Microchip electrophoresis-electrochemical detection platform for analysis of drugs and their metabolites

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DIPLOMA THESIS

Submitted to The University of Zagreb, Faculty of Pharmacy and Biochemistry

In Zagreb, 2015

This diploma thesis has been reported at the course Pharmaceutical Analysis, at the University of Zagreb, Faculty of Pharmacy and Biochemistry under the supervision of Professor Biljana Nigović Ph.D. The experimental work was carried out in the Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki under the expert guidance of Associate Professor Tiina Sikanen Ph.D.

Hvala profesorici Biljani Nigović na velikoj pomoći u pisanju ovog diplomskog rada i što je s velikim entuzijazmom prihvatila ovaj projekt!

Hvala mami, tati i bratu što su me podržavali bez obzira na sve, prebrodili samnom svaki pad i proslavili svaki uspjeh!

Hvala mojim dragim prijateljima i kolegama. Zbog vas ću pamtiti studentske dane kao najljepše razdoblje u životu!

Special gratitude goes to my supervisor in Helsinki, Docent Tiina Sikanen! Thank you for accepting me in your group and providing me the opportunity to work on this interesting project! Thanks goes to whole CheMiSys group for all the assistance and fun time spent in your lab and beyond!

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1 Introduction

1.1 Metabolic reactions - interactions with drugs and other chemicals

We are in a period of a rapidly growing research-based pharmaceutical industry. The improvement in drug research field and modern high-throughput techniques such as 3D modeling of protein structures, pharmacogenomics and combinatorial chemistry result in a large number of new entities discovered every year[1,2]. When a new molecule is a promising drug candidate, its physiological properties need to be determined as a part of preclinical trials, the first step before extensive clinical trials[3]. Absorption, distribution, metabolism and excretion (ADME) are the psychological properties used for describing the way in which a compound disposes within an organism. Drug concentration in blood depends on its ADME properties which influence pharmacological activity of a compound. Almost two-thirds of new chemical entities will fail in development as a result of poor ADME characteristic[4].
While most of the properties can be predicted early in the discovery phase using *in vitro* techniques and recently developed computational (*in silico*) modeling[5], the determination of metabolic reactions remains the most challenging task.

Hepatic metabolism is the main elimination mechanism for most of the drugs and non-drug xenobiotics. Cytochrome P-450 monooxygenase system (CYP-450) together with conjugation enzymes is responsible for the elimination of more than 90% drugs cleared by the liver[6]. Drugs are metabolised in the human liver by different forms of CYP, such as 1A2, 2A6, 2B6, 2C8, 2C18, 2C9, 2C19, 2D6, 2E1, 3A4 and the result is a reactive molecule further conjugated with endogenous molecules (for example, glutathione)[7]. In the early stage of drug development it is necessary to determine a metabolic profile of a new compound. The questions such as: How fast a molecule will be metabolised; which enzymes are involved; what metabolites will be formed; are there any toxic metabolites; does it have interactions with the existing, clinically relevant drugs, are some of the questions that need to be answered[8].
Furthermore, the existence of multiple enzyme forms ("isoforms") of CYP, the variability among human individuals (polymorphic enzymes), and major interspecies difference makes it difficult to accurately predict the metabolic pathway of a new compound.

Therefore, in order to get a vague idea about the metabolic pathway *in vivo*, a large amount of *in vitro* results is necessary.

Apart from drugs, humans are continuously exposed to compounds such as pesticides and household chemicals (detergents)[9]. These environmental chemicals are widely used, not only in agriculture and public health, but also in individual households. While physiological effects of drugs are widely tested, these chemicals are largely unfamiliar and understudied[10,11]. Humans are exposed through food, water, public places, houses or gardens and connection to chemical exposure continues to strengthen despite the latest efforts to limit the individual exposure. A number of published studies have shown that pesticides are linked to several health problems such as Alzheimer's disease[12], asthma[13], cancer[14,15] and many more[16]. Health hazards of environmental and agricultural chemicals are determined from short-term studies while long-term effects remain unknown or become evident after epidemiological follow-up studies.

The biotransformation of xenobiotics enables higher organisms to eliminate lipophilic substances that might otherwise cause toxic effects by accumulating in their tissues. As well as drugs, they use xenobiotic-metabolizing cytochrome (CYP) enzymes as the primary site for phase I metabolism. As a result, a large number of interactions with clinically relevant drugs is possible. Similar to pharmaceutical drug research, it is important to explore the full metabolic profile of common environmental chemicals to get an idea about their metabolic pathways and possible interactions.

Recently, metabolic profiling of these chemicals has been gaining in importance, however, amount of data today is very limited due to lack of the fast *in vitro* screening assays.

1.2 Selected CYP P450 reactions

Nonsteroidal anti-inflammatory drugs (NSAID) are a class of most prescribed medications worldwide. Since their synthesis in late 1800s, phenacetin and paracetamol have become the most popular non-narcotic analgesic (antipyretic) drugs. By using CYP1A2, phenacetin is cleared through conversion to paracetamol and this reaction is used as a marker for CYP1A2 *in vitro* and *in vivo* reactions.

In addition to phenacetin, another widely used NSAID diclofenac and its conversion to 4'-hydroxydiclofenac has been used for the determination of the enzymatic activity of CYP2C9.

Other drugs metabolized by CYP2C9 include ifosfamide, warfarin, phenytoin, and cyclophosphamide.
Coumarin (1, 2-benzopyrone) is a xenobiotic used in treatment of edema and inflammation of venous and lymphatic vessels[17]. With its metabolite, 7'-hydroxycoumarine (umbelliferone), coumarin is used as marker reaction for CYP2A6 enzymatic activity, especially due to umbelliferone's strong fluorescence properties.

These three reactions were selected for this work since they have been used as common marker reactions for the determination of enzymatic activity in numerous *in vitro* and *in vivo* assays.
Common CYP inducers among commonly used drugs are phenobarbital, phenytoin and rifampin. Inhibitors of CYP enzymes are ketoconazole, diltiazem, verapamil, cyclosporine and many more. With the available knowledge about CYP enzymes and the use of marker reactions for *in vitro* experiments useful predictions about interactions can be made.

1.3 Micro Total Analysis Systems

Since its introduction in the 1990s until today, the area of "Micro Total Analysis System" (μTAS), also called "lab on a chip", has been growing rapidly[18]. Its development started in the early 1980s with progressive development of silicon microfabrication processes and nowadays it is considered to be one of the most promising technologies in modern bioanalysis. The approach combines several functional elements integrated onto a single microfluidic platform, followed by fast analysis and very small amounts of samples. Usually, the analytical process follows a well-defined procedure form sample collecting, sample pretreatment and a long lasting analysis with a conventional analytical instrument. The ability of μTAS to perform injection, separation and detection within the same device has opened new doors in modern analytical chemistry, medicine, pharmacy, cell biology and many more. Besides the low sample volumes, expensive reagents can be saved and the amount of toxic waste minimized.

Microfluidic devices have gained great interest since capillary electrophoresis (CE) microchips were presented[19,20]. Although glass and silicon dominated the early years, polymers such as poly(dimethylsiloxane) (PDMS)[21], poly(methylmethacrylate) (PMMA)[22],

glycidly ether of bisphenol A (SU-8)[23] as substrate materials have been employed due to their chemical and mechanical properties, low cost and ease of fabrication.
Undoubtedly, the integration and compactness of microfluidic devices hold great promise for analytical chemistry and high-speed screening of a large number of new chemical entities.

1.3.1 Microchip Capillary Electrophoresis (MCE)

One especially exciting use for microanalytical devices is in the analysis and separation of chemical and biological substances. The electrophoresis of today may be one of the main technique for separation of both large and small molecules. Despite a number of different types of electrophoresis, they all include the same principle of generating an electric field between two points and migration of ions through matrix in-between. Basic instrumentation is simple. Except for capillary, a controllable high voltage supply is needed and a detector to detect the components of the mixture. Capillary electrophoresis combines a family of techniques with different performing characteristics. These are: capillary gel electrophoresis, capillary zone electrophoresis, isoelectric focusing, isotachophoresis and micellar electrokinetic capillary chromatography.

Capillary electrophoresis (CE) employs narrow capillaries where the electroosmotic flow (EOF) of buffer solutions and ionic species is a fundamental process. The EOF in conventional silica capillaries is generated as a result of ionization of the acidic silanol groups of the inside capillary wall. At high pH groups get dissociated and negatively charged, causing cations from the solution to build up near the surface forming an "electric double layer". The application of high voltage results in cations moving towards cathode, carrying water with them. The result is net flow in the direction of negative electrode which could be considered an "electric pump" of CE[24].

In polymer chips the origin of surface charge is different. Surface charge is a result of the enchanted autolysis of water with the preferential adsorption of hydroxyl ions. In that way, solid, hydrophobic material develops negative charge similar to silica capillary[25].

The properties of separation in capillary are a hybrid between traditional polyacrylamide gel electrophoresis (PAGE) and high performance liquid chromatography (HPLC). It has efficiency on the order of gas chromatography (GC) or higher, it is better for quantification, requires small amounts of samples and reagents, it can be easily automated and uses modern

detector technologies. Additionally, the ability to use higher electric fields in a capillary results in a separation lasting only a few minutes rather than several hours[26].

Capillary electrophoresis is a highly popular analytical system that can be easily integrated on a chip. The Microchip capillary electrophoresis (MCE) provides a number of advantages. In the miniaturized form, a device requires even smaller quantities of reagents and the separation takes 2-3 minutes. The most important aspect lays in the fact that efficiency of on-chip electrophoretic separations is not behind the larger counterparts, but it can also perform highly integrated and more complex analyses. The ultimate microchip platform can be easily transferred to the place where analysis is needed and can be operated with ease, which makes it a great improvement in a term of rapid screening of numerous new biological, environmental and pharmaceutical entities.

1.3.2 Electrochemical detection (EC)

Separation techniques (electrophoresis, chromatography) on a microchip provide many advantages over conventional methods. On the other hand, outcomes of size reduction will occur in demanding detection systems. To detect modest concentrations of samples in microchips, highly sensitive detectors should be constructed. Some of common detection methods include laser-induced fluorescence (LIF) and mass spectrometry (MS), yet the robustness of related instrumentation and high cost analysis are incompatible with μTAS concept. However, low cost, easy operation, compatibility with microfabrication, selectivity and high sensitivity of electrochemical detection (EC) have recently raised attention[27,28].

The amperometric electrochemical detection measures electrical current resulting from oxidation or reduction of species after applying constant voltage to the working electrode. After the zones of analytes are separated inside a column they pass over a planar electrode held at a fixed potential. The potential of a working electrode is slightly higher than analyte potential serving as driving force for redox reaction of the analyte. Current peak, caused by electron transfer, is visible on the amperometry diagram as a function of time and is directly related to the concentration of the compound.

To all amperometric measurements, crucial is Faraday's law of electrolysis:

$$\mathbf{Q = n*F*N}$$

N moles of material are converted under *Q* number of coulombs. *F* is Faraday's constant (96500 C mol^{-1}) and *n* number of moles of electrons gained or lost in process per mole of material.

With respect to time, there is a need for another equation:

$$\mathbf{dQ/dt = n*F*dN/dt = i}$$

This equation represents the measurable quantity, the current released during the redox process occurring on the surface of electrode. Electropherograms after analysis represent the detector response (current) as a function of time.

In microchips, the electrochemical detector can be integrated at the end of the separation channel (*end*-channel detection) or inside it (*in*-channel detection) using microfabrication techniques and metals such as gold, platinum or carbon. Different modifications of electrode surface (coatings) can enhance the power of amperometric detectors[28].

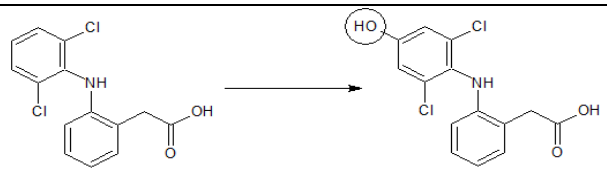
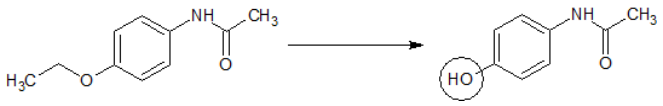
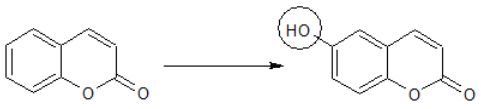
Potentiometry, contact and contactless conductivity are other common detection methods combined with microchip electrophoresis.

2 Aim of the thesis

Progress and development of the current analytical techniques is by far too slow to keep up with continuous discoveries of a large amount of new chemical entities (e.g., drugs, household and environmental chemicals). New, sensitive and primarily fast methods are needed to speed up metabolic characterization (*in vitro* metabolic assays), drug discovery processes and, in general, the screening process of different samples.

In this work, portable microchip capillary electrophoresis (MCE) platform with electrochemical detection (EC) was developed as a system that can improve rapid analysis of the selected CYP P450 substrates and metabolites listed in Table 1.
MCE-EC platform was examined as practical and easily handled device ready to compete with traditional analytical instruments in terms of sensitivity and speed, as well as to offer alternative to massive and expensive instrumentation.

Table 1. List of the important CYP enzymes along with their specific substrates and metabolites.

CYP2C9 Diclofenac 4-hydroxylation	
CYP1A2 Phenacetin O-de-ethylation	
CYP2A6 Coumarin 7-hydroxylation	

In addition to drug metabolism assays, the feasibility of the MCE-EC approach was examined in determination of the dissolved drug concentration following the rapid dissolution of nanoparticle based drug formulations.

3 Materials and methods

3.1 Chemicals

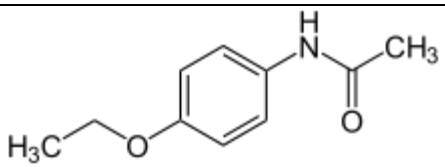
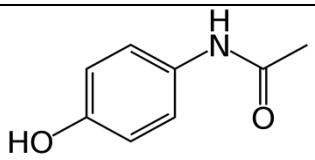
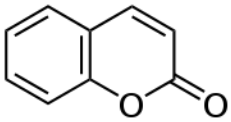
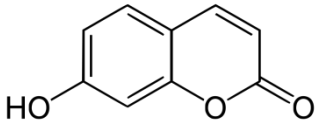
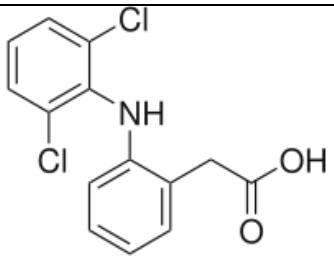
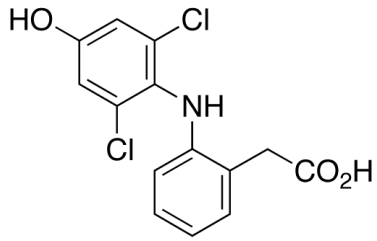
All chemicals used in this work are listed in Table 2.

Table 2. List of chemicals with suppliers and control numbers used in the research.

Chemical	Supplier	CAS
1-octanol	Sigma-Aldrich, Steinheim, Germany	111-87-5
4-hydroxydiclofenac	Toronto Chemicals, Toronto, ON	n/a
Boric acid	Riedel-de Haën, Seelze, Germany	31146
Cloroform	n/a	n/a
Coumarin	Sigma-Aldrich, Steinheim, Germany	91-64-5
Dichloromethane (DCM)	n/a	n/a
Diclofenac	Sigma-Aldrich, Steinheim, Germany	239-346-4
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Steinheim, Germany	67-68-5
Dopamine	Sigma-Aldrich, Steinheim, Germany	200-445-2 (EC)
Hydrochloric acid	Riedel-de Haën, Seelze, Germany	n/a
Indomethacin	Sigma-Aldrich, Steinheim, Germany	n/a
Acid 2-morpholino-ethanesulfonic hydrate (MES hydrate)	Sigma-Aldrich, Steinheim, Germany	1266615-59-1
Methanol	n/a	n/a
MQ water, deionized	Mili-Q water purification system	-
NADPH	Sigma-Aldrich, Steinheim, Germany	2646-71-1
Paracetamol (Acetaminophen)	Sigma-Aldrich, Steinheim, Germany	103-90-2
Phenacetin	Sigma-Aldrich, Steinheim, Germany	n/a
Umbelliferone	Sigma-Aldrich, Steinheim, Germany	202-240-3 (EC)

The molecular structures and pK_a values of the test compounds are listed in Table 3.

Table 3. List of selected substrate and metabolite pairs with pK_a values.

SUBSTRATE	METABOLITE	pK_a
 PHENACETIN	 PARACETAMOL	PHENACETIN **15** (-NH-) PARACETAMOL **9.5** (-OH)
 COUMARIN	 UMBELLIFERONE	COUMARIN no ionizable atoms UMBELLIFERONE **7.8** (-OH)
 DICLOFENAC	 4'-OH-DICLOFENAC	DICLOFENAC **4.0** (-COOH) 4'-OH-DICLOFENAC **8.6** (-COOH) **3.8** (-OH)

3.2 Preparation of stock solutions and buffers

Stock solutions were prepared by direct weighing of the correct amount of solid chemical (phenacetin, paracetamol, umbelliferone, coumarin, diclofenac, 4-hydroxydiclofenac). Compounds were dissolved in DMSO, solutions were protected from light and stored in freezer over the night. The concentration of all the compounds was 10 mM, except phenacetin and coumarin which were prepared in a 20 mM concentration. The run samples were diluted from DMSO stock solutions, except for the MCE separations of the metabolite-substrate pairs and LLE (liquid-liquid extraction). For that purpose stock solutions were prepared in 20 mM sodium borate buffer (pH 10.0) in the same way, by weighing and dissolving solids in buffer. Phenacetin and umbelliferone were prepared in 1 mM concentration due to poor solubility in water. Coumarin was prepared in 5 mM concentration and paracetamol in 10 mM concentration solution. All stock solutions prepared in the buffer (except paracetamol with

very good solubility) were mixed with Vortex machine for longer period and left for at least 15 minutes before analysis.

Sodium borate buffer was prepared by weighing of solid boric acid and dissolving in MQ-water. The pH was adjusted to 10 using 2 M NaOH solution.
MES-NaOH buffer was prepared by weighing of solid MES hydrate and dissolving in MQ-water. The pH was adjusted to 6 using 2 M NaOH solution.

3.3 Instrumentation

Portable and compact analytical instrument for microfluidic and electrophoresis applications purchased from MicruX Technologies (Oviedo, Spain) is shown on Figure 1. The instrument has "HVStat" (HVSTAT2010) with LiPo battery (3300 mAh) as a high voltage (HV) supply with maximum voltage of ±3000 V and a biopotentiostat for dual amperometric detection. Microchips are placed inside the platform (MCE-HOLDER-DC02), for easier use. Holder integrates the electrical contacts for detection and integrated platinum wires as HV electrodes which enable the use of MicruX reusable electrophoresis chips (38 x 13 mm). The system is controlled by MicruX Manager Software v2.1.

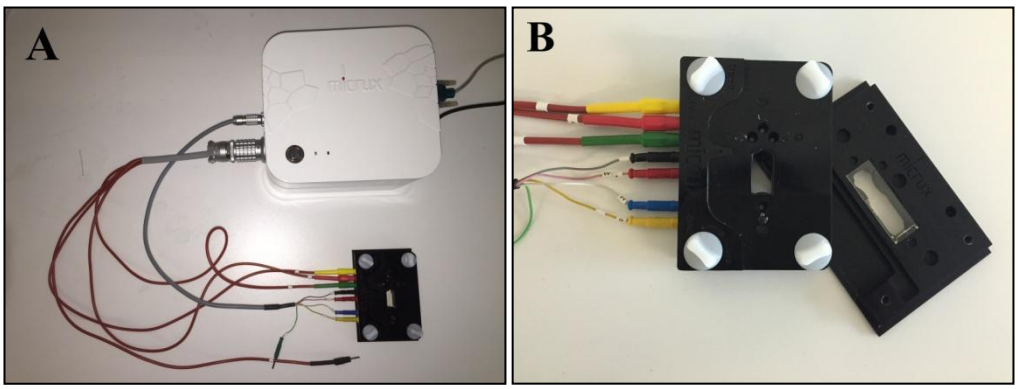


Figure 1. MicruX microchip electrophoresis instrument with EC detection (A); opened microfluidic platform with microchip inside (B).

For this research, single channel SU-8/glass microchips manufactured by MicruX (Oviedo, Spain) were used. 150 nm thin-film electrodes were integrated on the glass cover plate and placed at the end of separation channel. External dimensions of each chip are 38 x 13 x 0.75 mm, which is the size that can fit inside platform (holder). Microchannel cross-section

dimensions are 20 x 50 µm (height x width) and the layout features a simple cross injection geometry (Figure 2). Separation channel length is 35 mm (A → B) and sample injection channel length is 10 mm (C → D).

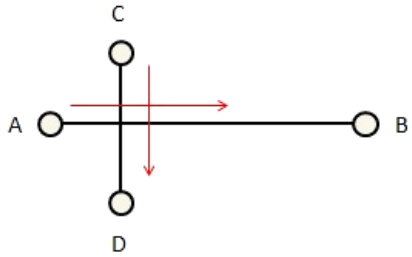


Figure 2. Schematic view of a typical microchip electrophoresis system with separation and injection channels.

Three different detector designs were used in this research.

SU-8-Pt001T has a thin 50 µm platinum working electrode (WE) and a 250 µm platinum auxiliary (AE) and reference electrode (RE).

SU-8-Pt002T has a 100 µm width of all three platinum electrodes (WE, RE, AE).

SU-8-Au001T has the same dimensions as in SU-8-Pt001T but with gold electrodes.

In all three designs, the gap between individual electrodes is 100 µm.

Extra chip design was used for separation purposes, also manufactured by MicruX Technologies. In custom made microchip, the separation channel length (A → B) was prolonged to 55 mm and detector design is the same as in SU-8-Pt001T. (Figure 3.)

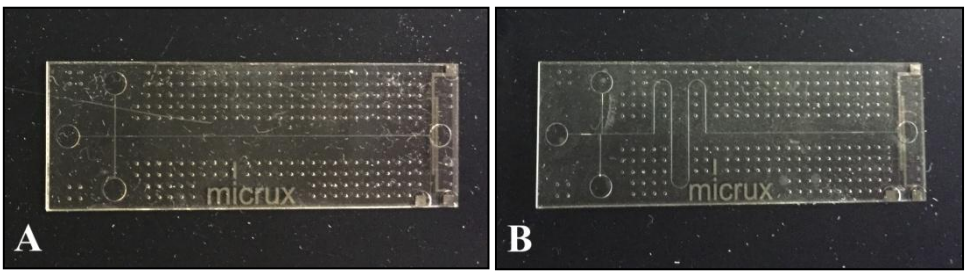


Figure 3. Short channel (35 mm) SU-8-Pt001T microchip (A); custom, long channel (55 mm) SU-8-Pt001T microchip (B).

3.4 MCE separations with EC detection

Separations of substrates and metabolites were performed on custom made long channel microchips with 5.5 cm separation channel length, using conditions described in Table 4. All samples were prepared in 20 mM sodium borate buffer (pH 10.0), which was also used as running buffer.

Table 4. Conditions for MCE-EC substrates / metabolites separations

INJECTION (C → D)		SEPARATION (A → B)	
Voltage [V/cm]	Time [s]	Voltage [V/cm]	Time [s]
+ 800	6	≈ 545 V/cm	60

3.5 Sample preparation by liquid phase microextraction

Liquid phase microextraction (LPME) was performed using SU-8-Pt001T chip. The chip was removed from the holder and filled with 20 mM sodium borate buffer (pH 10.0) (acceptor solution) using vacuum suction. On sample inlet “C” one more drop was added to decrease the possibility of air bubble retention under membrane. A small piece (3 mm x 3 mm) of Celgrade 2500 membrane (Celgrade, Charlotte, NC, USA) with 55% porosity, 25 µm thickness and 0.21 µm x 0.05 µm pores previously wetted with 1-octanol was placed on the top of buffer drop. The chip was returned to the holder and connected back to the HV supply. 100 µM umbelliferone and paracetamol samples were prepared in 100 mM HCl. Finally, 8 µL of acidic sample solution was added to the top of membrane to initiate extraction. After extraction times of 30 s, 60 s or 5 min, injection voltage of +800 V was applied for 6 s and 20 s, respectively, followed by separation voltage of +1000 V.

3.6 Sample preparation by electrokinetic sample stacking

Electrokinetic sample stacking was performed in SU-8-Pt001T microchip. 100 mM sodium borate (pH 10.0) was used as running buffer. Samples of dopamine and paracetamol were prepared in 10 mM sodium borate buffer (pH 10.0) To obtain a sample plug inside of the separation channel injection was performed from sample inlet "C" to separation outlet "B"

("gated" injection). The injection time was approximately half of the migration time for each compound. After the "filling" of the separation channel, a voltage of 1000 V was applied from separation inlet "A" to separation outlet "B" (normal separation). Detection voltage was + 0.9 V for dopamine and paracetamol.

3.7 Liquid-liquid extraction

100 µM standards of paracetamol and umbelliferone were prepared in acid environment. The total volume of standard was 100 µl. It was composed of 10 µl 2M HCl, MQ-water and stock solutions of paracetamol and umbelliferone prepared dissolving solids in 20 mM sodium borate buffer pH 10. In Eppendorf tubes with standards, organic solvents DCM, chloroform and 1-octanol were added in a 1:1 ratio (100 µl of organic phase), respectively. Each sample was mixed using the Vortex machine for 5 minutes and left 5-10 minutes for separation of phases. Carefully, using a pipette, phases were separated. After evaporation of the organic (solvent) phase, the precipitate was resuspended in 100 µl 20 mM sodium borate buffer pH 10 and analyzed by MCE-EC. Water phase was kept for extra analysis in case of unsuccessful extraction into organic phase.

4 Results and discussion

4.1 Choice of buffer

Buffer is one of the most important feature of electrophoresis. The selection of optimal buffer requires a few parameters to be considered, such as pKa value that should be close to desired pH. In that way a buffer will have the greatest capacity to absorb or release protons. Apart from pKa, formal charge of buffer is important feature, same as molecular size. The electrophoretic mobility of the counter ion (cation) determinates the magnitude of the cathodic electroosmotic flow (EOF).
In capillary electrophoresis buffer concentration influence electroosmotic flow. EOF is reversely proportional to the square root of buffer concentration, therefore, dilute buffers provide fastest separation.

In this research, two buffers were used for all studies: MES-NaOH (pH 6.0) and sodium borate (pH 10.0). Both buffers showed least interaction with oxidation of analytes, providing clear peaks with less background noise. The stabilization of baseline took shorter time with sodium borate buffer compared to MES buffer. MES-NaOH required extra runs after every washing of microchannel or buffer change. In that way, the use of sodium borate buffer shortened analysis time and made it easy to work with.

The influence of buffer concentration on EC signal was studied in this work. Three different concentrations of sodium borate buffer were used: 20 mM, 50 mM and 100 mM (pH 10.0). Results are shown in the Table 5 and Figure 4.

Table 5. Average peak areas, migration times and plate numbers for **100 µM** dopamine and **50 µM** paracetamol in different concentration sodium borate buffers pH 10. *Conditions*: V_{sep} = +1500 V, V_{inj} = +800 V for 6 s, E_d (dopamine, paracetamol) = +0.9 V.

	20 mM sodium borate			**50 mM sodium borate**			**100 mM sodium borate**		
	Area	t_M [s]	N	Area	t_M [s]	N	Area	t_M [s]	N
Dopamine	9.46	14.4	634.6	5.99	18.6	1053.9	0.85	21.8	1365.7
Paracetamol	2.51	16.8	870.2	1.84	22.7	1406.8	0.23	31.7	1582.5

As expected, lower concentration buffers provided fastest separation of both paracetamol and dopamine (Figure 4). The peak areas became smaller and an increase in plate number was noticed for both compounds with an increase of buffer concentration. High concentration of buffer molecules interacts with oxidation of species causing decrease in peak area and slower migration of analytes towards separation outlet.

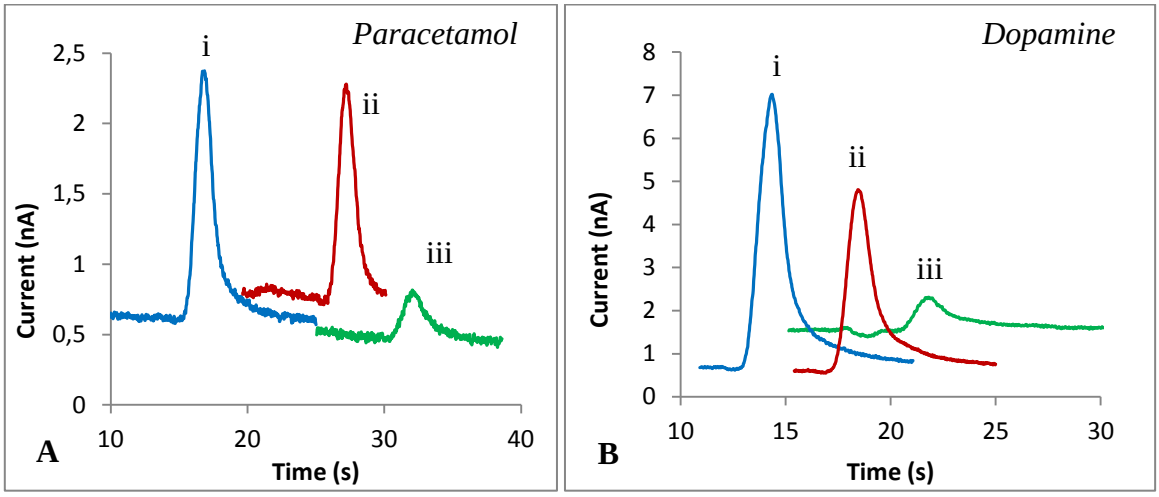


Figure 4. Electropherograms of 50 µM paracetamol (A) and 100 µM dopamine (B) in 20 mM sodium borate (i), 50 mM sodium borate (ii), 100 mM sodium borate (iii). *Conditions*: V_{sep} = +1500 V, V_{inj} = +800 V for 6 s, E_d (dopamine, paracetamol) = +0.9 V. L_{eff} (effective separation length) = 30 mm.

The difference between two buffers used in this work was tested on 10 µM dopamine sample. Results are given in Figure 5. The longer migration time of compound in sodium borate buffer was made the main difference between the two runs. Under optimized conditions there was no significant difference in peak shape and peak area. The same peak area for dopamine was obtained in both buffers, though the detection potential was optimized separately for each buffer and was +0.7 V for MES-NaOH and +0.9 V for Na-borate buffer (Figure 5).

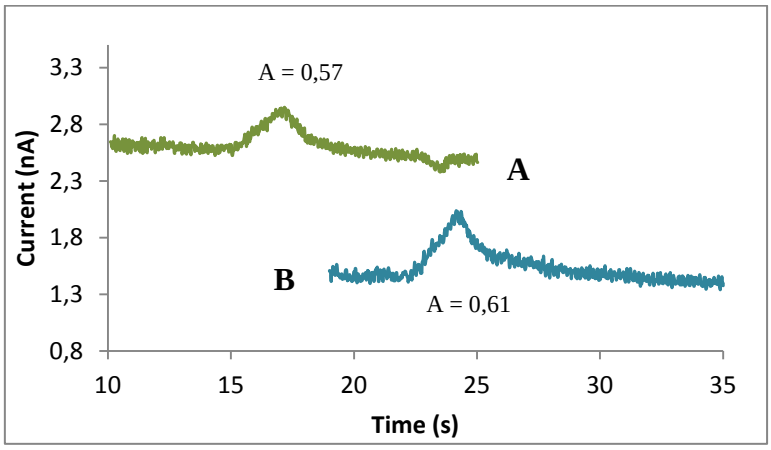


Figure 5. Electropherograms of dopamine 10 µM in 20 mM MES-NaOH (pH 6.0) (A) and 20 mM sodium borate (pH 10.0) (B). *Conditions:* V_{inj} = +800 V for 6 s, V_{sep} = +1000 V, E_d (A) = +0.7 V, E_d (B) = +0.9 V. L_{eff} = 30 mm.

All things considered, the use of low molar concentration (20 mM) both MES and borate buffer are acceptable options for drug and metabolite analyses using the MCE-EC device. In addition to this, conductivity of dilute buffers is lower, therefore higher electric fields can be used.

If a drug is showing degradation in high pH buffer solution (like diclofenac and its metabolite 4'-hydroxydiclofenac), lower pH MES buffer should be used as primary option.

4.2 Effect of solvents on obtained signal

The standard solvent for the preparation of stock solutions is dimethyl sulfoxide (DMSO). It is a polar, aprotic solvent that dissolves non-polar and polar compounds and it can be mixed

with a wide range of organic solvents and water. If a compound is water-soluble or unstable in DMSO, other solvents such as Milli -Q water can be used.

Dimethyl sulfoxide (DMSO) was the solvent used for the preparation of all stock solutions so its electroactivity was studied as a part of this work (Figure 6).

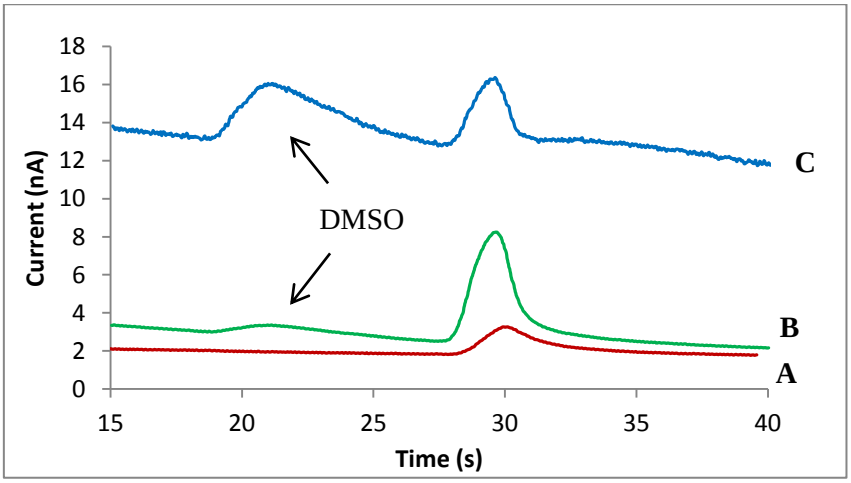


Figure 6. Electropherograms of paracetamol 100 µM in 20 mM sodium borate (pH 10.0) obtained with different detection potentials. E_d = +0.8 V (A), E_d = +1.1 V (B), E_d = +1.3 V (C). *Conditions*: V_{inj} = 800 V for 6 s, V_{sep} =1000 V. L_{eff} = 30 mm.

The results indicate that with increase of detection potential above + 1,0 V, the new peak starts to appear before the main paracetamol peak. Separation of the same volume of blank sample confirmed the peak as DMSO solvent (DMSO: buffer = 1:100). DMSO is visible with E_d higher than +1.0 V and it can interfere with detection of other compounds. If the detection potential (E_d) of a drug or metabolite in borate buffer exceeds +1.0 V, other solvent should be considered as stock preparation solvent.

One of the methods reported to influence EOF, viscosity, inductivity of separation buffer and selectivity of separation is the addition of organic modifiers[29]. The addition of methanol as second solvent in buffer solution was studied in this work. New buffers were prepared with 10% and 30% of methanol, respectively, in 20 mM MES-NaOH buffer (pH 6.0). Results are showed in Figure 7.

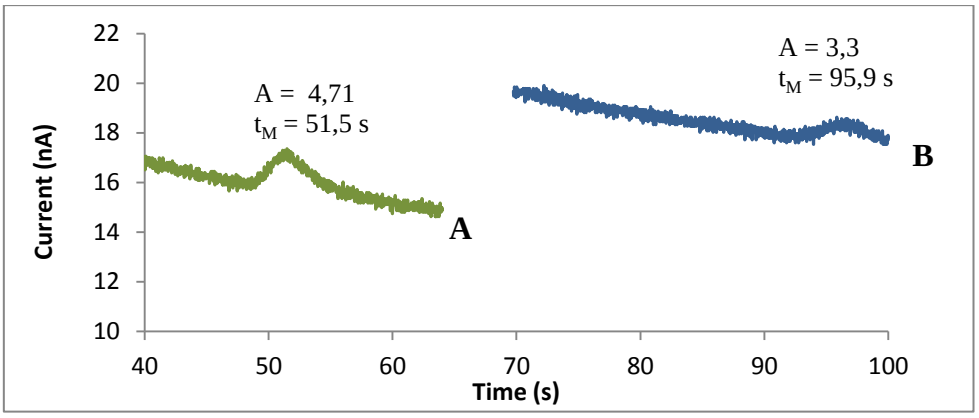


Figure 7. Electropherogram of dopamine 100 µM in 20 mM MES-NaOH with 10 % Methanol (A) and 30 % Methanol (B). *Other conditions*: V_{inj} = 800 V for 6 s, V_{sep} = 1000 V, E_d = 0.7 V. L_{eff} = 30 mm.

The results indicate that the peak area was decreased with an increase in methanol concentration in the buffer. Migration time was increased dramatically, especially with the 30% concentration of solvent in buffer.

In conclusion, the addition of methanol did not increase sensitivity, but did lead to a decrease of peak height and negatively interfered with detection of dopamine and, therefore, it should be avoided as solvent in further MCE-EC tests.

4.3 Assembling the MCE-EC setup

The effect of Micrux microchip holder configuration was studied as there are two possible ways of connection mode between HV supply and microchip holder reported by the manufacturer. The connection “mode 1”, in which all the inlets (sample “C” and buffer “A”) and outlets (sample “D” and buffer “B”) are used, same as three electrodes for detection: working, reference and auxiliary electrode. In connection “mode 2”, the auxiliary electrode is not used, but its power supply is used as ground “B” or separation outlet. Although there is a possibility that non-usage of auxiliary electrode can reduce signal significantly, as well, “mode 2” provides faster stabilization of baseline, less baseline noise, thus, clearer peaks in almost every run with no significant decrease in peak intensity and area. Therefore, this connection configuration was used in this research.

The procedure of microfabrication on SU-8 microchips allows obtaining a large number of designs. Moreover, the integration of electrodes for amperometric detection during same procedure is made possible. In this research three microchip EC detection designs with end-channel electrode detector were tested. SU-8-Pt001 and SU-8-Pt002 are commercial microchips with platinum detector integrated at the end of the separation channel. SU-8-Au001T is the same design microchip with integrated gold electrodes. Firstly, the difference between gold and platinum electrodes was studied using SU-8-Au001T and SU-8-Pt001T microchips and dopamine as highly electroactive compound. 20 mM MES-NaOH pH 6.0 was employed as a running buffer, a separation voltage of +1000 V and an injection voltage of +800 V and +700 V for 4 s, for gold and platinum electrode, respectively.

The platinum electrode chip provided better results considering the peak area, symmetry and migration time (Figure 8). The peak area obtained with platinum electrode microchip was 6,6-fold larger and a decrease in migration time of about 10 s was noticed. The decrease in migration time is most probably a result of differences in microfabrication processes between Au and Pt patterning, that have an effect on the final surface properties.

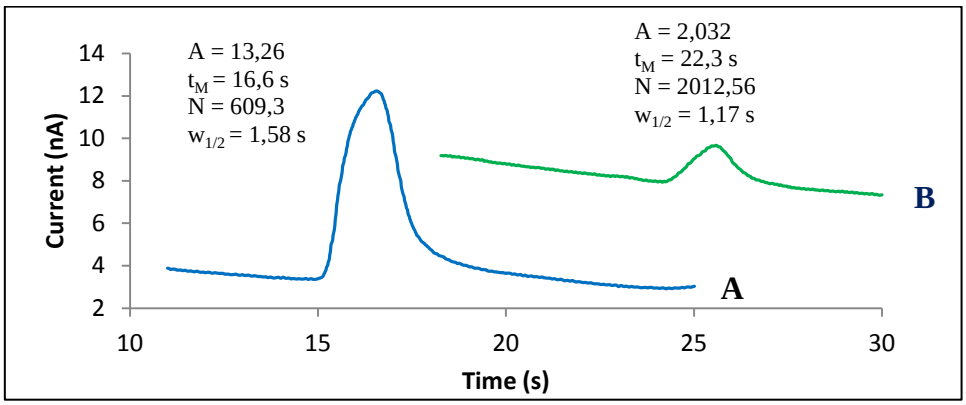


Figure 8. Electropherograms for 100 µM dopamine in SU-8-Pt001T microchip (A) and SU-8-Au001T microchip (B). *Conditions*: running buffer 20 mM MES-NaOH pH=6, V_{sep}= +1000 V, V_{inj}= +800 V for 4 s; E_d= +1.3 V. L_{eff} = 30 mm.

The effect of EC electrode geometry was tested using SU-8-Pt001T and SU-8-Pt002T chips (Table 6). Dopamine, paracetamol, phenacetin and umbelliferone, 100 µM each, were the compounds used in the tests. Sodium borate buffer (20 mM, pH 10.05) was employed as running buffer. Using the same potential for detection of each compound at two chips, the

peak areas were 1-1.3-fold bigger using SU-8-Pt002T chip for dopamine, paracetamol and phenacetin. Almost a 5-fold increase was observed with umbelliferone sample. However, peak symmetry, same as peak height, was better using SU-8-Pt001T chip for two highly electroactive compounds dopamine and paracetamol (Figure 9) which is probably due to narrower working electrode. On the other hand, wider SU-8-Pt002T working electrode provided a larger area. Umbelliferone, however, demonstrated different behavior, better shape, a larger area and height, same as big difference in migration time with SU-8-Pt002T chip layout.

Table 6. EC electrode geometry effect on peak area, migration times and average plate number in 100 µM samples. *Conditions*: running buffer 20 mM sodium borate pH 10.0; V_{sep} = 1000 V, V_{inj} = 800 V for 6 s.

		SU-8-Pt001T			**SU-8-Pt002T**		
COMPOUND	E_d [V]	Average peak area	t_M [s]	Average N	Average peak area	t_M [s]	Average N
Dopamine	0.9	17.96	26	1179.75	19.17	22	428.05
Paracetamol	0.7	8.30	28	1541.26	10.933	25	811.4
Phenacetin	1.3	26.22	25	541.82	32.715	22	368.63
Umbelliferone	1.0	1.205	66	2701.23	5.827	47	2256.55

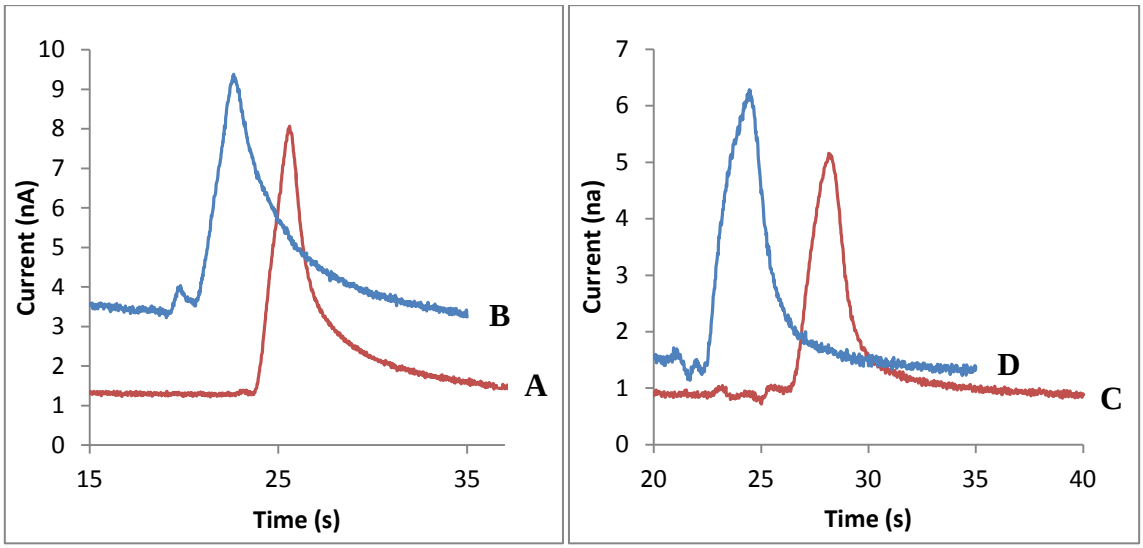


Figure 9. Electropherograms of dopamine 100 µM in SU-8-Pt001T (A) and SU-8-Pt002T (B) and paracetamol 100 µM in SU-8-Pt001T (C) and SU-8-Pt002T (D). L_{eff} = 30 mm.

As expected, better sensitivity is achieved with narrower platinum working electrode, hence the choice of SU-8-Pt001T electrode microchip for detection of low drug concentrations.

MicruX Manager software, besides controlling electrophoresis processes, offers the possibility of reducing baseline noise by changing filter parameters. It is possible to change *frequency* and *order* parameters to obtain the best signal for each compound. To obtain their effect on signal, a few different frequency and order values were tested. The study has indicated no significant difference between frequencies from 1 to 100. At the frequency of 0.1 the peak area was 2-fold lower, wider on the baseline and the background noise was reduced to minimum. A frequency lower than 1 provides more filtering, but causes filtering and reduction of the main signal. For further research the frequency 5 was chosen with order value 1. The increase in order value was tested with same 50 µM paracetamol sample under same conditions. The results showed a spreading of peaks on the baseline with an increase in order parameter. For further studies order value 1 was used. In all runs showed on Figure 10 the order value was set to 1 and the frequency value was changed.

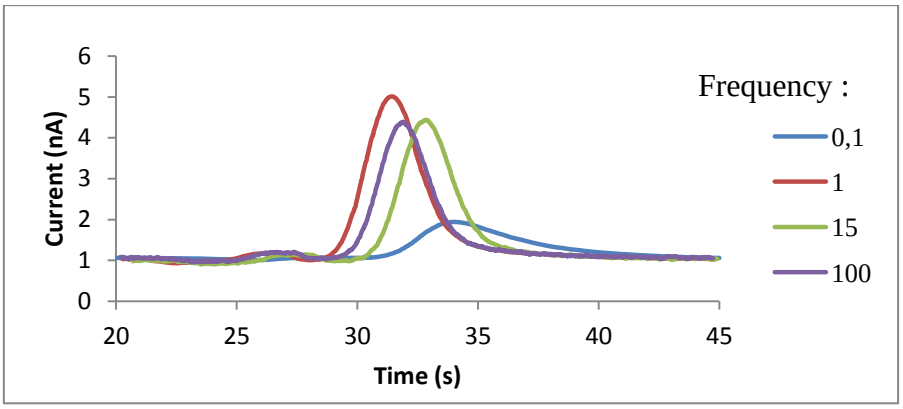


Figure 10. Electropherogram of 50 µM paracetamol in 20 mM sodium borate as running buffer. Testing filter parameters was performed with V_{sep} = +1500 V, V_{inj} = 800 V for 6 s. L_{eff} = 30 mm. In all runs showed on graph order value was set to 1 and frequency value was changed.

4.4 Analytical method development

A successful application of microchip electrophoresis (MCE) with electrochemical (amperometric) detection for metabolic assays requires finding optimal parameters such as injection time and voltage. Sample injection, in this case, was electrokinetic, which means that a sample is injected by applying a voltage to sample inlet. A schematic view is shown on Figure 2, where voltage is applied to inlet "C", and outlet "D" was used as ground, zero voltage site. The injected volume depends on electroosmotic flow (EOF), electrophoretic mobility of molecules and temperature.

In this research, different injection times and voltages were tested and the results are shown in Figure 11. 20 mM MES-NaOH buffer was applied as running buffer and 100 µM dopamine was used as the test compound.

As shown in Figure 11, applying higher voltages and injection times results in an increase of peak area as a result of bigger volume in channels intersection. After a certain point, a broadening of dopamine peak was observed. Applying too high of a voltage resulted in great peak area but also in loss of symmetry. It was necessary to compromise conditions in order to get best performance. Shorter injection times of 4, 5 and 6 seconds with the injection voltage of 700 to 900 V/cm were the best choice for most of the compounds.

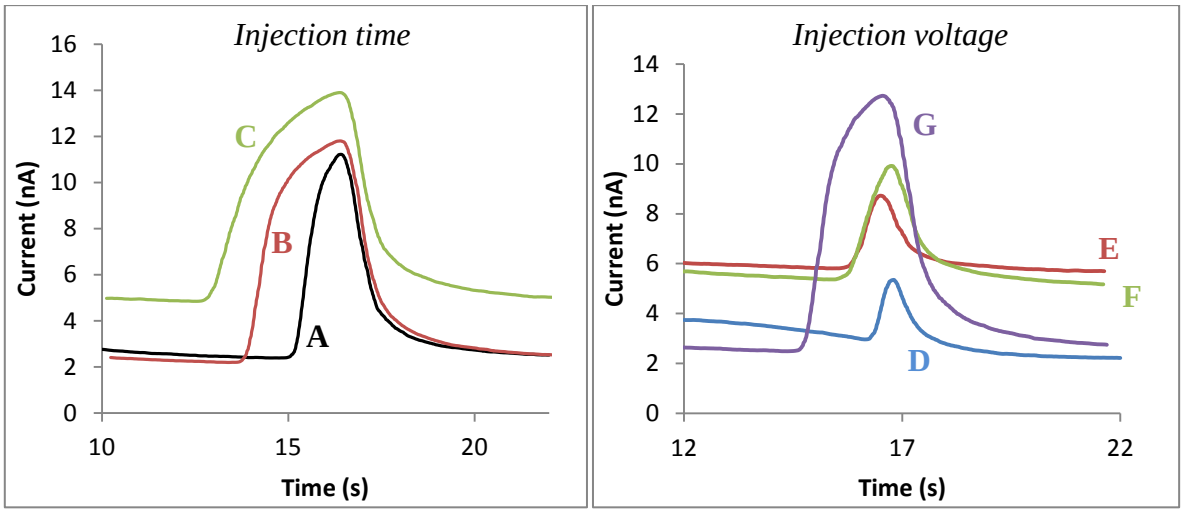


Figure 11. Electropherograms of dopamine 100 µM in 20 mM MES-NaOH pH 6.0. Effect of injection time on signal shape: 4 seconds (A), 7 seconds (B) and 10 seconds (C), V_{inj} = +750 V; and effect of injection voltage on peak shape: +250 V (D), +500 V (E), +700 V (F) and 1000 V (G); injection time 4 s. *Other conditions* V_{sep} = +1000 V, E_d = +1.3 V. L_{eff} = 30 mm.

Linear dynamic range was established for dopamine, paracetamol and umbelliferone and given in Figure 12. Due to fast degradation of 4-hydroxydiclofenac its dynamic range was excluded from results.

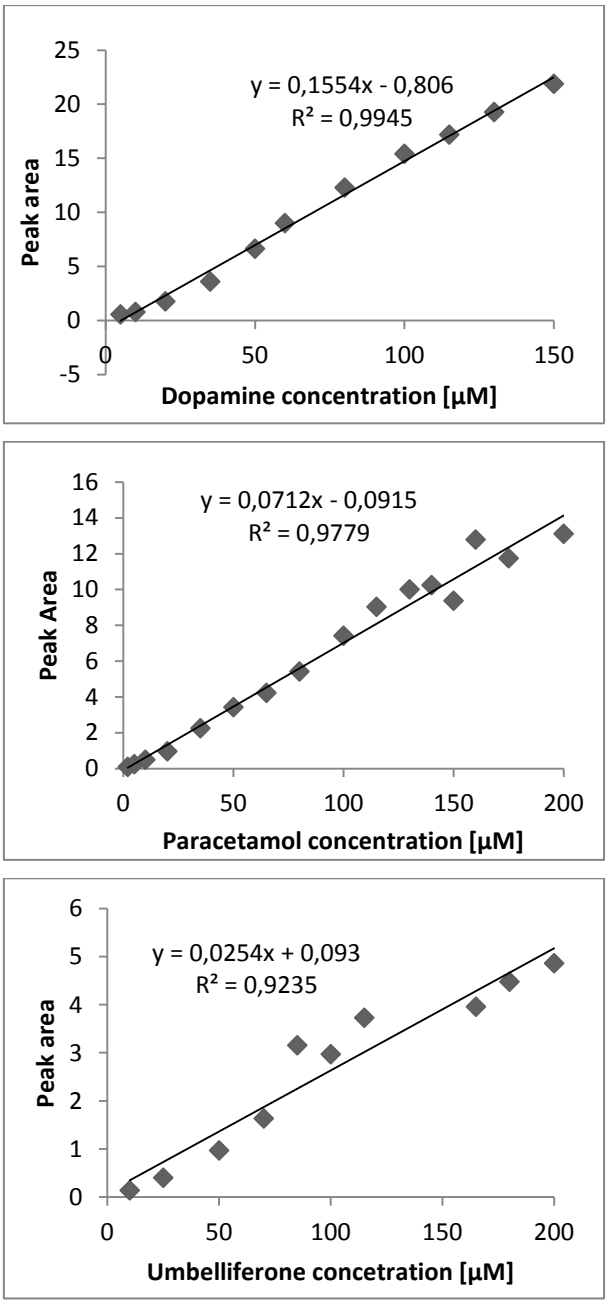


Figure 12. Linear dynamic ranges for dopamine, paracetamol and umbelliferone in 20 mM sodium borate pH 10.0. Microchip: SU-8-Pt001T. *Conditions (dopamine):* V_{sep} = +1000 V, V_{inj} = +800 V for 6 s, E_d = +0.9 V; *conditions (paracetamol):* V_{sep} = +1000 V, V_{inj} = +800 V for 6 s, E_d = +0.8 V; *conditions (umbelliferone):* V_{sep} = +1000 V, V_{inj} = +800 V for 6 s, E_d = +0.8 V. L_{eff} = 30 mm.

The limit of detection (LOD) and the limit of quantification (LOQ) are important validation characteristics used to describe the lowest concentration of an analyte that can be detected and measured in the analytical process. There are a few common methods for determining the LOD and LOQ[30]. In this assay, the calculation from calibration curve at low concentrations[31] was used as a method. Commercial SU-8-Pt001T chip was used in all of the tests. Dopamine was chosen as test electroactive compound. LOD and LOQ for dopamine, paracetamol, umbelliferone and 4'-hydroxydiclofenac were estimated in 20 mM sodium borate buffer pH 10.0 and 20 mM MES-NaOH buffer pH 6.0, respectively (Table 7).

Table 7. LOD and LOQ values for tested compounds in different buffers

	20 mM sodium borate pH 10.0		**20 mM MES-NaOH pH 6.0**	
COMPOUND	**LOD [µM]**	**LOQ [µM]**	**LOD [µM]**	**LOQ [µM]**
Dopamine	1.67	5.07	0.92	2.77
Paracetamol	0.64	1.94	n/a	n/a
Umbelliferone	3.03	9.18	n/a	n/a
4'-OH-diclofenac	5.37	16.29	n/a	n/a

For all the compound tested with MCE-EC method, a low micromolar concentration could be detected which is the base point for the application of this method to drug inhibition screening.

Dynamic ranges of paracetamol, dopamine and umbelliferone exceed ranges 0.302-0.232 µg/ml, 0.766-22.977 µg/ml and 1.492-32.428 µg/ml, respectively. The method is suitable for *in vitro* screening of metabolites, and linearity ranges are follow the ranges reported by conventional analytical methods such as tandem GC-MS and HPLC[32].

However, sample enrichment and purification method (preferably implemented on-chip) are needed in addition to the MCE-EC method to increase its sensitivity and improve peak shape and height. Some of them are tested and reported in this work.

4.5 On-chip sample preparation experiments

4.5.1 Liquid phase microextraction

Liquid phase microextraction (LPME) under totally stagnant conditions and combined with MCE was reported for the first time in 2009 as an on-line sample preparation method[33]. The main principle is extraction of analytes from acidic, aqueous sample droplet through porous membrane wetted with organic phase, into basic, aqueous acceptor solution (buffer). pH of donor solution was adjusted to 1-2 by preparing samples in 100 mM HCl. pH of acceptor solution (buffer in sample inlet) was 10. Both pH values were adjusted in the way to ensure that paracetamol and umbelliferone are in neutral form in the sample droplet, which allows them to pass organic phase in membrane, and fully protonated in acceptor solution. The ionization of analytes in acceptor solution should prevent them from reentering the organic phase membrane.

For both compounds tested with different extraction times (from 30 seconds to 5 minutes) and different injection times (from 6 s to 20 s) there was no analyte peak. Applying the sample directly under the membrane produced a peak after injection of 6 seconds and separation with +1000 V, which confirmed that the problem is not in the injection and electrophoresis process, but in the extraction of the compound through the membrane. The extraction could be completely unsuccessful or very slow, which causes most of the compounds to stay close to the membrane and diffuse very slowly to the channel inlet.

4.5.2 Electrokinetic sample stacking

Electrokinetic sample stacking was developed as an enrichment method in this work and it is based on the difference in electric field strength (and conductivity) between sample zone and separation zone. The samples are prepared in 10-fold lower concentration buffer and therefore a sample zone has low conductivity and high electric field strength. As a separation buffer, 10-fold higher concentration buffer (higher conductivity and lower electric field strength) was employed. Analyte migrates faster in sample region (sample plug) and moves to the boundary between sample and separation buffer which results in narrowing of the analyte zone. A compound will have higher concentration than the original concentration in the sample (Figure 13).

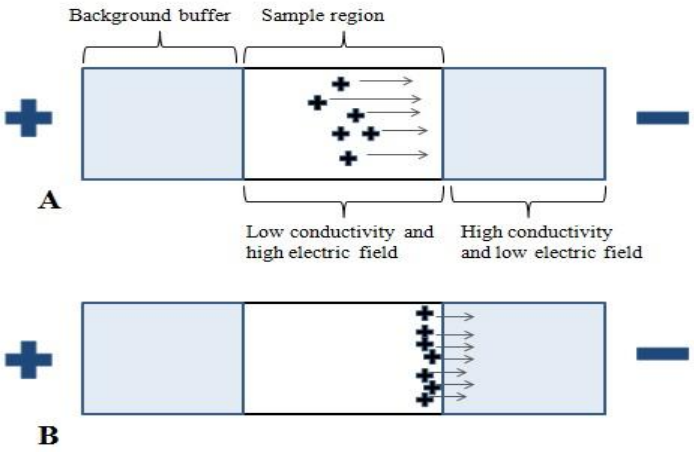


Figure 13. Schematic view of electrokinetic sample stacking. After "gated" injection, sample plug is formed inside capillary (A). Analytes migrates faster in high electric field (sample) region forming narrow analyte zone (B).

Electrokinetic sample stacking was developed for dopamine and paracetamol (Figure 14 and Figure 15).

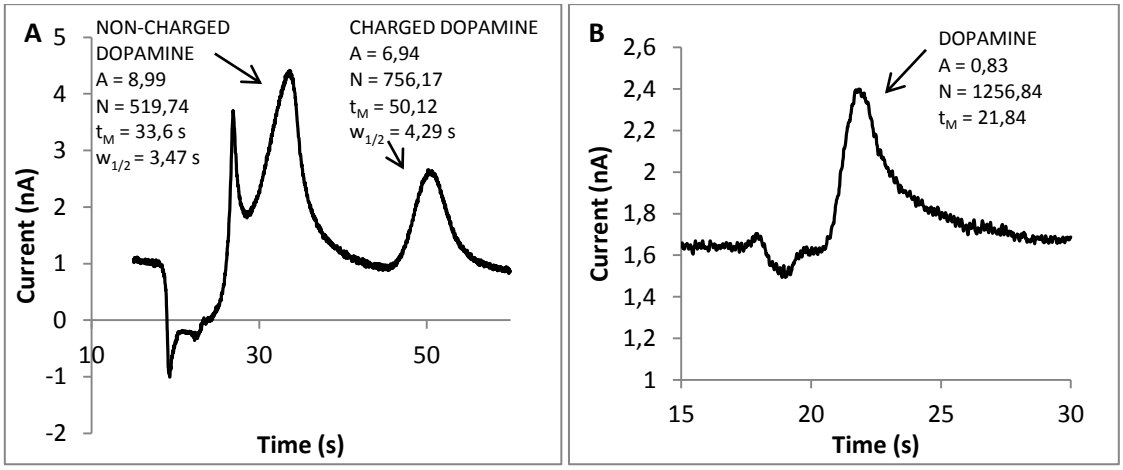


Figure 14. Electropherogram of dopamine 100 µM obtained after preconcentration of sample prepared in 10 mM sodium borate buffer pH 10.0 with 100 mM sodium borate pH 10.0 employed as running buffer (A). *Conditions*: V_{sep} = +1000 V, V_{inj} ("gated" injection C → B) = +800 V for 15 s, E_d = +0.9 V; and electropherogram of "normal" injection of same concentration dopamine with 100 mM sodium borate pH 10.0 as running buffer (B). *Conditions*: V_{sep} = +1500 V, V_{inj} ("floating" injection C → D) = +800 V for 6 s, E_d = +0.9 V. L_{eff} = 30 mm.

In the case of the dopamine, the electropherogram showed two separated peaks after performing the electrokinetic sample stacking. The first peak was concluded to originate from neutral dopamine and the second from negatively charged dopamine. pK_a value of dopamine is close to 10 (pH of buffer used in run) which resulted in neutral charge of some of the dopamine molecules in the sample. The use of pH higher than 10 resulted in fast degradation of dopamine sample in buffer and whole dopamine could not be transformed to charged species.

Paracetamol (pK_a = 9.5) electrokinetic sample stacking enrichment is shown in Figure 15. All molecules are negatively charged and collected in one narrow zone which resulted in about 8-fold bigger peak area compared to run in 100 mM buffer under same conditions.

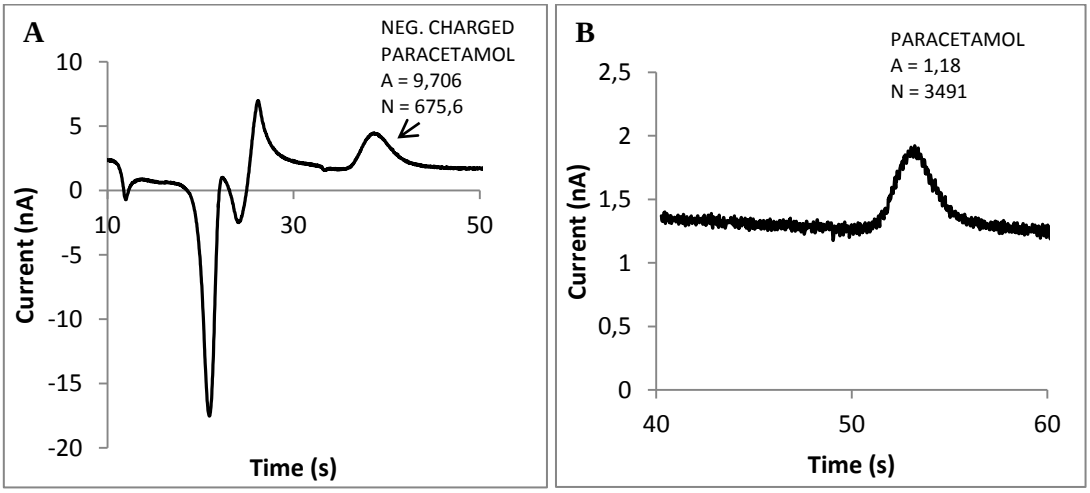


Figure 15. Electropherograms of paracetamol 50 µM with electrokinetic sample stacking with 10 mM sodium borate pH 10.0 as a sample buffer and 100 mM sodium borate as running buffer (A); and paracetamol 50 µM without sample stacking with 100 mM sodium borate pH 10.0 as running buffer (B). *Other conditions*: V_{sep} = +1000 V, V_{inj} (A) 800 V for 24 s (gated C → B injection); V_{inj} (B) +800 V for 6 s (C → D injection), E_d = +0.9 V. L_{eff} = 30 mm.

Electrokinetic sample stacking proved to be fast and practical on-chip method which has increased peak area multiple times, thus it is feasible for use in combination with this technique.

4.6 Liquid-liquid extraction (off-chip sample purification)

In vitro metabolism assays are performed by using liver microsome fractions from humans or experimental animals. Using human liver microsomes as *in vitro* systems allows evaluation of the main phase I metabolites formed by cytochrome P450. They are a useful tool for determination of metabolic pathways, as well of possible inhibitions of CYP enzymes by commonly used drugs or different environmental chemicals. The motivation for this work is the validation of MCE-EC as a screening method for metabolic assays performed on *in vitro* human liver microsomes. Except for the successful separation of drugs from metabolites formed in incubation process, the main goal is their separation from electroactive compounds inside the incubation matrix. For this reason, liquid-liquid phase extraction (LLE) was tested as a sample purification method.

LLE is a method based on solubility of compounds in two different liquid phases, water and organic phase. By acidifying the standard sample, paracetamol and umbelliferone were in neutral form which allowed them to transfer into organic phase and separate from extra electroactive compounds from matrix left in water phase. Redissolution of the precipitate after evaporation of organic phase was the most important step of extraction. The sample tested immediately after redissolution was negative for analytes (Table 8). After one day and extra mixing, same samples were positive for paracetamol and umbelliferone (Figure 16).

Table 8. List of solvents used in LLE with compounds and their peak areas after extraction and MCE analysis. Peak areas were obtained after 24 hour of redissolution.

	PEAK AREA	
	PARACETAMOL	UMBELLIFERONE
CLOROFORM	2.35	n/a
Dichloromethane (DCM)	3.094	3.71

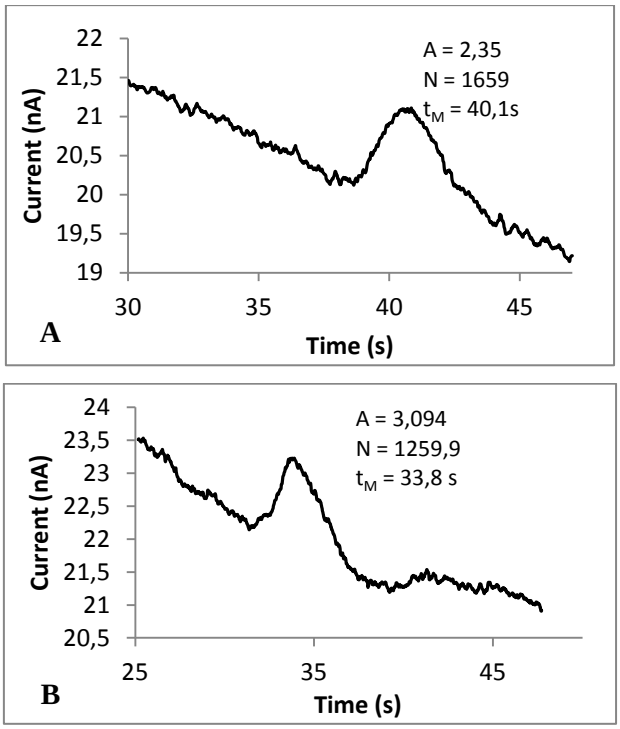


Figure 16. Electropherograms after redissolution of residue left after evaporation of organic phase. Paracetamol after LLE and evaporation of chloroform (A), paracetamol after LLE and evaporation of DCM (B). *Conditions*: V_{sep} = +1500 V, V_{inj} = +800 V for 6 s, E_d = + 1.1 V. Buffer: 20 mM sodium borate pH 10.0. L_{eff} = 30 mm.

4.7 Microchip electrophoresis separations

MCE-EC platform was tested as a practical and fast screening platform for some CYP P450 substrates and metabolites. To determine the feasibility of method for environmental purposes mentioned in the introduction, substrates and metabolites needed to be separated on the baseline from each other. For that purpose, long channel microchips were used. Electrode geometry of microchips is the same as in SU-8-Pt001T.

To determine if the method can be used for *in vitro* metabolism assays, substrate and metabolite have to be sufficiently resolved from each other during the CE separation and detected in concentration needed for Michaelis-Menten kinetics.

CYP1A2 - Km ≈ 1-116 µM
CYP2A6 - Km ≈ 0.3-2.4 µM
CYP 2C9 - Km ≈ 3.1-13 µM

In Michaelis-Menten kinetics, the substrate concentration is very much larger than the concentration of a product. With this in mind, the detection of metabolites is a challenge when it comes to this method. Paracetamol concentration produced in enzyme incubations is 1-5 µM and umbelliferone concentration is 0.1-10 µM.

Both phenacetin/paracetamol and coumarin/umbelliferone pairs were separated successfully in 20 mM borate buffer (pH 10.0), using the highest separation voltage possible with Microx platform, i.e., at +3000 V, which gave approximately 545 V/cm. The results showed that 100 µM substrate and 20 µM metabolite concentration could be easily separated and detected with custom long channel microchip in both cases, coumarin/umbelliferone and phenacetin/paracetamol (Figure 17). The lower concentration of analytes, 50 µM substrate and 10 µM metabolite, were tested after that. Phenacetin and paracetamol could be separated and detected in lower concentration (Figure 17. C), but in case of coumarin/umbelliferone metabolite the peak was not visible anymore.

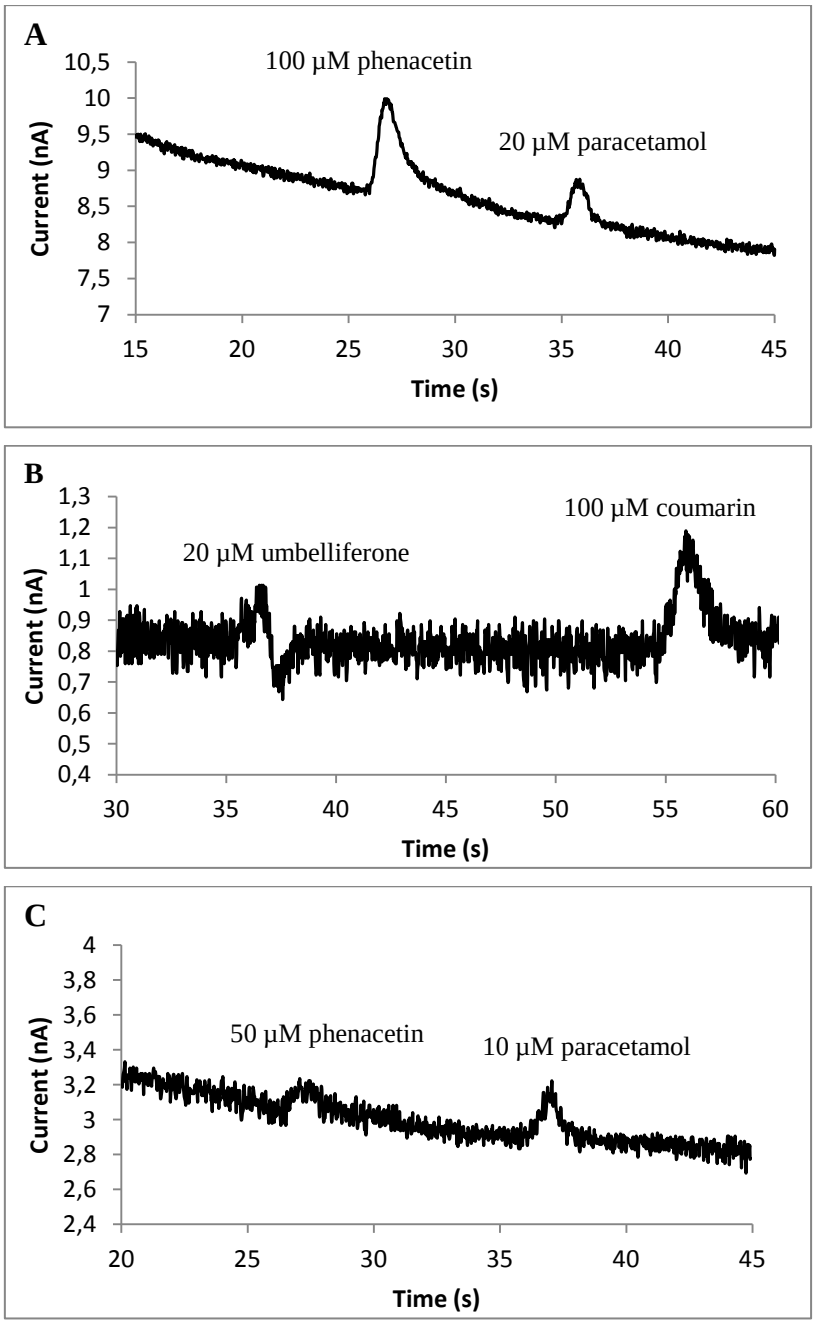


Figure 17. Separation of substrates and metabolites. Running buffer 20 mM sodium borate (pH 10.0). Separation voltage +3000 V (545 V/s). Detection potential: +1,1 V (A), +0.9 V (B), +1.0 V (C). L_{eff} = 50 mm.

Altogether, the separation and detection of phenacetin and paracetamol is successful and it could be performed even without any sample enrichment protocol. Coumarin and umbelliferone are successfully separated, but the detection of umbelliferone in such low concentration requires an extra enrichment technique.

4.8 Dissolution assays with indomethacin

Apart from finding the optimal conditions for determination of CYP P450 substrates and metabolites, a technique was tested as a potential method for the examination of very poor soluble drugs, such as indomethacin. Indomethacin was tested in bulk form, firstly dissolved in DMSO as 10 mM stock solution, then prepared in 20 mM sodium borate buffer (pH 10.0) in a certain concentration. Afterwards, it was tested in nanoparticle formulation, prepared with Poloxamer 407 (Lutrol F127) using milling technique.

Results indicated a weak signal of indomethacin in bulk form with E_d from 0,7 to 0,9 V which was confirmation of indomethacin electroactivity and first step in dissolution assays.

Indomethacin nanoparticle formulation was tested in the same way, by dissolving in 20 mM sodium borate (pH 10.0). Nanoparticles are prepared in order to increase solubility of indomethacin and in contact with borate buffer all nanoparticles were quickly dissolved. The analysis of nanoparticle sample with same detection potential gave a negative peak with different migration time in every run. This unusual outcome could be a result of the interference of polymer and indomethacin in nanoparticle form of drug. Polymer alone was also analyzed (in same concentration as in nanoparticle formulation) and results showed no peak at E_d from 0.7 to 0.9 V.

Because of the interference and impossibility of analyte detection inside nanoparticles, conclusion can be made that this method is not feasible for this type of dissolution assays. Nevertheless, dissolution assays in combination with MCE-EC represent an interesting topic and further research in this direction can yield more promising results.

5 Conclusions

The aim of this research was to examine the feasibility of MCE-EC platform for metabolism assays using CYP probe reactions, together with optimizing conditions and parameters for metabolism and enzyme inhibition studies. Firstly, MCE-EC technique proved to be a very practical and fast screening method following the μTAS ("lab-on-chip") concept. It requires very small volumes for analyses of low concentrations of drugs and their metabolites. For all of tested compounds, paracetamol, umbelliferone and 4'-hydroxydiclofenac, low micromolar concentrations could be detected with MicruX platform which, together with successful separation of drug and metabolite pairs, forms basis for metabolism assays.

Limits of detection and quantification for all the compounds were reached in low micromolar concentrations. To obtain even lower LOD and LOQ values, a few sample enrichment methods were tested. Electrokinetic sample stacking proved to be a useful method for increasing detection sensitivity and it can be easily applied on MCE-EC instrument used. LPME (liquid phase microextraction), on the other hand, turned out to be an unsuccessful sample enrichment method, so one of the challenge still remains in finding an "on-line" sample preparation protocol which can be implemented directly on the platform.

Metabolism assays with CYP probe reactions are imitating physiological conditions and they should be performed in biological matrix, which leads to another challenge of detecting metabolites in mixture of many electroactive compounds. Liquid - liquid extraction was tested as simple purification method for that purpose and proved to be successful a answer to the extraction of metabolites from incubation matrix. The extraction still needs to be combined with an enrichment method in order to obtain a clear and strong signal of metabolites, which remains a challenge for further MCE-EC research.

Enzyme reactions are initiated by adding a high concentration of cofactors like NADPH into drug-matrix probe and stopped by adding inhibitors such as furafylline (CYP1A2 inhibitor). The interference of both was tested as a part of this research. The NADPH did not give a signal with all detection potentials which leads us to conclude that this cofactor is not an electroactive compound. Same as NADPH, inhibitor furafylline did not interfere with detection of any of analytes. From these results, a conclusion can be drawn that this kind of

electrophoresis instrument with electrochemical detection is feasible for *in vitro* metabolism assays.

Apart from metabolism assays, MicruX instrument appears to be a very practical, fast and handy device for any kind of analysis that requires higher sensitivity. Apart from that, it has shownd to be a good tool for learning about electrophoresis on microchip in an easy way, providing a wide range of possibilities, such as implementation of different ideas directly on platform, the use of microchips of different geometries and materials, and manipulation with voltages, currents and many more.

6 References

1. Mullard, Asher. "EMA recommended approval for 40 new drugs in 2014."*Nature Reviews Drug Discovery* 14.2 (2015): 82-82.
2. Mullard, Asher. "2014 FDA drug approvals." *Nature Reviews Drug Discovery*14.2 (2015): 77-81.
3. Kostiainen, R., et al. "Liquid chromatography/atmospheric pressure ionization–mass spectrometry in drug metabolism studies." *Journal of Mass Spectrometry*38.4 (2003): 357-372..
4. Sinko, P. J. "Drug selection in early drug development: screening for acceptable pharmacokinetic properties using combined in vitro and computational approaches." *Current opinion in drug discovery & development*2.1 (1999): 42-48.
5. Boobis, Alan, et al. "In silico prediction of ADME and pharmacokinetics: Report of an expert meeting organised by COST B15." *European Journal of Pharmaceutical Sciences* 17.4 (2002): 183-193.
6. Smith, P. A., et al. "Towards integrated ADME prediction: past, present and future directions for modelling metabolism by UDP-glucuronosyltransferases."*Journal of Molecular Graphics and Modelling* 22.6 (2004): 507-517.
7. Rendic, Slobodan. "Summary of information on human CYP enzymes: human P450 metabolism data." *Drug metabolism reviews* 34.1-2 (2002): 83-448.
8. Gómez-Lechón, Maria José, et al. "Human hepatic cell cultures: in vitro and in vivo drug metabolism." *Alternatives to laboratory animals: ATLA* 31.3 (2002): 257-265.
9. Alavanja, Michael CR, Jane A. Hoppin, and Freya Kamel. "Health Effects of Chronic Pesticide Exposure: Cancer and Neurotoxicity* 3." *Annu. Rev. Public Health* 25 (2004): 155-197.
10. Hodgson, Ernest, and Randy L. Rose. "Human metabolic interactions of environmental chemicals." *Journal of biochemical and molecular toxicology* 21.4 (2007): 182-186..
11. Hodgson, Ernest, and Randy L. Rose. "Metabolic interactions of agrochemicals in humans." *Pest management science* 64.6 (2008): 617-621.
12. Hayden, Kathleen M., et al. "Occupational exposure to pesticides increases the risk of incident AD The Cache County Study." *Neurology* 74.19 (2010): 1524-1530.

13. Salam, Muhammad Towhid, et al. "Early-life environmental risk factors for asthma: findings from the Children's Health Study." *Environmental Health Perspectives* 112.6 (2004): 760.
14. Teitelbaum, Susan L., et al. "Reported residential pesticide use and breast cancer risk on Long Island, New York." *American journal of epidemiology* 165.6 (2007): 643-651.
15. Settimi, Laura, et al. "Prostate cancer and exposure to pesticides in agricultural settings." *International Journal of Cancer* 104.4 (2003): 458-461.
16. Owens, Kagan, Jay Feldman, and John Kepner. "Wide range of diseases linked to pesticides." *Pestic. You* 30 (2010): 13-21
17. Casley-Smith, John R., Robert Gwyn Morgan, and Neil B. Piller. "Treatment of lymphedema of the arms and legs with 5, 6-benzo-[alpha]-pyrone." *New England Journal of Medicine* 329.16 (1993): 1158-1163
18. Reyes, Darwin R., et al. "Micro total analysis systems. 1. Introduction, theory, and technology." *Analytical chemistry* 74.12 (2002): 2623-2636.
19. Manz, Andréas, N. Graber, and H. áM Widmer. "Miniaturized total chemical analysis systems: a novel concept for chemical sensing." *Sensors and actuators B: Chemical* 1.1 (1990): 244-248.
20. Manz, Andreas, et al. "Planar chips technology for miniaturization and integration of separation techniques into monitoring systems: capillary electrophoresis on a chip." *Journal of Chromatography A* 593.1 (1992): 253-258.
21. Schöning, Michael J., et al. "Amperometric PDMS/glass capillary electrophoresis-based biosensor microchip for catechol and dopamine detection." *Sensors and Actuators B: Chemical* 108.1 (2005): 688-694.
22. Muck, Alexander, et al. "Fabrication of poly (methyl methacrylate) microfluidic chips by atmospheric molding." *Analytical chemistry* 76.8 (2004): 2290-2297.
23. Sikanen, Tiina, et al. "Performance of SU-8 microchips as separation devices and comparison with glass microchips." *Analytical chemistry* 79.16 (2007): 6255-6263.
24. Altria, Kevin D. *Capillary electrophoresis guidebook: principles, operation, and applications*. Vol. 52. Springer Science & Business Media, 1996.
25. Beattie, James K. "The intrinsic charge on hydrophobic microfluidic substrates." *Lab Chip* 6.11 (2006): 1409-1411.
26. Breadmore, Michael C. "Capillary and microchip electrophoresis: challenging the common conceptions." *Journal of Chromatography A* 1221 (2012): 42-55.

27. Zeng, Yong, et al. "Microchip capillary electrophoresis with electrochemical detection." *Analytical chemistry* 74.10 (2002): 2441-2445.
28. Wang, Joseph, Baomin Tian, and Eskil Sahlin. "Micromachined electrophoresis chips with thick-film electrochemical detectors." *Analytical chemistry* 71.23 (1999): 5436-5440.
29. Zhang, Qian-Li, et al. "Separation of caffeine and theophylline in poly (dimethylsiloxane) microchannel electrophoresis with electrochemical detection." *Journal of Chromatography A* 1098.1 (2005): 172-176.
30. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: Text and Methodology. ICH-Q2B, Geneva; 1996.
31. Shrivastava, Alankar, and Vipin B. Gupta. "Methods for the determination of limit of detection and limit of quantitation of the analytical methods." *Chronicles of Young Scientists* 2.1 (2011): 21.
32. Belal, Tarek, Tamer Awad, and Randall Clark. "Determination of paracetamol and tramadol hydrochloride in pharmaceutical mixture using HPLC and GC-MS." *Journal of chromatographic science* 47.10 (2009): 849-854.
33. Sikanen, Tiina, et al. "Implementation of droplet-membrane-droplet liquid-phase microextraction under stagnant conditions for lab-on-a-chip applications."*Analytica chimica acta* 658.2 (2010): 133-140.

7 Summary

In a period of rapidly growing pharmaceutical industry development of the current analytical techniques is by far too slow to fulfill requests of demanding metabolic analysis of new pharmaceuticals and new chemical entities (environmental and household chemicals).

Portable Microchip Capillary Electrophoresis (MCE) platform with amperometric electrochemical detection (EC) was developed in this work, offering a new approach to analytical chemistry. By following Micro Total Analysis System (μTAS) concept, MCE-EC platform combines rapid analysis, low price and practicality with low requirements of reagents.

Research was made by using compact platform for MCE-EC purchased from MicruX Techologies. It was developed for chosen drug/metabolite pairs (phenacetin/paracetamol, coumarin/umbelliferone and diclofenac/4'-hydroxydiclofenac). Different voltages, analysis times and system setups were tested. In addition to this, different *on-chip* sample preparation and purification methods (liquid phase microextraction and electrokinetic sample stacking) were implemented on this easy-handle platform. The linearity of an analytical procedure, as well as the limit of detection (LOD) and the limit of quantification (LOQ) were determined.

8 Sažetak

UVOD

Nalazimo se u razdoblju ubrzanog rasta istraživačkog područja farmaceutske industrije. U dugotrajnim pretkliničkim istraživanjima novih kemijskih entiteta najveći izazov još uvijek ostaje određivanje metaboličkih reakcija, inhibicije i indukcije metaboličkih enzima i interakcija s ostalim ksenobioticima. Osim lijekovima, ljudi su konstantno izloženi raznim kemikalijama iz okoliša i kućanstva. I dok su učinci lijekova uglavnom poznati, fiziološki učinci ostalih kemikalija su vrlo malo istraživani. Kapilarna elektroforeza implementirana na mikročip (MCE) te niska cijena, kompatibilnost s mikrofabrikacijom i visoka osjetljivost elektrokemijske detekcije (EC) su donijeli novi pristup u području analitike lijekova i ksenobiotika.

OBRAZLOŽENJE TEME

Svrha rada je razvoj metode elektroforeze na mikročipu s elektrokemijskom detekcijom u svrhu brzih analiza izabranih CYP 450 supstrata i metabolita (fenacetin/paracetamol, kumarin/umbeliferon, diklofenak/4-hidroksidiklofenak). Na taj način omogućilo bi se brzo i olakšano analiziranje metabolizma lijekova i ksenobiotika u kombinaciji s *in vitro* testovima.

MATERIJALI I METODE

Matične otopine pripremljene su vaganjem krutina (fenacetina, paracetamola, umbeliferona, kumarina, diklofenaka i 4-hidroksidiklofenaka) i otapanjem u dimetilsulfoksidu (DMSO). Boratni i MES pufer pripremljeni su vaganjem krute borne kiseline i MES hidrata, otapanjem u deioniziranoj (MQ) vodi. pH je podešen korištenjem 2 M NaOH.
Prijenosni i kompaktni analitički instrument nabavljen je od tvrtke MicruX Technologies (Oviedo, Španjolska), a rad mu se temelji na elektroforezi na mikročipu s elektrokemijskom detekcijom. Kao mikročipovi korišteni su SU-8/staklo čipovi od istog proizvođača. Tanke film elektrode smještene su na kraju separacijskog kanala, a dimanzije mikrokanala su 20 x 50 µm s jednostavnom "križnom" geometrijom.

REZULTATI I RASPRAVA

Izbor otopine pufera

U ovom radu dvije otopine pufera su korištena za sva ispitivanja: MES-NaOH (pH 6.0) i boratni pufer (pH 10.0). Utjecaj koncentracije pufera na elektrokemijski signal ispitan je korištenjem tri različite koncentracije boratnog pufera 20 mM, 50 mM i 100 mM. Prema očekivanju, niža koncentracija pufera rezultirala je kraćim migracijskim vremenom. Površina pika smanjivala se s povećanjem koncentracije pufera. Razlika između dva korištena pufera ispitana je na uzorku dopamina. Duže migracijsko vrijeme (oko 10 s) zabilježeno je korištenjem boratnog pufera. Uz optimizaciju uvjeta nije bilo značajnih razlika između dva pufera te su oba kompatibilna za korištenje u analizi lijekova i metabolita.

Utjecaj otapala na dobiveni signal

Dimetilsulfoksid (DMSO) je standardno otapalo za pripremu matičnih otopina pa je ispitana njegova elektroaktivnost. Rezultat je pokazao kako s povećanjem detekcijskog potencijala iznad +1.0 V dolazi do nastajanja pika koji je potvrđen kao DMSO dodatnim analizama te može negativno interferirati s detekcijom drugih spojeva. Dodatak metanola kao drugog

otapala u otopinu pufera je također ispitan. Novi puferi su pripremljeni s 10% i s 30% metanola. Rezultat pokazuje značajno produljenje migracijskog vremena, smanjenje površine pika te zbog svega navedenog metanol treba izbjegavati u daljnjim ispitivanjima s MCE-EC tehnikom.

Podešavanje postavki instrumenta
Razlike između čipova sa zlatnim i platinastima elektrodama utvrđene su korištenjem SU-8-Au001T i SU-8-Pt001T mikročipova analizom dopamina. Platinasta elektroda pokazala je bolje rezultate uzimajući u obzir površinu pika, simetriju pika i migracijsko vrijeme. Osim toga, ispitan je i utjecaj geometrije elektrode na signal. Šira radna elektroda na SU-8-Pt002T čipu dala je veću površinu pika, ali je uska radna elektroda SU-8-Pt001T, očekivano, rezultirala s boljom simetrijom i visinom dobivenih pikova.

Razvoj analitičke metode
Kao dio istraživanja i razvoja metode ispitani su različiti naponi i vremena injektiranja uzorka. Primjena visokih napona te duljih vremena injektiranja rezultira s većim površinama dobivenih pikova zbog većeg volumena uzorka u području križanja mikrokanala. Kraća vremena injektiranja (4-6 s) s naponima 700-900 V/cm izabrani su kao optimalni uvjeti.
Područja linearnosti za paracetamol, dopamin i umbeliferon su redom 0.302-0.232 µg/ml, 0.766-22.977 µg/ml i 1.492-32.428 µg/ml. Limiti detekcije (LOD) i limiti kvantifikacije (LOQ) u boratnom puferu su 0.64 i 5.07 µM za paracetamol, 1.67 i 5.07 µM za dopamin, 3.03 i 9.18 µM za umbeliferon te 5.37 i 16.29 µM za 4-hidroksidiklofenak.

Metode pripreme uzorka
Mikroekstrakcija na tekućoj fazi je implementirana na MCE platformu, ali nije pokazala uspješan rezultat. Metoda nije rezultirala pikom zbog neuspješne ili vrlo spore ekstrakcije analita kroz polupropusnu membranu zbog čega molekule ispitivane tvari nisu difundirale do otvora mikrokanala.
Elektrokinetičko slaganje uzorka, s druge strane, je metoda koja je uspješno primijenjena jer je uočeno višestruko povećanje površine pikova dopamina i paracetamola.
Ekstrakcija tekuće-tekuće se pokazala kao uspješna metoda za pročišćavanje uzorka iz inkubacijskog matriksa korištenih za *in vitro* metaboličke reakcije.

Razdvajanje lijekova i metabolita elektroforezom na mikročipu
Oba para lijek/metabolit, fenacetin/paracetamol i kumarin/umbeliferon uspješno su razdvojeni korištenjem navedene metode u kombinaciji s modificiranim SU-8-Pt001T mikročipom s dugim separacijskim kanalom. Uspješno razdvajanje ovih parova temelj je korištenja MCE-EC metode u svrhu brzih, metaboličkih *in vitro* analiza.

ZAKLJUČAK

Metoda koja koristi MCE-EC instrument, razvijana u ovom istraživanju, pokazala je dobre rezultate u analizi lijekova i metabolita. Niske koncentracije metabolita uspješno su detektirane nakon optimizacije svih parametara, a metaboliti su razdvojeni od lijekova na baznoj liniji. Međutim, izazov ostaje u pronalasku metode za pročišćavanje i ukoncentriravanje uzorka, po mogućnosti implementirane direktno na mikročip, kako bi se zadržala koncepcija mikro sustava za kompletnu analizu (µTAS).

Basic documentation card

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Diploma thesis

MICROCHIP ELECTROPHORESIS-ELECTROCHEMICAL DETECTION PLATFORM FOR ANALYSIS OF DRUGS AND THEIR METABOLITES

Ines Lenić

SUMMARY

In a period of rapidly growing pharmaceutical industry development of the current analytical techniques is by far too slow to fulfil requests of demanding metabolic analysis of new pharmaceuticals and new chemical entities (environmental and household chemicals).
Portable Microchip Capillary Electrophoresis (MCE) platform with amperometric electrochemical detection (EC) was developed in this work, offering a new approach to analytical chemistry. By following Micro Total Analysis System (μTAS) concept, MCE-EC platform combines rapid analysis, low price and practicality with low requirements of reagents.

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The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 43 pages, 17 figures, 8 tables and 33 references. Original is in English language.

Keywords: microchip, electrophoresis, electrochemical detection, μTAS, screening, substrates, metabolites

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The thesis was accepted: September 2015.

Temeljna dokumentacijska kartica

Sveučilište u Zagrebu
Farmaceutsko-biokemijski fakultet
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Diplomski rad

KAPILARNA ELEKTROFOREZA NA MIKROČIPU S ELEKTROKEMIJSKOM DETEKCIJOM ZA ANALIZU LIJEKOVA I NJIHOVIH METABOLITA

Ines Lenić

SAŽETAK

U razdoblju ubrzanog rasta farmaceutske industrije razvoj postojećih analitičkih metoda je prespor i ne može zadovoljiti potrebe zahtjevnih metaboličkih analiza novih lijekova i novih kemijskih entiteta (kemikalija iz okoliša i kućanstva).
Prenosiva platforma za mikročipnu kapilarnu elektroforezu (MCE) s amperometrijskom elektrokemijskom detekcijom ispitana je u ovom radu i nudi novi pristup u području analitičke kemije. Prateći koncept Micro Total Analysis System (μTAS) ili mikro sustava za kompletne analize kombinira brzinu, nisku cijenu i praktičnost s niskim potrebama reagenasa.

Istraživanje je provedeno na kompaktnoj platformi za MCE s elektrokemijskom detekcijom nabavljenoj od tvrtke Micrux Technologies. Metoda je razvijena za odabrane parove lijekova i metabolita (fenacetin/paracetamol, kumarin/umbeliferon, diklofenak/4'-hidroksidiklofenak). Ispitani su različiti naponi, vremena analiza i postavke sustava. Također, različite metode implementirane su na mikročip s ciljem pripreme i pročišćavanja uzorka (mikroekstrakcija na tekućoj fazi i elektrokinetičko slaganje uzorka). Određeno je područje linearnosti analitičke metode te limit detekcije i limit kvantifikacije za svaki od supstrata.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

Rad sadrži: 43 stranica, 17 grafičkih prikaza, 8 tablica i 33 literaturnih navoda. Izvornik je na engleskom jeziku.

Ključne riječi: Mikročip, elektroforeza, elektrokemijska detekcija, analiza lijekova i metabolita

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Rad prihvaćen: rujan 2015.