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New approaches to the electrophoretic determination of drugs in samples with complex matrices using multifunctional coatings of a quartz capillary based on ionic liquids

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TABLE OF CONTENTS

INTRODUCTION

Relevance of the research topic. The use of modifiers in the method of capillary electrophoresis (CE) is one of the forward-looking areas and enables to significantly expand the potential of this method. Different compounds can be both immobilized on the inner wall of the quartz capillary as well as introduced into the background electrolyte (BGE) as a pseudostationary phase, that results in the growth of efficiency and separation selectivity. Along with the advantages of this method (rapidity, low sample and solvent consumption, ease of implementation, possibility of intracapillary preconcentration), this makes CE relevant in the field of pharmaceuticals and medicinal chemistry for the separation of structurally similar biologically active compounds, including enantiomers.

Despite the variety of compounds used in CE, modifiers based on the imidazolium cation are among the most universal and suitable for separating a large number of biologically active compounds, since they have a positive charge in the structure, an aromatic system, ease of injection various substituents into the imidazolium fragment, and also can participate in the processes of intracapillary preconcentration. Introducing β-cyclodextrin (CD), a frequently used chiral selector, into the structure of the imidazole cation could provide both the presence of own electrophoretic mobility of the macrocycle and additional interactions with analytes (enantiomers, hydrophobic steroid hormones), which would make it possible to control the selectivity of their separation. In addition, the fact of a synergistic effect was established with the simultaneous injection of ionic liquids (IL) and cyclodextrins into the composition of the background electrolyte during the separation of enantiomers, which also indicates the cation as stationary and pseudostationary phases in the electrokinetic determination of biologically active compounds what this research paper is dedicated to.

Extent of prior research

Alkyl imidazolium ionic liquids have already proven to be successful in various fields of CE as modifiers for the formation of dynamic and covalent coatings of the quartz capillary walls, as well as components for the formation of pseudostationary phases (micellar or microemulsion), including in chiral separation [1]. However, the injection of substituents that provide additional interactions with analytes enables to expand the scope of such modifiers. At present, the combination of CD and imidazolium IL [2, 3] is of great interest to researchers. Their combined use can improve the separation parameters, which has been shown by high performance liquid chromatography (HPLC). In the field of capillary electrophoresis, there are many works devoted to the use of both cyclodextrins and imidazolium ionic liquids. However, the potential of the latter, where one of the substituents is cyclodextrin (i.e., the imidazolium cation is covalently bound to CD), has not been revealed. There are only a small number

of works that discuss the separation of tetracyclines [4] and enantiomers of dansyl derivatives of amino acids [5, 6] when such compounds are addeded into BGE.

Objectives and tasks of the research

The *objective* of this research paper is to develop approaches to modifying the electrophoretic system with the use of compounds based on cyclodextrin and imidazolium ionic liquid, applying intracapillary preconcentration methods for further determination of biologically active substances of different nature (hydrophobic, hydrophilic) including chiral in biological liquids.

To achieve this objective, the following research *tasks* have been set:

1. Development of synthesis of a covalent coating based on imidazolium cation with the possibility of varying substituents and revealing the analytical capabilities of such coatings in the determination of analytes of various nature (hydrophobic, hydrophilic, and enantiomers).
2. Characterization of the obtained covalently bonded coatings and determination of the factors influencing the electrophoretic separation (pH, composition and ionic strength of the background electrolyte).
3. Synthesis of a modifier based on imidazolium cation and β-CD followed by the identification of its potential as a pseudostationary phase in the separation of hydrophobic analytes (corticosteroid hormones) and drug enantiomers.
4. Obtaining estimated characteristics in terms of limits of detection, separation efficiency and selectivity of the proposed approaches.
5. Search for various options for intracapillary preconcentration in order to reduce the limits of detection of analytes determination and development of hybrid methods of enantiomers on-line preconcentration.
6. Approbation of the regularities established on model systems in the analysis of biological liquids.

Scientific novelty

In the course of the dissertation research, a method for forming covalent coatings of quartz capillary walls with the ability to vary the substituent in the imidazolium cation affecting the functionality of the coating was proposed. The presence of β-CD as such a substituent enabled the simultaneous determination of hydrophobic and hydrophilic analytes (steroid hormones, biogenic amines, amino acids). The injection of a modifier based on imidazole and β-CD as a pseudostationary phase allowed for the separation of important corticosteroid hormones with high efficiency (70-90 thousand theoretical plates). With the participation of this modifier, a new method of intracapillary preconcentration of uncharged analytes was proposed: electrophoretic injection (field amplified sample

injection) of their complexes with positively charged CD derivatives. The introduction of an imidazolium fragment into β-CD allowed the use of the macrocycle as a chiral selector and for the first time performed electrophoretic separation of ketorolac enantiomers in the mode of electrokinetic chromatography (resolution factor 1.4).

A hybrid variant of intracapillary preconcentration (combination of sweeping and pH gradient) of individual enantiomers of ketoprofen and ketorolac was proposed. The achieved values of concentration factors were 295-395. A fact of migration order change of ketoprofen enantiomers in dual chiral systems, where antibiotics acted as the second selector alongside the studied chiral selector, was revealed.

Theoretical and practical significance of the work

The results explaining the mechanisms of electrophoretic separation and intracapillary preconcentration of analytes, including enantiomers of non-steroidal drugs, using modifiers based on imidazolium cation, have been obtained and systematized. The developed methods of intracapillary preconcentration provided a significant reduction in the limits of detection of biologically active compounds (up to 1-2 pg/mL for biogenic amines, 30-50 ng/mL for corticosteroid hormones, and 12-57 ng/mL for individual enantiomers of ketoprofen and ketorolac), sufficient for their determination in biological liquids (urine, human blood plasma).

The provisions submitted for defense:

1. The formation of covalent coatings of quartz capillary walls by varying the substituent in the imidazolium cation determines their multifunctionality in capillary electrochromatography.
2. Alkylimidazolium coatings of the capillary are capable of simultaneous electrophoretic determination of basic and acidic biologicaly active compounds. The presence of β-CD as a substituent in the imidazolium ring allows for the simultaneous determination both of hydrophilic (biogenic amines and aminoacids) and hydrophobic (steroid hormones) in one run, as well as acts as a chiral stationary phase.
3. The modifier based on imidazole and β-CD can act as a pseudostationary for high-efficiency separation of important corticosteroid hormones (70-90 thousand theoretical plates). The presence of own electrophoretic mobility of β-CD derivative, that is caused by imidazolium cation, can be used to implement new approaches to intracapillary preconcentration of uncharged analytes.
4. A positive charge of the imidazolium cation in β-CD structure provides additional electrostatic interactions with analytes, and subsequently, an increase in resolution factors during the separation of acidic racemates enatiomers, e.g. nonsteroidal anti-inflammatory drugs.

5. The combination of different options of intracapillary preconcentration allows for the concentration of individual enantiomers of ketorolac and ketoprofen in 290-390 times, while traditional methods are ineffective in this case.

6. The injection into the background electrolyte of a macrocyclic antibiotic vancomycin as a second chiral selector affects the mechanism of enantiomer recognition and can lead to a change in the migration order of enantiomers.

Reliability and validation of the results

The reliability of the obtained results is due to a significant amount of experimental data, the representativeness of the analyzed material sample, the use of modern methods of analysis and scientific equipment for chromatographic and electrophoretic studies. The results of the work have been published in high-ranking international and Russian journals indexed in the RSCI, Web of Science, and Scopus databases, and have been widely validated at scientific research conferences. The results have been presented at 14 international and 8 all-Russian conferences, with 7 presentations awarded first-degree diplomas.

The article "Kravchenko A.V., Kolobova E.A., Kartsova L.A. Usage of 3-methyl-1-β-cyclodextrinimidazole tosylate for electrophoretic separation and preconcentration of corticosteroids by capillary electrophoresis" was awarded a diploma by the Russian Academy of Sciences as part of the competition for scientific works by young scientists in chromatography in honor of the 150th anniversary of M.S. Tsvet.

Correspondence to the scientific specialty

The dissertation paper corresponds to item 2 "*Methods of Chemical Analysis"*, item 8 "*Masking, separation and concentration methods"*, item 10 "*Analysis of Organic Substances and Materials*" and item 15 "*Analysis of drugs"* of the specialty passport 1.4.2. Analytical Chemistry (Chemical Sciences). The tasks solved in the dissertation paper also fully correspond to the specified specialty.

Charpter I. LITERATURE REVIEW

I.1 Application of the capillary electrophoresis method in the field of medicinal chemistry and pharmaceuticals

New trends in the capillary electrophoresis are increasingly attracting the attention of scientists to this method. Among the main advantages of the method there are following ones: the ease of modification of the electrophoretic system, small amounts of the analyzed sample, and low cost (compared to HPLC). All this has led to the spread of CE in various fields of research: biomedicine, ecology, food analysis, pharmaceuticals, forensic science, etc [7, 8].

The high efficiency of capillary electrophoresis provides the determination of a large number of components in biological fluids in a short analysis time. For example, in [9] the analysis of 46 drugs in human blood was carried out during 20 minutes (Figure 1).

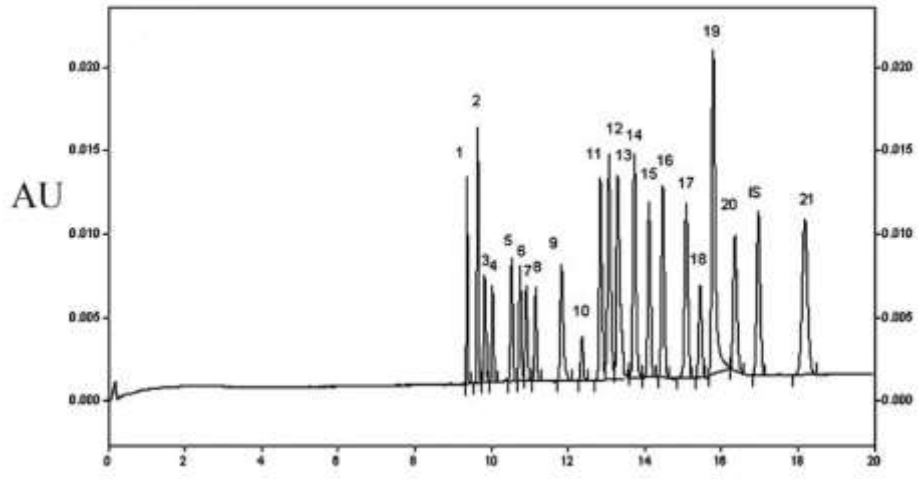


Figure 1. Electropherogram of drugs and toxic substenses.

Conditions: 150 mM phosphate buffer (pH 2.4), containind 20 % methanol. Analytes: 1-Chloroquine, 2-Pholcodine, 3-Amphetamine, 4-Methamphetamine, 5-3,4-Methylenedioxyamphetamine, 6. 3,4-Methylenedioxymethamphetamine, 7-Pseudoephedrine, 8-Ephedrine, 9-Ketamine, 10-Pethidine, 11-Promethazine, 12-Imipramine, 13-Chlorprothixene, 14-Thebaine, 15-Codeine, 16-Morphine, 17-O6-monoacetylmorphine, 18-Heroin, 19-Noscapine, 20 Haloperidol, 21-Diphenoxylate [9].

In recent years, a tendency of the active use of electrophoretic methods for the determination of drugs and their metabolites in biological fluids into biomedicine and pharmaceuticals has been noticed, which is an alternative to traditionally used chromatographic methods. The principal feature of this approach is the possibility of separating different groups of chemical comp ounds in one analytical cycle. A significant role in the evolution of CE was played by the possibility of modifying the electrophoretic

system: the introduction of various modifiers as additives, stationary or pseudo-stationary phases turned out to be a powerful resource in regulating the efficiency and selectivity separation of structurally similar compounds (metabolites, enantiomers), as well as the implementation of screening systems in the development of drugs.

I.1.1 Screneeng of biologically active compound: medicines and their metsbolites

The CE method is suitable for screening both biotherapeutics (macromolecules, protein preparations) [10, 11] and small molecules. For the first one, capillary gel electrophoresis (CGE) [12], isotachophoresis [13], and capillary zone electrophoresis (CZE) [14, 15] modes are used for qualitative and quantitative analysis of the biotherapeutics, as well as for monitoring in dosage forms their aggregates (dimers and oligomers), which may cause side effects in patients.

The CE method turned out to be extremely useful and helpful for the analysis of small molecules, since along with the determination of the main component of the drug, it allows you to control the presence of possible impurity compounds, organic and inorganic ions, to identify the enantiomeric purity of drugs, preservatives added to prevent the reproduction of fungi and bacteria [16], as well as to measure various physicochemical parameters, such as pKa values [17], lipophilicity, and the ability to associate with proteins [18]. Often, the detection of the main component in biological fluids is accompanied by the determination of metabolites [19, 20]. For these purposes, the following modes are used: capillary zone electrophoresis, capillary electrochromatography (CEC), micellar electrochromatography (MECC), electrokinetic chromatography, ligand-exchange capillary electrophoresis (LECE), etc.

Nevertheless, the CE method is still considered by a number of researchers as technically complex and insufficiently reproducible. To refute this erroneous opinion, the publication [21] is extremely useful, where the reproducibility and reliability of migration times measured by different laboratories in different countries are evaluated.

One of the approaches to achieve reproducibility of the migration times of analytes is the formation of stationary phases on the walls of a quartz capillary, which makes it possible to overcome the sorption of analytes on the walls of a quartz capillary. The choice of a specific modifier is influenced by the presence and nature of functional ionogenic groups in the analyzed analytes, hydrophobicity/hydrophilicity, and the size of the molecules. The use of various modification options using a wide range of compounds is periodically considered in detailed reviews, for example, for 2017-2019 [22] for 2019-2022 [23], and their targeted use for the determination of biologically active compounds in [24]. Reviews [25, 26] summarized the material on nanoparticles (NPs) as modifiers of electrophoretic systems, and in [1, 27] – on ionic liquids (ILs). There has been a significant increase in

publications on the use of porous materials, such as organometallic frameworks (MOFs), which can be adsorbed on the surface of capillaries in the form of ready-made structures [28, 29] or synthesized *in situ* inside a capillary. Table 1 shows examples of work over the past 5 years using various modifiers for the electrophoretic determination of low molecular weight biologically active compounds.

Table 1. Examples of the use of various compounds for the formation of coatings on the quartz capillary walls.

	Modifier	Type of coating	Determined analytes	Reference
polymers	copolymers of acrylamide and 3-acrylamidopropyltrimethylammonium	Covalently bound	Isoxsuprine, miconazole, bupropion, citalopram, verapamil, ketamine	[30]
	copolymer of acrylamide and 2-acrylamido-2-methylpropanesulfonate	Covalently bound	low molecular weight organic acids: acetoacetate, lactate, pyroglutamate, 3-hydroxybutyrate, aspartate	[31]
	copolymer of acrylamide and 2-acrylamido-2-methyl-1-propanesulfonate	Covalently bound	phosphorylated monosaccharides: ribose-5-phosphate; glucose-1-phosphate; fructose-6-phosphate; glucose-6-phosphate	[32]
antibiotic	Vancomycin	Covalently bound	Enantiomers of promethazine	[33]
IL	1-hexyl-3-methylimidazolium chloride and tetrafluoroborate	Monolayer	metanephrine, normetanephrine, vanillinmandelic acid, and homovanillic acid	[34]
nanoparticle	nanoparticles of gold NPs, L-cysteine, ionic liquid tetramethylammonium lactobionate	Monolayer	enantiomers of β-blockers propranolol, esmolol, bisoprolol and sotalol	[35]

Continuation of table 1

	Gold NPs, thiol-β-cyclodextrin	sol-gel with subsequent covalent modification	enantiomers of essential amino acids	[36]
MOF	*(HQA)($ZnCl_2$)(2.5H_2O)}$_n$* HQA=6-methoxyl-(8S,9R)-cinchonan-9-ol-3-carboxylic acid	Monolayer	Enantiomers of amino acids (AA)	[37]
	L-Cys-PCN-224 PCN: $Zr_6(\mu_3\text{-}O)_8(OH)_8(TCPP)_2$, TCPP = meso-tetrakis(4-carboxyphenyl)porphyrin	Covalently bound	AA enantiomers: histidine, methionine, valine, leucine, phenylalanine, proline, glutamine, tryptophan, aspartic acid.	[38]
	Bio-MOF-1 ($Zn_8(ad)_4(BPDC)_6O$•2Me_2NH_2,8DMF, 11H_2O, (ad= adeninate, BPDC=biphenyl dicarboxylate)	Monolayer	Non-steroidal anti-inflammatory drugs (NSAIDs): ketoprofen, flurbiprofen, ibuprofen Sulfa drugs: sulfamethazine, sulfadiazine, sulfamethoxazole	[39]
	HKUST-1 type $Cu_3(BTC)_2$ (dimeric metal units connected by linker molecules of benzene-1,3,5-tricarboxylate (BTC))	Covalently bound	enantioseparation of propranolol, esmolol, metoprolol, amlodipine and isotalol	[40]

Continuation of table 1

Mixed	*ZIF-8-poly(IL-co-EDMA)* copolymer of 1-allylmethylimidazolium chloride and ethylene dimethacrylate with inclusions of zeolite imidazolate framework-8	Monolith	Phloroglucinum; hydroquinone; resorcinol; pyrocatechin; phenol	[41]
	MOFs consisting of octakis-[6-deoxy-6-(3-mercaptopropane sodium)]-γ-cyclodextrin (sugammadex) and a transition metal ion (Cu^{2+}) with polydopamine as a linker	Monolayer	dansyl derivatives of DL-AA	[42]
	MOF [Mn(cam)(bpy), cam = (1R,3S)-(+)- camphoric acid, bpy = 4,4'- dipyridyl.	Covalently bound	Sulfonamides: sulfamerazine, sulfanitran, sulfadiazine, sulfadimethoxine, sulfamonomethoxine, sulfachloropyridazine, sulfamethoxazole, sulfamethizole, phthalylsulfathiazole	[28]

Modification of the walls of a quartz capillary

There are three types of stationary phase capillaries used in CE: packed, open-tubular and monolithic (Figure 2). Due to the complexity of preparing frits, as well as the frequent formation of bubbles and the poor reproducibility of packed columns, in recent years, most of the work has been devoted to open-tubular and monolithic options for modifying capillaries.

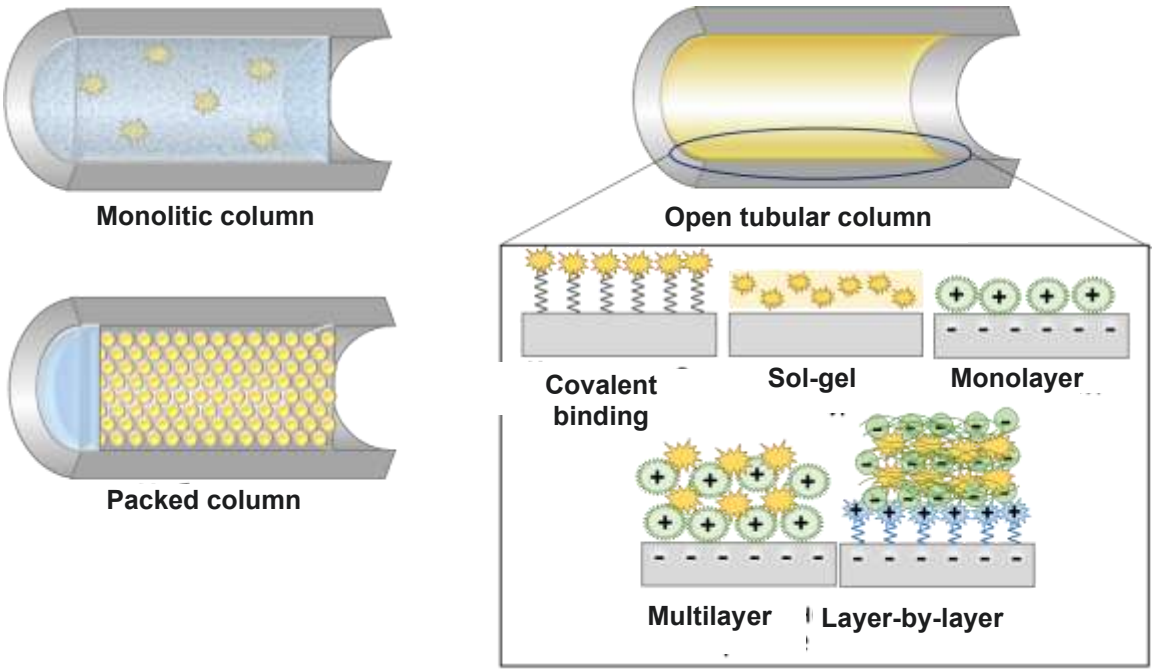


Figure 2. Sketcchy of popular types of stationary phases that are using in CE [43].

When producing monolithic columns, macroporous material as a result of polymerization is made *in situ*, however, this approach requires more modifiers compared to open-tubular columns. For the latter there are several approaches proposed to form them on the capillary surface: covalent bonding or sorption (dynamic coatings, mono- and multilayer coatings) (figure 2). In case of covalent bonding, the activation of capillary walls with obtaining of silanol bonds and further performance of reactions is a compulsory stage: polymer layer is formed by polymerization or copolymerization [44, 45], including the use of silylation agents, which have become widespread due to low toxicity and the ease of making bond Si-O [46, 47]; sol-gel method (sol, which is a highly dispersed system, forms gel where solvent molecules are in ordered 3D net) [48–51], and layer-by-layer self-assembly [52–55], with gradual rinsing of a quartz capillary with solutions of modifiers and layers cross-linking. In most cases, subsequent functionalization of the obtained coatings takes place for the targeted determination of specific compounds through non-covalent interactions such as ionic, π-π-stacking, hydrogen bonds.

Methods for obtaining covalently bound stationary phases are based on chemical reactions, but their progress is difficult to control, as the capillary is a closed system. The modification can be often confirmed by the characteristics obtained by physico-chemical methods [56]:

- *Surface charge of quartz capillary walls.* Coating formation is often followed by the change of the surface charge and, consequently, the change of value and direction of electroosmotic flow (EOF): if a modifier on the capillary surface belongs to a cation functional group, EOF will become anodic; if the capillary is unmodified, EOF will be cathodic. The value of EOF will also be influenced by coating density and pH of a background electrolyte, and the repeatability of EOF velocity indicated coating stability from analysis to analysis.

- *Surface morphology and coating thickness* is determined by scanning electron microscopy (SEM), which has become an indispensable method for visual confirmation of the modification on the inner wall of the quartz capillary (figure 3). The photographs of the inner surface of capillaries with a covalently bound stationary phase show roughness, hilly and uneven surfaces, missing in unmodified capillaries which have smooth surface. In addition, SEM photographs can be used to evaluate the density and uniformity of the obtained coating. Evaluation of coating stability by obtaining the pictures before and after the run of analyses is a noteworthy way of SEM application.

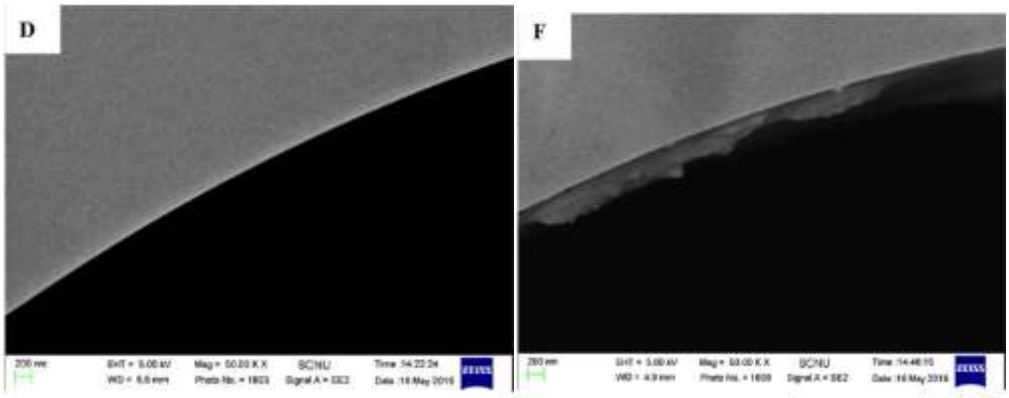


Figure 3. The photographs of unmodified and modified capillaries obtained by SEM [55].

- *Elemental analysis, chemical structure* (oscillation and absorption spectroscopy). Elemental analysis carried out by X-Ray Energy Dispersive Spectroscopy method enables to obtain information about materials, consisting of certain elements, *such as metallic zinc, gold or carbon in grapheme, metal-organic frameworks and nanoparticles [35-37, 57]. Fourier transform* infrared spectroscopy is required to confirm expected covalent bonds and, as a consequence, the actual attachment of the modifier to capillary inner walls.

- Crystalline structure (X-ray structural analysis). Recently, X-ray structural analysis and X-ray photoelectron spectroscopy [57] have been used to confirm chemical structures. The first method is important for identifying and confirming the crystalline structure of the modifiers used, while the second allows for the detection of the formation of covalent bonds, including when attached to the walls of the capillary. Confirmation of the coating structure, as well as its stability, is necessary for controlling

the reproducibility of coating characteristics from capillary to capillary and is particularly important when using modified capillaries in routine analyses.

Injection of modifiers into the background electrolyte

Modifiers injected into the background electrolyte are not only capable of forming dynamic coatings on capillary walls (mono- and multilayered), but can also act as a pseudostationary phase, implementing MEKC, microemulsion electrokinetic chromatography (MEEKC), or EKC modes depending on the type of modifiers used and their interactions with the analytes. One of the main advantages of such variants is the ability to vary the concentration of the modifier in the background electrolyte, providing more interaction sites with the analytes, while the number of sites on capillary walls may be limited by the specific surface area of the coating. In addition, such methods are indispensable for separating neutral lipophilic compounds, such as fat-soluble vitamins [58] and steroid hormones [59]. The absence of restrictions on the structure and quantity of modifiers used in the background electrolyte expands the analytical possibilities for finding separation conditions for structurally similar compounds, including enantiomers. It is important to note that CE can solve very complex problems in the field of chiral separation. For example, a ligand-exchange micellar electrokinetic chromatography with copper(II)-L-isoleucine complexes as chiral selectors incorporated into sodium dodecyl sulfate (SDS) micelles has been developed for the enantioseparation of ofloxacin and four related substances (Figure 4) [60].

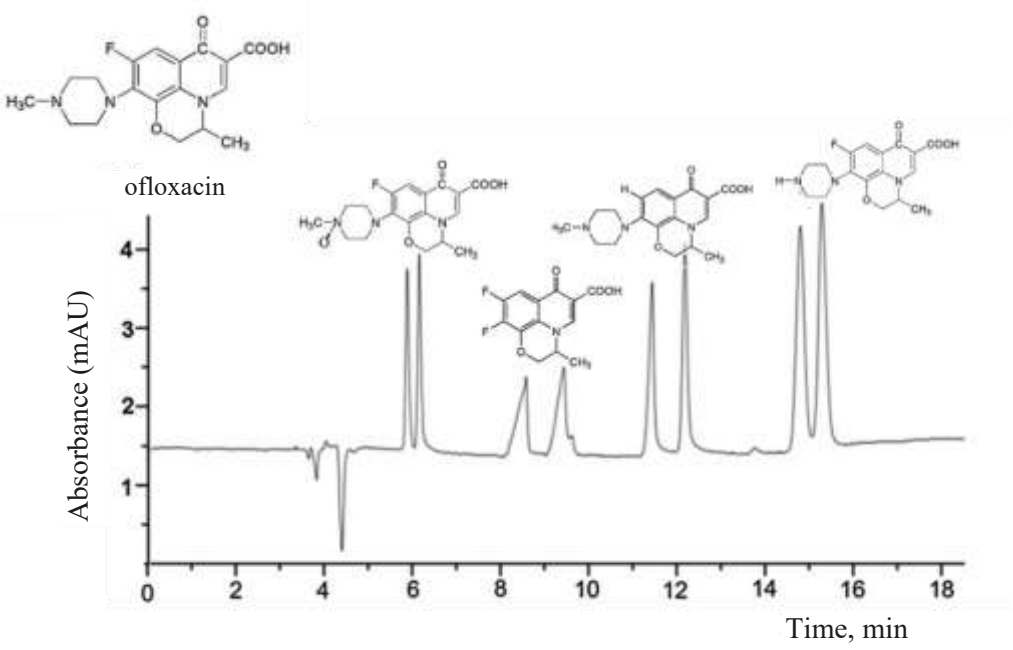


Figure 4. Electropherogramm of simultaneous enantiomer determination of impurities of oflaxicine and their structure [60].

At the same time, cyclodextrins still remain dominating modifiers in separation of enantiomers. (see Chaper 1 section 2).

I.1.2 Other applications of the CE method in modifying electrophoretic system

An interesting application of CE is the ability to study metabolism, including enzymatic reactions, using one part of the capillary as a microreactor and the other for separating reaction products [61, 62]. The first such experiment was carried out in 1991 by Banke for the analysis of alkaline protease [43].

As an example, let us consider the glucuronidation of acetaminophen - an analgesic and antipyretic drug. Microsomes extracted from rat liver were encapsulated in tetramethoxysilane-based matrices to perform drug metabolism with subsequent determination of metabolites. Substrates were electrokinetically injected into the microreactor and metabolized by uridine-5'-diphosphoglucuronyltransferase (UDPGT). Then, metabolites and unreacted substrates were separated and detected by electrophoresis (Figure 5). The encapsulated column with microsomes can be reused without loss of activity for at least two days, allowing for high-throughput screening [63].

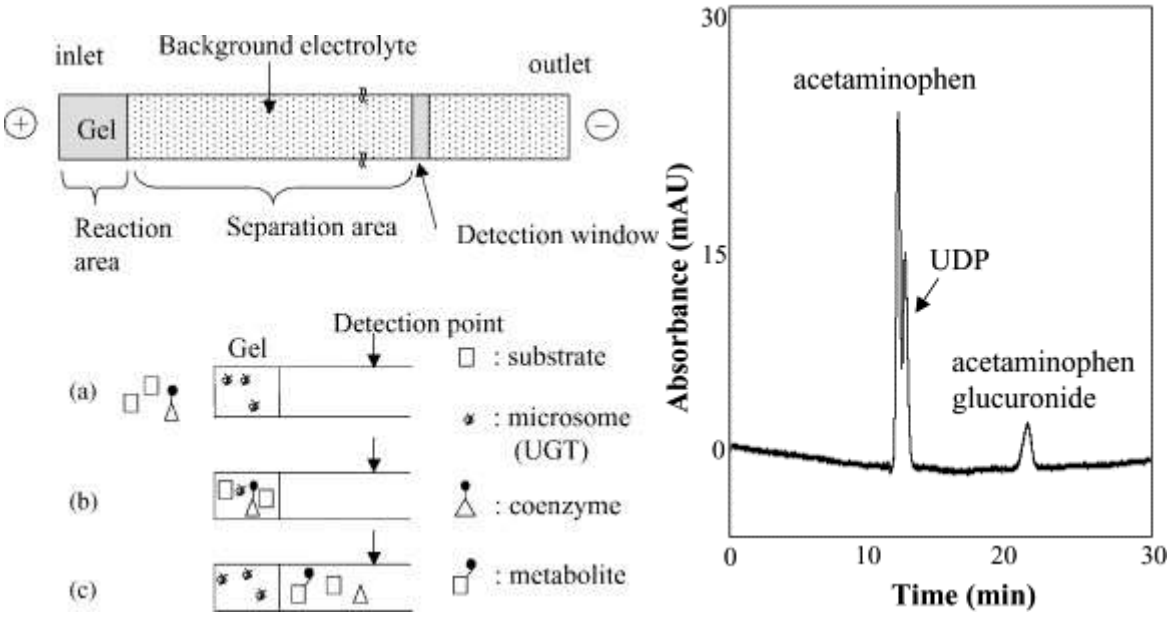


Figure 5. Scheme of analytical system for determination of metabolites of medicenes (a) and electropherogram of on-line screneeng of metabolism of acetaminophen (b).

Conditions: sample 2 mM, 20 мМ UGT и 10 mM $MgCl_2$; injection: 4 kV, 20 s; length of gel with incaplusated microsomes: 2.0 cm; total lenght: 34 cm; background electrolyte: 20 mM phosphate buffer solution (pH 7,5), applied voltage: 4 kV; wavelenhgt: 254 nm.

Modification of quartz capillary walls allows for screening the effectiveness of lactose derivatives as antagonists of the interaction between leukocytes and endothelial cells. Overexpressing cells NEK 293 (Human Embryonic Kidney 293) – a cell line derived from human embryonic kidneys – were immobilized on the inner walls of capillaries as a stationary phase, imitating endothelial cells lining the inner surface of blood vessels; (N-[2-(1,3-dilactosyl)-propanyl]-2-amino-pentandiamide (Gu-4)) was added to the background electrolyte to imitate the drug in the blood. The shape of the electrophoretic peak was used to evaluate the interaction between lactose derivatives and stationary phases (Figure 6) [64].

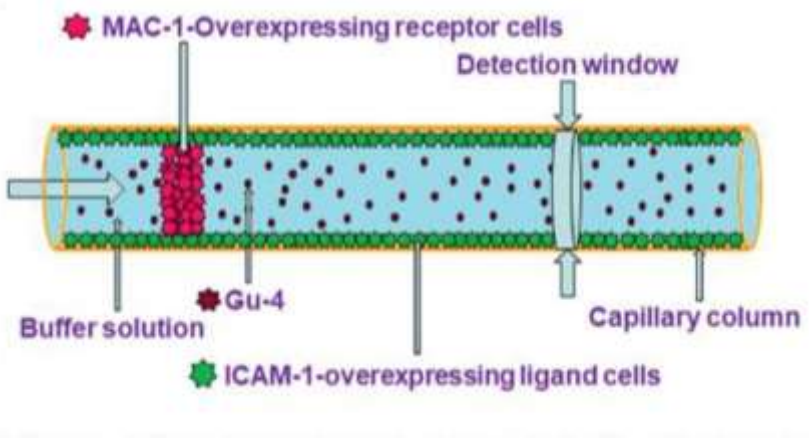


Figure 6. The imitation of blood system [64].

I.2. Chiral separation by capillary electrophoresis using cyclodextrins

Enantiomers separation is one of the challenging, complex, and important tasks in the field of pharmaceutical and medicinal chemistry. Enantiomers of a drug can differ significantly in activity, toxicity, and their effects on living organisms. One enantiomer usually has the desired therapeutic activity (eutomers), while the other (distomer) may be inactive or even exhibit antagonist effects, causing undesirable side effects and toxicity. This is why it is necessary to control the enantiomeric purity of drugs using separation methods to ensure the required resolution factors for the analyte and its enantiomeric impurity. A well-thought-out on-line preconcentration strategy can increase sensitivity and detect this impurity at low concentrations. Furthermore, the migration sequence of enantiomers during determination of enantiomeric impurity is also important [65]: it is desirable for the impurity component to migrate first to avoid overlapping with the main peak. The most commonly used separation and preconcentration methods for enantiomers are HPLC, CE, and gas chromatography. HPLC is the leader in this field (figure 7), but it requires expensive columns with chiral stationary phases, while capillary electrophoresis has higher efficiency, minimal sample volume requirements, and solvent type flexibility.

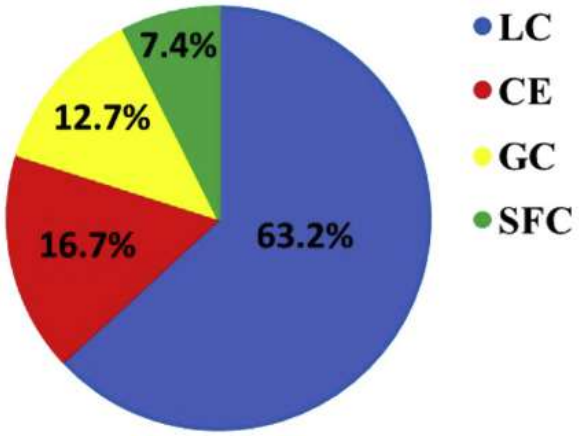


Figure 7. The number of papers published during 2015-2019 years on chiral separation with the use of different separation techniques. Data were obtained from database Web of Science (August 2019) using keywords "chiral analysis", "Liquid chromatography", "capillary electrophoresis", "gas chromatography", "supercritical fluid chromatography" [65].

Another advantage of CE in the separation of enantiomers is the presence of ionogenic compounds with their own electrophoretic mobility when voltage is applied, which is determined by the characteristics of the method itself. This contributes to the mechanisms of separation and allows surpassing the thermodynamic selectivity of chiral recognition, which cannot be achieved with HPLC, where the selectivity of chiral separation is solely determined by the difference in affinity of enantiomers to the chiral selector [66].

Cyclodextrins (CDs) [67-72], which are cyclic oligosaccharides formed by 6 (α-CD), 7 (β-CD), or 8 (γ-CD) α-1,4-D-glucopyranoside units in a toroidal structure with hydrophobic cavities inside and hydrophilic surfaces on the outside, have proven to be successful chiral selectors in CE. It is the presence of the hydrophobic cavity that promotes the formation of inclusion complexes with enantiomers. The different spatial structures of enantiomers lead to differences in their interaction with the CD cavity, as well as with the hydroxyl groups on the CD surface. The affinity of an enantiomer to the CD cavity can be numerically characterized by complexation constants, but this does not provide information about the structure of the complex itself. Computer modeling is often used to solve such problems and the hypothetical structures are confirmed by nuclear magnetic resonance spectroscopy [69, 73, 74]. For example, for ibuprofen and β-CD, it has been shown that the enantiomers of ibuprofen in the CD cavity are oriented with their nonpolar group towards the wider β-CD cavity; the R-enantiomer moves more freely inside the macrocycle cavity, while the S-enantiomer demonstrates more restricted movement. In addition, the S-enantiomer forms a significant number of hydrogen bonds with the secondary hydroxyl groups of β-CD, while the R-isomer does not possess this characteristic. All these factors lead to

differences in complexation constants and electrophoretic mobilities of the corresponding complexes and, as a result, to chiral separation (Figure 8).

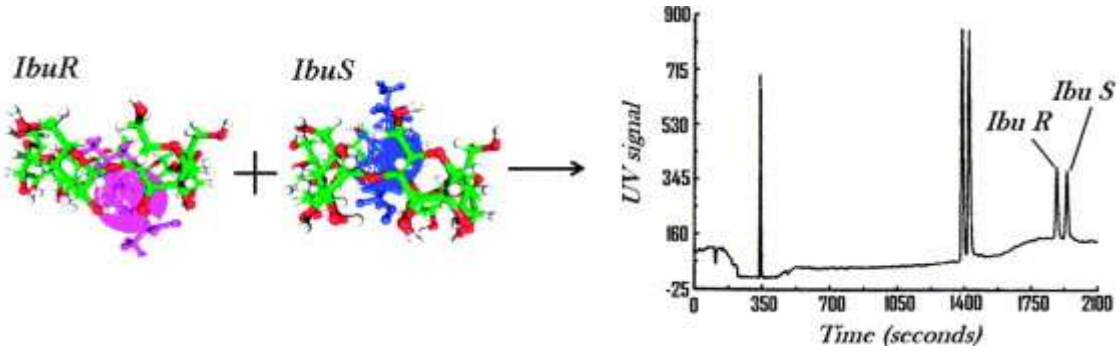


Figure 8. The scheme of the formation of complexes between ibuprofen enantiomers and CD [68].

Attempts are being made to study complexation processes and separation methods. The authors [75] were able to assess the contribution of inclusion complex formation to enantioselectivity when analytes approach from the narrow or wide side of the cyclodextrin (CD). Covalent attachment of CD to silica gel allows for the fixation of the oligosaccharide in a way that only one side (narrow or wide) remains accessible for complexation with the enantiomer. Mono-substituted azide-CDs, where the substitution occurs on the primary (narrow side) or secondary (wide side) hydroxyl group, were used for further click reaction with alkyne-functionalized silica gel surface (Figure 9). Analysis using two such types of stationary phases, where interaction is only available on one side of the CD, allows to conclude that for dansyl derivatives of leucine enantiomers, the complex formed when the analyte approaches from the wide side of the CD contributes more to enantioselectivity, resulting in higher enantioselectivity factors. In the case of flavone, the migration order of enantiomers even changes depending on the orientation of CD in the stationary phase (Figure 9).

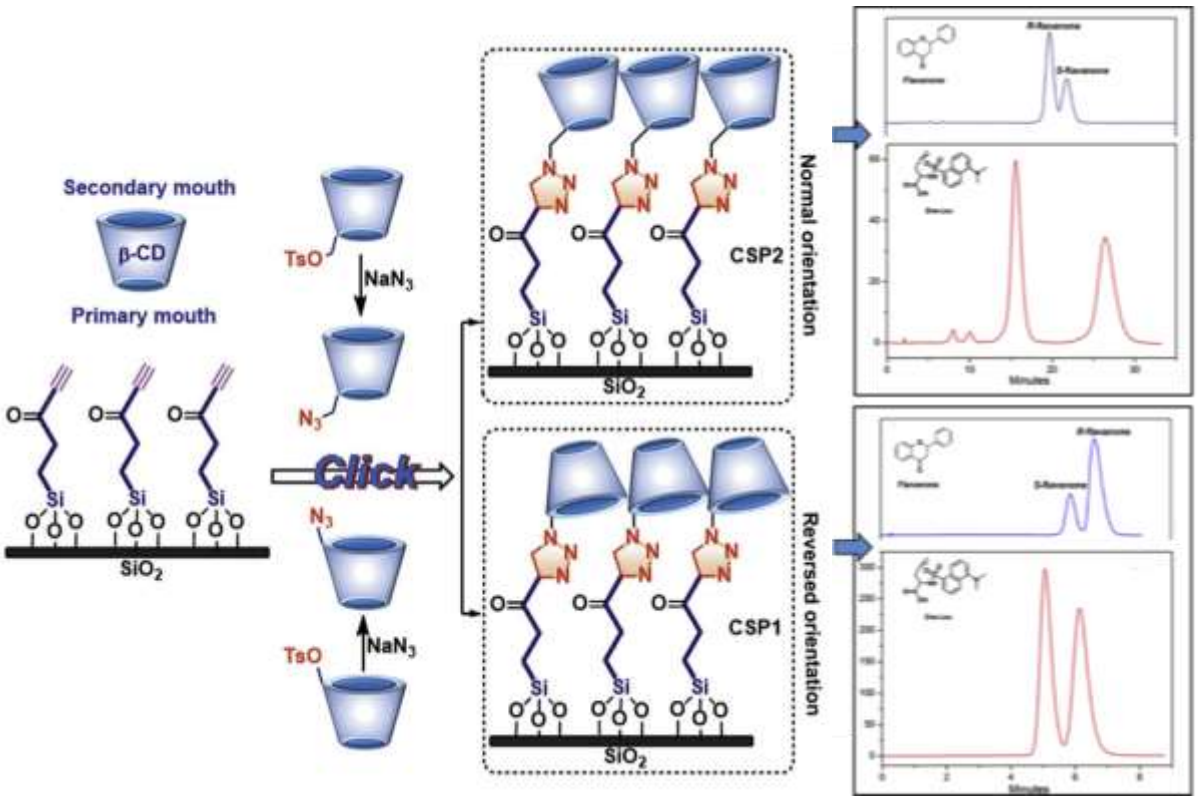


Figure 9. The scheme of syntesis stationary phase based on CD with normal and reversed orientation and chromatograms of dansyl derivatives of D,L-leucine and R,S-flavone [75].

Conditions: 40 % MeOH, 60% triethylammonium acetate buffer (pH 5.01), 1 mL/min, 30 ^{0}C

However, the use of classical CDs as chiral selectors does not always allow for complete separation of enantiomers. In some cases, it is necessary to increase enantioselectivity factors with the help of additives into the composition of BGE along with the existing chiral selector, which contribute to the implementation of an additional mechanism of interaction with analytes. Another forward-looking direction is the modification of cyclodextrins themselves: injection different charged groups into the macrocycle can not only affect resolution factors for neutral analytes but also enhance selectivity against oppositely charged enantiomers relative to CD derivatives through additional electrostatic interactions. These two directions are described in more detail below.

I.2.1 Substituting the hydroxyl groups in cyclodextrin molecules

Substituting the hydroxyl groups in cyclodextrin molecules with various positively or negatively charged functional groups expands the analytical capabilities of these macrocycles for enantiomer separation [76, 77]. Uncharged CDs (native and derivatized) can only be used for separating charged analytes, as they migrate together with EOF. The electrophoretic mobility of such a complex is only provided by the electrophoretic mobility of the analytes themselves [70, 71]. In turn, charged CDs can not only provide chiral separation of racemates but also exhibit good resolving power against oppositely

charged analytes through both inclusion complex formation and electrostatic interactions [78-81]. The counter-directional migration of enantiomers and the chiral selector allows for the use of low concentrations of the latter, reducing the solution viscosity and preventing high current values. Furthermore, introducing substituents into CDs can be considered as a way to change the size of their cavities, which also affects the efficiency of "host-guest" complex formation and chiral recognition.

Cyclodextrins have 18, 21, and 24 free hydroxyl groups in the case of α, β, and γ-CD, respectively: one primary and two secondary in each glucopyranose unit, which can undergo chemical modifications (Figure 10). The primary hydroxyl groups (OH-6) are the most basic, nucleophilic, and sterically accessible, and can be easily modified by various electrophiles. Hydrogen bonding between the OH-2 and OH-3 of neighboring glucopyranose units and the adjacent electron-accepting anomeric acetal function result in the highest acidity of OH-2. These hydroxyl fragments can be selectively deprotonated, for example, under anhydrous basic conditions. The OH-3 hydroxyl groups are less reactive and least sterically accessible. Modification at this position requires the use of protecting groups or reagents capable of firmly binding to the CD cavity [69].

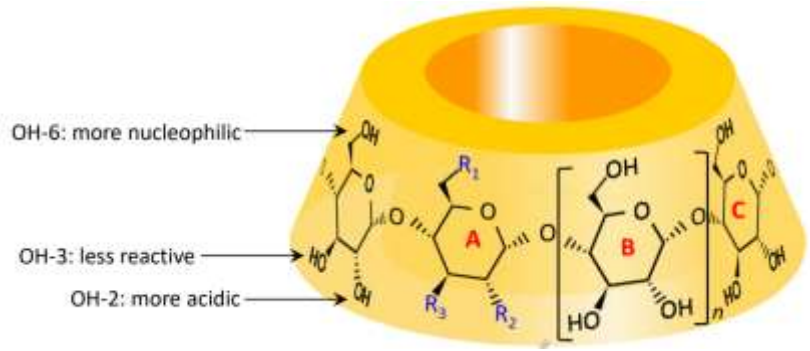


Figure 10. The scheme of cyclodextrine with possible modification cites [69]

Control of the CD substitution degree is important during modification. The use of mono-substituted CDs compared to commercially available randomly substituted CDs is most preferable: such CDs provide greater reproducibility and enantioselectivity. Currently, a significant number of mono-substituted CD derivatives with descriptions of their synthesis methods are known. In this series, among negatively charged ones, sulfated, carboxymethylated, and sulfobutyl ether CDs can be distinguished, the synthesis and application of which are the subject of a comprehensive review [83]. Cationic derivatives of CD usually include pyrrolidine [84, 85], amino groups [79, 86-89], or imidazole [5, 90]. The latter have several advantages: an aromatic system, ease of substituents injection into the structure of the imidazole ring, additional interactions with analytes. However, their full potential has not yet been fully realized. Imidazole derivatives of CD can be effective chiral selectors for the separation of

negatively charged analytes that contain an aromatic system in their molecules. These include, for example, nonsteroidal anti-inflammatory drugs due to the presence of an aromatic imidazole ring in their molecules, contributing to enantioseparation through π-π interactions between the imidazolium cation and the drug [6].

Additional contribution to chiral recognition can also be made by hydrogen bonds between analytes and substituents in CDs, the nature of which is crucial. For example, it has been shown in [69] that out of two homologues – mono-6A-(2-methoxyethylamino)-β-CD (MEtAMCD) and mono-6A-(4-methoxybutylamino)-β-CD (MBuAMCD) – it is MEtAMCD that provides better enantioselectivity towards dansyl derivatives of amino acids, as well as phenoxyalkanes and hydroxy acids, due to the formation of hydrogen bonds, as confirmed by NMR spectroscopy (Figure 11).

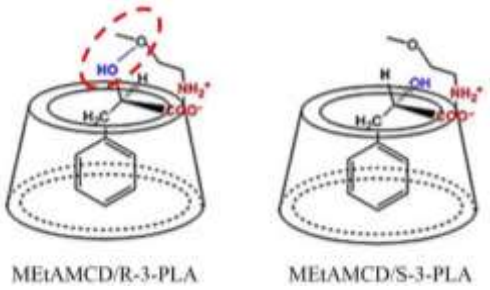


Figure 11. Steric differences in complexes formation between MEtAMCD and 3-phenyllactic acid (3-PLA). Additional hydrogen bond responsible for chiral selectivity is highlighted red line [69].

Substituents into the structure of cyclodextrin not only enhances chiral recognition through additional interactions but can also completely change the mechanism of enantiomer separation, up to the implementation of ligand-exchange CE mode. β-CD, whose hydroxyl group is substituted with an imidazole-linked histamine was successfully used for the separation of non-derivatized racemate of tryptophan in the presence of copper (II) ion by ligand exchange mechanism. In this case, cyclodextrin itself acted as a ligand in the first coordination sphere [91]. The proposed structures of complexes are presented in Figure 12A. Stable complexes were formed at CD concentrations above 0.6 mM, and enantiomer separation was not observed (Figure 12B). Complete separation was achieved at low modifier concentrations (0.10 and 0.25 mM), when the equilibrium shifted towards complex

dissociation. Slightly higher stability of the L-enantiomeric complex led to different migration times of these two enantiomers and better resolution (Figure 12B).

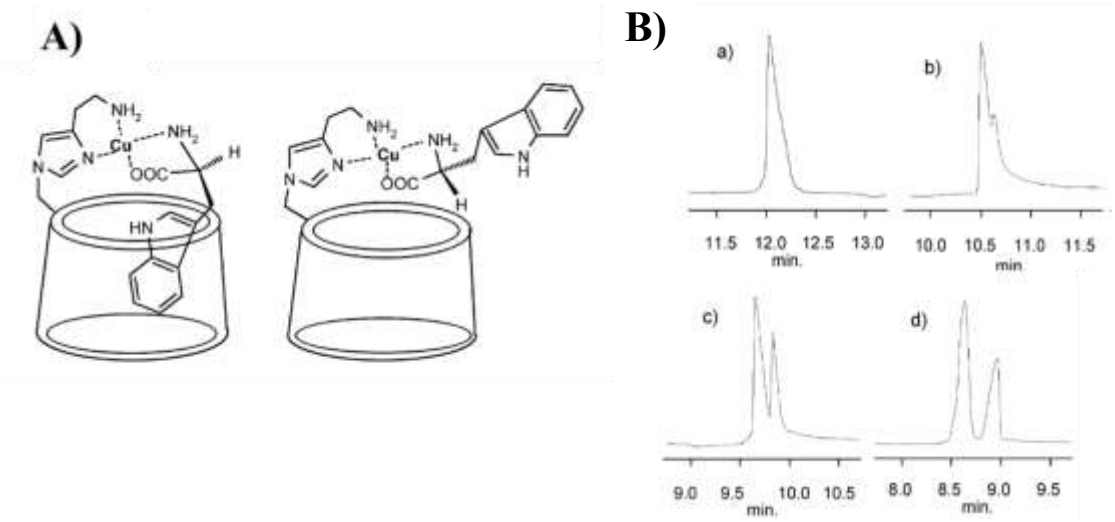


Figure 12. The estimated structures of complexes between L-(left) or D-(right) tryptophan with β-CD substituted with an imidazole-linked histamine (A) and electrophoregram of separation of tryptophan enantiomers in LECE mode with modifier concentraton 1.8 (a), 0.8 (b), 0.6 (c), 0.25 mM (d) (B) [91].

I.2.2 System of two modifiers in the background electrolyte for enantiomer separation

In some cases, enantioselectivity can be improved by injecting a second modifier into the background electrolyte, if it is also a chiral selector. Such a system is called a *dual chiral system*. One of the most studied systems is neutral CD (native, derivatized) in combination with charged (anionic, cationic, zwitterionic) CD. In such systems, the increase in enantioselectivity is achieved due to the difference in complexation mechanisms of two different CDs with enantiomers [82]. Despite the fact that such combinations remain one of the most commonly used approaches in chiral CE, the search for alternative additives is still relevant. The emergence of new functional materials, such as ionic liquids, deep eutectic solvents (DES), nanoparticals, molecularly imprinted polymers (MIPs), MOFs, etc. [92], has significantly influenced the analytical capabilities of CE, where they have been actively used as additives in the background electrolyte, including for enantiomer separation (Figure 13A).

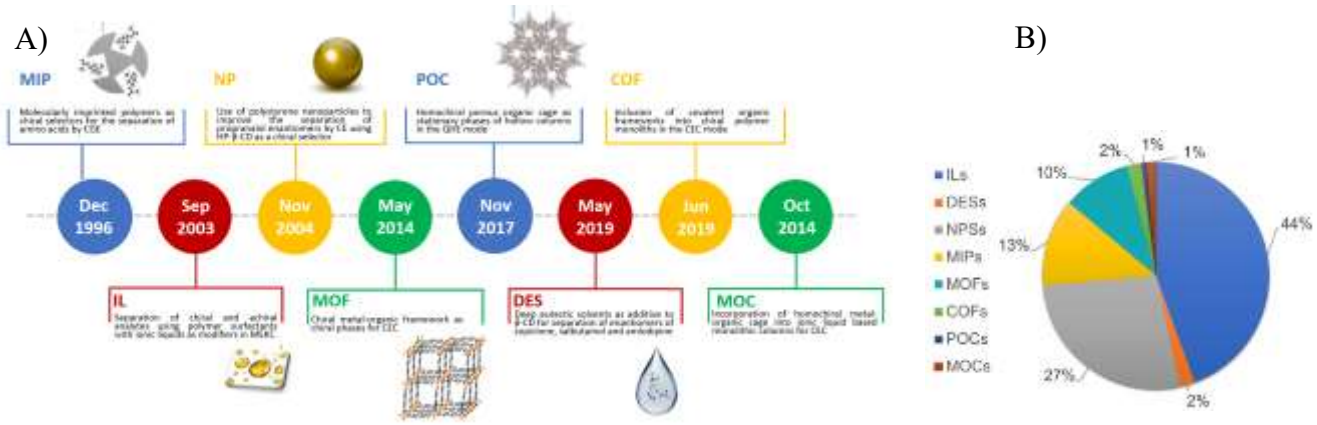


Figure 13. New functional materials as chiral selectors and their first application in CE (A) and the number of their mentions in the field of chiral CE (B) (based on [92].

Ionic liquids (~44% among other new functional materials) have found wide application as additives in the background electrolyte for chiral separation (Figure 13B) [92]. Despite the large number of publications [93-100], research in this area continues, which is due not only to the variety of IL structures (variability of cations and anions) but also to the unique properties of these compounds: solubility in organic solvents and water, high conductivity, electrostatic interactions with analytes [101]. Ionic liquids have already found active application in capillary electrophoresis: when introduced into the background electrolyte, they can act as a pseudostationary phase [93-95], modifier of quartz capillary walls [96,97], or chiral selector [98-100]. Whether they remain ionic liquids as such in this case or whether they are a mixture of independent cations and anions is a subject of current terminological discussions [65].

Both achiral and chiral ILs are used, with the cation, anion, or both ions being chiral in the latter case [102]. In typical achiral ILs used for enantiomer separation in CE, the cation is usually tetraalkylammonium, alkylimidazolium, or alkylpyridinium, and the anion is usually an inorganic anion (e.g., OH-, Cl-, Br-, [BF4]-, [PF6]-). Achiral ILs in the background electrolyte in the presence of a chiral selector can act as modifiers, contributing to increased selectivity of separation due to the following factors:

- change in the ionic strength of the background electrolyte, which can affect the magnitude of the electroosmotic flow and current strength; this, in turn, will lead to changes in the migration characteristics of analytes and efficiency;

- adsorption of IL cations on the inner surface of the capillary walls, which prevents the adsorption of analytes, suppresses cathodic EOF, and even reverses it to anodic: migration parameters of enantiomers change, and efficiency increases;

- influence on chiral recognition through additional interactions with analytes and the existing chiral selector.

Among the first examples of using achiral ILs as additional modifiers for chiral separation, the work [103] can be noted, where chiral naphthols (±)-1,1'-bi-2-naphthol (BOH), ((±)-1,1'-bi-2-naphthyl-2,2'-diyl hydrogen phosphate (BNP), and 1,1'-bi-2-naphthyl-2,2'-diamine (BNA) (Figure 14) were separated in MEKC mode using poly(oleoyl-L-leucylvaline sodium) as a micelle-forming and chiral agent. Only when an achiral IL was introduced into the background electrolyte as a modifier of the chiral system, it was possible to separate all three pairs of enantiomers in one cycle of electrophoretic analysis. When studying the influence of the nature of ILs, including tetrafluoroborate 1-butyl-3-methylimidazolium ($BMImBF_4$), tetrafluoroborate 1-ethyl-3-methylimidazolium (EMImBF4), hexafluorophosphate 1-ethyl-3-methylimidazolium ($EMImPF_6$), 1-ethyl-3-trifluoromethanesulfonate methylimidazolium ($EMImSO_3F_3$), and chloride 1-ethyl-3-ethylimidazolium (EMIMCl)), on the resolution factors of enantiomers in the simultaneous separation of three racemates, it was found that the ionic liquid $EMIMPF_6$ provided the best separation of all racemates except BOH (Figure 14, electropherogram II). Successful resolution was achieved using ILs $BMImBF_4$ and $EMImPF_6$ (Figure 14, electropherograms III, IV).

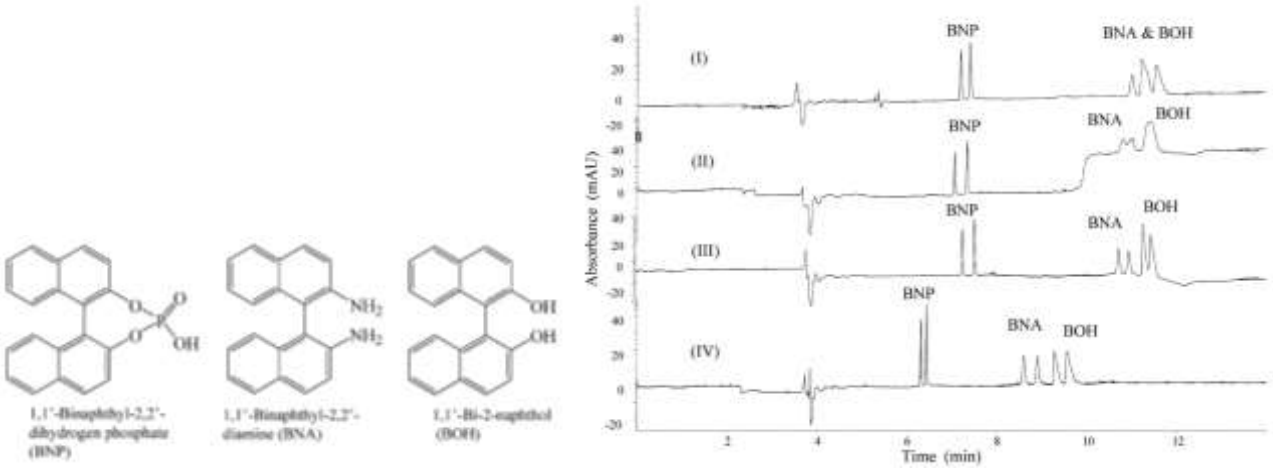


Figure 14. Structures of three racemic mixures binaphtyl derivatives (left) and electropherograms of their sepration (right) whithout IL (I); with addition 5 mM $EMImPF_6$ (II); 1 mM $BMImBF_4$ (III); 2 mM $EMImBF_4$ (IV).

Conditions: 0.5% poly(oleoyl-L-leucylvaline sodium), 10 mM TRIS, 10 мМ borat buffer (pH 10.0); sample injection: 3 s 30 mbar, 15 °C, 30 kV, 245 nm [103].

Despite the fact that the use of achiral ILs allows for improved enantiomer separation, chiral recognition is achieved through interactions between enantiomers and the chiral selector. Therefore, the addition of achiral ILs can only slightly increase resolution (Rs). The use of chiral ILs is more promising,

as unlike achiral ILs, they can contribute to enantiorecognition being able to modify the electrophoretic system at the same time. In dual chiral systems, where both a chiral selector and a chiral IL are present in the background electrolyte, a synergistic effect is observed in the enantiomer separation process. Confirming this synergistic effect and demonstrating that chiral ILs not only modify the electrophoretic system but also participate in the chiral recognition process has become an important task.

The synergistic effect of using chiral ILs and cyclodextrins for enantiomer separation was first demonstrated in [104, 105]. The authors thoroughly studied the possibility of this phenomenon using a model system consisting of three components: an analyte, chiral IL, and a derivative of CD (Figure 15).

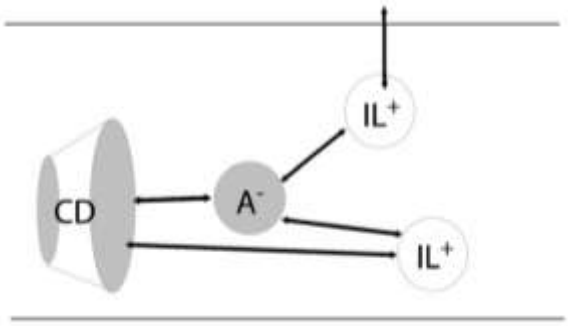


Figure 15. Scheme of possible interaction in the system: analyte, chiral IL (chiral cation in BGE composition or absorbed on capillary wall) and CD derivative [105].

Ethyl- and phenyl-holin bis(trifluoromethylsulfonyl)imide were used as chiral IL cations. To determine the specific effect of the chiral cation, parallel experiments were conducted where $LiNTf_2$ was introduced into the background electrolyte instead of the chiral IL under the same conditions. Additional experiments on a capillary coated with polybrene were performed to evaluate the influence of adsorbed IL on the capillary walls [105]. The model mixture included arylpropionic acids (naproxen, ibuprofen, ketoprofen, suprofen, carprofen, indoprofen). It was found that the nature of the CD affects the competition between the analyte and the IL cation through complexation with the CD cavity. The introduction of the IL increases the resolution factors of enantiomers due to the salt effect (the IL acts as a modifier of the electrophoretic system). The increase in resolution factors through selective separation confirms the existence of a synergistic effect when both selectors are present. In the case of chiral ILs, specific ion-pair interactions can be involved in chiral recognition.

There are reports of using chiral ILs as single chiral selectors [99, 100], but there are far more studies where they are used in combination with traditional chiral selectors, most commonly CDs.

Unfortunately, finding regularities in chiral separation is extremely complex. Examples of enantiomer separation in dual IL-CD systems are provided in Table 2 with some comments that can be taken into account when working in this direction.

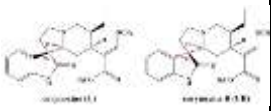
Table 2. Enantiomers separation when using dual chiral selectors in the background electrolyte (chiral IL and CD)

Nature of CD	Used chiral IL	Determined enantiomers	Comments	Ref.
α-CD	Tetramethylammonium-L-Arginine ([TMA][L-Arg]), Tetraethylammonium-L-Arginine ([TEA][L-Arg]), Tetrabutylammonium-L-Arginine ([TBA][L-Arg]), Tetramethylammonium-L-Proline ([TMA][L-Pro]), Tetramethylammonium-L-Glutamic Acid ([TMA][L-Glu], Tetramethylammonium Hydroxide ([TMA][OH])	amlodipine, citalopram, nefopam; sulconazole	In optimized conditions, enantiomeric separation of five model drugs with different structural types was performed	[106]
β-CD	[TMA][L-Arg], [TEA][L-Arg] and [TBA][L-Arg]	Dansyl derivatives of amino acids	A synergistic effect of chiral ionic liquids (CILs) and cyclodextrins (CDs) in nonaqueous capillary electrophoresis mode was observed. The participation of CILs in enantio-recognition was confirmed by experiments where CILs were used as sole chiral selectors. .	[107]

Continuation of table 2

hydroxypropyl-β-CD (HP-β-CD)	[TMA][L-Arg], [TEA][L-Arg] and [TBA][L-Arg] [TMA][L-Arg], tetramethylammonium-L-hydroxyproline ([TMA][L-Hyp]) And tetramethylammonium-L-isoleucine ([TMA][L-Ile])	Amlodipine, nefopam, duloxetine, propranolol	As in the case of two dual systems TMA-L-Arg/HP-β-CD and TMA-D-Arg/HP-β-CD, the influence of IL stereocenter configuration on enantiomeric separation was studied. The values of the EOF velocity were the same in both systems, but the type of stereoconfiguration of the cation affected the results: a noticeable increase in Rs for drug enantiomers in the HP-β-CD/TMA-D-Arg system was not observed, while in the HP-β-CD/TMA-L-Arg system, a positive synergistic effect was observed for all drugs.	[108]
	tetramethylammonium-L-lysine ([TMA][L-Lys]), tetramethylammonium-L-lysine ([TBA][L-Lys]) and tetrabutylammonium L-glutamic acid ([TBA][L-Glu]), [TMA][L-Glu]	duloxetine, verapamil, terbutaline, econazole, sulconazole, metoprolol, nadolol	The synergistic effect was observed in the dual chiral system CIL/HP-β-CD only for five compounds (duloxetine, verapamil, terbutaline, econazole, sulconazole) for which incomplete separation of enantiomers was previously achieved in a system with a single CD.	[104]
	[TBA][L-Lys], [TBA][L-Glu]	econazole and sulconazole	The first analytical method for determining the enantiomers of sulconazole in pharmaceutical preparations (antifungal ointment) was developed. The dual system [TBA][L-Lys]/HP-β-CD was used for enantiomeric separation (Rs 3.4 and 2.4 for econazole and sulconazole, respectively), and the analytical characteristics of the developed methods were evaluated. Analysis of real objects was performed.	[109]

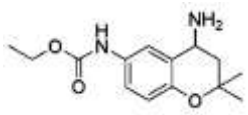
Continuation of table 2

hydroxypropyl-β-CD (HP-β-CD)	1-ethyl-3-methylimidazolium-L-lactate	ofloxacin, propranolol, dioxo-prometazine, isoprenaline, chlorpheniramine, liarozol, tropicamide, amlodipine, brompheniramine, homatropine	A synergistic effect between CDs and ILs was confirmed. The separation factors were increased in the dual chiral system for all 10 analytes (Rs=1.43-5.45).	[85]
	[TBA][L-Glu]	corinoxin and corinoxin B 	Separation of two enantiomers, corinnoxin and corinnoxin B, was achieved in the CD/IL dual chiral system. Preconcentration enhancement (with field-amplified sample stacking and stacking with a water plug) with concentration factors of 700-900 was additionally implemented.	[110]
	N,N,N-trimethyl-1-valinol-bis(trifluoromethanesulfone)imide; N,N-dimethyl-1-prolinol-bis(trifluoromethanesulfone)imide; N,N,N-trimethyl-1-phenylalaninol-bis(trifluoromethanesulfone)imide	nefopam, econazole, sulconazole, ketoconazole and amlodipine	The influence of the IL structure on enantiomeric separation efficiency was shown. Among the three studied ILs, the most pronounced synergistic effect was observed in the dual CD system with IL based on L-valine, presumably due to less competition between drug enantiomers for the hydrophobic cavity of CDs compared to the five-membered ring of IL based on proline and the benzene ring of IL based on phenylalanine.	[111]

Continuation of table 2

	1-butyl-3-methylimidazolium (T-4)-bis[(2S)-2-(hydroxy-kO)-3-methylbutanoato-kO]borate and 1-butyl-3-methylimidazolium (T-4)-bis[(αS)-α-(hydroxy-κO)-4-methylbenzeneaceto-κO]borate	propranolol, amlodipine, nefopam, tropicamide, and duloxetine	The first application of spirocyclic chiral ionic liquids as a second chiral selector for enantiomeric separation was achieved. The synergistic effect in the CD/IL system significantly improved the enantiomeric separation of analytes compared to conventional separation systems with CDs only.	[112]
γ-cyclodextrin	(R)-N,N,N-trimethyl-2-aminobutanol-bis(trifluoromethanesulfone)imidate (EtCholNTf$_2$)	homocysteine	The synergistic effect in the dual chiral system of IL/CD was confirmed by replacing the IL with LiNTf$_2$, which resulted in lower resolution factors compared to EtCholNTf$_2$. A change in enantiomer migration order was observed: when using EtCholNTf2 or γ-CD as the sole chiral selector, the D-enantiomer migrated first, while in the dual system of these two chiral selectors, an inversion of migration order was observed. NMR experiments showed the presence of intermolecular interactions between the internal hydrogen atoms of γ-CD and the aromatic group of homocysteine, confirming the formation of an inclusion complex with CDs. Interactions between γ-CD and CILs were also observed, indicating the formation of inclusion complexes of EtCholNTf$_2$ with the CD cavity.	[113, 114]

Continuation of table 2

	L-carnitine methyl ester bis(trifluoromethane)sulfonimide; L-(+) carnitine methyl ester lactate	Cysteine and homocysteine derivatized with 9-fluorenylmethoxy-carbonyl	Two new chiral ionic liquids based on the non-protein amino acid carnitine as the cation were studied, and their specificity towards cysteine and homocysteine was evaluated using a single chiral selector and in dual chiral systems with CDs.	[115-117]
Monosubstituted sulfated CDs	(R)-N,N,N-trimethyl-2-aminobutanol-bis(trifluoromethanesulfone)imidate (EtCholNTf2)	 R/S-4-amino-2,2-dimethyl-6-ethoxycarbonylamino-3,4-dihydro-2H-1-benzopyran	A significant increase in resolution factors was observed when determining the enantiomeric purity of benzopyran derivatives, which was crucial for determining enantiomeric impurities. In the presence of a very high concentration of the R-enantiomer (1 mg/mL), it became possible to quantitatively determine very low concentrations of the S-enantiomer with the introduction of a chiral electrolyte with the resolution factor of 22.6.	[118]

Continuation of table 2

glucosyl-β-CD (Glu-β-CD)	trifluoroacetate-L-hydroxyproline, nitrate-L-hydroxyproline, trifluoroacetate-L-threonine	Amlodipine, nefopam, econazole	The influence of organic solvents in the background electrolyte (methanol, ethanol, and isopropyl alcohol) on enantiomeric resolution was shown to be ambivalent. In the L-HPro+TFA-/Glu-β-CD system, the resolution factor for enantiomers of nefopam and econazole increased with the introduction of organic solvents (up to 30% v/v) due to changes in solution viscosity, and then decreased at higher concentrations (40% v/v) due to peak broadening caused by prolonged analysis time. For amlodipine, a significant decrease in the resolution factor for enantiomers was observed with the addition of organic solvents to the background electrolyte. The authors explain this fact by the less effective complexation process due to the difference in physico-chemical properties between CDs and amlodipine: the latter is hydrophobic, while Glu-β-CD is more soluble in water.	[119]
sulfated-CD	[TBA][L-Asp], [TMA][L-Asp], [TBA][L-Arg]), [TMA][L-Arg], tetrabutylammonium-L-isoleucine ([TBA][L-Ile]), ([TMA][L-Ile])	ivabradine	Increasing the concentration of the IL in the BGE led to a significant increase in enantioselectivity factors, but also increased analysis time.	[120]

A synergistic effect was also found in the dual chiral system chiral IL/antibiotic. Along with CD, the latter is often introduced into the background electrolyte as a chiral selector due to the presence of a great number of stereocenters in its molecule [121]. Thus, the authors [122] showed that such dual systems demonstrated an improved separation of nefopam enantiomers compared to the use of only a chiral selector (clarithromycin or CD derivative), where enantiomers separation was not found (figure 16).

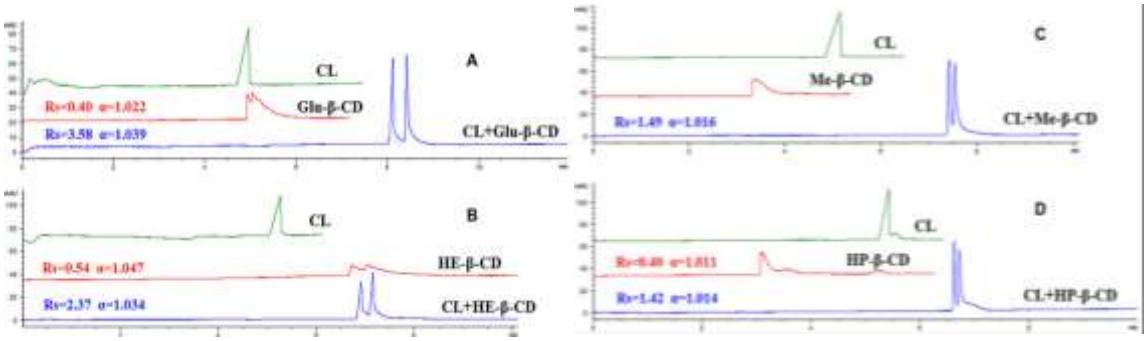


Figure 16. Electropherogramms of nefopam in four chiral systems and single systems with the use of CD derivatives [122].

Conditions: (**A**) 50 mM clarithromycin, 40 mM Glu-β-CD, 25 mM borate buffer (BBS) solution adjusted to pH 5.5 (**B**) 50 mM clarithromycin, 20 мМ hydroxyethyl-β-CD, 25 mM BBS adjusted to pH 4.5 (**C**) 40 mM clarithromycin, 20 mM Me-β-CD, BBS adjusted to pH 6.5 (**D)** 50 mM clarithromycin, 15 mM HP-β-CD, 25 mM BBS adjusted to pH 7.0; capillary 33 см (effective lenght 24,5 cm) × internal diametr 50 μM; BGE: 12.5 mM BBS containing MeOH (50% v/v.); 20 kV; 25 °C.

It is known that the tasks in the field of chiral analysis not always can be solved with the use of common systems based on CD, therefore, there is a need in new approaches. It has been shown that the modification of the CD structure, as well as the injection of modifiers of different nature into the BGE can affect the processes of enantiorecognition and significantly expand the potential of chiral separation using CE. One of the noteworthy combinations is CD and imidazolium IL. A synergistic effect of the joint injection of these modifiers into the background electrolyte during chiral separation was observed. The optimization of separation conditions is followed by the search for the approaches to online preconcentration, which contributes to a decrease in the limits of detection, which are sufficient to determine analytes in biological liquids.

I.3 On-line preconcentration of biologically active compounds in capillary electrophoresis

One of the advantages of the CE method is the possibility of implementing *intracapillary*, or *on-line* preconcentration without stopping the process of electrophoretic analysis itself. On-line preconcentration is based on the injection of a large volume of sample with subsequent focusing of the analyte into a narrower zone due to the difference in the electrophoretic mobilities of the compounds under study in the zone of the sample and the background electrolyte, which, in turn, can be determined by the difference in the conductivity of their solutions [7].

The quantitative characteristic is the preconcentration factor (SEF_h - Sensitivity Enhancement Factor), which is defined as:

$$SEF_h = \frac{h_1}{h_2} \cdot r \quad , \qquad (1)$$

where: h_1 is the peak height of the corresponding analyte upon preconcentration;

h_2 is the peak height under standard conditions (injection 2s 30 mbar or 2s 10 kV);

r is the dilution factor.

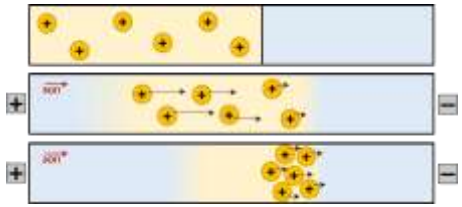
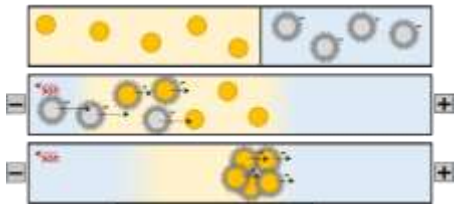
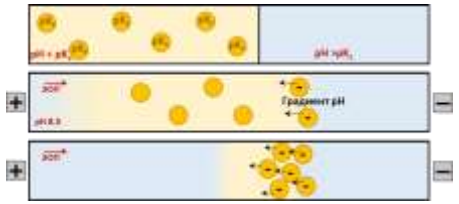
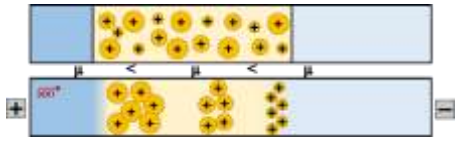
The analysis of various techniques of online preconcentration has become a solution to one of the main limitations of the CE method: low concentration UV-sensitivity. Currently, there is a wide variety of approaches, ranging from the simplest methods, where preconcentration occurs due to the different conductivity of the sample matrix and the BGE (*field-amplified stacking*), to hybrid methods with the combined use of two or more techniques. The driving force behind the development of online preconcentration in CE was not only the requirement for high sensitivity for practical applications, but also the complexity of the composition of the sample matrix.

At the beginning of this paragraph, we will briefly consider techniques of on-line preconcentration, which can be nominally classified as foundamental. Their combinations (hybrid preconcentration methods) are discussed in the second paragraph. The preconcentration of enantiomers and the use of new approaches for this purpose are considered separately, since these issues are not sufficiently represented in the literature.

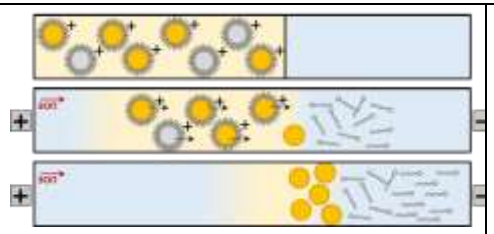
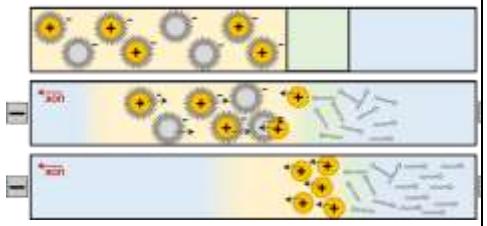
I.3.1 Mechanisms of on-line preconcentration

Field-amplified sample stacking, sweeping, isotachophoresis, and field-amplified sample injection are among the commonly used techniques of on-line preconcentration, which can be called fundamental. These methods are defined in a large number of publications, in which the principles and mechanisms are described in detail [123]. Their summary is presented in Table 3 with brief explanatory schemes and key features.

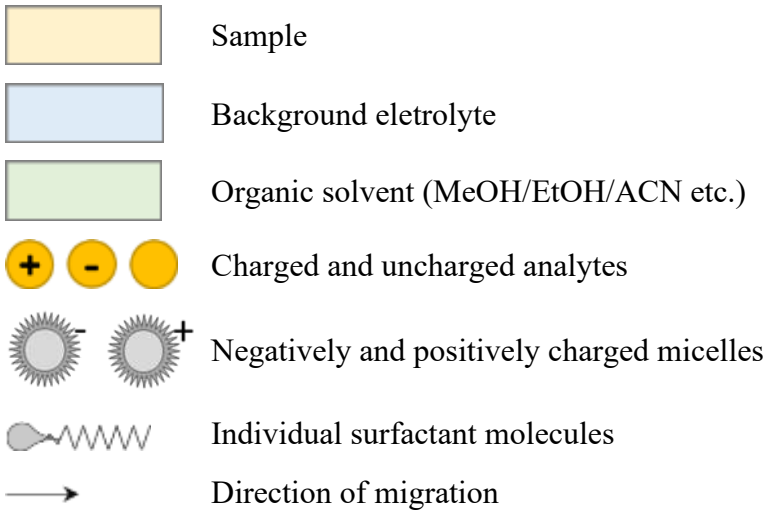
Table 3. Schemes of online preconcentration.

Techniques	Scheme	Conditions	Key Features of Preconcentration
Field amplified sample stacking ***(FASS)***		The conductivity of the sample matrix is less than that of the background electrolyte	The test ions in the sample matrix will have a greater electrophoretic mobility when electric field is amplified; slowing down on the boundary with the background electrolyte, the analytes are concentrated.
Sweeping		The conductivity of the sample matrix and the BGE is comparable; micelles formed by surface-active substances (surfactants) are in the composition of the BGE	Penetrating into the sample matrix, charged micelles interact with uncharged analytes, forming associates, the migration of which is slower than that of the analytes themselves, which contributes to preconcentration.
Dynamic pH-junction		Changes in the electrophoretic mobility of the analyte at different levels of pH, determined by the pKa values of the functional groups. pH-junction is formed when a sample is injected in a matrix with a different pH value compared to the BGE.	For weak acids, bases and ampholytes. It is based on a change in the ionic form of the analyte in the pH gradient zone on the boundary of the break *BGE-sample* when voltage is applied to the ends of the capillary. An uncharged ionic analyte migrating along with the EOF enters the gradient zone, changes its analytical form to ionic, and moves in the opposite direction to the EOF, which contributes to preconcentration. EOF mobility is greater, and the analytes migrate to the detector.
Isotachophoresis		The sample is injected between the leading and terminal electrolytes; the sample components have a greater mobility than the ions of the terminal electrolyte, but less than the leading one.	When voltage is applied, the analyzed analytes are divided into separate zones in order of the decrease of their electrophoretic mobilities; when equilibrium condition is reached, all ions migrate at constant velocity, which ensures not only preconcentration, but also separation of analytes.

Continuation of table 3

Analyte focusing by micelle collapse (AFMC)		Micelles are injected into the sample solution with high conductivity. The buffer solution with low conductivity and not containing micelles is used as the BGE.	When voltage is applied, micelles carry neutral analytes towards the detector. On the boundary with the BGE, the surfactant concentration becomes lower than the value of the critical micelle concentration (CMC). As a result, micelles collapse and analytes are released and preconcentrated.
Micelle to solvent stacking (MSS)		Micelles in the solution of the analyzed sample should have the opposite charge to the analyzed analytes. The organic solvent is injected into the BGE or as a plug between the sample and the BGE.	Ionogenic charged analytes are transferred by micelles to the zone with an organic solvent; on the boundary, the collapse or the decrease in the concentration of micelles in the solution occurs. Free analytes migrate electrophoretically back into the zone of micelles, interacting with them again. The process is repeated until all micelles in the sample collapse. This, in turn, leads to preconcentration.

Keys:



One of the most frequently used methods of intracapillary preconcentration in CE is sweeping, where micelles play the role of a pseudostationary phase and participate not only in preconcentration, but also contribute to an increase in separation selectivity. Along with the classic sweeping technique, where the sample matrix has the same conductivity as the BGE, we describe variations in which the matrix has greater or less conductivity than that of the background electrolyte [124], which in some cases leads to higher preconcentration efficiency. It should be noted that the concentration of micelles in the sample zone in the process of detection can affect the signal, reducing the sensitivity.

One of the solutions to this problem is the collapse of micelles before the sample reaches the detection window. Such approaches, which are currently successfully used for on-line preconcentration, have a more complex mechanism and are called *Analyte focusing by micelle collapse (AFMC)* and *Micelle to solvent stacking (MSS)* (Table 3). Both techniques include the use of micelles, however, unlike the classic sweeping technique, micelles are injected only into the sample solution with subsequent collapse. In this case, the BGE conductivity should be less than that of the sample matrix. The main difference between these two techniques lies in the nature of the preconcentrated analytes and the reason for the collapse of micelles. AFMC was developed for neutral analytes carried by micelles to the boundary with the BGE, where micelles collapse and analytes are released due to a decrease in the surfactant concentration to values below CMC [125, 126]. Therefore, the MSS technique is used for charged analytes, while the charge of micelles should be opposite to the charge of analytes. In this case, the collapse of micelles occurs due to the presence of an organic solvent in the BGE composition or in the form of a plug injected between the analyzed sample and the BGE; as micelles are collapsing, the effective electrophoretic mobility of charged analytes first decreases and then changes in the opposite direction [127]. Cyclodextrins are widely used as a pseudostationary phase, and the interaction with CD (the so-called *micelle to cyclodextrin stacking (MCDS*)) can be the reason for the collapse of micelles [128, 129]. Sequentially, a sample containing micelles is injected into the capillary, and then the plug of the background electrolyte with uncharged CD injection follows. Under voltage, analytes associated with charged micelles migrate to the boundary with CD, where inclusion complexes of the macrocycle with micelles are formed. The reduction of the amount of free surfactant becomes insufficient for the formation of micelles, which leads to the release of analytes on the boundary. Further, concentrated analytes migrate driven by their own electrophoretic mobilities.

When making a selection of preconcentration techniques for a specific task, not only the nature of the analytes should be taken into account, but also in which real samples the approbation of the measured conformities is planned, namely: the nature of the matrix of the analyzed sample, which can be conventionally divided into low- and high-conductive in relation to the buffer electrolytes used in the CE method. If problems do not occur with low-conductive matrices in most cases, there are limitations

in the use of on-line preconcentration techniques with high-conductive matrices. Sometimes matrix conductivity can be reduced by diluting the sample, but if the concentration of the analized analytes is initially low, as in the case of biological fluids, then dilution should be avoided. The gathered experience has made it possible to generalize the applicability of the main preconcentration techniques, taking into account the nature of the analytes and the sample matrix (Table 4) [130].

Table 4. Main preconcentration techniques and their compatibility with high-conductive saline matrices [130].

On-line preconcentration technique	**Charge of the analyte**	**Compatibility with high-conductive saline matrices**	**Achieved SEF**
Field-amplified sample stacking	⊕⊖	incompatible	10^1-10^2
Large volume sample stacking	⊕⊖	incompatible	10^2-10^3
Field-amplified sample injection	⊕⊖	incompatible	10^2-10^3
Dynamic pH-junction \ pH mediated stacking	⊕⊖	compatible	10^1-10^3
Sweeping	⊕⊖◯	limited compatibility	10^1-10^5
Isotachophoresis	⊕⊖	compatible	10^1-10^2
AFMC	◯	limited compatibility	10^1-10^2
MSS	⊕⊖	limited compatibility	10^1-10^2

I.3.2 Combination of different online preconcentration methods (hybrid methods)

Each of the intracapillary concentration approaches described above has limitations that can be neutralized by a combination of two or more preconcentration techniques. Such techniques can act synergistically [131, 132] or sequentially, when the appropriate separation mechanism is triggered after the completion of the previous one [133-136]. In addition to improving sensitivity, this association allows to concentrate and separate analytes with different physical and chemical properties within a single analysis.

At this point, various hybrid methods of on-line preconcentration have already been described; nevertheless, there is a general trend, namely, the combination of two approaches that differ in mechanism. In most cases, these are modes based on distribution and complexing (micelles participation: sweeping or AFMC and MSS variants) in combination with methods where preconcentration occurs due to changes in the electrical conductivity of sample solutions and the BGE (FASS, FASI, dynamic pH-junction, etc.).

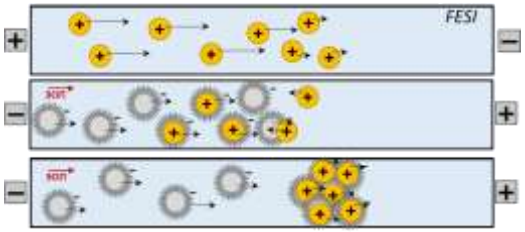
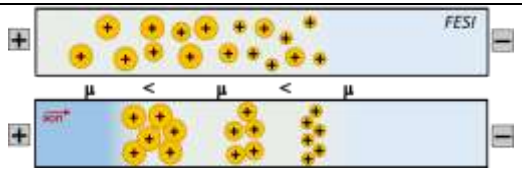
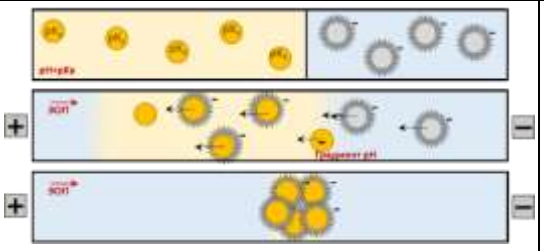
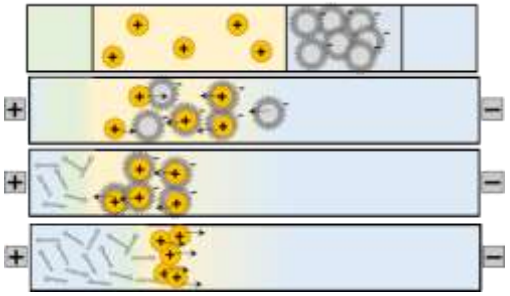
Some of these variants have acquired their own name, for example, the combination of long-term electrokinetic injection and sweeping is called *cation / anion-selective exhaustive injection - sweeping (CSEI-sweeping, ASEI-sweeping).* The method is considered to be the first combination of methods of electrophoretic online preconcentration. It was proposed by J.P. Quirino and S. Terabe in 2000 for preconcentration of cations (for anions, the selection of the appropriate surfactant is required), providing an increase in sensitivity by almost a million times [133]. Along with the requirements of each of them, namely, electrokinetic injection from a matrix of low conductivity (*FASI*) and the absence of micelles in the sample matrix (*sweeping*), for such hybrid variant, the following condition should be observed: the analytes are charged and migrate towards EOF. After electrokinetic injection at the second stage of preconcentration, the polarity of the voltage changes, the analytes migrate in the opposite direction, being concentrated by micelles migrating towards them from the background electrolyte (*sweeping mode*). This approach makes it possible to concentrate not only active analytes which are charged biologically [137, 138] but also individual enantiomers [139, 140]. A special paragraph is dedicated to the preconcentration of the latter below.

Another variant of intracapillary preconcentration is called *electrokinetic superconcentration*. It is a combination of electrostaking and isotahophoresis. Its essence is in the electrokinetic injection of a sample between the leading and terminal electrolytes, followed by the implementation of isotachophoresis when voltage is applied. Although the method was first described in 2003 [134] for the preconcentration of rare-earth metal cations, subsequently, it was widely used in the preconcentration of biologically active compounds: alkaloids [141], cathinones [142], nucleosides [143], and neurotransmitters [144].

The combination of *dynamic pH-junction and sweeping* not only ensured the simultaneous separation of hydrophobic and hydrophilic compounds [132, 145-147], but also proved to be useful in the determination of drugs and their metabolites. Since a large number of metabolites are weakly ionic, the pH gradient of the buffer electrolyte contributes to both separation selectivity and preconcentration. A special review [148] is dedicated to this problem.

One of the unusual combinations, which is *sweeping followed by micelle to solvent stacking (sweeping - MSS)*, ensures the extraction of analytes from the matrix, their preconcentration, and then the extraction from micelles [135, 149, 150]. The main idea of this approach is that a micellar plug is injected before the sample, while maintaining the MSS conditions: analytes and micelles have opposite charges, and the background electrolyte contains an organic solvent. When voltage is applied, micelles penetrate into the sample zone and concentrate oppositely charged analytes (*sweeping mechanism*), which have the observed electrophoretic mobility, that changes to reversed, and they are transferred by micelles to the boundary with the BGE containing an organic solvent. Upon reaching this boundary, the micelles collapse due to the presence of organic solvent and concentrate again (*MSS mechanism*). The discussed hybrid methods are summarized in Table 5.

Table 5. Schemes of hybrid online preconcentration methods.

Method	Scheme	Conditions	Key Features of Preconcentration
Cation/anion-selective exhaustive injection – sweeping (CSEI-sweeping, ASEI-sweeping)		Electrokinetic injection from a matrix of low conductivity (*FASI*) and absence of micelles in the sample matrix (*sweeping)* analytes are charged and migrate against the EOF.	After electrokinetic injection, the polarity of voltage changes, the analytes migrate in the opposite direction, being concentrated by micelles migrating towards them from the background electrolyte
Electrokinetic supercharging		The conditions of electrostacking and isotachophoresis are preserved	Electrokinetic injection of the sample between the leading and terminal electrolytes, followed by the implementation of isotachophoresis when voltage is applied.
Dynamic pH-junction and sweeping		The dynamic pH-junction and sweeping conditions are maintained in one analytical cycle.	The capillary is filled with the BGE containing micelles, then the sample comes. When voltage is applied, a local change in pH and preconcentration occur in the dynamic pH-junction mode. At the same time, SDS micelles migrate from the BGE and concentrate analytes in the sample zone (*sweeping*).
Sweeping-micelle to solvent stacking (sweeping - MSS)		The MSS conditions are preserved: analytes and micelles have opposite charges, and an organic solvent is injected into the composition of the BGE	A micellar plug is injected before the sample. When voltage is applied, micelles penetrate the sample area and concentrate analytes (*sweeping mechanism*). Upon reaching the boundary with the BGE containing an organic solvent, the micelles collapse and release concentrated analytes.

The combination of on-line preconcentration options has proved useful for non-aqueous capillary electrophoresis mode (NACE), where the working organic solvents have low conductivity, making stacking difficult. For example, when determining tamoxifen and its metabolites for preconcentration, the combination of FESI-MSS was used [151]. However, electrokinetically injected analytes concentrated due to interaction with micelles, and not due to difference in conductivity between the sample and the BGE. This made it possible to use a longer injection time, and the preconcentration factors for tamoxifen and its metabolites were 214–625. The approach was successfully applied to determine concentrations of the drug and its metabolites in human plasma. The approaches used for on-line preconcentration in NACE are described in detail in the review [152].

To date, there are publications on the combination of even three online preconcentration techniques in CE, for example, FASI-sweeping-MSS [153, 154], dynamic pH-junction-sweeping-LASS [155], FASI-sweeping-AFMC [156, 157]. It was possible to concentrate analytes by several thousand times, which can be successfully used for the sensitive determination of trace amounts of analytes in complex biological samples (Figure 17).

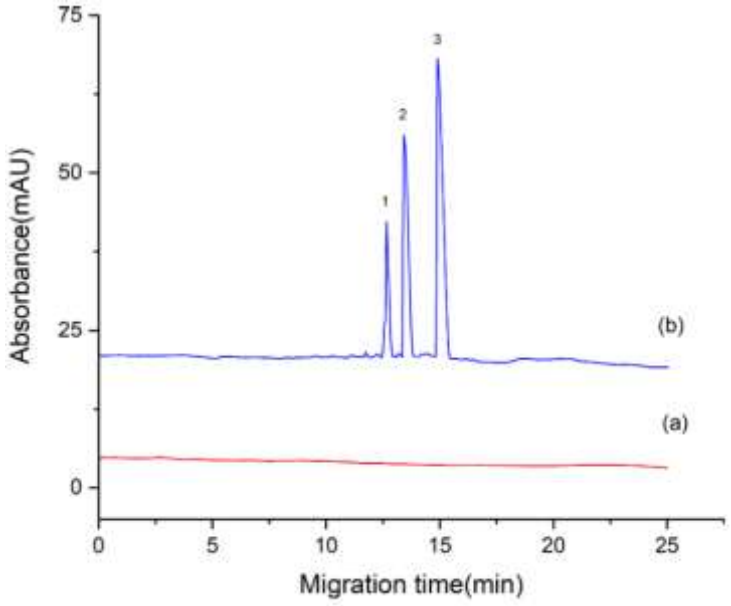


Figure 17. Standard sample injection (a) and three-step preconcentration (FASI-sweeping-MSS) of real sample (excrements).

Conditions: 50 mM phosphate buffer solution (pH 2.5). Sample injection 3 s 50 mbar (a) and 120 s 10 kV (b); voltage +20 kV.

Analytes: 1-vinblastine sulphate, 2-katarantin sulfate, 3-vindoline sulfate [154].

Such options provide high preconcentration factors, but careful selection of conditions is required [15, 158], since each of the parameters can strongly affect the final result: the volume of injected micellar plug, the volume of sample injection, the type and concentration of micelles, and the amount of organic solvent. There are researches where the design of the experiment and chemometric processing are used to optimize the preconcentration conditions [156].

I.3.3 Preconcentration of enantiomers

Intracapillary preconcentration of individual enantiomers is one of the understudied and difficult problems in the field of CE due to additional interactions with a chiral selector during electrophoretic separation. At the same time, the general patterns of on-line preconcentration are preserved: a decrease in resolution with an increase in the time of sample injection, which can be especially critical in the case of enantiomers separation [159, 160].

The analysis of the available publications has shown that for enantiomers, mainly hybrid preconcentration methods are used, which makes it possible to maintain separation selectivity; sweeping with electrokinetic injection (FESI-sweeping, CSEI-sweeping) dominates (Table 6). It is reported about the preconcentration of the cations of the individual enantiomers by a factor of 10,000, while maintaining the achieved enantiselectivity factors [159, 161].

Thus, the mentioned hybrid variant (*CSEI - sweeping*) is modified, namely, an anionic CD is added as a chiral selector to a micellar buffer solution containing sodium dodecyl sulfate [159], or micelles are completely replaced by an anionic derivative of CD [161], which performs the functions of micelles and a chiral selector, the so-called *CSEI-sweeping-CD* which is a modified version of on-line preconcentration.

Table 6. Methods for online preconcentration of enantiomers in real samples

Analytes (racemates)	Sample Matrix	On-line preconcentration method	SEF	Reference
Pheniramine	Rat plasma	LVSS-sweeping; CSEI-sweeping	160 4000	[162]
Propranolol	Saliva, urine, plasma	FESI-sweeping	21 000	[160]
9 pairs of derivatized amino acids	Beer samples	Poly(ethylene oxide) stacking	110-220	[163]
Chlorpheniramine	Rat plasma	CSEI-sweeping	190	[139]
Fenoprofen	River water	LVSS–ASEI	1000	[164]
Fluoxetine, Norfluoxetine	Urine, plasma	FASS	20	[165]
Glycopyrrolates	Rat plasma	CSEI-sweeping	495	[140]
Mirtazapine	Plasma	FASS	50-60	[166]
Alprenolol	Model mixture	FESI-sweeping	100	[167]
Methamphetamine	Hair	CSEI- sweeping modified CD	10000	[159]
Citalopram	Dosage form, rat plasma	LVSS- sweeping modified CD CSEI- sweeping modified CD	663 2163	[161]
Methylone, mephedrone, 4-methylephedrine, methylenedioxypyrovalerone;	Urine	Electrokinetic supercharging	940-1310	[142]

I.3.4 Use of modifiers in on-line preconcentration

Modifiers for on-line preconcentration are relevant in various aspects:

- The formation of dynamic and covalently attached coatings enables to control the speed and direction of EOF, which is important for some preconcentration techniques, for example, MSS (analytes should migrate in the opposite direction from EOF). In addition, coatings reduce the adsorption of analytes on the capillary walls, contributing to an increase in sensitivity [153, 164, 168, 169].

- Surfactants traditionally used in on-line preconcentration (SDS and cetyltrimethylammonium bromide (CTAB)) are replaced by modifiers such as ionic liquids at a concentration higher than CMC [59, 127, 153, 170, 171], gemini with the formation of visicles [172], charged derivatives of CD [161]. All these modifiers can also play the role of micelles and concentrate analytes. The selection of one or another variant is determined by the possible additional contribution to the separation selectivity.

-The use of polymers to increase the BGE viscosity [145, 173, 174]. Analytes in the sample solution migrate rapidly in the sample matrix zone and slow down, reaching the boundary with the BGE due to the high viscosity of the latter; the velocity of test molecules decreases, and preconcentration, which is similar to field-amplified sample stacking, occurs.

Therefore, the method of capillary electrophoresis is relevant and promising in solving the most important medical and biological problems. The presence of additional driving force due to the application of voltage; high efficiency and rapidity, less stringent requirements for analyzed analytes and working solutions represent the advantages of this method. The possibility of modifying the electrophoretic system makes it possible to separate hydrophilic and hydrophobic biologically active compounds, including enantiomers, in one analytical cycle. Cyclodextrins and their derivatives have successfully proven themselves as modifiers. At the same time, a number of limitations of traditionally used systems based on CD were discovered. The search for new approaches to their modification is required. For example, the use of ionic liquids for this purpose will significantly expand the range of analytes in CE, including chiral separation, and contribute to the increase of the resolution factors of enantiomers due to the synergistic effect. Furthermore, polyfunctional systems of this type are promising in controlling the separation selectivity of analytes and in intracapillary preconcentration processes, helping to reduce the limits of detection of analytes. There are few publications concerning the injection of imidazolium cation into CD molecule, however, in our opinion, it is these structures that can provide additional effective interactions with analytes, expanding analytical potential of the electrophoretic

method in chiral separation as well. The problem of intracapillary electrophoretic preconcentration of individual enantiomers has not been sufficiently studied, which is an important and nontrivial task.

All these questions are considered in this research work.

Chapter II. EXPERIMENTAL SECTION

II.1 Instrumentation

Electrophoretic determination was performed using the system of capillary electrophoresis Capel'-105M (Lumex, Russia) with UV-spectrophotometric detector and quartz capillaries with the external polyimide coating with the following characteristics: total capillary length 60 cm; effective capillary length 50 cm; outside diameter 360 μm, and inner diameter 50 μm.

Sample preparation for analysis was carried out using

measuring instruments: analytical balance OHAUS Pioneer (PA214C, readability 0.0001 g) pH meter HI 2210 (Germany) (readability pH 0.01); automatic pipettes 20-200 μl (volume increment 1 μl); 100-1000 μl (volume increment 5 μl) from Satorius; and

auxiliary equipment: centrifuge ELMI CM-50MP (Latvia), ultrasonic bath Branson Ultrasonic Bath 2510 (USA) and multi-vortex Multi V-32 BioSan (Latvia), (USA); laboratory drying cabinet "LOIP LF" (St. Petersburg).

Experiments for independent evaluation of the proposed electrophoretic approaches were carried out with HPLC method on liquid chromatograph LC-40 Nexera (Shimadzu, Japan) with Agilent Zorbax SB-C8 2.1 mm × 150 mm 3.5-micron column.

The presence and thickness of the covalent coating on the inner wall of the quartz capillary were assessed from images obtained with SEM analysis on Carl Zeiss Merlin scanning electron microscope with field emission cathode.

The results were processed using the software Elforan for Windows. Limits of detection were calculated as signal-to-noise ratio (S/N)x3. The noise values were obtained in the Elforan program automatically by selecting the area without the signal of the analytes. The limit of quantification was calculated as the signal-to-noise ratio (S/N)x10. Precision was assessed by calculating the standard deviation of the results of three parallel measurements of analyte migration times. Statistical processing was carried out using Excel software. Validation parameters of the proposed approaches for determining biologically active compounds using the proposed methods in real objects and assessment of precision are described in II.9

II.2 Reagents

Standards: (-)-adrenaline (A, Sigma), L-(-)-noradrenaline (NA, Aldrich), DL-normetanephrine hydrochloride (NMN, Sigma), dopamine hydrochloride (DA, Sigma), D,L-Metanephrine hydrochloride (NMN, Sigma), serotonin hydrochloride (5-HT, Sigma), vanilmandelic acid (VMA, Sigma),

homovanillic acid (HVA, Sigma), 3,4-Dihydroxybenzylamine hydrobromide (3,4-DHBA, Sigma), 3-Methoxytyramine hydrochloride (3-MTA, Sigma), homogentisic acid (HHA, Sigma), 5-Hydroxyindole-3-acetic acid (5-HIAA, Sigma), corticosterone (B, Sigma-Aldrich), cortisone (E, Sigma-Aldrich), hydrocortisone (F, Sigma-Aldrich), 11-Deoxycortisol (S, LGC), 11-Deoxycorticosterone (DOC, Dr. Ehrentorfer), progesterone (PR, Sigma-Aldrich), *S,R*-ketoprofen (Sigma), *S*-ketoprofen (Sigma), *S,R*-ketorolac (Sigma), *S*-ketorolac (Sigma).

Synthesis of modifiers and covalent coatings: 1-bromobutane (99%, ReagentPlus), 1-bromooctane, imidazole (> 99%, Sigma-Aldrich), (3-glycidoxypropyl)trimethoxysilane (GPTMS, Sigma), 1 ,1-diphenyl-2-picrylhydrazyl, (DPPH) (Sigma), acetone (>99.8%, Merck), N,N-dimethylformamide (DMF, >99.9%, J.T. Baker), β-cyclodextrin (β-CD, >98%, «Sigma-Aldrich»), p-toluenesulfonyl chloride («Sigma-Aldrich»), 1-methylimidazole («Sigma-Aldrich»), benzyltriethylammonium chloride (TEBAC, Sigma-Aldrich), dimethyl sulfoxide (DMSO, ACS reagent, ≥99.9%), n-butanol (suitable for HPLC, ≥99.7%), 2-propanol (ACS reagent, ≥99.5%)

Preparation of working solutions: acetonitrile (ACN, Fluka, HPLC grade), methanol (Fluka, HPLC grade), trichloroacetic acid (ACS reagent, ≥99.0%), formic acid (Supelco, ≥98%), sodium hydroxide (analytical grade, «Khimreaktiv»), hydrochloric acid (cp grade) («Reachem»); sodium dihydrogen phosphate dihydrate (cp grade) («Reachem»); sodium hydrogen phosphate dihydrate (cp grade) («Reachem»); 1-Dodecyl-3-methylimidazolium chloride (C_{12}MImCl, Acros Organics); 1-Hexadecyl-3-methylimidazolium chloride (C_{16}MImCl, Acros Organics); cetyltrimethylammonium bromide (CTAB, Acros Organics); sodium dodecyl sulfate (SDS, ≥98.5%, ReagentPlus), (2-Hydroxypropyl)-β-cyclodextrin (HP-β-CD, Sigma-Aldrich); ethylenediaminetetraacetate dihydrogen disodium dihydrate (Na_2EDTA, Sigma), sulfated β-cyclodextrin 12-15 mol per mol β-CD, s-β-CD Sigma), α-cyclodextrin (α-CD, ≥98%, Sigma-Aldrich); sodium carbonate (analytical grade, «Khimreaktiv»), glacial acetic acid (Sigma-Aldrich), aluminium oxide (Al_2O_3, >98%, Sigma-Aldrich), vancomycin (European Pharmacopoeia Reference Standard), amikacin (European Pharmacopoeia Reference Standard), amoxicillin (European Pharmacopoeia Reference Standard), chloroform (≥99.8%, Sigma-Aldrich), dextran (Mr~40,000, Sigma-Aldrich), deionized water.

II.3 Synthesis of modifiers

The method of nuclear magnetic resonance spectroscopy and the comparison of the spectra with the literature data were used to confirm the structures of all the obtained compounds.

II.3.1 Synthesis of 6A-O-p-toluenesulfonyl-β-cyclodextrin

0.42 M sodium hydroxide solution (0.5 L), 14.29 g of β-cyclodextrin, 6.67 g of *p*-toluenesulfonyl chloride were placed into a three-neck flask with a volume of 1 L and were stirred at a temperature of 0–5 ℃ for 2 h. Then another portion of p-toluenesulfonyl chloride (10 g) was added and the mixture was stirred further at a temperature of 0-5 ℃ for 3 hours. The solution was cooled and filtered through Schott filter, then 100 ml of 10% hydrochloric acid solution were added to the filtrate and stored overnight in a refrigerator at 0°C. The precipitated product was dried under vacuum and purified by double recrystallization from water. *6A-O-p*-Toluenesulfonyl-β-cyclodextrin (Ts-β-CD) weighing 4.20 g was obtained. A brief scheme is shown in Figure 18. The structure of the obtained compound was confirmed by NMR and the obtained spectrum was compared with the literature data presented in [175].

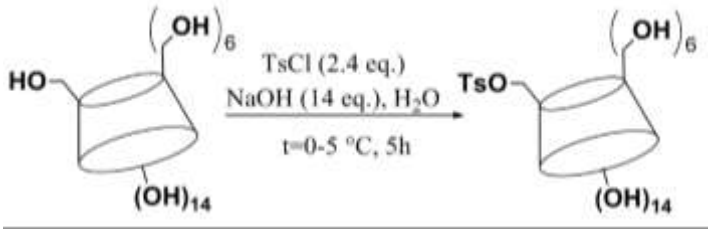


Figure 18. The scheme of syntesis of *6A-O-p*-toluenesulfonyl-β-cyclodextrin.

II.3.2 Synthesis of 3-alkyl-1-β-cyclodextrinimidazole tosylates

Synthesis of two derivatives of 3-methyl-1-β-cyclodextrinimidazolium tosylate (3-Me-1-β-CDImOTs) and 1-butyl-3-β-cyclodextrinimidazolium tosylate (3-Bu-1-β-CDImOTs) was made from Ts- β-CD, the synthesis of which is described above; the scheme is shown in Figure 19.

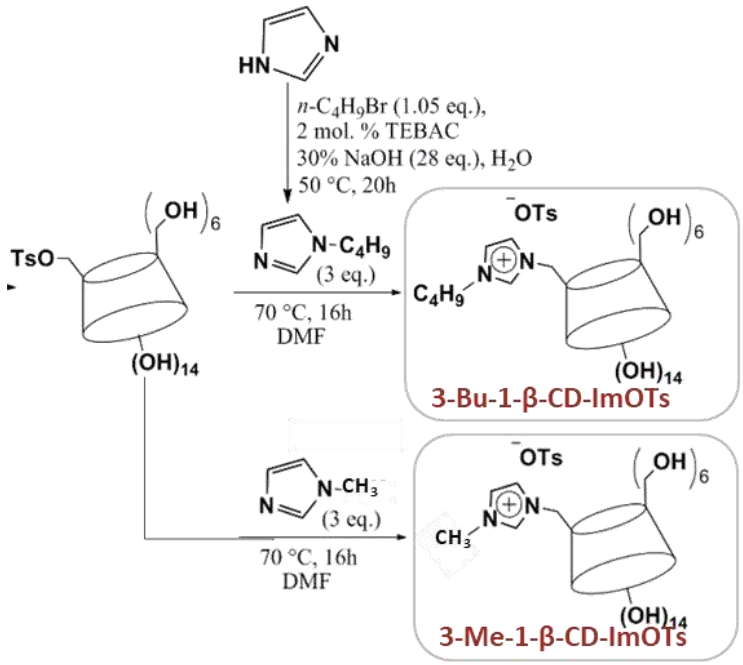


Figure 19. The scheme of syntesis of modifiers based on imidazole and β-CD.

3-methyl-1-β-cyclodextrinimidazolium tosylate

1 g of Ts-β-CD was dissolved in 2 ml of DMF, 0.19 g of 1-methylimidazole was added and stirred at 90 ^{0}C for 48 h, then cooled to room temperature and 7.8 ml of acetone was added and stirred for 30 min. The precipitate was filtered off, washed with acetone and dried under vacuum to give a white solid.

3-butyl-1-β-cyclodextrinimidazolium tosylate

N-butylimidazole [176] was preliminarily synthesized to obtain the butyl derivative according to the following algorithm: imidazole (0.107 mol) and triethylbenzylammonium chloride (2 mol %) were dissolved in 30% NaOH water solution (20 ml) and stirred vigorously. Then 1-bromobutane (0.113 mol) was added dropwise at room temperature and the mixture was stirred at 50° C for 20 hours until two phases appeared. The upper organic phase was extracted, washed and used in the next step. The product was characterized by a refractive index at 22°C, which was in good agreement with data from the literature.

Then Ts-β-CD (1.4 g, 0.001 mol) and *N*-butylimidazole (0.4 g, 0.003 mol) were heated in DMF (2.6 ml) and stirred at 70°C for 16 h [177]. The precipitate was filtered off, washed with acetone, and dried on rotary evaporator. A white solid weighing 1.3 g was obtained.

II.4 Preparation of working solutions

II.4.1 Preparation of standard solutions of analytes

The preparation of standard solutions was performed by dissolving weighed portions of 1-10 mg in the volume of the solvent required to obtain the final concentration of 1 mg/ml (table 7). All standard solutions were stored at -20°C until they were used (up to 2 moths). The required working solutions were prepared by diluting the standard solutions with distilled water or an appropriate buffer solution and stored at +4 ^{0}C for no more than 5 days.

Table 7. Preparation of standard solutions

Standards	**Solvent used**
Biogenic amines	0.1 M HCl
Phenyl-indole carboxylic acids	deionized water
Corticosteroid hormones	Acetonitrile
Ketoprofen, ketorolac and ibuprofen	acetonitrile/water (10/90 vol.)
Proteins	deionized water

II.4.2 Preparation of buffer solutions

Electrophoretic separation was carried out in background electrolytes (BGE) in the pH range of 2.0–12; initial phosphate buffer solutions (PBS) with a higher concentration and appropriate pH values were used for the BGE preparation.

Initial phosphate buffer solutions (100 mM) were prepared by dissolving 4.45 g of disodium hydrogenphosphate dihydrate (M=177.99 g/mol) or 3.90 g of sodium dihydrogen phosphate dihydrate (M=156.01 g/mol) in 20 ml of deionized water and sonicated for 10 min. Then the pH was adjusted to the required value (2.0, 2.5, 3.2, 3.8, 4.5, 6.4, 8.0, 9.5) by 0.5 M NaOH or 1 M HCl . After that, distilled water was added to reach the total solution volume (25 ml). All the buffer solutions were filtered through a 0.45 μm syringe-type Millipore membrane filter, sonicated and centrifuged prior to use. Background electrolytes were obtained by diluting buffer solutions with distilled water.

II.5 Covalent coatings based on the imidazolium cation

II.5.1 Synthesis of covalent coatings

Algorithm for the synthesis of covalent coatings based on the imidazolium cation (Figure 20):

- *activation of the capillary walls* with 2 M NaOH solution at 90℃ for 1 hour;
- *washing* with acetone and storage at a temperature of 160℃ to remove water adsorbed on the surface of the capillary;
- *silylation* for 6 h (the total time spent on obtaining the oligomeric layer is indicated: the capillary was successively washed, filled four times with a silylating agent and left for 2 h, 1.5 h, 1.5 h and 1 h at 110℃ in thermostat); to prepare a solution of the silylation agent 0.1 wt. % DPPH was dissolved in DMF:GPTMS (7:3, v/v).
- *functionalization*; in order to obtain structures similar to ionic liquids based on imidazolium. The first functionalization step was a nucleophilic substitution reaction with imidazole dissolved in DMF (25 mg/mL) at 90°C for 6 h, and the second step was post-functionalization with the use of 1-bromobutane, 1-bromooctane (10 h at 80°C) or Ts -β-CD. For the latter, the conditions were optimized. The parameters were varied: reaction time (from 10 to 22 hours), temperature (from 80 to 100 °C) and concentration of the modifying agent (from 25 to 750 g).

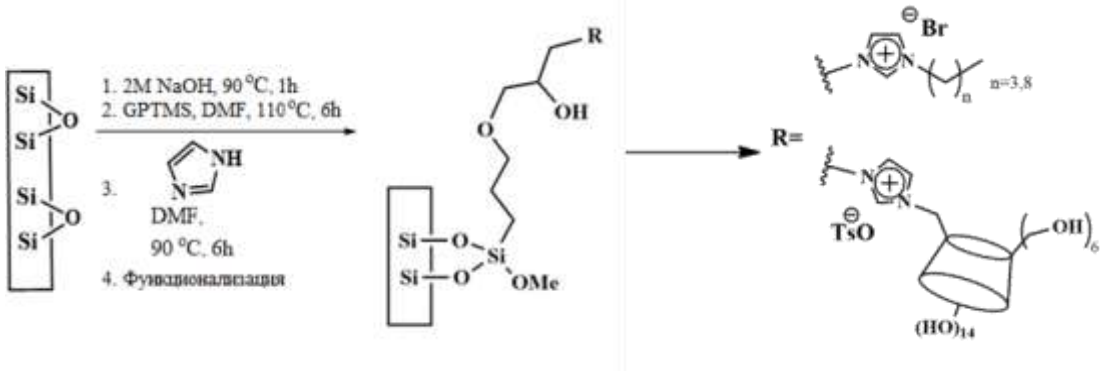


Figure 20. The scheme of synthesis of covalent coatings based on imidazole with following postmodification.

II.5.2 Description of coatings with physico-chemical methods of analysis

Confirmation of the presence and structure of the coating

The direction and velocity of EOF was measured to evaluate the formed coating, and the selectivity of the separation of the model mixture of steroid hormones was additionally calculated for the coating based on CD. The anodic EOF indicates the presence of a positive charge on the surface of the capillary, and the separation of the mixture of steroid hormones indicates the presence of cyclodestrin on the surface of the quartz capillary and the implementation of the CEC mode. In order to confirm the presence of the coating, pictures of the inner surface of the capillary were made using the SEM method, where the presence of roughness indicated the formation of the coating.

Evaluation of stability of covalent coatings

From analysis to analysis and *from capillary to capillary* we assessed the stability of covalent coatings by measuring the EOF velocity during electrophoretic experiments under the following conditions: 10 mM phosphate buffer solution (pH 2.0), -20 kV; 220 nm; 5 % (vol.) DMF-water solution as the EOF marker.

We evaluated the stability of the coating in *an organic solvent medium* (acetonitrile) by measuring EOF after running acetonitrile through the capillary with the covalent coating for 5 min and by comparing the obtained value with that obtained before the injection of acetonitrile. Next, the capillary filled with acetonitrile with the covalent coating was left for 2 days, and then the EOF velocity was measured again. The conditions were following: 10 mM phosphate buffer solution (pH 2.0), -20 kV; 220 nm; 5% (vol.) DMF-water solution as the EOF marker.

To determine *the pH working range*, we assessed the stability of the coating by measuring the EOF velocity in the pH range of 2.0–4.0; 5.4; 6.4; 7.9; 9, 11; 12.3 with the buffer concentration of 10 mM. We performed the measurement three times at each point in the direction of decrease in acidity, and then - its increase, the average value and standard deviation were calculated. The procedure was repeated twice.

II.5.3 Analysis of the main analytes: biogenic amines

To determine the conditions for the separation of biogenic amines (concentration 0.5–25 μg/ml) using the covalent coating, the following conditions were varied: the concentration of phosphate buffer solution (pH 2.0, 5–25 mM); voltage: -20 kV; UV-detection at 220 nm; sample injection 2-80 s at 30-50 mbar; temperature: 20^0C.

II.5.4 Analysis of acid analytes: phenyl- and indolecarboxylic acids

To determine the conditions for the separation of phenyl- and indolecarboxylic acids with the use of the covalent coating (0.5–120 μg/ml), the following conditions were varied: the concentration of phosphate buffer solution (5–25 mM), the pH of phosphate buffer solution (2.0–5.0).

II.5.5 Analysis of hydrophobic analytes: corticosteroid hormones

To determine the conditions for the separation of steroid hormones (concentration 0.5–10 μg) using the covalent coating, the following conditions were varied: concentration of phosphate buffer solution (5–25 mM), the pH of phosphate buffer solution (2.0; 3.0; 4.0; 7.0), content of organic solvent (methanol or acetonitrile, 0-25%); voltage -20 kV; UV-detection at 254 nm; sample injection: 2-20 s at 30-50 mbar; temperature: 20°C.

II.6 Injection of a modifier based on imidazolium cation into the BGE composition

II.6.1 Preparation of modifier solutions

Preparation of 3-butyl-1-β-cyclodextinimidazolium tosylate solution

To obtain 2 mM 3-β-CD-1-butylimidazolium tosylate solution, a weighed portion of 15 mg was dissolved in 5.36 ml of water and kept in the ultrasound bath for 20 min at 40°C.

Preparation of a 3-methyl-1-β-cyclodexrinimidazolium tosylate solution

To obtain 10 mM 3-methyl-1-β-cyclodexrinimidazolium tosylate solution, a weighed portion of 67.8 mg was dissolved in 5 ml of water and kept in the ultrasound bath for 20 min at 40°C.

II.6.2 Influence of modifier concentration on the EOF velocity (dynamic modification of the quartz capillary walls)

To study the influence of modifier concentration in the background electrolyte on the degree of dynamic modification of the quartz capillary walls, we measured the EOF velocity in the presence of a modifier with different concentration (0.25, 0.5, 0.7, 1.0, 1.25, 1.5 mM) in the background electrolyte. After each series of analyzes with the given modifier concentration, the capillary was washed with alkali, acid, and water to remove the adsorbed modifier. Conditions: 10 mM phosphate buffer solution (pH 6.4), -20 kV; 220 nm; 5% (vol.) DMF-water solution as the EOF marker.

II.6.3 Analysis of steroid hormones

To determine the conditions for the separation of corticosteroid hormones in the CEC mode using the synthesized modifier as a pseudostationary phase, the following parameters were varied: the pH of the background electrolyte (2.0, 2.8, 3.2, 3.8, 4.5, 6.4, 7.5, 8.0) and the concentration of phosphate buffer solution (5–50 mM), concentration of the modifier based on imidazolium cation and IL (0.1–2.25 mM), content of organic solvent (0–20 vol.%)

II.6.4 Enantioseparation of drugs

To determine the conditions for the separation of drug enantiomers, we varied the following parameters: the pH of the background electrolyte (2.0, 2.8, 3.2, 3.8, 4.5, 6.4, 7.5, 8.0) and the concentration of phosphate buffer solution (5–50 mM), the concentration of the modifier based on imidazolium cation and IL (0.1 - 2.25 mM), content of organic solvent (0-20% vol.) and its nature (acetonitrile, methanol, ethanol, 2-propanol, 1-butanol)

II.6.5 Calculation of complex formation constants of CD derivative with individual enantiomers

To calculate the complex formation constants, we used the algorithm proposed in [178] with some modifications. To take into account the possible influence of the solution viscosity, which can be caused by the injection of oligosaccharides into the BGE, the dependence of the EOF velocity on the dynamic viscosity of the solution was preliminarily set (the solvents methanol, deionized water, ethanol, dimethyl sulfoxide, and 2-propanol were taken as reference viscosity points. Next, the EOF velocity and electrophoretic mobilities of enantiomers were measured at different concentrations of the chiral selector in the composition of the BGE, taking into account corrections for viscosity of the solutions.

II.7 Dual chiral systems

II.7.1 Investigation of the analytical capabilities of 1-Bu-3-β-CDImOTs as a chiral selector in dual chiral systems

Test experiments to study the effect of using two chiral selectors in the separation of the model mixture of enantiomers of ketoprofen (10 μg/ml) and ketorolac (50 μg/ml) were carried out with varying the concentration of the second modifier in the composition of the BGE, while the concentration of 1-Bu-3-β-CDImOTs remained constant (1 mM), i.e. the second chiral selector was taken at concentrations of 0.5 mM, 1.0 mM, and 1.5 mM (or 2.0 mM). Conditions: injection 2 s 30 mbar; 25 mM PBS (pH 6.4), 254 nm, 20 kV

The following compounds were studied as the second chiral selector: uncharged CDs (β-CD, HP-β-CD, α-CD), as well as sulfo-β-CDs, dextran, and antibiotics (vancomycin, amoxicillin, and amikacin). Stock solutions at concentration of 10 mM (for amoxicillin and 4 mM for β-CD due to the low solubility in water) were prepared by dissolving the required weighed portion in water and then used them for inroduction into the background electrolyte.

II.8 On-line preconcentration

II.8.1 Application of covalent coatings based on the imidazolium cation

Field-amplified sample stacking for biogenic amines

We carried out preconcentration of biogenic amines (concentration 5×10^{-3}-25 μg) in the field-amplified sample stacking under the following conditions: background electrolyte 5-25 mM, phosphate buffer solution (pH 2.0); voltage: -20 kV; UV- detection 220 nm; sample injection: 2-20 s at 30-50 mbar; temperature: 20 ^{0}C

Large volume sample stacking for corticosteroid hormones

Preconcentration of steroid hormones (concentration 0.5–10 μg) in the large volume sample stacking mode was carried out under the following conditions: background electrolyte 10 mM, phosphate buffer solution (pH 2.0); voltage: -20 kV; UV-detection 254 nm; sample injection: 2-80 s at 30-50 mbar; temperature 20 °C.

Field-amplified sample injection and sweeping of biogenic amines

For the preconcentration of biogenic amines in FASI-sweeping mode, SDS was used as a micelle forming agent (CMC = 8.2 mM) under the following conditions: background electrolyte: 10 mM, phosphate buffer solution (pH 2.0), SDS (15-50 mM); voltage +20 kV; wavelength: 220 nm; sample injection: 2-100 s at 10-15 kV; temperature: 20 ^{0}C.

Use of modifier plug for steroid hormones preconcentration

Two types of plug injection were used: hydrodynamic (2-20 s 30 mbar) before the injection of the analyzed sample into a quartz capillary preliminarily filled with background electrolyte, and electrokinetic (2-40 s 5-15 kV) after it. Solutions of ionic liquids were used as a micelle-forming agent: 15–20 mM C_{12}MImCl and 1 mM C_{16}MImCl, 1 mM CTAB.

II.8.2 Preconcentration of steroid hormones separated in electrokinetic chromatography mode

The preconcentration of steroid hormones (concentration 0.5-10 μg) in the large volume sample stacking mode was carried out under the following conditions: 5 mM phosphate buffer solution (pH 6.4), 0.7 mM 3-Me-1-β-CD-ImOTs, 10 (vol.) % ACN. Model mixture of 5 steroids (0.1-10 μg/ml): sample injection - 2-50 s 30 mbar, voltage +20 kV, wavelength 254 nm.

The preconcentration of steroid hormones *electrokinetically injected in the form of complexes with CD* was carried out under the following conditions: 5 mM phosphate buffer solution (pH 6.4), 0.7 mM 3-Me-1-β-CD-ImOTs, 10 (vol.) % ACN, model mixture containing five steroid hormones (0.1-10 μg/ml) in water solution, 3-Me-1-β-CD-ImOTs (0.15 mM); sample injection 2-50 with 20 kV.

II.8.3 Preconcentration of individual enantiomers separated in electrokinetic chromatography mode

The preconcentration of individual enantiomers was performed using both classic approaches and a combination of several forms of online preconcentration (hybrid methods) (Table 8).

Table 8. Tested conditions for intracapillary preconcentration of enantiomers of ketoprofen and ketorolac.

Type of online preconcentration	BGE composition	Sample matrix composition	Enantiomer concentration range	Sample injection	Wavelength, voltage
Field-amplified stacking	25 mM phosphate buffer pH 6.4, 1 mM 3-Bu-1-β-CD-ImOTs	2.5 mM PBS (pH 6.4)	0.5-50 μg/ml	2-80 s 30 mbar	254 nm, +20 kV
Stacking with large injection volume	25 mM PBS pH 6.4, 1 mM 3-Bu-1-β-CD-ImOTs	25 mM PBS pH 6.4	0.5-50 μg/ml	40 s 30 mbar	254 nm, +20 kV
Sweeping	25 mM PBS pH 6.4, 5-20 mM SDS, 1 mM 3-Bu-1-β-CD-ImOTs	25 мМ ФБР pH 6.4	0.5-50 μg/ml	2-40 s 30 mbar	254 nm, +20 kV
	25mM C_{12}MImCPBS pH 6.4, 5-2 mM l, 1 mM 3-Bu-1-β-CD-ImOTs	25 mM PBS pH 6.4	0.5-50 μg/ml	2-40 s 30 mbar	254 nm - 20kV
Dynamic pH - junction	25 mM PBS pH 6.4, 1 mM 3-Bu-1-β-CD-ImOTs	25 mM PBS pH 2.5 or 3.2	0.5-50 μg/ml	2-40 s 30 mbar	254 nm, +20 kV
Hybrid variant	25 mM PBS pH 6.4, 1 mM 3-Bu-1-β-CD-ImOTs	25 mM PBS pH 2.5 or 3.2, prior to sample injection micellar plug 10 mM SDS	0.5-50 μg/ml	2-40 s 30 mbar	254 nm, +20 kV

II.9 Analysis of real samples

Blood and urine samples, as well as drug-free human plasma, were obtained from the Federal State Institute of Public Health “The Nikiforov’s NRCERM” (St. Petersburg, Russia).

II.9.1 Determination of biogenic amines in urine samples

Sample pretreatment of urine samples was performed according to the previously described method of sorption preconcentration using activated alumina [179]. To prevent oxidation of analytes, 1.2 ml of a 50 mM water solution of Na_2EDTA were added to 5 ml of a urine sample, then the pH of the sample was adjusted to 8.5 with a 1 M water solution of Na_2CO_3, 25 mg of activated alumina was added,

mixed, and filtered using the Preppy system with a membrane pump. At the last stage, the sorbent was washed with distilled water (twice, 3 ml each) and the analytes were eluted with washing with 0.5 ml of a 1 M acetic acid solution.

II.9.2 Determination of steroid hormones in human plasma samples

Venous blood samples were collected from healthy volunteers after fasting in 5 ml heparin tubes. After centrifugation at 3000 rpm for 5 min at 4°C, the plasma was transferred into propylene tubes and stored at -20°C until analysis.

The liquid-liquid extraction method was used for pretreatment of plasma samples. 10 µl of the working solution of the internal standard were added into 1.0 ml of plasma or calibration samples. After vortex mixing for about 2 minutes, chloroform (5 ml) was added and thoroughly vortexed for 5 minutes, then the mixture was centrifuged at 15,000 rpm at 4°C for 10 minutes. After centrifugation, the upper layer was removed, and the lower organic layer was cleaned up twice with 0.5 ml of 0.1 M NaOH and 1.0 ml of H_2O. Then, when most of the water was removed, sodium sulfate was added and left for 20 minutes. The dehydrated chloroform layer was transferred into 0.6 ml eppendorf tubes and evaporated to dryness. The residue was dissolved in 200 ml of water with the addition of 0.05 mM 3-Me-1-β-CD-ImOTs, and centrifuged at 3000 rpm for 3 min, and the clear supernatant was injected into the capillary for analysis.

The content of steroid hormones was determined by the method of absolute calibration. To prepare calibration standards, phosphate-buffered saline was used as a surrogate matrix: 10 µl of the appropriate working solution were added to 1 ml of phosphate-buffered saline to obtain calibration solutions of steroids equivalent to 5.0, 10.0, 25.0, 50.0, 100.0 and 250.0 ng/ml. To obtain working solutions, stock solutions of corticosteroids (1 mg/ml) were diluted with deionized water to the required concentration (50, 100, 250, 500, 1000, 2500 ng/ml). The working solution of the internal standard (20 µg/ml) was obtained by diluting the stock solution in deionized water.

II.9.3 Determination of ketorolac enantiomers in the samples of human plasma

Venous blood samples were collected from healthy volunteers in 5 ml vacutainer tubes containing K_3EDTA 45 minutes after taking one Ketorol Express tablet. After centrifugation at 3000 rpm for 5 min at 4°C, the plasma was transferred to polypropylene tubes and stored at -20°C until analysis.

Working solutions of *R,S*-ketorolac 5.0, 10.0, 15.0, 20.0, 25.0, and 50.0 µg/ml were prepared by diluting the stock solution with distilled water. The concentration ratio of enantiomers in the racemic

mixture was considered 1:1; therefore, the concentrations of the individual enantiomers were 2.5, 5.0, 7.5, 10.0, 12.5, and 25.0 μg/ml.

Calibration samples were prepared by adding 20 μl of working solution to 180 μl of drug-free plasma. The final concentrations of calibration standard in plasma were 0.25, 0.50, 0.75, 1.00, 1.25, 2.5 μg/ml.

For electrophoretic analysis, 20 μl of working solution of internal standard (10 μg/ml *S*-ketoprofen) were added to the calibration samples, then 50 μl of 20% solution of trichloroacetic acid were added to precipitate the protein. After that, we vortexed the samples for 5 min and centrifuged them at 7000 rpm for 5 min. Then 100 μl of supernatant were transferred to a 1.5 ml eppendorf tube and diluted with 100 μl of distilled water.

For chromatographic analysis, 400 μl of 0.2% (wt.) solution of trichloroacetic acid into methanol were added to the calibration standards. After that, the samples were vortexed for 5 min and centrifuged at 7000 rpm for 5 min. Then 100 μl of the supernatant was transferred to vials and analyzed with HPLC.

Patients' plasma samples were thawed at room temperature and added 20 μl of a working solution of internal standard (10 μg/ml *S*-ketoprofen) to 200 μl of plasma samples. Protein precipitation and further samples pretreatment were performed according to the described procedures of electrophoretic or chromatographic analysis.

II.9.4 Sample preparation of tablets with the active ingredient ketorolac

A tablet containing ketorolac as an active ingredient (Ketorol express, Dr. Reddy`s Laboratories, Ltd, 10 mg) was dissolved in 10 ml of solution of water and acetonitrile (1:1 vol.), sonicated for 10 min and centrifuged at 7000 rpm for 10 min. Before electrophoretic analysis, the supernatant was diluted 100 times with deionized water.

II.10 Experiments without modification of the electrophoretic system

To evaluate the effectiveness of the proposed modifications of electrophoretic systems, we compared the obtained values of efficiency and separation selectivity with the values obtained in zero experiments, which we carried out at the same concentrations and pH values of the background electrolyte.

Chapter III. RESULTS AND DISCUSSIONS

Capillary electrophoresis (CE) is one of the forward-looking methods to determine biologically active compounds and medical drugs in complex matrices samples. It carries out both an active search of effective modifiers of electrophoretic systems to form the coatings (dynamic and covalent) of the quartz capillary walls, and hybrid variants of intracapillary *(on-line)* preconcentration, that enables to reduce the limits of determination to the values which are sufficient to analyze biological liquids.

As studied modifiers, we opted for imidazolium ionic liquids and modifiers based on imidazolium cation, which appeared to be universal in the CE field and suitable to separate a great number of biologically active compounds due to their positive charge, aromatic system and simple way to introduce different substituents into the imidazolium ring. Cyclodextrins, able to perform as a complexing agent with respect to hydrophobic analytes and a chiral selector for drug enantiomers, can be used as such substituents.

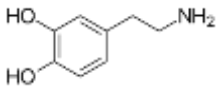
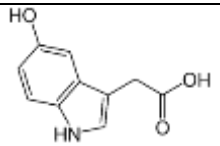
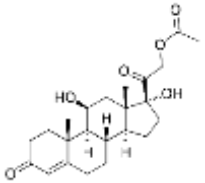
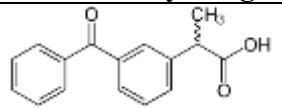
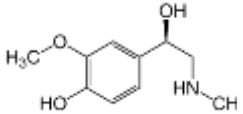
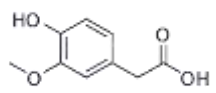
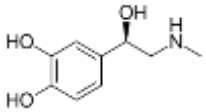
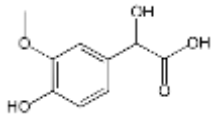
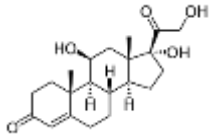
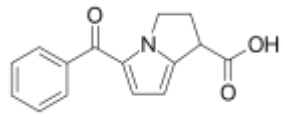
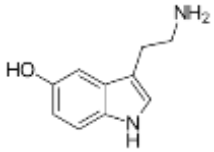
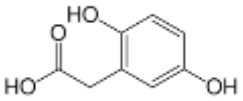
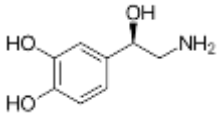
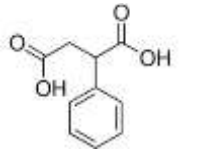
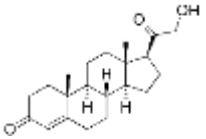
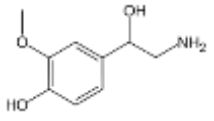
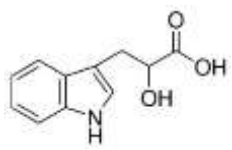
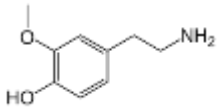
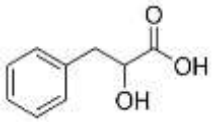
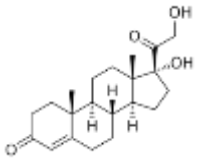
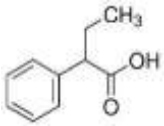
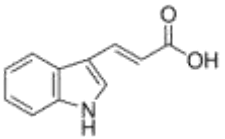
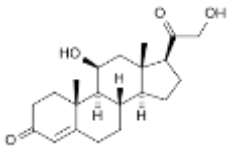
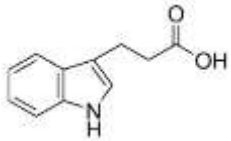
Therefore, polyfunctional modifiers, being the most noteworthy, can perform several functions: to modify the walls of a quartz capillary (including EOF reversal), to increase the efficiency and selectivity of separation due to interaction with analytes (π-π, electrostatic, hydrogen bonds formation), to facilitate separation of structurally similar compounds by implementing additional interaction mechanisms (CD complexation), as well as to be involved in online preconcentration processes.

In the course of the research, we identified three areas how to use such compounds:

- as pseudostationary phases
- as chiral additives
- as a modifier of quartz capillary inner walls with covalent coatings formation and implementation of the capillary electrochromatography mode

Using model mixtures of analytes belonging to different classes (table 9), we identified the analytical possibilities of the studied modifiers as stationary and pseudostationary phases.

Table 9. Structures of the studied compounds and their pKa values.

Biogenic amines	Acid derivatives of biogenic amines, phenyl-indole carboxylic acids	Corticosteroid hormones	Racemates of non-steroidal anti-inflammatory drugs
 **Dopamine (DA)** pKa=8.93	 **5-Hydroxyindoleacetic acid (5-HIAA)** pKa = 4.22	 **Cortisone (E)**	 ***R,S*-ketoprofen (ket)**
 **Metanephrine (MN)** pKa= 9.25	 **Homovanillic acid (HVA)** pKa = 3.74		
 **Adrenaline (A)** pKa=8.91	 **Vanillylmandelic acid (VMA)** pKa = 3.42	 **Cortisol (F)**	 ***R,S*-ketorolac (ketor)**
 **Serotonin (5-HT)** pKa = 9.31	 **Homogentisic acid** pKa = 3.57		
 **Noradrenaline (NA)** pKa = 8.58	 **Phenylsuccinic acid** pKa1 = 3.78; pKa2 = 5.55	 **Deoxycorticosterone (DOC)**	
 **Normetanephrine (NMN)** pKa = 9.25	 **3-indole acrylic acid** pKa = 4.59		
 **3-Methoxytyramine (3-MTA)** pKa = 9.64	 **3-phenyl-lactic acid** pKa = 4.02	 **11-Deoxycortisol (S)**	
	 **2-phenylbutyric acid** pKa = 4.66		
	 **3-indole lactic acid** pKa = 3.86	 **Corticosterone (B)**	
	 **3-indole propionic acid** pKa = 4.39		

III.1 Covalent coatings based on imidazolium cation

III.1.1 Covalent coatings formation

Pretreatment of capillaries with linker formation

We proposed the scheme for the synthesis of covalent coatings based on imidazolium cation, including activation of quartz capillary walls with alkali, silylation and functionalization. To obtain the structures of imidazolium ionic liquids type, the first stage of functionalization was a nucleophilic substitution reaction with imidazole, whereas the second stage included the variation of reagents structures used for the subsequent reaction.

First of all, we optimized the capillary pretreatment step to the synthesis of covalent coatings in the following way: the capillary was etched and the absorbed water was removed from its surface. During the activation of the capillary's surface, we considered two variants: etching the capillary with 2 M Sodium hydroxide solution (keeping the capillary filled with alkali at 90 °C for 1 h) and long flushing at high pressure (1000 mbar). The first way showed more effectiveness.

Desorption of the water, absorbed on the inner wall of the capillary, is an essential step before silylation. For this purpose, the capillary was preliminary flushed with acetone, purged with air and then kept at a high temperature in a thermostat, leaving the ends of the capillary unsealed. The range of the studied temperatures is 100-180 ^{0}C with a 20 ^{0}C step. After keeping at a high temperature for 1 hour, the capillary was silylated, and then we assessed the degree of modification comparing the values of electroosmotic flow on the capillaries, modified with a silylating agent, with an unmodified capillary [180]:

$$\varphi = 2\frac{(\mu_{OC} - \mu_{OSi})}{(\mu_{OC} + \mu_{OSi})}, \quad (2)$$

Where μ_{OC} is EOF mobility in unmodified capillary, sm^2/V*s;

μ_{OSi} is EOF mobility in the capillary with a covalently sealed silylating agent, sm^2/V*s.

The highest modification degree was achieved at dehydration temperature of 160 °C and is fully consistent with the result obtained in [180] for 1-oct-7-enyltrimethoxysilane.

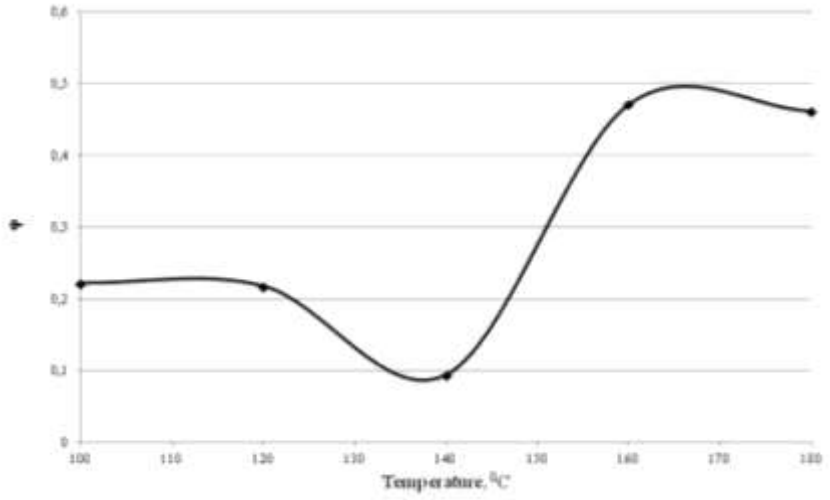


Figure 21. The modification degree of quartz capillary on silylation stage after desorption of the absorbed water at elevated temperatures.

Conditions: capillary electrophoresis system “Capel’-105M”, 10 mM phosphate buffer solution (pH 7); 10 kV; UV-detection 220 nm; EOF marker is 5 (vol.) % solution of DMF in water.

Silylation reaction was performed with the solution containing degassed 30% (3-glycidyloxypropyl) trimethoxysilane (v/v) and 0.1% DPPH in DMF. After the capillary had been sealed on both ends, it was kept in the thermostat at 120 °C for 6 h.

The next stage included the reaction of functionalization with imidazolium with the opening of 3-glycidyloxypropyltrimethoxysilane oxirane cycle, proceeding, as Krasuski rule says, according to the S_{N2} mechanism (substitution proceeds near the least substituted atom of oxirane cycle). The capillary was filled with imidazolium solution in DMF (25 mg/ml) and kept at 90 °C for 6 h [181].

Figure 22 shows the general scheme of synthesis.

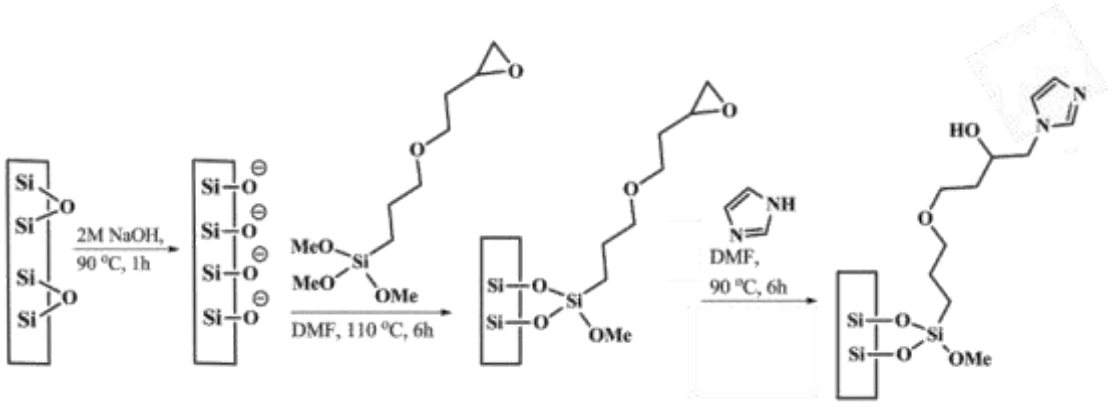


Figure 22. The scheme of synthesis of linker for covalent coating based on imidazole.

Covalent coatings postfunctionalization

Variation of the imidazolium cation substituent enables to change the functionality of the coating and considerably expand analytical potential of synthesized covalent coatings. Depending on reagents, used on the second stage of functionalization, we obtained covalent coatings with different structures – alkylimidazolium and based on β-CD. The latter was chosen as a functionalizing object due to its hydrophobic cavity that makes it very promising for selective electrophoretic determination of steroid hormones and drugs enantiomers.

To obtain alkylimidazolium coatings alkylbromides were used. Interaction of 1-bromobutane or 1-bromooctane with imidazolium, covalently immobilized on the inner capillary wall, resulted in 1, 3-dialkylimidazolium salts.

To perform the in the reaction, the capillary was filled with 1-bromoalkane and kept for 10 hours at 80^0C. For modification with cyclodextrine, we supposed (similarly to functionalization with alkylbromides) to perform the nucleophilic substitution reaction with imidazolium covalently sealed to the capillary walls, however, hydroxyl groups of cyclodextrine are poorly leaving groups and cannot enter into a nucleophilic substitution reaction given conditions. To obtain functionalized β-CD coatings we used a preliminary synthesized β-cyclodextrine derivative, where the primary hydroxyl group was replaced with a good leaving tosyl group. Then we filled the capillary with tosyl derivative solution in DMF and optimized the conditions of this postfunctionalization stage reaction. We varied the concentration of a modifying agent (25-750 mg/ml), temperature (80-100 ^{0}C) and reaction time (10-22 hours). We ascertained that the intended effect could be achieved with 500 mg/ml modifier concentration, 22 hours of reaction, and at 100^0C: the formed coatings showed high repeatability of more than 150 electrophoretic analyses of mixtures of hydrophobic and hydrophilic analytes [182].

Figure 23 shows the general scheme of synthesis and obtained structures of covalent coatings.

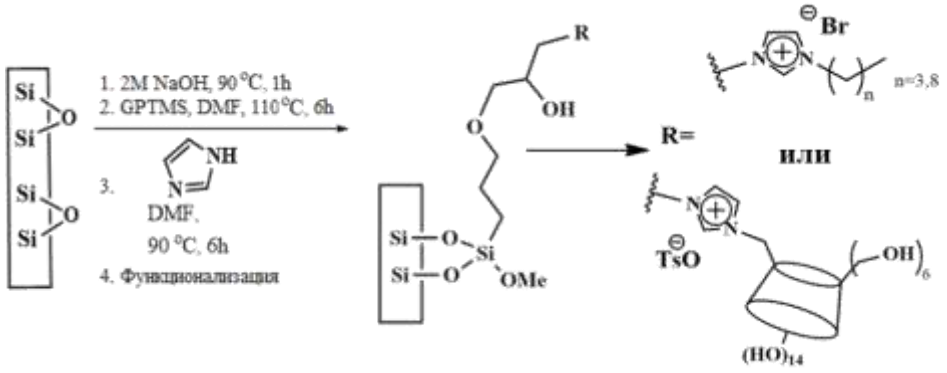


Figure 23. The general scheme of synthesis and obtained structures of covalent coatings.

III.1.2. Characteristics of synthesized covalent coatings

Positively charged covalent coatings on the inner wall of the quartz capillary lead to EOF reversal, with an intensive anodic EOF being observed at pH 2.0 of the background electrolyte.

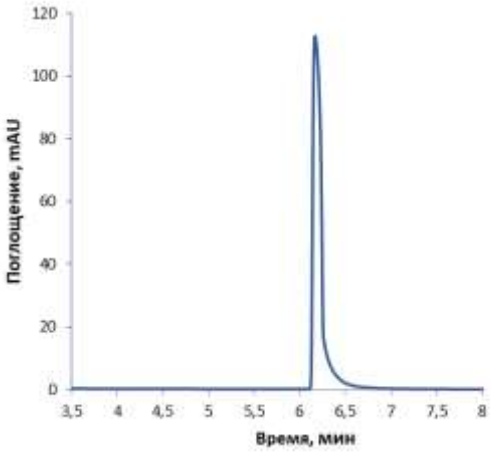


Figure 24. Electropherogram of the EOF marker (EOF marker - 5% (vol.) solution of DMF in water) on alkylimidazolium covalent coatings.

Conditions: capillary electrophoresis system "Capel'-105M", 10 mM phosphate buffer solution (pH 2.0); -20 kV; 220 nm.

SEM photographs also confirm the presence of covalent coating on the inner wall of the quartz capillary (figure 25). The pictures show that the surface of the modified capillary has a layer of covalent coating. Measured at several points, its thickness is 18-20 nm.

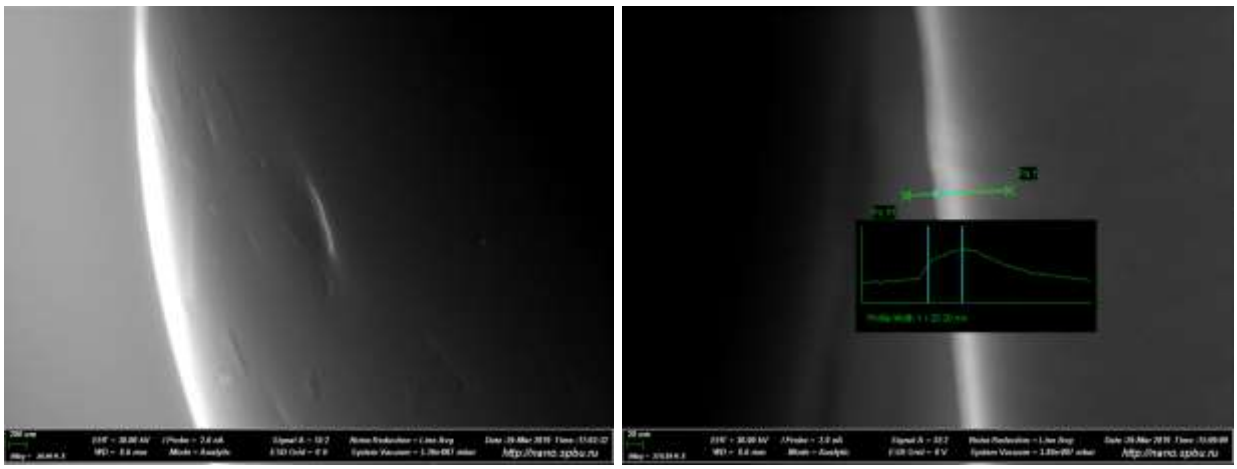


Figure 25. Photograph of the modified inner surface of the quartz capillary (left) and determination of the coating thickness (right). Conditions: Carl Zeiss Merlin device, accelerating voltage 30 kV; capillaries with a covalent coating based on imidazole and β-CD (functionalization conditions: $C_{Ts\text{-}\beta\text{-}CD}$=500 mg/mL; 22 h, 100 ^{0}C).

The stability of covalent coatings from analysis to analysis and from capillary to capillary was studied. Conclusions concerning capillary stability were based on the EOF velocity measurements (table 10). Synthesized coatings show high repeatability of more than 150 analyses.

Table 10. Reproducibility of the EOF velocity from capillary to capillary and from analysis to analysis.

	From capillary to capillary		From analysis to analysis	
	μ_e, $10^9 m^2V^{-1}s^{-1}$	RSD, %	μ_e, $10^9 m^2V^{-1}s^{-1}$	RSD, %
based on CD	-34.7±2.7	7.7	-36.4±0.5	1.4
butylimidazolium	-43.4±1.4	3.2	-43.7±0.4	0.9

In addition, by measuring the EOF velocity, the stability of covalent coatings when an organic solvent is added into the composition of the background electrolyte was assessed. In the search for separation conditions, organic solvents can be useful in case the change of hydrophobic-hydrophilic balance is required. Before adding acetonitrile, EOF velocity on the analyzed capillary was -40.7×10^9, $m^2V^{-1}s^{-1}$ and was measured in the following conditions: 10mM phosphate buffer solution (pH 2.0), -20 kV, EOF marker – 5% DMF water solution. Then, we passed acetonitrile through the capillary for 5 minutes and measured the EOF velocity again in the conditions similar to the initial measurement of the EOF velocity. The process was repeated 15 times. The change of EOF velocity values after acetonitrile total exposure within 1 hour was no more than 4%, which meets the standard error in measuring EOF velocity after flushing with acetonitrile ($-38.9\pm1.5\times10^9$, $m^2V^{-1}s^{-1}$, RSD, n=13, P=95%) and may be related to a longer recovery of the diffusion layer near the coating surface. Thus, we can postulate the stability of the coating when acetonitrile is being added to the BGE. However, keeping the capillary in acetonitrile for 2 days leads to a more considerable change of the EOF velocity (~15%), that is why when working with organic solvents it is best to wash up the capillary with working buffer solution, without leaving it filled with an organic solvent for a long time.

To evaluate the coating stability at different pH values of the background electrolyte and determine the working pH range we controlled the EOF velocity and compared with the values, obtained on the unmodified capillary. We performed the measurements on the capillaries with covalent coating three times at each point firstly decreasing acidity and then increasing it. The process was performed twice. In light of this, we can conclude that partial destruction of the coating occurs in alkaline medium and caused by the hydrolysis of silanol bonds, which are directly involved in fixing the covalent coating on the inner wall of the quartz capillary. An increase in the number of free silanol groups due to coating degradation results in the increase of the cathode EOF velocity in alkaline medium and decrease of the

anodic EOF velocity in acidic medium, however, even after the second hysteresis loop we registered anodic EOF, which indicates the presence of the coating (Figure 26).

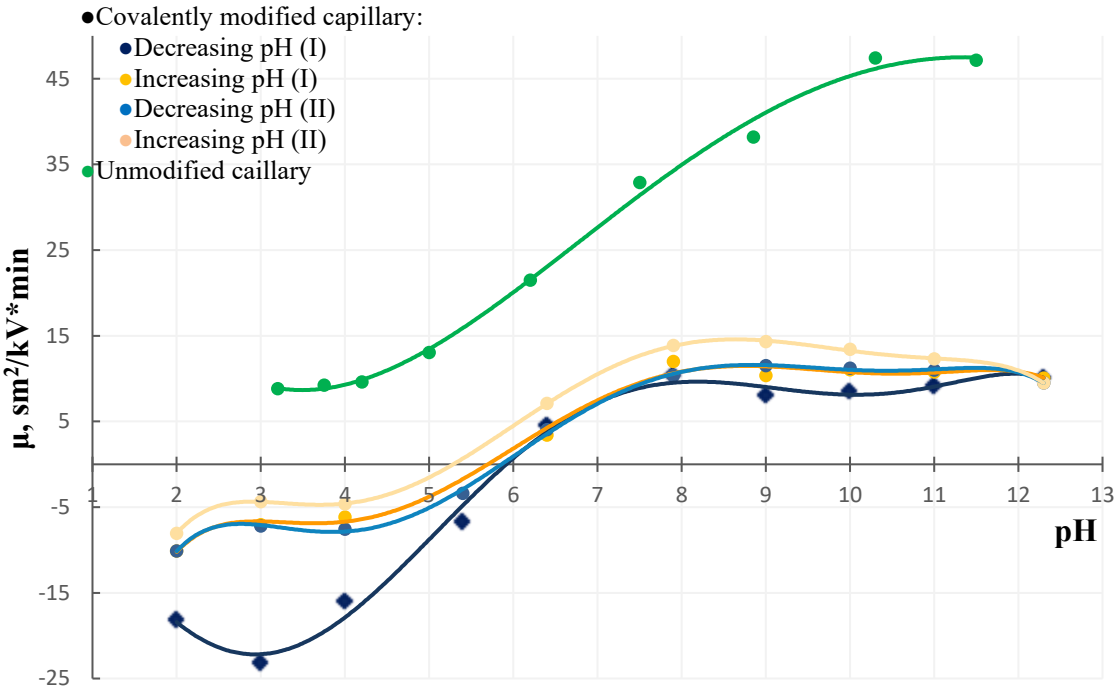


Figure 26. The evaluation of the stability of covalent coatings on the walls of a quartz capillary in a wide pH range using a cyclodextrinimidazolium coating as an example.

To evaluate the analytical possibilities of the synthesized coatings we chose different compounds: biogenic amines as hydrophilic analytes, and corticosteroid hormones as hydrophobic analytes; and we used ketoprofen racemate to determine whether cyclodextrine imidazolium coating could facilitate chiral separation.

III.1.3 Analysis of biogenic amines on covalently modified capillaries

A positive charge of the covalent coating on quartz capillary walls prevents the sorption of main analytes – biogenic amines, providing their separation with R_s high values (R_s=0.9–2.7). We found a massive increase in separation efficiency and selectivity which is largely caused by π-π interactions between analytes and imidazolium ring that can be seen in the example of the dopamine-serotonin pair (Figure 27A)

We studied the influence of imidazolium ring substituent in the structure of covalent coating on the results of electrophoretic determination of biogenic amines. The time of biogenic amines analysis on the coating with β-CD being a substituent, increased in comparison with *N*-alkylimidazolium coatings (figure 27). One of the possible reasons is decrease in efficiency of electrostatic repulsion of positively

charged analytes from capillary walls with a similar charge due to the lower availability of the imidazolium cation.

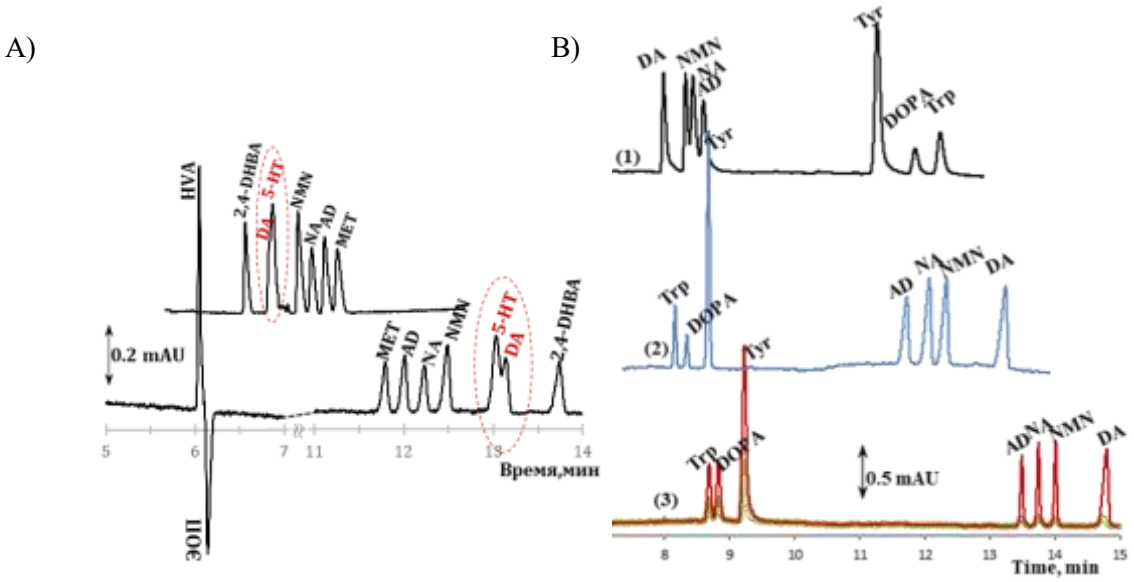


Figure 27. Electropherograms of a model mixture of 7 biogenic amines on an unmodified capillary (top) and a capillary covalently modified with butylimidazole (A) and catecholamines and absorbing amino acids in the UV region of the spectrum (from top to bottom) on an unmodified capillary (1) and capillaries covalently modified with butylimidazole (2) and cyclodextrinimidazole (3) (B).

Conditions: capillary electrophoresis system "Capel'-105M", 10 mM NaH_2PO_4 solution (adjusted to pH 2.0 with 0.1 M HCl solution), sample injection 5 s 30 m bar, - 20 kV (on covalently modified capillaries) and +20 kV (on unmodified capillaries), 220 nm.

Sample: model mixture of biogenic amines and absorbing amino acids (25 μg/ml): NA - norepinephrine, DA - dopamine, NMN - normetanephrine, AD - epinephrine, Trp - tryptophan, Tyr - tyrosine, DOPA - 3,4-dihydroxyphenylalanine.

Alkylimidazolium coatings showed their effectiveness in determination of acidic derivatives of biogenic amines, as well as phenyl-and indolecarboxylic acids. In spite of the fact that butylimidazolium coating allows simultanious determination of acidic, zwitterionic and basic analytes (figure 28), electrophoretic separation of structurally similar compounds requires optimization of special conditions.

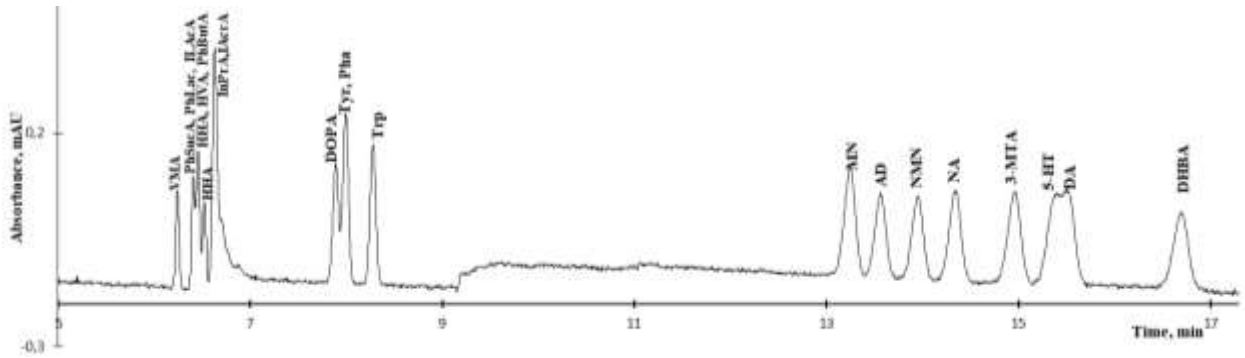


Figure 28. Electrophoregram of the simultanious determination of acidic and basic analytes on a butylimidazolium covalent coating.

Conditions: capillary electrophoresis system "Capel'-105M", 10 mM NaH_2PO_4 solution (adjusted to pH 2.0 with 0.1 M HCl solution), sample injection: 2.0 s 30 mbar; -20 kV; 220 nm. The stationary phase is a covalent coating based on the butylimidazolium cation.

To find the separation conditions of all 10 carboxylic acids, we studied the influence of pH value and BE ionic strength on the selectivity of their separation. At pH 2 the dissociation of acids is suppressed (pKa of these acids is in the range of 3 – 5; see table 9), an increase in the pH value of the background electrolyte results in the dissociation of acids and change in their migration velocity. We observed a non-linear change in the acids separation selectivity: an increase in pH from 2 to 3.8 showed the growth of all acids separation selectivity (the maximum values were reached at pH 4.2); a further increase of the background electrolyte pH to 4.5 resulted in the decrease in some acids separation selectivity (figure 29); and for indoleakrylic acid the peak asymmetry increased significantly. An increase in the background electrolyte ionic strength to 25 mM did not lead to a marked increase in separation selectivity factors.

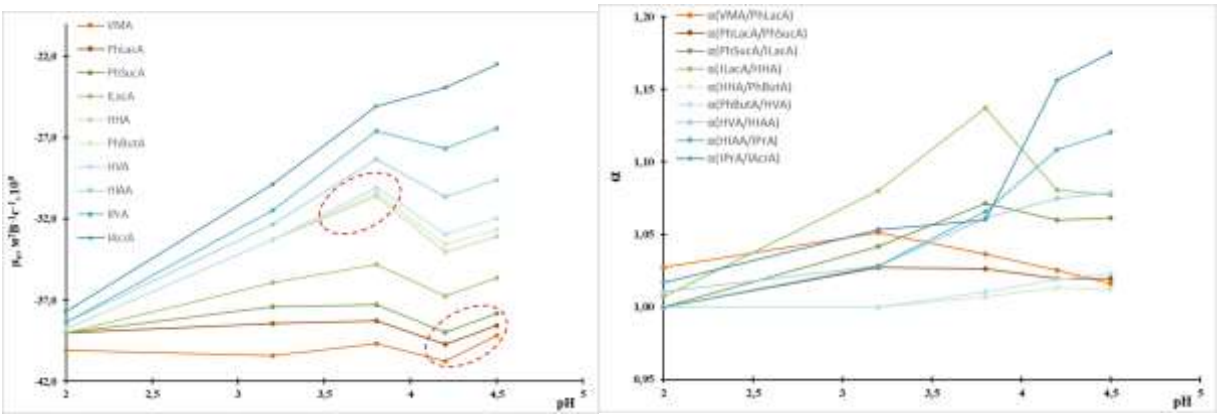


Figure 29. Dependence of changes in electrophoretic mobilities (left) and selectivity coefficients (right) of ten aromatic acids on the pH value of the background electrolyte.

Separation of all ten acids was achieved under the following conditions: 10 mM NaH_2PO_4 solution (adjusted to pH 2.0 with 0.1 M HCl solution), pH 4.2, -20 kV, 220 nm (Figure 30)

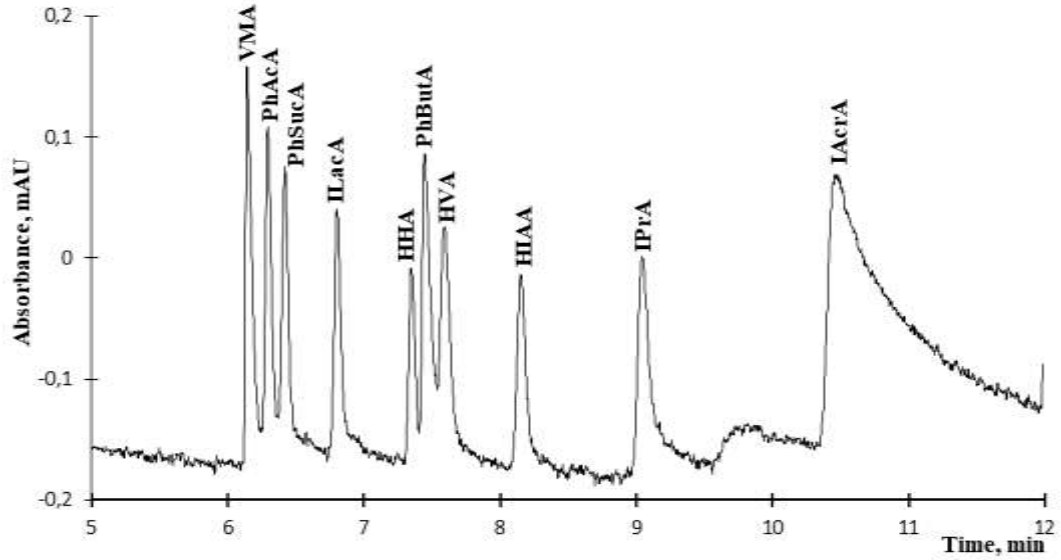


Figure 30. Electropherogram of a mixture of phenyl and indole carboxylic acids on a butylimidazolium covalent coating.

Conditions: 10 mM NaH_2PO_4 (adjusted to pH 4.2 with 0.1 M HCl, inlet: 2.0 s 30 mbar; -20 kV; 220 nm. Stationary phase - covalent coating based on butylimidazolium cation.

III.1.4 Preconcentration of biogenic amines on covalently modified capillaries

We performed the preconcentration of biogenic amines in large volume sample stacking mode on all three types of covalent coatings. In case of butylimidazolium coating, the more accessible positive charge on the capillary surface leads to more efficient concentration; the limits of detection for octyl and cyclodextrin coatings proved to be substantially higher than for butyl coatings, which can be explained by less effective electrostatic repulsion from the capillary walls with the increase in positive charge screening (table 11).

Table 11. Comparison of the limits of detection and concentration factors of biogenic amines obtained using intracapillary concentration in large volume sample stacking mode on various covalently bound coatings.

	Butylimidazolium coating		**Octylimidazolium coating**		**Cyclodextrinimidazolium coating**	
	SEF_h	LODs, μg/ml	SEF_h	LODs, μg/ml	SEF_h	LODs, μg/ml
DA	110	12	7	120	3	160
NA	140	11	4	230	5	170
NMN	120	14	8	150	5	170
A	120	17	8	170	6	240

We obtained noteworthy results when a micelle forming agent SDS was added into BGE. Due to hydrophobic interactions, SDS was sorbed on the surface of the modified capillary, which led to a change in its charge to negative. A cathodic EOF was generated at a background electrolyte pH 2.0, which allowed to implement a hybrid preconcentration method – a combination of sweeping with FASI, and to considerably reduce LOD (table 12). A brief scheme of preconcentration is shown in Figure 31. The codirectional migration of analytes and cathodic image intensifier tube, generated due to the formation of a bilayer by SDS molecules, made it possible to implement the FASI mode: a large volume of a sample dissolved in a low-conductivity matrix was introduced under voltage into a capillary preliminarily filled with a micellar buffer. At the interface with BGE, analytes interacted with the cavity of micelles, which led to the formation of associates and, as a result, slowing down the migration of analytes and their preconcentration.

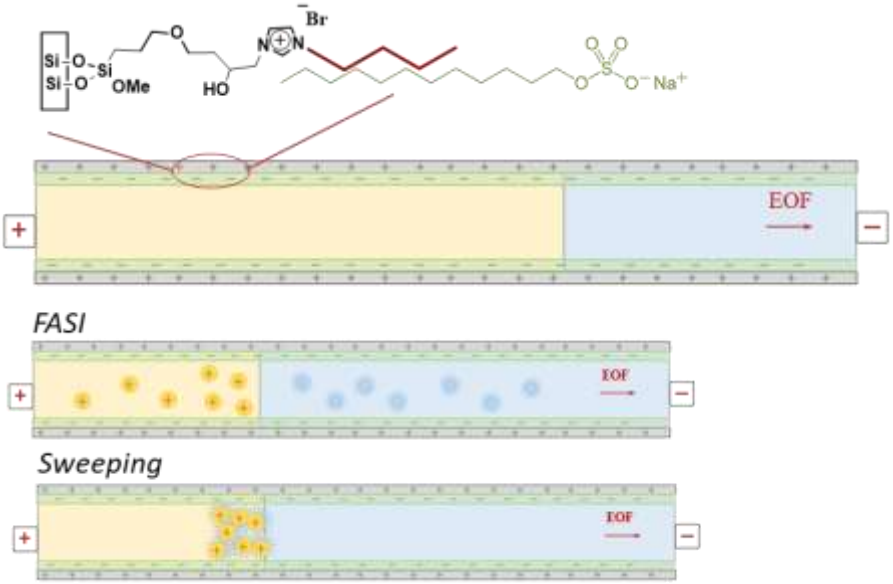


Figure 31. The scheme of hybrid preconcentration of biogenic amines (combination of sweeping and FASI).

Table 12. On-line preconcentration of biogenic amines on capillaries covalently modified with alkylimidazolium ionic liquid

	N-butylimidazolium coating				**N-octylimidazolium coating**			
Type of preconcentration technique	*Field-amplified sample stacking*		Sweeping + FASI		*Field-amplified sample stacking*		Sweeping + FASI	
Sample injection	1500 mbar*s		750 kV*s		1500 mbar*s		750 kV*s	
Background electrolyte	10 mM NaH_2PO_4 (pH 2.0)		10 mM NaH_2PO_4 (pH 2.0), 50 mM SDS		10 mM NaH_2PO_4 (pH 2.0)		10 mM NaH_2PO_4, (pH 2.0), 50 mM SDS	
	SEF_h	LODs, ng/ml	SEF_h	LODs, ng/ml	SEF_h	LODs, ng/ml	SEF_h	LODs, ng/ml
DA	110	12	1030	2.1	7	120	3500	0.3
NA	140	11	1460	1.5	4	230	980	1.1
NMN	120	14	930	2.3	8	150	2200	0.5
A	135	17	1500	1.4	8	170	2600	0.6

SDS micelles in the background electrolyte also performed as a pseudostationary phase, which led to the implementation of micellar electrokinetic chromatography and change in analytes migration with increase in separation efficiency and selectivity (figure 32).

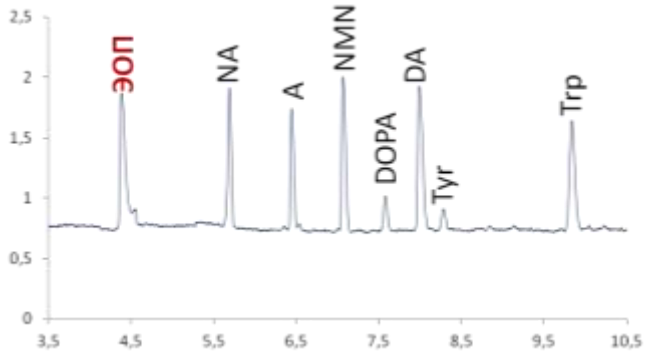


Figure 32. Electropherogram of a model mixture of catecholamines and amino acids.

Conditions: capillary electrophoresis system "Capel'-105M", 10 mM NaH_2PO_4 solution, pH 2.0 (adjusted to the required pH value with 0.1 M HCl solution) and 50 mM SDS, -20 kV, 220 nm. Analytes: NA - norepinephrine, A - epinephrine, NMN - normetanephrine, DA - dopamine, Tyr - tyrosine, Trp - tryptophan, DOPA - 3,4-dihydroxyphenylalanine.

III.1.5 Electrophoretic analysis of steroid hormones

The formation of a positive charge on the capillary surface during the functionalization of the CD coating allows not only the separation of hydrophilic biogenic amines, but also the implementation of the capillary electrochromatography mode due to the formation of “guest-host” complexes. From research literature we know that CD is introduced into the background electrolyte to separate hydrophobic corticosteroid hormones, therefore, the presence of β-CD in the covalent coating structure could provide the separation of these analytes due to their interaction with the hydrophobic cavity of the macrocycle. At the same time, in capillary zone electrophoresis mode neutral steroid hormones are eluted together with the anodic EOF on alkylimidazolium coatings without separation (Figure 33).

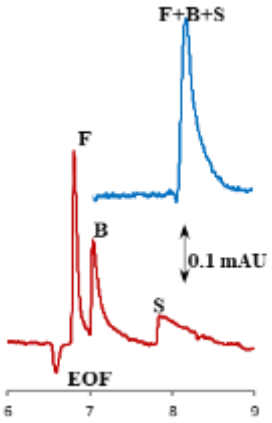


Figure 33. Electropherogram of hydrophobic corticosteroid hormones on butylimidazolium and cyclodextrinimidazolium coatings.

Conditions: capillary electrophoresis system "Capel’-105M", 10 mM NaH_2PO_4 solution (adjusted to pH 2.0 with 0.1 M HCl, -20 kV; 254 nm, model mixture of steroid hormones (10 μg/ml): F-cortisol, S-11- deoxycortisol, B-corticosterone, injection 5 s 30 mbar.

It was possible to separate three corticosteroid hormones, while structurally similar cortisone and cortisol were not separated in the given conditions and migrated together. We tested various relevant approaches to increase the separation selectivity of this pair of analytes: different additives were introduced into the background electrolyte, such β-CD, HP-β-CD, β-CD-MImOTs, C_{16}MImCl и C_{12}MImCl at concentrations exceeding the critical micelle concentration, and the hydrophobic-hydrophilic balance was changed by introducing organic solvents into the background electrolyte. However, instead of the intended effect the decrease in separation selectivity was observed (Figure 34).

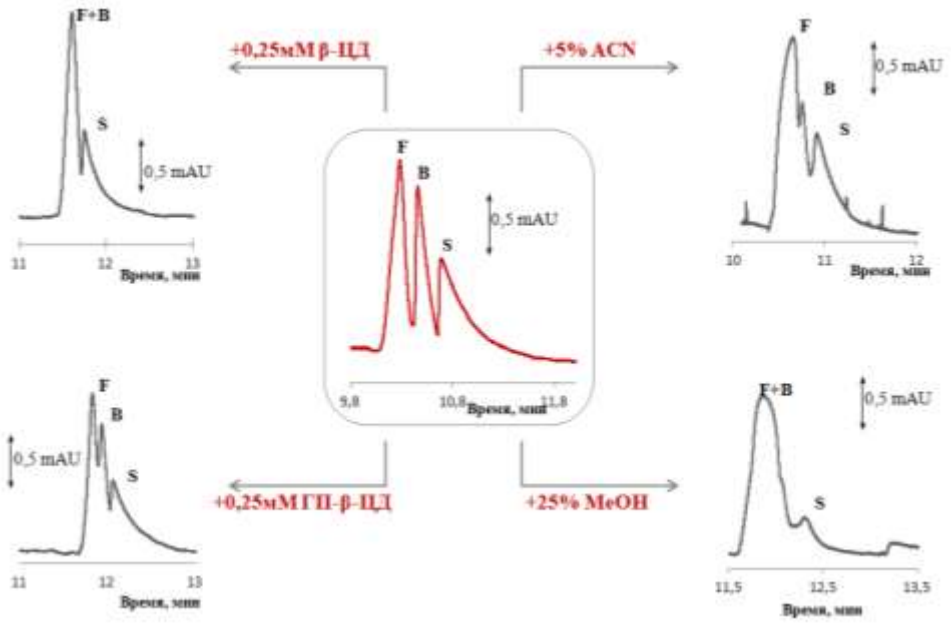


Figure 34. Effect of additions to the composition of the background electrolyte on the separation selectivity of steroid hormones.

Conditions: capillary electrophoresis system "Capel-105M", 10 mM phosphate buffer solution (pH 2); quartz capillary coated with imidazole and β-CD (functionalization conditions: $C_{Ts\text{-}\beta\text{-}CD}$=500 mg/ml; 22 h, 100 ^{0}C), -20 kV, 254 nm; model mixture of steroid hormones (10 μg/ml): F-cortisol, S-11-deoxycortisol, B-corticosterone, injection 5 s 30 mbar.

This situation can be explained as follows: additives block the cavities of cyclodextrins hindering this way the access of steroid hormones to macrocycles. When cyclodextrins are injected into the background electrolyte, such blocking can be performed due to the formation of their dimmers together with hydrogen bonds between the hydroxyl groups of cyclodextrins in the stationary phase and background electrolyte [183].

However, a remarkable feature of such coatings is the discovered potential of simultanious electrophoretic determination of hydrophobic (steroid hormones) and hydrophilic analytes (biogenic amines and amino acids) in one analytical cycle (figure 35) with high repeatability values of migration characteristics (table 13).

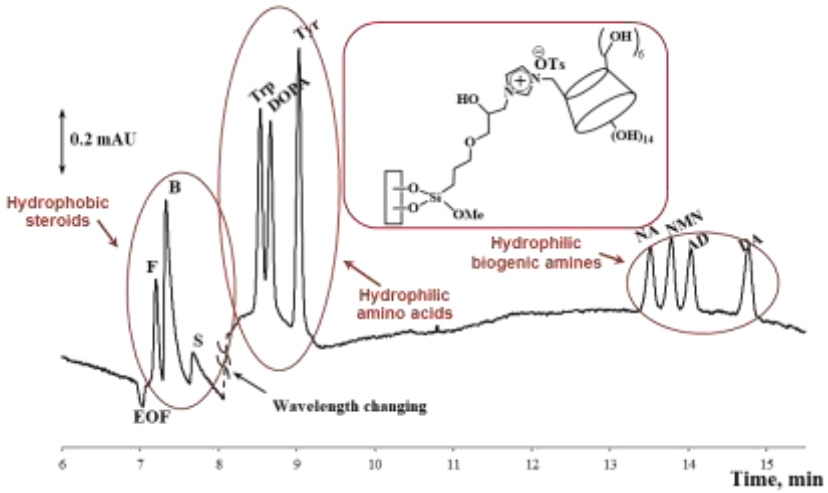


Figure 35. Electropherogram of the joint determination of hydrophobic corticosteroid hormones and hydrophilic biogenic amines on a covalently modified capillary functionalized with β-CD [182].

Conditions: 10 mM NaH_2PO_4 solution (adjusted to pH 2.0 with 0.1 M HCl solution; sample injection: 2.0 s 30 mbar; -20 kV; 254 nm (1-8 min) and 220 nm (8-15 min), 0 mbar (1-10 min) and 40 mbar (10-15 min).

Sample: Model mixture of steroid hormones (10 μg/ml) F-cortisol, S-11-deoxycortisol, B-corticosterone, biogenic amines and absorbing amino acids (25 μg/ml); NA - norepinephrine, DA - dopamine, NMN - normetanephrine, AD - epinephrine, Trp - tryptophan, Tyr - tyrosine, DOPA - 3,4-dihydroxyphenylalanine.

Table 13. Reproducibility of the migration characteristics of corticosteroid hormones on a cyclodextrinimidazolium coating of a quartz capillary.

	Capillary to capillary		Run to run	
	μ_e, $10^9 m^2V^{-1}s^{-1}$	RSD (%)	μ_e, $10^9 m^2V^{-1}s^{-1}$	RSD (%)
Cortisol (F)	-36.5±2.0	5.4	-34.2±0.5	1.5
Corticosterone (B)	-35.7±2.3	6.4	-32.5±0.5	1.5
11-Deoxycortisol (S)	-33.8±2.3	6.8	-30.3±0.2	0.7

III.1.6 Preconcentration of steroid hormones

Similarly to biogenic amines, the search for acceptable and effective options for intracapillary preconcentration of steroid hormones required a special set of preliminary experiments. Large volume sample stacking mode proved to be ineffective as a decrease in the separation selectivity was observed. The introduction of additives into the background electrolyte to implement sweeping also led to a decrease in the separation selectivity due to CD cavities blocked with surfactant molecules.

We required an approach, which could allow us to concentrate steroid hormones without affecting the capillary electrochromatography mechanism. It was supposed that the introduction of the pseudostationary phase in the plug form before or after sample injection could provide additional interaction with analytes, facilitating concentration and without blocking macrocycles cavities in the stationary phase.

The addition of CTAB, C_{16}MImCl, and C_{12}MImCl into the background electrolyte in concentration higher than CMC did not provide the required characteristics of electrophoretic separation. We managed to maintain the selectivity of steroid hormones separation with an increase in the sample injection volume only when positively charged 3-Me-1-β-CDImOTs was injected as a plug (figure 36). The counterdirectional migration of this compound allowed concentrating analytes without blocking CD cavities on the capillary surface.

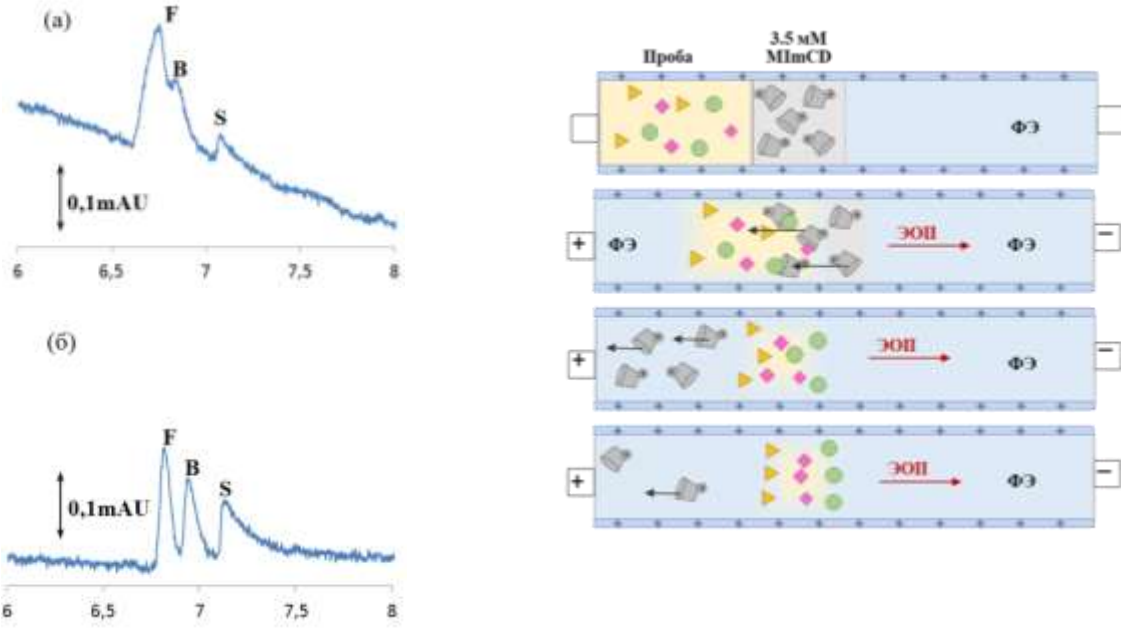


Figure 36. Electropherograms of a model mixture of steroid hormones without (top left) and with (bottom left) a concentration stopper; proposed scheme for preconcentrating steroid hormones with the introduction of a pseudo-stationary phase plug (right).

Conditions: capillary electrophoresis system "Capel-105M", 10 mM phosphate buffer solution (pH 2) (a) + hydrodynamic injection of a 3-Me-1-β-CDImOTs plug (0.5 mg/ml) 5 s 30 mbar before entering the analyte sample; -20 kV; 254 nm; input 10 s 30 mbar.

In order to increase the concentration factors, we varied the plug injection time and the concentration of 3-Me-1-β-CDImOTs. Varying the concentration did not significantly affect the controlled parameters, whereas the time ratio of steroid hormones and plug injection affected the

obtained values of separation efficiency and selectivity. During the steroid hormones sample injection (10 s 30 mbar and plug – 5 s 30 mbar) we managed to concentrate steroids only 2-3 times, and a subsequent increase in the injection time resulted in destacking.

Own electrophoretic mobility of the modifier used for preconcentration allows its electrokinetic injection after the sample (figure 37). At the same time, the factors of concentration proved to be lower than expected (SEF 2-10 during the plug injection 2s 10 kV), and the limits of detection were 120-160 ng/ml.

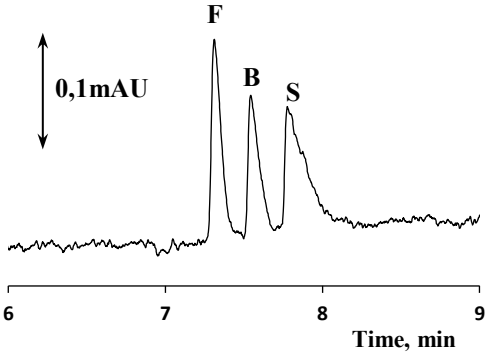


Figure 37. Electropherogram of a model mixture of steroid hormones

Conditions: capillary electrophoresis system "Capel'-105M", 10 mM phosphate buffer solution (pH 2); electrokinetic injection of pseudo-stationary phase (0.5 mg/ml) after analytes 2 s 10 kV; -20 kV, 254 nm. Model mixture of steroid hormones 5 µg/ml, 15 s 30 mbar.

III.1.7 Chiral separation of ketoprofen on covalent coatings

In separation and concentration methods, cyclodextrins can perform as chiral selectors in the determination of enantiomers, however, there was no separation of enantiomers in the course of preliminary experiments on covalently modified capillaries. This could be explained by the insufficient number of active sites for chiral separation, which required returning to the optimization step of the cyclodextrin functionalization stage. By varying the conditions for the synthesis of covalent coatings and increasing the number of CD on the capillary surface, we obtained the coatings which ensured the separation of ketoprofen enantiomers. In addition to the increase in the BGE ionic strength, it resulted in the growth of resolution factors of ketoprofen enantiomers with 1.03 enantioselectivity (figure 38). To establish the order of their migration, we performed an electrophoretic experiment with an individual enantiomer (*S*-ketoprofen).

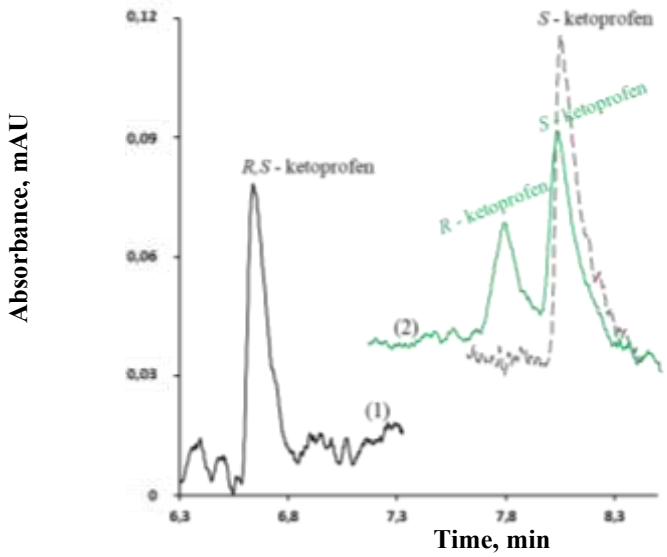


Figure 38. Electropherograms of the model mixture of ketoprofen racemate before (1) and after (2) optimization of separation conditions (dashed line – electropherogram of S-ketoprofen to identify the migration order).

Conditions: capillary electrophoresis system "Capel'-105M", 10 mM (a) and 20 mM (b) PBS (pH 2); -20 kv; 254 nm; cyclodextrinimidazolium coating (functionalization conditions: $C_{Ts\text{-}\beta\text{-}CD}$=750 mg/ml; 22 h, 100 ^{0}C), model mixture of ketoprofen racemate (25 μg/ml), sample injection: 5 s 30 mbar.

It should be noted that the use of high concentrations of tosyl-β-CD at the postfunctionalization stage is difficult due to the high viscosity of the solution, thus, further optimization with an increase in the concentration of the functionalizing agent was not performed.

III.2 Modifiers based on imidazolium and CD as pseudostationary phases in the separation of hydrophobic analytes

Analytes of different polarity (corticosteroid hormones, amino acids, and biogenic amines) were separated with high selectivity in one analytical cycle on the capillaries with a covalently bonded stationary phase based on imidazole and β-CD. It has been shown that an increase in the number of active sites of such coatings allows using them for chiral separation of drug products as well.

It seemed noteworthy to test the potential of such structures as a pseudostationary phase, where the amount of the modifier and, consequently, the active sites could be easily controlled by changing its concentration in the background electrolyte.

For this purpose, we obtained cyclodextrin derivatives structurally similar to the previously analyzed covalent coatings - 1-Me-3-β-CDImOTs.

When revealing the potential of such a compound to facilitate the formation of dynamic coatings, it was established that the introduction of IL with a bulky framework substituent (1-Me-3-β-CDImOTs) into the background electrolyte does not lead to the modification of the quartz capillary walls with the formation of positively charged dynamic coatings and EOF reversal in spite of having a positive charge in its structure. The same situation is relevant to imidazolium IL with a small alkyl radical, e.g. C_4MImCl: the sorption of the modifier on the capillary walls is confirmed by comparing the initial velocity of EOF ($\mu_{ЭОП}=47.3\times10^{-9}m^2V^{-1}s^{-1}$) after activation of the capillary walls before the introduction of the modifier into the background electrolyte with the EOF velocity after its introduction ($\mu_{EOF}=36.8\times10^{-9}m^2V^{-1}s^{-1}$) (figure 39). A long washing of the capillary with 0.5 M NaOH solution or 0.1 M HCl solution does not allow to remove the adsorbed modifier, therefore, at least three blank electrophoretic experiments should be performed to establish the balance between the BGE modifier and the sorbed modifier on the capillary walls.

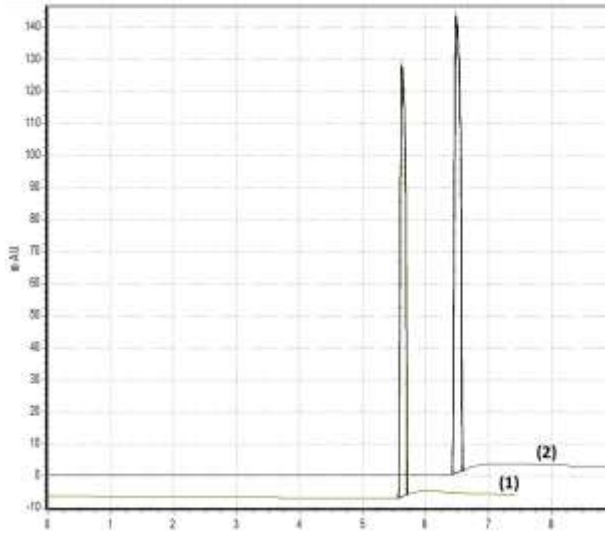


Figure 39. Electropherogram of the EOF before the addition of the modifier into the BGE (1) and with the addition of a positively charged derivative of β-CD.

Conditions: capillary electrophoresis system "Capel'-105M", 5 mM PBS (pH 6.4) 0 mM (1) and 2 mM (2) 3-Me-1-β-CD-ImOTs, 20 kV, 220 nm, EOF marker – 3 (vol.) % solution of DMF in water.

Own electrophoretic mobility of the synthesized modifier, which is $\mu_e = 10.5 \times 10^{-9}m^2V^{-1}s^{-1}$, was determined at pH 6.4.

III.2.1 Separation of steroid hormones in electrokinetic chromatography mode

The structure of the synthesized 3-Me-1-β-CDImOTs has two functional centers: a hydrophobic cavity of the macrocycle and a positively charged imidazolium ring, which allows this modifier to perform as a pseudostationary phase in the electrophoretic separation of uncharged hydrophobic

analytes. Corticosteroid hormones were chosen as model analytes. It was previously proven, that they form guest-host complexes with the CD cavity [184].

However, the use of only uncharged CD as a pseudostationary phase did not ensure the separation of corticosteroid hormones: uncharged macrocycles migrate together with the EOF; their complexes with these analytes do not have electrophoretic mobility [185]. Most often, charged micelles are added to such systems, which provide a difference in the electrophoretic mobilities of the components being determined [59]. Due to the positively charged imidazolium fragment, neutral cyclodextrins acquire their own electrophoretic mobility: uncharged analytes, interacting with the CD cavity, migrate as positively charged associates towards the cathode.

Thus, with the injection of additives into the BGE, a change in the mode of electrophoretic separation can be caused by the sorption of the modifier on the quartz capillary walls and the processes of complexation between the analytes and the modifier sorbed on the walls of the capillary and present in the BGE (Figure 40). When 3-Me-1-β-CD-ImOTs is added into the background electrolyte, the dominant process is the formation of complexes with the modifier in the BGE, since, as we have shown earlier, its sorption on the capillary walls is extremely insignificant.

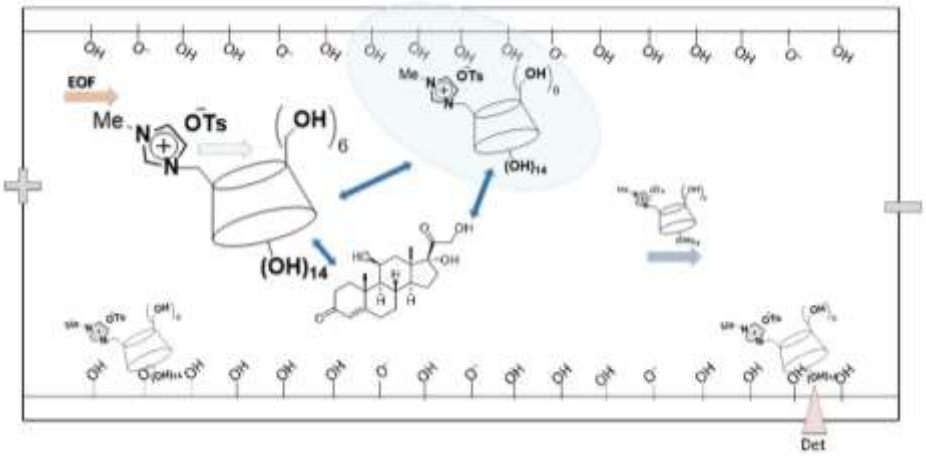


Figure 40. Scheme of three proposed mechanisms explaining the processes of electrophoretic separation of steroid hormones when 3-Me-1-β-CD-ImOTs are added into the background electrolyte.

Separation of corticosteroids required the presence of a modifier in the BGE, and the search for conditions for the separation of analytes included varying the pH (2.0–8.0) and ionic strength (5–50 mM) of the background electrolyte, as well as the concentration of 1-Me-3-β-CDImOTs (0.1– 2.25 mM).

When a modifier was added into the BGE, even at low concentrations (0.25 mM), 11-deoxycortisol, corticosterone, and 21-hydroxyprogesterone were separated, and cortisone and hydrocortisone (cortisol) migrated together. The increase of the modifier concentration did not improve the result (Figure 41).

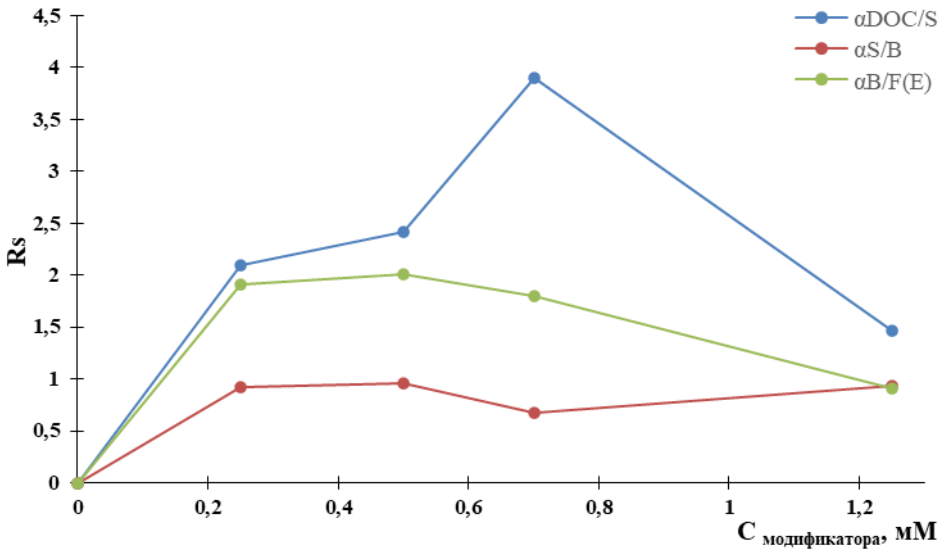


Figure 41. Change in resolution under varying the concentration of the modifier in the composition of the BGE.

Conditions: capillary electrophoresis system "Capel'-105M", 5 mM PBS (pH 3.2), 0-1.25 mM 3-Me-1-β-CD-ImOTs. Sample: model mixture of corticosteroid hormones (10 μg/ml): cortisone (E), hydrocortisone (F), 11-deoxycortisol (S), corticosterone (B), 21-hydroxyprogesterone (DOC), sample injection: 2 s 30 mbar; 20 kV, 254 nm.

Varying the pH value of the background electrolyte (2.0; 2.8; 3.2; 3.8; 4.5; 6.4; 7.5; 8.0) affected the rate of analytes migration and the change in efficiency: an increase in pH led to a decrease in efficiency, while the separation selectivity of corticosteroids increased. Nevertheless, the separation of structurally similar cortisone and cortisol has not been achieved. The efficiency growth with increasing pH of BGE is caused by faster migration of analytes due to an increase in the EOF velocity. At the same time, it was found that the absorption of the modifier, due to the presence of the imidazolium ring, affects the baseline signal: with the growth of pH, it increases and complicates analytes separation (Figure 42).

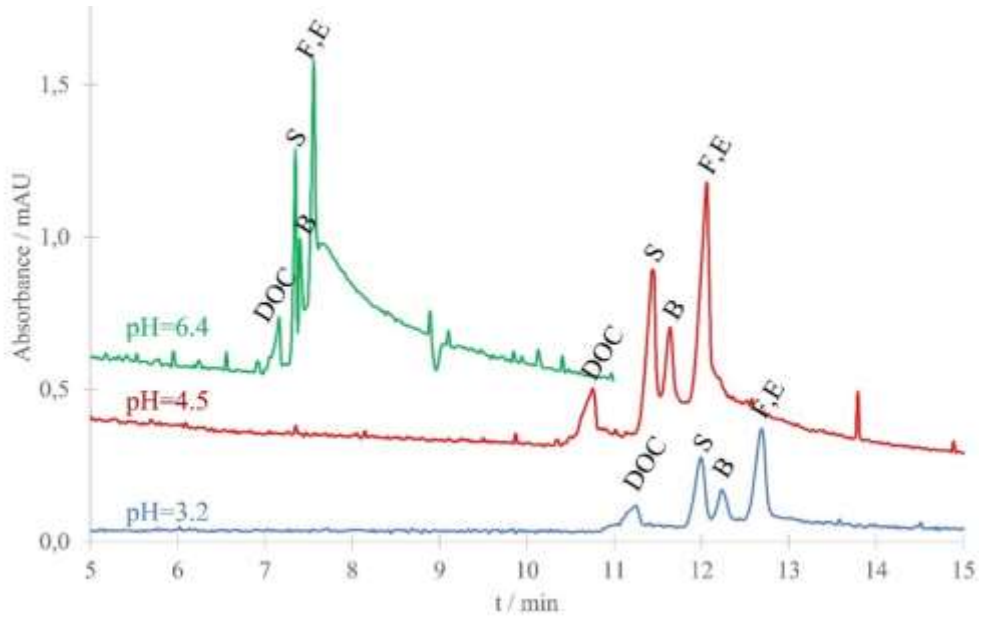


Figure 42. Electropherogram of a mixture of corticosteroid hormones at different pH values of BGE.

Conditions: capillary electrophoresis system "Capel'-105M", 5 mM PBS (pH 3.2, 4.5, or 6.4), 0.7 mM 3-Me-1-β-CD-ImOTs. Sample: model mixture of corticosteroid hormones (10 μg/ml): cortisone (E), hydrocortisone (F), 11-deoxycortisol (S), corticosterone (B), 21-hydroxyprogesterone (DOC), sample injection: 2 s 30 mbar; 20 kV, 254 nm.

An increase in ionic strength (up to 50 mM), which could reduce the EOF velocity and neutralize the found effect, did not lead to the expected results: an increase in the buffer concentration led to a noticeable decrease in resolution. An acceptable pH value (3.2) and concentration (5 mM) of the phosphate buffer solution were found to achieve the required separation selectivity. Differences in the complexation constants and own electrophoretic mobility of the β-CD derivative led to the separation of four corticosteroids in the electrokinetic chromatography mode (Figure 43) with the injection of a 0.7 mM modifier into the BGE.

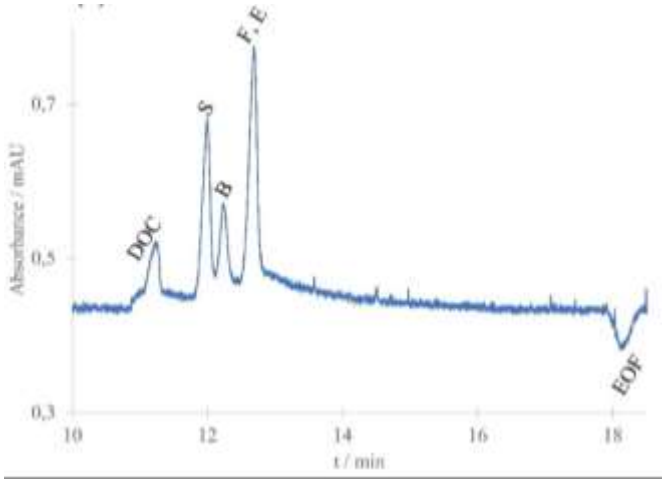


Figure 43. Separation of corticosteroids upon the addition of 1-Me-3-β-CDImOTs into the background electrolyte.

Conditions: capillary electrophoresis system "Capel'-105M", 5 mM phosphate buffer solution (pH 3.2), 0.7 mM 3-Me-1-β-CD-ImOTs. Model mixture of corticosteroids (10 μg/ml): cortisone (E), hydrocortisone (F), 11-deoxycortisol (S), corticosterone (B), 21-hydroxyprogesterone (DOC), injection 2 s 30 mbar; 20 kV, 254 nm.

Varying the pH and ionic strength of the buffer, as well as the concentration of the modifier in the background electrolyte, proved to be insufficient for separating structurally similar cortisone and hydrocortisone. Only a change in the hydrophobic-hydrophilic balance on the addition of organic solvent to the BGE affected the process of complex formation and separation of these steroid hormones. The effect of acetonitrile and methanol on corticosteroid resolution factors was studied. At a concentration of 10% (v/v) acetonitrile, analyte separation (unlike methanol) was achieved (Figure 44).

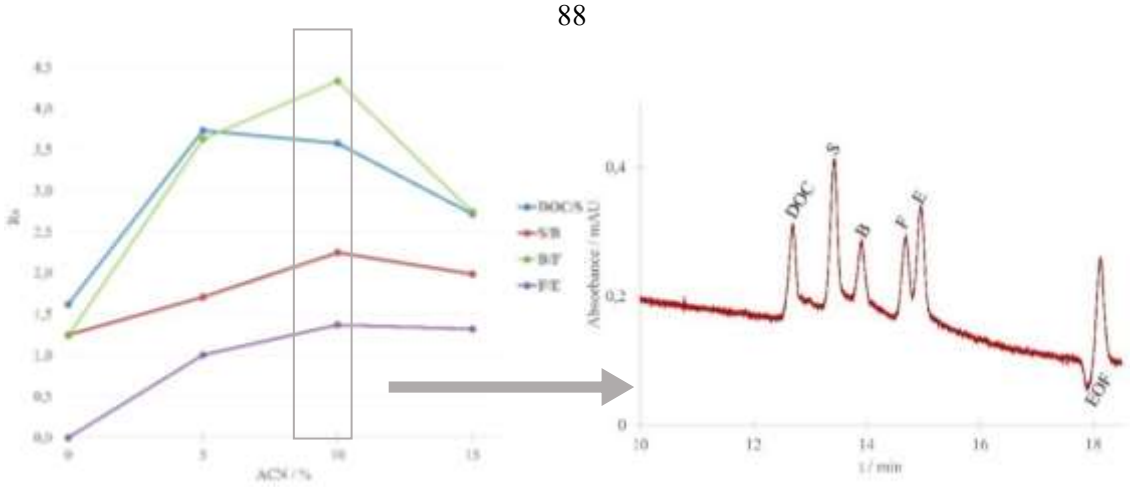


Figure 44. The evaluation of the effect of acetonitrile in the composition of the background electrolyte on the resolution (Rs) of corticosteroids (left); electrophoregram of corticosteroids with 3-Me-1-β-CD-ImOT and 10% (vol.) acetonitrile in BGE (right).

Conditions: capillary electrophoresis system "Capel'-105M", 5 mM phosphate buffer solution, 0.7 mM 3-Me-1-β-CD-ImOT, 0-15 (vol.) % acetonitrile. Model mix of corticosteroids (10 μg/ml): cortisone (E), hydrocortisone (F), 11-deoxycortisol (S), corticosterone (B), 21-hydroxyprogesterone (DOC); sample injection 2 with 30 mbar; 20 kV, 254 nm.

The analytical potential of mono-6-deoxy-6-butylimidazole-β-cyclodextrin tosylate (1-Bu-3-β-CDImOTs) in the separation of steroid hormones was revealed. Similar patterns were found, however, complete separation of corticosteroids is achieved at a higher concentration and ionic strength of the buffer solution in the case of 1-Bu-3-β-CDImOTs compared to the values found for 3-Me-1-β-CD-ImOTs as a pseudostationary phase. The analysis time under such conditions increases to 30 minutes (Figure 45).

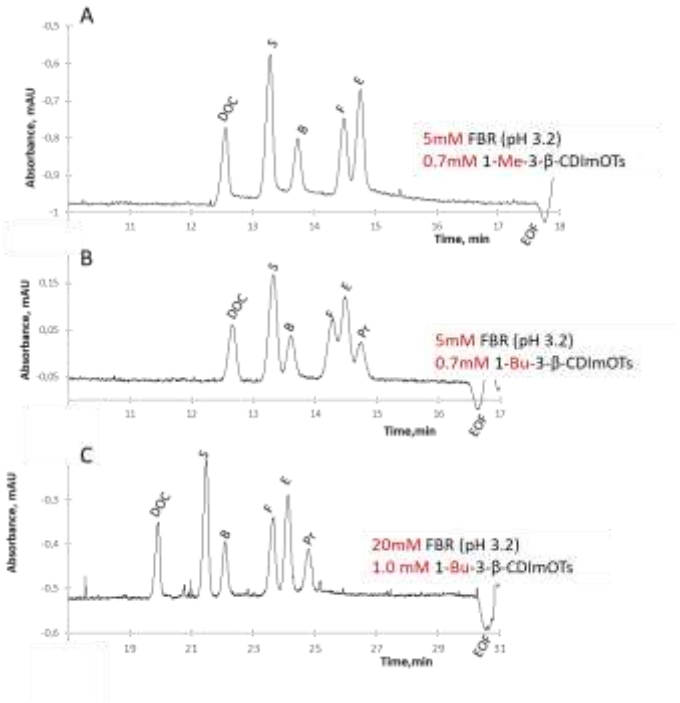


Figure 45. Electropherograms of corticosteroid hormones obtained by adding derivatives based on imidazole and β-CD into the background electrolyte as a pseudostationary phase.

Conditions: capillary electrophoresis system "Capel'-105M", 5 mM (A, B) and 20 mM (C) phosphate buffer solution (pH 3.2), 0.7 mM 3-Me-1-β-CDImOTs (A) or 0.7 (B) and 1 mM (C) 1-Bu-3-β-CDIm, 10 (vol.) % ACN, 20 kV, 254 nm. Model mixture of steroid hormones (10 μg/ml): cortisone (E), hydrocortisone (F), 11-deoxycortisol (S), corticosterone (B), 21-hydroxyprogesterone (DOC), injection 2 s 30 mbar.

Thus, the following conditions were found for the selective separation of a model mixture of steroid hormones: 5 mM phosphate buffer solution (pH 3.2), 0.7 mM 3-Me-1-β-CD-ImOTs, 10 (vol.) % ACN, voltage 20 kV, 254 nm. The corticosteroids LOD values, defined as a signal-to-noise ratio of 3:1, under these conditions were 1.25–2.73 μg/ml, which is too high to detect these analytes in plasma samples. In order to reduce the LOD, the potential of various methods of intracapillary concentration has been studied.

III.2.2 Preconcentration of steroid hormones

The use of such a variant of intracapillary preconcentration as field-amplified sample stacking (FASS), based on differences in the conductivity of the sample solution and the background electrolyte, requires the presence of own electrophoretic mobility of the analyzed analytes, which is absent in steroid hormones. However, the ability of the 3-Me-1-β-CD-ImOTs modifier to form inclusion complexes with corticosteroids allows not only to separate the latter, but also to apply preconcentration methods based on changes in the electrophoretic mobility of charged "modifier-corticosteroid" associates. In this case,

3-Me-1-β-CD-ImOTs was added to the sample. The required concentration of 3-Me-1-β-CD-ImOTs in the sample was calculated according to the concentration of steroids and the assumption that steroid hormones and cyclodextrin form complexes in a ratio of 1:1. A stock solution of steroids (10 μg/ml) was prepared with the addition of 3 -Me-1-β-CD-ImOTs (0.15 mM) in slight excess. Along with the implementation of FASS, a positively charged associate could be electrokinetically injected into a capillary for preconcentration in FASI mode (Figure 46).

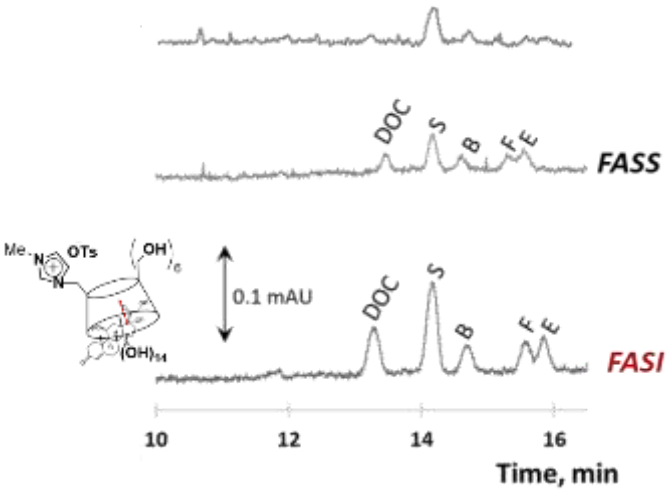


Figure 46. Electropherograms (from top to bottom) obtained without concentration during hydrodynamic (FASS) and electrokinetic (FASI) administration of corticosteroid hormone associates with 3-Me-1-β-CD-ImOTs.

Conditions: capillary electrophoresis system "Capel'-105M", 5 mM phosphate buffer solution, 0.7 mM 3-Me-1-β-CD-ImOTs, 10 (vol.) % acetonitrile. Model mixture containing five steroid hormones (100 ng/ml): cortisone (E), hydrocortisone (F), 11-deoxycortisol (S), corticosterone (B), 21-hydroxyprogesterone (DOC); sample injection - 50 s 30 mbar (FASS) or 50 s 20 kV (FASI); 20 kV, 254 nm.

In the electrostacking mode we managed to concentrate steroid hormones 16-25 times. The results are summarized in Table 14.

Table 14. Summarizing of the main parameters obtained for two preconcentration techniques

	FASS	**FASI**
Corticosteroids model mixture concentration, ng\ml	100	
Sample injection	900 mbar*s	1000 kV*s
SEF	7-11	16-25
LOD, ng\ml	100-123	35-50

III.3 Separation of nonsteroidal anti-inflammatory drugs enantiomers in the electrokinetic chromatography mode

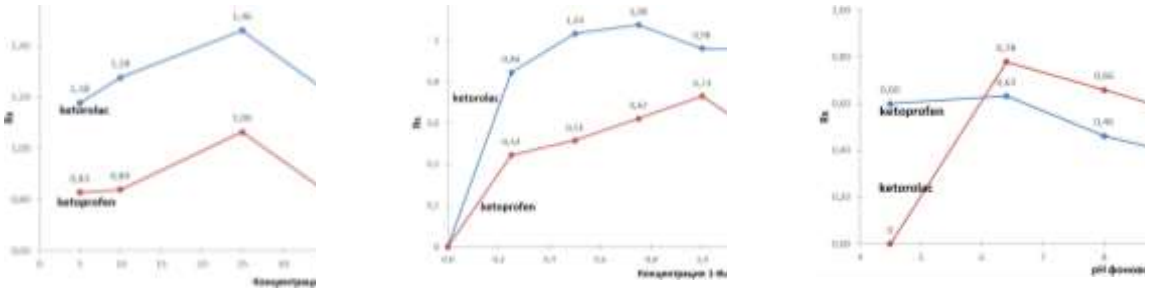
The presence of a hydrophobic cavity in CDs allows them to act as a chiral selector in the separation of drug enantiomers, while the positively charged imidazolium ring in the selector structure can provide an additional contribution to the separation efficiency and selectivity. In the course of test experiments, it was found that the discussed modifier can be used to separate NSAIDs (ketoprofen and ketorolac), but not for adrenoblockers. The reason is the basic nature of the latter. The study of the potential of the synthesized β-CD derivatives as chiral selectors was carried out on the example of a model mixture of racemates of acidic non-steroidal anti-inflammatory drugs – ketoprofen and ketorolac (the structures are shown in Table 9).

III.3.1 Electrophoretic separation of ketoprofen and ketorolac enantiomers

Due to the lack of clear algorithms for choosing the conditions for the separation of enantiomers, a series of preliminary test experiments was required to determine the most significant parameters, which included varying the pH of the background electrolyte (2.0, 4.5, 7.5, 11.0), the ionic strength of the buffer solution (5, 10, 25, 50 mM), concentrations of the chiral selector 1-Bu-3-β-CDImOTs (0.25, 0.50, 1.00 mM).

The most significant parameters were the concentration of 1-Bu-3-β-CDImOTs and the pH of the background electrolyte. The latter influenced the analytical form of the determined compounds. After a series of preliminary experiments, these parameters were varied with a narrower step to find the optimal separation conditions. At the pH of the background electrolyte exceeding the pKa values of the analytes (3.5–4.5), we managed to achieve the highest values of the resolution factors. With a further increase in pH (>6.5), the EOF velocity noticeably increased, which led to a decrease in the selectivity of NSAID separation. The dependence of enantiomeric resolution factors on the concentration of 1-Bu-3-β-CDImOTs also turned out to be non-linear (Figure 47). Most likely, because the excess of β-CD

derivatives in the composition of the background electrolyte contributed to the formation of their complexes with each other via hydrogen bonds. At the same time, the availability of macrocycles cavities decreased, which also caused a decrease in the selectivity of NSAIDs enantiomers separation.



PBS (5-40mM), pH 6.4 + 1mM 1-Bu-3-β-CDImOTs, 254nm, 20kV

5mM PBS + 1-Bu-3-β-CDImOTs (0.25-1.25mM), 254nm, 20kV

5mM PBS (pH 4.5 - 9.5) + 1mM 1-Bu-3-β-CDImOTs, 254nm, 20kV

Figure 47. Graphs of the enantioselectivity factors of the enantiomers of ketoprofen and ketorolac as a function of the ionic strength of the background electrolyte, the concentration of 1-Bu-3-β-CDImOTs, and the pH of the background electrolyte.

Thus, the addative of the modifier based on β-CD and imidazolium allows separating the enantiomers of NSAIDs; the highest enantioselectivity factors (1.46 and 1.06 for ketorolac and ketoprofen, respectively) were achieved with the following background electrolyte composition: 25 mM phosphate buffer solution (pH 6.4), 1 mM 1-Bu-3-β-CDImOTs (Figure 48) [186].

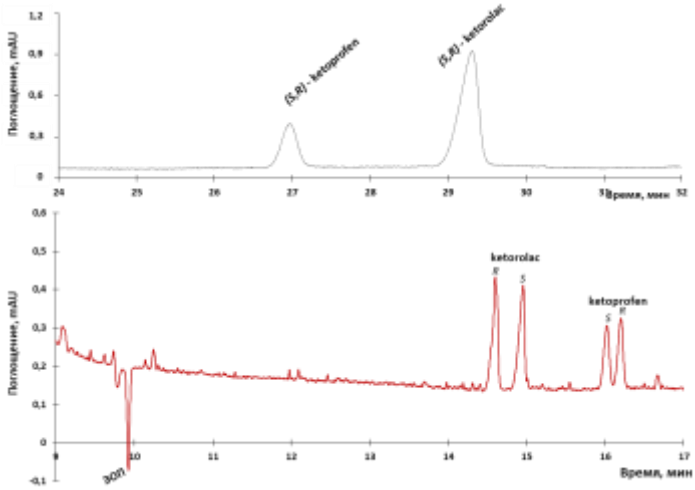


Figure 48. Electropherograms of enantiomers of ketoprofen and ketorolac in the absence (top) and presence (bottom) of 1-Bu-3-β-CDImOTs into BGE.

Conditions: capillary electrophoresis system "Capel'-105M", 25 mM PBS (pH 6.4), 0 mM (top) and 1 mM (bottom) 1-Bu-3-β-CDImOTs, 254 nm, 20 kV; model mixture (rac-ketoprofen 10μg/ml and rac-ketorolac 50 μg/ml); sample injection 2 s 30 mbar;

When identifying the analytical possibilities of the second synthesized positively charged chiral selector (1 mM in BGE), separation of ketoprofen and ketorolac enantiomers was also achieved, while the values of enantioselectivity factors practically did not differ (Figure 49). Due to the lack of significant differences between two modifiers, subsequent experiments were performed using 1-Bu-3-β-CDImOTs: the value of its electrophoretic mobility turned out to be higher, which reduces the analysis time.

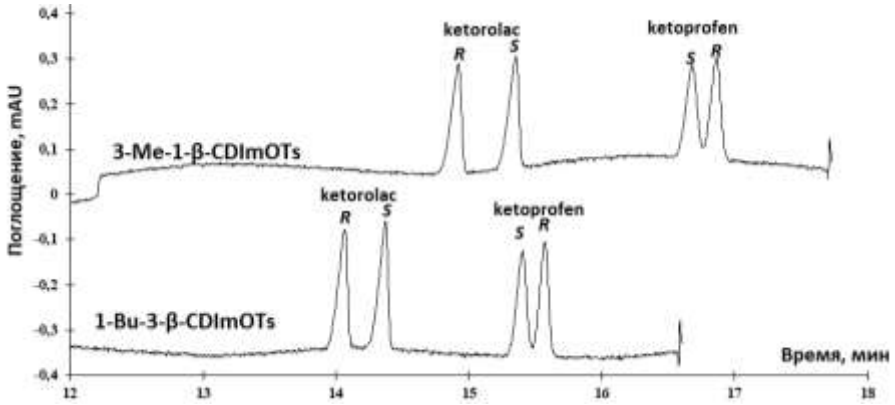


Figure 49. Electropherograms of the enantiomers of ketoprofen and ketorolac with the additive of 1-Bu-3-β-CDImOTs or 3-Me-1-β-CDImOTs into BGE.

Conditions: capillary electrophoresis system "Capel'-105M", 25 mM PBS (pH 6.4), 1 mM 3-Me-1-β-CDImOTs or 1-Bu-3-β-CDImOTs, 254 nm, 20 kV; model mixture (rac-ketoprofen 10 μg/ml and rac-ketorolac 50 μg/ml); sample injection 2 s 30 mbar.

One of the approaches to increase the selectivity of structurally similar analytes separation, which is often used in the field of chiral CE, is to change the hydrophobic-hydrophilic balance by adding an organic solvent into the BGE composition. In the case of steroid hormones separation, a positive effect was obtained by injecting acetonitrile into the BGE, however, the addition of organic solvents during the separation of ketoprofen and ketorolac enantiomers did not lead to an increase in enantioselectivity factors (Figure 50).

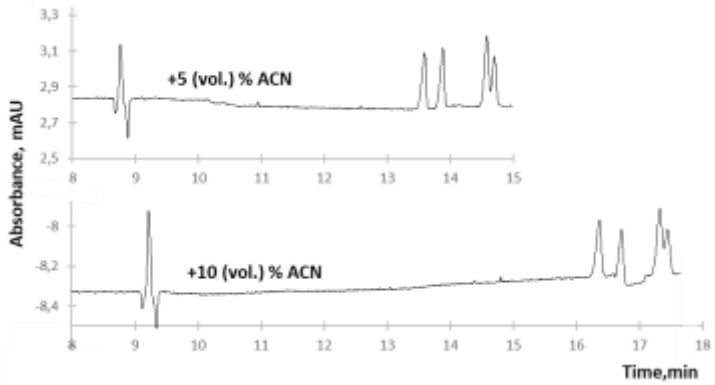


Figure 50. Electrophoregrams of the enantiomers of ketoprofen and ketorolac with the additive of acetonitrile into the background electrolyte.

Conditions: capillary electrophoresis system "Capel'-105M", model mixture (rac-ketoprofen 10 μg/ml and rac-ketorolac 50 μg/ml); sample injection 2s 30mbar; 25mM (pH 6.4), 1mM 1-Bu-3-β-CDImOTs 5 or 10 (vol.) % ACN, 254 nm, 20 kV.

The effect of other organic solvents used to change the hydrophobic-hydrophilic balance on the electrophoretic separation of enantiomers was evaluated. The addition of methanol and ethanol also did not affect the separation selectivity, while more hydrophobic substances (propan-2-ol and butan-1-ol) prevented the process of complex formation of analytes with CD cavities, solvating NSAID molecules in the BGE and reducing separation selectivity; in the case of butanol, it approached almost zero (Figure 51).

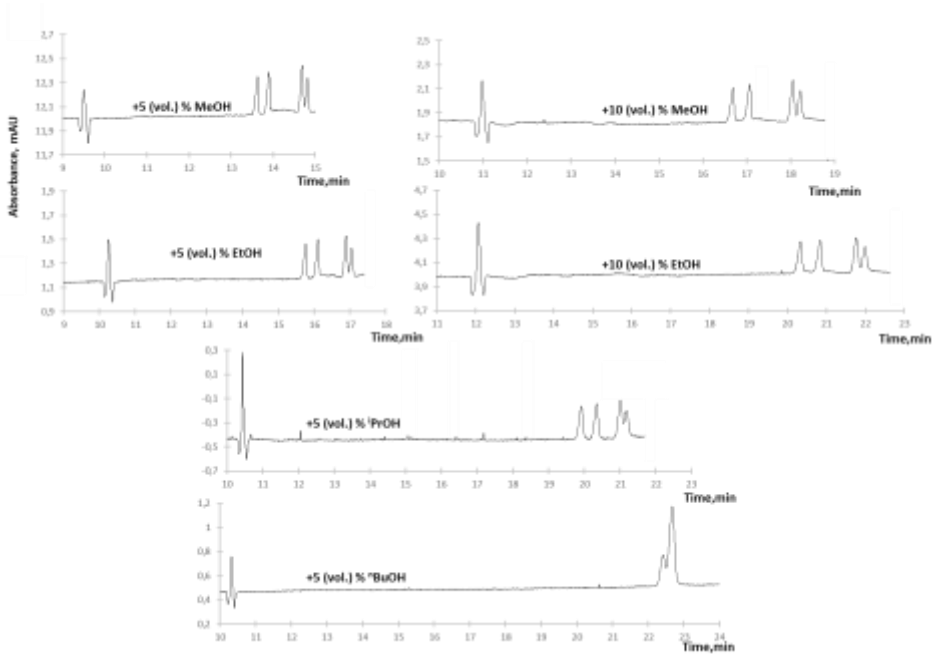
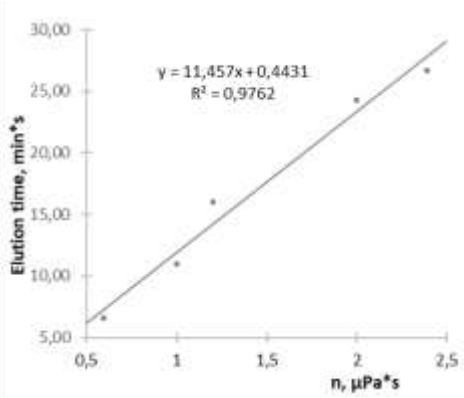


Figure 51. Electropherograms of enantiomers of ketoprofen and ketorolac with the additive of alcohols into the background electrolyte.

Conditions: capillary electrophoresis system "Capel'-105M", 25 mM PBS (pH 6.4), 1 mM 1-Bu-3-β-CDImOTs, 5 or 10 (vol.)% MeOH, EtOH, i-PrOH, n-BuOH (see. in Fig.), 254nm, 20kV. model mixture (rac-ketoprofen 10μg/ml and rac-ketorolac 50 μg/ml); sample injection 2 s 30 mbar.

III.3.2 Determination of the complex formation constants of enantiomers with the synthesized cyclodextrin derivative

To characterize the process of complexation of individual enantiomers with the synthesized CD derivative, the complexation constants were calculated according to the algorithms proposed in [178] with some modifications. The calculation of the complexation constants values is based on measurements of the electrophoretic mobilities of enantiomers at various concentrations of the modifier in the composition of the BGE. However, when large molecules such as cyclodextrins are used as modifiers, a change in migration times can occur due to an increase in the viscosity of the solution. To confirm this conclusion, calibration curves of the analytes elution time and the values of their dynamic viscosity at 20°C were made, with water, isopropyl alcohol, methanol, ethyl alcohol, and dimethyl sulfoxide acting as solvents of the identified dynamic viscosity. The elution time was measured as follows: the capillary was filled with a single solvent at a pressure of 1000 mbar for 5 min, after which an EOF marker (0.1% aqueous solution of DMF, at 30 mbar for 2 s) was introduced and the analysis was performed without applying voltage at a constant pressure 50 mbar. For each solvent, the procedure was repeated three times, and then the calibration curve of the elution time and viscosity was made (Figure 52).



Solvent	EOF elution time, min	Viscosity at 20 °C, μPa*s	Accuracy, %
MeOH	6,6	0,597	94
H_2O	11,0	1,002	93
EtOH	16,0	1,200	113
DMSO	24,3	1,996	104
iPrOH	26,7	2,390	95

Figure 52. Calibration curve for determining the viscosity of background electrolyte solutions.

Then, the same measurement of the elution time was carried out for all BGE solutions with different CD concentrations at 20°C. However, the values of the elution time did not differ much from the values obtained by measuring water; therefore, further recalculation was carried out by the method of proportion with reference to the values of water (table 15).

Table 15. Obtained values of the background electrolytes viscosity with the injection of the CD derivative.

Concentration of 1-Bu-3-β-CDImOTs in BGE	EOF elution time, min	Dynamic viscosity at 20 °C, μPa*s
0.10 mM	11.16	1.02±0.02
0.25 mM	11.15	1.02±0.01
0.50 mM	11.11	1.01±0.03
0.75 mM	11.18	1.02±0.02
1.0 mM	11.21	1.02±0.02
1.25 mM	11.22	1.03±0.03
1.50 mM	11.25	1.03±0.02

The obtained values of viscosity were used to correct enantiomers electrophoretic mobilities (formula (3), table 16)

$$\mu^{+} = \mu^{exp}\left(\frac{\eta_{exp}}{\eta_{w}}\right), \quad (3)$$

where μ^{+} - corrected mobility,

μ_{exp}- observed mobility,

η_{exp} and η_{w}- BGE viscosity and water viscosity, respectively.

Table 16. Values of the corrected electrophoretic mobilities of enantiomers at different concentrations of the modifier in the background electrolyte

$C_{1\text{-Bu-3-}\beta\text{-CDImOTs}}$, 10^3M	pC	Corrected mobility (μ^+), 10^9 $m^2*V^{-1}s^{-1}$			
		R-ketorolak	*S*-ketorolak	*S*-ketoprofen	*R*-ketoprofen
0.10	4.00	-16.21	-16.21	-16.32	-16.32
0.25	3.60	-13.58	-13.88	-14.37	-14.46
0.50	3.30	-11.23	-11.61	-12.51	-12.64
0.75	3.12	-9.77	-10.17	-11.25	-11.40
1.00	3.00	-8.59	-8.99	-10.17	-10.34
1.25	2.90	-7,75	-8.13	-9.31	-9.48
1.50	2.82	-7.76	-8.14	-9.32	-9.49
		Complexation constants			
		2260±160	2070±150	1680±80	1620±80

The calculation of the complexation constants is based on the assumption that the stoichiometric ratio between the analyte and the complexing agent corresponds to 1:1. For mathematical treatment, instead of CD concentration in the composition of BGE, the negative logarithm of the concentration is used (similarly to the acid-base balance (pC = -log[$C_{1\text{-Bu-3-}\beta\text{-CDImOTs}}$]). The obtained nonlinear regression was the dependence of the corrected mobility on the logarithm of the concentration 1-Bu-3-β-CDImOTs. The pC value at the inflection point obtained by solving the equation is equal to the logarithm of the complexation constant (pK, red line in Figure 53). Mathematical operation, which is the inverse of the logarithm, was used to obtain the complexation constants of the individual enantiomers with the used modifier (Table 16).

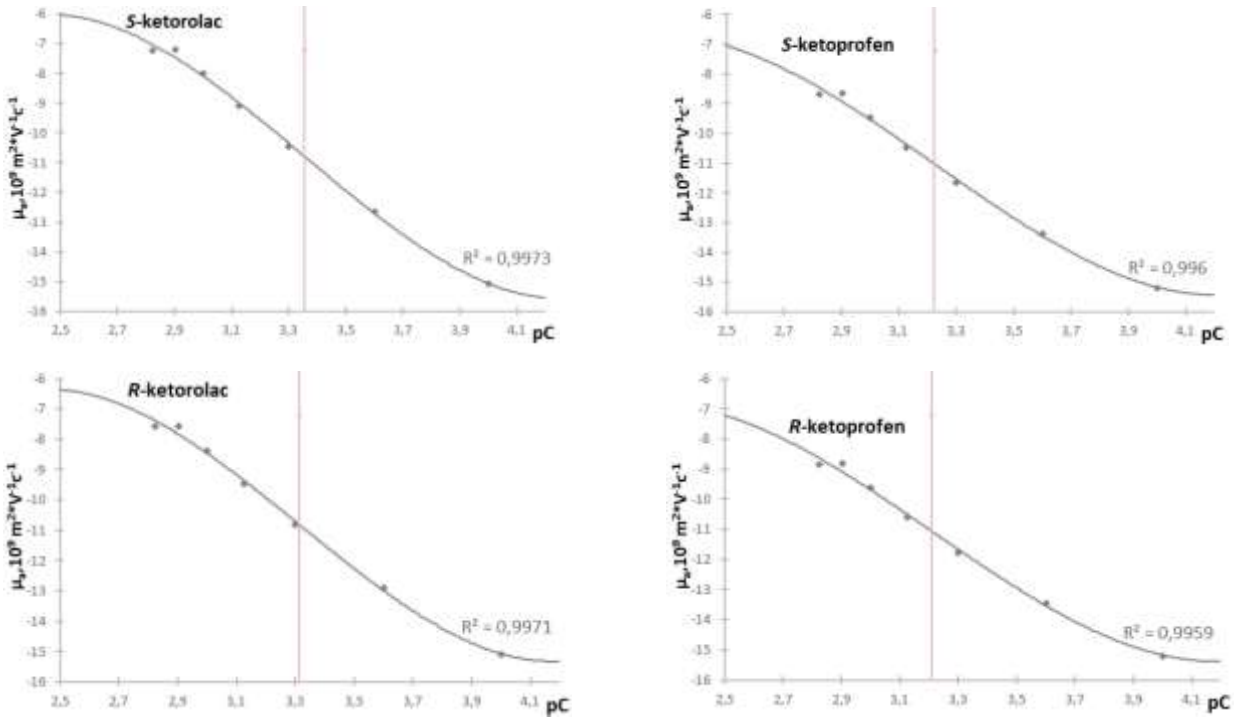


Figure 53. Dependences of the corrected electrophoretic mobilities of individual enantiomers of ketoprofen and ketorolac at various concentrations of 1-Bu-3-β-CDImOT, expressed as the inverse logarithm of pC.

High values of the complexation constants may result from additional electrostatic interactions between positively charged imidazolium in CD and negatively charged NSAID.

III.3.3 Dual chiral systems

One of the little studied, but actively developing areas in chiral capillary electrophoresis is dual chiral systems, which in some cases can significantly increase enantioselectivity factors. It seemed noteworthy to test the potential of the positively charged chiral selector synthesized by us, which is a derivative of β-CD in such systems. Along with 1-Bu-3-β-CDImOTs, a number of compounds with different structures and properties were used as the second chiral selector for dual chiral systems: β-CD, HP-β-CD, sulfo-β-CD, dextran, amikacin, amoxicillin and vancomycin.

The joint effect of two chiral selectors was studied using a model mixture of ketoprofen and ketorolac. In test experiments, the concentrations of modifiers in the background electrolyte were chosen in such a way as to compare three options: 1. Excess of 1-Bu-3-β-CDImOTs in the background electrolyte; 2. Excess of the second chiral selector in the background electrolyte; 3. Same mole ratio of 1-Bu-3-β-CDImOTs and the second chiral selector, i.e. the concentrations of two modifiers in the prepared working buffer solutions were in the following ratio (mol): 1:1.5 (or 1:2), 1:1, 2:1.

In the initial experiments on the electrophoretic separation of the enantiomers of ketoprofen and ketorolac, the concentration of 1-Bu-3-β-CDImOTs was 1.0 mM; this was taken as a basis when calculating the concentration of the second chiral selector (0.5 mM; 1.0 mM and 1.5 mM or 2.0 mM, respectively, the concentration of 1-Bu-3-β-CDImOTs remained constant - 1.0 mM.

First of all, we studied the systems with cyclodextrins acting as the second chiral selector, since combinations of two different cyclodextrins are the most common. Initially, 1-Bu-3-β-CDImOTs were considered together with uncharged CDs (β-CD, HP-β-CD and α-CD), and oppositely charged CD (sulfo-β-CD). The system with a high molecular weight oligosaccharide – dextran (MM ~ 30-40 thousand) was irrespectively studied. The selected dual systems did not show the expected synergistic effect and an increase in enantioselectivity factors, and in the case of β-CD and HP-β-CD, on the contrary, they even led to a decrease in separation selectivity (Figure 54).

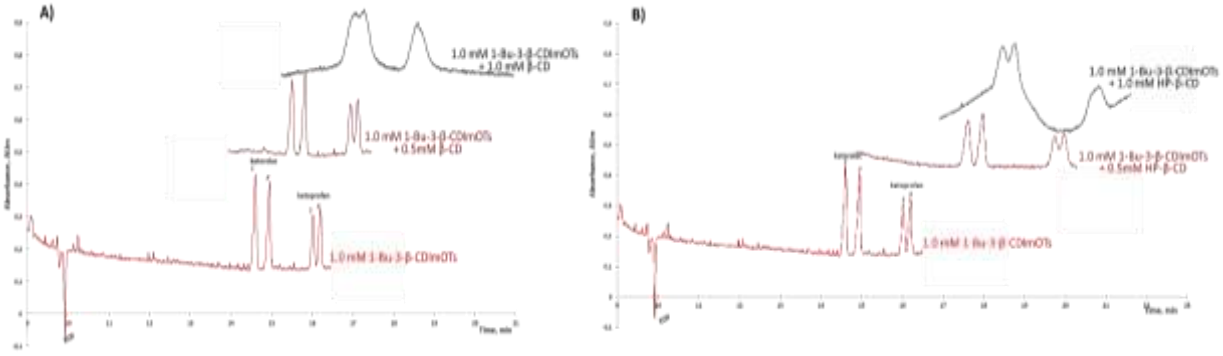


Figure 54. Electropherograms of separation of NSAID enantiomers using a double chiral system with varying concentrations of chiral selectors 1-Bu-3-β-CDImOTs + β-CD (A) and 1-Bu-3-β-CDImOTs + HP-β-CD (B).

Conditions: capillary electrophoresis system "Capel'-105M", 25 mM PBS (pH 6.4), 1.0 mM 1-Bu-3-β-CDImOTs and 0.5 mM or 1.0 mM β-CD (A) and HP-β-CD (B), 254nm, 20kV; model mixture (rac-ketoprofen 10 μg/ml and rac-ketorolac 50 μg/ml); sample injection 2 s 30 mbar.

This could be caused by the formation of cyclodextrins dimers and, as a result, a decrease in the availability of cavities for complexation with NSAID enantiomers. A decrease in selectivity to almost zero can be seen even at the modifier concentrations ratio of 1:1 (mol.). In systems with an excess of β-CD or HP-β-CD in the BGE, the analysis time increased significantly, and the selectivity decreased almost to zero. Thus, in this variant of dual chiral systems, there is a broad trend, which is a decrease in separation selectivity and efficiency with an increase in the concentration of the second selector.

The use of α-CD, dextran and sulfo-β-CD did not affect the enantioselectivity factors; there was only a slight decrease in the values of the resolution factors due to a decrease in the EOF velocity (Figure 55).

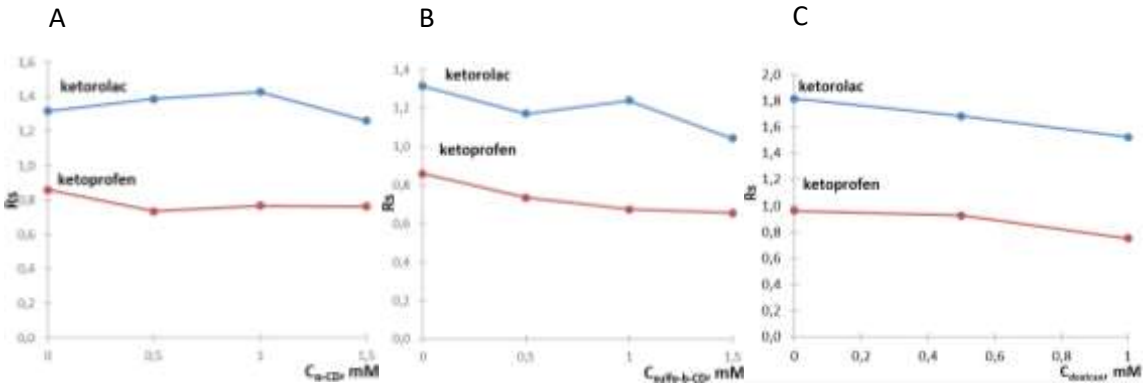
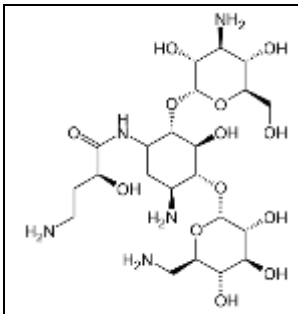
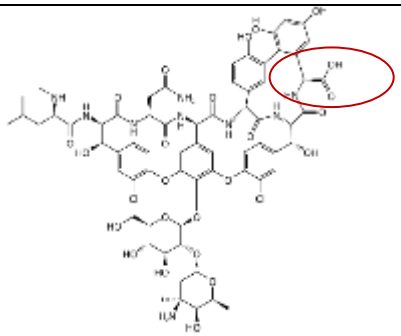
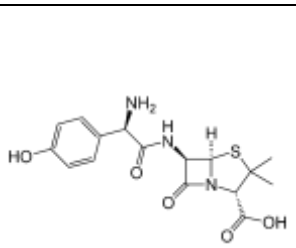


Figure 55. Change in the resolution of the enantiomers of ketoprofen and ketorolac when α-CD (A), sulfo-β-CD (B) and dextran (MM=30-40 thousand) (C) are added to the background electrolyte as the second chiral selector.

Conditions: capillary electrophoresis system "Capel'-105M", 25 mM PBS (pH 6.4), 1.0 mM 1-Bu-3-β-CDImOTs and 0.5 mM or 1.0 mM β-CD (A) and HP-β-CD (B) , 254 nm, 20 kV; model mixture (rac-ketoprofen 10 µg/ml and rac-ketorolac 50 µg/ml), injection 2 s 30 mbar

Another variant of dual chiral systems was also studied, where, along with 1-Bu-3-β-CDImOTs, we used the following antibiotics: vancomycin, amoxicillin, and amikacin (Table 17). In test experiments, the second chiral selector was also injected at concentrations of 0.5 mM, 1.0 mM, and 1.5 mM to reveal the general trend in changes in enantioselectivity factors.

Table 17. Antibiotics selected as the second chiral selector and their acid-base characteristics.

		
Amikacin (pKa= 8.1)	**Vancomycin** (pKa_1 = 2.6; pKa_2 = 7.2; pKa_3 = 8.6; pKa_4 = 9.6; pKa_5=10.5; pKa_6 = 1.7)	**Amoxicillin** pKa_1=3.2, pKa_2=7.4

In the course of preliminary experiments, where antibiotics were injected into the background electrolyte at concentrations of 0.5; 1.0 and 1.5 mM in addition to 1 mM 1-Bu-3-β-CDImOTs, a noteworthy pattern was found: the use of vancomycin as the second chiral selector leads to a non-linear change in the enantioselectivity factors of ketoprofen. There is a decrease in the value of this parameter

to zero with a subsequent increase. It has been suggested that two chiral selectors have a competitive interaction, which may lead to a change in the order of migration of ketoprofen enantiomers.

To test this assumption, a special series of electrophoretic analyses was carried out with varying the concentration of vancomycin in the BGE with a smaller step (0.10, 0.25, 0.50, 0.75, 1.00, 1.20 mM), and in order to identify a change in the order of migration of enantiomers, an additional amount of *S*-ketoprofen was injected into the analyzed mixture of NSAIDs at a concentration of 2.5 µg/ml (which was sufficient to distinguish between two enantiomers by different peak areas) (Figure 56).

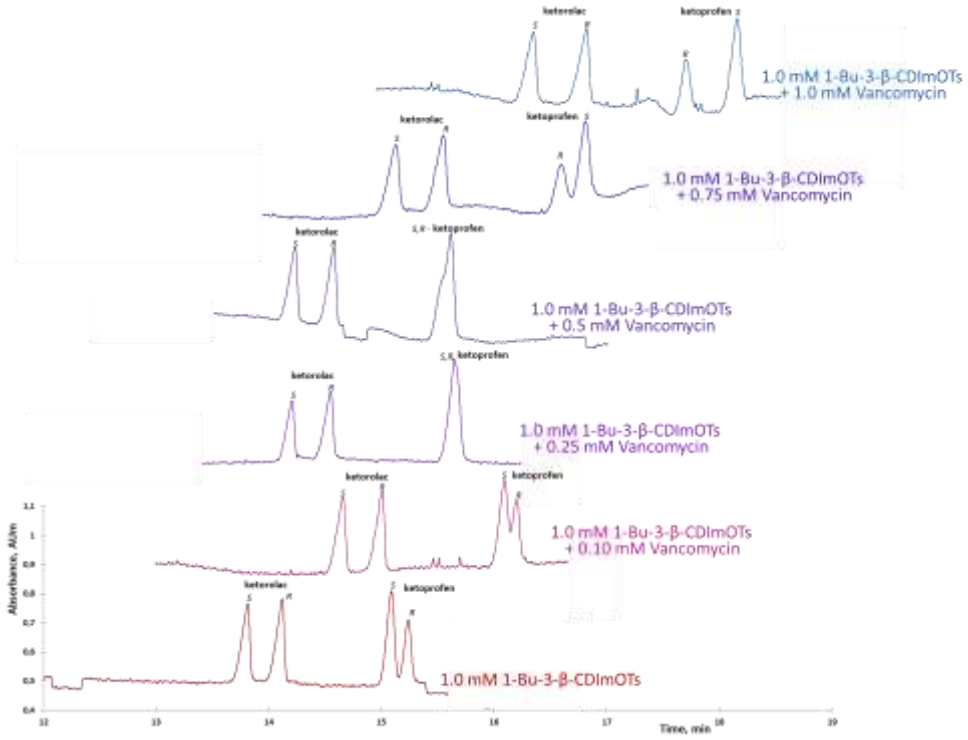


Figure 56. Electropherograms of separation of NSAID enantiomers at different ratios of two chiral selectors in the background electrolyte.

Conditions: model mixture (rac-ketoprofen 10 µg/ml + S-ketoprofen 2.5 µg/ml and rac-ketorolac 50 µg/ml), injection 2 s 30 mbar; 25 mM phosphate buffer solution (pH 6.4), 1.0 mM 1-Bu-3-β-CDImOTs and 0-1.0 mM vancomycin, 254 nm, 20 kV.

When shaping the curve of the dependency of the enantioselectivity factors for ketorolac and ketoprofen on the concentration of vancomycin in the BGE composition, it can be seen that for ketoprofen there is a local minimum in the concentration region of 0.25 mM (Figure 57A), where the change in the order of migration of ketoprofen enantiomers occurs, fixed due to the standard addition of S-ketoprofen (Figure 57B).

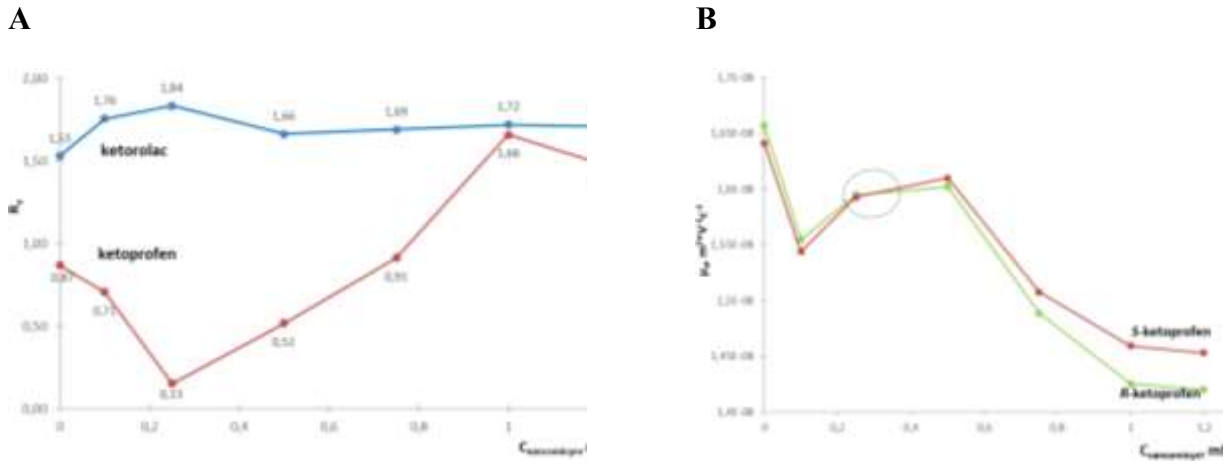


Figure 57. Graph of the enantioselectivity factors of ketorolac and ketoprofen (A) and the observed electrophoretic mobility of individual enantiomers of ketoprofen (B) depending on the concentration of vancomycin introduced into the supporting electrolyte as the second chiral selector.

Conditions: model mixture (rac-ketoprofen 10 µg/ml + S-ketoprofen 2.5 µg/ml and rac-ketorolac 50 µg/ml), injection 2s 30 mbar; 25 mM phosphate buffer solution (pH 6.4), 1.0 mM 1-Bu-3-β-CDImOTs and 0-1.0 mM vancomycin, 254 nm, 20 kV.

A change in the migration order of enantiomers upon the introduction of a second chiral selector into the background electrolyte is a rare topic in research literature, which makes it difficult to interpret the results comparatively. On the one hand, two chiral selectors can have a competitive effect, while on the other hand, a change in the order of migration in a dual chiral system (chiral IL and γ-CD) can be explained by the formation of a "new chiral object" [113], which represents a complex between CD and chiral IL, thus changing the mechanism of chiral recognition in the dual system.

Upon introduction of vancomycin as the second chiral selector (concentrations of both chiral modifier selectors at 1 mM, i.e. ratio 1:1), the enantioselectivity factors of ketoprofen increase to 1.66 with the reversal of enantiomeric migration. A decrease in the concentration of modifiers while maintaining their molar ratio (0.5 and 0.5 mM) does not allow maintaining the same separation selectivity, indicating the necessity of a sufficient number of active chiral centers.

A similar series of experiments was carried out with two other antibiotics, amoxicillin and amikacin. Despite the fact that amoxicillin is similar in acid-base properties (pKa) to vancomycin, the expected change in the order of migration did not occur. Enantioselectivity factors changed insignificantly when amoxicillin was added to the composition of the background electrolyte (Figure 58).

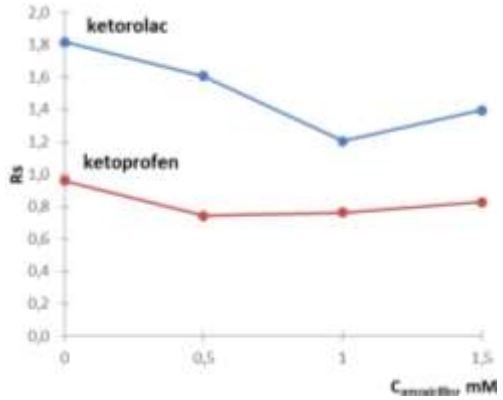


Figure 58. Changes in the resolution of the enantiomers of ketoprofen and ketorolac when the antibiotic amoxicillin is added into the background electrolyte as the second chiral selector.

Conditions: model mixture (rac-ketoprofen 10 µg/ml + S-ketoprofen 2.5 µg/ml and rac-ketorolac 50 µg/ml), injection 2s 30 mbar; 25mM PBS (pH 6.4), 1mM 1-Bu-3-β-CDImOTs and 0-1.5mM amoxicillin, 254nm, 20kV.

With amikacin positively charged at pH 6.4, a slight synergistic effect was observed in the dual chiral system and an increase in enantioselectivity factors for both pairs of NSAID enantiomers at an amikacin concentration of 0.5 mM in the BGE (Figure 59), however, we did not find a change in the order of migration of enantiomers.

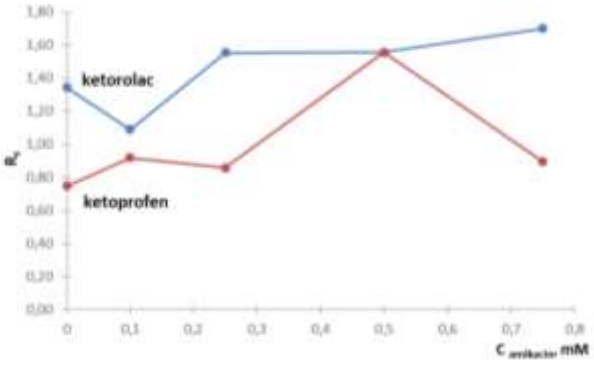


Figure 59. Graph of the enantioselectivity factors of ketorolac and ketoprofen with the additive of amikacin into BGE as the second chiral selector.

Conditions: model mixture (rac-ketoprofen 10 µg/ml + S-ketoprofen 2.5 µg/ml and rac-ketorolac 50 µg/ml), injection 2s 30 mbar; 25 mM PBS (pH 6.4), 1 mM 1-Bu-3-β-CDImOTs and 0-0.75 mM amikacin, 254 nm, 20 kV.

The above graph shows that maximum enantioselectivity factors are observed at a ratio of two chiral selectors of 2:1 (mol.), i.e. when the BGE contains 1.0 mM of 1-Bu-3-β-CDImOTs and 0.5 mM of amikacin.

The electropherogram of the enantiomers separated under these conditions is shown in Figure 60.

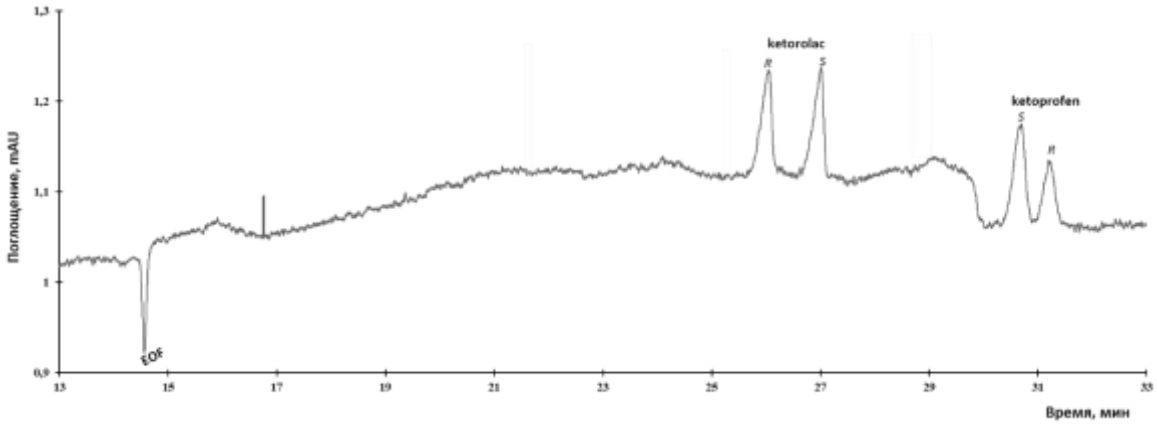


Figure 60. Electrophoregram of the enantiomers of ketoprofen and ketorolac in the dual chiral system with the additive of amikacin into the background electrolyte.

Conditions: model mixture (rac-ketoprofen 10 µg/ml + S-ketoprofen 2.5 µg/ml and rac-ketorolac 50 µg/ml), injection 2s 30 mbar; 25mM PBS (pH 6.4), 1mM 1-Bu-3-β-CDImOTs and 0.5mM amikacin, 254nm, 20kV.

To reveal the features of the synthesized chiral selector based on imidazole and β-CD, a series of comparative electrophoretic experiments was performed, where classical uncharged cyclodextrins (β-CD and HP-β-CD), vancomycin acted as a chiral selector; options with dual chiral selectors were also implemented in the form of combinations of β-CD + *vancomycin* and HP-β-CD + *vancomycin*. For correct comparison with the results obtained in systems with 1-Bu-3-β-CDImOTs, the same concentration of modifiers, 1 mM, was chosen (Figure 61).

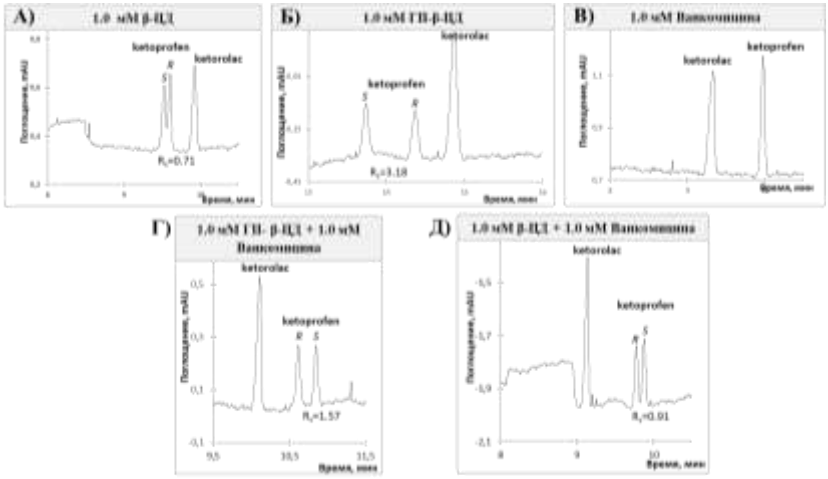


Figure 61. Electropherograms of the enantiomers of ketoprofen and ketorolac with the additive of one or two chiral selectors into the background electrolyte.

Conditions: model mixture (rac-ketoprofen 10mcg/ml and rac-ketorolac 50mcg/ml), injection 2s 30mbar; 25mM PBS (pH 6.4), 1mM β-CD (A) or 1mM HP-β-CD (B), or 1mM vancomycin (C), or 1mM β-CD+ 1mM vancomycin (D), or 1 mM HP-β-CD + 1 mM vancomycin (D), 254nm, 20kV.

It can be seen from the obtained electropherograms that uncharged CDs do not allow the separation of ketorolac enantiomers, in contrast to the synthesized positively charged 1-Bu-3-β-CDImOTs. It should be noted that upon careful examination of the available publications, we were unable to find studies on the electrophoretic separation of ketorolac enantiomers with the introduction of CDs or their derivatives into the background electrolyte. In dual chiral systems, reversal of enantiomer migration is also observed for ketoprofen.

III.3.4 Separation and preconcentration of individual enantiomers of ketoprofen and ketorolac

The possibilities of on-line concentration of individual ketoprofen and ketorolac enantiomers for their subsequent determination in blood plasma have been studied. In the field of CE, on-line preconcentration of individual enantiomers is a poorly studied and complex task. Firstly, peak overlap is observed with increasing sample injection time. Secondly, mechanisms of on-line preconcentration can involve additional interactions (such as distribution between the BGE and micelle cavity in sweeping mode), which compete with interactions between analytes and chiral selectors. Disruption of the latter leads to a decrease in enantioselectivity.

During the experiments, a limitation for ketoprofen and ketorolac enantiomers in the application of an effective concentration method such as sweeping was identified. Two types of surfactants (SDS and 1-dodecyl-3-methylimidazolium chloride (C_{12}MImCl)) were studied at concentrations above their critical micelle concentrations (CMC). After adding surfactants to the background electrolyte, no enantioseparation was observed (Figure 62). Most likely, sweeping is not suitable for concentrating

ketoprofen and ketorolac enantiomers under these conditions due to the competition between two processes: the distribution of analytes between the BGE and the hydrophobic core of the micelles, and the formation of complexes between analytes and the chiral selector (a derivative of β-cyclodextrin). Additionally, micelles can block the hydrophobic cavity of the chiral selector, as was the case with on-line concentration on cyclodextrin-imidazolium covalent coating.

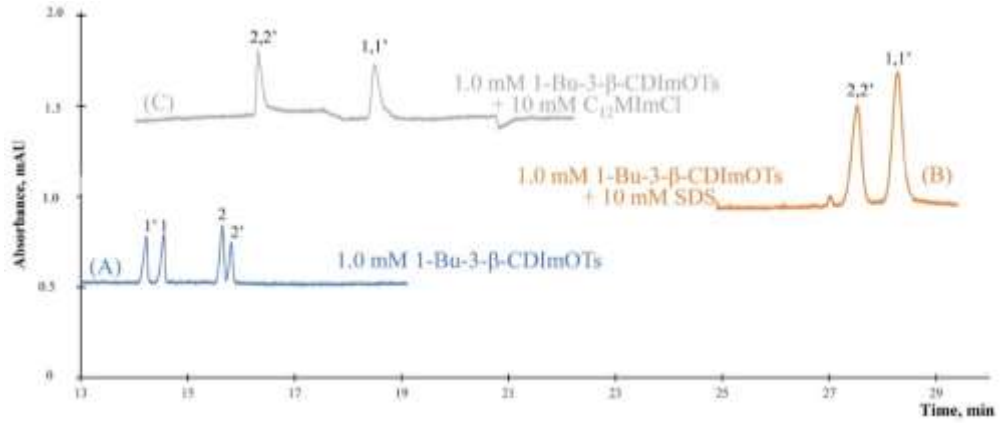


Figure 62. Electropherograms of enantiomers of ketorolac and ketoprofen without preconcentration and with the additive of surfactants into the composition of BGE for sweeping.

Conditions: 25 mM PBS (pH 6.4) + 1 mM 1-Bu-3-β-CDIm + 10 mM SDS or C_{12}MImCl, 254 nm, ±20 kV; model mixture (R,S-ketoprofen 10 μg/ml and R,S-ketorolac 50 μg/ml); injection 2 s 30 mbar. 1' - R-ketorolac, 1 - S-ketorolac, 2 - S-ketoprofen, 2' - R-ketoprofen.

In the case of large volume sample stacking (LVSS), the peaks of enantiomers overlapped, and increasing the sample injection time reduced the separation selectivity. In the field-amplified sample stacking (FASS) mode, where analytes are speeded up in the sample zone, it was possible to concentrate enantiomers by 14-25 times, but furthere increasing the sample injection volume led to peak interference (figure 63).

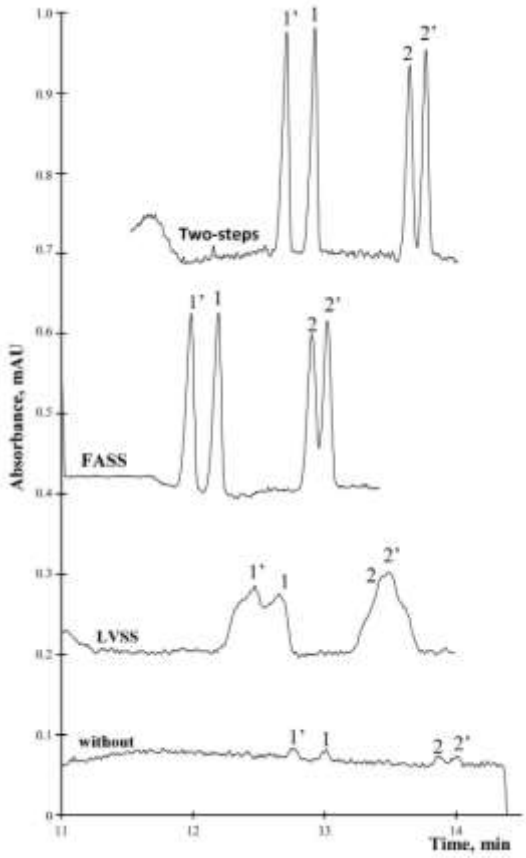


Figure 63. Electropherograms of enantiomers of ketorolac and ketoprofen (from bottom to top) without preconcentration and with preconcentration in LVSS, FASS and in the proposed two-step method.

Conditions: 25 mM PBS (pH 6.4) + 1 mM 1-Bu-3-β-CDIm, 254 nm, 20 kV; model mixture (R,S-ketoprofen 0.5 μg/ml and *R,S*-ketorolac 2.5 μg/ml); injection 40 s 30 mbar. 1' - R-ketorolac, 1 - S-ketorolac, 2 - S-ketoprofen, 2' - R-ketoprofen.

After identifying the limitations of traditional on-line preconcentration methods, hybrid approaches were explored. We developed a hybrid on-line concentration method based on changing the direction of effective electrophoretic mobility of analytes (by varying the dissociated and undissociated forms of the analyte) and sweeping in the sample zone. This proposed method allowed for the concentration of individual enantiomers by 295-395 times, reducing the limit of detection to 12-57 ng/ml (Figure 63, upper electrophoregram).

The proposed mechanism is based on changing the direction of effective electrophoretic mobility of analytes and is similar to the one described in [187], where analytes accumulated at the boundary between a background electrolyte with an organic solvent additive and the sample solution. However, in [187], this change was caused by the disruption of micelles due to the presence of an organic solvent

in the background electrolyte, while in our proposed method, the change in effective electrophoretic mobility is achieved by varying the ionic form of the analyte.

Thus, the algorithm for intracapillary preconcentration is as follows: a capillary filled with a background electrolyte (25 mM phosphate buffer, pH 6.4 + 1 mM 1-Bu-3-β-CD-ImOTs) is hydrodynamically injected with a 10 mM micellar solution of SDS as a short plug (2-10 s at 30 mbar), followed by the injection of a model mixture of ketoprofen and ketorolac enantiomers (dissolved in 25 mM phosphate buffer, pH 3.2; the selected analytes are predominantly in the undissociated form at this pH) (Figure 64, line 1).

We also proposed the mechanism of the developed on-line preconcentration, which involves the migration of anionic micelles towards the anode through the sample zone, concentrating the analytes in a narrow zone at applied voltage. At this stage, the micelle velocity determines the electrophoretic mobility of the neutral enantiomers. Simultaneously, at the interface between the sample (pH 3.2) and the background electrolyte (pH 6.4), a pH gradient is formed under the influence of voltage, causing analytes to dissociate and release from the micelles (negatively charged analytes have a lower affinity for anionic micelles) (Figure 64, line 3). At the same time, analytes interact with the positively charged derivative of β-cyclodextrin in the background electrolyte, forming inclusion complexes. The effective electrophoretic mobilities of analytes change from anodic to cathodic, leading to additional preconcentration. In the final step, the concentrated enantiomers are separated in the electrokinetic chromatography mode (figure 64, line 4).

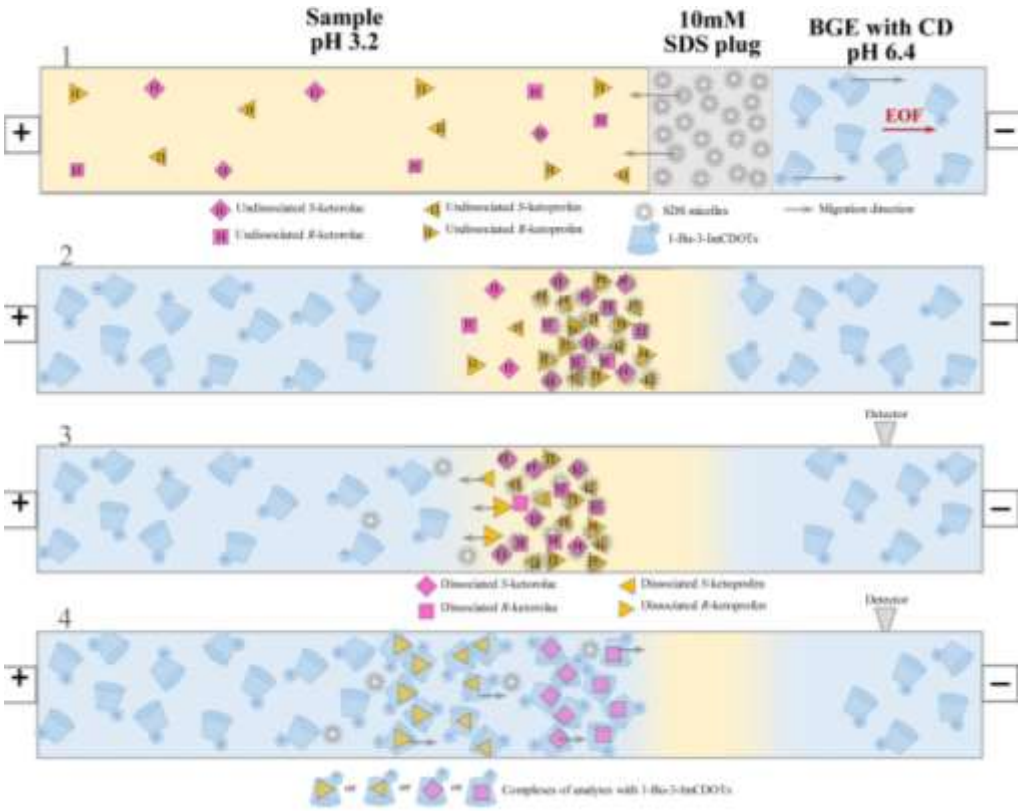


Figure 64. Proposed mechanism of the suggested two-step mode of on-line preconcentration.

The achieved preconcentration factors (SEF) and LOD for different on-line preconcentration methods are presented in Table 18.

Table 18. Conditions, preconcentration factors and LOD for different on-line preconcentration methods.

	Without preconcentration		**Field-amplified sample stacking**		**Two-step preconcentration method**	
	R,S-ketorolac	*R,S*-ketoprofen	*R,S*-ketorolac	*R,S*-ketoprofen	*R,S*-ketorolac	*R,S*-ketoprofen
Sample	5 μg/ml in 25 mM NaH_2PO_4(pH 6.4)	1 μg/ml	1.0 μg/ml in 2.5 mM NaH_2PO_4(pH 6.4)	0.20 μg/ml	250 μg/ml in 25 mM NaH_2PO_4(pH 3.2)	100 μg/ml
Injection	2 s 30 mbar		40 s 30 mbar		60 s 30 mbar + 6 s 50 mbar 10 mM SDS	
SEF	-	-	24-25	14-15	**295-325**	**385-390**
$N\times10^{-3}$, t.p.	210-253	253-297	185-135	158-220	450-460	395-402
LOD (*for each enantiomer)*	2.13-2.15 μg/ml	0.48-0.52 μg/ml	300-320 μg/ml	110-130 μg/ml	45-57 μg/ml	11-12 μg/ml

III.4 Validation of the developed approaches for the analysis of real objects

III.4.1 Determination of biogenic amines in urine samples using covalent coatings

The hybrid on-line concentration method (FASI + sweeping) on alkylimidazolium covalent coatings allowed for the analysis of urine samples without prior (off-line) preconcentration. However, the matrix effect of components presents in biological fluids (creatinine, urea, pigments) at relatively high concentrations (μg/mL) hindered the determination of analytes. Therefore, sample preparation was performed with sorption preconcentration of biogenic amines on activated alumina, followed by electrophoretic analysis under the identified conditions (figure 65).

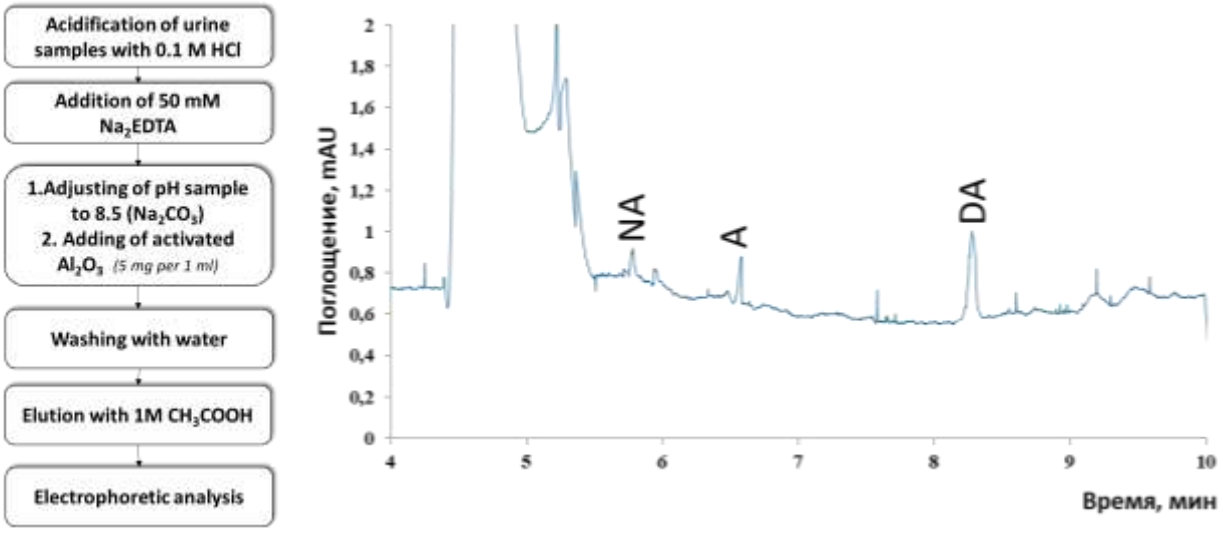


Figure 65. Short scheme of sample preparation of urine samples for the determination of biogenic amines (left) and electropherogram of biogenic amines in urine samples (right).

Conditions: capillary electrophoresis system "Capel'-105M", 10 mM NaH_2PO_4 (adjusted to the required pH value with 1 M HCl solution) and 50 mM SDS; -20kV, 220nm, sample injection 50 s 50 mb.

Calibration curves were shaped for the quantitative determination of biogenic amines in urine samples with individual analyte concentrations ranging from 10-100 ng/mL (Figure 66).

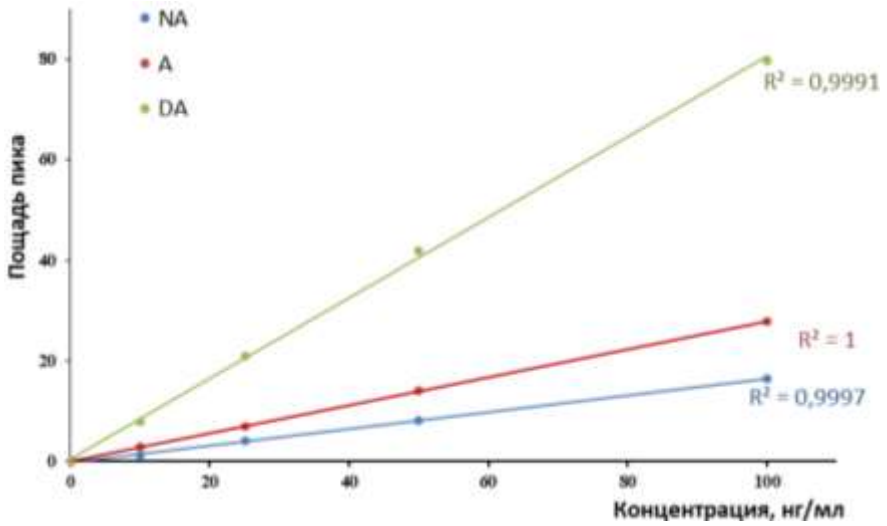


Figure 66. Calibration curves for determining the concentration of biogenic amines in urine samples.

Conditions: capillary electrophoresis system "Capel'-105M", 10 mM NaH_2PO_4 (adjusted to the required pH value with 1 M HCl solution) and 50 mM SDS; -20kV, 220nm, sample injection 50s 50mb.

Found concentrations of biogenic amines are presented in Table 19.

Table 19. Concentrations of catecholamines in urine samples.

Biogenic amines	Concentration, ng/ml
NA	15±4
A	29± 4
DA	62± 8

III.4.2 Determination of steroid hormones in human blood plasma

To demonstrate the applicability of the proposed approach for the electrophoretic determination of corticosteroid hormones using 3-Me-1-β-CD-ImOTs, the levels of five steroid hormones were monitored in human blood plasma. Prior to analysis, corticosteroids were extracted from plasma samples using liquid-liquid extraction with a 5-fold concentration step: 10 μL of internal standard (prednisolone) was added to 1.0 mL of plasma; then chloroform (5.0 mL) was added, thoroughly mixed, and centrifuged; the lower organic layer was washed twice with 0.10 M NaOH solution (0.5 mL each) and 1.0 mL of water. Residual water was removed by adding sodium sulfate, followed by evaporation of chloroform to dryness, and the precipitate was dissolved in 200 μL of water with the addition of 0.05 mM 3-Me-1-β-CD-ImOTs for analysis using CE under the proposed conditions. The content of cortisol and hydrocortisone was determined using an internal standard method (prednisolone acetate as the

internal standard). It was found that the levels of cortisol and hydrocortisone in plasma were 9.20±0.5 ng/mL and 192.8±0.7 ng/mL, respectively (Figure 67). The remaining steroid hormones were found to be below the limit of detection, as expected in normal variants.

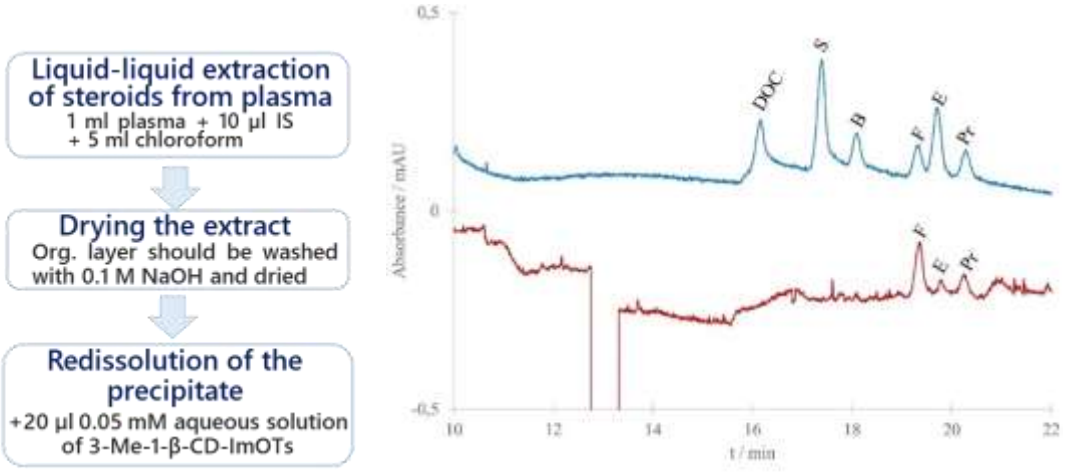


Figure 67. Scheme of sample preparation of plasma samples for the determination of corticosteroid hormones (left) and electropherograms of a mixture of steroid standards (100 ng/ml) in phosphate buffered saline (top right) and plasma (bottom right).

Conditions: 5 mM phosphate buffer, 0.7 mM 3-Me-1-β-CD-ImOTs, 10 (vol.) % ACN. Sample injection: 60 s 20 kV; voltage 20 kV, wavelength 254 nm.

III.4.3 Determination of ketorolac enantiomers in human blood plasma

For the analysis of individual enantiomers of ketorolac in human blood plasma, linearity was determined in the concentration range of 0.25-2.5 µg/mL, as well as the limits of detection, limits of quantification, and precision (Table 20) were calculated as recommended by the Food and Drug Administration[1].

[1] Document number ORA-LAB.5.4.5

Table 20. Validation parameters for individual enantiomers of ketorolac using the two-step on-line preconcentration method

	Analysis in human plasma (with two-step preconcentration mode)	
	***R*-ketorolac**	***S*-ketorolac**
Concentration range (μg/ml)	0.25-2.50	0.25-2.50
Coefficient of determination (R^2)	0.9905	0.9921
LOD (μg/ml)	0.04	0.06
LOQ (μg/ml, relative error 20 %)	0.170	0.190
Repeatability (n=5), %RSD	5-8	5-8
Intermediate precision (n=10), %RSD	7-13	7-13
Accuracy (n=5), %	90-110	93-110

The proposed approach was tested for the analysis of ketorolac enantiomers in human blood plasma 45 minutes after taking the "Ketorol Express" tablet, which is a racemate (Figure 68A). After sample preparation, which involved protein precipitation, electrophoretic analysis of the supernatant was carried out under the found conditions using the proposed two-step online preconcentration method. To reduce the influence of the high-salt matrix, the sample was diluted twofold, and the concentration of SDS in the micellar plug was increased to 20 mM (15 s injection at 50 mbar) to maintain the achieved concentration factors. Absolute calibration method was used for concentration determination. The found values were 610-660 ng/mL for the R-enantiomer and 280-380 ng/mL for the S-enantiomer of ketorolac (Figure 68 B).

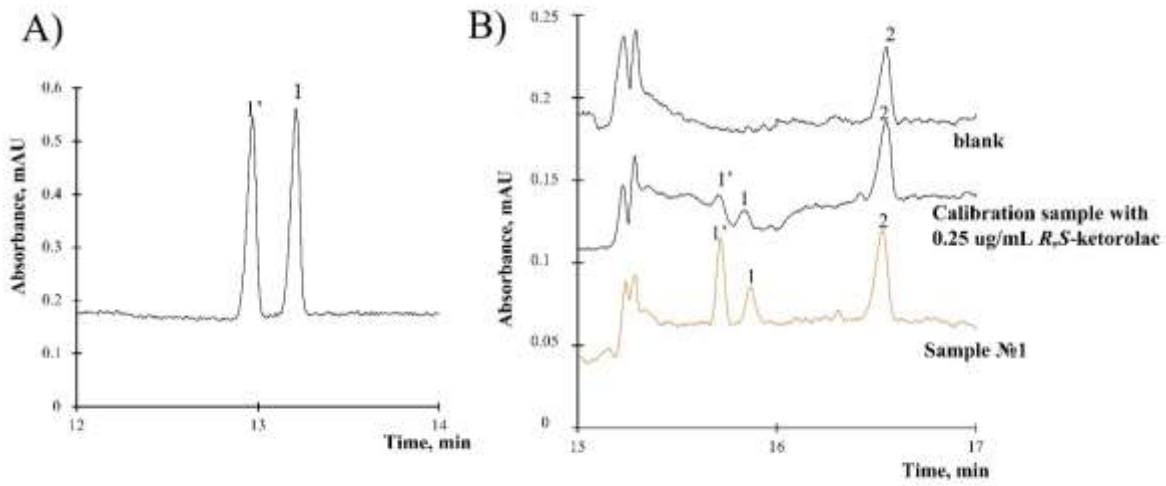


Figure 68. Electropherograms of Ketorol Express tablet solution (A) and electropherograms (from top to bottom) of a plasma sample, a calibration sample (1 μg/ml) of *R,S*-ketorolac, and a plasma sample 45 minutes after taking Ketorol Express (B).

Conditions: 25 mM PBS, pH 6.4, 1 mM 1-Bu-3-β-CD-ImOTs, 20 kV, 310 nm. Stopper SDS in front of sample: 20 mM SDS in 25 mM PBS, pH 6.4, inject 15 s, 50 mbar; sample injection 10 s 30 mbar (A) and 80 s 30 mbar (B). 1' - *R*-ketorolac, 1 - *S*-ketorolac, 2 - *S*-ketoprofen.

To evaluate the accuracy of the proposed ketorolac determination approach, the same plasma samples were analyzed by HPLC, and the found values of the total enantiomer concentrations were compared with the sum of enantiomer concentrations obtained by the CE method. Chromatograms obtained from the analysis of plasma samples and calibration samples are presented in Figure 69. The CE data corresponded to the values obtained by the HPLC method (Table 22).

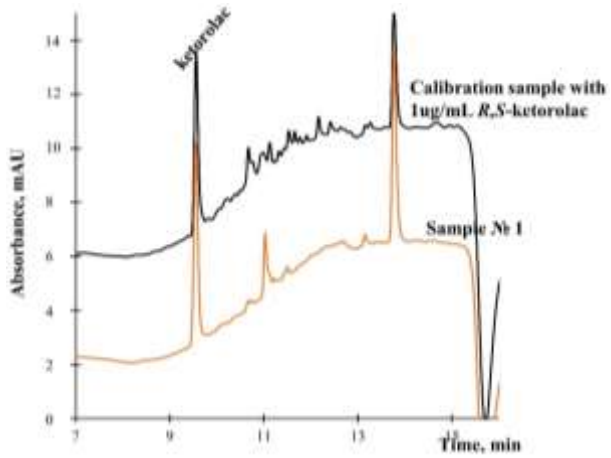


Figure 69. Chromatograms of a calibration sample (1 μg/ml) of R,S-ketorolac and a plasma sample.

Conditions: Nexera LC-40 (Shimadzu) with diode array detection; column Agilent Zorbax SB-C8 2.1 mm × 150 mm, 3.5 μm; mobile phases: A (H_2O with 0.1% (vol.) formic acid) and B (MeOH); flow rate 0.3 ml/min, gradient elution: 0 min - 40% eluent B, 5 min - 40% eluent B, 10 min - 90% eluent B, 13 min - 90% eluent B, 13.50 min 40% eluent B. Total analysis time 17 min. Sample injection volume 10 μl, thermostat temperature 40 °C, wavelength 315 nm.

Table 21. Ketorolac concentrations in plasma determined with HPLC and CE

		Sample 1	Sample 2
CE	$C_{R\text{-ketorolac}}$, μg/ml	0.66±0.03	0.61±0.03
	$C_{S\text{-ketorolac}}$, μg//ml	0.38±0.02	0.28±0.02
		1.04±0.05	0.89±0.05
HPLC	$C_{rac\text{-ketorolac}}$, μg/ml	1.17±0.03	0.99±0.03

Table 22. The comparison of proposed approaches with the literature data

Modification of electrophoretic system	Analytes	LOD	Ref.
Biogenic amines			
no (CE-MS/MS)	NA, A, DA	4,5-140 ng/mL	[188]
N-buthylimidazolium cavalent coatig		1.4-2.1 ng/mL	
N-octhylimidazolium cavalent coatig		0.3-1.1 ng/mL	
Corticisteroid hormones			
25 mM SDS+ 0.5 мМ C_{16}MImCl	F, E, B, S, DOC	200 ng/mL	[189]
covalent coatig based on imidazole and β-CD	F, B, S	120-160 ng/mL	
EKC mode with pseudostashionary phase based on imidazole and β-CD	F, E, B, S, DOC	35-50 ng/mL	
Enantiomers of NSAIDs			
	кетопрофен	кеторолак	
0.05 M heptakis 2,3,6-tri-O-methyl-β-cyclodextrin	0.25 mg/mL	-	[190]
EKC mode with pseudostashionary phase based on imidazole and β-CD	12 (*S*), 18 (*R*) ng/mL	44 (*R*), 55 (*S*) ng/mL	
covalent coatig based on imidazole and β-CD	8 (*R*) 10 (*S*) mg/mL	-	

CONCLUSION

1. An approach to the synthesis of coatings covalently bound to quartz capillary walls by varying the substituent in the imidazolium ring has been proposed. It has been established that the formed alkylimidazolium covalent coatings provide high-efficiency and selective electrophoretic separation of both basic biogenic amines and phenyl- and indolecarboxylic acids. The introduction of β-CD into the coating structure allows for the simultaneous determination of hydrophobic corticosteroid hormones and hydrophilic biogenic amines, as well as acting as a chiral stationary phase (capillary chiral electrokinetic chromatography mode) for the separation of ketoprofen enantiomers.

2. It has been shown that the combination of N-alkylimidazolium covalent coatings with the proposed hybrid on-line preconcentration method (sweeping + FASI, with sodium dodecyl sulfate as the micelle-forming agent) allows for a significant reduction in the detection limits of biogenic amines (down to 0.6-2.0 ng/mL). A variant of intracapillary preconcentration of steroid hormones with electrophoretic introduction of a "plug" of a positive derivative of β-CD after the introduction of the analyzed sample has been proposed, providing an efficiency of up to 147,000 theoretical plates and a 10-16 fold decrease in the detection limit of steroid hormones.

3. A modifier based on alkylimidazole and β-CD has been synthesized, and its analytical capabilities as a pseudostationary phase for the separation of corticosteroid hormones have been revealed. It has been shown that the additive of acetonitrile (10 (vol.) %) into the background electrolyte allows for rapid separation of five important corticosteroids.

4. The formation of positively charged complexes "ionic liquid-analyte" forms the basis of a newly developed approach to intracapillary preconcentration of neutral steroid hormones: positively charged associates enable the injection and preconcentration of analytes with factors of 16-25 in the FASI mode. The detection limits of steroid hormones have been reduced to 35-50 ng/mL, allowing for their electrophoretic determination in human blood plasma.

5. Conditions for the electrophoretic separation of ketoprofen and ketorolac enantiomers have been found when introducing a chiral selector based on butylimidazole and β-cyclodextrin into the background electrolyte. This approach allowed for the first electrophoretic separation of ketorolac enantiomers in the electrokinetic chromatography mode. Complexation constants with positively charged derivatives of β-CD have been calculated for each enantiomer pair.

6. A hybrid variant of intracapillary preconcentration (*combining sweeping and dynamic pH junction)*of individual enantiomers of ketoprofen and ketorolac has been developed, with

preconcentration factors ranging from 290 to 390, resulting in a reduction of the detection limits of individual ketorolac enantiomers to 50 ng/mL. The proposed approach has been tested for the determination of ketorolac enantiomer concentrations in human blood plasma after taking the "Ketorol-express" tablet. The obtained values are in good agreement with the data obtained by RP- HPLC method.

7. The analytical capabilities of different dual chiral systems have been revealed. An interesting regularity was found when combining 1-Bu-3-β-CDImOTs and vancomycin: with an increase in vancomycin concentration in the background electrolyte, a nonlinear change in the enantioselectivity factors of ketoprofen with a reversal of their migration order was observed.

The work was carried out using the equipment of "Chemical Analysis and Materials Research Centre" and "Interdisciplinary Resource Centre for Nanotechnology" of the St. Petersburg State University Research Park with financial support from the Russian Science Foundation grant No. 19-13-00370.

LIST OF ABBREVIATIONS

A– adrenaline

AFMC– Analyte focusing by micelle collapse

ASEI-sweeping –anion-selective exhaustive injection- sweeping

B – corticosterone

3-Bu-1-β-CDImOTs – 1-butyl-3-β-cyclodextrinimidazolytosylate

BMImBF$_4$ – 1-butyl-3-methylimidazolium tetrafluoroborate

BNA – 1,1'-bi-2-naphthyl-2,2'-diamine

BNP - ((±)-1,1'-bi-2-naphthyl-2,2'-diyl hydrogen phosphate

BOH – (±)-1,1'-bi-2-naphthol

C$_{12}$MImCl – 1-dodedecyl-3-methylimidazolium chloride

C$_{16}$MImCl– 1-hexadecyl-3-methylimidazolium chloride

CSEI-sweeping – cation-selective exhaustive injection – sweeping

DA– dopamine

3,4-DHBA– 3,4-dihydroxybenzylamine

Doc – 11-deoxycorticosterone

DOPA – 3,4-dihydroxyphenylalanine

DPPH – 1,1-diphenyl-2-picrylhydrazyl

E - cortisone

EMImBF$_4$– 1-ethyl-3-methylimidazolium tetrafluoroborate

EMImPF$_6$– 1-ethyl-3-methylimidazolium hexafluorophosphate

EMImSO$_3$F$_3$– 1-ethyl-3-trifluoromethanesulfonate methylimidazolium

EtCholNTf$_2$– (R)-N,N,N-trimethyl-2-aminobutanol-bis(trifluoromethanesulfone)imidate

EMIMCl – 1-ethyl-3-ethylimidazolium chloride

F – hydrocortisone

FASI – Field amplified stacking injection

FASS – Field amplified sample stacking

Glu-β-CD – glucosyl-β-CD

GPTMS - (3-glycidoxypropyl)trimethoxysilane

HHA – homogentisic acid

HIAA – 5-hydroxyindoleacetic acid

5-HT– serotonin

HVA – homovanillic acid

InLacA – 3-indole lactic acid

InPrA – 3-indole propionic acid

LVSS– large volume sample stacking

MBuAMCD – mono-6A-(4-methoxybutylamino)-β-CD

MEtAMCD – mono-6A-(2-methoxyethylamino)-β-CD

3-Me-1-β-CDImOTs- methyl-1-β-cyclodextrinimidazolium tosylate

MCDS – Micelle to cyclodextrin stacking

MN– metanephrine

MSS – Micelle to solvent stacking

3-MTA – 3-methoxytyramine

NA – noradrenaline

NMN– normetanephrine

S – 11-deoxycortisol

SEF_h – Sensitivity enhancement factor

3-PLA – 3-phenyl lactic acid

Pr – progesterone

[TBA][L-Arg] – tetrabutylammonium-L-arginine

[TBA][L-Glu] – tetrabutylammonium L-glutamic acid

[TBA][L-Ile] – tetrabutylammonium-L-isoleucine

[TBA][L-Lys] – tetramethylammonium-L-lysine

[TEA][L-Arg] – tetraethylammonium-L-arginine

TEBAC – benzyltriethylammonium chloride

[TMA][L-Arg] – tetramethylammonium-L-arginine

[TMA][L-Glu] – tetramethylammonium -L-glutamic acid

[TMA][L-Hyp] – tetramethylammonium-L-hydroxyproline

[TMA][L-Ile] – tetramethylammonium-L-isoleucine

[TMA][L-Lys] – tetramethylammonium-L-lysine

[TMA][OH] – tetramethylammonium hydroxide

[TMA][L-Pro] – tetramethylammonium-L-proline

Trp – tryptophan

Ts-β-CD – 6A-O-p-toluenesulfonyl-β-cyclodextrin

Tyr – tyrosine

VMA – vanillylmandelic acid

PhBuA – 2-phenylbutyric acid

PhSucA - 3-indoleacrylic acid

PhLacA – 3-phenyl lactic acid

AA – amino acid

BBS– borate buffer solution

HPLC - High Performance Liquid Chromatography

DES – deep eutectic solvents

HP-β-CD - hydroxypropyl-β-CD

SDS - sodium dodecyl sulfate

DMF – dimethylformamide

IL - ionic liquids

CGE - capillary gel electrophoresis

CZE - capillary zone electrophoresis

CMC - critical micelle concentration

CE - capillary electrophoresis

CEC - capillary electrochromatography

LECE – ligand exchange capillary electrophoresis

MIP - molecularly imprinted polymers

MOF - organometallic framework

MEKC – micellar electrochromatography

MEEKC– microemulsion electrokinetic chromatography

NACE – non-aqueous capillary electrophoresis

NSAID - non-steroidal anti-inflammatory drug

NP – nanoparticle

SAA – surface active agent

LOQ - limit of quantitative detection

LOD – limits of detection

SEM - scanning electron microscopy

UDPG - uridine-5'-diphosphoglucuronosyltransferase

PBS - phosphate buffer solution

BGE – background electrolyte

CTAB - cetyltrimethylammonium bromide

CD – cyclodextrin

EKC - electrokinetic chromatography

EOF - electroosmotic flow

LIST OF THE ARTICLES BASED ON THE DISSERTATION RESEARCH

Articles

1. ***Kolobova E., Kartsova L., Kravchenko A., Bessonova E***. Imidazolium ionic liquids as dynamic and covalent modifiers of electrophoretic systems for determination of catecholamines. *Talanta.* 2018. V. 188 P. 183–191 DOI: 10.1016/j.talanta.2018.05.057.

2. ***Kolobova E.A., Kartsova L.A., Bessonova E.A., Kravchenko A.V.*** On-line concentration of biogenic amines by capillary electrophoresis method using the synthesized covalent coating based on the imidazolium ionic liquids. *Analytics and Control*. 2017. V. 21, N. 1, P. 57-64. DOI: 10.15826/analitika.2017.21.1.006.

3. ***Kravchenko A, Kolobova E, Kartsova L.*** Multifunction covalent coatings for separation of amino acids, biogenic amines, steroid hormones, and ketoprofen enantiomers by capillary electrophoresis and capillary electrochromatography. *Sep Sci plus*. 2020. V. 3. P. 102–111. DOI: 10.1002/sscp.201900098.

4. ***Kravchenko A.V., Kartsova L.A.,*** Effect of Modifiers on the Electrophoretic Separation and Intercapillary Preconcentration of Biologically Active Compounds. *J. Analyt.l Chem.,* 2021, 76, 1043-1050. DOI 10.1134/S1061934821090069.

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6. ***Kravchenko A.V., Kolobova E.A., Kechin A.A., Kartsova L.A.*** Development of a capillary electrophoretic method for determination of ketorolac enantiomers in human plasma using cationic β-cyclodextrin derivative as a chiral selector. *Journal of Separation Science,* doi: 10.1002/jssc.202200601.

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