psi	Pounds per inch (1 psi = 6894.76 Pa)
PST	Phenol sulfotransferase
RSD	Relative standard deviation
SDS	Sodium dodecyl sulphate or sodium lauryl sulphate
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultra violet
UV-Vis	Ultra violet-visible

TABLE OF CONTENTS

PREFACE

Enzymes are biological catalysts [1] that play an important role in biochemical reactions necessary for normal growth, maturation and reproduction through the whole living world. They catalyze virtually all chemical reactions in living systems and the assay of enzyme activity is probably one of the most frequently encountered procedures in biochemistry and molecular biology [2]. There is no ideal assay for any enzyme and in general, appropriateness of an assay will depend on the nature of the enzyme, its purity and the purpose of the assay. To be acceptable an assay should be specific, sensitive, quantitative, simple and rapid. And just capillary electrophoresis (CE) fulfils almost all these demands.

CE is a powerful and relatively new analytical tool [3,4]. The small dimensions of the CE separation systems utilized are a primary advantage for the bioresearch. A recent trend is the use of CE in the area of immunoassays and enzyme assays, due to its fast analysis time and extremely small amount of sample. Especially in-capillary approaches to study enzyme activity such as Electrophoretically Mediated MicroAnalysis (EMMA) of particular interest since the assay, and all its necessary operations, completely occurs within the capillary, thus reducing the volume of the assay from the microliter level to the nanoliter level.

This work aims to develop EMMA-based assays for different enzymes, thereby exploring the capabilities of CE-UV regarding this application. The first chapter starts with a general introduction of the employed technique, capillary electrophoresis. The basic separation principle is explained, as well as some expressions and equations related to CE separations. Then, in two reviews, in-capillary enzymatic reactions studied by CE are covered; special emphasis is given to an EMMA technique. The reviews contain a number of parts: besides the nomenclature, a basic principle, a procedure, and a detailed literature overview is provided, discussing recent developments and findings. In the second chapter, the experimental finding concerning the first enzyme, rhodanese are discussed. The first part of this chapter deals with the determination of the Michalis constants, the effect of temperature, and the type of kinetic mechanism of rhodanese reaction by EMMA in partial filled

capillary. The second part of the second chapter deals with the inhibition study of rhodanese by 2-oxoglutarate. Chapter 3 introduces the next enzyme, phenol sulfotransferase, and the results obtained with the developed EMMA methodology. This chapter describes determination of the Michaelis constant and the substrate inhibition by EMMA with preseparation step that solved the problem of the presence of the very strong inhibitor in co-substrate solution. In chapter 4, the enzyme angiotensin converting enzyme (ACE) is introduced. Capillary electrophoresis after enzymatic reaction at capillary inlet was used to determine inhibitory properties of known ACE inhibitors such as captopril. The general summary and conclusion at the end of this dissertation summarize the experimental work and discuss the capabilities of CE regarding intedrated enzyme reaction.

[1] Boyer, P. D., in Boyer, P. D. (Ed), *The Enzymes*, Academic Press Inc., New York, 1970.

[2] Bergmeyer, H. U., *Methods of Enzymatic Analysis*, Verlag Chemie, Weinheim, 1983.

[3] Li, S. F. Y., Capillary electrophoresis, principles, practice and applications, Elsevier Science B.V., Amsterdam 1993.

[4] Heiger, D., High Performance Capillary Electrophoresis, An Introduction, Agilent Technologies, Germany 2000.

CHAPTER 1
GENERAL INTRODUCTION

1.1 Capillary electrophoresis

1.1.1 Introduction

Tiselius introduced electrophoresis as a separation technique in 1937 [1]. Placing protein mixtures between buffer solutions in a tube and applying an electric field, he found that proteins migrated in a direction and at rate determined by their charge and mobility. For his work in separation science Tiselius was awarded a Nobel Prize.

Electrophoresis has been defined as the differential movement of electrically charged compounds in solution under the influence of an electric field [2]. For decades, electrophoresis has been the standard for clinical protein analysis and has become an important tool in DNA separations such as mutation screenings. The most common format of electrophoresis is the slab gel format. However, despite its ability for high-resolution analysis of complex systems, it suffers from several disadvantages. Slab gel electrophoresis is time consuming and not readily amenable to complete automation and quantitation [3]. Capillary electrophoresis offers clear advantages over slab gel electrophoresis in terms of speed, ease of automation, and quantitation [4].

Capillary electrophoresis can be broadly described as high-efficiency separations of sample ions in narrow bore capillary tube that is filled with an electrolyte solution [5]. The narrow capillary diameter facilitates the dissipation of heat generated by the electrical resistance of the electrolyte inside the capillary [6]. Because of the high field strengths short analysis times and high peak efficiencies are achieved [7]. Moreover, the large surface area-to-volume ratio of the capillary efficiently dissipates the heat. Other advantages directly arise from the micro scale dimensions of the capillary itself: the total capillary volume ranges in the order of magnitude of a few

microliters [8]. Therefore, solvent consumption is reduced in CE and sample load is typically very low since only nanoliter amounts of sample are injected.

CE is a relatively new separation technique with the first commercial equipment available in the late 1980s and early 1990s. The future of CE seems assured as CE methods are now accepted in forensic analysis laboratories, law courts and in submissions to drug regulatory authorities [9]. However, high-performance liquid chromatography (HPLC) remains to be the standard analytical technique in many industries as it offers precise, sensitive and reliable means of analyzing a very wide range of analytes. CE provides efficiencies up to two orders of magnitude greater then HPLC. Currently CE is increasingly seen as being either an alternative separation method capable of faster analysis and higher efficiency than HPLC or as complementary to augment the information obtained from the analysis [4]. Furthermore, the quantities injected are normally too low for real preparative purposes and fraction collection is not as straightforward as in HPLC.

Classic capillary electrophoretic instruments do not offer the capability of parallel analysis as in slab gel electrophoresis. To solve this problem, two approaches were obvious: a multicapillary approach and a multichannel system etched into the surface of microchips. Originally, multiplexed or array electrophoresis was developed and described for DNA sequencing but arrays have been applied in other areas as well [10]. Microfabricated electrophoresis chips offer not only volumetric reduction of reagents but separation times in as little as 1s. However, microchips are not limited to simple separations. The ability to design and construct complex channel systems has facilitated the use of microchips in biochemical analyses [11].

1.1.2 Principles of CE

Electrophoresis is the movement of sample ions under the influence of an applied voltage. The ion will move toward the appropriate electrode and pass through the detector. The migration rate, or mobility, of the solute ion is governed largely by its size and number of ionic charges. The electrophoretic mobility (μ_e) of a charged compound is determined by its charge over mass ratio and can be expressed by [12]:

$$\mu_e = \frac{q}{6 \pi \eta r} \qquad \textit{Equation 1.1}$$

where q is the charge of the particle, η is the viscosity of the buffer and r is the hydrodynamic ion radius.
The dimension of the mobility in SI units is $m^2\ V^{-1}\ s^{-1}$, however, in electrophoresis, the data given in $cm^2\ V^{-1}\ s^{-1}$ are frequently used [20].

The actual electrophoretic velocity, or speed of the solute ions, is related to their mobilities and the magnitude of the applied voltage:

$$v_e = \mu_e E \qquad \textit{Equation 1.2}$$

where v_e is the electrophoretic velocity, E is the applied electric field.

1.1.2.1 Electroosmotic flow

A fundamental force in CE is the electroosmotic flow (EOF). EOF or electroosmosis refers to the movement of uncharged liquid relative to the stationary charged surface (e.g. capillary inner wall) due to applied potential gradient [20]. Above pH 3, the silanol groups of the fused silica wall start to ionize and counterions (cations) from the buffer solution tend to adsorb onto the wall to balance the surface charge. The result is a rigid double layer of tightly adsorbed cations immediately adjacent to the wall, which is superposed by a diffuse double layer (Gouy-Chapman layer) with more loosely associated cations (Fig. 2). The potential drop over this diffuse double layer is termed the zeta potential (ζ). The application of an electric field results in the movement of the more loosely bound cations towards the cathode, and since they are hydrated the consequence is a bulk flow of liquid in the same direction [1,2].

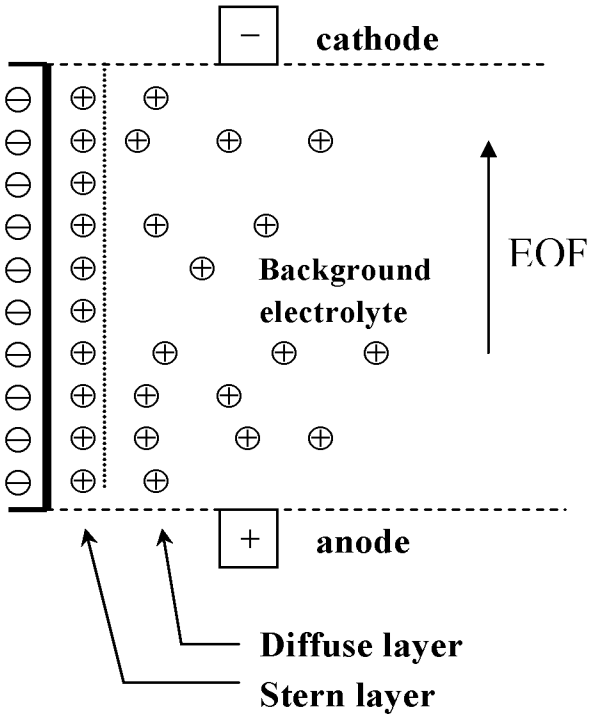

Fig. 2. The principal of electroosmotic flow inside the silica capillary.

The electroosmotic velocity (v_{eo}) can be expressed as [5]:

$$v_{eo} = \frac{\varepsilon \zeta}{4 \pi \eta} E \qquad \textit{Equation 1.3}$$

where ε is the dielectric constant of the solvent and ζ is the zeta potential. The zeta potential is essentially determined by the surface charge on the capillary wall. Since this charge is strongly pH dependent, the magnitude of the EOF varies with pH.

Under the conditions of a negatively charged capillary wall, the EOF is oriented from the anode to the cathode. Anions will be swept towards the cathode since the magnitude of the flow can be more than an order of magnitude greater than their electrophoretic mobilities. Thus cations, neutral compounds and anions can be analyzed in a single run since they all move in the same direction. When electrophoresis is applied at 'normal' polarity, i.e. injection at the anode and detection at the cathode, the positively charged

solutes will migrate first, followed by the neutral species and finally the negatively charged compounds.
The apparent mobility of a compound is then the sum of the electrophoretic and the electroosmotic mobility [5]:

$$\mu_{app} = \mu_e + \mu_{eo}$$ *Equation 1.4*

The apparent mobility ($cm^2\ V^{-1}\ s^{-1}$) can be determined directly from an electropherogram as shown in the following equation [2]:

$$\mu_{app} = \frac{L_D}{E\ t_r}$$ *Equation 1.5*

where E is the applied field strength, t_r is the migration time of a compound (r) and L_T is the effective length of the capillary. The μ_{eo} can be determined with EOF markers such as acetone, DMSO, mesityl oxide and benzylalcohol.

This electrically driven transport of liquid through the capillary exhibits a flat flow profile, unlike in HPLC where a parabolic flow profile is obtained because transport of liquid is obtained by a high-pressure pump. This flat flow profile of the EOF limits band broadening in CE and contributes to the high efficient separations in CE.
While the EOF is usually beneficial, it often needs to be controlled. Fundamentally, control of EOF requires alteration of the capillary surface charge or buffer viscosity. There are several methods to accomplish this, such as changes in electric field, buffer pH, ionic strength or buffer concentration, temperature, or EOF can be controlled by modification of the capillary wall by means of dynamic (buffer additives) or covalent coating.

1.1.2.2 Joule heating

The heat generated by the passage of electrical current is called Joule heat. The temperature increase depends on the power (product of voltage and current) generated and is determined by capillary dimensions, conductivity of the buffer, and the applied voltage. The main advantage of performing electrophoresis in narrow-bore capillaries is reduction of the effects of heating which have traditionally limited electrophoretic techniques. Heating is problematic since it can cause nonuniform temperature gradients, local changes in viscosity, and subsequent zone broadening.

Although the application of high field strengths leads to fast and efficient separations, currents above 100 μA are avoided. In addition, highly conductive electrolytes at high concentrations increase heat production. Therefore, the biological buffers (or Good buffers [13]) such as TRIS, MES and HEPES are commonly used in CE because they can be used at much higher concentrations than inorganic electrolytes due to their low conductivity.
Temperature regulation is important in CE due to the strong viscosity dependence of sample injection and migration time. In order to obtain reproducible conditions, temperature control of the capillary is commonly achieved by forced air cooling or by liquid cooling. The most efficient approach is liquid cooling. Joule heating can also be limited by decreasing of electric field, or by reducing of capillary inner diameter.

1.1.3 Modes of operation in CE

Electrophoretic techniques may be classified according to various aspect, here is presented a classification according to the characteristic course of the separation process. The basic modes are listed in table 2 together with their specific basis of separation. Mostly, the different modes are accessed simply by altering the buffer composition and can be carried out by using the same equipment.

Table 2. Different modes of operation in CE.

Mode of CE	Basis of separation
Capillary zone electrophoresis (CZE)	Mobility of ion in free solution
Micellar electrokinetic chromatography (MEKC)	Hydrophobic / ionic interactions with micelle
Capillary gel electrophoresis (CGE)	Size and charge
Capillary isoelectric focusing (CIEF)	Isoelectric point
Capillary isotachophoresis (CITP)	Moving boundaries
Capillary electrochromatography (CEC)	Electrophoretic + chromatographic mechanism of separation

Capillary gel electrophoresis (CGE) is the capillary format of classical PAGE where electrophoresis is performed using gel-based support media. Capillary isotachophoresis (CITP) and capillary isoelectric focusing (CIEF) are essentially identical to the classical isotachophoresis and isoelectric focusing. The only difference lies in the fact that the processes take place in capillary dimensions.

1.1.3.1 Capillary zone electrophoresis (CZE)

The principles of separation in CZE, or "free solution capillary electrophoresis", are discussed in chapter 1.1.3. CZE is characterized by migration of the sample in a background electrolyte (BGE) which conducts practically all the electric current. The composition of background electrolyte remains constant along the migration path and does not change with time [20]. Currently CZE is the most commonly used technique in CE. Separation of both anionic and cationic solutes is possible by CZE due to the electroosmotic flow. Neutral solutes can not be separated by CZE as they all coelute with the EOF.

1.1.3.2 Micellar electrokinetic chromatography (MEKC)

MEKC is a unique mode of CE since it can separate neutral as well as charged solutes [14]. MEKC is a separation technique combining some of the operational principles of micellar liquid chromatography and CZE. This technique can be considered as a type of chromatography with a moving "stationary micellar phase" and with electroosmotically pumped mobile phase [15].

In MEKC, a surfactant is added to the background electrolyte. When the surfactant concentration is higher than the its critical micelle concentration (CMC), micelles are formed. The use of MEKC was firstly described by Terabe and co-workers [16].

At present micellar phases of SDS and cetyltrimethylammonium bromide (CTAB) are mostly used for electrophoretic separation [17]. Micelles of ionic (or cationic) surfactants migrate electrophoretically due to their surface charge, e.g. negatively charged SDS micelles to the anode. Because in most

cases the electroosmotic mobility (μ_{eo}) is higher than the electrophoretic mobility of the micelle ($\mu_{e,MC}$), the net velocity of micelles is towards the anode. Nonionic solutes partition between the hydrophobic micellar phase and the aqueous buffer phase is depending on the polarity of the compound. For these substances, the separation is based on the degree of partitioning (*i.e.* a chromatographic separation mechanism). Micelles can also interact with solutes through electrostatic interactions. For ionic solutes, separation is therefore based on chromatography and charge effects, including electrophoresis [18].

The selectivity can easily be manipulated in MEKC. Varying the physical nature of the miccelle by using different surfactants can yield dramatic changes in selectivity, similar to those obtained by changing the stationary phase in LC. Surfactants can be anionic, non-ionic, zwitterionic, or mixtures of each (Table 3).

MEKC is a dynamic mode of CE since it can be used for charged and uncharged analytes and for wide range of substances with hydrophilic or hydrophobic characteristics.

Table 3. Surfactants.

	Biological detergent	**CMC (mM)**
Anionic	SDS	8.2
Cationic	DTAB	14
	CTAB	1.3
Non-ionic	n-Dodecyl-β-D-maltoside	0.16
	Triton X-100	0.24
Zwitterionic	CHAPS	8
	CHAPSO	8
Bile salt	Cholic acid	14
	Deoxycholic acid	5
	Taurocholic acid	10-15

1.1.3.3 Capillary electrochomatography (CEC)

CEC is a hybrid of both CE and HPLC [19] and should offer itself as a superior analytical technique as it takes the advantage of the benefits of CE miniaturization and the selectivity of HPLC. However, the technique is far from mature and currently it is the least exploited electrophoretically driven separation technique.

CEC uses electrically driven flow to transport the solutes through a capillary packed with stationary phase material. The electrically driven flow should reduce band broadening associated with pressure-driven parabolic-flow profiles. Separations are achieved by the use of both electrophoretic and chromatographic mechanisms.

CEC can offer unique selectivities and generate highly efficient separations but it suffers from technical problems. The main operating problem with CEC occurs due to localized heating and air bubble formation inside the capillary, leading to current (and EOF) breakdown [19]. Considerable research efforts are paid to the development of novel packing materials that could enhance the performance of the technique [9].

1.1.4 Instrumentation

1.1.4.1 Instrumental set-up

The applicability of any analytical method lives and dies with the access to a suitable instrumentation [20]. One key feature of CE is the overall simplicity of the instrumentation. A typical schematic of an instrument set-up is shown in Fig. 1. The principal components are a high voltage power supply, a capillary that passes trough the optical center of a detection system connected to a data acquisition device, a sample introduction system, and an autosampler. Typically, the CE instrument is controlled by a personal computer. In CE, capillaries typically have internal diameters (ID) ranging from 25 to 100 μm and are 20 to 100 cm long. They are mostly made of fused silica because of its good UV transparency and thermal conductivity. To make them strong and easy to handle, the capillaries are coated with polyimide. For optical detection, a detection window can easily be created, by the removal of

a small section of the protective polyimide coating. Capillaries made of other materials, e.g., glass or Teflon, are used seldom [20].

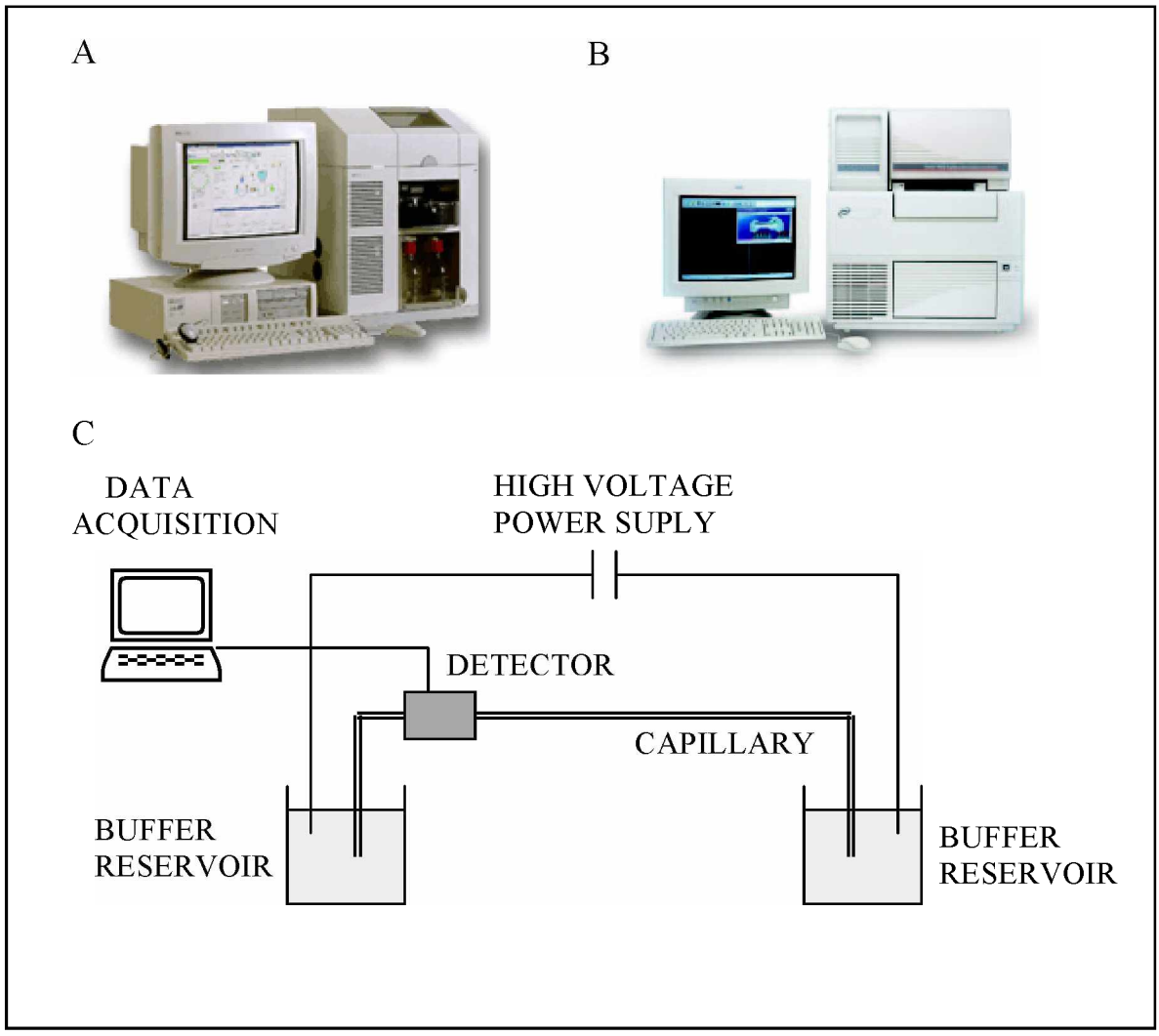

Fig. 1 A the Agilent CE Instrument (from Agilent Technologies, Waldbronn, Germany), B the P/ACE MDQ capillary electrophoresis instrument (from Beckman Instruments, Fullerton, CA, USA), C schematic overview of a CE instrument.

1.1.4.2 Sample injection

In CE only minute volumes of sample are loaded into the capillary in order to maintain high efficiency. With respect to sample overloading, the injection plug length is more critical parameter than volume. As a rule of thumb, the sample plug length should be less then 1 to 2 % of the total length of capillary

[1]. When the initial sample pulse occupies 1 % of the capillary length, then no more then 120 000 plates can be attained [20].

In CE the sample can be injected hydrodynamicaly [21,22] or electrokineticaly [23]. Hydrodynamic sample injection is the most widely used method. It can be accomplished by application of pressure at the injection end of the capillary, vacuum at the exit end of the capillary, or by siphoning action obtained by elevating the injection reservoir. The injected volume v_i (m^3) will be a function of the applied pressure difference Δp (Pa) along the capillary and can be calculated by Poiseuille's law for liquid flow through a circular tube [5]:

$$v_i = \frac{\Delta p \, \pi \, d^4 \, t_i}{128 \, \eta \, L_T} \qquad \textit{Equation 1.6}$$

where d is the capillary inside diameter (or the ID, expressed in m), t_i is the injection time (s), η is the viscosity (Pa s) and L_T is the total capillary length (m). As can be seen in the equation, the volume of sample loaded is a function of the capillary dimensions and the viscosity of the buffer in the capillary.

The sample can also be injected electrokineticaly where the voltage is applied to sample vial and the combination of analyte mobility and EOF drives the sample into the capillary. Usually field strength 3 to 5 times lower than that used for separation is applied.

1.1.4.3 Detection

Detection in CE is a significant challenge as a result of the small dimensions of the capillary. As in HPLC, UV-Vis detection is by-far the most common. The major advantage of UV-Vis detection is its nearly universal nature since most organic molecules exhibit at least some absorption in the lower UV. However, in the small ID capillaries in which CE detection is typically performed, the path length dependency of absorbance detection limits the sensitivity. This path length dependency directly results from Beer's law [2]:

$$A = \varepsilon \, b \, c \qquad \textit{Equation 1.7}$$

where A is the measured absorbance, ε is the molar extinction coefficient of the compound, b is the optical path length (cm) and c is the concentration of the sample (M). As a result of the small path length in CE (< 100 μm), the concentration detection limits are relatively poor, generally falling in the 10^{-6} M range. Several attempts have been made to improve these detection limits by increasing the detection path length through the use of a bubble, a Z-shaped, or a multireflection detection cell. Unfortunately, the theoretically predicted increases in absorbance have not been achieved and often a reduction in resolution was seen [24].

Other detection techniques have been described, i.e. indirect absorbance detection, fluorescence, electrochemical and refractive index detection. A special class, of course, is represented by the detection systems used in so called hyphenated techniques, e.g. combination of CE with mass spectrometry, CE-MS. In CE-MS, the efficiency and speed of analysis in CE are combined with the high selectivity of the MS detector. However, the interface between the CE instrument and the MS detector is complicated [24].
Fluorescence detectors represent another widely used class of optical detectors in CE. Fluorescence detection is more sensitive than light absorbance but it is more selective, too. The most sensitive on-line detection scheme for CE is laser-induced fluorescence detection (LIF). Compared with UV absorption detection, sensitivity of enhancement when using fluorescence and LIF detection is about 10-fold to 1000-fold, respectively.

Indirect detection represents almost universal detection alternative in capillary electrophoresis. By term "indirect" is meant here the detection technique where some characteristic property of the background electrolyte constituent is monitored (UV absorbance, fluorescence, electrochemical transformation, etc.) and the detection signal is obtained as a decrease of this background electrolyte signal due to the migration of the zone of separand molecules which replace the detection active component of the BGE [20].

Table 1. Different detection methods in CE [1].

Method	Mass detection limit (moles)	Concentration detection limit (molar)	Advantages/disadvantages
UV-Vis	10^{-13}-10^{-15}	10^{-5}-10^{-8}	• Universal • Diode array offers spectral information
Fluorescence	10^{-15}-10^{-17}	10^{-7}-10^{-9}	• Sensitive • Requires sample derivatization
Laser-induced fluorescence	10^{-18}-10^{-20}	10^{-14}-10^{-16}	• Extremely sensitive • Requires sample derivatization • Expensive
Amperometry	10^{-18}-10^{-18}	10^{-10}-10^{-11}	• Sensitive, selective • Useful only for electroactive analyses • Requires special electronics, capillary modification
Conductivity	10^{-15}-10^{-16}	10^{-7}-10^{-8}	• Universal • Requires special electronics, capillary modification
Mass spectrometry	10^{-16}-10^{-17}	10^{-8}-10^{-9}	• Sensitive • offers structural information • Interface between CE and MS
Indirect UV, fluorescence, amperometry	10-100 times less than direct method	-	• Universal • Lower sensitivity than direct methods

1.1.5 Biomedical application of CE

The rapid and reproducible analysis of extremely low volume samples makes CE ideal for clinical use. An interesting feature of CE is the possibility of directly injecting a tiny amount of biofluid (serum, urine, cerebrospinal fluid,...) onto the capillary. This can reduce sample pretreatment which is an advantage in clinical chemistry where sample throughput is high. One of the major barriers with transplanting CE from the research environment to the clinical laboratory is throughput. Even though some commercial CE units have the ability to analyze a large number of samples in an automated fashion, analysis is sequential and not simultaneous. Therefore, for routine analyses, "multicapillary clinical CE" units will need to be devised; however, the work on it has already begun [3].

CE has found many applications in the carbohydrate area and in the areas of metal ions, small inorganic ions, proteins and peptides and pharmaceuticals [9]. CE will also find a use in the clinical laboratory with specialized analysis, for example, determination of the specific DNA fragments linked with diagnosis of certain disease states. To success of CE for the analysis of small organic and inorganic ions has made it perfectly suited for certain forensic analyses. Forensic DNA analysis represents an exciting and challenging new area for CE as well. Within the field of molecular biology, the use of CE for the sequencing of genes is not only feasible but appears to be superior to standard technique [3].

The use of CE to study enzyme–substrate interaction is discussed on the following pages.

1.2 In-capillary enzymatic assays

1.2.1 Introduction

Enzymes are often identified and quantitated by measuring their biological activity, *i.e.* their catalytic activity. In enzyme assays, the catalyzed conversion of a substrate by a specific enzyme provides information about enzyme activity and reaction kinetics involved. In addition to studying kinetics of the free enzyme, kinetics in the presence of an inhibitor are

studied. Inhibitors can reversibly or irreversibly deactivate an enzyme. The influence of inhibitors on enzyme kinetics is used for determination of the type of inhibition and of the inhibition constants. This characterization is extremely important in the design and development of new drugs as well as in clinical chemistry [25].

Initially, CE was only used as a separation tool in the determination of enzyme activities. In these off-line enzyme assays, the reaction between enzyme and substrate occurs outside the capillary in reaction vial. After the incubation step, the reaction mixture is injected into the capillary and CE is used to separate the compounds. As a separation technique, CE offers advantages over other techniques such as the high speed of analysis and small sample volumes. However, in order to further scale down the quantity of enzyme required for analysis and thus to obtain a real nanoliter scale assay, the reaction should be conducted directly in the capillary [26].

1.2.2 In-line set-up: heterogeneous and homogeneous assays

In the in-line approach, the enzymatic reaction is integrated in the CE separation. For an in-line enzyme assay, it is necessary to have conditions that are able to (a) mix the necessary reagents together, (b) carry out the enzymatic reaction, (c) separate the product(s) from the substrate(s) and the enzyme, and (d) detect the results of the reaction. Two approaches can be distinguished in this set-up: heterogeneous and homogeneous enzyme assays.

1.2.2.1 Heterogenous assay

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. Advantages of immobilized enzyme capillary microreactors include picoliter to nanoliter volume requirements, higher stability, and the ability to reuse the enzymes. The group of El Rassi [27] developed a coupled format, which they referred to as enzymophoresis. Different enzymes, such as hexokinase and adenosine deaminase were successfully immobilized on the inner wall of short fused-silica capillaries. These open-tubular enzyme reactors were coupled in tandem

with a normal CZE capillary via a Teflon tube. The substrates first entered the capillary enzyme reactor and were converted to products that were further separated in the separation capillary for finally on-capillary detection by CE-UV. This system allowed on-line digestion and mapping of minute amounts of ribonucleic acids and the simultaneous synthesis and separation dinucleotides.

Some drawbacks are associated with heterogeneous capillary enzyme reactors. When the enzymes are immobilized on a surface certain properties such as the activity *vs.* pH dependency can be altered. Only limited conditions can be explored without affecting the immobilized enzymes and these reactors have only limited use since most enzymes are specific to only one or a few reactions. Additionally, the preparation of enzyme microreactors is labor-intensive and time consuming. Moreover, it is difficult to quantitate the number of enzymes actually immobilized on the capillary and to reproduce the same capillary reactor. Therefore, quantitation and reproducibility is a problem in the heterogeneous enzyme reactor approach [26].

1.2.2.2 Homogenous assay

In homogeneous enzymatic assays, both the enzyme and substrate(s) are present in solution, *i.e.* in the liquid state. This approach eliminates the above-mentioned drawbacks associated with immobilized enzymes [26]. Homogeneous enzyme assays can be roughly divided in two groups: the orthogonal approach (1) and the linear approach (2). In the first format, more than one electrophoretic capillary or channel is involved in the enzymatic reaction and separation. They are called orthogonal assays because the structural organization of the channels/capillaries is most often orthogonal. Most of the assays that belong to the orthogonal approach are performed on planar CE chips. The second format, the linear approach, comprises all assays that are performed in only one channel or capillary. Almost all assays that fall into the latter category are based on electrophoretically mediated microanalysis (EMMA) and are discussed in detail on the following pages in two reviews.

REFERENCES

[1] Heiger, D., High Performance Capillary Electrophoresis, An Introduction, Agilent Technologies, Germany 2000.

[2] Kuhn, R. and Hoffstetter-Kuhn S., *Capillary Electrophoresis: Principles and Practice*, Springer-Verlag, Berlin 1993.

[3] Landers, J. P., in Landers, J. P. (Ed.) *Handbook of capillary electrophoresis*, CRC Press, Inc., Boca Raton 2000.

[4] Li, S. F., Y., Capillary electrophoresis, principles, practice and applications, Elsevier Science B.V., Amsterdam 1993.

[5] Altria, K.D., in Altria, K.D. (Ed.) *Capillary Electrophoresis Guidebook*, Humana press, Totowa 1996.

[6] Jandik, P., Bonn, G., *Capillary electrophoresis of small molecules and ions*, VCH Publishers, Inc., New York 1993.

[7] Kemp, G., *Biothechnol. Appl. Biochem.* 1998, 27, 9-17.

[8] Kovichi, N.J. in P. Camilleri (Ed.) *Capillary Electrophoresis: Theory and Practice*, CRC Press, Boca Raton 1993.

[9] Altria, K.D., *J. Chromatogr. A* 1999, 856, 443-463.

[10] Ma, L., Gong, X. and Yeung, E.S., *Anal. Chem.* 2000, 72, 3383-3387.

[11] Zugel, A. A., Burke, B. J., Regnier F. E., Lytle, F. E., *Anal. Chem.* 2000, 72, 5731-5735.

[12] Karger, B.L., Foret, F., in Guyman, N.A. (Ed.), *Capillary Elecrophoresis Technology*, Marcel Dekker Inc., New York 1993.

[13] Good, N.E., Izawa, S., in San Pietro, A. (Ed.), *Methods in Enzymology, Volume XXIV*, Academic Press, New York 1972.

[14] Khaledi, M.G., *J. Chromatogr. A* 1997, 780, 3-40.

[15] Terabe, S., Otsuka, K., Ando, T., *Anal. Chem.* 1985, 57, 834-841.

[16] Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A. and Ando T., *Anal. Chem.* 1984, 56, 111-113.

[17] Burton, D.E., Sepaniak, M.J., Maskarinec, M.P., *J. Chromatogr. Sci.* 1987, 25, 514-518.

[18] Thormann, W., *Therap. Drug Monit.* 2002, 24, 222-231.

[19] Altria, K.D., Smith, N.W. and Turnbull, C.H., *Chromatographia* (1997), 46, 664-674.

[20] Foret, F., Křivánková, L., Boček, P., *Capillary zone electrophoresis*, VCH Publishers, Inc., New York 1993.

[21] Gross, L., Yeung, E.S., *Anal. Chem.* 1990, 62, 427-431.
[22] Hjertén, S., *Chromatogr. Rev.* 1967, 9, 122-219.
[23] Aguilar, M., Huang, X., Zare R.N., *J. Chromatogr.* 1989, 480, 427-431.
[24] Swinney, K. and Bornhop, D.J., *Electrophoresis* 2000, 21, 1239-1250.
[25] Guijt, R.M., Baltussen E. and van Dedem G.W.K., *Electrophoresis* 2002, 23, 823-835.
[26] Bao, J.J., Fujima, J.M. and Danielson, N.D., *J. Chromatogr. B* (1997) 699, 481-497.
[27] Nashabeh, W. and El Rassi, Z., *J. chromatog.*. 1992, 596, 251-264.
[28] Van Dyck, S., Vissers, S., Van Schepdael, A., Hoogmartens, J., *J. Chromatogr. A* (2003) 986, 303-311.

1.2.3 Electrophoretically mediated microanalysis

1.2.3.1 REVIEW: Electrophoretically Mediated Microanalysis

NOVÁKOVÁ S., VAN DYCK S., VAN SCHEPDAEL A., HOOGMARTENS J., GLATZ Z. (2004), J. Chromatogr. A, 1032, 173-184.

EMMA_review1.pdf

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Review

Electrophoretically mediated microanalysis

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Abstract

This review describes the existing developments in the use of the capillary electrophoretic microanalytical technique for the in-line study of enzyme reaction, electrophoretically mediated microanalysis (EMMA). The article is divided into a number of parts. After an introduction, the different modes, basic principle, procedure, and some mathematical treatments of EMMA methodology are discussed and illustrated. The applications of EMMA for enzyme assay and for non-enzymatic determination are summarized into two tables. In addition to classical capillary electrophoresis (CE) instrument EMMA, special emphasis is given to a relatively new technique: EMMA on CE microchip. Finally, conclusions are drawn.

Keywords: Reviews; Electrophoretically mediated microanalysis; Chip technology; Enzymes

Contents

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0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.12.025

1. Introduction

Enzymes are biological catalysts [1] that play an important role in biochemical reactions necessary for normal growth, maturation and reproduction through the whole living world. They catalyze virtually all chemical reactions in living systems and the assay of enzyme activity is probably one of the most frequently encountered procedures in biochemistry and molecular biology [2]. Due to their low concentrations in samples containing a large amount of other proteins, direct measurements of enzymes by mass are impossible. However, enzymes can be measured more easily by their catalytic activities, which are the most relevant properties of enzymes in the biochemical context.

There is no ideal assay for any enzyme and in general, appropriateness of an assay will depend on the nature of the enzyme, its purity and the purpose of the assay. To be acceptable an assay should be specific, sensitive, quantitative, simple and rapid. It should also be unaffected by side reactions and by presence of drugs or antimetabolites in both crude and purified samples. And just capillary electrophoresis (CE) with its different modes—capillary zone electrophoresis, micellar electrokinetic capillary chromatography, capillary isoelectric focusing, capillary electrochromatography, etc. fulfils almost all these demands.

CE is a powerful and relatively new analytical tool [3,4]. The small dimensions of the CE separation systems utilized are a primary advantage for the bio-research. A recent trend is the use of CE in the area of immunoassays and enzyme assays, due to its capabilities and advantages over other techniques. First, CE offers fast analysis time and requires extremely small amounts of sample. Second, CE provides the capability of highly efficient separations of the reaction products from the substrates. Third, several relatively sensitive detection methods, such as UV-Vis spectrophotometry, laser-induced fluorescence (LIF) and mass spectrometry, are available for CE. Therefore, detection can be accomplished without the use of radiolabeled materials. Currently, the availability of sophisticated and automated CE equipment makes the technique suitable to be implemented in routine laboratories.

CE enzyme assays can be divided into three distinct categories [5]:

(i) a "pre-capillary" assay which is performed by sampling from an assay solution and separating substrate(s) and product(s) by means of CE;
(iii) an "on-capillary" assay which is performed by electrophoretically mixing enzyme and substrate(s) within the capillary and consecutive on-capillary detection of the product(s);
(iii) a "post-capillary" assay which is performed by mixing substrate(s) with enzyme or vice versa, previously separated by CE.

The second alternative—the on-capillary assay—is of particular interest since the assay, and all its necessary operations, completely occurs within the capillary, thus reducing the volume of the assay from μl to nl.

2. Electrophoretically mediated microanalysis

In CE, the separations are generally based upon differences in electrophoretic mobility. The first work, in which the variability in electrophoretic mobilities among enzyme and its substrate(s) was used not only for the initiation of an enzymatic reaction inside the capillary but also for the separation and the detection of the reaction product(s), was published by Bao and Regnier in 1992 [6]. These authors initially named the new method "ultramicro enzyme assays in capillary electrophoretic system". A year later, the article dedicated to mathematical treatment of this new concept in enzyme assay was published by the same group (Professor F.E. Regnier, Department of Chemistry, Purdue University, West Lafayette, IN, USA) and the methodology was denominated as electrophoretically mediated microanalysis (EMMA) [7]. Since this time, the term EMMA has been accepted by other authors and it has become "terminus technicus". In relation with other methods applied for enzyme assay, Bao et al. classified the EMMA methodology as *homogeneous enzyme assay by CE* [8], whereas in *heterogeneous enzyme assay by CE* one of the reactants (most often the enzyme) is immobilized onto the wall of the capillary.

In general, enzyme assay requires a number of operations such as the mixing of reagents and the initiation of the reaction, the incubation of the reaction mixture, and consequently detection of the reaction product(s). The EMMA methodology couples all these operations in one integrated technique utilizing the different electrophoretic mobilities of enzyme and substrate(s) to initiate reaction inside the capillary and to separate the components of the reaction mixture from each other for the final on-capillary quantification [7].

There are basically two ways to mix the reaction components in a capillary under electrophoretic conditions [9–11]. In the continuous mode of EMMA (long contact mode), the capillary is initially completely filled with one of the reactants while the second reactant is introduced. In contrast, the plug–plug mode of EMMA (transient format or short contact mode) is based on a plug–plug interaction of reactants in the capillary.

2.1. Continuous modes of electrophoretically mediated microanalysis

The continuous mode of EMMA can be divided into two variants on the basis of the difference in introduction of the second reactant.

2.1.1. Zonal sample introduction method

In this variant, the capillary is initially filled with one of the reactants and upon injection of the second reactant,

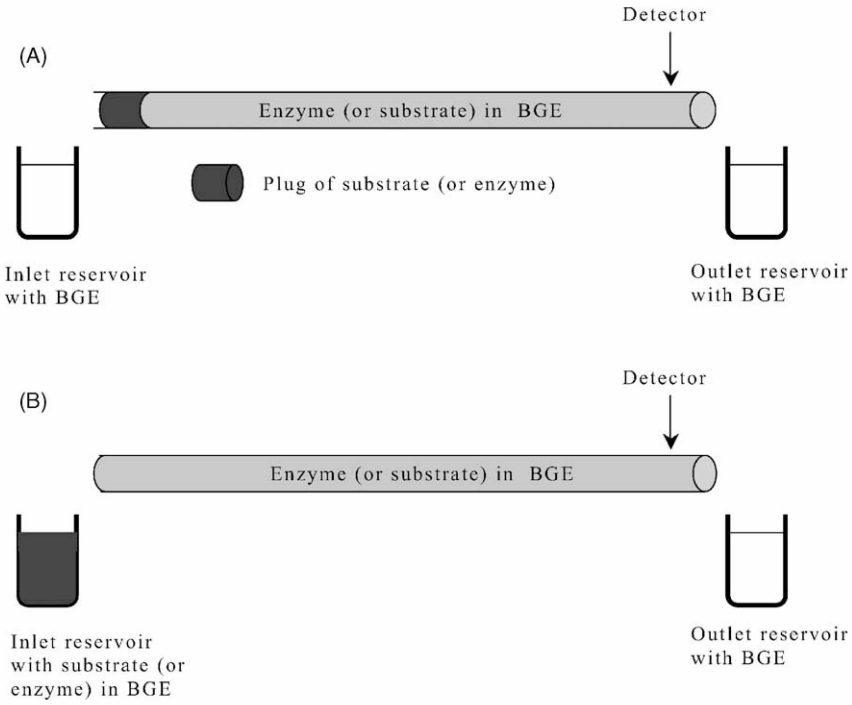

Fig. 1. Schematic illustration of the continuous modes of EMMA: (A) zonal sample introduction; (B) moving boundary sample introduction.

product(s) are formed during the electrophoretic mixing of the enzyme and the substrate(s) (Fig. 1A) [6,7,12–21]. This type of electrophoretically mixing of the reactants was used in the pioneering work of Bao and Regnier [6]. In their experiments, the running buffer (background electrolyte (BGE)) contained the substrate (glucose-6-phosphate) and coenzyme (NADP) required for the catalyzed reaction. The enzyme—glucose-6-phosphate dehydrogenase (G-6-PDH)—was injected into the capillary, and the formation of product (NADPH) was monitored at a downstream absorbance detector at 340 nm (Fig. 2).

2.1.2. *Moving boundary introduction method*

Although CE has traditionally employed zonal injection mode, moving boundary CE has been reported by Pawliszyn and Wu as an alternative sample introduction technique [22,23]. This alternative injection mode was first used in EMMA methodology by Harmon and co-workers for a microsomal leucine aminopeptidase assay [24,25]. In this variant, the capillary is initially filled with the slower migrating reactant (enzyme or substrate) while the faster migrating complementary reactant is maintained in the inlet buffer reservoir (Fig. 1B). Upon the application of an electric field, the faster migrating reactant electrophoretically enters the capillary from the inlet reservoir and interpenetrates the slower migrating species present in the capillary. This variant results in greater reactant overlap than with the zonal injection mode and, consequently, typically yields an order of magnitude higher concentration sensitivity, which can be seen from the electropherograms in Fig. 3.

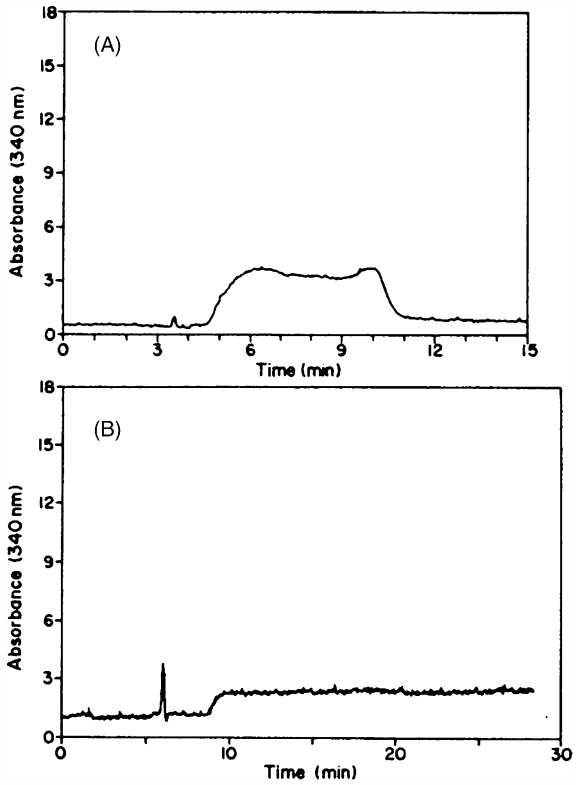

Fig. 2. Typical electropherograms, after zonal sample introduction, showing the formation of NADPH during the process of G-6-PDH migration through the capillary: (A) short separation length or high potential situation; (B) long separation length or low potential situation. From [6], with permission.

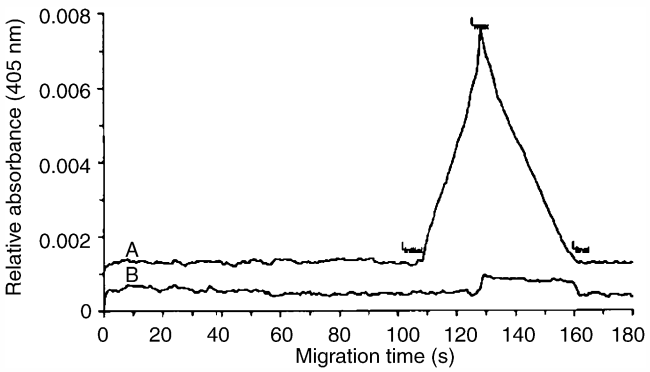

Fig. 3. Moving boundary (A) and 5 nl zonal injection kinetic EMMA (B) determinations of microsomal leucine aminopeptidase (LAP). LAP was assayed by hydrolysis of L-leucine–*p*-nitroanilide to L-leucine and *p*-nitroaniline, which was monitored on basis of unique absorbance at 405 nm. From [24], with permission.

2.2. Plug–plug modes of electrophoretically mediated microanalysis

The plug–plug mode can be divided into four different variants.

2.2.1. Classical plug–plug mode

In this variant, enzyme and substrate(s) are introduced into the capillary as distinct plugs, the first reactant injected being the one with the lower electrophoretic mobility [5,26–39]. Upon the application of an electric field, the two zones interpenetrate due to differences in their electrophoretic mobilities. Enzymatic reaction takes place and the resultant reaction product(s) and the unreacted substrate(s) are electrophoretically transported towards the detector, where they

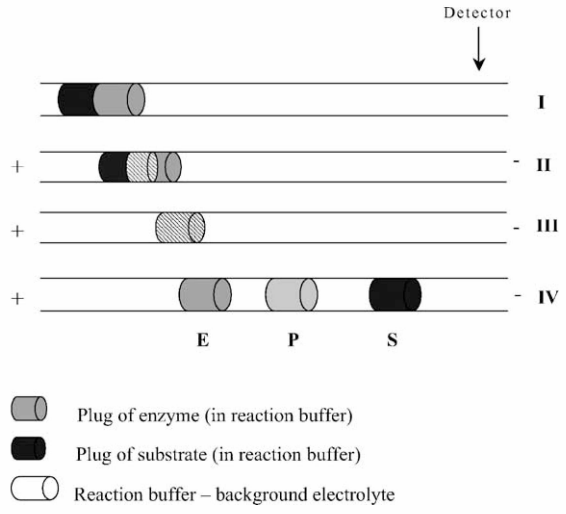

Fig. 4. Schematic illustration of the classical plug–plug mode of EMMA (E: enzyme; S: substrate; P: product of enzymatic reaction). (I) A plug of enzyme and substrate are introduced consecutively in the capillary. (II and III) Upon the application of an electric field the two zones interpenetrate due to differences in their electrophoretic mobilities, and enzymatic reaction takes place. (IV) The reaction product and the unreacted substrate are electrophoretically transported to the detector.

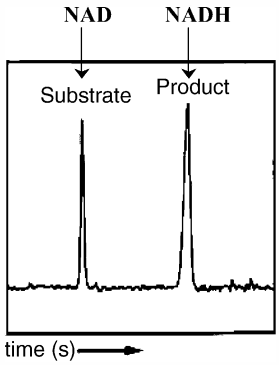

Fig. 5. Electropherogram of the classical plug–plug EMMA assay of G-6-PDH. This electropherogram shows a model system of the conversion of NAD to NADH, in the oxidation of glucose-6-phosphate to 6-phosphogluconate by G-6-PDH. From [5], with permission.

are individually detected (Fig. 4). As an example, Kwak et al. applied the plug–plug mode of EMMA to almost the same model system as Bao and Regnier—the conversion of NAD to NADH in the oxidation of glucose-6-phosphate to 6-phosphogluconate by G-6-PDH (Fig. 5) [5].

2.2.2. Partial filling technique

The most important factor for successful application of the EMMA methodology in the study of enzymes is, that the electrophoretic conditions, especially the composition and pH of the background electrolyte, are favorable for both the separation of substrate(s) and product(s), and the enzymatic reaction itself. Sometimes the conditions required for the enzymatic reaction and for the electrophoretic separation are totally different making it impossible to use the classical EMMA arrangement. Recently, Van Dyck et al. introduced the combination of the EMMA methodology with a partial filling technique [40]. In this set-up, part of the capillary is filled with the optimum buffer for the enzymatic reaction whereas the rest of the capillary is filled with the background electrolyte optimal for the separation of substrate(s) and product(s) (Fig. 6). Van Dyck et al. combined the EMMA methodology with micellar electrokinetic capillary chromatography for determining bovine plasma amine

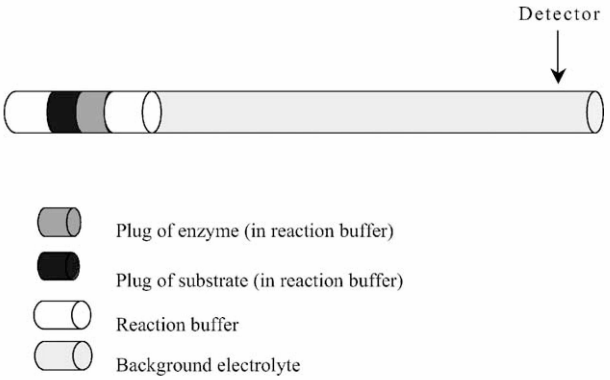

Fig. 6. Schematic illustration of plug–plug mode of EMMA with partial filling technique.

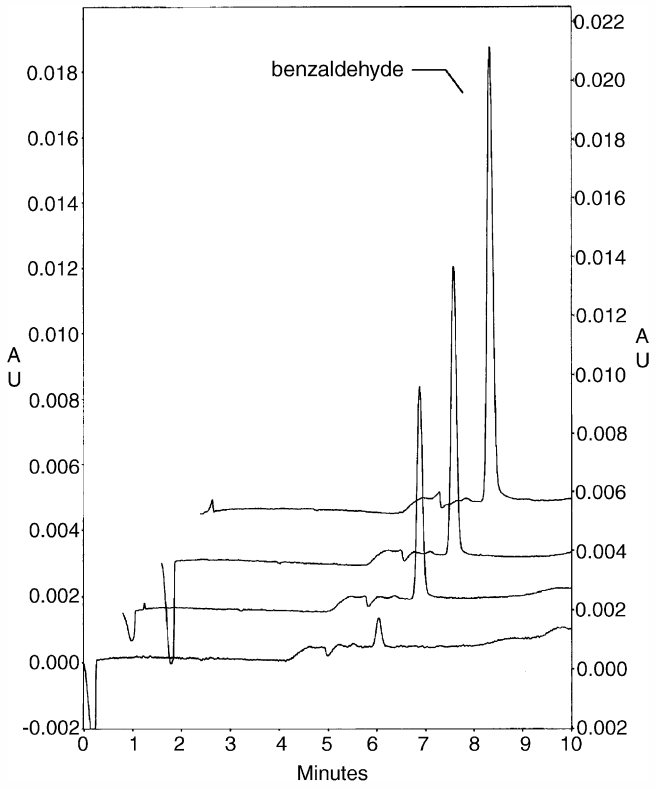

Fig. 7. Overlay of four electropherograms showing plasma amine oxidase assay with benzylamine as a substrate using partial filling EMMA combined with micellar electrokinetic chromatography. Two zones were used in this work: a zone filled with phosphate buffer that allows the reaction to proceed and a second zone, the separation zone, which separates the formed benzaldehyde from the other reaction products. From [40], with permission.

oxidase activity with benzylamine as substrate (Fig. 7). So far, several other background electrolytes have been applied in this innovative EMMA modification—low pH background electrolyte in combination with direct detection for adsorbing inorganic anions with high mobilities [41,42], 10 mM chromate–0.1 mM cetyltrimethylammonium bromide (CTAB) in combination with indirect detection for non-adsorbing inorganic anions with high mobilities [43] and 30 mM sorbic acid–0.1 mM CTAB in combination with indirect detection for non-adsorbing organic anions with moderate mobilities [11].

2.2.3. At-inlet reaction

The at-inlet technique has been described by Taga and Honda, who performed CE with derivatization reactions at the capillary inlet, using amino acids as model compounds [44]. Van Dyck et al. applied this technique on enzymatic reaction [45]. After a sandwich mode injection, enzyme–substrate(s)–enzyme, the overlayed plugs of enzyme and substrate(s) are allowed to react at the capillary inlet. Unlike in a typical EMMA analysis, enzyme and substrate(s) are not electrophoretically mixed prior to the reaction, their mixing is driven by simple diffusion. Subsequently, voltage is applied and the reaction compounds are separated and quantified (Fig. 8). This technique was used for kinetic study of angiotensin converting enzyme (Fig. 9). This variant is especially suitable for enzymes not resistant to an electric field.

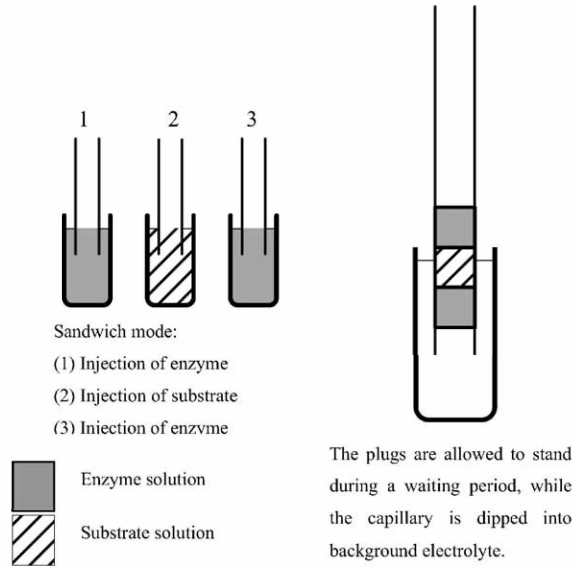

Fig. 8. Schematic illustration of the at-inlet reaction. After the injection of the individual reagents the overlayed plugs are allowed to stand during a predetermined waiting period; subsequently voltage is applied and the reaction compounds, together with the in-line generated reaction product, are transported to the detector.

2.2.4. Electroinjection analysis

The electroinjection analysis (EIA) was introduced by Andreev and co-workers as an alternative to the EMMA methodology [46–48]. In view of the fact that the principle is similar, EIA is covered in this review as a special variant of the plug–plug mode of EMMA.

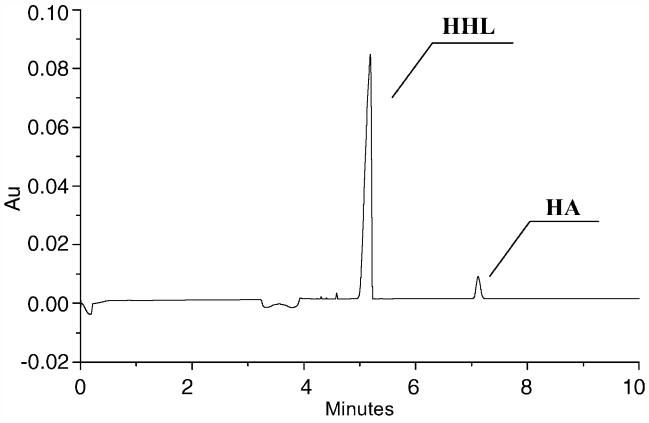

Fig. 9. Electropherogram after on-capillary reaction of angiotensin converting enzyme (ACE) at the capillary inlet. ACE activity was assayed by the conversion of hippuryl-L-histidyl-L-leucine (HHL) to hippuric acid (HA). From [45], with permission.

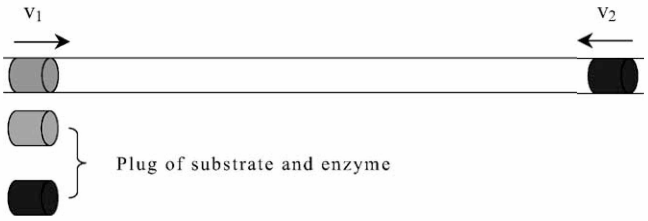

Fig. 10. Schematic illustration of electroinjection analysis (EIA), v_1 and v_2 are substrate and enzyme velocities, respectively.

In EIA, mixing of the reactants is also due to the difference of their electrophoretic mobilities in the applied electric field, but the reactants are simultaneously injected from the opposite ends of the capillary by using electrokinetic injection and they are moving in opposite directions (Fig. 10). Because of the presence of the electroosmotic flow, it is possible to mix not only oppositely charged reactants but even reactants with the same charge and different electrophoretic mobilities. EIA brought several advantages over classic EMMA methodology: (i) the possibility of using very short capillaries and consequently lower electric fields; and (ii) the prevention of cross-contamination of enzyme and its substrate(s) because they are injected through opposite ends of the capillary. Andreev et al. used this technique for non-enzymatic Cr(VI) determination as a red complex of Cr(III)–diphenylcarbazone (Fig. 11). This reaction was easily performed by EIA since Cr(VI) as CrO_4^{2-} has high mobility whereas the reagent—diphenylcarbazide is almost neutral [47].

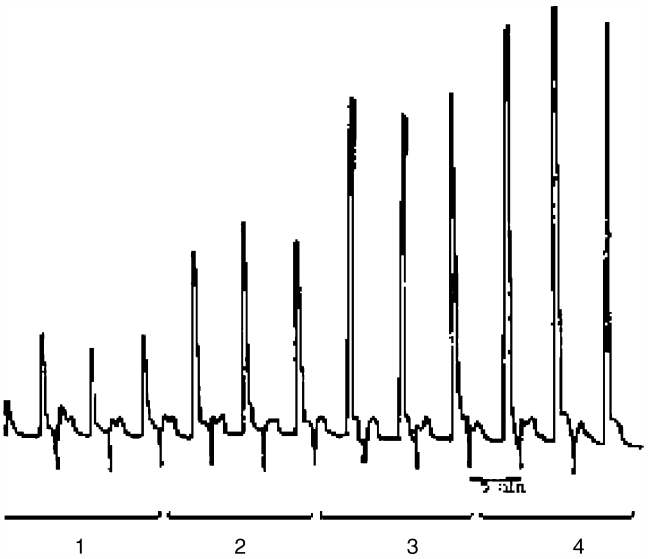

Fig. 11. Electropherogram of EIA determination of Cr(VI). This figure shows a series of product peaks in the determination of Cr(VI) with diphenylcarbazide corresponding to: (1) 50 ng/ml Cr; (2) 100 ng/ml Cr; (3) 150 ng/ml Cr; (4) 200 ng/ml Cr. From [47], with permission.

3. Electrophoretically mediated microanalysis methodology

At present, the plug–plug mode of EMMA is more advantageous compared to the continuous mode, owing to several reasons, e.g. less reagent is required for analysis since only small plugs of enzyme and substrate(s) are used in the experiment. Moreover, the electropherograms of all variants of the plug–plug mode are similar to that of conventional CE, in contrast with those of the continuous mode, and their evaluation can be performed by means of classic integration software. This is especially evident from the comparison of Figs. 2 and 5, in which the authors studied the same enzymatic system. For these reasons the following part, dedicated to the EMMA methodology, is oriented mainly on the plug–plug mode. Generally, the EMMA methodology includes three steps:

(i) reagent metering;
(ii) initiation and progress of reaction;
(iii) detection of reaction product.

3.1. Reagent metering

The EMMA methodology requires that the enzyme and its substrate(s) are sequentially introduced into the capillary. To preserve the efficient separation capabilities of CE, the injection system should not cause significant zone broadening. Therefore, it is important that the sample injection method employed is capable of delivering small volumes of sample efficiently and repeatedly into the capillary [3]. In EMMA, reactants are typically introduced as zones of finite width by traditional CE injection methods, such as hydrodynamic or electrokinetic injection. Hydrodynamic sample injection is the most widely used method, in which the volume of sample loaded is a function of the capillary dimensions, the viscosity of the buffer, the applied pressure, and the injection time. Electrokinetic, or electromigration, injection is performed by applying a voltage, which is typically three to five times lower than the voltage used for separation.

Injection volumes in CE are typically a few nl. These ultramicroinjection volumes allow the analysis of samples available in very small amounts, such as the interstitial and intercellular fluids, which is a very promising application field for EMMA methodology.

3.2. Initiation and progress of reaction

In traditional methods, chemical and enzymatic reactions are generally initiated by turbulent mixing of the solutions containing the reactants. However, in all EMMA modes, with exception of the at-inlet variant, the mixing of the enzyme and its substrate(s) is accomplished by exploiting the variability in transport velocity among the chemical species in the chosen electrophoretic medium [7]. Following the injection of the reactants into the capillary, electrophoretic

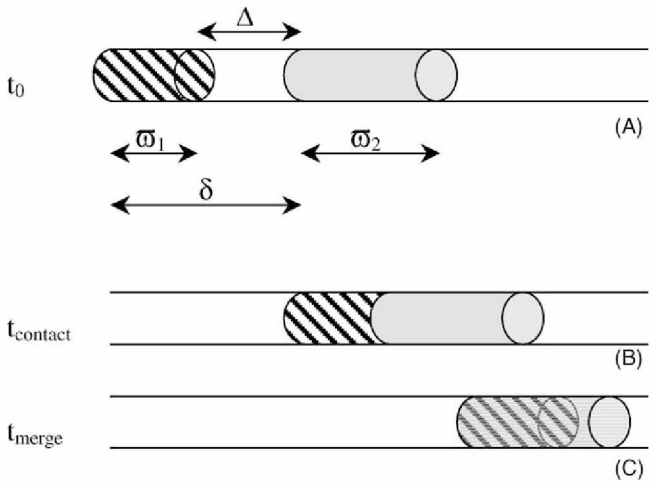

Fig. 12. An illustration of electrophoretic mixing of two zones: (A) spatially distinct zones; (B) beginning engagement; (C) completely mixed.

mixing is initiated by the application of an electric field. The reactant zones migrate at a differential velocity v_{DIFF} based on the difference in electrophoretic mobility between the two components of interest $\Delta\mu_{EP}$ and the applied electric field strength E:

$$v_{DIFF} = \Delta\mu_{EP} E \quad (1)$$

Knowledge of the difference in electrophoretic mobilities and distance between reactant zones allows to calculate the contact time $t_{contact}$ (Fig. 12B), neglecting the effect of longitudinal diffusion:

$$t_{contact} = \frac{\Delta}{\Delta\mu_{EP} E} \quad (2)$$

where Δ is the distance between the leading edge of the zone of greater transport velocity and trailing edge of the zone of lesser transport velocity (Fig. 12A). If the reactant zones are injected adjacently ($\Delta = 0$), interpenetration of the zones occurs immediately upon the application of an electric field. The interpenetration of the zones continues as the potential is maintained, and the reactant of greater transport velocity migrates through that of lower transport velocity. Furthermore, the total time of the enzymatic reaction $t_{reaction}$ can be estimated as:

$$t_{reaction} = \frac{\varpi_1 + \varpi_2}{\Delta\mu_{EP} E} \quad (3)$$

where ϖ_1 and ϖ_2 are the lengths of the reagent zones (Fig. 12A).

Prolonged on-capillary incubation of enzyme and substrate(s) can be achieved by turning off the voltage when the two zones are completely mixed. The time required for the two zones to completely merge t_{merge} (Fig. 12C), can be estimated from the formula:

$$t_{merge} = \frac{\delta}{\Delta\mu_{EP} E} \quad (4)$$

where δ is the distance between the trailing edge of the zone of greater transport velocity and the trailing edge of the zone of lesser transport velocity (Fig. 12A). Turning off the voltage at this point (t_{merge}) allows the reaction to continue in the absence of an electric field. The technique is called "zero potential amplification". This built-in step is usually necessary to accumulate enough product(s) for the spectrophotometric detection. Occasionally, the EMMA methodology without turning off the voltage is called "constant potential EMMA" and the one with turning off the voltage is called "zero potential EMMA".

3.3. Detection of reaction product

Detection in the EMMA methodology is generally performed by electrophoretically transporting the detectable species to the detection system. The small capillary dimensions and the tiny injection volumes present a real challenge to achieve sensitive detection without introducing zone dispersion. Zone broadening normally caused by joints, fittings and connectors as in LC is eliminated by on-capillary detection. The lower limit of detection in CE varies with the detection method. With the nl volumes used in CE, it is often possible for on-capillary UV-Vis absorbance detection to achieve a limit of detection of 10^{-7} M (i.e. 10^{-16} mol) [49]. Although UV-Vis spectrophotometry is probably the most used detection technique in EMMA methodology, several applications of LIF were also published. LIF detection can provide highly sensitive detection and allows a very low concentration detection limit of 10^{-12} M (i.e. 10^{-21} mol) [49]. For example, Lee et al. reported a method for the determination of picomolar concentration of proteins, in which EMMA methodology was used to label proteins on-capillary with a fluorogenic reagent [50]. In this particular case, the method was called EMMA–CE–LIF. Chemiluminescence is yet another detection technique that is highly sensitive and often highly specific. Regehr and Regnier described chemiluminescent detection in EMMA methodology for three different enzymes and demonstrated a limit of detection for catalase of 15 zmol (9300 molecules) [49]. However, LIF and chemiluminescence detections require expensive equipment and are rather difficult to operate. Electrochemical detection is another method, which can provide comparable limits at lower cost, but special equipment, not always familiar with commercial CE instruments is also necessary [17].

As mentioned earlier, the drop in the detection limits could be achieved by chemical amplification and zero potential modes.

4. Application of electrophoretically mediated microanalysis

Since its discovery by Bao and Regnier, EMMA methodology has been applied in a number of biochemical systems—for assays of enzyme activity, determination of substrates, Michaelis constants, inhibitors and inhibition constants. In addition, it has also been used in several

Table 1
Overview of the applications of the EMMA methodology on enzyme systems

Enzyme	Type of EMMA	Detection	Note	Reference
Glucose-6-phosphate dehydrogenase	Plug–plug	UV (260 nm)		[5]
Glucose-6-phosphate dehydrogenase	Zonal	UV (340 nm)	First work using EMMA	[6]
Alcohol dehydrogenase	Zonal	UV (340 nm)	Mathematical treatment of EMMA	[7]
Alkaline phosphatase	Combination zonal and plug–plug	LIF	Inhibition study	[9]
β-Glucosidase	Plug–plug	UV (214 nm)	EMMA in coated capillary with linear polyacrylamide	[10]
Fructose-biphosphate aldolase, fructose-1,6-diphosphatase	Partial filling mode	Indirect UV (254 nm)	30 mM sorbic acid–0.1 mM CTAB	[11]
Alcohol dehydrogenase	Zonal	UV (340 nm)	EMMA of ethanol	[12]
Leucine aminopeptidase	Zonal	LIF		[13]
Alkaline phosphatase, β-galactosidase	Zonal	Vis (405 nm)	Gel filled capillaries	[14]
Alcohol dehydrogenase, leucine aminopeptidase, alkaline phosphatase, β-galactosidase	Zonal	UV (340 nm) Vis (405 nm)	Selectivity in EMMA by control of product detection time	[16]
Alkaline phosphatase	Zonal	Electrochemical: carbon electrode held at +180 mV vs. Ag/AgCl, Vis (405 nm)	Electrochemical detection is more sensitive than spectrophotometric detection	[17]
Lactate dehydrogenase	Zonal	LIF	Detection limit: 1.3×10^{-21} mol of LDH	[18]
Hexokinase/apyrase, lactate dehydrogenase/glucose-6-phosphate dehydrogenase	Zonal	UV (260 nm)	Double enzyme-catalyzed microreactors using CE	[20]
Leucine aminopeptidase	Moving boundary	Vis (405 nm)		[24]
Leucine aminopeptidase	Zonal and moving boundary	Vis (405 nm)	Dynamic modeling of EMMA	[25]
Glucose-6-phosphate dehydrogenase, alcohol dehydrogenase	Zonal and plug–plug	UV (260 and 340 nm)	According to authors: an enzymatic CE microreactor	[26]
Pepsin	Plug–plug	LIF	Peptide mapping	[27]
Adenosine deaminase	Plug–plug	UV (254 nm)		[28]
Adenosine deaminase	Plug–plug	UV (254 nm)	Inhibition study	[29]
Phosphodiesterase I	Plug–plug	UV (260 nm)	Oligonucleotide analysis	[30]
Phosphodiesterase I	Plug–plug	UV (260 nm)	Degradation of oligonucleotides with capillary polymer sieving electrophoresis	[31]

Glucose-6-phosphate dehydrogenase, acid phosphatase	Plug–plug	UV (200 nm)		[32]
Alkaline phosphatase	Plug–plug	UV (230 nm)	Michaelis–Menten analysis of ALP	[33]
Creatine kinase	Plug–plug	UV (256 nm)		[34]
α-Amylase, glucoamylase	Plug–plug	UV (280 nm)	Measurement of α-amylase and glucoamylase activities in sake rice koji	[35]
Cyclophilin (peptidyl-prolyl-*cis*/*trans*-isomerase)	Plug–plug	UV (200 nm)	Combined with affinity capillary electrophoresis	[36]
α-Glucosidase, β-galactosidase, β-*N*-acetylglucosamidase	Plug–plug	UV (214 nm), Vis (405 nm)		[37]
Catechol-*O*-methyltransferase	Plug–plug	UV (200 nm)		[38]
γ-Glutamyltransferase	Plug–plug	UV (380 nm)	EMMA combined with MEKC	[39]
Amine oxidase	Partial filling mode	UV (254 nm)	EMMA combined with MEKC	[40]
Rhodanese	Partial filling mode	UV (200 nm)		[41]
Rhodanese	Partial filling mode	UV (200 nm)	Inhibition study	[42]
Haloalkane dehalogenase	Partial filling mode	Indirect UV (315 and 375 nm)	10 mM chromate–0.1 mM CTAB	[43]
Angiotensin converting enzyme	At-inlet reaction	UV (230 nm)		[45]
Glucose oxidase, galactose oxidase, catalase	Zonal	Chemiluminescent		[49]
Leucine aminopeptidase	Plug–plug	Two-photon excited fluorescence detection	EMMA on microchip	[51]
Alkaline phosphatase	Zonal	LIF	EMMA on microchip	[52]
β-Galactosidase	Plug–plug	Fluorescence	EMMA on microchip	[53]
β-Galactosidase	Zonal	LIF	EMMA on microchip-inhibition study	[54]
Protein kinase	Zonal	Fluorescence	EMMA on microchip-inhibition study	[55]
Acetylcholinesterase	Zonal	LIF	EMMA on microchip-inhibition study	[56]
Lactate dehydrogenase	Zonal	UV (280 and 340 nm)		[57]
Glucose oxidase	Zonal	LIF		[58]
Alkaline phosphatase	Combination zonal and plug–plug	LIF	Inhibition study of ALP by theophylline	[59]
Lactate dehydrogenase	Zonal	Electrochemical: carbon electrode held at +0.8 V vs. SCE		[60]
N-Acetylneuramidase	Zonal	UV (200 nm)	According to authors: throughout-capillary derivatization	[61]
Alkaline phosphatase, acid phosphatase	Plug–plug and partial filling mode	UV (270 nm)	Determination of water-soluble vitamins	[62]

MEKC: micellar electrokinetic chromatography; SCE: saturated calomel electrode.

Table 2
Overview of the applications of the EMMA methodology on non-enzymatic determinations.

Determination	Type of EMMA	Detection	Note	Reference
EMMA of calcium	Zonal	Vis (575 nm)	Complexometric reaction of calcium with *o*-cresolphtalein	[15]
Redox activities of microorganisms	Zonal	Vis (610 nm)	Characterization of microorganisms	[21]
Determination of Cr(VI) and Co(II)	EIA	Vis (500 and 540 nm)	Mathematical model	[47]
Analysis of proteins	Plug–plug	LIF	EMMA was used to label proteins on-capillary with fluorogenic reagent	[50]
Analysis of catalytic role of *Monascus* pigment	Zonal plug–plug	UV (264 nm)	On-capillary MEKC	[63]
Quality control of gentamicin	Plug–plug	UV (330 nm)	EMMA is used for on-capillary derivatization	[64]
Protein fingerprinting of six *Staphylococcus* species	Plug–plug	LIF	Manipulation of protein fingerprints during on-capillary fluorescent labeling	[65]
Jaffé reaction between creatinine and picrate	Plug–plug	Vis (485 nm)	EMMA with small molecules	[66]
Quality control of kanamycin	Plug–plug	UV (335 nm)	EMMA is used for on-capillary derivatization	[67]
Determination of glutathione, DTT, cysteine, homocysteine	Plug–plug	UV (200 and 343 nm)	Specific thiol determination by on-capillary reaction with 2,2′-dipyridyldisulfide	[68–71]

special non-enzymatic determinations. Most of the applications together with the type of EMMA, detection technique and special remarks are summarized in Tables 1 and 2.

5. Electrophoretically mediated microanalysis on microchip

Miniaturized CE systems for chemical, especially for biochemical and clinical analysis are attractive for a number of reasons: low sample and reagent consumption, decreased analysis times, automated control of dilution, mixing and separation and integration on a single device (Fig. 13). The EMMA methodology in a chip format easily combines the operations of reactants loading, merging and mixing, execution of enzymatic reactions for a fixed time, product(s) separation, and detection in a single channel. The fact that multiple operations are combined in one channel greatly simplifies device fabrication and operation. Given the high throughput of microfabricated systems and many advantages of the EMMA methodology, it seems highly advantageous to combine these two techniques. As a result, the EMMA methodology was transferred to different microchip formats. Zugel et al. reported determination of leucine aminopeptidase by means of two-photon excited fluorescence detection via the EMMA methodology on a microchip [51]. Murakami et al. described the EMMA assay of alkaline phosphatase on a glass chip [52], and Burke and Regnier published EMMA methodology of β-galactosidase on microchip [53] (Fig. 14). Microchip devices have also been used for inhibition studies of β-galactosidase [54], protein kinase A [55], and acetylcholinesterase [56].

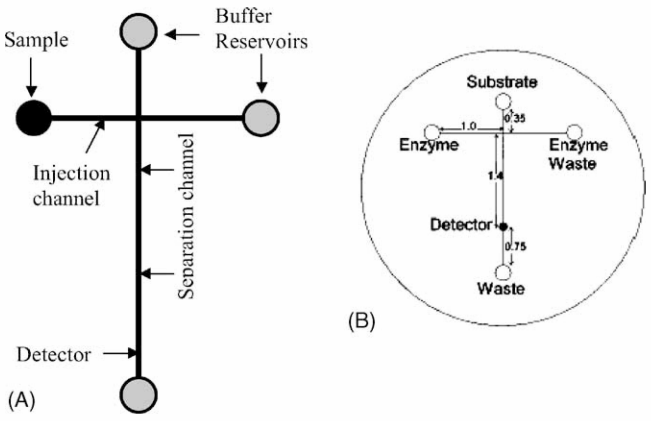

Fig. 13. Schematic illustration of simple CE microchip devices: (A) typical cross-channel topography of miniaturized CE chip; (B) schematic diagram of cross-shaped microchip, which was used for EMMA assay of leucine aminopeptidase. All dimensions are in cm. From [51], with permission.

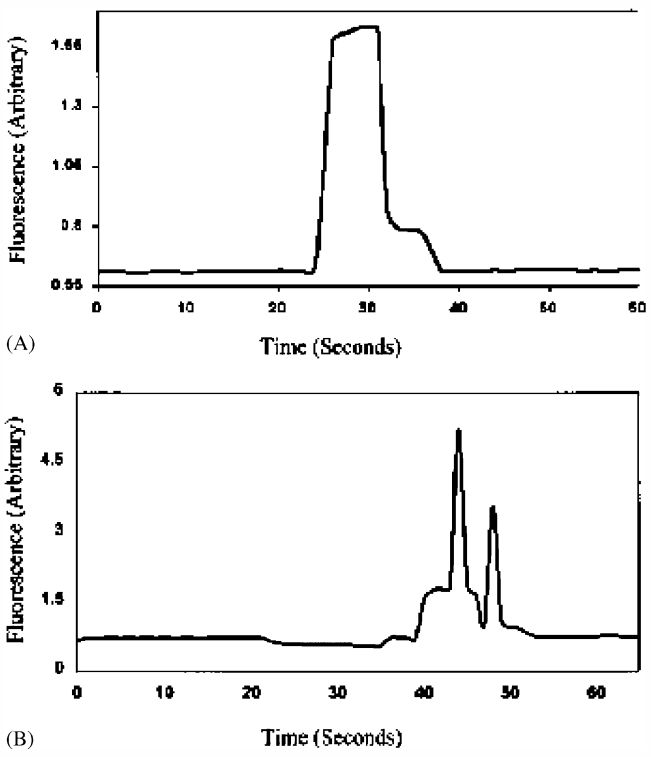

Fig. 14. The overlayed electropherograms of EMMA assay of β-galactosidase on borosilicate microchip: (A) constant potential EMMA of β-galactosidase; (B) zero potential EMMA of β-galactosidase. From [53], with permission.

6. Conclusion

The EMMA methodology offers numerous advantages over traditional enzymatic assays. The use of electrophoretic mixing allows reagent zones to be merged without the concurrent dilution experienced in bulk methods, does not cause turbulence and the resulting band spreading. Moreover, it allows homogeneous kinetic enzyme assays to be performed and detected entirely on-capillary with very high mass sensitivity due to the small dimension of CE separation systems and the amplifying nature of enzymatic reactions. Compared with spectrophotometric and other discontinuous assays, the method is rapid, can be automated, and requires only small amounts of reagents, which is especially important in the case of enzymes. EMMA could become a very powerful method when it is combined with the microchip capillary electrophoresis systems or the multichannel electrophoretic microchips.

Acknowledgements

The authors thank the Grant Agency of Czech Republic (grant No. 203/03/1125) and the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT grant No. 993194).

References

[1] P.D. Boyer (Ed.), The Enzymes, Academic Press, New York, 1970.
[2] H.U. Bergmeyer, Methods of Enzymatic Analysis, Verlag Chemie, Weinheim, 1983.
[3] S.F.Y. Li, Capillary Electrophoresis—Principles, Practice and Applications, Elsevier, Amsterdam, 1992.
[4] D. Heiger, High Performance Capillary Electrophoresis—An Introduction, Agilent Technologies, Waldbronn, 2000.
[5] E.S. Kwak, S. Esquivel, F.A. Gomez, Anal. Chim. Acta 397 (1999) 183.
[6] J. Bao, F.E. Regnier, J. Chromatogr. 608 (1992) 217.
[7] B.J. Harmon, D.H. Patterson, F.E. Regnier, Anal. Chem. 65 (1993) 2655.
[8] J.J. Bao, J.M. Fujima, N.D. Danielson, J. Chromatogr. B 699 (1997) 481.
[9] A.R. Whisnant, S.D. Gilman, Anal. Biochem. 307 (2002) 226.
[10] Y. Kanie, O. Kanie, Electrophoresis 24 (2003) 1111.
[11] Y. Zhang, M.R. El-Maghrabi, F.A. Gomez, Analyst 125 (2000) 685.
[12] B.J. Harmon, D.H. Patterson, F.E. Regnier, J. Chromatogr. A 657 (1993) 429.
[13] K.J. Miller, I. Leesong, J. Bao, F.E. Regnier, F.E. Lytle, Anal. Chem. 65 (1993) 3267.
[14] D. Wu, F.E. Regnier, Anal. Chem. 65 (1993) 2029.
[15] D.H. Patterson, B.J. Harmon, F.E. Regnier, J. Chromatogr. A 662 (1994) 389.
[16] B.J. Harmon, I. Leesong, F.E. Regnier, Anal. Chem. 66 (1994) 3797.
[17] D. Wu, F.E. Regnier, M.C. Linhares, J. Chromatogr. B 657 (1994) 357.
[18] C. Xue, E.S. Yeung, Anal. Chem. 66 (1994) 1175.
[19] F.E. Regnier, D.H. Patterson, B.J. Harmon, Trends Anal. Chem. 14 (1995) 177.
[20] D.S. Zhao, F.A. Gomez, Electrophoresis 19 (1998) 420.
[21] M. Torimura, S. Ito, K. Kano, T. Ikeda, Y. Esaka, T. Ueda, J. Chromatogr. B 721 (1999) 31.
[22] J. Pawliszyn, J. Wu, J. Chromatogr. 559 (1991) 111.
[23] J. Wu, J. Pawliszyn, Talanta 39 (1992) 1281.
[24] B.J. Harmon, I. Leesong, F.E. Regnier, J. Chromatogr. A 726 (1996) 193.
[25] D.H. Patterson, B.J. Harmon, F.E. Regnier, J. Chromatogr. A 732 (1996) 119.
[26] L.Z. Avila, G.M. Whitesides, J. Org. Chem. 58 (1993) 5508.
[27] H.T. Chang, E.S. Yeung, Anal. Chem. 65 (1993) 2947.
[28] J. Saevels, A. Van Schepdael, J. Hoogmartens, Electrophoresis 17 (1996) 1222.
[29] J. Saevels, K. Van den Steen, A. Van Schepdael, J. Hoogmartens, J. Chromatogr. A 745 (1996) 293.
[30] J. Saevels, A. Van Schepdael, J. Hoogmartens, J. Capillary Electrophoresis 4 (1997) 167.
[31] J. Saevels, K. Huygens, A. Van Schepdael, J. Hoogmartens, Anal. Chem. 69 (1997) 3299.
[32] D.S. Zhao, F.A. Gomez, Chromatographia 44 (1997) 514.
[33] Y. Xu, X. Liu, M.P.C. Ip, J. Liq. Chromatogr. Relat. Technol. 21 (1998) 2781.
[34] J.M. Fujima, N.D. Danielson, Anal. Chim. Acta 375 (1998) 233.
[35] T. Watanabe, A. Yamamoto, S. Nagai, S. Terabe, Electrophoresis 19 (1998) 2331.
[36] S. Kiessig, H. Bang, F. Thunecke, J. Chromatogr. A 853 (1999) 469.
[37] Y. Kanie, O. Kanie, Carbohydr. Res. 337 (2002) 1757.
[38] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 23 (2002) 1341.
[39] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 23 (2002) 2854.
[40] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 22 (2001) 1436.
[41] S. Nováková, Z. Glatz, Electrophoresis 23 (2002) 1063.
[42] S. Nováková, M. Telnarová, Z. Glatz, J. Chromatogr. A 990 (2003) 189.
[43] M. Telnarová, S. Vytisková, R. Chaloupková, Z. Glatz, Electrophoresis 25 (2004) 290.
[44] A. Taga, S. Honda, J. Chromatogr. A 742 (1996) 243.
[45] S. Van Dyck, S. Vissers, A. Van Schepdael, J. Hoogmartens, J. Chromatogr. A 986 (2003) 303.
[46] V.P. Andreev, A.G. Kamenev, N.S. Popov, Talanta 43 (1996) 909.
[47] V.P. Andreev, N.B. Ilyina, E.V. Lebedeva, A.G. Kamenev, N.S. Popov, J. Chromatogr. A 772 (1997) 115.
[48] V.P. Andreev, N. Pliss, J. Chromatogr. A 845 (1999) 227.
[49] M.F. Regehr, F.E. Regnier, J. Capillary Electrophoresis 3 (1996) 117.
[50] I.H. Lee, D. Pinto, E.A. Arriaga, Z. Zhang, N.J. Dovichi, Anal. Chem. 70 (1998) 4546.
[51] S.A. Zugel, B.J. Burke, F.E. Regnier, F.E. Lytle, Anal. Chem. 72 (2000) 5731.
[52] Y. Murakami, T. Morita, T. Kanekiyo, E. Tamiya, Biosens. Bioelectron. 16 (2001) 1009.
[53] B.J. Burke, F.E. Regnier, Electrophoresis 22 (2001) 3744.
[54] A.G. Hadd, D.E. Raymond, J.W. Halliwell, S.C. Jacobson, J.M. Ramsey, Anal. Chem. 69 (1997) 3407.
[55] C.B. Cohen, E. Chin-Dixon, S. Jeong, T.T. Nikiforov, Anal. Biochem. 273 (1999) 89.
[56] A.G. Hadd, S.C. Jacobson, J.M. Ramsey, Anal. Chem. 71 (1999) 5206.
[57] J.M. Fujima, N.D. Danielson, J. Capillary Electrophoresis 3 (1996) 281.
[58] Z. Jin, R. Chen, L.A. Colón, Anal. Chem. 69 (1997) 1326.
[59] A.R. Whisnant, S.E. Johnston, S.D. Gilman, Electrophoresis 21 (2000) 1341.
[60] W.C. Yang, A.M. Yu, H.Y. Chen, Chem. J. Chin. Univ. 22 (2001) 547 (in Chinese).
[61] A. Taga, M. Sugimura, S. Suzuki, S. Honda, J. Chromatogr. A 954 (2002) 259.

[62] H. Okamoto, T. Nakajima, Y. Ito, J. Chromatogr. A 986 (2003) 153.
[63] T. Watanabe, T.K. Mazumder, A. Yamamoto, S. Nagai, S. Arimoto-Kobayashi, H. Hayatsu, S. Terabe, Mutat. Res. 444 (1999) 75.
[64] E. Kaale, E. Van Goidsenhoven, A. Van Schepdael, E. Roets, J. Hoogmartens, Electrophoresis 22 (2001) 2746.
[65] Z. Zhang, E. Carpenter, X. Puyan, N.J. Dovichi, Electrophoresis 22 (2001) 1127.
[66] C.J. Kochansky, S. Koziol, T.G. Strein, Electrophoresis 22 (2001) 2518.
[67] E. Kaale, A. Van Schepdael, E. Roets, J. Hoogmartens, Electrophoresis 24 (2003) 1119.
[68] Z. Glatz, H. Mašlaňvá, J. Chromatogr. A 895 (2000) 179.
[69] Z. Glatz, P. Ševčíková, J. Chromatogr. B 770 (2002) 237.
[70] P. Ševčíková, Z. Glatz, J. Sep. Sci. 26 (2003) 734.
[71] P. Ševčíková, Z. Glatz, J. Tomandl, J. Chromatogr. A 990 (2003) 197.

1.2.3.2 REVIEW: Advances in Capillary Electrophoretically Mediated Microanalysis

VAN DYCK S., KAALE E., NOVÁKOVÁ S., GLATZ Z., HOOGMARTENS J., VAN SCHEPDAEL A. (2003) Electrophoresis, 24, 3868-3878.

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Review

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Advances in capillary electrophoretically mediated microanalysis

Recent developments in the use of capillary electrophoretic techniques for the in-line study of enzyme reactions and derivatization protocols are reviewed. The article is divided into two parts: (i) in-line enzyme reactions and (ii) in-line derivatization. The first part introduces electrophoretically mediated microanalysis (EMMA) and discusses and illustrates the different modes of EMMA. A literature overview is provided, starting from 1996, and the investigated enzymes are classified into two tables based on the mode of engagement (*i.e.*, continuous or transient) of the developed EMMA-based assay. The second part starts with an introduction of the procedures and the nomenclature used in the area of in-line derivatization protocols based on EMMA. Reported derivatization procedures are discussed and classified in tables, according to the functional group that is derivatized.

Keywords: Capillary electrophoresis / Derivatization / Electrophoretically mediated microanalysis / Enzyme / Review
DOI 10.1002/elps.200305636

Contents

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Abbreviations: EMMA, electrophoretically mediated microanalysis; **G-6-PDH**, glucose-6-phosphate dehydrogenase; **LAP**, leucine aminopeptidase; **LDH**, lactate dehydrogenase; **pNA**, *p*-nitroaniline; **OPA**, 1,2-phthalic dicarboxaldehyde

1 Introduction

Enzymes are often identified and quantitated by measuring their biological activity, *i.e.*, their catalytic activity. In enzyme assays, the catalyzed conversion of a substrate by a specific enzyme provides information about enzyme activity and reaction kinetics involved [1]. In addition, the substrate can also be quantified and in these substrate assays, the enzymes are used to enable detection. Initially, CE was only used as a separation tool in the determination of enzyme activities. In these off-line enzyme assays, the reaction between enzyme and substrate occurs outside the capillary and after incubation, the reaction mixture is injected into the capillary. As a separation technique, CE offers advantages over other techniques such as high speed of analysis and small sample volumes. However, in order to further scale-down the quantity of enzyme required for analysis and thus to obtain a real nanoliter scale assay, the reaction should be conducted directly in the capillary [2].

Bao and Regnier [3] were the first to report an in-line homogeneous enzyme assay in which they studied the model enzyme glucose-6-phosphate dehydrogenase (G-6-PDH). This approach was later referred to as "electrophoretically mediated microanalysis" or EMMA [4]. In the EMMA technique, the reagents (enzyme and substrate) are introduced into the capillary as distinct zones. Upon the application of an electric potential, these zones electrophoretically merge, due to differences in their electrophoretic mobilities. The reaction proceeds during the

mixing process in the presence or absence of an electric field. The resultant product is transported to the detector under the influence of an applied voltage. In EMMA, the capillary is not only used as a separation tool but also as an enzymatic reaction vessel. Therefore, enzymatic reactions can be performed at a nanoliter scale, whereas the limit in reaction volume for traditional methods is the microliter scale level. Since its introduction in 1992, EMMA has become a general approach for ultra-microenzyme assays in CE, it has been used in a number of biochemical systems (see Section 2.2: literature overview of EMMA assays) and mathematical models have been developed [5–7] to simulate diverse analyses.

UV-Vis absorbance and fluorescence are frequently used detection techniques in CE. However, analytes must have either chromophores or fluorophores in order to be detected. Especially in fluorescence detection this is a major limitation since only few organic molecules exhibit intense native fluorescence. Therefore, compounds often need to be chemically transformed into fluorescent or UV-Vis absorbing derivatives. Traditionally, derivatization is performed in two ways: either pre- or postcapillary. The most simple form is precapillary derivatization, where the sample is labeled prior to separation. Alternatively, the label can be attached to the compounds after the separation step. In this postcapillary approach, the CE instrument has to be modified in order to incorporate a reaction cell between the capillary outlet and the detector. In general, solute derivatization is normally not preferred as it involves additional sample handling [8].

Because of the interesting results achieved by EMMA in the determination of enzymatic activities and enzyme (substrate) concentrations, the in-capillary reaction technique EMMA was also applied to nonenzymatic (derivatization) reactions in recent years. In such an in-line derivatization technique, pre- or postcapillary derivatization steps are replaced by automated electrophoretic procedures in which the capillary is used as a microreaction chamber for the derivatization of analytes. The advantages are basically identical to those described for enzymatic reactions: full automation of the derivatization step and low consumption of sample and reagents [9].

2 In-capillary enzymatic reactions by EMMA

2.1 Modes of EMMA

EMMA can be performed in many modes based on the mechanisms of zone engagement, sample introduction, and the temporal pattern of the applied voltage. Basically, there are two ways to mix the reaction components in a capillary under electrophoretic conditions: continuous engagement EMMA (A) and transient engagement EMMA (B), which is also referred to as the plug-plug format [10]. The two basic EMMA modes are illustrated in Fig. 1.

2.1.1 Continuous engagement EMMA

In continuous engagement EMMA, the capillary and the buffer reservoirs are filled with reagent, which is most often the substrate. Enzyme is introduced as in any CE system and the subsequent application of the voltage mixes the reactants inside the capillary. Product formation continues until the enzyme exits the capillary. An illustration of continuous engagement EMMA is shown in Fig. 1A. First, the capillary is filled with a buffer containing a reagent, *e.g.*, the substrate (I). When a plug containing the enzyme is then injected (II) and a potential is applied (III), the enzyme will continuously mix and react with substrate molecules during its migration through the capillary, which leads to a continuous formation and separation of product (IV).

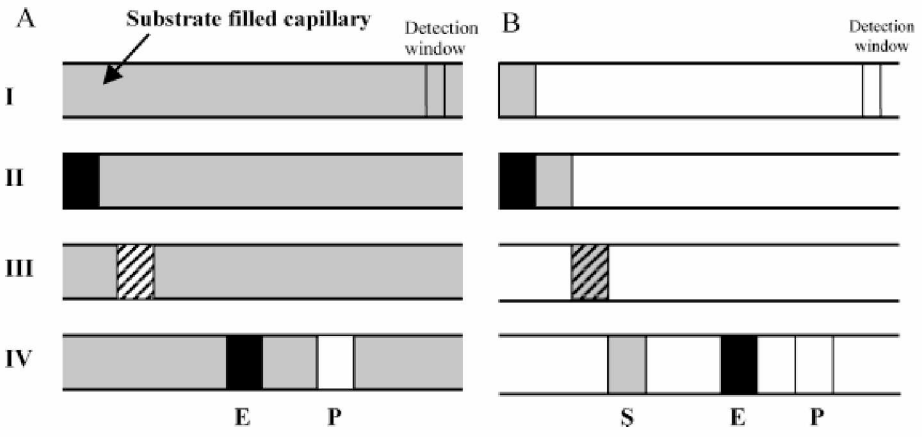

Figure 1. Schematic illustration of the two basic EMMA modes: (A) continuous engagement mode, (B) transient engagement mode (= the plug-plug reaction). S, substrate; E, enzyme; P, product.

EMMA assays can be run in two ways: at zero or at constant potential. When the assay is carried out under constant potential, the reactants are mixed and separated continuously from the products. Although the products are swept away from the enzyme by the electrophoresis process, the rate at which the product is produced is much higher than that of the enzyme-product separation and product accumulates. Thus, the product formed at constant potential appears as a flat plateau (Fig. 2). In the zero potential mode the product is allowed to accumulate in the absence of the electric field and is then electrophoretically transported to the detector. The potential can be reduced to zero at any time prior to detection. The profile of this mode is a peak on top of the plateau (Fig. 2). This in-capillary incubation step (= zero-potential amplification) enhances the sensitivity [3].

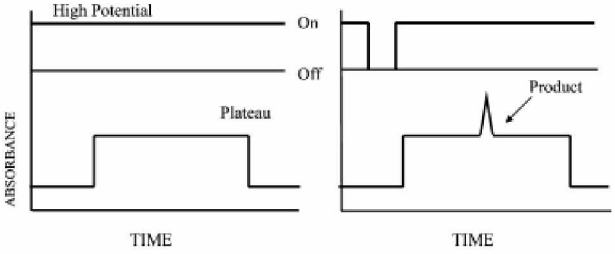

Figure 2. Schematic illustration of the constant potential mode (left) and the zero potential mode (right) in continuous engagement EMMA. The potential programs are shown on top and the corresponding electropherograms are illustrated at the bottom part of the figure.

Continuous engagement assays in which the analyte is introduced into the capillary as a distinct zone (plug) are also referred to as zonal injection methods. The majority of the continuous engagement EMMA assays use zonal injection to introduce the analyte in the capillary. However, Harmon *et al.* [11] used a moving boundary sample introduction to assay leucine aminopeptidase (LAP) by continuous engagement EMMA. In their study, the capillary was initially filled with the analyte solution (LAP) while the faster migrating substrate, Leu-*p*-nitroanilide, was maintained in the inlet reservoir. Upon the application of the voltage, substrate entered the enzyme-filled capillary and the resulting reaction product, *p*-nitroaniline (pNA), was transported to the detector. This technique was characterized by a triangular product peak and resulted in a greater reagent overlap and, thus, in higher product yields.

Continuous engagement EMMA is a sensitive technique and therefore most often used if complete reaction or maximum reactivity is desired, for instance for very dilute enzyme solution. The sensitivity of this technique was illustrated by the group of Yeung [12] who were able to detect single molecules of lactate dehydrogenase (LDH) by zero potential EMMA (continuous mode) with LIF detection.

2.1.2 Transient engagement EMMA (= plug-plug reaction)

In the plug-plug format, small plugs of each reagent are injected separately into the capillary that is filled with separation buffer. Upon the application of an electric field, differential mobility in CE is used to bring the two plugs into contact (Fig. 1B). The slowest reagent, *e.g.*, the substrate (S), is injected first on the capillary (I) and when the faster moving reagent, the enzyme (E), is injected (II), it will overtake the first band and mix with it upon the application of the voltage (III). Reaction occurs when the two plugs overlap and product is generated. Finally, the compounds are separated as they are transported to the detector (IV). As shown in Fig. 3, the electropherogram of the plug-plug reaction is similar to that of conventional CE and thus no steplike increase of the baseline (*i.e.*, the plateau in the case of constant potential) is seen in this format. The implementation of a zero-potential amplification step is more difficult in this mode, because the electrophoretic flow must be stopped precisely when the two bands overlap (Fig. 1B III), whereas in continuous engagement EMMA the flow can be stopped at any time.

The limited reaction time (at constant potential) in transient engagement EMMA can be an advantage if the enzymatic reaction is fast: this produces very broad peaks in the continuous engagement mode. Furthermore, the electrophoresis process prior to the contact of enzyme and substrate inside the capillary enables to separate potential interfering substances [13]. In addition, fewer amounts of reagents are used in the plug-plug approach since only small plugs of samples are required in the experiment.

2.2 Literature overview of EMMA assays

2.2.1 Continuous engagement EMMA

Table 1 summarizes reported applications of EMMA applied in the continuous engagement mode. In the study of Fujima *et al.* [14], the LDH enzyme system was investigated in both directions by CE-UV for the on-line determination of both substrates (lactate and pyruvate), as well as LDH activity. Yang *et al.* [15] investigated LDH activity *via* reduced nicotinamide adenine dinucleotide (NADH) production that was monitored by means of electrochemical detection. Regehr and Regnier [16] utilized the

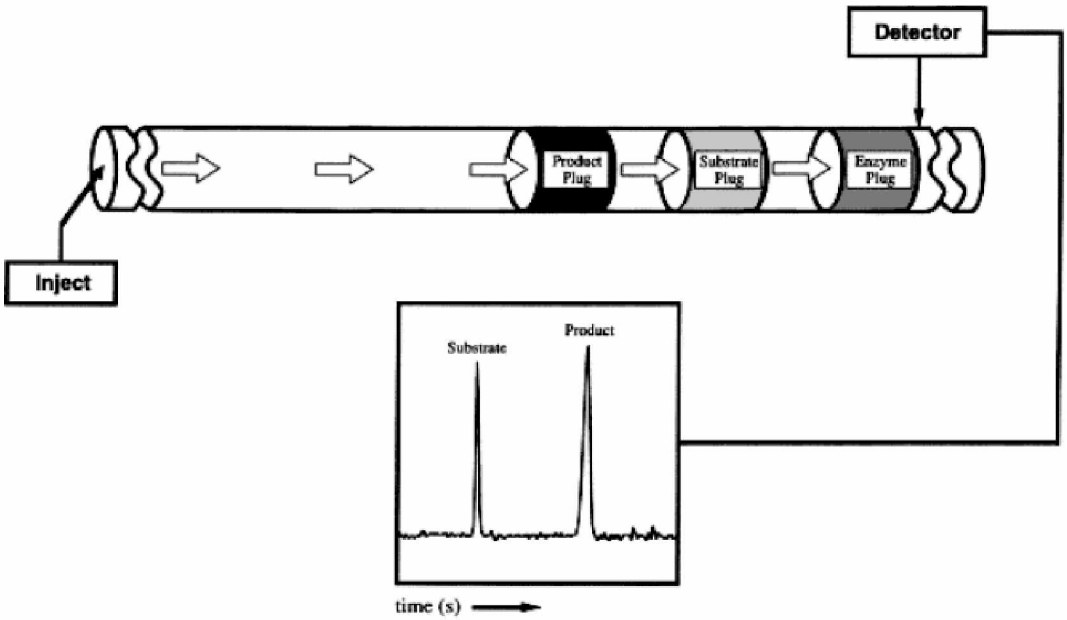

Figure 3. Typical electropherogram of a plug-plug reaction. The substrate is NAD and the product NADH, in the G-6-PDH conversion of glucose-6-phosphate. Reprinted from [27], with permission.

Table 1. Literature overview on continuous engagement EMMA

Enzyme	Detectable species	Note	E/S	Ref.
LAP	pNA	CE-Vis, moving boundary sample introduction	E	[11]
LDH	NAD(H)	CE-UV	E/S	[14]
LDH	NADH	Electrochemical detection	E	[15]
G oxidase Gal oxidase Catalase	H_2O_2 H_2O_2 H_2O_2	Chemiluminescence detection in a postcolumn reaction chamber	E	[16]
G oxidase	H_2O_2	CE-LIF, glucose determined in tear fluid	S	[17]
Creatine kinase	ATP	CE-UV, single and multiple injection protocol	E/S	[18]
Sialidase	NANA	CE-UV, two-step voltage program	E/S	[19]
ALP	Fluorescent product	CE-LIF, inhibition studies (K_i): activation, reversible and irreversible inhibition	E	[20] [21]

E, enzyme assay; S, substrate assay
For abbreviations see Section 2.2.1.

enzymatic production of H_2O_2 by glucose oxidase (G oxidase) and galactose oxidase (Gal oxidase) in their studies of chemiluminescence detection in continuous EMMA assays. A third enzyme, catalase, was assayed by its consumption of H_2O_2. Detection was achieved by the chemiluminescent reaction of luminol and H_2O_2 (catalyzed by peroxidase) in a postcolumn reaction chamber. Jin *et al.* [17] measured the concentration of glucose in a submicroliter sample of teardrops by in-capillary reaction with glucose oxidase, thereby producing H_2O_2. A second reaction, involving the fluorogenic compound homovanillic acid and peroxidase, produced a fluorescent species that was detected by CE-LIF.

Fujima and Danielson [18] described assays for phosphocreatine and creatine kinase. Both substrate and enzyme assays were based on the phosphorylation reaction involving the conversion of adenosine diphosphate (ADP)

to adenosine triphosphate (ATP), which was separated and monitored by CE-UV. In order to increase the sample throughput, they proposed a multiple injection protocol, which was compared to the normal single injection setup. In the single injection method, the enzyme or substrate was injected into a capillary filled with substrate or enzyme, respectively, whereas in the multiple injection mode mixtures of both enzyme and substrate were successively injected into a buffer-filled capillary. Sample throughput increased in the multiple injection setup, but linearity and quantitation were only fair, and therefore, the multiple injection protocol was proposed for rapid profiling of samples.

Recently, Taga *et al.* [19] reported in-capillary sialidase (*N*-acetylneuraminidase) digestion, an important reaction in glycobiology. The substrate, sialoglycan or sialoglycoprotein, was introduced in a capillary filled with sialidase-containing buffer. The resulting product, *N*-acetylneuraminic acid (NANA), was determined by CE-UV. A lower voltage (*e.g.*, 5 kV) was more favorable for digestion but required longer analysis times. In order to decrease the analysis time, a two-step voltage program (5 kV followed by 20 kV) was used to mix the compounds and to transport the product to the detection window.

Whisnant *et al.* [20, 21] developed an on-capillary inhibition assay with fluorescence detection for the enzyme alkaline phosphatase (ALP). The capillary was filled with a fluorogenic substrate, AttoPhos, and zones of inhibitor (theophylline) and enzyme were injected into the substrate-filled capillary [20]. This setup could be considered to be a combination between continuous and transient engagement EMMA. As the zones of inhibitor and enzyme migrated through the capillary at constant potential, the product was continuously formed. When the zones of inhibitor and enzyme mixed, the product formation decreased. In the electropherograms, the inhibition caused a negative peak on the irregular, but highly reproducible product plateau. The calculated inhibition constant (K_i) was in agreement with literature values. In their next paper [21] they studied different classes of ALP inhibitors and a distinction could be made between reversible and irreversible inhibition of ALP, and ALP activation in the in-capillary system.

2.2.2 Transient engagement EMMA

Reported applications of EMMA applied in the transient engagement mode are summarized in Table 2. Saevels *et al.* [22] studied the deamination of adenosine to inosine by adenosine deaminase (ADA) and developed a transient engagement assay. In this study, the EMMA technique was applied for the first time to determine the Michaelis constant (K_m) of an enzymatic conversion. The kinetic study revealed a K_m value consistent with previously reported values. In a next paper, ADA inhibition was studied [23]. In these experiments, the buffer contained *erythro*-9-(2-hydroxy-3-nonyl)adenine, a competitive inhibitor of ADH. The product, inosine, was formed when the individually injected enzyme and substrate plugs mixed electrophoretically in the inhibitor-filled capillary. When the two plugs of enzyme and substrate were fully engaged, the enzyme was allowed to incubate with the substrate at zero potential for 5 min. Lineweaver-Burk plots were used to determine the inhibition constant (K_i). This group also investigated phosphodiesterase I activity from snake venom to assess the susceptibility to 3-exonuclease degradation of oligonucleotides by in-line CE-UV [24, 25]. The enzymatic reaction was integrated in a polymer-filled capillary that acted as a sieving matrix for the products. The enzyme activity was quantitated spectrophotometrically by the mononucleotide deoxyadenosine monophosphate (dAMP).

The group of Gomez [26, 27] described a plug-plug reaction to study and optimize two model systems quantitatively: the conversion of nicotinamide adenine dinucleotide (NAD) to NADH in the oxidation of glucose-6-phosphate (G-6-P) by G-6-PDH and the conversion of ADP into adenosine monophosphate (AMP) by acid phosphatase (AP). This group reported in another study [28] the use of transient engagement EMMA in dual enzyme assays. They studied two model systems: hexokinase (HK) and apyrase (1) in the conversion of ATP to ADP and AMP in the presence of glucose, and LDH and G-6-PDH (2) in the conversion of NADH to NAD in the presence of pyruvate and G-6-P.

Xu *et al.* [13] reported a Michaelis-Menten analysis of ALP by CE-UV. A separately injected substrate plug of *p*-nitrophenyl phosphate passed the previously injected enzyme plug under constant voltage and the resulting product, *p*-nitrophenol (pNP), was monitored at 230 nm. Polyacrylamide-coated capillaries were used in order to prevent possible enzyme adsorption and the presence of a soluble polymer in the assay buffer reduced diffusion inside the capillary. The assay procedure was able to quantitatively measure the K_m value of the ALP-catalyzed reaction.

An in-capillary analysis of an enzyme product was useful for the activity measurement of glucoamylase in sake rice koji extract, as described by the group of Terabe [29]. The sake rice koji is the raw material used for the brewing of sake, which is a popular beverage in Japan. The extract that contained the enzyme was injected first in the capillary. Then, the substrate, pNP-β-maltoside, was injected and upon the application of the voltage, the faster migrat-

Table 2. Literature overview on transient engagement EMMA

Enzyme	Detectable species	Note	E/S	Ref.
ADA	Inosine	CE-UV, kinetic study (K_m) and inhibition study (K_i)	E	[22] [23]
Phosphodiesterase	dAMP	CE-UV, polymer sieving matrix	S	[24] [25]
G-6-PDH AP	NAD(H) A(D)MP	CE-UV, quantitative studies	E	[26] [27]
HK, apyrase LDH, G-6-PDH	A(M)(D)TP NAD(H)	CE-UV, dual enzyme assays	E	[28]
ALP	pNP	CE-UV, kinetic study (K_m)	E	[13]
Glucoamylase	pNP-β-gluco-pyranoside	CE-UV, glucoamylase activity in sake rice koji extract	E/S	[29]
FBPase ALD	Fructose-6-P DihydroxyacetoneP	Indirect UV detection	E	[31]
PAO	Bal	CE-UV, partially filled capillary, kinetic study (K_m)	E	[32]
COMT	Van, Iso	CE-UV, off-line and in-line study	E	[33]
GGT	pNA	CE-UV, use of a micellar solution, kinetic study (K_m)	E	[34]
Rhodanese	Thiocyanate	CE-UV, partially filled capillary, kinetic study (K_m) and inhibition study (K_i)	E	[35] [36]
α-Glucosidase β-Gal β-*N*-Acetyl-glucosaminidase	pNP pNP pNP	CE-UV, kinetic study (K_m)	E	[37]
β-Glucosidase β-Gal	pNP (o)pNP	CE-UV, kinetic study (K_m) and dual enzyme assay	S	[38]
ALP	Riboflavin	CE-UV, two-step voltage program	E	[39]

E, enzyme assay; S, substrate assay
For abbreviations see Section 2.2.2.

ing substrate plug passed the extract inside the capillary. The product, pNP-β-glucopyranoside, was separated from the other components by MEKC using sodium cholate micelles as the pseudostationary phase. According to the authors, the described in-capillary plug-plug reaction could be useful for analysis in the food industry [30].

Zhang *et al.* [31] demonstrated indirect UV detection to quantitate in-capillary enzyme-catalyzed microreactions. This technique was applied using two model systems: the conversion of fructose-1,6-biphosphate (FBP) to fructose-6-phosphate (Fructose-6-P) by fructose-1,6-biphosphatase (FBPase) and the conversion of FBP into dihydroxyacetone phosphate (dihydroxyacetoneP) by fructose-biphosphate aldolase (ALD). Van Dyck *et al.* [32] studied plasma amine oxidase (PAO) activity by measuring the reaction product, benzaldehyde (Bal), by means of CE-UV. The capillary was only partially filled with the separation solution, because enzymatic activity was lost in the presence of the surfactant SDS. The basic limitation of EMMA, *i.e.*, the necessity to have conditions compatible with both the reaction and the separation, was thus overcome. This system was successful in determining the kinetic parameter K_m. In a next study, catechol-*O*-methyltransferase (COMT) was investigated [33]. The closely related reaction products, vanillic (Van) and isovanillic acid (Iso) were separated in a buffer containing cyclodextrins. The in-line assay with CE-UV was not sensitive enough to allow for a full kinetic study of COMT activity. This group also studied γ-glutamyltransferase (GGT) [34] in combination with the synthetic substrate glutamyl-*p*-nitroanilide. The separation of the reaction product pNA was achieved by the use of a micellar solution of deoxycholic acid sodium salt. The micelles did not alter the enzymatic activity and a kinetic study was carried out which yielded a K_m value consistent with literature findings.

The group of Glatz [35] studied the rhodanese-catalyzed reaction of thiosulfate into thiocyanate in the presence of cyanide, the co-substrate. They used CE-UV in a partially filled capillary of which one part was filled with a buffer optimal for the enzymatic reaction, whereas the second part was filled with the background electrolyte optimal for the separation of the inorganic substrates and products. The EMMA method was applied to determine the kinetic parameters of the enzyme. Moreover, the mechanism of the enzymatic reaction could be elucidated. Recently, this group presented another paper [36], in which the previously developed EMMA method was applied to study the inhibition of rhodanese by 2-oxoglutarate, a competitive inhibitor. The inhibition constants (K_i) were determined with respect to the substrates, thiosulfate and cyanide, and the type of inhibition was also evaluated.

Kanie and Kanie [37] studied different glycosidase enzymes (α-glucosidase, β-galactosidase (β-Gal) and β-*N*-acetylglucosaminidase) by CE-UV. The substrates used were pNP-α-glucopyranoside, pNP-β-galactopyranoside and pNP-β-*N*-acetylglucosaminide, respectively, and for all three reactions the product pNP was used to quantitate the enzymatic activity. These three enzymes were successfully analyzed and for all three reactions, the determined K_m values were similar to those obtained by a photometric assay. In another, recent paper [38], they investigated β-glucosidase and β-Gal with the substrates pNP-β-glucopyranoside and pNP-β-galactopyranoside, which yielded the product pNP. In this study, the conditions for the EMMA assay were optimized and the kinetic parameters for β-glucosidase were obtained. They also reported a mixed enzymatic reaction in order to evaluate the potential use of a mixture of functionally and/or structurally related enzymes. β-Glucosidase and β-Gal were assayed in the same analysis with the substrates pNP-β-glucopyranoside and oNP-β-galactopyranoside, respectively.

Recently, a study was published in which the simultaneous determination of water-soluble vitamins by an in-capillary reaction method was described [39]. The in-line reaction method was used to determine riboflavin phosphate in a vitamin-enriched drink based on its conversion into riboflavin by ALP. Simultaneously, three other vitamins and caffeine were subjected to quantitative analysis. The zones containing the substrate and the enzyme were electrophoretically mixed in the presence of a weak field and the product, riboflavin, was separated and detected by CE-UV. The authors reported a good agreement between the newly developed method and traditional LC-based methods.

3 In-capillary derivatization reactions by EMMA

Analytical derivatization can be applied in a wide range of disciplines. In addition to the traditional area of organic chemistry, derivatization methods are an integral part of most of the chemical analysis practiced in medical, pharmaceutical, and food science. In general, analytical derivatization is employed for two reasons: (i) to improve sensitivity or chromatographic/ electrophoretic selectivity behavior and (ii) to permit analysis of compounds with inadequate volatility or stability. Traditionally derivatization has been done using pre- and postcapillary procedures. In the former, the product instability could introduce imprecision between repeated analyses while in the latter the need for postcapillary hardware modification complicates this approach. We are currently witnessing successful applications of in-capillary reaction, after its first introduction a decade ago [3] as EMMA, in the field of enzyme study as reviewed in Section 2.2 above. Applications in chemical derivatization in various fields will be shortly reviewed hereafter. This section will survey most recent findings and discuss them under three catagories: transient engagement EMMA, throughout technique and zone passing technique.

3.1 Types of in-capillary derivatization reactions by EMMA

In-capillary derivatization by EMMA can be subclassified into three formats, depending on where and how the derivatization reaction occurs. In the first format, transient engagement EMMA, the plug-plug reaction starts with separate plugs of analyte and reagent in tandem, or the plugs could as well be introduced as sandwich *i.e.*, reagent/analyte/reagent. Between the plugs, sometimes a short plug of the separation buffer is placed. Reagent(s) and analyte(s) zones merge under the influence of an electric field by a process known as electrophoretic mixing, a process in which a zone of higher electrophoretic mobility is caused to overtake and merge with a zone of lower electrophoretic mobility [40]. The reaction is then allowed to proceed for as long as the two zones overlap. A zero-potential amplification (ZPA) is possible whereby the voltage is stopped when the two zones have merged, to allow accumulation of more product. After the reaction completion, the remaining reagent is separated from the product(s), and both migrate towards the capillary end by application of a separation voltage. This approach is quite popular to study reactions in which one of the reactants is not stable enough to be incorporated in the background electrolyte, as it is the case for most enzymatic reactions or if the reagent is strongly absorbing in UV [41].

In the second format, Regnier and co-workers [4, 7, 11] developed a methodology designated as the throughout-capillary technique for kinetic studies of slow enzyme reactions. Using this technique, Oguri *et al.* [42] described the derivatization of some amino acids with 1,2-phthalic dicarboxaldehyde (OPA). The separation capillary is filled with a reagent buffer and the analyte is introduced as a plug by means of traditional capillary electrophoretic injection techniques such as hydrodynamic or electrokinetic injection. Upon voltage application, electrophoretic mixing permits the analyte to encounter many times its own volume of reagent buffer and it is converted to a derivative, separated and detected.

The third EMMA possibility, the zone passing technique, is based on derivatization in the middle of a capillary by passing either a sample or a reagent zone through the other in the electric field [43]. This approach is similar to the plug-plug approach, but the only difference is that with zone passing the reagent/analyte zones are pushed to the middle of the capillary by introduction of a long plug of the background electrolyte. In plug-plug and zone-passing EMMA, the order and site of sample/reagent introduction is determined by the magnitude and sign of the mobility, and peak intensity varies with the passing time period. These techniques are useful for kinetic studies of rapid reactions and reactions yielding products, which are unstable in presence of excess reactants [43].

Andreev *et al.* [44–47] presented another variant of EMMA, which they referred to as electroinjection analysis (EIA). In this approach, the two reactants of interest are injected simultaneously from opposite ends of the capillary using electrokinetic injection. They meet in the capillary, pass through each other and react. The ions $Fe3^{+}$ and Ti^{4+} were determined with this approach, which can be considered to be a variation of the zone-passing technique.

3.2 Applications of in-capillary derivatization reactions by EMMA

3.2.1 Transient engagement EMMA

The applications of EMMA for chemical derivatization are summarized in Table 3. In 1996, Oguri *et al.* [48] developed a plug-plug sandwich derivatization, in which the analytes were sandwiched between two reagent plugs. The method involved in-capillary derivatization of a 16-component mixture of amino acids using 1-methoxycarbonylindolizine-3,5-dicarbaldehyde (IDA) as reagent. Its derivatization efficiency appeared to be intermediate between the throughout and precapillary techniques, on the other hand its precision was poor, compared to the other two techniques.

Kaale *et al.* [49] reported a sandwich plug-plug derivatization, which was successfully applied to perform derivatization and separation of the multicomponent aminoglycoside antibiotic gentamicin using 1,2-phthalic dicarboxaldehyde and mercaptoacetic acid (MAA) as labeling reagents. Full automation of the derivatization and analytical procedure, high derivatization efficiency, high sample throughput and precision were the excellent features of this method. It offered short analysis time, as well as selectivity and sensitivity suitable for impurities determination in bulk samples. Separation of gentamicin C_1, C_{1a}, C_2, C_{2a}, C_{2b}, sisomicin and several minor components was achieved. For the first time separation and identification of three impurities: garamine, 2-deoxystreptamine, and paromamine, was reported.

In another paper [50], the plug-plug type EMMA method was used for electrophoretic mixing with subsequent reaction of nanoliter plugs of kanamycin samples and OPA/MAA. An incubation time of up to 5 min allowed the reaction to proceed prior to the application of a separation voltage and UV detection was at 335 nm. A simple borate background electrolyte containing methanol was used. The quantitative aspects of this method showed that it was suitable for analysis of kanamycin bulk samples. Kang *et al.* [51] studied the in-capillary derivatization of homocysteine and other thiols with 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F). The background electrolyte was composed of 20 mM Trizma®-phosphate, pH 2.1. They monitored both UV and LIF signals; with the former more repeatable migration times were obtained. However, LIF detection was 1000 times more sensitive compared to UV, which made this method suitable to determine thiols in clinical samples.

Lee *et al.* [52] described a method for the analysis of picomolar concentration of proteins using plug-plug EMMA to label the proteins with 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) to generate highly fluorescent products. They claimed that compared to UV detection this EMMA with LIF detector provides a 7 000 000-fold improvement in sensitivity. Zhang *et al.* [53] described the use of an in-capillary procedure for derivatization of amino acids, peptides and alkyl amines by anhydrides using capillary electrophoresis. This group demonstrated the potential applications of EMMA to synthesize and analyze molecular targets. The method was claimed to be both economically sound and environmentally appealing.

Taga *et al.* [41] demonstrated an extended application of in-capillary derivatization to a much slower reaction such as the condensation of reducing carbohydrates with 1-phenyl-3-methyl-5-pyrazolone (PMP) which would otherwise require 30 min at elevated temperatures. Although the theoretical plate number, and accordingly the

Table 3. Literature overview on in-capillary derivatization reactions by EMMA

Technique[a)]	Reagent	Reactive group	Notes on analytes	Detection λ (nm)	Ref.
PPE/TE	IDA	1°-NH_2	16-Components mixture of amino acids, and 4 aminoglycoside antibiotics	UV 409	[48]
PPE	OPA	1°-NH_2	Gentamicin	UV 330	[49]
PPE	OPA	1°-NH_2	Kanamycin	UV 335	[50]
PPE	ABD-F	-SH	Homocysteine	UV 220 LIF λ_{ex} = 488, λ_{ex} = 514	[51]
PPE	FQ	1°-NH_2	Protein extracts	LIF λ_{ex} = 488, λ_{ex} = 514	[52]
PPE	Phthalic anhydrides/ acetic anhydrides	1°-NH_2	Amino acids, alkylamines, and peptides	UV 200	[53]
PPE	PMP	-OH	Carbohydrates	UV 245	[42]
PPE	OPA	1°-NH_2	Amino acids	UV 230	[43]
TE	OPA	1°-NH_2	Histamine and other biological amines	LIF λ_{ex} = 340, λ_{ex} = 450	[40]
TE	OPA	1°-NH_2	Amino acids	UV 230	[54]
TE	OPA	1°-NH_2	Amino acids	LIF λ_{ex} = 340, λ_{ex} = 450	[56]
TE	OPA	1°-NH_2	Histamine and other biological amines	UV 340 LIF λ_{ex} = 340, λ_{ex} = 450	[40]
TE	OPA	1°-NH_2	Histamine	LIF λ_{ex} = 340, λ_{ex} = 450	[57]
TE	OPA	1°-NH_2	Biogenic amines	UV 340	[58]
TE	*N*-Arylamino-naphthalene-sulfonic acids	Specific protein binding sites	Bovine whey proteins	LIF	[59]
ZPE	OPA	1°-NH_2	Amino acids	UV 230	[43]

a) PPE, plug- plug EMMA; TE, throughout technique EMMA; ZPE, zone passing EMMA
For other abbreviations see Section 3.2.1.

resolution, was significantly lower than the corresponding values in precapillary derivatization, a reasonable repeatability was ensured for both migration time and peak area. Notwithstanding such inferiority in separation capability, in-capillary derivatization can be done with only 1/1000 of the sample amount required in precapillary derivatization. It was emphasized that this technique is suitable for automated analysis. Once the appropriate values for the introduction times of the sample as well as the reagent solutions, the standing time, the voltages to be applied for plug driving and product analysis, and the duration of voltage application are determined and put into program, the introduced samples can be automatically analyzed. It was found that zone passing and throughout technique were not applicable for such slow reactions.

3.2.2 Throughout technique

Oguri *et al.* [48] developed a simple assay method for amino acids using IDA as a labelling reagent. The derivatization efficiency and precision of the precapillary, plug-plug and throughout techniques were compared using standardized peak areas. The throughout technique performed superiorly in both tests followed by plug-plug, with precapillary being the last. The same method was applied to the determination of a 4-component mixture of aminoglycoside antibiotics. Taga *et al.* [54] explored the characteristic features of the throughout technique of in-capillary derivatization for analysis of selected amino acids (phenylalanine, glycine, and glutamic acid) as a rapid derivatization model. The amino acids were converted into their corresponding OPA derivatives, while they were moving through a solution containing OPA under the influence of an applied electric field. In the throughout technique, it was possible to roughly estimate the reaction rate constant and the reaction order for the derivatization reaction of the individual amino acids. Qualitative features like peak symmetry, sharpness, and resolution ranked as follows: precapillary > at-inlet > zone passing > throughout technique. In the at-inlet technique of derivatization [55], a sample and a reagent zone are introduced successively to the capillary inlet. These reac-

tants are then mixed by diffusion and react during a specific standing period. Immediately afterwards, the derivatives formed are analyzed by applying a voltage. Since the mixing in this technique does not occur by electrophoresis, applications are not further covered here.

Oguri *et al.* [56] reported an in-capillary derivatization method where derivatization and separation are performed simultaneously in a capillary tube filled with a run buffer containing the derivatization reagent. The method appeared to provide a further improvement in the sensitivity by using OPA/*N*-acetylcysteine (NAC) instead of IDA, reported previously as a derivatization reagent [48]. Direct determination of amino acid from soybean protein hydrolysate without need for sample cleanup was also achieved. The same group reported an in-capillary method for determination of histamine and other biological amines in spoiled sea food samples [42]. In another study [57], endogenous histamine was directly evaluated in rat peritoneal mast cells using in-capillary derivatization. The individual mast cell was injected into the front end of the separation capillary. The mixture of lysing solution and derivatization reagent was injected at the same time to lyse the mast cell to release the histamine and label it with fluorescent tag. All steps were automated and LIF detection was used with excitation at 340 nm and emission at 450 nm. This method holds the possibility of performing analysis on single cells. In a recent paper [58], a selective detection method for biogenic amines present in highly complex matrices was devised by employing both electrokinetic injection and on-capillary-derivatization with capillary electrochromatography (CEC).

Benito *et al.* [59] developed an on-capillary derivatization, to determine some bovine whey proteins in raw milk samples. Although the *N*-arylaminonaphthalene sulfonate derivatives used have sufficient sensivity for UV detection at the concentrations present in milk samples, a fluorescent probe based on LIF was used because of fear of other interfering substances with UV activity which are present in the milk samples. It was demonstrated that compared to other LIF detection methods used for CE of proteins, on-capillary derivatization is rather inexpensive and easy to implement because of the full automation.

It can be concluded from these findings that despite the advantages offered, such as rapid kinetic studies, full automation, and high precision, the throughout technique is still at its infancy. A lot has to be done to mature it to routine applications. If the reagent involved is UV-active, high baseline noise and high detection limits can be expected.

3.2.3 Zone passing technique

Taga *et al.* [43] successfully applied the zone passing technique to the analysis of several amino acids using OPA as the derivatization agent. This technique of derivatization was demonstrated to be very useful for selective determination of species present in complex matrices because the analyte is separated from other sample ions before the derivatization. Thus, it was demonstrated that observation of the zone-passing mode of in-capillary derivatization gave useful information on the estimation of the rate constant of the derivatization reaction of amino acids with OPA. This technique will be generally useful for kinetic studies of chemical reactions, especially rapid reactions, and will be used widely in physical chemistry.

4 Conclusions

In-capillary enzymatic and derivatization reactions achieved by EMMA in three formats (*viz*: transient engagement technique, throughout technique, and zone passing technique) have been reviewed. It was demonstrated that this technique allows to perform the reaction entirely in the capillary acting as a microreactor vessel. Full automation of all reaction steps: sampling, mixing, reaction, separation, detection, and quantitation are the advantages of the in-capillary reactions. Based on the recent findings surveyed, major advances in capillary electrophoretically mediated microanalysis have been demonstrated especially in the fields of enzymology, chemical derivatization, and synthesis and analysis of molecular target compounds.

Received July 14, 2003

5 References

[1] Guijt, R. M., Baltussen, E., van Dedem, G. W. K., *Electrophoresis* 2002, *23*, 823–835.

[2] Bao, J. J., Fujima, J. M., Danielson, N. D., *J. Chromatogr. B* 1997, *699*, 481–497.

[3] Bao, J., Regnier, F. E., *J. Chromatogr.* 1992, *608*, 217–224.

[4] Regnier, F. E., Patterson, D. H., Harmon, B. J., *Trends Anal. Chem.* 1995, *14*, 177–181.

[5] Harmon, B. J., Patterson, D. H., Regnier, F. E., *Anal. Chem.* 1993, *65*, 2655–2662.

[6] Harmon, B. J., Leesong, I., Regnier, F. E., *Anal. Chem.* 1994, *66*, 3797–3805.

[7] Patterson, D. H., Harmon, B. J., Regnier, F. E., *J. Chromatogr. A* 1996, *732*, 119–132.

[8] Kuhn, R., Hoffstetter-Kuhn, S., *Capillary Electrophoresis: Principles and Practice*, Springer-Verlag, Berlin 1993, pp. 3–109.

[9] Molina, M., Silva, M., *Electrophoresis* 2002, *23*, 3907–3921.

[10] Chu, Y., Avila, L. Z., Gao, J., Whitesides, G. M., *Acc. Chem. Res.* 1995, *28*, 461–468.

[11] Harmon, B. J., Leesong, I., Regnier, F. E., *J. Chromatogr. A* 1996, *726*, 193–204.

[12] Xue, Q., Yeung, E. S., *Nature* 1995, *373*, 681–683.

[13] Xu, Y., Liu, X., Ip, M. P. C., *J. Liq. Chromtogr. Rel. Technol.* 1998, *21*, 2781–2797.

[14] Fujima, J. M., Danielson, N. D., *J. Capil. Electrophor.* 1996, *6*, 281–285.

[15] Yang, W. C., Yu, A. M., Chen, H. Y., *Chem. J. Chin. Univ.* 2001, *22*, 547–551.

[16] Regehr, M. F., Regnier, F. E., *J. Capil. Electrophor.* 1996, *3*, 117–124.

[17] Jin, Z., Chen, R., Colon, L. A., *Anal. Chem.* 1997, *69*, 1326–1331.

[18] Fujima, J. M., Danielson, N. D., *Anal. Chim. Acta* 1998, *375*, 233–241.

[19] Taga, A., Sugimura, M., Suzuki, S., Honda, S., *J. Chromatogr. A* 2002, *954*, 259–266.

[20] Whisnant, A. R., Johnston, S. E., Gilman, S. D., *Electrophoresis* 2000, *21*, 1341–1348.

[21] Whisnant, A. R., Gilman, S. D., *Anal. Biochem.* 2002, *307*, 226–234.

[22] Saevels, J., Van Schepdael, A., Hoogmartens J., *Electrophoresis* 1996, *17*, 1222–1227.

[23] Saevels, J., Van den Steen, K., Van Schepdael, A., Hoogmartens, J., *J. Chromatogr. A* 1996, *745*, 293–298.

[24] Saevels, J., Huygens, K., Van Schepdael, A., Hoogmartens, J., *Anal. Chem.* 1997, *69*, 3299–3303.

[25] Saevels, J., Van Schepdael, A., Hoogmartens, J., *J. Capil. Electrophor.* 1997, *4*, 167–172.

[26] Zhao, D. S., Gomez, F. A., *Chromatographia* 1997, *44*, 514–520.

[27] Kwak, E. S., Esquivel, S., Gomez, F. A., *Anal. Chim. Acta* 1999, *397*, 183–190.

[28] Zhao, D. S., Gomez, F. A., *Electrophoresis* 1998, *19*, 420–426.

[29] Watanabe, T., Yamamoto, A., Nagai, S., Terabe, S., *Electrophoresis* 1998, *19*, 2331–2337.

[30] Watanabe, T., Terabe, S., *J. Chromatogr. A* 2000, *880*, 295–301.

[31] Zhang, Y., El-Maghrabi, M. R., Gomez, F. A., *Analyst* 2000, *125*, 685–688.

[32] Van Dyck, S., Van Schepdael, A., Hoogmartens, J., *Electrophoresis* 2001, *22*, 1436–1442.

[33] Van Dyck, S., Van Schepdael, A., Hoogmartens, J., *Electrophoresis* 2002, *23*, 1341–1347.

[34] Van Dyck, S., Van Schepdael, A., Hoogmartens, J., *Electrophoresis* 2002, *23*, 2854–2859.

[35] Nováková, S., Glatz, Z., *Electrophoresis* 2002, *23*, 1063–1069.

[36] Nováková, S., Telnarová, M., Glatz, Z., *J. Chromatogr. A* 2003, *990*, 189–195.

[37] Kanie, Y., Kanie, O., *Carbohydr. Res.* 2002, *337*, 1757–1762.

[38] Kanie, Y., Kanie, O., *Electrophoresis* 2003, *24*, 1111–1118.

[39] Okamoto, H., Nakajima, T., Ito, Y., *J. Chromatogr. A* 2003, *986*, 153–161.

[40] Harmon, B. J., Regnier, F. E., in: Khaledi, M. G. (Ed.), *HPCE Theory, Techniques and Applications*, John Wiley and Sons, New York, NY, USA 1998, 925.

[41] Taga, A., Suzuki, S., Honda, S., *J. Chromatogr. A* 2001, *911*, 259–267.

[42] Oguri, S., Watanabe, S., Abe, S., *J. Chromatogr. A* 1997, *790*, 177–183.

[43] Taga, A., Sugimura, M., Honda, S., *J. Chromatogr. A* 1998, *802*, 243–248.

[44] Andreev, V., Kamenev, A. G., Popov, N. S., *Talanta* 1996, *43*, 909–914.

[45] Andreev, V., Ilyina, N., Lebedeva, E., Kamenev, A., Popov, N. S., *J. Chromatogr. A* 1997, *772*, 115–127.

[46] Andreev, V., Pliss, N. S., *J. Chromatogr. A* 1999, *845*, 227–236.

[47] Andreev, V., Makarova, E., Pliss, N. S., *Anal. Chem.* 2001, *73*, 1316–1323.

[48] Oguri, S., Fujiyoshi, T., Miki, Y., *Analyst* 1996, *121*, 1683–1688.

[49] Kaale, E., Van Goidsenhoven, E., Van Schepdael, A., Roets, E., Hoogmartens, J., *Electrophoresis* 2001, *22*, 2746–2754.

[50] Kaale, E., Van Schepdael, A., Roets, E., Hoogmartens, J., *Electrophoresis* 2003, *24*, 1119–1125.

[51] Kang, S. H., Wei, W., Yeung, E. S., *J. Chromatogr. B* 2000, *744*, 149–156.

[52] Lee, I. H., Pinto, D., Arriaga, E. A., Zhang, Z. R., Dovichi, N. J., *Anal. Chem.* 1998, *70*, 4546–4548.

[53] Zhang, Y., Gomez, F. A., *Electrophoresis* 2000, *21*, 3305–3310.

[54] Taga, A., Nishino, A., Honda, S., *J. Chromatogr. A* 1998, *822*, 271–279.

[55] Taga, A., Honda, S., *J. Chromatogr. A* 1998, *802*, 243–248.

[56] Oguri, S., Yokoi, K., Motohase, Y., *J. Chromatogr. A* 1997, *787*, 253–260.

[57] Oguri, S., Ohta, Y., Suzuki, C., *J. Chromatogr. B* 1999, *736*, 263–271.

[58] Oguri, S., Yoneya, Y., Mizunuma, M., Fujiki, Y., Otsuka, K., Terabe, S., *Anal. Chem.* 2002, *74*, 3463–3469.

[59] Benito, I., Marina, M. L., Saz, J. M., Diez-Masa, J. C., *J. Chromatogr. A* 1999, *841*, 105–114.

1.3 Rationale and aims

Electrophoretically Mediated Microanalysis (EMMA) is a CE technique that allows reaction-based chemical analysis to be performed and monitored entirely in-capillary. The EMMA methodology utilizes the electrophoretic mobilities of enzyme, substrate and product to initiate enzyme-substrate reaction during CE and to separate the reaction compounds from each other for final on-capillary quantitation. The different assay steps (mixing, reaction, separation and detection) occur in the same micro vessel, *i.e.* the capillary. As a result, the whole assay is integrated and miniaturized and can be fully automated.

In this work, CE-UV will be used to develop enzyme assays based on EMMA methodology. The enzymatic systems included in this project are clinically relevant but highly differential with respect to the type and number of substrates, products and cofactors. In order to investigate these enzymes, model substrates that are converted into UV-detectable products will be used. For each enzymatic system, the electrophoretic parameters, which are convenient to perform EMMA assays, will be investigated; the composition of the background electrolyte (type and concentration), protocol of injection and incubation, applied voltage, capillary dimensions (length and diameter) and temperature.

The first enzyme to be studied is rhodanese, followed by phenol sulfotransferase (PST) and angiotensin converting enzyme (ACE). These are all water soluble and commercially available enzymes. The developed EMMA assays will then be used to investigate whether the kinetic parameters can be derived for the enzyme reactions. In a next step, physiological inhibitor of rhodanese, 2-oxoglutarate, will be studied and it will be determined if it is possible describe inhibition of rhodanese by means of the previously developed EMMA methodology. In the case of ACE, Van Dyck et. al. have already developed the in-capillary methodology [28], thus the next step is an inhibition study in which the influence of known ACE inhibitors will be evaluated by means of the previously developed in-capillary assay.

The capabilities of CE regarding integrated enzymatic reactions will be explored in this work and general conclusions will be drawn with regard to the integration of enzyme reactions and separations by CE-UV.

CHAPTER 2
RHODANESE

2.1 Determination of Kinetic Parameters of Rhodanese by EMMA in Partially Filled Capillary

NOVÁKOVÁ S., GLATZ Z. (2002) **Electrophoresis**, 23, 1063-1069.

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Determination of the kinetic parameters of rhodanese by electrophoretically mediated microanalysis in a partially filled capillary

Electrophoretically mediated microanalysis (EMMA) was applied for the study of kinetic parameters of the bisubstrate enzymatic reaction of rhodanese. The Michaelis constants (K_m) for both substrates and the effect of temperature on rhodanese reaction were evaluated by means of the combination of the EMMA methodology with a partial filling technique. In this setup, the part of the capillary is filled with the buffer best for the enzymatic reaction whereas, the rest of the capillary is filled with the background electrolyte optimal for separation of substrates and products. The enzymatic reaction was performed in 25 mM *N*-(2-hydroxymethyl)piperazine-2'-(2-ethanesulfonic acid) (HEPES) buffer (pH 8.5) while the low pH background electrolyte 100 mM β-alanine-HCl (pH 3.5) was used for separation of substrates and products that are the inorganic anions. The estimated value of K_m for thiosulfate of 1.30×10^{-2} M was consistent with previously published values; the K_m for cyanide of 7.6×10^{-3} M was determined for the first time. In addition, the type of kinetic mechanism of enzymatic reaction was also elucidated. The finding of the double displacement (ping-pong) mechanism is in accordance with previous literature data. Also, the experimentally determined temperature optimum of the rhodanese-catalyzed reaction around 20–25°C agreed with literature values.

Keywords: Electrophoretically mediated microanalysis / Partial filling technique / Rhodanese
EL 4842

1 Introduction

Natural exposure to cyanide during evolution has resulted in a complex mechanism for its detoxication [1]. In the major pathway cyanide is metabolized to the less toxic thiocyanate after reaction with a sulfur donor, such as thiosulfate [2–4]. This reaction is catalyzed by the enzyme rhodanese [5, 6]. Rhodanese (thiosulfate: cyanide sulfur transferase, EC 2.8.1.1) was discovered in 1933 by Lang [7]. It is responsible for the transfer of the sulfane sulfur of thiosulfate to an acceptor, which is likely to be cyanide under some physiological conditions: $S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}$. Rhodaneses are widespread in the biological world, their activity has been detected in several species ranging from microorganisms through fungi, plants, and animals to man [8]. Its physiological role has been debated for many years with proposals ranging from the detoxication of cyanide to the suggestion that rhodanese is important in the bioenergetic oxidation of thiosulfate [9], in generating iron-sulfur protein complexes [10], in lipoate metabolism [11], and in the reactivation of nitrogenase [12].

Due to the significance of rhodanese for applied and environmental toxicology, many assay methods have been developed for this enzyme. Assay methods include spectrophotometric [13, 14] and spectrofluorimetric [15] assays, and measuring cyanide consumption or thiocyanate production using ion-selective electrodes [16, 17]. Recently, a new sensitive method was developed for the determination of rhodanese activity by means of CZE [18]. The enzymatic reaction is carried out directly in the thermostatted autosampler vial, and CZE is used for the separation of substrates and products. The determination of thiocyanate by CZE consequently enables the rhodanese enzymatic activity to be measured.

Ten years ago, a new application for the evaluation of enzymatic reactions in capillary electrophoresis was proposed and developed: electrophoretically mediated microanalysis (EMMA) [19]. In this method, substrate(s) and enzyme are introduced in the capillary as distinct plugs, the first analyte injected being the one with the lower electrophoretic mobility. Upon the application of an electric field, the two zones interpenetrate due to the differences in their electrophoretic mobilities. Enzy-

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Abbreviations: EMMA, electrophoretically mediated microanalysis; **PVA**, polyvinyl alcohol

0173-0835/02/07-08–04–1063 $17.50+.50/0

matic reaction takes place and the resultant reaction product(s) and the unreacted substrate(s) are electrophoretically transported towards the detector where they are individually detected. Since its discovery by Bao and Regnier, EMMA methodology has been utilized in a number of biochemical systems, for assays of enzyme activities [19–24], determination of substrates [24–26], Michaelis constants [27–29], inhibitors and inhibition constants [30, 31], *etc.* In this communication, the EMMA method was applied to determine the kinetic parameters of rhodanese – Michaelis constants (K_m) for thiosulfate and cyanide and the temperature optimum of the enzymatic reaction. Moreover, the kinetic mechanism of enzymatic reaction was also elucidated. To the best of our knowledge it is the first application of EMMA for this purpose.

2 Materials and methods

2.1 Materials

Rhodanese from beef liver, HEPES, and potassium cyanide were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical reagent grade, supplied from Fluka (Buchs, Switzerland). The background electrolyte was prepared by adding hydrochloride acid (~ 50 mM final concentration) to 0.1 M β-alanine solution up to pH 3.5. The HEPES buffer was prepared by adding of 0.1 M sodium hydroxide to 0.1 M HEPES solution up to pH 8.5. All solutions were prepared with Milli-Q water (Millipore, Milford, MA, USA) and filtrated through a 0.45 μm membrane filter. Enzyme and substrates solutions were freshly prepared in 25 mM HEPES buffer (pH 8.5) each day.

2.2 Instrumentation

A Hewlett-Packard ^{3D}Capillary Electrophoresis System (Waldbronn, Germany) with a diode-array UV-VIS detector was used to carry out all CZE separations. The CZE system autosampler was heated using a recirculating water bath. Data were collected on a HP Vectra VL5 166 MHz personal computer using the Hewlett-Packard ^{3D}CE ChemStation Software. A Polymicro Technologies (Phoenix, AZ, USA) 75 μm fused-silica capillary was used for all separations in uncoated capillaries. A polyvinyl alcohol (PVA)-coated silica capillary (50 μm inner diameter, 64.5 cm total length, 56.0 cm effective length) was obtained from Agilent Technologies (Waldbronn, Germany).

2.3 Monitoring of rhodanese reaction by EMMA method

A 75 μm fused-silica capillary (64.5 cm total length, 56.0 cm effective length) was washed with 0.1 M β-alanine-HCl (pH 3.50) as a background electrolyte for 3 min. The on-column enzymatic reaction was performed by injection of 25 mM HEPES buffer (pH 8.5), the enzyme solution, the substrate solution, 25 mM HEPES buffer (pH 8.5), the background electrolyte all at 50 mbar for 4.0 s consecutively into the capillary. The reaction was initiated by the application of the separation voltage −18 kV (negative polarity). The temperature of the capillary was 25°C unless otherwise specified. Samples were detected at 200 nm with a bandwidth of 20 nm. The peak areas were measured using ChemStation software and the concentrations of thiocyanate were calculated from the calibration graphs.

2.4 Monitoring of rhodanese reaction by discontinuous precolumn CZE method

An aliquot of 0.45 mL of 25 mM HEPES buffer (pH 8.5) containing the given concentration of one substrate and 25 μL of enzyme solution (0.5 mg/mL protein) were placed in a polypropylene vial and incubated in the CZE system autosampler. The reaction was initiated by the addition of 25 μL of the second substrate solution at the fixed concentration. The enzymatic reaction was stopped after 3 min by addition of 10 μL of 38% formaldehyde. A 75 μm fused-silica capillary (64.5 cm total length, 56.0 cm effective length) was applied for all separations. 0.1 M β-alanine-HCl (pH 3.5) was used as a background electrolyte. Before each run the capillary was washed with the background electrolyte for 3 min. Injection was accomplished by an application of 50 mbar pressure to the inlet vial for 4 s. Separation was performed at −18 kV (negative polarity). The temperature of the capillary was 25°C. Samples were detected at 200 nm with a bandwidth of 20 nm. The peak areas were measured by means of ChemStation software and the concentrations of thiocyanate were calculated from the calibration graphs.

3 Results and discussion

The most important factor for successful application of the EMMA method for the study of enzymes is that electrophoretic conditions, especially the composition and pH of the background electrolyte, are favorable for both the separation of substrate(s) and product(s) of the enzymatic reaction, and the enzymatic reaction itself. As mentioned above, rhodanese is an enzyme utilizing

inorganic anions and so several strategies developed for the separation of inorganic anions by means of CZE could be applied. Since the pH optimum of the enzyme has been estimated to be around pH 8.0–8.7, the preliminary experiments were performed using a PVA-coated separation capillary. In this case, the enzyme reaction buffer (25 mM HEPES, pH 8.5) could be directly used as the background electrolyte. Unfortunately, the electrophoretic mobilities of cyanide and thiocyanate were close at this pH and although the cyanide is not adsorbing at a detection wavelength of 200 nm, its presence had a disturbing effect on the shape of thiocyanate peaks. That was especially evident at high concentrations (data not shown). The possibility of using an uncoated fused-silica capillary and the background electrolyte containing tetradecyltrimethylammonium bromide as a flow modifier was also tested. However, the identical problem occurred with the high concentrations of cyanide. Furthermore, the thiocyanate peaks were broad because of the moderate electrophoretic mobility and consequently the sensitivity and reproducibility of the analyses were poor. Recently, van Dyck *et al.* [29] introduced the combination of EMMA methodology with a partial filling technique. In this setup, part of the capillary is filled with the optimum buffer for the enzymatic reaction whereas the rest of the capillary is filled with the background electrolyte optimal for the separation of substrates and products. A similar approach was adopted for the study of rhodanese. The enzymatic reaction was performed in 25 mM HEPES buffer (pH 8.5) [18] while the low pH background electrolyte (100 mM β-alanine-HCl, pH 3.5) was used for the separation of substrates and products [18, 32]. The uncoated fused-silica capillary was used in this modification. By working at low pH the problem with the high concentrations of cyanide is eliminated because cyanide is protonated to an acid pH ($pK_{a\,HCN}$ = 8.68) [33].

The principle of the EMMA method as carried out in this study is schematically described in Fig. 1. The capillary was first filled with the background electrolyte. Subsequently, plugs of HEPES buffer, of the enzyme solution, of substrate solution, of HEPES buffer, and finally a plug of the background electrolyte were injected hydrodynamically into the capillary. The exact injection parameters are described in Section 2.3. The order of the enzyme and substrate plugs was estimated experimentally because the electrophoretic mobility and p*I* of rhodanese were unknown and could not be determined since the crude enzyme preparation was used during the whole study. In order to prevent the preseparation of substrates before contact with the enzyme, the intermediate buffer plug between the enzyme and substrates plugs was omitted. Small buffer plugs were injected before the enzyme and after the substrate solutions to shield the enzymatic reaction from the denaturating effect of the low pH background electrolyte.

The knowledge of mobilities of the enzyme and substrates is essential to estimate when the zones completely merge and so to calculate the total enzymatic reaction time. In view of the fact that the lengths of the enzyme and substrate plugs were identical during all experiments, the reaction time should be taken as a constant and its exact value is not important. Turning off the separation voltage at the time when the zones completely merge has been used in several EMMA applications. It allowed the enzymatic reactions to continue resulting in the accumulation of products and consequently in the increase of the detection limit. This so-called "zero potential amplification" was not necessary in this study since a measurable amount of thiocyanate was formed during the zone interpenetration.

The typical electropherogram of the on-column enzymatic reaction with the enzyme solution (0.5 mg/mL protein) and the substrate solution containing 2.5 mM sodium thiosulfate and 25 mM potassium cyanide is shown in Fig. 2A. Under the low pH of background electrolyte, only the thiosulfate and thiocyanate peaks are detectable, since cyanide and sulfite are present in molecular forms – HCN and SO_2 – and do not migrate under given separation conditions. The identity of the enzyme product – thiocyanate was confirmed by spiking the enzyme solution with potassium thiocyanate (Fig. 2B). The same result was observed when the substrate solution was spiked (data not shown). The small peak between the thiosulfate and thiocyanate peaks was an unknown compound from the enzyme sample that could be used as an internal standard. Reproducibility of the EMMA method for thiocyanate was tested by repeated analyses using the same

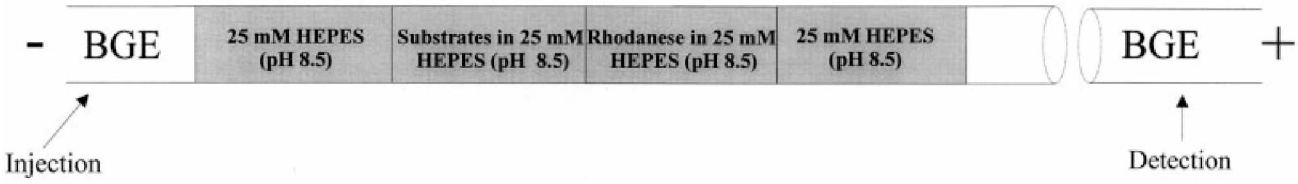

Figure 1. Schematics of the combination of EMMA methodology with a partial filling technique.

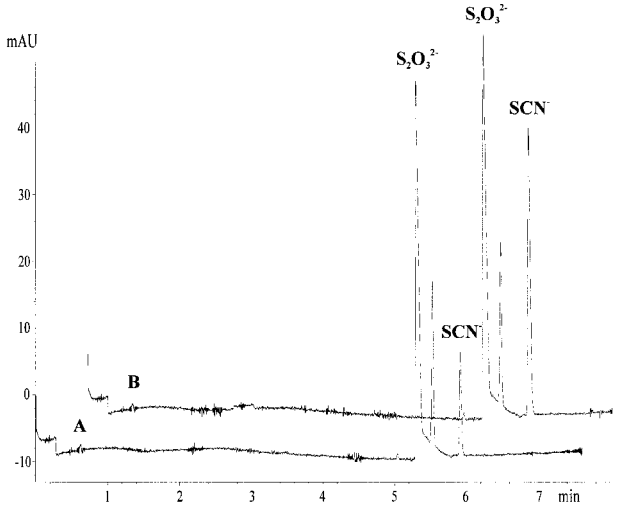

Figure 2. (A) Typical electropherogram of the on-column rhodanese reaction with the enzyme solution (0.5 mg/mL) in 25 mM HEPES buffer (pH 8.5) and the substrate solution of 2.5 mM sodium thiosulfate and 25 mM potassium cyanide in 25 mM HEPES buffer (pH 8.5). (B) The same system as described above with the enzyme solution spiked with 1 mM potassium thiocyanate. Separation conditions: background electrolyte, 0.1 M β-alanine-HCl (pH 3.5); separation voltage, −18 kV (negative polarity); 75 μm fused-silica capillary (64.5 cm total length, 56.0 cm effective length); direct detection at 200 nm; capillary temperature 25°C; injection, 25 mM HEPES buffer (pH 8.5), enzyme solution, substrate solution, 25 mM HEPES buffer (pH 8.5), and background electrolyte at 50 mbar for 4.0 s consecutively into the capillary.

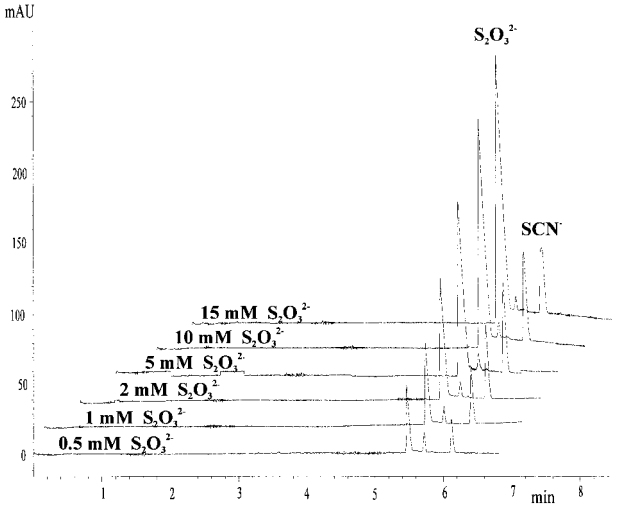

Figure 3. The overlay electropherograms of on-column rhodanese reactions at fixed concentration of potassium cyanide (10 mM) and six different concentrations of sodium thiosulfate between 0.5 and 15 mM. Separation and injection conditions as in Fig. 2.

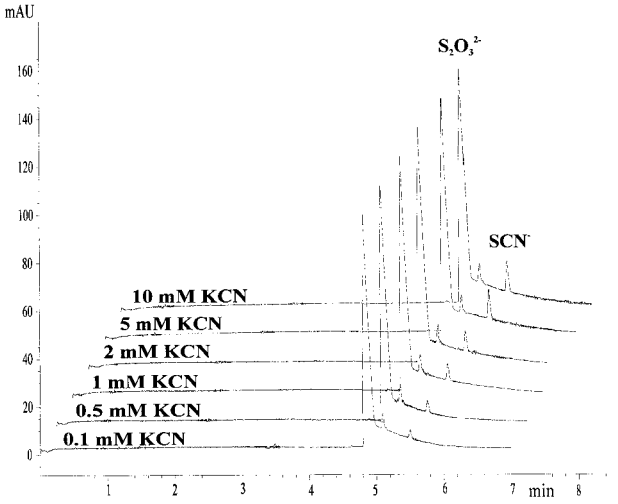

Figure 4. The overlay electropherograms of on-column rhodanese reactions at fixed concentration of sodium thiosulfate (20 mM) and six different concentrations of potassium cyanide between 0.1 and 10 mM. Separation and injection conditions as in Fig. 2.

system as described above. The excellent reproducibility for migration time (RSD = 0.23%, n = 10) was estimated, the reproducibility for peak area (RSD = 3.88%, n = 10) was a little worse because this parameter is affected not only by the reproducibility of the injections of the enzyme and substrates solutions but also by the enzymatic reaction itself.

Using the optimal conditions obtained from the above investigations, the EMMA method was used to evaluate some kinetic parameters of rhodanese. Since rhodanese is a bisubstrate enzyme, its kinetics are more complicated than at one-substrate reaction and cannot be described by a general rate equation. The type of the equation depends on the nature of the bisubstrate reaction. The determinations of K_m for both substrates were performed by changing the concentration of one substrate and maintaining the concentration of the second substrate constant, usually saturating concentration and *vice versa*. The bisubstrate reaction is thus reduced to single-substrate case. Each substrate combination was analyzed in triplicate. Figures 3 and 4 show the overlay electropherograms, which were obtained at six different concentrations of thiosulfate or cyanide and fixed concentration of cyanide or thiosulfate correspondingly.

The discontinuous precolumn CZE method for the determination of rhodanese activity was used as a control (data not shown). The graphic method of Eisenthal and Cornish-Bowden [34] using the direct linear plot of initial velocity and substrate concentration was applied for data evaluation in both cases. The initial reaction velocities were measured from the thiocyanate peak areas. As

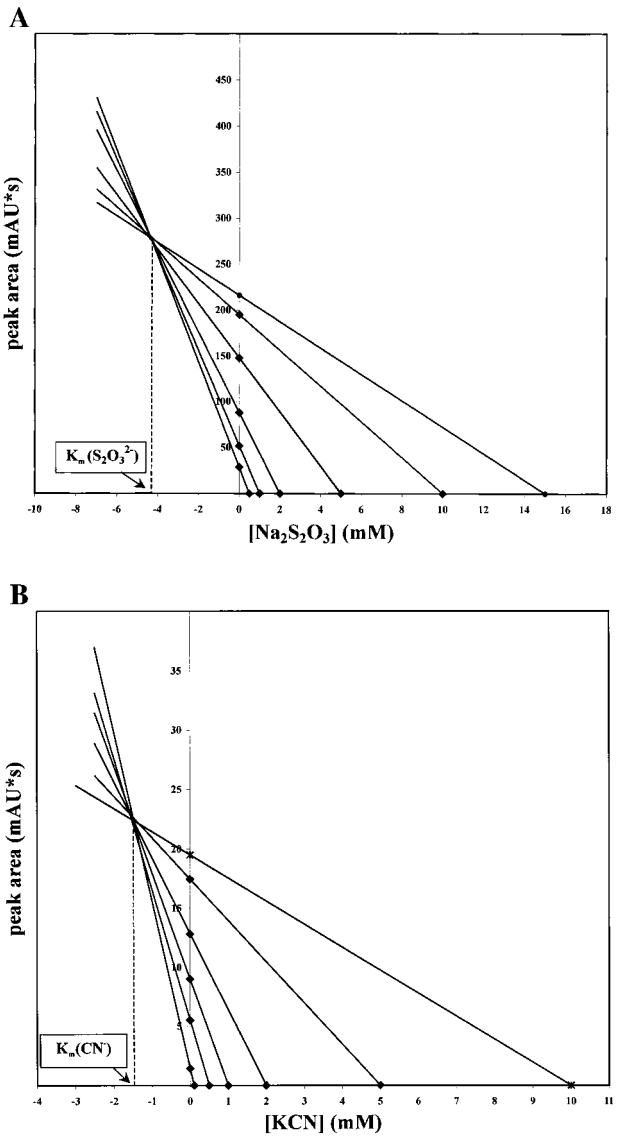

Figure 5. Direct linear plots of (A) initial velocity and substrate concentration for varied concentration of sodium thiosulfate and a fixed concentration of potassium cyanide and (B) for varied concentration of potassium cyanide and a fixed concentration of sodium thiosulfate.

peak areas have no physical units, the reaction velocities are scaled arbitrarily. The concentrations of substrates in the sample plug were taken to be equal to the concentrations in the vial before sampling. The K_m for cyanide and thiosulfate as determined by the EMMA method were 1.48×10^{-3} M and 4.12×10^{-3} M respectively (Figs. 5A and B), which agreed with the values of 1.22×10^{-3} M and 6.48×10^{-3} M determined by the discontinuous precolumn CZE method (data not shown). It is necessary to point out that these values are only "apparent" since the value of K_m depends on the fixed concentration of the second sub-

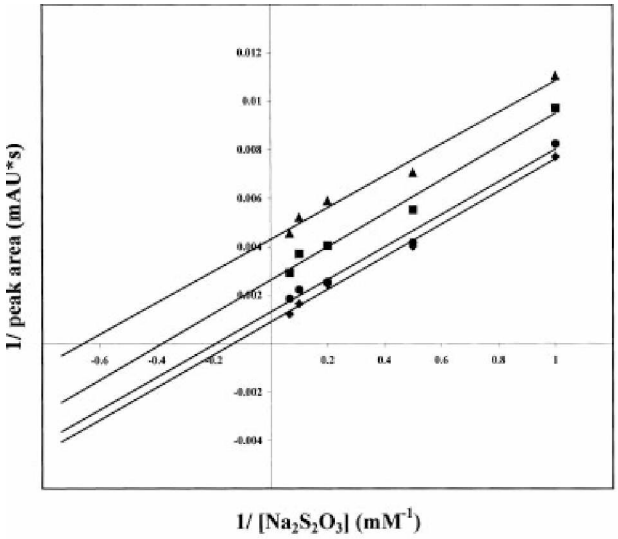

Figure 6. Primary double reciprocal plots of initial velocity and substrate concentration obtained with rhodanese. Sodium thiosulfate concentration varied between 1.0 and 15 mM, while potassium cyanide was at different fixed concentrations: (▲) 1 mM, (■) 2 mM, (●) 5 mM and (◆) 10 mM.

strate that was used. Moreover, as seen the selected concentrations of 10 mM potassium cyanide and 20 mM thiosulfate, respectively, are lower than the saturation concentrations that should be ten times higher than K_m. The other increase of these concentrations was impossible because of the deterioration effect of high ionic strength of the sample on the peak shapes (data not shown).

In order to solve this problem a different approach was used. The initial reaction velocities of the rhodanese enzymatic reaction were measured at the varying concentrations of the first substrate – sodium thiosulfate, and at the fixed concentration of the second substrate – potassium cyanide. These measurements were repeated at the several different fixed concentrations of the second substrate. Also in this case each substrate combination was analyzed in triplicate. Using this approach the "true" values of K_m should be obtained. Since the direct linear plot became rather complex with this experimental setup, any one of the linear transformations could be more convenient. The application of double reciprocal plots of the initial velocity and the substrate concentration was one of the choices because their slopes or intercepts could be used directly in subsequent secondary plots for K_m evaluation. In addition the different types of bisubstrate reaction could be distinguished by the nature of the double reciprocal plots. As can be seen from Fig. 6, the set of parallel lines was obtained and the graphic analysis thus provides strong evidence for a double displacement (ping-pong) mechanism (Fig. 7). This finding is in accor-

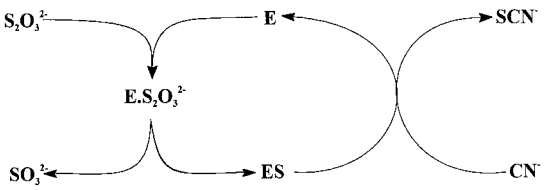

Figure 7. An illustration of double displacement (ping-pong) mechanisms of rhodanese-catalyzed reaction. $E.S_2O_3^{2-}$, noncovalent complex between enzyme and thiosulfate; ES, covalent complex between enzyme and sulfur.

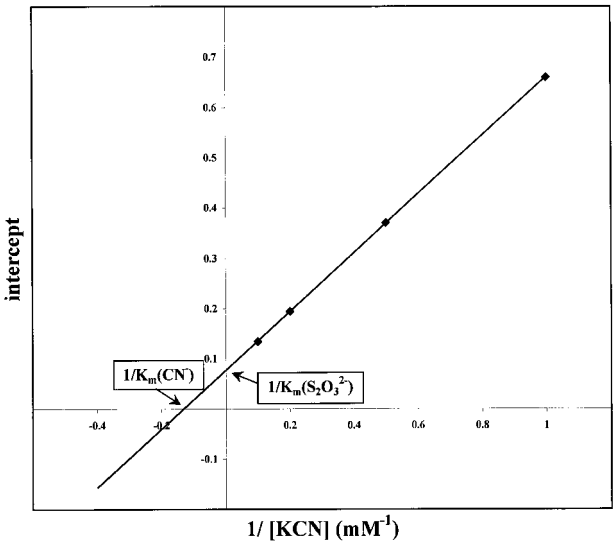

Figure 8. Secondary plot of the intercept of the lines in Fig. 6 *versus* reciprocal concentration of potassium cyanide.

dance with previous literature data [35]. The secondary plot of intercept on the concentration of potassium cyanide (Fig. 8) gave the K_m for thiosulfate and cyanide of 1.30×10^{-2} M and of 7.6×10^{-3} M. The values of K_m for thiosulfate for bovine liver rhodanese and for its recombinant form of 1.68×10^{-2} M and 1.59×10^{-2} M, respectively, reported by Miller *et al.* [36], are similar to the values reported here. To the best of our knowledge the K_m for cyanide has not been published.

The EMMA method was also used to evaluate the effect of the temperature on the enzymatic reaction. Fresh enzyme solution (25 μL) in a microvial was added to the autosampler, the temperature of the capillary and CZE autosampler were set at the same value and a short temperature preincubation step for 5 min was applied before the analysis. The internal standard potassium bromide was added to the enzyme solution for the normalization of the temperature effect on the thiocyanate peak. The analyses were performed as in the case of K_m determination (Fig. 9). The experimentally determined optimum was observed around 20–25°C, which agreed with literature values [8]. It should be noted that the pH of the enzyme reaction buffer and the background electrolyte, their viscosities, the electrophoretic mobilities of the enzyme and substrates, and so the reaction time would also be affected by temperature.

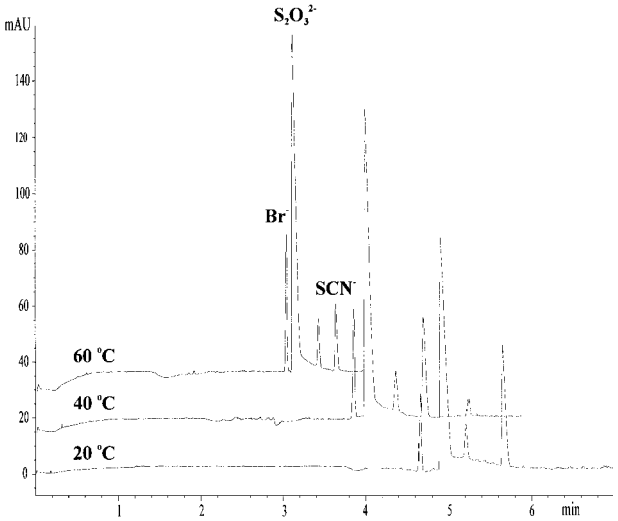

Figure 9. The overlay electropherograms of on-column rhodanese reactions at fixed concentrations of sodium thiosulfate (5 mM) and potassium cyanide (20 mM) and different capillary temperatures. Other separation and injection conditions as in Fig. 2.

4 Concluding remarks

Determination of the kinetic parameters of the bisubstrate enzymatic reaction of rhodanese by the combination of EMMA methodology with a partial filling technique is described. The basic limitation of EMMA methodology – the necessity to have the electrophoretic conditions compatible with both the separation of substrate(s) and product(s) of the enzymatic reaction, and the enzymatic reaction – is thus overcome. Compared to spectrophotometric and other discontinuous assays, the EMMA method is rapid, can be automated, and requires only small amount of reagents, which is especially important in the case of enzymes. This method has great potential for such determinations in other enzyme-substrate(s) systems.

This work was supported by grant No. 766/2002 from Czech Ministry of Education and grant No. 525/00/0785 from Grant Agency of Czech Republic.

Received October 2, 2001

5 References

[1] Westley, J., in: Vennesland, B., Conn, E. E., Knowles, C. J., Westley, J., Wissing, F. (Eds.), *Cyanide in Biology*, Academic Press, London 1981, pp. 61–76.

[2] Westley, J., in: Van Tamelen, E. E. (Ed.), *Bioorganic Chemistry*, Vol. 1, Academic Press, New York 1977, pp. 371–390.

[3] Westley, J., in: Jacoby, W. B. (Ed.), *Enzymatic Basis of Detoxication*, Vol. II, Academic Press, New York 1980, pp. 245–262.

[4] Sörbo, B., in: Senning, A. (Ed.), *Sulfur in Organic and Inorganic Chemistry*, Vol. 2, Marcel Dekker, New York 1972, pp. 143–169.

[5] Sörbo, B., in: Greenberg, D. M. (Ed.), *Metabolic Pathways*, Vol. 7, Academic Press, New York 1975, pp. 433–456.

[6] Westley, J., Adler, H., Westley, L., Nishida, C., *Fundam. Appl. Toxicol.* 1983, *3*, 377–382.

[7] Lang, K., *Biochem. Z.* 1933, *259*, 243–256.

[8] Westley, J., *Adv. Enzymol. Relat. Areas Mol. Biol.* 1973, *39*, 327–368.

[9] Suzuki, I., *Annu. Rev. Microbiol.* 1974, *28*, 85–101.

[10] Bonimi, F., Pagani, S., Cerletti P., *FEBS Lett.* 1977, *84*, 149–152.

[11] Villarejo, M., Westley J., *J. Biol. Chem.* 1963, *238*, 4016–4020.

[12] Pagani, S., Elddridge, M., Eady, R. R., *Biochem. J.* 1987, *244*, 485–488.

[13] Lu, W. P., Kelly, D. P., *FEMS Microbiol. Lett.* 1983, *18*, 289–292.

[14] Burrous, M. R., Westley, J., *Anal. Biochem.* 1985, *149*, 66–71.

[15] Aird, B. A., Lane, J., Westley, J., *Anal. Biochem.* 1987, *164*, 554–558.

[16] Llenado, R. A., Rechnitz, G. A., *Anal. Chem.* 1972, *44*, 1366–1370.

[17] Hussein, W. R., Von Storp, L. H., Guilbault, G. G., *Anal. Chim. Acta* 1972, *61*, 89–97.

[18] Glatz, Z., Bouchal, P., Janiczek, O., Mandl, M., Češková, P., *J. Chromatogr. A* 1999, *838*, 139–148.

[19] Bao, J. J., Regnier, F. E., *J. Chromatogr.* 1992, *608*, 217–224.

[20] Miller, K. J., Leesong, I. K., Bao, J. M., Regnier, F. E., Lytle, F. E., *Anal. Chem.* 1993, *65*, 3267–3270.

[21] Wu, D., Regnier, F. E., Linhares, M.C., *J. Chromatogr. B* 1994, *657*, 357–363.

[22] Harmon, B. J., Leesong, I., Regnier, F. E., *J. Chromatogr. A* 1996, *726*, 193–204.

[23] Watanabe, T., Yamamoto, A., Nagai, S., Terabe, S., *Electrophoresis* 1998, *19*, 2331–2337.

[24] Fujima, J. M., Danielson, N. D., *J. Capil. Electrophor.* 1996, *3*, 281–285.

[25] Jin, Z., Chen, R., Colon, L. A., *Anal. Chem.* 1997, *69*, 1326–1331.

[26] Harmon, B. J., Patterson, D. H., Regnier, F. E., *J. Chromatogr.* 1993, *657*, 429–434.

[27] Saevels, J., Van Schepdael, A., Hoogmartens, J., *Electrophoresis* 1996, *17*, 1222–1227.

[28] Xu, Y., Liu, X. H., Ip, M. P. C., *J. Liq. Chromatogr. Rel. Technol.* 1998, *21*, 2781–2797.

[29] Van Dyck, S., Van Schepdael, A., Hoogmartens, J., *Electrophoresis* 2001, *22*, 1436–1442.

[30] Saevels, J., Stehen, K. V. D., Van Schepdael, A., Hoogmartens, J., *J. Chromatogr. A* 1996, *745*, 293–298.

[31] Whisnat, A. R., Johnston, S. E., Gilma, S. D., *Electrophoresis* 2000, *21*, 1341–1348.

[32] Glatz, Z., Nováková, S., Štěrbová, H., *J. Chromatogr. A* 2001, *917*, 273–277.

[33] Cotton, F. A., Wilkinson, G., *Advanced Inorganic Chemistry*, Wiley New York 1966, pp. 313–313.

[34] Eisenthal, R., Cornish-Bowden, A., *Biochem. J.* 1974, *139*, 715–721.

[35] Westley, J., Nakamoto T., *J. Biol. Chem.* 1962, *237*, 547–549.

[36] Miller, D. M., Kurzban, G. P., Mendoza, J. A., Chirgwin, J. M., Hardies, S. C., Horowitz, P. M., *Biochim. Biophys. Acta* 1992, *1121*, 286–292.

2.2 Inhibition Study of Rhodanese by means of EMMA

NOVÁKOVÁ S., TELNAROVÁ M., GLATZ Z. (2003). **J. Chromatogr. A**, 990, 189-195.

Rhodanese_2.pdf

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Journal of Chromatography A, 990 (2003) 189–195
JOURNAL OF CHROMATOGRAPHY A
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Inhibition study of rhodanese by means of electrophoretically mediated microanalysis

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Abstract

A combination of the electrophoretically mediated microanalysis methodology with a partial filling technique was applied for the inhibition study of bovine liver rhodanese by 2-oxoglutarate. In this set-up, part of the capillary is filled with the best buffer for the enzymatic reaction, while the rest of the capillary is filled with the optimal background electrolyte for separation of substrates and products. The estimated value of K_I for 2-oxoglutarate was $3.62 \cdot 10^{-4} \pm 1.43 \cdot 10^{-4}$ M with respect to cyanide and $1.40 \cdot 10^{-3} \pm 1.60 \cdot 10^{-4}$ M with respect to thiosulfate. In addition, the type of inhibition was also evaluated. The findings of 2-oxoglutarate as the competitive inhibitor with respect to cyanide and as the uncompetitive inhibitor with respect to thiosulfate are in accordance with previous literature data.

Keywords: Electrophoretically mediated microanalysis; Partial filling electrophoretically mediated microanalysis; Rhodanese; Oxoglutarate; Enzymes; Enzyme inhibitors

1. Introduction

The enzyme rhodanese (thiosulfate: cyanide sulfur transferase, EC 2.8.1.1) discovered by Lang in 1933 [1] is involved in the major route of biological cyanide detoxification [2–5]. It catalyses transfer of the sulfane sulfur of thiosulfate to an acceptor, which is normally cyanide in the standard assay, and is likely to be cyanide under some physiological conditions [6,7]:

$$S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}.$$

Rhodaneses are widespread in the biological world: their activity has been detected in several species ranging from microorganisms through fungi, plants and animals to man [8]. Their physiological role has been debated for many years with proposals ranging from the detoxication of cyanide to the suggestion that rhodanese is important in bioenergetic oxidation of thiosulfate [9], in generating iron–sulfur protein complexes [10], in lipoate metabolism [11] and in reactivation of nitrogenase [12].

Because of the significance of rhodanese to fundamental and applied toxicology, many studies have been performed to elucidate its kinetic mechanism [13–15]. Most of them utilized spectrophotometric assays having associated limits such as consumption of relatively large amount of enzyme, no possibility of automation, etc. In order to overcome these limitations a new method based on capillary zone electrophoresis (CZE) has been recently applied to determine the kinetic parameters of rhodanese–electrophoretically mediated microanalysis (EMMA)

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01818-6

[16]. In this method, substrate(s) and enzyme are introduced in the capillary as a distinct plug, the first analyte injected being that with the lower electrophoretic mobility. Upon the application of an electric field, the two zones interpenetrate due to differences in their electrophoretic mobilities. Enzymatic reaction takes place and the resultant reaction product(s) and the unreacted substrate(s) are electrophoretically transported towards the detector, where they are individually detected.

The Michaelis constants for both substrates and the effect of temperature on rhodanese reaction have been evaluated utilizing the EMMA approach. In addition the type of kinetic mechanism of enzymatic reaction has also been elucidated. The purpose of this study was to apply the EMMA methodology to the inhibition study of rhodanese by 2-oxoglutarate. To the best of the authors' knowledge it is the first application of EMMA method for an inhibition study of bi-substrate enzymatic reaction.

2. Experimental

2.1. Materials and reagents

Rhodanese from beef liver, 2-oxoglutarate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and potassium cyanide were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical reagent grade, supplied from Fluka (Buchs, Switzerland). The background electrolyte was prepared by adding hydrochloride acid to 0.1 *M* β-alanine solution up to pH 3.5. The HEPES buffer was prepared by adding 0.1 *M* sodium hydroxide to 0.1 *M* HEPES solution up to pH 8.5. All solutions were prepared with Milli-Q water (Millipore, Milford, MA, USA) and filtered through a 0.45 μm membrane filter. Enzyme, substrates and inhibitor solutions were freshly prepared in 25 m*M* HEPES buffer (pH 8.5) each day. The mixing of substrates and inhibitor was performed immediately before the measurements to prevent their reaction.

2.2. Instrumentation

A Hewlet-Packard ^{3D}CE system (Waldbronn, Germany) with diode-array UV–Vis detector was used to carry out all CZE separations. Data were collected on an HP Vectra VL5 166-MHz personal computer using the Hewlet-Packard ^{3D}CE ChemStation Software. A Polymicro Technologies (Phoenix, AZ, USA) 75 μm fused-silica capillary was used for all separations.

2.3. Monitoring of rhodanese reaction by EMMA method

A 75 μm fused-silica capillary (64.5 cm total length, 56 cm effective length) was washed with 0.1 *M* β-alanine–HCl (pH 3.5) as a background electrolyte for 3 min. The on-capillary enzymatic reaction was performed by injection of 25 m*M* HEPES buffer (pH 8.5), the enzyme solution in 25 m*M* HEPES buffer (pH 8.5), the substrates solution in 25 m*M* HEPES buffer (pH 8.5) containing 2-oxoglutarate as an inhibitor, 25 m*M* HEPES buffer (pH 8.5), and the background electrolyte all at 50 mbar for 4.0 s consecutively into the capillary. The temperature of the capillary was 25 °C. The reaction was initiated by application of −18 kV (negative polarity) separation voltage. Samples were detected at 200 nm with a bandwidth of 20 nm. The peak areas were measured using ChemStation software. Evaluation and calculation of inhibition constants were done by means of the SigmaPlot 2001 software.

3. Results and discussion

3.1. Enzyme inhibition

The inhibition of enzymatic activity by specific molecules and ions is important because it serves as a major control mechanism in biological systems. Also, many drugs and toxic agents act by inhibiting enzymes. Furthermore enzyme inhibition can provide insight into the mechanism of enzyme action.

Enzyme inhibition can be either a reversible or irreversible process. In irreversible inhibition, the inhibitor is covalently linked to the enzyme or bound so tightly that its dissociation from the enzyme is very slow. In contrast, reversible inhibition is char-

acterised by a rapid equilibrium of the enzyme and inhibitor.

Three main types of reversible inhibition — competitive, uncompetitive and non-competitive — are known.

A competitive inhibitor resembles the shape and size of an enzyme substrate. It competes for substrate binding sites on the enzyme surface and so decreases the number of bound substrate. Michaelis–Menten equation for competitive inhibition can be described as

$$v_0 = \frac{V_{max}[S]}{K_M \cdot (1 + \frac{[I]}{K_I}) + [S]} \quad (1)$$

where v_0 and V_{max} are the initial and maximum velocities, respectively, K_M is the Michaelis constant, $[S]$ is the concentration of substrate, $[I]$ is the concentration of inhibitor, and K_I is the inhibition constant. The Lineweaver–Burk (double reciprocal) plots of initial velocity on the concentrations of substrate at the different concentration of a competitive inhibitor are intersected at the point $1/V_{max}$, which is typical for competitive inhibition.

An uncompetitive inhibitor is incapable of binding to the free enzyme. It can only bind to the enzyme–substrate complex. Once the inhibitor binds, it prevents the enzyme from turning the substrate into the product. Michaelis–Menten equation can be described as

$$v_0 = \frac{V_{max}[S]}{K_M + [S] \cdot (1 + \frac{[I]}{K_I})} \quad (2)$$

The series of the Lineweaver–Burk plots at the different concentrations of an uncompetitive inhibitor form the set of parallel lines.

A non-competitive inhibitor can bind both the enzyme and the enzyme–substrate complex. It binds at a site separate from the active site and modifies the enzyme conformation to inhibit the formation of the product. Michaelis–Menten equation can be described as

$$v_0 = \frac{V_{max}[S]}{K_M \cdot (1 + \frac{[I]}{K_I}) + [S] \cdot (1 + \frac{[I]}{K_I})} \quad (3)$$

The Lineweaver–Burk plots at the different concentration of a non-competitive inhibitor are intersected at the point $1/K_M$, which is typical for non-competitive inhibition [17].

It must be emphasised that all these formulas and plots are derived for uni-substrate enzymatic reactions. For multi-substrate reactions the algebraic origins of the plots are more complex, but essentially the same patterns arise and can be analysed to yield true K_I values.

3.2. Electrophoretically mediated microanalysis (EMMA) of rhodanese

The EMMA methodology was described by Bao and Regnier in 1992 [18]. Since its discovery it has been applied in a number of biochemical systems — for assays of enzyme activities [18–24], determination of substrates [23,25,26], Michaelis constants [16,27–30], inhibitors and inhibition constants [31,32], etc. As mentioned above, EMMA utilizes the different electrophoretic mobilities of enzyme, substrate(s) and product(s) to initiate enzymatic reaction inside the separation capillary and to separate the given compounds. Its original arrangement — the same buffer used for the enzymatic reaction and the electrophoretic separation — fundamentally restricts its applicability: the electrophoretic conditions, especially the composition and pH of background electrolyte, must be favorable for both the separation of substrate(s) and product(s) of the enzymatic reaction and the enzymatic reaction itself. To solve this problem Van Dyck et al. introduced the combination of the EMMA methodology with a partial filling technique [27]. In this set-up part of the capillary is filled with the best buffer possible for the enzymatic reaction while the rest of the capillary is filled with the optimal background electrolyte for separation of substrates and products. A similar approach has been adopted for the study of kinetic parameters of rhodanese [16]. The enzymatic reaction was performed in 25 m*M* HEPES buffer (pH 8.5) [33] while the low pH background electrolyte (100 m*M* β-alanine–HCl, pH 3.5) was used for separation of substrates and products [33,34].

An identical set-up with only minor modification — the inhibitor added to the substrate solution — was used in this work. The capillary was first filled

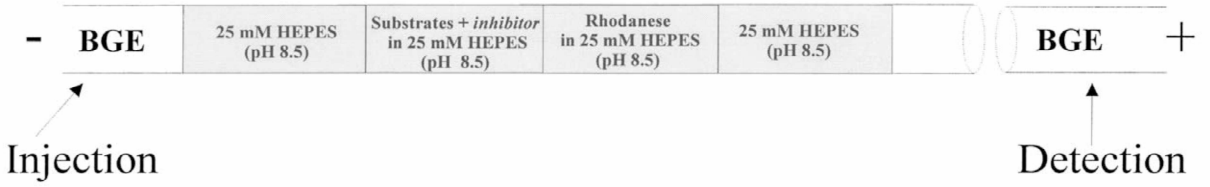

Fig. 1. Illustration of the combination of the EMMA methodology with a partial filling technique.

with the background electrolyte. Subsequently, a plug of the HEPES buffer, a plug of the enzyme solution, a plug of the substrates solution with or without the inhibitor, a plug of the HEPES buffer and finally a plug of the background electrolyte were injected hydrodynamically into the capillary (Fig. 1). The exact injections parameters are described in the Experimental section. The typical electropherogram of the on-column enzymatic reaction with the enzyme solution (0.5 mg of protein per ml) and the

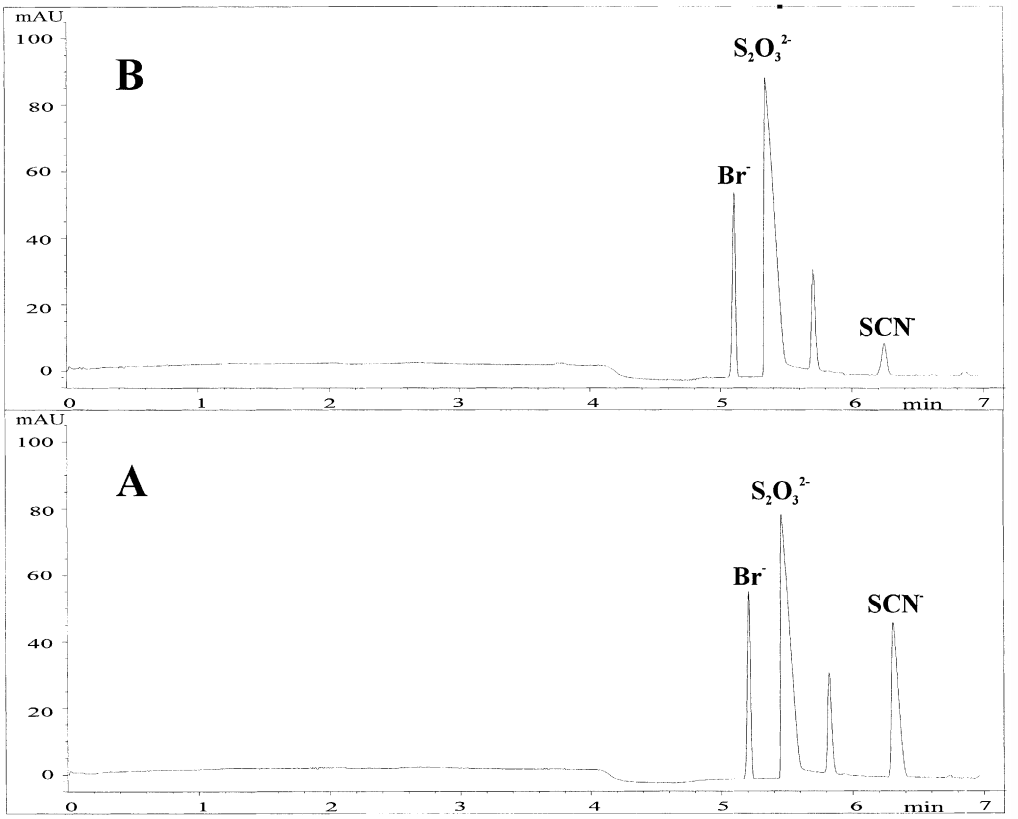

Fig. 2. Typical electropherogram of the on-column rhodanese reaction without (A) and with (B) 2-oxoglutarate as inhibitor added into the plug of substrates. The concentration of substrates was 5 m*M* thiosulfate and 5 m*M* cyanide, while the concentration of 2-oxoglutarate was 5 m*M*. The 0.4 m*M* bromide was added as an internal standard. Separation conditions: background electrolyte 0.1 *M* β-alanine–HCl (pH 3.50), separation voltage −18 kV (negative polarity), 75 μm fused-silica capillary (64.5 cm total length, 56.0 cm effective length), direct detection at 200 nm, temperature of capillary 25 °C. Injection: 25 m*M* HEPES buffer (pH 8.5), 50 mbar for 4.0 s; the enzyme solution, 50 mbar for 4.0 s; the substrates solution without or with inhibitor, 50 mbar for 4.0 s; 25 m*M* HEPES buffer (pH 8.5), 50 mbar for 4.0 s; and the background electrolyte, 50 mbar for 4.0 s, consecutively into the capillary.

substrates solution containing 5.0 m*M* sodium thiosulfate and 5.0 m*M* potassium cyanide is shown in Fig. 2A. The 0.4 m*M* bromide was added as an internal standard; the small peak between the thiosulfate and thiocyanate peaks was an unknown compound from the enzyme sample. The inhibition activity of 2-oxoglutarate on rhodanese reaction can be seen from the electropherogram measured under the same conditions but with the addition of 5.0 m*M* 2-oxoglutarate to the plug of substrates (Fig. 2B).

Since rhodanese is a bi-substrate enzyme, the inhibitory behavior of 2-oxoglutarate against each substrate was determined individually by measuring the initial velocities of the enzymatic reaction at the varying concentrations of the one substrate and the inhibitor, and at the fixed concentration of the second substrate and vice versa. Each substrate and inhibitor combination was analysed in triplicate. The initial reaction velocities were measured from the thiocyanate peak areas. As peak areas have no physical units, the reaction velocities are scaled arbitrarily.

The Michaelis–Menten plots with the potassium cyanide as varied substrate, sodium thiosulfate as fixed substrate (10.0 m*M*) and at different concentrations of 2-oxoglutarate are given in Fig. 3. The Lineweaver–Burk plots are linear intersecting the $1/v_0$ axis at the point $1/V_{max}$ and show that 2-oxoglutarate is a competitive inhibitor with respect to cyanide (Fig. 4). The K_I value for 2-oxoglutarate with respect to cyanide, $3.62 \cdot 10^{-4} \pm 1.43 \cdot 10^{-4}$ *M*, was computed from these data.

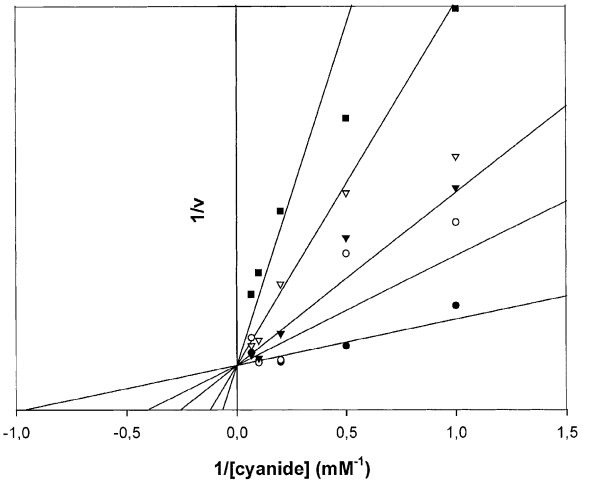

Fig. 4. The Lineweaver–Burk plots showing competitive inhibition by 2-oxoglutarate with the cyanide as the varied substrate and thiosulfate (10.0 m*M*) as the fixed substrate. The family of lines was obtained by varying the 2-oxoglutarate concentration as follows: (●) 0 m*M*; (○) 0.5 m*M*; (▼) 1.0 m*M*; (▽) 2.5 m*M*; (■) 5.0 m*M*.

On the other hand, Fig. 5 shows the Michaelis–Menten plots with the sodium thiosulfate as varied

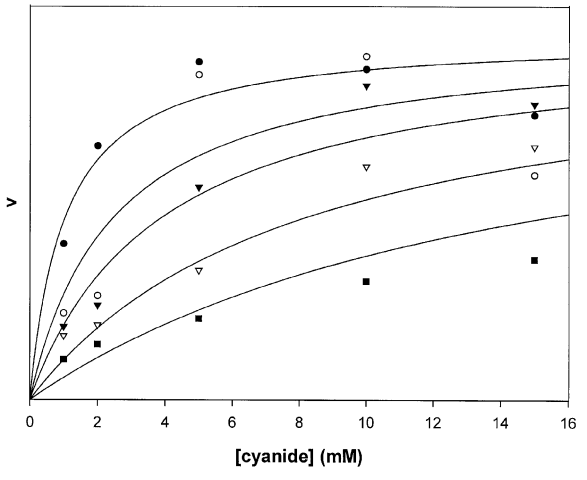

Fig. 3. The Michalis–Menten plots for the enzymatic reaction of rhodanese inhibited by: (●) 0 m*M*; (○) 0.5 m*M*; (▼) 1.0 m*M*; (▽) 2.5 m*M*; and (■) 5.0 m*M* 2-oxoglutarate with the cyanide as the varied substrate and thiosulfate (10.0 m*M*) as the fixed substrate. The separation conditions were the same as in Fig. 2.

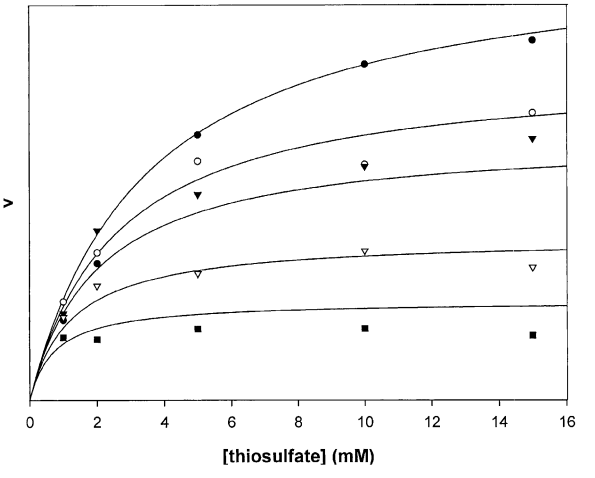

Fig. 5. The Michalis–Menten plots for the enzymatic reaction of rhodanese inhibited by: (●) 0 m*M*; (○) 0.5 m*M*; (▼) 1.0 m*M*; (▽) 2.5 m*M*; and (■) 5.0 m*M* 2-oxoglutarate with the thiosulfate as the varied substrate and cyanide (10.0 m*M*) as the fixed substrate. The separation conditions were the same as in Fig. 2.

substrate, potassium cyanide as fixed substrate (10.0 m*M*) and at different concentrations of 2-oxoglutarate. The Lineweaver–Burk plots are linear and parallel (Fig. 6) indicating 2-oxoglutarate as an uncompetitive inhibitor with respect to thiosulfate. The K_I value for 2-oxoglutarate with respect to thiosulfate, $1.40 \cdot 10^{-3} \pm 1.60 \cdot 10^{-4}$ *M*, was computed as well.

The inhibition of rhodanese activity by tricarboxylic acid cycle intermediates was first observed by Lawrence [35]. Susumu [36] later demonstrated that bovine liver rhodanese was inhibited competitively by 2-oxoglutarate with respect to cyanide and uncompetitively with respect to thiosulfate and proposed the reaction mechanism shown in Fig. 7, which includes the inhibition reaction by 2-oxoglutarate (2-OG) as well as the non-enzymatic formation of cyanohydrin (Y). All our results and conclusions are in accordance with this schematic. The inhibition study in the present paper thus provide further important evidence for elucidation of the action of rhodanese. In consequence the EMMA methodology could serve as a progressive tool of modern enzymology in the context of metabolomic research.

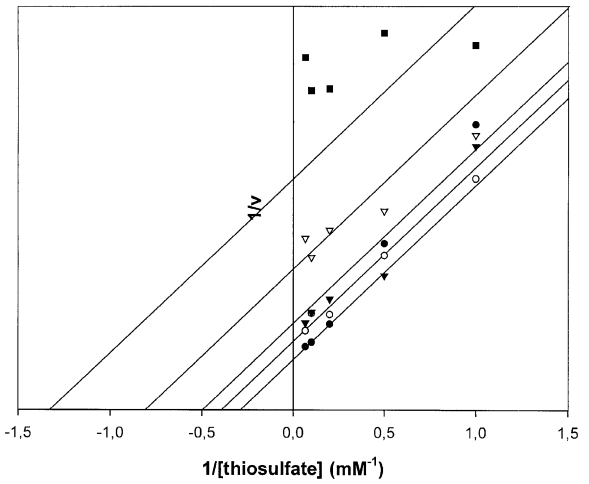

Fig. 6. The Lineweaver–Burk plots showing uncompetitive inhibition by 2-oxoglutarate with the thiosulfate as the varied substrate and cyanide (10.0 m*M*) as the fixed substrate. The family of lines was obtained by varying the 2-oxoglutarate concentration as follows: (●) 0 m*M*; (○) 0.5 m*M*; (▼) 1.0 m*M*; (▽) 2.5 m*M*; (■) 5.0 m*M*.

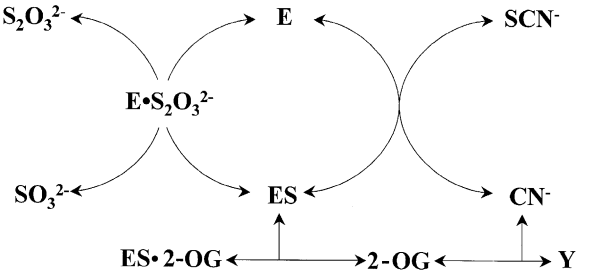

Fig. 7. Illustration of the inhibition of rhodanese by 2-oxoglutarate: 2-oxoglutarate is competitive inhibitor with respect to cyanide and uncompetitive inhibitor with respect to thiosulfate. (E, enzyme; E·$S_2O_3^{2-}$, non-covalent complex between enzyme and thiosulfate; ES, covalent complex between enzyme and sulfur; 2-OG, 2-oxoglutarate; Y, cyanohydrin).

4. Conclusions

This work shows that the inhibition study of bovine liver rhodanese can be easily performed by EMMA methodology combined with partial filling technique. The method can be used not only to estimate K_I but also for the determination of the inhibition type. Compared to spectrophotometric and other discontinuous assays, the method is rapid, can be automated, and requires only small amounts of reagents, which is especially important in the case of enzymes. Consequently the method has great potential for such determinations in other enzyme-inhibitor systems.

Acknowledgements

This work was supported by grant No. 766/2002 from the Czech Ministry of Education and grant No. 203/03/1125 from the Grant Agency of the Czech Republic.

References

[1] K. Lang, Biochem. Z. 259 (1933) 243.
[2] J. Westley, in: B. Vennesland, E.E. Conn, C.J. Knowles, J. Westley, F. Wissing (Eds.), Cyanide in Biology, Academic Press, London, 1981, p. 61.

[3] J. Westley, in: E.E. Van Tamelen (Ed.), Bioorganic Chemistry, Vol. 1, Academic Press, New York, 1977, p. 371.
[4] J. Westley, in: W.B. Jacoby (Ed.), Enzymatic Basis of Detoxication, Vol. II, Academic Press, New York, 1980, p. 245.
[5] B. Sörbo, in: A. Senning (Ed.), Sulfur in Organic and Inorganic Chemistry, Vol. 2, Marcel Dekker, New York, 1972, p. 143.
[6] B. Sörbo, in: D.M. Greenberg (Ed.), Metabolic Pathways, Vol. 7, Academic Press, New York, 1975, p. 433.
[7] J. Westley, H. Adler, L. Westley, C. Nishida, Fundam. Appl. Toxicol. 3 (1983) 377.
[8] J. Westley, Adv. Enzymol. Relat. Areas Mol. Biol. 39 (1973) 327.
[9] I. Suzuki, Annu. Rev. Microbiol. 28 (1974) 85.
[10] F. Bonimi, S. Pagani, P. Cerletti, FEBS Lett. 84 (1977) 149.
[11] M. Villarejo, J. Westley, J. Biol. Chem. 238 (1963) 4016.
[12] S. Pagani, M. Eldridge, R.R. Eady, Biochem. J. 244 (1987) 485.
[13] J. Westley, T. Nakamoto, J. Biol. Chem. 237 (1962) 547.
[14] J. Westley, D. Heyse, J. Biol. Chem. 246 (1971) 1468.
[15] R. Mintel, J. Westley, J. Biol. Chem. 241 (1966) 3386.
[16] S. Nováková, Z. Glatz, Electrophoresis 23 (2002) 1063.
[17] D. Voet, J.G. Voet, Biochemistry, Wiley, New York, 1995.
[18] J.M. Bao, F.E. Regnier, J. Chromatogr. 608 (1992) 217.
[19] K.J. Miller, I.K. Leesong, J.M. Bao, F.E. Regnier, F.E. Lytle, Anal. Chem. 65 (1993) 3267.
[20] D. Wu, F.E. Regnier, M.C. Linhares, J. Chromatogr. B 657 (1994) 357.
[21] B.J. Harmon, I.K. Leesong, F.E. Regnier, J. Chromatogr. A 726 (1996) 193.
[22] T. Watanabe, A. Yamamoto, A. Nagai, S. Terabe, Electrophoresis 19 (1998) 2331.
[23] J.M. Fujima, N.D. Danielson, J. Cap. Electrophoresis 3 (1996) 281.
[24] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 23 (2002) 1341.
[25] Z. Jin, R. Chen, L.A. Colon, Anal. Chem. 69 (1997) 1326.
[26] B.J. Harmon, D.H. Patterson, F.E. Regnier, J. Chromatogr. A 657 (1993) 429.
[27] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 22 (2001) 1436.
[28] J. Saevels, A. Van Schepdael, J. Hoogmartens, Electrophoresis 17 (1996) 1222.
[29] Y. Xu, X.H. Liu, M.P.C. Ip, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 2781.
[30] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 23 (2002) 2854.
[31] J. Saevels, K.V.D. Stehen, A. Van Schepdael, J. Hoogmartens, J. Chromatogr. A 745 (1996) 293.
[32] A.R. Whisnat, S.E. Johnston, S.D. Gilma, Electrophoresis 21 (2000) 1341.
[33] Z. Glatz, P. Bouchal, O. Janiczek, M. Mandl, P. Češková, J. Chromatogr. A 838 (1999) 139.
[34] Z. Glatz, S. Nováková, H. Štěrbová, J. Chromatogr. A 917 (2001) 237.
[35] P. Lawrence, Ph.D. Thesis, University of Wisconsin, 1967.
[36] O.I. Susumu, J. Biochem. 76 (1974) 455.

CHAPTER 3
PHENOL SULFOTRANSFERASE

3.1 Study of Enzyme Kinetics of Phenol Sulfotransferase using Electrophoretically Mediated Microanalysis

NOVÁKOVÁ S., VAN DYCK S., VAN SCHEPDAEL A., HOOGMARTENS J., GLATZ Z. (2004) **J. Chromatogr. A**, 1032, 319-326.

Phenol sulphotransferase.pdf

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Study of enzyme kinetics of phenol sulfotransferase by electrophoretically mediated microanalysis

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Abstract

Electrophoretically mediated microanalysis (EMMA) was applied for the study of the kinetic parameters of the enzymatic reaction of phenol sulfotransferase SULT1A1 isoenzyme with 4-nitrophenol as a substrate. The SULT1A1 activity was determined by the quantitation of the product, 4-nitrophenyl sulfate, at 274 nm by using different injection and separation steps. This new approach solved the problem of the presence of the very strong inhibitor, adenosine 3′,5′-bisphosphate (PAP), in the co-substrate solution (adenosine 3′-phosphate 5′-phosphosulfate, PAPS) which is unstable at room temperature. The inhibitor PAP was electrophoretically separated from the co-substrate PAPS before the injection of enzyme and substrate inside the capillary (and thus before their in-capillary encountering). With the developed in-capillary SULT1A1 activity assay an average Michaelis constant (K_m) for 4-nitrophenol was calculated to be 0.84 μM, a value which is consistent with a previously reported value. Strong substrate inhibition (above a 4-nitrophenol concentration of 2.5 μM) was observed, and this is also in accordance with literature values.

Keywords: Kinetic studies; Electrophoretically mediated microanalysis; Enzymes; Phenol sulfotransferase

1. Introduction

Sulfation, or more precisely sulfonation, represents a major pathway for the biotransformation/detoxication of drugs and xenobiotics, as well as endogenous compounds such as cholesterol, catecholamines, steroid and thyroid hormones, and bile acid [1]. The responsible enzymes are called the sulfotransferases (STs). Two general classes of these enzymes exist in tissue fractions: the cytosolic enzymes that are important in drug metabolism, and the membrane bound enzymes that are involved in the sulfonation of glycoproteins and glycosaminoglycans [2]. Cytosolic sulfotransferases play an important role in the second-phase metabolism of xenochemicals, and are also involved in the inactivation of endogenous signal molecules such as neurotransmitters [3]. The family of cytosolic sulfotransferases catalyzes the sulfonation reaction involving the transfer of an electrophilic sulfonate group from a biologically active form of inorganic sulfate, adenosine 3′-phosphate 5′-phosphosulfate (PAPS), to a nucleophilic acceptor substrate [4–8]. Structural analogues of the sulfate donor PAPS are effective inhibitors of these enzymes [9–12].

Spectrophotometric assays [13–15] and LC-based methods with UV detection [16,17] have been reported for assaying sulfotransferase activity. However, the most common assay of sulfotransferase activity involves the monitoring of the transfer of radioisotopic sulfate from [^{35}S] PAPS to the reaction product [1]. The separation of [^{35}S] PAPS from the sulfated reaction products can be performed by a precipitation reaction with barium salts [17] or by use of thin layer chromatography [18] or LC techniques [2]. Alternatively, radioactively labeled sulfate acceptors (substrates) have been used to assay ST activity [18,19]. In general, the use of

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doi:10.1016/j.chroma.2003.11.082

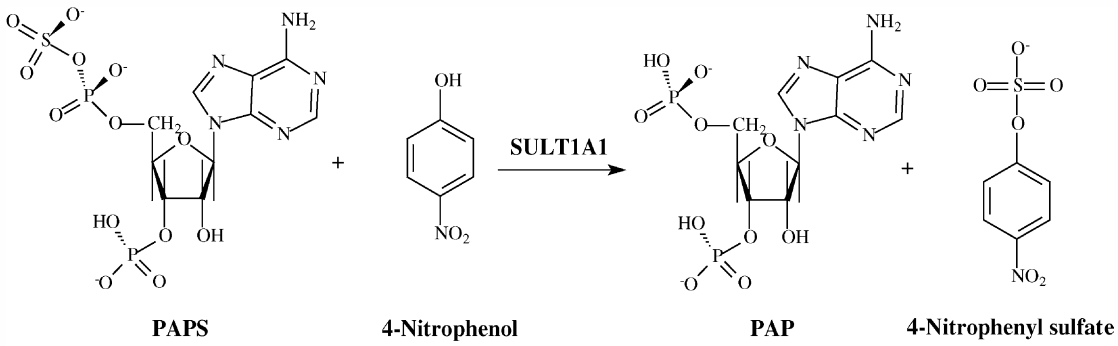

Fig. 1. The sulfonation reaction catalyzed by phenol sulfotransferase.

radioactively labeled PAPS or radioactive sulfate acceptor provides assays with a high degree of sensitivity [1].

Capillary electrophoresis is a powerful and relatively new analytical tool, characterized by high resolution separations, short analysis times and low sample load. This implies that the capillary, the separation tool, can also be used as a small reaction vessel. In this manner, all the different assay steps (i.e. reaction, separation, quantitation) can be combined in one automated, microscale assay.

CE systems have been successfully applied for on-line enzyme-catalyzed reactions by a methodology known as electrophoretically mediated microanalysis (EMMA), firstly described by Bao and Regnier [20]. EMMA utilizes the different electrophoretic mobilities of enzyme, substrate, and product to initiate reaction inside the capillary and to separate the components from each other for final in-capillary quantitation. There are basically two ways to mix reaction components [21–23] in a capillary under electrophoretic conditions. The first one is the continuous format of EMMA ("long contact mode"). In this format the capillary is initially filled with one of the reactants while the second analyte is introduced. The second one is the plug–plug format of EMMA (transient format or "short contact mode"). In contrast, this format is based on a plug–plug interaction. One of the advantages of the plug–plug format of EMMA is the electrophoresis process prior to the contact of enzyme and substrate inside the capillary. This process permits the separation of potential interfering substances, which may be advantageous when assaying enzyme activity in crude biological samples [24]. EMMA methodology has been used for different biochemical systems: enzyme activity assays [21,25–27], kinetic studies with the determination of Michaelis constants [28–31], or the study of inhibitors and inhibition constants [32–34].

In this work we used the isoenzyme SULT1A1 that is responsible for the sulfonation of small phenolic substrates such as 4-nitrophenol (Fig. 1). The purpose of this study was to determine whether it is possible to assay SULT1A1 activity with 4-nitrophenol as a model substrate by capillary electrophoresis, more precisely by using the EMMA methodology.

2. Materials and methods

2.1. Materials and reagents

Phenol sulfotransferase SULT1A1*2 cytosolic extract (human, recombinant) 60 μl, 4.4 mg prot./ml, 15766 units/mg prot. (one unit conjugates one picomole of sulfate to 4-nitrophenol at pH 6.5 at 37 °C), 4-nitrophenol (pNP), 4-nitrophenyl sulfate (pNPS), adenosine 3′,5′-bisphosphate (PAP), bovine serum albumin (BSA), dithiothreitol (DTT) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Sigma (St. Louis, MO, USA). Adenosine 3′-phosphate 5′-phosphosulfate (PAPS, purity ≥80% by LC) was purchased from Calbiochem (San Diego, CA, USA) and cholic acid (sodium salt) was obtained from Acros Organics (Geel, Belgium). In the off-line mode the background electrolyte consisted of a 150 mM HEPES buffer of pH 6.5 (adjusted with 1 M NaOH at 37 °C). In the in-line mode the background electrolyte consisted of a 150 mM HEPES buffer (pH 6.5 at 37 °C) with 20 mM cholic acid (sodium salt), which was freshly prepared each day. All solutions were prepared with Milli-Q water (Millipore, Milford, MA, USA) and filtered through 0.2 μm nylon filters (Alltech, Lokeren, Belgium).

Solutions of SULT1A1 were prepared in 25 mM potassium phosphate buffer, pH 6.5 (at 37 °C), that contained 1.5 mg/ml BSA, 10 mM DTT, and 8 mM $MgCl_2$. The final SULT1A1 solution contained 8.32 U/μl and was stored at −70 °C. Before use, the SULT1A1 solution was thawed rapidly at 37 °C (water bath) and stored on ice. Solutions of the substrate 4-nitrophenol (0.05–20 μM) and of the co-substrate PAPS (115 μM), were prepared in 25 mM potassium phosphate buffer, pH 6.5 (at 37 °C) and were kept in the thermostated storage room of the CE instrument at 5 °C in order to prevent degradation of the PAPS and the enzyme during the analyses.

2.2. CE instrumentation

All experiments were carried out on a P/ACE MDQ CE system (Beckman Instruments, Fullerton, CA, USA). On-line detection was performed at 260 and 274 nm with

a diode array detection system. Data collection and peak area analysis were performed by P/ACE MDQ 32 Karat software (Beckman Coulter, version 5.0). Calculation of the Michaelis constant was done by means of SigmaPlot 2001 software (version 7.101). Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 31.2 cm (21 cm from the injection side to the detector) ×75 μm i.d. were used. The capillary was thermostated by liquid cooling at 37 °C and the sample tray was thermostated at 5 °C.

3. Results and discussion

3.1. Strategy for the development of a CE-based assay

The human recombinant phenol sulfotransferase (PST) enzyme was studied. Therefore, the thermostable sulfotransferase SULT1A1 that conjugates sulfate to simple phenols such as pNP was purchased. This expensive human recombinant enzyme has only become recently available on the market. The co-substrate PAPS is also expensive, and unstable at room temperature. Therefore, commercial PAPS always contains a certain amount of PAP. The reaction product PAP is a competitive inhibitor [10,11] of the sulfonation reaction, having a K_i of 0.4 μM [12] and should be avoided in the reaction mixture. Firstly, separation conditions were established for the model substrate pNP and the reaction products pNPS and PAP. Secondly, the reaction was carried out outside the capillary in an off-line study. Finally, the previously developed separation step was combined with the enzymatic reaction step in order to develop an in-line method.

3.2. Off-line mode

The reaction mixture contained 50 μl SULT1A1 solution (8.32 U/μl), 100 μl of pNP solution (1 mM), 250 μl of PAPS solution (115 μM) and 600 μl of a 25 mM potassium phosphate buffer (pH 6.5 at 37 °C) that contained 8 mM $MgCl_2$, 1.5 mg/ml BSA and 10 mM DTT. The electrophoresis ran at 5 kV.

The human recombinant SULT1A1 enzyme was incubated at 37 °C with the substrate pNP and the corrected peak area of pNPS was determined at 274 nm. The mixture was directly injected into the capillary by a pressure injection (0.3 p.s.i., 5 s; 1 p.s.i. = 6894.76 Pa). A linear relationship was found when the reaction product was determined at regular time intervals (up to 100 min). A correlation coefficient (r) of 0.9914 was determined for the following regression equation: $y = 2.023x + 177$ with y the corrected peak area of pNPS and x the time expressed in minutes. Unfortunately, there was no direct relationship between the quantity of SULT1A1 and the enzyme activity and moreover, the results were not repeatable (data not shown). A possible explanation is the formation of PAP that strongly inhibits the reaction. PAP is a reaction product, and moreover, commercial PAPS typically contains significant amounts of PAP and other impurities [19] since PAPS is very unstable at room temperature. Therefore, a certain amount of PAP can not be excluded from the reaction mixture.

3.3. In-line mode

3.3.1. Preseparation of PAP from commercial PAPS

One of the advantages of transient engagement EMMA is the electrophoresis process prior to the contact of enzyme and substrate inside the capillary. This process permits the separation of potential interfering substances, which may be advantageous when assaying enzyme activity in crude biological samples [24]. This means that the inhibitor PAP can be electrophoretically separated from the co-substrate PAPS before the injection of enzyme and substrate inside the capillary (and thus before their in-capillary encountering).

PAP and PAPS are well separated from each other in a 150 mM HEPES buffer of pH 6.5 (37 °C) that contained 20 mM cholic acid (sodium salt). The electrophoretic mobility of PAP was determined to be -3.300×10^{-4} cm^2 V^{-1} s^{-1} and that of PAPS -4.029×10^{-4} cm^2 V^{-1} s^{-1}. This difference in electrophoretic mobility was determined to be large enough to allow a fast separation of PAP from the PAPS co-substrate, e.g. during 1 min at 15 kV.

3.3.2. In-capillary assay based on EMMA with PAP preseparation step

The co-substrate PAPS was used at a 115 μM concentration. The solutions of SULT1A1, PAPS and pNP were kept in the thermostated storage room of the CE instrument at 5 °C in order to prevent degradation of the PAPS and the enzyme during the analyses. The electrophoresis ran at 10 kV. According to our calculations, about 37 nl of the SULT1A1 solution (8.32 U/μl) was injected in each EMMA analysis. The activity of the SULT1A1 enzyme was determined by measuring the corrected peak area of the product pNPS at 274 nm.

First, the co-substrate PAPS was injected in the capillary by a pressure injection (0.5 p.s.i., 5 s). Then, a 15 kV electric field was applied during 1 min in order to separate the inhibitor PAP from the co-substrate PAPS. Subsequently, the substrate pNP and the enzyme were injected by pressure injections (0.3 p.s.i., 5 s). The injection order of enzyme and substrate was determined experimentally. Then, the electrophoresis ran at 5 kV (1 min) in order to mix the enzyme, substrate and the co-substrate PAPS inside the capillary. In a next step, the voltage was turned off, e.g. for 2 min, in order to let enzyme and substrate react in the absence of the electric field (i.e. zero-potential amplification). Again, this in-capillary incubation protocol was determined experimentally. Finally, the electrophoresis ran at 10 kV in order to sweep the reaction product to the detector for on-line quantitation. The product passes the detector within 4 min. After each injection step, the electrodes (and the capillary ends) were dipped into water to prevent carry over of sample. An

Table 1
Different steps of the EMMA-based reaction for PST activity

Steps	Plugs	Pressure p_i (p.s.i.)	Time t_i (s)	Voltage (kV)
(1) Injection of PAPS	PAPS	0.5	5	/
(2) Preseparation PAP from PAPS	/	/	60	15
(3) Injection of S	pNP	0.3	5	/
(4) Injection of E	SULT1A1	0.3	5	/
(5) Mixing of the compounds and subsequent reaction of E and S	/	/	60	5
(6) Incubation	/	/	120	0
(7) Separation	/	/	300	10

overview of the different steps of the EMMA assay for recombinant PST activity is provided in Table 1. An electropherogram after in-capillary reaction of the SULT1A1 enzyme and its substrate pNP is shown in Fig. 2.

3.3.3. Repeatability of the EMMA-based assay and quantitation of pNP

The within-day repeatability of the in-capillary formation and the subsequent analysis and quantitation of the reaction product pNPS was determined for 12 consecutive analyses of the SULT1A1 solution (8.32 U/μl). A substrate concentration of 20 μM was used and a 115 μM concentration of PAPS. The R.S.D. value determined for the corrected peak area of pNPS was 5.2% ($n = 12$) and the R.S.D. of the peak migration time was 2.2% ($n = 12$). This R.S.D. value is not only affected by the injections of the enzyme and substrate solutions, but also by the enzymatic reaction itself.

The determination of SULT1A1 activity was achieved by measuring the corrected peak area of the product pNPS generated during the in-capillary reaction. Therefore, a strict linear correlation between the pNPS concentration and its corrected peak area is required. The background electrolyte consisted of 150 mM HEPES (pH 6.5 at 37 °C) with 20 mM cholic acid (sodium salt) and a 10 kV potential was applied. A stock solution of pNPS was prepared in a 25 mM potassium phosphate buffer (pH 6.5) and a series was made by diluting the stock solution with the same buffer over a concentration range between 0.78 μM and 12.5 μM. In the regression equation, $y = 0.591x + 0.373$, y represents the corrected peak area and x represents the concentration of pNPS in μM. A correlation coefficient (r) of 0.9932 was observed. The limit of detection (LOD) was found to be 0.05 μM. According to our calculations, 37.2 nl was injected into the capillary (0.3 p.s.i., 5 s), which corresponds to an injected amount of 1.9×10^{-15} mol of pNPS.

3.3.4. Effect of the amount of SULT1A1 enzyme on product formation

The injection of different concentrations of the SULT1A1 enzyme was impossible due to the high cost of the recombinant enzyme. Thus, in order to measure the effect of the amount of enzyme on the reaction, the extent of the sulfation reaction was controlled by injecting longer or shorter enzyme plugs. Fig. 5 (Section 3.3.6) shows an overlay of six electropherograms obtained after in-capillary reaction between pNP and varying amounts of SULT1A1.

As shown in Fig. 3, the sulfonation reaction inside the capillary was proportional to the injection time of SULT1A1.

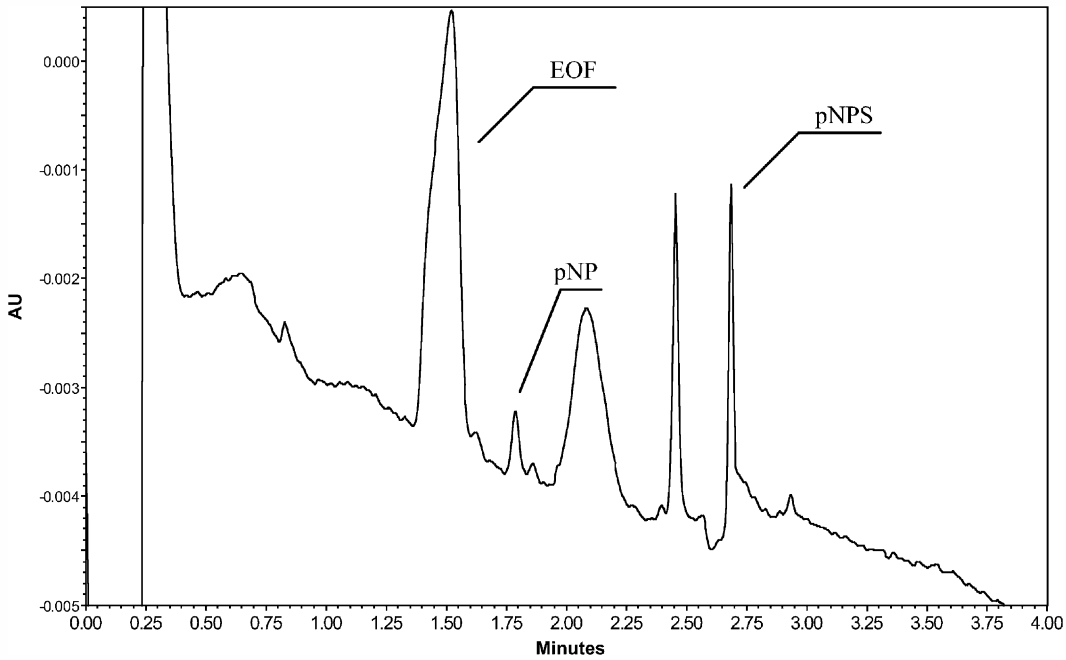

Fig. 2. Electropherogram after in-capillary reaction between the SULT1A1 enzyme and pNP. Incubation at zero potential during 2 min. Concentration of SULT1A1: 8.32 U/μl, pNPS: 115 μM and pNP: 20 μM. CE conditions: run buffer: 150 mM HEPES (pH 6.5) with 20 mM sodium cholate; voltage: 10 kV (45 μA); detection at 274 nm.

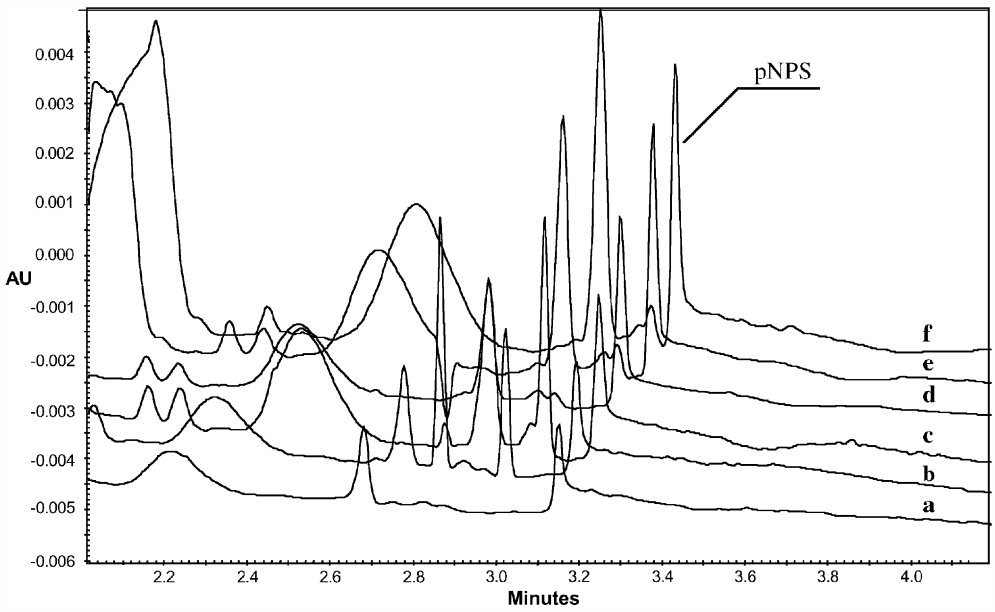

Fig. 3. Overlay of six electropherograms after in-capillary reaction of the SULT1A1 enzyme with increasing enzyme injection times of 2, 3, 4, 5, 6 and 7 s (a, b, c, d, e and f, respectively) for an injection pressure of 0.3 p.s.i. Incubation at zero potential during 2 min. Concentration of SULT1A1: 8.32 U/μl, concentration of pNPS: 115 μM and concentration of pNP: 20 μM. CE conditions: 150 mM HEPES (pH 6.5) with 20 mM sodium cholate; voltage: 10 kV (45 μA); detection at 274 nm.

The injection time of the enzyme plug was increased (injection pressure at 0.3 p.s.i.) from 2 to 7 s and after each run, the amount of pNPS generated during the in-capillary encountering of enzyme and substrate was determined. A linear relationship was found: a correlation coefficient (r) of 0.9984 for the regression equation $y = 285x + 116$ with y the corrected peak area of pNPS and x the injection time of the enzyme plug in seconds.

3.3.5. *Effect of incubation time on the in-capillary SULT1A1 reaction*

According to Michaelis–Menten kinetics, we assume that what is being measured is the initial rate of product formation (v), in such a way that products have not significantly accumulated [35]. Therefore, we expect the amount of product to be linear with time. Fig. 4 shows an overlay of four electropherograms, obtained after in-capillary reaction

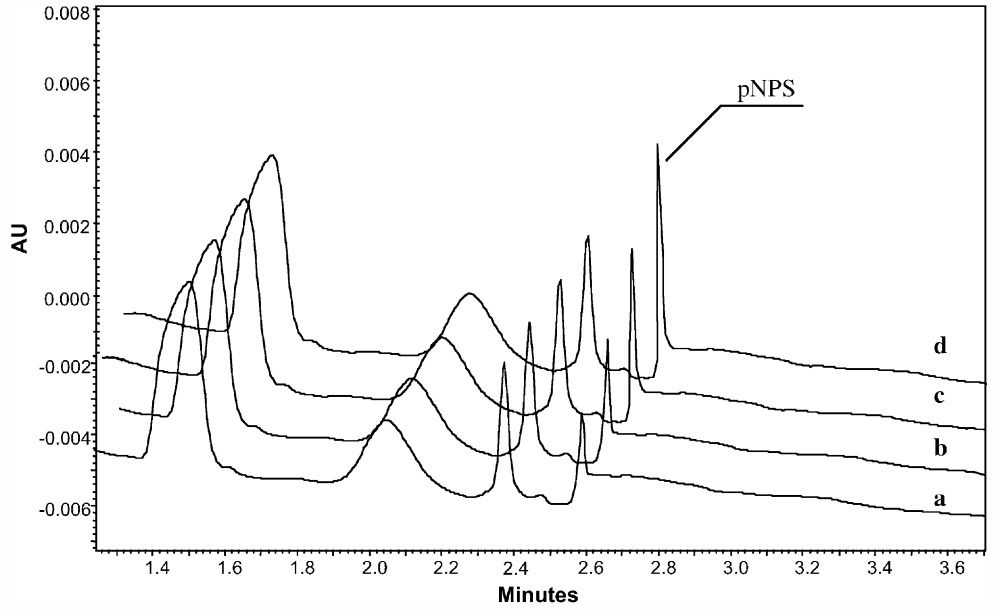

Fig. 4. Overlay of four electropherograms after in-capillary reaction of the SULT1A1 enzyme with increasing in-capillary incubation times of 0.5, 1, 2 and 3 min (a, b, c and d, respectively). Concentration of SULT1A1: 8.32 U/μl, concentration of pNPS: 115 μM and concentration of pNP: 0.25 μM. CE conditions: run buffer: 150 mM HEPES (pH 6.5) with 20 mM sodium cholate, voltage: 10 kV (current 45 μA); detection at 274 nm.

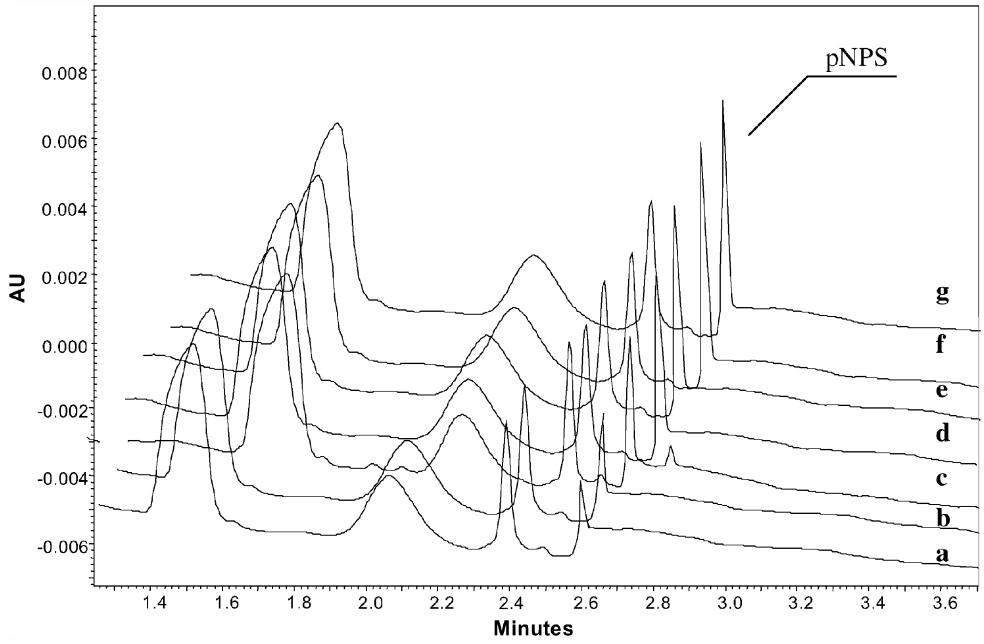

Fig. 5. Overlay of seven electropherograms after in-capillary reaction between SULT1A1 and pNP. Incubation at zero potential during 2 min. Concentration of SULT1A1: 8.32 U/μl, concentration of pNPS: 115 μM and concentration of pNP: 0.05, 0.10, 0.25, 0.50, 1.00, 1.50 and 2.50 μM (a, b, c, d, e, f, g, respectively). CE conditions: see Fig. 5.

during varying times of incubation at zero potential: 0.5, 1, 2 and 3 min.

The corrected peak areas of pNPS were plotted against incubation time and subjected to linear regression analysis. A correlation coefficient (r) of 0.9957 was found, for the regression equation $y = 394x + 275$ with y the corrected peak area of pNPS and x the incubation time in minutes.

3.3.6. Determination of the Michaelis constant

In order to investigate the effect of the pNP concentration on the activity of SULT1A1, seven different pNP concentrations were used, ranging from 0.05 μM to 2.50 μM. The SULT1A1 solution contained 8.32 U/μl and a PAPS solution of 115 μM was used. Fig. 5 shows an overlay of seven electropherograms, in which the substrate concentration was varied from run to run.

The Michaelis–Menten plot that corresponds to the electropherograms in Fig. 4 is shown in Fig. 6A. This line is a fit of the data to the Michaelis–Menten equation. The insert (Fig. 6B) shows the enzyme activity over a substrate concentration range from 0.05 up to 10 μM. Clearly, above a pNP concentration of 2.5 μM, substrate inhibition occurs. This is in agreement with literature findings [8].

The Michaelis–Menten constant for the pNP sulfation was computed from the Lineweaver–Burk plot or the double reciprocal plot. The K_m value for SULT1A1 (8.32 U/μl) was

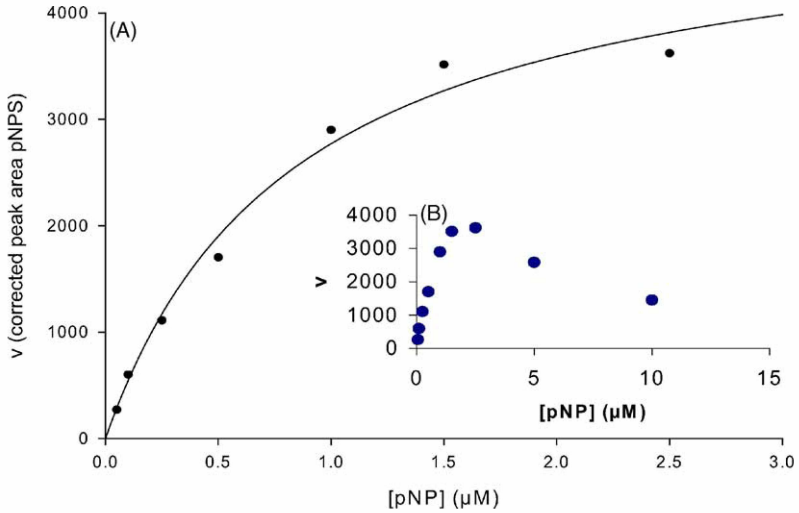

Fig. 6. (A) The Michaelis–Menten plot for the sulfonation of pNP by SULT1A1. The pNP concentration was varied between 0.05 μM and 2.50 μM. Concentration of SULT1A1: 8.32 U/μl and concentration of pNPS: 115 μM. (B) Plot identical to (A) except for two extra pNP concentrations.

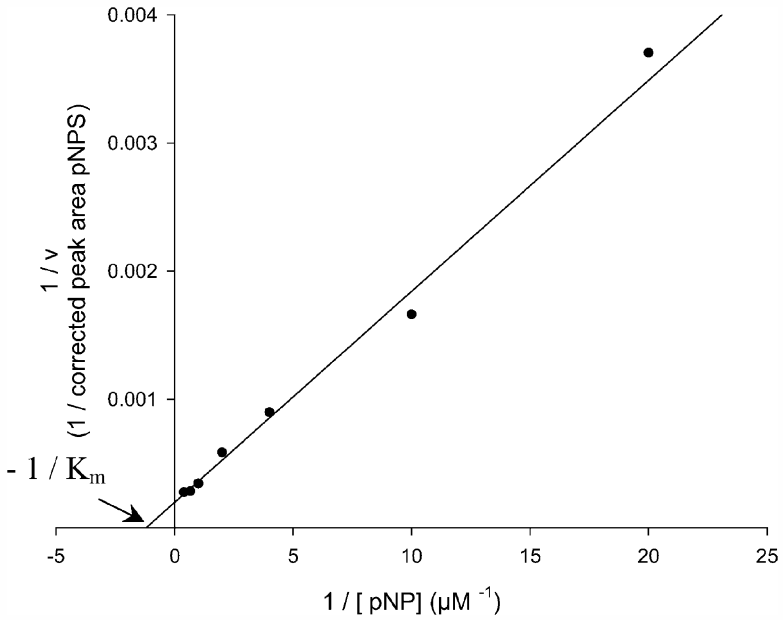

Fig. 7. Lineweaver–Burk plot for a SULT1A1 concentration of 8.32 U/μl. The pNP concentration was varied between 0.05 μM and 2.50 μM, and concentration of pNPS: 115 μM.

determined to be 0.84 ± 0.04 μM. This value corresponds to literature values for the SULT1A1 isoenzyme. Gamage et al. reported a K_m value of 1.0 ± 0.2 μM for the recombinant SULT1A1 enzyme, determined with the substrate pNP [8]. The computed Lineweaver Burk plot of these data is shown in Fig. 7.

4. Conclusion

A new method based on electrophoretically mediated microanalysis (EMMA) was developed for SULT1A1 assay. The kinetic study yielded a K_m value of 0.84 ± 0.04 μM, a value consistent with literature findings. The strong substrate inhibition observed (above a 4-nitrophenol concentration of 2.5 μM) is also in accordance with literature values. Due to the small path length, concentrations in the nanomolar range or lower cannot be determined with CE–UV. In this plug–plug EMMA method, the electrophoresis process prior to the in-capillary contact of enzyme and substrate permitted the preseparation of the inhibitor PAP from the sulfate donor PAPS. Compared to spectrophotometric assays, the EMMA method is rapid, automated and requires only small amounts of the expensive reagents (nanoliter injection volumes). Moreover, the EMMA assay does not need expensive radiolabeled compounds and their disposal.

Acknowledgements

This work was supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT) Grant No. 993194 and partially supported by grant No. 203/03/1125 from the Grant Agency of the Czech Republic. Dr. R. Gilissen (ADMETox Department of Johnson and Johnson Pharmaceutical Research and Development, Beerse, Belgium) is acknowledged for fruitful discussions.

References

[1] R. Weinshilboum, D. Otternes, in: F.C. Kaufmann (Ed.), Handbook of Experimental Pharmacology: Conjugation–Deconjugation Reactions in Drug Metabolism and Toxicity, Springer, Berlin 1994, pp. 45–78.
[2] R.M. Weinshilboum, D.M. Otternes, I.A. Aksoy, T.C. Wood, C. Her, R.B. Raftogianis, FASEB J. 11 (1997) 3.
[3] M. Negishi, L.G. Pedersen, E. Petrochenko, S. Shevtsov, A. Gorokhov, Y. Kakuta, L.C. Pedersen, Arch. Biochem. Biophys. 390 (2001) 149.
[4] C. Tsoi, R. Morgenstern, S. Swedmark, Arch. Biochem. Biophys. 401 (2002) 125.
[5] W.B. Jacoby, D.M. Zeigler, J. Biol. Chem. 265 (1990) 20715.
[6] M.V.H. Coughtrie, S. Sharp, K. Maxwell, N.P. Innes, Chem. Biol. Interact. 109 (1998) 3.
[7] M.V.H. Coughtrie, L.E. Johnston, Drug. Metabol. Disp. 29 (2001) 522.
[8] N.U. Gamage, R.G. Duggleby, A.C. Barnett, M. Tresillian, C.F. Latham, N.E. Liyou, M.E. McManus, J.L. Martin, J. Biol. Chem. 278 (2003) 7655.
[9] S.S. Rens-Domiano, J.A. Roth, J. Neurochem. 48 (1987) 1411.
[10] C.J. Marcus, R.D. Sekura, W.B. Jakoby, Anal. Biochem. 107 (1980) 296.
[11] E. Lin, Y. Yang, Anal. Biochem. 264 (1998) 111.
[12] R.T. Borchardt, C.S. Schasteen, Biochim. Biophys. Acta 708 (1982) 272.
[13] L.T. Frame, S. Ozawa, S.A. Nowell, H. Chou, R.R. Delongchamp, D.R. Doerge, N.P. Lang, F.F. Kadlubar, Drug. Metabol. Disp. 28 (2000) 1063.
[14] M.D. Burkart, C. Wong, Anal. Biochem. 274 (1999) 131.
[15] E.C.A. To, P.G. Wells, J. Chromatogr. 301 (1984) 282.
[16] T. Honkasalo, E. Nissinen, J. Chromatogr. 424 (1988) 136.

[17] R.J. Anderson, R.M. Weinshilboum, Clin. Chim. Acta 103 (1980) 79.
[18] S.G. Ramaswamy, W.B. Jakoby, Methods Enzymol. 143 (1987) 201.
[19] M.K. Sim, T.P. Hsu, J. Pharmacol. Methods 24 (1990) 157.
[20] J. Bao, F.E. Regnier, J. Chromatogr. 608 (1992) 217.
[21] J.J. Bao, J.M. Fujima, N.D. Danielson, J. Chromatogr. B 699 (1997) 481.
[22] A.R. Whisnant, S.D. Gilman, Anal. Biochem. 307 (2002) 226.
[23] Y. Kanie, O. Kanie, Electrophoresis 24 (2003) 1111.
[24] Y. Xu, X. Liu, M.P.C. Ip, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 2781.
[25] K.J. Miller, I. Leesong, J. Bao, F.E. Regnier, F.E. Lytle, Anal. Chem. 65 (1993) 3267.
[26] D. Wu, F.E. Regnier, Anal. Chem. 65 (1993) 2029.
[27] T. Watanabe, A. Yamamoto, S. Nagai, S. Terabe, Electrophoresis 19 (1998) 2331.
[28] J. Saevels, A. Van Schepdael, J. Hoogmartens, Electrophoresis 17 (1996) 1222.
[29] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 22 (2001) 1436.
[30] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 23 (2002) 2854.
[31] S. Nováková, Z. Glatz, Electrophoresis 23 (2002) 1063.
[32] J. Saevels, K. Van den Steen, A. Van Schepdael, J. Hoogmartens, J. Chromatogr. A 745 (1996) 293.
[33] A.R. Whisnant, S.E. Johnston, S.D. Gilman, Electrophoresis 21 (2000) 1341.
[34] S. Nováková, M. Telnarová, Z. Glatz, J. Chromatogr. A 990 (2003) 189.
[35] A. Fersht, Structure and Mechanism in Protein Science, W.H. Freeman & Co., New York 1999, p. 104.

CHAPTER 4

ANGIOTENSIN CONVERTING ENZYME

4.1 Inhibition Study of Angiotensin Converting Enzyme by Capillary Electrophoresis after Enzymatic Reaction at Capillary Inlet

VAN DYCK S., NOVÁKOVÁ S., VAN SCHEPDAEL A., HOOGMARTENS J. (2003) **J. Chromatogr. A**, 1013, 149-156.

Angiotensin converting enzyme.pdf

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JOURNAL OF CHROMATOGRAPHY A
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Inhibition study of angiotensin converting enzyme by capillary electrophoresis after enzymatic reaction at capillary inlet

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Abstract

Capillary electrophoresis was used to study the inhibition of angiotensin-converting enzyme (ACE) by different inhibitors. Reaction occurred at the capillary inlet during a predetermined waiting period, followed by the electrophoretic separation of the reaction compounds. ACE activity was determined by the quantification of the reaction product, hippuric acid, at 230 nm. The technique was used to study the potency of five different inhibitors (captopril, lisinopril, perindoprilat, quinaprilat and benazeprilat). During a kinetic study, the K_i value of captopril was estimated to be 55.4±8.8 n*M*, a value consistent with previously reported values.

Keywords: Kinetic studies; At-inlet technique; Angiotensin-converting enzyme; Enzymes; Enzyme inhibitors; Hippuric acid

1. Introduction

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a relatively non-selective dipeptidyl carboxypeptidase that accepts various substrates, including angiotensin I and bradykinin. ACE plays an important role in the regulation of blood pressure by converting angiotensin I into the vasoconstrictor angiotensin II and also by inactivating the vasodilator bradykinin. The enzyme is found principally on the luminal surface of endothelial cells in contact with the blood, but it is also expressed at lower levels in other cell types [1].

An interest exists in ACE both as a drug target and regarding its role in drug metabolism interactions. Early mechanism studies on angiotensin I conversion and bradykinin hydrolysis, led to the isolation (from snake venom) and the synthesis of small peptide inhibitors. Based on this work and on the modeling studies of carboxy-peptidase active sites, orally active small-molecule inhibitors were then synthesized, such as captopril and lisinopril [1]. These compounds proved to be highly successful in the treatment of hypertension and related target-organ damage, including heart failure and renal insufficiency. Up to now, 17 ACE inhibitors have been developed for clinical use; they are structurally heterogeneous compounds with different pharmacokinetic properties [1]. Furthermore, the serum ACE activity level is a well-established marker for the diagnosis of pathologies such as sarcoidosis, in which increased serum ACE activity has been reported [2].

Due to its importance, many methods have been developed for the determination of ACE activity,

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00575-2

such as spectrophotometric assays, radioisotopic and fluorimetric methods [3–5].

In spectrophotometric methods for determination of ACE activity, the synthetic tripeptide substrate, hippuryl-L-histidyl-L-leucine (HHL), has been used most widely [2]. ACE releases L-histidyl–L-leucine (HL) and hippuric acid (HA) from this substrate, the latter one can be easily detected by UV at 228 nm:

$$\text{hippuryl-histidyl-leucine} \xrightarrow{\text{ACE}} \text{histidyl-leucine} + \text{hippuric acid} \quad (1)$$

HPLC-based [6,7] and CE-based [8,9] assays were introduced, since the hippuric acid released by the enzymatic reaction cannot be completely separated from the substrate by solvent extraction.

Most of these methods have drawbacks such as the consumption of a relatively high amount of enzyme and the lack of automation. To overcome these limitations, a new CE based method has been developed, in which enzyme and substrate react at the capillary inlet part prior to the electrophoretic separation of the reaction compounds [10]. ACE activity towards the tripeptide substrate HHL was determined by CE with spectrophotometric quantification of the reaction product, hippuric acid, at 230 nm. By using this miniaturized method, a Michaelis–Menten constant was determined for the ACE-assisted cleavage of HHL.

CE is a powerful and relatively new analytical tool, characterized mainly by high resolution separations, short analysis times and low sample load. In general, separations applied to biochemical systems are well suited to miniaturization because samples typically have low volumes.

In this work, we performed a kinetic study of the inhibitor captopril and we investigated the relative inhibitory potency of five different ACE inhibitors (i.e. captopril, lisinopril, perindoprilat, quinaprilat and benazeprilat) by means of the previously developed in-capillary system.

2. Materials and methods

2.1. CE instrumentation

All experiments were carried out on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA). On-line detection was performed at 230 nm with a diode array detector. Data collection and peak area analysis were performed by 32 Karat software (version 5.0). Calculation of inhibition constants was done by means of SigmaPlot 2001 software (version 7.101). Uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 31.2 cm (21 cm from the injection side to the detector)×75 μm I.D. were used. The capillary was thermostated by liquid cooling at 37 °C unless specified otherwise.

2.2. Materials and reagents

ACE from rabbit lung, HHL and captopril were purchased from Sigma–Aldrich (Steinheim, Germany). Lisinopril dihydrate was donated by Merck Sharp and Dohme (Brussels, Belgium) and AstraZeneca (Brussels, Belgium). Perindoprilat was synthesized by Servier (Paris, France). Quinaprilat was a gift from Pfizer (Brussels, Belgium) and benazeprilat was donated by Novartis (Basel, Switzerland). Solutions of ACE, HHL and the inhibitors (captopril, lisinopril, perindoprilat, benazeprilat and quinaprilat) were prepared in a 10-m*M* HEPES buffer {2-[4-(2-hydroxyethyl)-1-piperazine]ethane sulfonic acid, Sigma–Aldrich} adjusted with 1 *M* NaOH (BDH Laboratory Supplies, Poole, UK) to pH 8.0 at 37 °C, that contained 150 m*M* NaCl (BDH). All solutions were prepared with Milli-Q water (Millipore, Milford, MA, USA) and filtered through 0.2-μm nylon filters (Alltech, Lokeren, Belgium).

2.3. At-inlet reaction

The CE run buffer consisted of 150 m*M* HEPES adjusted with 1 *M* NaOH to pH 8.0 at 37 °C. Before use, a new capillary was treated with 0.1 *M* NaOH for 2 h. Prior to analysis, the capillary was conditioned by a wash cycle at 20 p.s.i. starting with 0.1 *M* NaOH for 5 min, followed by a 2-min rinse with Milli-Q water and a 10 min rinse with run buffer (1 p.s.i. = 6894.76 Pa). After each analysis of reaction mixture the capillary was rinsed with 0.1 *M* NaOH, water and run buffer for 1, 1 and 3 min, respectively.

The enzyme solution and the substrate solution, with or without inhibitor, were introduced into the

inlet part of the capillary by a sandwich injection mode, i.e. enzyme solution (0.3 p.s.i., 5 s)–substrate solution (0.3 p.s.i., 5 s)–enzyme solution (0.3 p.s.i., 5 s). After each injection step of substrate or enzyme, the capillary ends (and electrodes) were dipped into water in order to prevent sample carry over. Before and after the sandwich injection mode a small plug of water was injected hydrodynamically (0.1 p.s.i., 5 s). The consecutively injected plugs were allowed to stand during a predetermined waiting period and then a voltage of 6 kV was applied. The mixing of substrate and a specific inhibitor was performed immediately before the measurements to prevent any reaction. ACE activity (or the extent of reaction) was determined by the corrected peak area, i.e. the peak area divided by the migration time, of the product HA. As peak areas have no physical units, the reaction velocities are scaled arbitrarily.

3. Results and discussion

3.1. Enzyme inhibition by CE

Capillary electrophoretic systems have been successfully applied for in-line enzymatic reactions by a methodology known as electrophoretically mediated microanalysis (EMMA), firstly described by Bao and Regnier [11]. EMMA utilizes the different electrophoretic mobilities of enzyme and substrate to initiate reaction inside the capillary, which is used as the reaction vessel. Subsequently, the different reaction compounds are transported to the detector by electrophoresis, where they are individually detected. EMMA methodology has been applied in a number of biochemical systems, including assays of enzyme activity [12–14], determination of Michaelis constants [15–18] and inhibition constants of enzyme inhibitors [19–21].

A related, but different approach has been adopted for the kinetic study of ACE activity towards the peptide substrate HHL [10]. The compounds were introduced successively to the capillary inlet part by hydrodynamic injection steps and the injected plugs were then allowed to stand for an adequate period of time. The compounds reacted inside the capillary, while no voltage was applied. Immediately after the reaction, the compounds were separated by the application of the voltage and analyzed. Unlike in EMMA analysis, the compounds were not electrophoretically mixed prior to reaction in this method.

An identical setup was used in this study, the only modification was the addition of the inhibitors to the substrate solution. Fig. 1 provides a schematic overview of the different injection steps of the at-inlet reaction. In a first step (1) a plug of water (W) is injected on the capillary. Subsequently, a plug of enzyme (E) solution, a plug of substrate (S) solution (with or without inhibitor) and a plug of enzyme (E) solution are injected hydrodynamically on the capillary (2). Finally, a plug of water (W) is injected on the capillary (3). The plugs are then allowed to react while the capillary end is dipped into the run buffer. After reaction, a voltage of 6 kV is applied and the reaction compounds are swept towards the detector end of the capillary.

A typical electropherogram of the enzymatic reaction is shown in Fig. 2A, in which an ACE solution of 0.42 U/ml and a HHL (substrate) concentration of 4.92 mM were used. This substrate concentration is about 4.5 times the K_M (Michaelis–Menten constant), higher concentrations cause substrate inhibition. The inhibitory activity of captopril on the ACE induced cleavage of HHL can be seen from the electropherogram measured under identical

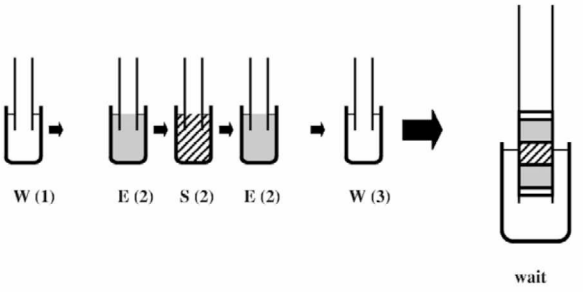

Fig. 1. Schematic illustration of the introduction of the different plugs to the inlet of a capillary: a plug of water is introduced at 0.1 p.s.i. (5 s) before (1) and after (3) the sandwich injection of enzyme and substrate; (2) sandwich injection of enzyme and substrate (3×0.3 p.s.i. during 5 s); the consecutively injected plugs are then allowed to stand during a predetermined waiting period while the capillary end is dipped in run buffer; subsequently voltage is applied and the reaction compounds are transported to the detector. □ water (W); ▭ enzyme solution (E); ▨ substrate solution with our without inhibitor (S).

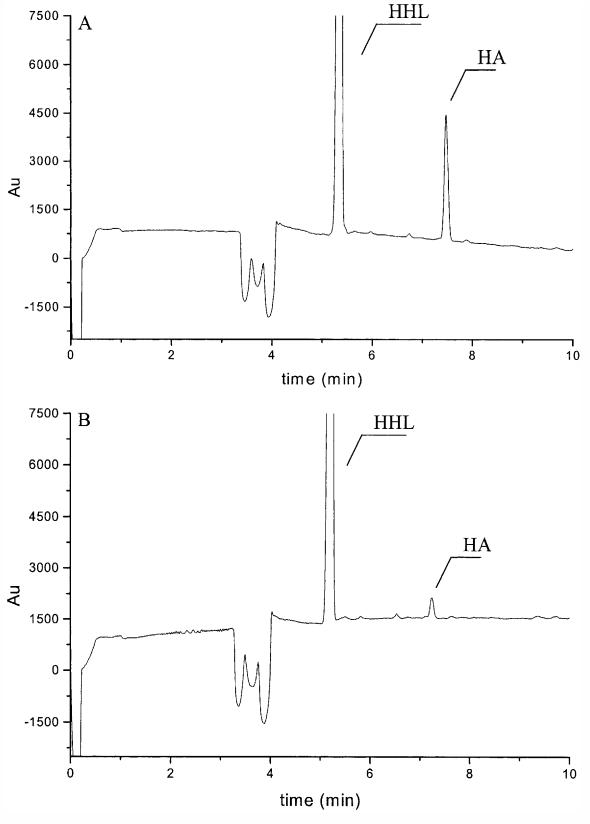

Fig. 2. Typical electropherogram obtained after on-line reaction at the capillary inlet, without (A) and with (B) the inhibitor captopril added to the substrate plug. The concentration of ACE: 0.42 U/ml, HHL: 4.92 m*M* and captopril: 800 n*M*. Waiting period of 0 min. CE conditions: run buffer: 150 m*M* HEPES (pH 8.0); applied voltage: 6 kV; current: 76 μA; detection at 230 nm, capillary cartridge temperature: 37 °C.

conditions, but with the addition of 800 n*M* of captopril to the substrate solution (Fig. 2B).

3.2. Apparent mobility of the ACE inhibitors

Since the velocity of the enzymatic reaction is measured by the peak area of the formed HA, it is important that no peaks are located underneath the product peak at 230 nm. The inhibitors are acids, as well as the reaction product HA. The apparent mobility of HA was determined to be $2.162 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$ in the 150 m*M* HEPES (pH 8.0; 37 °C) background electrolyte. As shown in Table 1, all the inhibitors had different apparent mobilities and migrated faster than the product HA. The apparent mobility was calculated from the migration time of the compound, taking into account the capillary length and the applied voltage.

Table 1
Apparent mobilities and percent of inhibition for the different inhibitors

Inhibitor	Apparent mobility ($cm^2\ V^{-1}\ s^{-1}$)	Percent of inhibition (%)
Perindoprilat	$2.389 \cdot 10^{-4}$	44.2
Lisinopril	$3.390 \cdot 10^{-4}$	42.6
Benazeprilat	$2.287 \cdot 10^{-4}$	42.5
Quinaprilat	$2.391 \cdot 10^{-4}$	41.0
Captopril	$2.473 \cdot 10^{-4}$	35.9

Mobility determined at a concentration of 1 mg/ml.
Inhibition (%) is the average value for three determinations at a $5 \cdot 10^{-8}$ *M* concentration.

3.3. Stability of captopril

In aqueous solution captopril undergoes an oxygen facilitated oxidation at its thiol group to yield captopril disulfide. The degradation of captopril could be followed in our system, since the inhibitory activity of the compound decreased over time which led to an increase in product formation. Fig. 3 shows the amount of HA determined after in-capillary reaction in the presence of 50 n*M* of captopril, measured at time intervals of 24 h. As can be seen in Fig. 3, the amount of HA increased by 73% over the 4-day stability experiment.

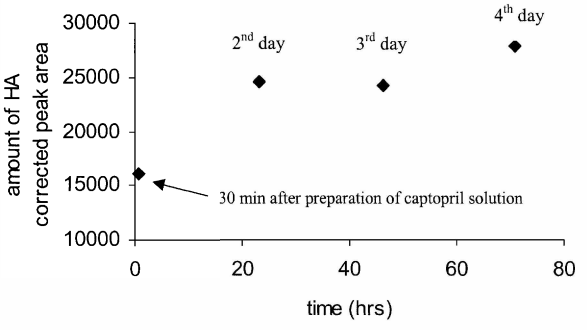

Fig. 3. The amount of HA after at-inlet reaction between ACE (0.42 U/ml) and HHL (5 m*M*) in the presence of captopril (50 n*M*), determined at regular time periods of 24 h after the preparation of the captopril solution. Waiting period of 1 min. CE conditions: see Fig. 2.

Captopril is optimally stable below pH 3.5, but this is not an ideal pH for the ACE enzymatic activity. The addition of water-soluble antioxidants can affect the oxidation mechanism, but we chose not to incorporate extra compounds in the reaction mixture except for the inhibitors. Therefore, captopril solutions were freshly prepared and analysis was always done 30 min after the preparation.

3.4. Inhibition curve of captopril

To verify that the developed method can generate comparable results to those obtained with other methods, an enzyme inhibition study was performed with the inhibitor captopril. Six different captopril concentrations were tested: 0, 1.26, 5.07, 25.3, 792 n*M* and 39.6 μ*M* and the amount of HA was determined after in-capillary reaction. The percentage of inhibition was determined according to the following equation:

$$\% = 100 - \left(\frac{x}{\text{blank}} \cdot 100\right) \quad (2)$$

where x represents the amount of HA determined at a given concentration of captopril and the blank or negative control value is the amount of HA determined without captopril being present (concentration of 0 n*M*). In Fig. 4, the percentage of inhibition (right y-axis: solid line) is expressed against the captopril concentration (μ*M*). The product curve (left y-axis: dotted line) represents the amount of product determined after in-capillary reaction between ACE (0.42 U/ml) and HHL (2.50 m*M*) in the presence of captopril. At the highest captopril concentration of 39.6 μ*M*, a 92.6% inhibition was obtained. This means that complete inhibition was not reached, even at high captopril concentrations. A possible explanation is the fact that the enzyme was not preincubated with the inhibitor, prior to the injection on the capillary and the start of the reaction. Another reason might be the dilution factor, which originates from the mixing process of the plugs during the at-inlet reaction [10]. The measured IC_{50} (concentration of compound at which the reaction was inhibited by 50%) with the in-capillary assay was approximately 0.33 μ*M*. This

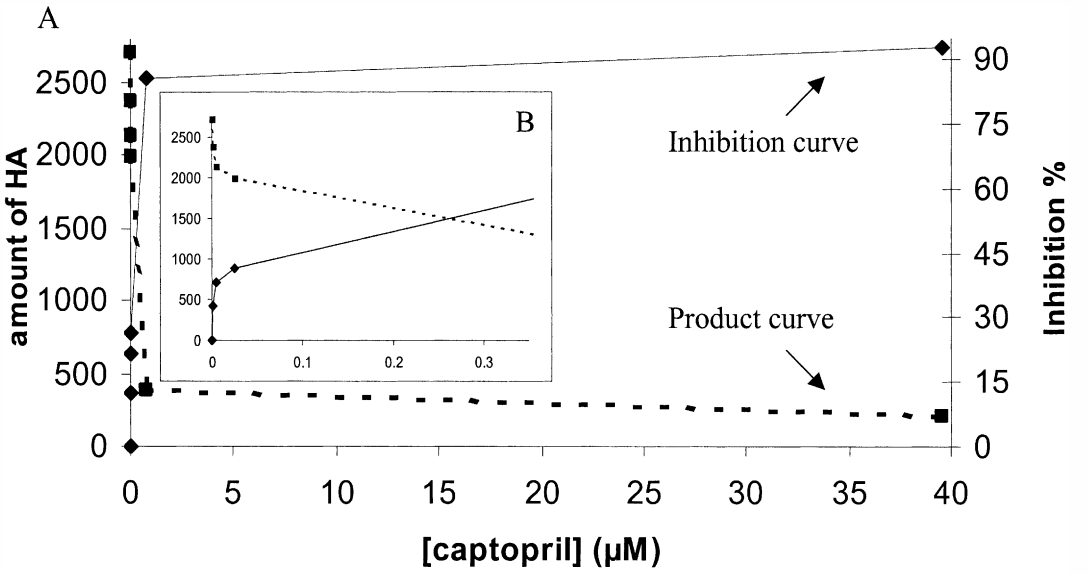

Fig. 4. (A) Left y-axis, product curve (dotted line), the amount of HA determined after at-inlet reaction between ACE (0.42 U/ml) and HHL (2.50 m*M*), when the captopril concentration was varied between 0 and 39.6 μ*M*. The extent of the reaction was determined by the corrected peak area of HA. Waiting period of 0 min. The right y-axis (solid line) represents the percentage of inhibition (%) for each captopril concentration. CE conditions: see Fig. 2. The insert (B) shows an enlarged view of the lower concentration range.

determined value is similar to literature values where IC_{50} values for captopril range from 2.27 nM to 0.58 μM [22–24].

3.5. K_i determination of captopril

We assumed a competitive mechanism for the inhibitor captopril [23] in order to estimate the enzyme inhibition constant or the K_i value of this compound. The Michaelis–Menten plots for the enzymatic cleavage of HHL at different concentrations of the inhibitor captopril (0, 50, and 100 nM) are given in Fig. 5A. Each concentration point was analyzed in triplicate. From these plots, the Lineweaver–Burk plots (double reciprocal plots) were constructed (shown in Fig. 5B). From these data, a K_i value for captopril of 55.4±8.8 nM could be computed. Depending on the nature of the assay and on the origin of the enzyme, reported values range from 0.33 to 72 nM [23,25,26], which means that the described method can estimate the K_i value of a given inhibitor.

Furthermore, this K_i value corresponds to the calculated value of 62.1 nM, derived from the IC_{50} value of the inhibition curve (see Section 3.4), calculated by Cheng and Prusoff's equation [27] giving the relationship between K_i and the concentration of inhibitor for 50% inhibition (IC_{50}):

$$IC_{50} = K_i(1 + [S]/K_M) \quad (3)$$

The K_M value of 1.16 mM for ACE with HHL as a substrate was determined previously [10] for three different enzyme concentrations (0.42, 0.33 and 0.25 U/ml), while a HHL concentration of 5.0 mM was used.

When the different concentrations of captopril were investigated, unpredictable migration time shifts and even current breakdown were sometimes seen. We do not have a straightforward explanation: the reactive thiol group of the inhibitor might react with the capillary wall or with other compounds of the reaction mixture.

3.6. Comparison of different inhibitors

To determine the inhibitory potency of the different ACE inhibitors with the developed at-inlet method, we compared the effect of captopril, lisinopril, quinaprilat, benazeprilat and perindoprilat, all at a concentration of $5 \cdot 10^{-8}$ M. None of the inhibitors were preincubated with the enzyme; the reaction was initiated by the consecutive injection steps of the enzyme and substrate solution on the capillary. The ACE concentration used was 0.42 U/ml and HHL was used at 5.00 mM. Table 1 shows the percentage of inhibition at a $5 \cdot 10^{-8}$ M concentration of inhibitor on the ACE induced cleavage of HHL, compared to a blank solution (substrate without inhibitor). Each analysis was performed in triplicate. Captopril showed to be less potent (36% inhibition at a concentration of $5 \cdot 10^{-8}$ M) than the other inhibitors, while perindoprilat (44% inhibition) showed to be the most potent inhibitor. These findings are in agreement with literature, where perindoprilat is known to be a very potent inhibitor (more potent than lisinopril and captopril) [28] and lisinopril is known to be more potent than captopril [30]. In other studies, however, quinaprilat is more potent than benazeprilat and lisinopril [29] and this was not found in our experiments, although the difference in inhibition at $5 \cdot 10^{-8}$ M was not very high.

Nevertheless, the difference in inhibitory potency of the five inhibitors determined after in-capillary reaction, implies that this method can be used for preliminary ACE inhibitor screening.

4. Conclusion

The inhibition of angiotensin-converting enzyme with different inhibitors was studied by means of CE. Reaction occurred at the capillary inlet between the enzyme and its substrate HHL in the presence of an ACE inhibitor. ACE activity was determined spectrophotometrically by the quantitation of the product HA after an electrophoretic separation of the reaction compounds. The method was not only used to compare inhibitory potencies of different inhibitors but also to estimate the K_i constant of captopril. The K_i of 55.4 nM determined for this inhibitor is in agreement with literature values. Since the capillary is used as a micro vessel in this technique, all the necessary steps (reaction, separation and quantitation) are combined in one fully automated and miniaturized assay. The obtained results can contribute to further applications of this

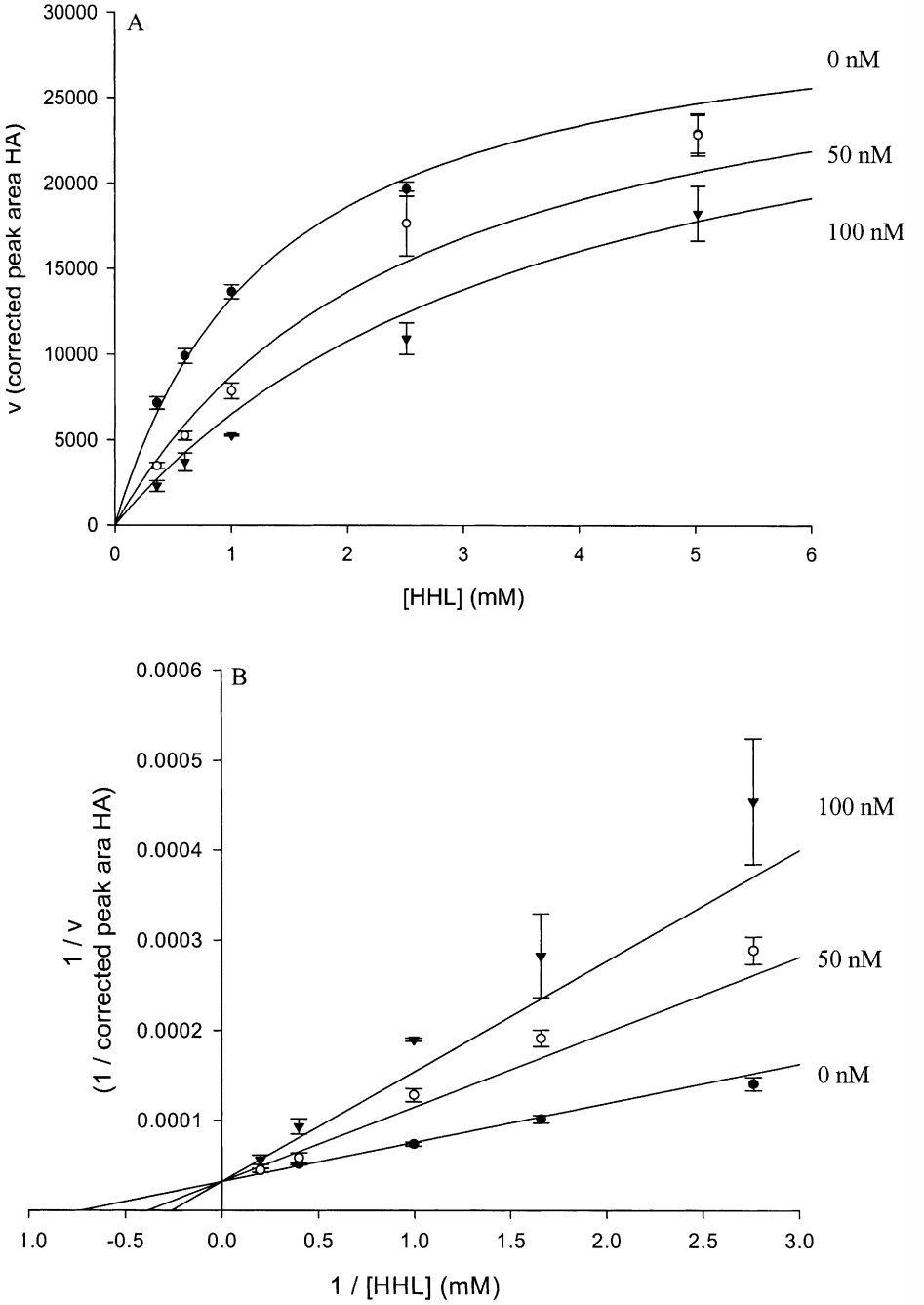

Fig. 5. (A) The Michaelis–Menten plots for the enzymatic reaction of ACE inhibited by (●) 0 nM; (○) 50 nM; and (▼) 100 n*M* captopril. The HHL concentration was varied between 0.361 and 5.02 m*M*. Each concentration point was analysed in triplicate. In-capillary reaction at capillary inlet, waiting period of 0 min. CE conditions: see Fig. 2. (B) The corresponding Lineweaver–Burk plots of ACE inhibition by captopril: (●) 0 nM; (○) 50 nM; and (▼) 100 nM.

approach as in the areas of drug development and inhibitor screening.

Acknowledgements

SVD is a recipient of a fellowship from the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT), grant No. IWT/SB/993194. The authors acknowledge with thanks the companies Merck, AstraZeneca, Servier, Pfizer and Novartis for the donation of the inhibitory compounds.

References

[1] M.A. Zaman, S. Oparil, D.A. Calhoun, Nat. Rev. Drug Discov. 1 (2002) 621.
[2] B. Bénéteau-Burnat, B. Baudin, Crit. Rev. Clin. Lab. Sci. 28 (1991) 337.
[3] D.W. Cushman, H.S. Cheung, Biochem. Pharmacol. 20 (1971) 1637.
[4] J.W. Ryan, A. Chung, C. Ammons, M.L. Carlton, Biochem. J. 167 (1977) 501.
[5] J. Friedland, E. Silverstein, Am. J. Clin. Pathol. 66 (1976) 416.
[6] S.G. Chiknas, Clin. Chem. 25 (1979) 1259.
[7] Q.C. Meng, E. Balcells, L. Dell'italia, J. Durand, S. Oparil, Biochem. Pharmacol. 50 (1995) 1445.
[8] Z.K. Shihabi, J. Chromatogr. A 853 (1999) 185.
[9] R.-Z. Zang, X.-H. Xu, T.-B. Chen, L. Li, P.-F. Rao, Anal. Biochem. 280 (2000) 286.
[10] S. Van Dyck, S. Vissers, A. Van Schepdael, J. Hoogmartens, J. Chromatogr. A 986 (2003) 303.
[11] J. Bao, F.E. Regnier, J. Chromatogr. 608 (1992) 217.
[12] K.J. Miller, I.K. Leesong, J.M. Bao, F.E. Regnier, F.E. Lytle, Anal. Chem. 65 (1993) 3267.
[13] D. Wu, F.E. Regnier, M.C. Linhares, J. Chromatogr. B 657 (1994) 357.
[14] T. Watanabe, A. Yamamoto, S. Nagai, S. Terabe, Electrophoresis 19 (1998) 2331.
[15] J. Saevels, A. Van Schepdael, J. Hoogmartens, Electrophoresis 17 (1996) 1222.
[16] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 22 (2001) 1436.
[17] S. Nováková, Z. Glatz, Electrophoresis 23 (2002) 1063.
[18] S. Van Dyck, A. Van Schepdael, J. Hoogmartens, Electrophoresis 23 (2002) 2854.
[19] J. Saevels, K. Van den Steen, A. Van Schepdael, J. Hoogmartens, J. Chromatogr. A 745 (1996) 293.
[20] A.R. Whisnant, S.E. Johnston, S.D. Gilman, Electrophoresis 21 (2000) 1341.
[21] S. Nováková, M. Telnárová, Z. Glatz, J. Chromatogr. A 990 (2003) 189.
[22] R.D. Carr, L. Higgs, P.G. Killingback, A.K. Nicol, S.E. O'Connor, A. Robson, E. Wells, W.T. Simpson, Br. J. Pharmacol. 100 (1990) 83.
[23] E.G.P. Udupa, N.M. Rao, Biochem. Mol. Biol. Int. 43 (1997) 1063.
[24] Y. Inada, Z. Terashita, Y. Imura, M. Tanabe, K. Nishikawa, S. Kikuchi, Jpn. J. Pharmacol. 42 (1986) 99.
[25] R. Shapiro, J.F. Riordan, Biochemistry 23 (1984) 5225.
[26] R.G. Almquist, W.R. Chao, M.E. Ellis, H.L. Johnson, J. Med. Chem. 23 (1980) 1392.
[27] Y.Ch. Cheng, W.H. Prusoff, Biochem. Pharmacol. 22 (1973) 3099.
[28] M. Barthelmebs, M. Devissaguet, J.L. Imbs, Clin. Exp. Theor. Pract. A 11 (1989) 471.
[29] D.W. Cushman, F.L. Wang, W.C. Fung, G.J. Grover, C.M. Harvey, R.J. Scalese, S.L. Mitch, J.M. Deforrest, Br. J. Clin. Pharmacol. 28 (1989) 115S.
[30] C.I. Johnston, B. Fabris, H. Yamada, F.A.O. Mendelsohn, R. Cubela, D. Sivell, B. Jackson, J. Hypertens. 7 (1989) S11.

SUMMARY AND CONCLUSIONS

Enzymes are often identified and quantitated by measuring their biological activity, i.e. their catalytic activity. In enzyme assays, the catalyzed conversion of a substrate by a specific enzyme provides information about enzyme activity and reaction kinetics involved. Assays are important tools, not only in fundamental research, but also in routine biochemistry and in clinical diagnosis. Consequently, there is a general demand for miniaturized assay methods that only need minute amounts of expensive reagents, integrated and automated assays that reduce the manipulation and lower the costs, and fast and parallel assays in order to obtain a high sample throughput.

Capillary electrophoresis is a powerful and relatively new analytical tool, characterized by high resolution separations, short analysis times and low sample load. CE methods have been developed in a number of application areas including pharmaceuticals, DNA and chiral compounds. CE can also be used to study enzyme-substrate interactions by technique known as electrophoretically mediated microanalysis (EMMA). In general, enzyme assays require a number of operations such as the mixing of reagents and the initiation of the reaction, the incubation of the reaction mixture, and consequently detection of the reaction product(s). The EMMA methodology couples all these operations in one integrated technique utilizing the different electrophoretic mobilities of enzyme and substrate(s) to initiate reaction inside the capillary and to separate the components of the reaction mixture from each other for the final on-capillary quantification.

Rhodanese was the first enzyme to be studied. The separation conditions were not compatible with the reaction conditions for this enzymatic system. In EMMA, however, a medium is needed in which the compounds migrated differently and in which catalytic activity can take place. This led to the concept of a partially filled capillary consisting of two distinct zones: (a) a zone containing a buffer optimal for the rhodanese reaction and (b) a zone

containing a buffer optimal for the separation of the reaction compounds. This was successful in assaying rhodanese activity and in determining kinetic parameters. The basic limitation of EMMA i.e. the necessity to have conditions compatible with both the reaction and the separation was thus overcome.

The second enzyme studied was phenol sulfotransferase (PST). Firstly, the PST reaction was carried out outside the capillary in an off-line study, but there was no direct relationship between the quantity of PST and the enzyme activity, most probably due to presence of the very strong inhibitor PAP in substrate solution PAPS. Next, a new in-capillary method was developed for PST assay. The electrophoresis process prior to the in-capillary contact of enzyme and substrate permitted the preseparation of the inhibitor PAP from the sulfate donor PAPS.

Finally, CE was used to study inhibition of angiotensin converting enzyme (by different inhibitors). Van Dyck et. al. showed in preliminary experiments that an EMMA based assay yielded extremely divergent product peak areas. Therefore, an at-inlet technique was developed in which the compounds were introduced to the capillary inlet and allowed to stand (and react) in absence of electric field. This work performed a kinetic study of the inhibitor captopril and determined the relative inhibitory potency of five different ACE inhibitors by means of the previously developed at-inlet technique. The difference in inhibitory potency of five ACE inhibitors implies that this methodology can be used for preliminary ACE inhibitor screening.

This dissertation shows that EMMA is a versatile tool for studying enzyme activity. When the conditions required for separation are not compatible with those required for the enzyme activity, a partially filled capillary can be used. Furthermore, MEKC can be combined with in capillary enzyme assay without altering the enzyme activity. This strongly increases the possibilities for developing integrated technique enzyme assay since MEKC can provide added selectivity. Finally, the electrophoresis process in transient engagement EMMA prior to the in-capillary contact of the compounds permits the preseparation of possible interfering components.

The described assay methods are based on a miniaturized and integrated technique, EMMA, which can be characterized by minimal reagent use, the absence of manual procedures and by complete automation. This minimizes the risk of cross contamination and strongly reduces the assay cost. The assays were developed in a single capillary CE instruments and therefore, parallel analysis of multiple samples was not possible. Another drawback is relatively low sensitivity associated with absorbance detection in CE. Therefore, future work should be focused on more sensitive detection modes and on the transfer the developed methods to multiplex CE instrument or to CE microchips. Notwithstanding these shortcomings, this work can be considered to be step forward in the development of more integrated and miniaturized assay technique.