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Novel application of capillary electrophoresis with a liposome coated capillary for prediction of blood-brain barrier permeability

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ABSTRACT

Profiling blood-brain barrier permeability of bioactive molecule is an important issue in early drug development, being a part of the optimization process of a compound's physicochemical properties, and hence pharmacokinetic profile. The study aimed to develop and optimize a new *in vitro* method for assessment of the compound's brain penetration. The tool is proposed as an alternative to the PAMPA-BBB (Parallel Artificial Membrane Permeability Assay for Blood-Brain Barrier) and based on a capillary electrochromatography (CEC) technique. It utilizes liposomes as structural substitutes of biological membranes, which are used as a capillary inner wall coating material. Following optimization of analysis conditions, migration times for a set of 25 reference drugs (mainly non-ionized in pH 7.4) were examined in a liposome coated capillary. On that basis, the retention factor (log k) was determined for each reference drug. Obtained log k values and experimentally received reference permeability parameters: log BB (*in vivo* data) and log P_e (PAMPA-BBB data) were compared with one another. Correlation coefficients were calculated, giving comparable results for CEC log k/log BB and analogical PAMPA-BBB log P_e/log BB analyses. Approximate ranges of log k for the central nervous system (CNS) permeable (CNS (+)) and non-permeable (CNS(−)) drugs were established.

1. Introduction

The drug development process requires both the evaluation of the pharmacological activity of a newly synthesized molecule and the optimization of its pharmacokinetic profile, defined mostly by physicochemical properties. Optimal physicochemical properties determine a compound's permeability through biological membranes, affecting all ADMET processes (absorption, distribution, metabolism, excretion, and toxicity). Interaction between a bioactive molecule and the biological membrane is essential in terms of both, its absorption from the gastrointestinal tract and its penetration through the other barriers in the body, including the particularly important blood-brain barrier (BBB). For potential central nervous system (CNS) drugs, profiling their BBB permeability is crucial for the further development of these molecules. A drug intended to interact with its target (receptor, enzyme or other) located in CNS must cross the BBB to induce the desired activity. A compound that lacks optimal physicochemical properties determining brain penetration is usually disqualified, even despite the strong *in vitro* activity toward its biological target. Information about BBB permeability at the early stage of the new molecule development process allows modification of the potential drug structure for its better CNS exposure. Brain penetration is important not only for drugs acting within the CNS; it is also essential for peripherally active compounds due to their possible adverse brain-related effects.

BBB is the continuous layer formed by the brain endothelial cells of brain capillaries. Transport through the endothelium is highly restricted because of the presence of complex tight junctions between the cells of the capillary wall, but also due to the active efflux transporters, mainly P-glycoprotein (P-gp, MDR1) [1,2]. The main function of the impermeability of BBB for many compounds is to protect the brain from the potentially toxic substances, and also to maintain a constant internal environment optimal for neuronal function. Thanks to the BBB, the composition of the extracellular fluid is quite independent of the composition of the circulating blood [3]. Penetration of the compounds through the BBB is carried out exclusively by transcellular passive diffusion or transcellular active/facilitated transport.

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To date, many diverse techniques have been utilized to quantify and assess the compounds' BBB permeability [4–18]. In general, these are *in vivo*, *in vitro* and *in silico* methods. *In vivo* techniques, usually performed to obtain the log BB (logarithm of brain and blood drug concentration ratio measured in a steady-state) or log PS (logarithm of the permeability-surface area coefficient) permeability parameters, provide results based on the real physiological conditions. However, *in vivo* measurements are complex, expensive, time-consuming, require animals and surgical expertise as well as a large amount of highly pure test compounds. To achieve a high throughput requirement and simultaneously to reduce the use of animal models, *in vitro* and *in silico* methods have been proposed extensively in the recent few decades. *In silico* techniques, often based on the quantitative structure-activity relationship and software calculations have become popular for BBB permeability prediction due to their many advantages. These are low cost, short time of analysis, environment-friendly character and high throughput; yet still some disadvantages limit the use of the *in silico* models in the drug discovery pipeline. The main point is that these methods involve a large number of molecular descriptors and complicated calculations, still being unable to take into account all the physiological processes occurring *in vivo*. As a result, experimental data and *in vitro* models are still more desired. Often, experimentally obtained *in vitro* permeability values are combined with calculated molecular descriptors related to CNS permeability, to achieve better prediction of drug-membrane transport. *In vitro* strategies utilize cell-based and non-cell-based assays. There are several *in vitro* cell-based methods for the assessment of both passive diffusion and active transport of compounds through the BBB. The best of these are those based on primary cultures of brain capillary endothelial cells, but surrogate BBB models based on non-cerebral cell lines are also often utilized. Surrogate BBB models employ epithelial-like cells, such as Madin–Darby Canine Kidney cells transfected with the human MDR1 gene (MDCK-MDR1 permeability assay) and the human colorectal adenocarcinoma cell line (Caco-2 permeability assay) [19,20]. However, methods predicting drug BBB passive and facilitated permeability, investigating possible influx and/or efflux ratios are costly and time-consuming and are not necessary for simple passive diffusion studies in the early phase of drug research for screening compounds. Besides, most CNS drugs, predominantly characterized as small molecules, are transported into the brain mainly through the passive diffusion [1,3,4,21]. Therefore, *in vitro* non-cell-based passive diffusion permeability assays are routinely performed at the early CNS drug development stage. Among these methods, separation techniques, as well as the Parallel Artificial Membrane Permeability Assay for Blood-Brain Barrier (PAMPA-BBB), are utilized for BBB permeability assessment. Separation methods include chromatographic and electrophoretic strategies, both of which meet low cost, high throughput, and high reproducibility criteria. In recent years many research groups have proposed their concepts of modeling drug-membrane transport based on chromatographic principles. Simple reversed-phase thin layer chromatography (RP-TLC) based model as well as the methods focused on the reversed-phase high-performance liquid chromatography (RP-HPLC) have been developed to obtain experimental retention parameters [5]. Often, experimental results have been combined with computed molecular descriptors [5,10,11]. To improve the model of a biological membrane, standard HPLC stationary phases have been replaced with immobilized artificial membranes (IAM) and utilized for brain permeability studies [12,13]. Also, microemulsion liquid chromatography (MELC) [14–16], as well as micellar liquid chromatography (MLC) [5,15] and a variant of MLC named biopartitioning micellar chromatography (BMC) [17] have been proposed, using micelles buffer solutions and microemulsions as mobile phases of a chromatographic system to more closely mimic the analytes – membranes interactions. G. Russo et al. [18] combined compounds' exposed polarity measured experimentally by supercritical fluid chromatography (SFC) with *in silico* calculated water-accessible surface area values and other descriptors to model the uptake of drugs through the BBB. Despite many advantages of LC-based methods, including high throughput and excellent repeatability of results, some limitations have also been reported. These are high consumption of organic solvents affecting the cost of analyses and often the requirement of the addition of *in silico* descriptors to the experimentally obtained permeability parameters (X. Subirats et al. work [14] is an exception here). In the case of IAM-based methods, utilized artificial membranes possess the monolayer structure. As a result, the test compounds only bind to them and do not pass through the membranes, unlike physiological conditions. In the last years the *in vitro* electromigration models, based on the capillary electrophoresis (CE), have been gaining importance in terms of the application for BBB permeability studies. CE is an analytical technique based on the separation principles; the analytes are separated under the influence of the applied voltage, according to their charge, mass, and structure. Separation of tested compounds in the capillary is an effect of two phenomena: electrophoretic migration (migration of charged particles in the electric field toward the oppositely charged electrode) and electroosmotic flow (EOF) [22]. The EOF is created in the capillary by the cations migrating toward the cathode, dragging the solvent (with all ions and neutral particles) along. The cations form a so-called double electrical layer, as a result of their interaction with negatively charged silanol groups of the inner wall of a typical silica-fused capillary. Electrophoretic strategies preserve all LC advantages. Also, they are characterized by simplicity, high separation efficiency, economical use of solvents and environment-friendly nature [6]. To date, CE based microemulsion electrokinetic chromatography (MEEKC) [7], biopartitioning micellar electrokinetic chromatography (BMEKC) [9] and liposomal electrokinetic chromatography (LEKC) [8] have been applied for assessment of compounds brain penetration.

Despite many proposed separation techniques, currently, the PAMPA-BBB tool is widely used as a high throughput *in vitro* BBB permeability testing method, including by our research team [23–25]. The assay was developed by L. Di et al. (2003 [4]), as a modification of the method for studying biological membranes permeability, discovered and described by M. Kansy et al. (1998 [26]). However, next to its many advantages, also in this technique some limitations do exist, primarily because of the non-bilayer lipid structure of the assay's artificial membrane and dodecane diluent. Besides, the test is quite expensive for a small academic research team. Therefore, there is a need for an alternative, rapid and relatively cheap *in vitro* model, mimicking human BBB, for assessment of permeability of new compounds with potential therapeutic use.

The presented study aimed to develop and optimize an *in vitro* method for studying BBB permeability of compounds, based on the CE technique, taking advantage of liposomes as structural analogs of the natural biological membranes. The developed method might be an alternative to the currently used PAMPA-BBB assay, as a tool for screening compounds at the early drug discovery stage.

The developed method uses a capillary electrochromatography technique (CEC) which is a variant of CE. CEC employs a packed or wall-coated capillary (open-tubular CEC). In an open-tubular CEC, a chromatographic phase is created in the capillary by covering its inner surface. Different phospholipids can be used as a capillary coating material, usually forming a semi-permanent layer, non-covalently attached to the silica wall. In the presented method, the capillary is covered internally with a phospholipid layer, composed of large unilamellar liposomes (large unilamellar vesicles, LUVs). The coating mimics BBB, as liposomes are structurally similar to the biological cell membrane. Once the capillary is well coated, the double electrical layer is still formed, but it is less effective when compared to an uncoated one because liposomes cover negatively charged silanol groups of the capillary wall. As a result, a suppressed EOF is observed (Fig. 1). Also, in a liposome coated capillary, after appropriate modifications of the analysis conditions, separation of the tested compounds is influenced by their interaction with the liposomal layer, due to their physicochemical properties determining permeation (*e.g.,* polar surface area and log P).

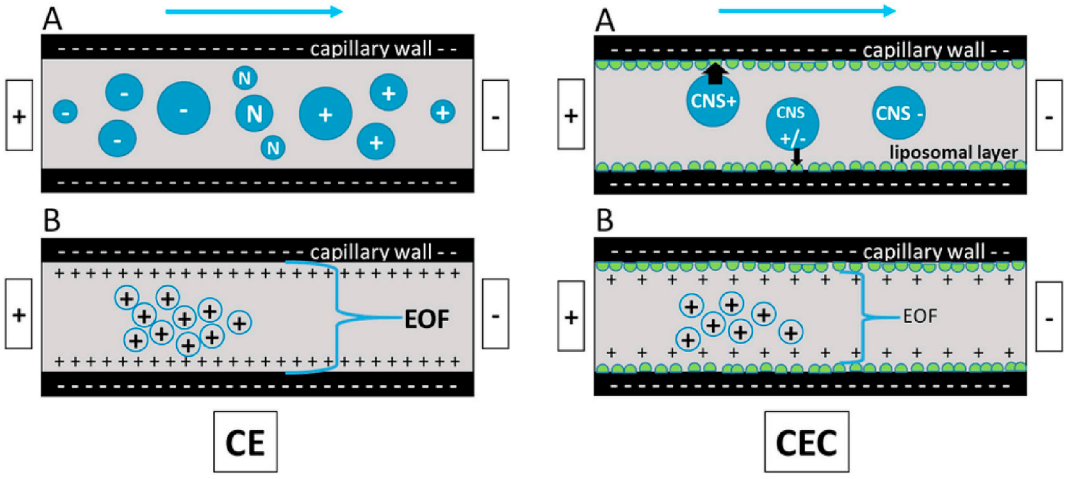

Fig. 1. Scheme of electrophoretic migration (A) and electroosmotic flow EOF (B) in CE and CEC (in the developed method). In CEC variant suppressed EOF is observed. The blue arrows indicate the direction of the ions and solvent migration in the capillary. The black arrows show the interaction of compounds with a liposomal layer. CNS(+); CNS(+/−); CNS(−) are compounds with good, uncertain and poor brain permeability, respectively; for simplicity only neutral in pH 7.4. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Simultaneously, separation is still dependent on compound charge or mass parameters which alone impact migration times in the uncoated capillary. Therefore, the CEC system is defined as a combination of compound electrophoretic migration and simultaneously its retention due to the presence of a liposomal coating. For neutral agents or those predominantly uncharged in the analysis conditions (also for zwitterions with a summary charge equal to 0), their charge related electrophoretic migration is minimized and separation is mainly dependent on the interaction with liposomes.

We assumed that neutral compounds with high BBB permeability should interact with the liposome layer mimicking the BBB (by entering the liposomes) and migrate toward the cathode with lower velocity. In contrast, molecules with low CNS penetration should quickly pass through the capillary due to the weak interaction with the liposomal coating (Fig. 1).

In the PAMPA-BBB assay, an extract from a porcine brain containing specific phospholipids is used to create an artificial membrane that mimics BBB [4]. In the developed method, phospholipids are utilized to prepare the liposomal layer of the inner capillary wall. However, unlike the PAMPA-BBB assay, phospholipids are dissolved in aqueous buffer solution, where they tend to self-assemble (due to their amphiphilic properties) into bilayer lamellar structures, spontaneously forming liposomes [27,28]. Thus, the created liposomal coating possesses a bilayer structure, being closely related to the phospholipid bilayer core of the cellular membrane. Liposomes are of great interest to investigate the interactions of compounds with natural phospholipid bilayers [29,30]. Studies utilizing liposomes in CE were initiated by Zhang et al., in 1995 [31]. Subsequently, many original papers have been published on the application of vesicles in the electrophoretic analysis. Liposomes have been used as a pseudo-stationary phase in the LEKC technique [8,32–34] or as a coating material of the inner wall of the capillary (CEC technique) [33,35–37]. The first successful liposome capillary coating was carried out by Yang et al., in 1998 [38]. Since that time, many diverse vesicle compositions and separating conditions have been studied in the field of the CEC technique [39–42]. So far, however, the LEKC technique is the only one liposomal chromatographic tool based on CE to be used for the prediction of gastrointestinal absorption of compounds [34], and also for testing skin permeability [43] and penetration across the BBB [8]. To the best of our knowledge, to date, there is no validated and optimized CEC method utilizing liposomes as the capillary inner wall coating material, which has been applied for the prediction of BBB penetration of bioactive molecules. Therefore, the development of such a model seems to be innovative, valuable and significant for the studies into drug discovery.

The presented CEC method, used for studying the interactions of compounds with biological membranes retains many CE advantages. These are the short time of analysis and the precision of the obtained experimental results, as well as the small amounts of tested compounds required for successful analysis. Also, substances of low purity can be used as the impurities are separated from the main product. The automated instruments make CEC an easy to perform technique; fused silica capillaries are durable, easy to clean and may be utilized repeatedly for different analyses. The amount of phospholipids needed to create liposomes and perform the study is small (when compared to methods, where the vesicles are used as a pseudo-stationary phase, like LEKC and other EKC models), which significantly reduces the cost of analysis. Besides, the structure and composition of liposomes can be easily modified, which is useful, considering the method optimization. Simultaneously, the proposed CEC method retains most of the good features of the LC-based strategies; it is even considered to be a hybrid between CE and HPLC techniques. However, as an electrophoretic tool it is characterized also by simplicity, high separation efficiency, economical use of solvents and environment-friendly nature – goals hard to achieve in case of many LC-based methods. Moreover, unlike HPLC and IAM-HPLC chromatographic stationary phases, the CEC liposomal layer mimicking BBB preserves the bilayer structure, characteristic for natural biological membranes.

The work plan for the development of the BBB permeability modeling method included optimization of the separation conditions, preparation of the appropriate calibration curves and finally method validation. In the first stage of analysis, described in this paper, after coating the capillary with a liposomal layer, separation parameters were optimized. Then, the migration times for the set of 25 selected reference drugs were examined. On this basis, a logarithm of the retention factor (log k) and the electrophoretic mobility were determined for each reference drug. Log k values were compared with experimentally obtained reference BBB permeability parameters, such as log BB (logarithm of brain and blood drug concentration ratio measured in a steady-state) or log P_e (logarithm of the effective permeability). Correlation coefficients were calculated on the basis of the prepared calibration curves. For comparison purposes, linear regression analysis of log P_e and log BB parameters was also done.

2. Materials and instruments

2.1. Chemicals

References (purity > 99.5%) were purchased as follows: acetaminophen, aminophenazone carbamazepine, cetirizine dihydrochloride, hexobarbital sodium, levodopa, norfloxacin, omeprazole, theobromine, theophylline (Sigma Aldrich, Steinheim, Germany); caffeine, phenytoin (Alfa Aesar, Kandel, Germany); antipyrine (Fluka, Seelze, Germany); barbital (Polfa Tarchomin, Warszawa, Poland); hydrocortisone (Fagron, Kraków, Poland); and progesterone (Caesar & Loretz, Hilden, Germany). The following drugs were extracted with chloroform from tablets: alprazolam (Zomiren SR; KRKA, Nove Mesto, Slovenia); diazepam (Relanium; GlaxoSmithKline Pharmaceuticals, Poznań, Poland); digoxin (Digoxin Teva; Teva Pharmaceuticals, Warszawa, Poland); fexofenadine (Fexofast; Galena, Wrocław, Poland); fluconazole (Fluconazole; Polfarmex, Kutno, Poland); lamotrigine (Lamilept; Teva Pharmaceuticals, Warszawa, Poland); midazolam (Dormicum; Roche, Warszawa, Poland); oxazepam (Oxazepam; Espefa, Kraków, Poland); and zolpidem (Zolsana; KRKA, Nove Mesto, Slovenia); the solvent (chloroform) was then evaporated to dryness.

Methanol (hypergrade for LC-MS), chloroform (HPLC purity), sodium hydroxide (NaOH, 1.0 M, CE purity), HEPES buffer (40 mM, pH adjusted to 7.4 by addition of 1.0 M NaOH), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (PS), and porcine Brain Polar Lipid Extract (Avanti, Alabaster, AL, USA) were obtained from Sigma Aldrich (Steinheim, Germany). Hydrochloric acid (HCl, 36%) was purchased from Stanlab (Lublin, Poland). Purified water was derived from the deionization unit (Hydrolab, Straszyn, Poland). HEPES buffer and HCl were filtered (0.45 μm pore size filter) before use.

2.2. Materials

Polycarbonate filter units of 0.1 μm pore size and the syringe filter units with a pore size of 0.45 μm were purchased from Sigma Aldrich (Steinheim, Germany). Uncoated fused-silica capillary (Beckman Instruments, Fullerton, CA, USA) 59.1 cm × 50 μm of internal diameter and 375 μm of external diameter, with an effective length of 49 cm was used throughout the study. Alternatively, we used a capillary of 20 μm in internal diameter (other parameters unchanged) obtained from Labstore (Warsaw, Poland).

2.3. Instruments

All CEC analyses were performed on a P/ACE MDQ instrument with a DAD detector (Beckman Instruments, Fullerton, CA, USA). The UV detection of the analytes was carried out at a wavelength of 220 nm. We used 32 Karat Software version 8.0 to record and analyze obtained electropherograms. Liposome extrusion was performed using LiposoFast Liposome Factory (Avestin Europe, Mannheim, Germany).

3. Methods – optimization of the new CEC method

3.1. Separation buffer

All experiments were performed using 40 mM HEPES buffer (pH 7.4) as the background electrolyte solution. According to the earlier studies [35,37] on CEC with phospholipid liposome coatings, HEPES buffer has been found to be the best one to achieve a good liposome layer in a fused-silica capillary. In pH 7.4, protonated piperazine amino groups of HEPES are able to act as linkers between a negatively charged fused-silica capillary wall and anionic liposomes, resulting in a stable capillary coating.

3.2. Liposome composition and preparation

The anionic liposomes used in the main study were composed of POPC and bovine brain PS in the molar ratio 80:20 mol% (POPC/PS liposomes). According to the literature [35], such composed vesicles cover the inner wall of the capillary in a stable way, thus enabling effective separation of tested compounds. It is also known, that a capillary coating composed of LUVs gives better repeatability of the EOF in comparison with the layer prepared of multilamellar vesicles (MLVs), due to the homogeneity of LUVs, which is hard to achieve in case of MLVs [44]. Therefore, in the developed method we utilized LUVs that were produced by extrusion of MLVs solution.

To prepare liposomes, appropriate amounts of phospholipids were dissolved in an organic solvent (chloroform, methanol) to form lipid stock solutions. Resultant stock solutions were mixed in order to give the desired composition with an appropriate molar ratio. The mixture was evaporated to dryness under a stream of argon and left for 24 h under the low pressure to evacuate traces of solvent. Lipid residues were next hydrated in separation buffer at 60 °C (lipid concentration of 4 mM) and maintained at this temperature for 1 h with vigorous vortexing. Obtained MLVs were processed into LUVs by 19 times extrusion through 0.1 μm pore size polycarbonate filters. The liposome solutions were stored in a refrigerator and used within 6 days.

Alternatively, we used anionic liposomes prepared from porcine Polar Brain Lipid (PBL) Extract (also utilized in the PAMPA-BBB assay [4]), according to the procedure described above. PBL Extract utilized in this study is composed of various phospholipids, including phosphatidylcholine (12.6%), phosphatidylethanolamine (33.1%), phosphatidylinositol (4.1%), PS (18.5%) and phosphatidic acid (0.8%). It also contains 30.9% of the unknown, mainly lipid components (cerebrosides, sulfatides), so the concentration of the prepared liposomal solution was defined as approximately 4 mM. The obtained liposomal solution was diluted to approximately 3 mM and used as a capillary coating material.

Since PBL Extract contains over 23% of negatively charged phospholipids (PS, phosphatidylinositol and phosphatidic acid), both PBL and POPC/PS liposomes were negatively charged.

3.3. Sample preparation

Each tested compound – in the amount of approximately 1.5 mg – was dissolved in 1 mL volume of methanol to give a stock solution. Prepared stock solutions were mixed with separating HEPES buffer (40 mM) in a 1:9 ratio and filtered through the 0.45 μm pore size filters to give final samples, ready for the injection into the capillary. Methanol, used as a solvent for the compounds, was also the EOF marker.

3.4. Coating procedure

The inner capillary wall was covered with a LUV layer, according to the known procedure [35], with only small modifications.

Liposomes may form two types of coatings on the silica surface of a capillary wall: a supported phospholipid bilayer (SLB) or a supported vesicular layer (SVL). The type of formation and the effectiveness of the capillary coating process has been studied, using atomic force microscopy [45–47], or the dissipative quartz crystal microbalance (QCM) technique. Viitala et al. [48] used the QCM technique to analyze the formation of a capillary layer, created by liposomes composed of the same phospholipid components (POPC/PS) as utilized in this study. The obtained coating was characterized as a viscoelastic and stable SVL type. The SVL layer is more suitable for the CEC technique, because it increases the probability of interaction between analytes and liposomes, due to its larger surface area in comparison to the SLB layer.

The coating process was conducted as follows: 10 min of rinsing the capillary with 0.5 M HCl under the pressure of 93.8 kPa; next, 15 min of

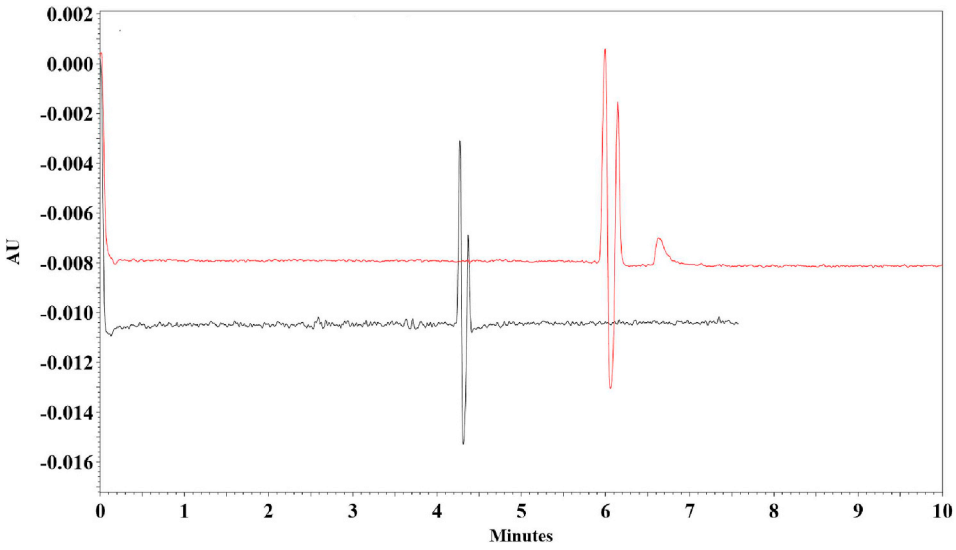

Fig. 2. Separation of hydrocortisone and progesterone mixture under applied voltage of 20 kV, in the uncoated capillary (black marked) and in the POPC/PS liposome coated capillary (red marked), both 50 μm of internal diameter. In the presence of the liposomal coating, separation of both steroids occurs. Due to the retention, progesterone migrates toward cathode with lower velocity. Hydrocortisone interaction with the phospholipid layer is weak. Black peaks in order: methanol, unseparated hydrocortisone and progesterone peak. Red peaks in order: methanol, hydrocortisone, progesterone. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

rinsing with water; and finally, 10 min of rinsing with the 3 mM liposome solution, both also under the pressure of 93.8 kPa. Then the capillary was left to stand filled with the liposome solution for another 15 min.

The effectiveness of the coating procedure was verified by measuring the EOF changes in the coated capillary in comparison to the uncoated one. The electroosmotic mobility μ_{eof} was determined using methanol as a neutral marker, as it displays almost no interaction with the capillary wall [49]. In a covered capillary, the EOF was suppressed, and the methanol μ_{eof} value was lower (see results section). The efficacy of the capillary coating was also confirmed by the separation of the mixture of tested drugs (Fig. 2).

In a liposome coated capillary, separation of references was influenced by their interaction with the liposomal layer. Compound migration times were prolonged for all references (due to suppressed EOF), yet calculated log k parameters varied according to drug affinity to liposomes. For molecules with weak liposome interaction, almost no differences in log k values were observed, between experiments conducted in coated and uncoated capillaries.

3.5. Separating conditions

Electrophoretic runs were conducted at the normal polarity mode, under the 20 kV voltage, resulting in a current of about 10 μA. Also, 13 kV and 10 kV voltages were tested to achieve the best compound separation. A lower voltage resulted in a small separation improvement, but an unfavorable prolonged time of analysis was observed (Fig. 3).

The temperature of the capillary chamber was set to 25 °C, according to the previously reported procedure [35], as it provides satisfactory repeatability of the electroosmotic flow both in the uncoated and the in the coated capillary. Prior to each analysis, the capillary was rinsed with 40 mM HEPES buffer pH 7.4 for 2 min (93.8 kPa), to eliminate any unbound liposomes and other contaminations. The sample was injected into the capillary at a pressure of 4.8 kPa with a duration time of 6 s (50 μm capillary) or at a pressure of 13.8 kPa with 7 s of duration time (20 μm capillary). After sample injection, the ends of the capillary were cleaned by immersion in water for 0.05 min, thus avoiding contamination of other solutions. 40 mM HEPES buffer pH 7.4 was used as a separation solute. The buffer was changed in vials every six runs to ensure its quality. The time of analysis was approximately 5–7 min for 20 kV voltage, and up to 11–14 min for 10 kV voltage. Each experiment was performed in triplicate. To remove the capillary liposomal coating, the cleaning procedure was followed by 10 min of water rinsing, then 40 min of flushing with a mixture of chloroform and methanol in a 2:1 ratio (v/v) and finally 5 min of air drying, all steps with 93.8 kPa pressure.

4. Methods – PAMPA-BBB assay

The penetration of the reference compounds across BBB was estimated using the PAMPA-BBB assay in order to obtain the experimental log P_e parameters. In this study, PAMPA-BBB was used as a non-cell-based *in vitro* assay [23,25] carried out in a coated 96-well membrane filter. The filter membrane of the donor plate was coated with PBL (Avanti, USA) in dodecane (4 μL of 20 mg/mL PBL in dodecane) and the acceptor well was filled with 300 μL of phosphate buffer saline, (PBS pH 7.4; V_A). The tested compounds were first dissolved in dimethylsulfoxide (DMSO) and then diluted with PBS pH 7.4 to reach the final concentrations of 50–500 μM in the donor well. The final concentration of DMSO did not exceed 0.5% (v/v) in the donor solution. A total of 300 μL of the donor solution (V_D) was added to the donor wells and the donor filter plate was carefully put on the acceptor plate so that the coated membrane was "in touch" with both donor solution and acceptor buffer. In principle, a test compound diffused from the donor well through the PBL membrane (Area = 0.28 cm^2) to the acceptor well. The concentrations of the tested compound in both donor and the acceptor wells were assessed after 3, 4, 5 and 6 h of incubation respectively in quadruplicate using a Synergy HT UV plate reader (Biotek, USA) at the maximum absorption wavelength of each compound (n = 3) or by HPLC/MS (n = 2), where a low signal was observed by UV-VIS spectrophotometry (*i.e.* barbital, cetirizine, digoxin, hexobarbital, phenytoin). In addition, a solution of theoretical compound concentration, simulating the equilibrium state established if the membrane were ideally permeable, was prepared and assessed as well. Concentrations of the compounds in the donor and acceptor wells and equilibrium concentrations were calculated from the standard curve and expressed as the permeability (P_e) according to Equation (1) [50,51],

$$\log Pe = \log\left\{C \times -\ln\left(1 - \frac{[drug]_{acceptor}}{[drug]_{equilibrium}}\right)\right\} \quad (1)$$

where V_A is the volume of the acceptor compartment, V_D is the donor well volume, *Area* is the accessible filter area and *Time* is the incubation time.

4.1. UHPLC-MS specification

UHPLC-MS analysis of selected donor and acceptor solutions was

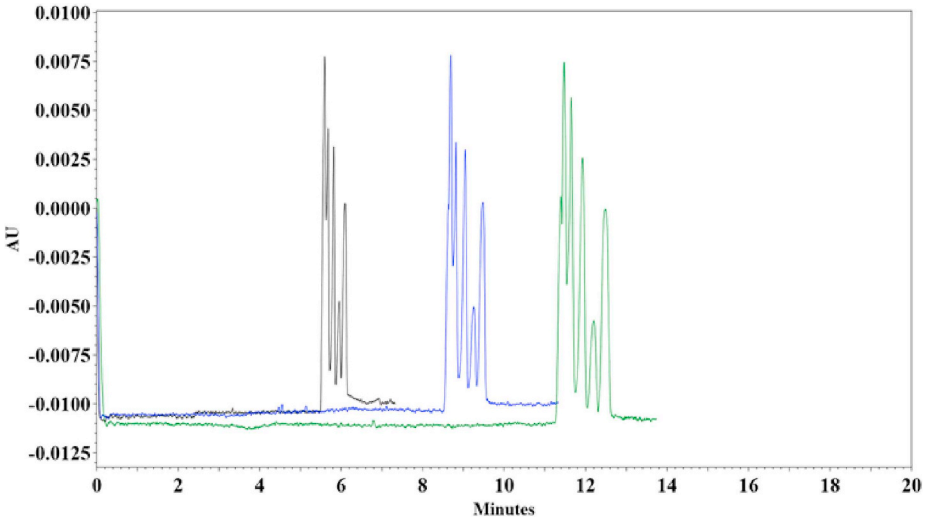

Fig. 3. Separation of a mixture of 5 reference drugs (peaks in order: methanol, norfloxacin, hydrocortisone, theophylline, hexobarbital, phenytoin), under applied voltage of 20 kV (marked in black), 13 kV (marked in blue) and 10 kV (marked in green). A 50 μm of internal diameter capillary, POPC/PS liposome coating. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

carried out using a Dionex UltiMate 3000 RS UHPLC analytical system coupled with a Q Exactive Plus hybrid quadrupole-orbitrap mass spectrometer (MS). This equipment was used to determine high-resolution mass spectra (HRMS) and unknown concentrations of five compounds resulting from the PAMPA-BBB assay (barbital, cetirizine, digoxin, hexobarbital, phenytoin). The chromatographic system included the following modules: HPG-3400RS binary pump, vacuum degasser, TCC-3000RS heated column compartment, and WTS-3000RS autosampler equipped with a 25 μL loop and DAD-3000 detector. A Phenomenex Kinetex C18 EVO column (3.0 × 150 mm/2.6 μm; 100 Å) was chosen as the stationary phase. The temperature in the column thermostat was set to 27 °C. The chromatographic analyses were performed by the reverse phase gradient elution method with mobile phase A: 0.1% (v/v) formic acid in ultrapure water of ASTM I type (resistance 18.2 MΩ × cm at 25 °C) prepared with a Barnstead Smart2Pure 3 UV/UF apparatus (Thermo Fisher Scientific, Bremen, Germany) and mobile phase B: 0.1% (v/v) formic acid in LC-MS grade acetonitrile. For the elution, a simple linear gradient program mixing ultrapure water (MPA) and acetonitrile (MPB), both acidified with 0.1% (v/v) of formic acid, was developed: 0–1.0 min 10% MPB, 1.0–4.0 min 10–100% MPB, 4.0–5.0 min 100% MPB, 5.0–5.0 min 100-10% MPB, 5.0–7.5 10% MPB. The flow-rate of the mobile phase was set to 0.4 mL/min and the injection volume to 5 μL. In MS, heated electro-spray ionization (HESI) was utilized (setting: sheath gas flow rate 55, aux gas flow rate 15, sweep gas flow rate 3, spray voltage 3.5 kV, capillary temperature 220 °C, aux gas temperature 220 °C, S-lens RF level 50). Ions were monitored in the range of 150–1000 *m/z* in positive mode with the resolution set to 140,000. The chromatograms and mass spectra were processed on Chromeleon 6.80 and Xcalibur 3.0.63 software, respectively. The substances were identified according to their high-resolution mass to charge ratio. In order to determine the unknown concentrations of the studied compounds in the donor and acceptor solutions, a calibration measurement spanning the range of 5–100 μg/mL was carried out. The linearity of calibration across its 5 points range was confirmed with $R^2 > 0.9910$ for all compounds.

5. Results and calculations

In the first stage of method development, we selected 25 reference drugs, with varying BBB permeability, diverse structure and lipophilicity, neutral or predominantly unionized (also few zwitterions with a summary charge equal to 0) in analysis conditions (pH 7.4). For these compounds, the influence of the electric field on their migration times was minimized and separation was mainly dependent on the interaction with liposomes. During method optimization, separation conditions were established in order to test all 25 references, preserving a reasonable time for their migration in the capillary. At first, we used a capillary with 50 μm of internal diameter and POPC/PS liposomes (80:20 mol%). In the preliminary research, the separation time was best for 20 kV voltage analysis, however, it was still acceptable for an experiment conducted under the voltage of 10 kV. Therefore, we decided to perform separation of all references under 20 kV and 10 kV voltage (other parameters of analysis were unchanged) and compare obtained correlation coefficients in both conditions. After coating the capillary with POPC/PS liposomes, 25 compounds were analyzed and their log k parameters were calculated on the basis of their migration times, according to the Equation (2),

$$\log\ k = \log\frac{t_R - t_{EOF}}{t_{EOF}} \quad (2)$$

where $t_{R,}$ t_{EOF} are the migration times (min) of the solute and the EOF marker (methanol), respectively.

The retention factor (k) is defined as the ratio of the total number of moles of analyte in the stationary and aqueous phase [52].

Equation (2) is a simplification of Equation (3) used by Terabe et al. [52] in micellar electrokinetic chromatography,

$$\log\ k = \log\frac{t_R - t_{EOF}}{t_{EOF}\left(1 - {}^{t_R}/_{t_{MC}}\right)} \quad (3)$$

where t_{MC} is the migration time of micelles *i.e.* pseudo-stationary phase. In the developed method liposomes, a stationary phase analogical to micelles, are attached to the capillary wall, so they never exit the capillary and their migration time becomes infinite($t_{MC} \rightarrow \infty$).

Determined log k parameters (Table 1) were used for quantitative and qualitative purposes. In terms of qualitative permeability assessment, references can be classified as easy brain penetrators CNS(+) and poor brain penetrators CNS(−), according to their log k values. Subsequently, approximate ranges of log k for CNS permeable and non-permeable drugs were established on the basis of their literature CNS (+)/CNS(−) classification [4,53–64]. References with log k values lower than −1.84 or higher than −1.61 (analysis under 20 kV voltage, with POPC/PS coating) were predominantly (75%) correctly assessed as CNS(−) or CNS(+) agents, respectively (Table 2). In the case of the PAMPA-BBB analogical log P_e ranges, there are 77% of the correct assessments of the CNS(+) and CNS(−) compounds; both CEC and PAMPA-BBB analyses are based on the experimentally obtained log k and log P_e values.

From the quantitative point of view, the model needs to be evaluated for its predictive ability. For this purpose, log k parameters were compared with experimental literature log BB data [65–69] (*in vivo*

Table 1
Log k and log P_e values determined by experimental methods (CEC and PAMPA-BBB, respectively). Log BB values are experimental data retrieved from the literature[65–69].

reference	CEC POPC/PS[a]		CEC PBL[b]	log BB[d]	PAMPA-BBB
	log k[c] ± SD	log k[c] ± SD	log k[c] ± SD		log P_e
	at 20 kV	at 10 kV	at 20 kV		
acetaminophen	−1.603 ± 0.001	−1.573 ± 0.005	−1.618 ± 0.010	−0.31	−6.081
alprazolam	−1.601 ± 0.008	−1.557 ± 0.002	−1.639 ± 0.020	0.044	−4.968
aminophenazone	−1.715 ± 0.069	−1.810 ± 0.013	−1.815 ± 0.003	0	−4.822
antipyrine	−1.635 ± 0.022	−1.597 ± 0.001	−1.700 ± 0.009	−0.097	−5.889
barbital	−0.656 ± 0.016	−0.639 ± 0.031	−0.696 ± 0.001	−0.25	−6.921
caffeine	−1.845 ± 0.026	−1.705 ± 0.008	−1.735 ± 0.010	−0.055	−5.405
carbamazepine	−1.495 ± 0.002	−1.525 ± 0.001	−1.535 ± 0.003	−0.14	−4.894
cetirizine	−1.020 ± 0.001	−1.086 ± 0.006	−1.041 ± 0.004	−1.3	−5.524
diazepam	−1.476 ± 0.087	−1.586 ± 0.014	−1.524 ± 0.008	0.52	−4.710
digoxin	−2.209 ± 0.070	−2.319 ± 0.063	−2.265 ± 0.035	−1.23	−7.097
fexofenadine	−2.208 ± 0.104	−2.089 ± 0.093	nd[e]	−0.98	−4.987
fluconazole	−1.701 ± 0.020	−1.648 ± 0.005	−1.645 ± 0.028	−0.22	−5.241
hexobarbital	−1.058 ± 0.001	−1.055 ± 0.003	−1.075 ± 0.004	0.1	−4.655
hydrocortisone	−1.610 ± 0.011	−1.702 ± 0.029	−1.543 ± 0.029	−0.9	−5.400
lamotrigine	−1.527 ± 0.010	−1.548 ± 0.017	−1.481 ± 0.004	0.48	−5.106
levodopa	−1.458 ± 0.009	−1.442 ± 0.011	−1.474 ± 0.008	−0.77	−5.325
midazolam	−1.550 ± 0.013	−1.451 ± 0.018	−1.600 ± 0.008	0.36	−4.695
norfloxacin	−2.193 ± 0.032	−2.032 ± 0.051	−2.350 ± 0.031	−1	−6.348
omeprazole	−1.387 ± 0.001	−1.395 ± 0.006	−1.421 ± 0.004	−0.82	−5.102
oxazepam	−1.080 ± 0.057	−1.390 ± 0.040	−1.526 ± 0.005	0.61	−4.659
phenytoin	−0.924 ± 0.005	−0.906 ± 0.011	−0.965 ± 0.015	−0.04	−5.043
progesterone	−1.283 ± 0.128	−1.406 ± 0.018	−1.438 ± 0.004	0.2	−5.075
theobromine	−1.631 ± 0.001	−1.701 ± 0.002	−1.700 ± 0.012	−0.28	−5.996
theophylline	−1.231 ± 0.004	−1.229 ± 0.003	−1.307 ± 0.001	−0.29	−5.719
zolpidem	−2.169 ± 0.024	−2.122 ± 0.037	nd	−0.54	−4.695

[a] Capillary with 50 μm internal diameter, coated with POPC/PS liposomes (80:20 mol%).
[b] Capillary with 50 μm internal diameter, coated with porcine PBL Extract liposomes.
[c] Means of three experiments.
[d] *In vivo* measurements from rats.
[e] Not defined due to overlapping peaks of methanol and reference.

measurements obtained from rats), and with log P_e values obtained experimentally in the PAMPA-BBB assay. All data are collected in Table 1.

The P_e value is expressed in [10^{-6}cm × s^{-1}] units and describes the penetration rate of the compound across the membrane. The log P_e parameters were calculated, according to Equation (1).

The obtained log k values and permeability parameters were plotted to give the calibration curves described by linear equations Equation (4) and Equation (5),

$$\log BB = a \log k + b \quad (4)$$

$$\log P_e = c \log k + d \quad (5)$$

where *a, c* represent the slopes and *b, d* are the intercepts of the calibration curves, respectively.

Correlation coefficients were determined for both 20 kV and 10 kV voltage separations. In the case of log k and log BB correlation (Fig. 4A), four compounds (caffeine, levodopa, cetirizine, and barbital) were excluded, as their log BB values were reported to be affected by processes other than only passive diffusion [4,70–75]. Linear regression for log k and log P_e parameters (Fig. 4B) was prepared on the basis of data for 22 compounds, with 3 more excluded from the final analysis (barbital, fexofenadine, and zolpidem) as outliers (see discussion section).

To compare PAMPA-BBB reference experimental results with *in vivo* permeability data, correlation analysis was prepared for log P_e and log BB parameters (Fig. 5). The same 4 drugs were excluded from linear regression as in case of analogical correlation of CEC retention factors and log BB (caffeine, levodopa, cetirizine, and barbital). The obtained R^2 coefficient was comparable to those calculated for log k and log BB parameters (Fig. 4A). Log k values obtained for 20 kV CEC analysis correlated even better with *in vivo* data than log P_e values from reference method (R^2 = 0.4258 versus R^2 = 0.3678, for CEC and PAMPA-BBB, respectively).

Total electrophoretic mobilities of reference compounds were also calculated (Fig. 6). The electrophoretic mobility μ_{tot} of an analyte is defined as the sum of the electroosmotic mobility in the capillary μ_{eof} and the electrophoretic mobility of the analyte itself μ_{ep} (Equation (6)).

$$\mu_{tot} = \mu_{ep} + \mu_{eof} \quad (6)$$

The total electrophoretic mobility μ_{tot} was obtained using Equation (7),

$$\mu_{tot} = \frac{V_{tot}}{E} \left[cm^2 \times V^{-1} \times s^{-1}\right] \quad (7)$$

where V_{tot} is electrophoretic velocity [cm × s^{-1}] obtained by dividing the effective capillary length by the migration time of the analyte, and *E* is the magnitude of the applied electrical field, calculated by dividing the applied voltage by total capillary length [V × cm^{-1}].

The electroosmotic mobility μ_{eof} (marked by methanol) was calculated analogically to total electrophoretic mobility, using Equation (7). The μ_{eof} parameter was used for assessment of the effectiveness of the capillary coating process (Table 3).

We also tested the separation of reference compounds in a capillary of 20 μm of internal diameter, with a coating composed of POPC/PS (80:20 mol%) liposomes. The liposomal layer was stable, as suppressed EOF was observed (Table 3), but no favorable changes in the separation of tested references were obtained. Unexpectedly, log k parameters of tested drugs were lower, indicating weaker interaction of those

Table 2
CNS(+)/CNS(−) classification of 25 references, based on literature data and *in vitro* experimental permeability parameters.

	acetaminophen	alprazolam	aminophenazone	antipyrine	barbital	caffeine	carbamazepine	cetirizine	diazepam	digoxin	fexofenadine	fluconazole
CNS + /− classification[a]	+	+	+	−	+	+	+	−	+	−	−	+
CEC[b]	+	+	±	±	+	−	+	+	+	−	−	±
PAMPA-BBB[c]	−	+	+	−	−	±	+	±	+	−	+	+

	hexobarbital	hydrocortisone	lamotrigine	levodopa	midazolam	norfloxacin	omeprazole	oxazepam	phenytoin	progesterone	theobromine	theophylline	zolpidem
CNS + /− classification[a]	+	−	+	−	+	−	+	+	+	+	±	−	+
CEC[b]	+	±	+	+	+	−	+	+	+	+	±	+	−
PAMPA-BBB[c]	+	±	+	+	+	−	+	+	+	+	−	−	+

[a] Literature data[4,53–64].
[b] Preliminary results; log k approximate ranges: CNS(+) log k > −1.61; CNS(−) log k < −1.84; uncertain data marketed as CNS(±) for −1.84 < log k < −1.61; analysis under voltage of 20 kV, in a 50 μm internal diameter capillary, with POPC/PS (80:20 mol%) liposomal coating.
[c] Log P_e ranges: CNS(+) log P_e > −5.398; CNS(−) log P_e < −5.699; CNS(±) −5.699 < log P_e < −5.398.

compounds with the liposomal coating (Table 4). We compared log k parameters calculated for 5 reference compounds according to their migration times in capillaries with 50 μm and 20 μm of internal diameter, under an applied voltage of 20 kV. As the obtained correlation coefficient was very high (R^2 = 0.9962), we did not conduct the analysis for all 25 references. Less effective separation of tested compounds was probably caused by the worse quality of the obtained liposomal layer. The type and effectiveness of this coating require verification by additional studies (*e.g.,* QCM technique).

In the course of method optimization, liposomes prepared from porcine PBL Extract were also utilized as a coating material for a capillary with 50 μm internal diameter. The coating was stable, and the EOF was suppressed, however not as well as in the case of POPC/PS vesicles (Table 3). Subsequently, the separation was less effective, as the interaction between the tested references and the covered capillary wall was in general slightly weaker in comparison to a POPC/PS liposomal coating (lower log k parameters obtained; comparison between 50 μm capillaries, separation at 20 kV voltage) (Table 1). The correlation coefficients calculated from linear regressions of log k and log BB as well as log k and log P_e parameters were also less satisfactory than the analogical ones obtained for POPC/PS coating (R^2 = 0.2601, F = 5.98, p = 0.0257, n = 19: for log k and log BB analysis and R^2 = 0.3447, F = 10.52, p = 0.0041, n = 22: for plotted log k and log P_e values). The PBL liposomal coating type and quality also need to be verified by additional studies (QCM or other), as well as the size of the PBL liposomes and their surface charge (*e.g.* zeta potential).

6. Discussion

Optimization of the operating conditions was the critical step for the new CEC method development. Many parameters were tested in order to obtain a satisfactory compounds' separation, simultaneously preserving a reasonable migration time for the tested references and mimicking, as far as possible, physiological conditions during analysis. As a result, we successfully covered the capillary with the liposomal layer, which enabled us to separate all 25 reference compounds and calculate their log k parameters based on their migration times. We tested two different liposome compositions, preparing LUVs composed of POPC/PS (80:20 mol%, according to literature data [35]) and vesicles composed of porcine PBL Extract. Both types of liposomes were able to cover the inner capillary wall, and the effectiveness of the created layer was confirmed by reduced EOF. However, the liposomes composed of POPC and PS phospholipids seemed to be more suitable for the developed method, due to their stronger interaction with the tested compounds in the capillary. Also, the composition of POPC/PS is constant, in contrast to brain lipid vesicles, which contain approximately 31% of unknown lipids. During method optimization, we used POPC/PS liposomes as a coating material for capillaries of 50 μm and 20 μm internal diameter. As the liposomal LUV coating has a thickness of only about 70 nm [38] (SVL type layer), it is expected that the narrower the capillary is, the better should be the interaction between liposomes and tested compounds. That was not confirmed by the study results. The log k parameters of tested drugs were lower in the 20 μm capillary (Table 4), so the compounds migrated with higher velocity toward the cathode, although EOF was more suppressed in comparison to the 50 μm capillary (Table 3). This was probably due to the poor quality of the obtained liposomal layer. Therefore, we decided to conduct the analysis in a capillary with 50 μm internal diameter, testing if the change of applied voltage enabled an improvement in the separation parameters. The voltage of 20 kV was the best to combine the short migration time of references with their satisfactory separation in the capillary. However, the time of separation was no longer than 14 min, even under the application of 10 kV voltage. That is why we decided to use data received under voltages of 20 kV and 10 kV in the presented analysis, to compare correlation coefficients obtained in both conditions. Initially, we plotted the received experimental log k parameters

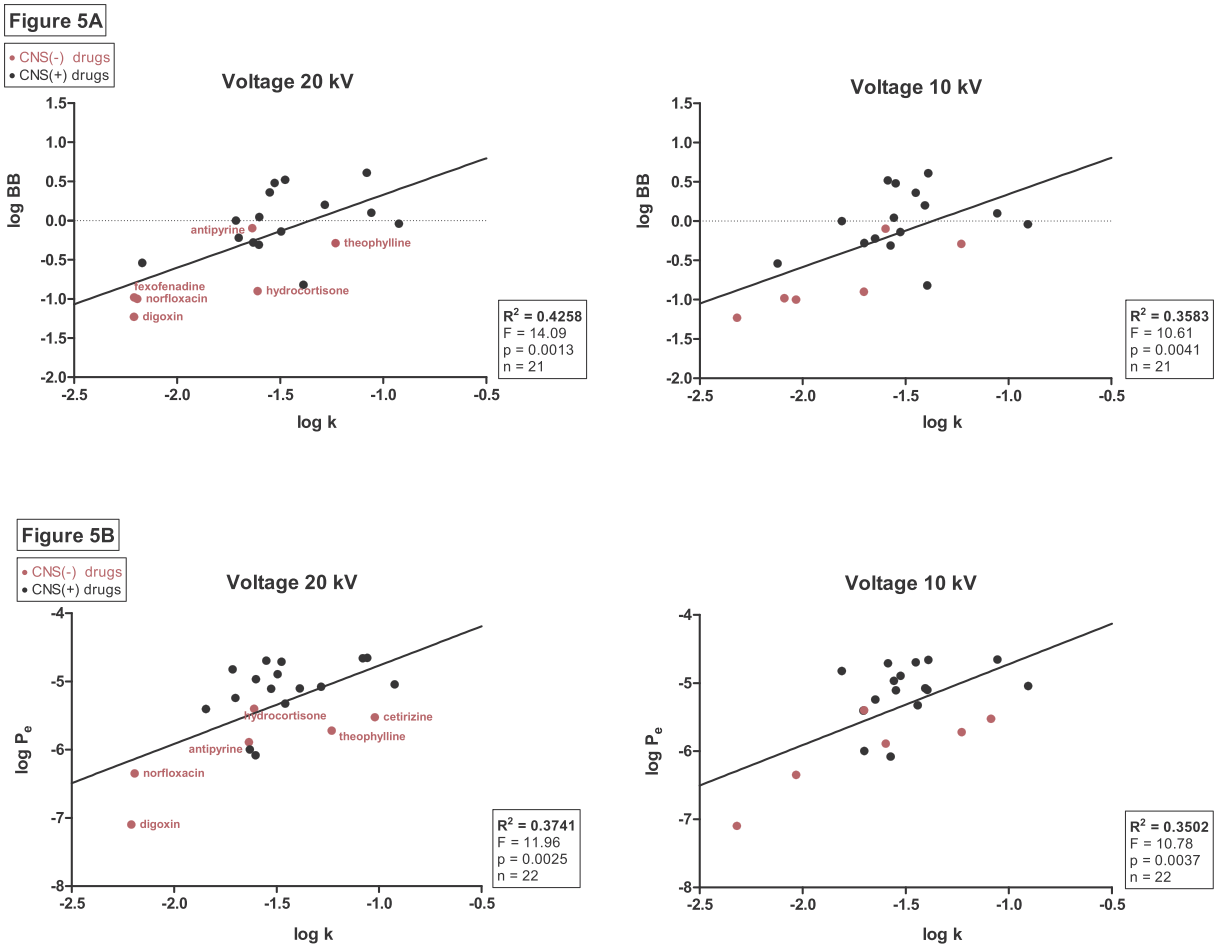

Fig. 4. Calibration curves obtained by linear regression for the reference compounds (Fig. 4A). Plotted values: log k and log BB parameters (Fig. 4B); Plotted values: log k and log P_e parameters. Electrophoretic analysis in POPC/PS (80:20 mol%) coated capillary with 50 μm internal diameter, under applied voltage of 20 kV and 10 kV; drugs defined as BBB non-permeable (CNS(−)) are marked in pink. Drugs with good BBB permeability (CNS(+)) are marked in black. CNS permeability classification based on literature data[4,53–64]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

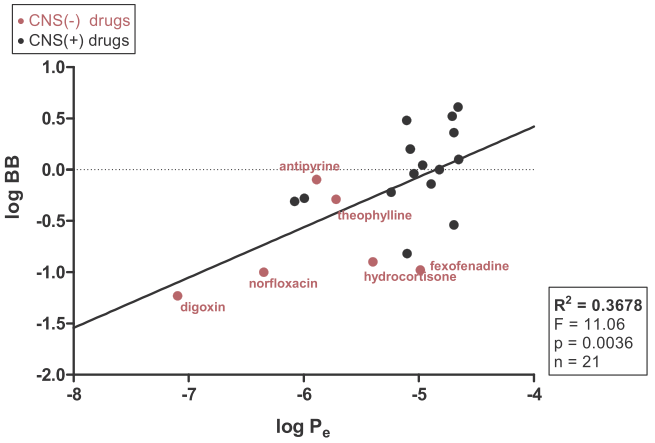

Fig. 5. Comparison of permeability data from PAMPA-BBB method and *in vivo* experiments from rats. Plotted values: log P_e and log BB. Drugs defined as BBB non-permeable (CNS(−)) are marked in pink. Drugs with good BBB permeability (CNS(+)) are marked in black. CNS permeability classification based on literature data[4,53–64]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

with log BB values obtained *in vivo*. The correlation between log k and log BB parameters was acceptable only when 4 references (caffeine, levodopa, cetirizine, and barbital) were excluded from the analysis, and better for separation under 20 kV voltage. This is in accordance with the literature data [76], recommending caution while correlating log BB values with *in vitro* obtained passive permeability parameters. Brain and blood drug concentration ratio measured *in vivo* in rats in a steady-state takes into account drug distribution through BBB by both passive diffusion and facilitated (influx and/or efflux) transport. The log BB value is also affected by other processes such as drug metabolism or plasma protein binding. Thus obtained CEC log k values should be correlated with log BB data only for compounds displaying a mainly passive BBB penetration route. Similar problems were noticed during the PAMPA-BBB method development. Reference drugs were mis-classified as false CNS(+) if they underwent rapid metabolism in systemic circulation or efflux by the P-gp protein, and as false CNS(−) if they were transported into the brain by both passive diffusion and carrier-mediated transport. Among the CEC tested references caffeine and levodopa undergo facilitated brain uptake [4,77] (in the case of levodopa, also opposite Na^+ - dependent transport from the brain to the bloodstream is reported [71]) and their permeability data decrease correlation significance (Fig. 7). In consequence, we decided to exclude them from the prepared log k and log BB calibration curve. Another issue concerns references which are easily permeable across BBB through passive diffusion but are eliminated from CNS by efflux

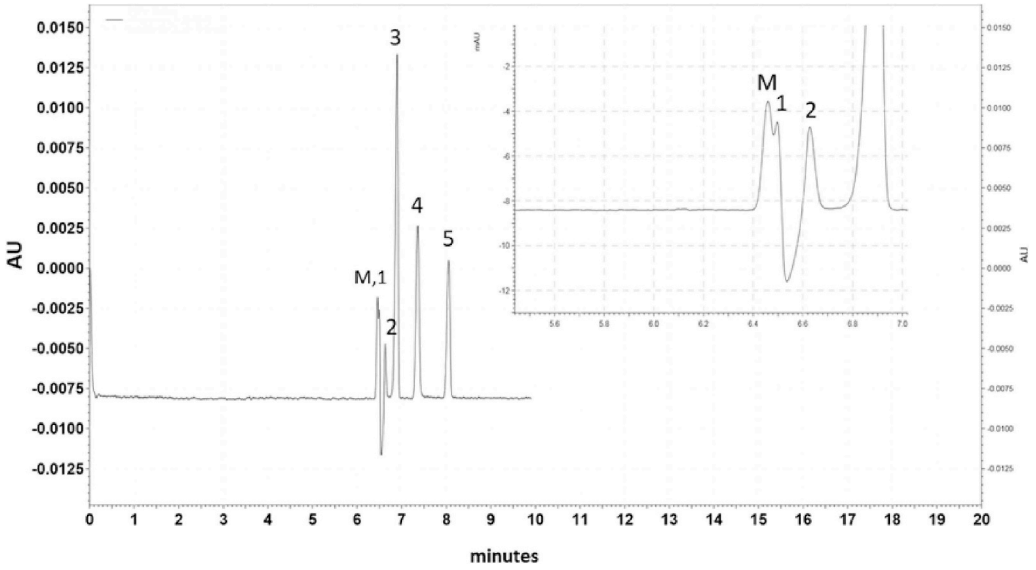

Fig. 6. Electropherogram presenting the separation of mixture of five reference drugs *i.e.* (1) theobromine (μ_{tot} = 3.71 × 10^{-8} m^2 × V^{-1} × s^{-1}), (2) hydrocortisone (μ_{tot} = 3.64 × 10^{-8} m^2 × V^{-1} × s^{-1}), (3) theophylline (μ_{tot} = 3.49 × 10^{-8} m^2 × V^{-1} × s^{-1}), (4) phenytoin (μ_{tot} = 3.28 × 10^{-8} m^2 × V^{-1} × s^{-1}) and (5) barbital (μ_{tot} = 2.99 × 10^{-8} m^2 × V^{-1} × s^{-1}); methanol (the EOF marker) peak is marked as M. Experimental conditions: 59.1 cm capillary (49 cm to detector window) with 50 μm internal diameter; POPC/PS liposome coating; temperature: 25 °C; separation buffer: 40 mM HEPES at pH 7.4; sample: 1:9 diluted methanol stock solution of compound (1.5 mg/mL) in separation buffer; applied voltage: +20 kV; direct UV detection at 220 nm.

transporters. According to the literature data [72,73], cetirizine undergoes efflux elimination, P-gp and not P-gp mediated. Its log BB parameter value determined *in vivo* is low (log BB = −1.3 [69]), indicating a low CNS drug concentration in a steady state. However, cetirizine is reported to be able to passively cross BBB and induce weak CNS related adverse effects (sedation) [73]. The *in silico* calculated drug log BB value, based on Abraham descriptors [67], is much higher (log BB = 0.1 [54]). The log k parameter, determined in the CEC method was also relatively high (log k = −1.01; Table 1), indicating the compound's interaction with the liposomal coating. As a result, the permeation was overestimated using the CEC method, and the drug was an outlier in the linear regression analysis (Fig. 7). Among the tested references, fexofenadine, digoxin, and norfloxacin are also eliminated from the brain by efflux transporters. Fexofenadine and digoxin undergo P-gp and non-P-gp mediated BBB efflux [72,78], but uptake transport is also involved in their distribution between blood and brain [61,79]. As a result, the correlation between fexofenadine and digoxin log k and log BB values was satisfactory in terms of the presented linear regression analysis (20 kV). However, for norfloxacin, a substrate for P-gp and non-P-gp efflux transporters [78], no active uptake mechanism has been reported. Nevertheless, the drug displayed weak *in vitro* interaction with the liposomal layer, probably due to its physicochemical properties (*i.e.* log P = −1.03), that also determine its poor *in vivo* membrane permeability, and it was not excluded from the correlation of log k and log BB parameters.

Log BB as a composite parameter also depends on factors other than passive and active transport. These are rapid drug metabolism, low partitioning to the brain tissue, sink effect of cerebrospinal fluid and high plasma protein binding. The low log BB value may be a signal of extensive drug binding to serum albumin or other carrier proteins in blood plasma [74]; on the other hand, the unbound compound's fraction may still be able to cross BBB and cause the desired pharmacological effect. Among the analyzed references, barbital displays low log BB value (log BB = −0.25), and as the acidic drug, it is reported to be bound by serum albumin [75]. Despite this fact, barbital is classified as CNS(+) agent [59], penetrating the brain by passive diffusion. Its determined CEC log k parameter was high (Table 1); when plotted with its low log BB value, the correlation coefficient was significantly weaker (R^2 = 0.3206, n = 22 and R^2 = 0.4258, n = 21; with and without barbital, respectively, the voltage of 20 kV; Fig. 7). Subsequently, barbital was excluded from the correlation analysis.

The same references (caffeine, levodopa, cetirizine, barbital) were excluded from linear regression prepared for PAMPA-BBB log P_e experimental data and log BB values (Fig. 5). Analogically to CEC method,

Table 4
Log k values of selected reference compounds, calculated on the basis of their migration times in capillary with 50 μm and 20 μm internal diameter, with POPC/PS liposomal coating, under applied voltage of 20 kV.

Compound	log k[a] ± SD for capillary/coating	
	50 μm POPC/PS[b]	20 μm POPC/PS[b]
barbital	−0.656 ± 0.016	−0.815 ± 0.010
diazepam	−1.476 ± 0.087	−1.768 ± 0.010
phenytoin	−0.924 ± 0.005	−1.074 ± 0.004
theophylline	−1.231 ± 0.004	−1.484 ± 0.001
theobromine	−1.631 ± 0.002	−1.898 ± 0.003

[a] Mean of three experiments.
[b] Capillary coated with POPC/PS liposomes (80:20 mol%).

Table 3
Suppression of the electroosmotic flow in a coated capillary with 50 and 20 μm internal diameter. Relative standard deviation (RSD) [%] indicates the EOF stability. Applied voltage: 20 kV.

Capillary/coating	50 μm uncoated	50 μm POPC/PS[a]	50 μm PBL[b]	20 μm uncoated	20 μm POPC/PS[a]
μ_{eof} x 10^{-8} [m^2 x s^{-1} x V^{-1}]	4.87	4.11	4.26	5.69	4.87
RSD [%]	7.18	8.40	8.61	0.41	2.75
number of measurements	45	100	70	3	15

[a] Capillary coated with POPC/PS liposomes (80:20 mol%).
[b] Capillary coated with porcine PBL Extract liposomes; the PBL liposomal coating quality as well as the size of the PBL liposomes and their surface charge need to be verified by additional studies.

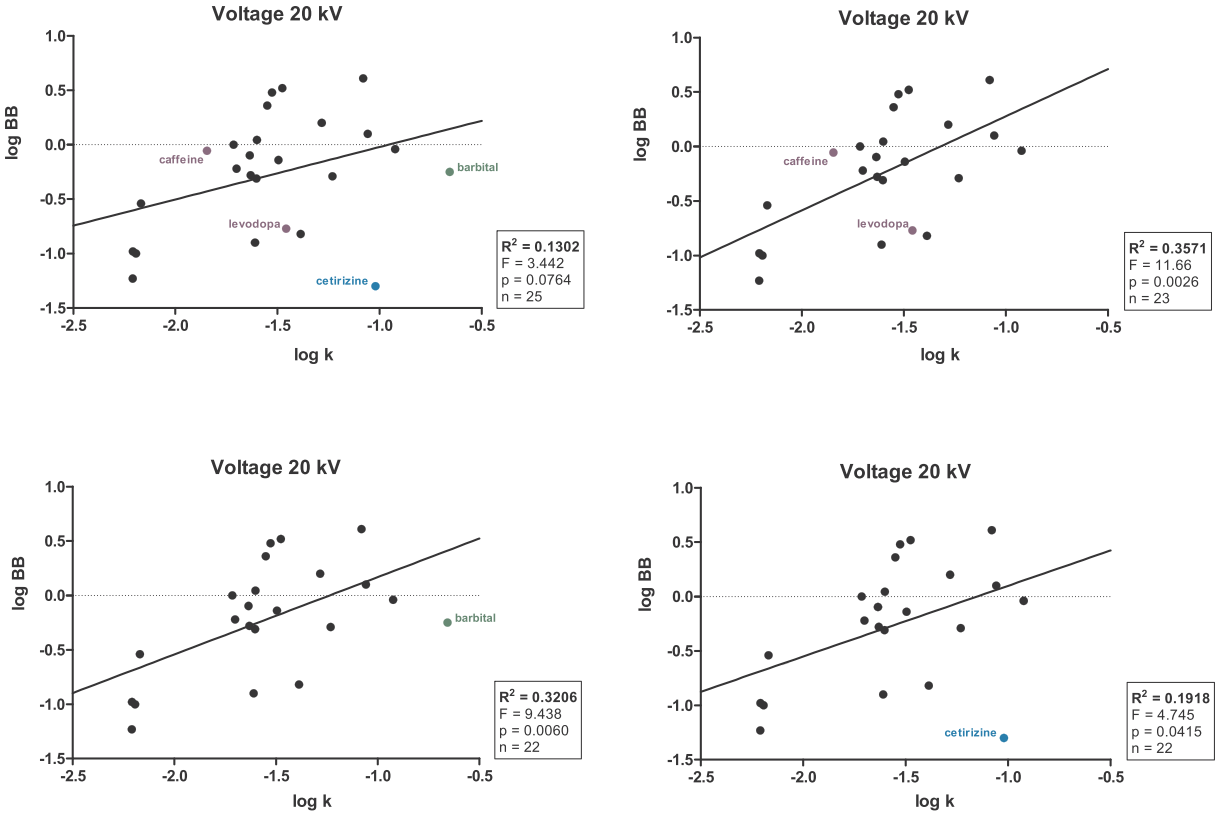

Fig. 7. Correlation coefficient analysis with four references excluded from linear regression presented in Fig. 4A, due to other than passive diffusion processes, affecting their log BB values. Cetirizine (marked in blue) is an outlier in the presented analysis. Caffeine and levodopa (both marked in violet), along with barbital (marked in green) diminish the significance of the correlation; capillary with 50 μm of internal diameter, POPC/PS (80:20 mol%) liposome coating. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

PAMPA-BBB data seemed not to correlate very well with log BB values for compounds with other than passive diffusion processes involved in their permeation (R^2 = 0.2472, F = 7.552, p = 0.0115 for all 25 analyzed drugs, and R^2 = 0.3678, F = 11.06, p = 0.0036, for 21 references).

Finally, we decided to compare log k and log P_e parameters determined by the passive diffusion models: CEC and PAMPA-BBB, respectively. Correlation coefficients were calculated (Fig. 4B); however, 3 compounds were classified as outliers and excluded from the analysis. Obtained R^2 values were comparable to those from corresponding log k and log BB regressions (Fig. 4A). It is worth emphasizing, that the PAMPA-BBB method gives information about the penetration rate of tested compounds, which are classified as CNS(+) or CNS(−) based on their P_e values. In turn, using the CEC method we can assess molecules' affinity to the phospholipid bilayer rather than their permeability rate. Low lipid-soluble drugs penetrate slowly through the membrane; still, they display interference with the lipid bilayer. Usually, they are also able to penetrate CNS and induce pharmacological effects. Such compounds may be characterized by low P_e values that do not correlate well with corresponding log k parameters. That was the reason why barbital was an outlier in the presented log k and log P_e correlation analysis. Barbital is a known CNS(+) agent but is reported to penetrate slowly across the membrane [59]. Indeed, its experimental log P_e value is more like this for CNS(−) drugs. The CEC method gives a better prediction of its CNS affinity. Determined log k parameter is high, indicating the compound's interaction with the liposomal layer. Hexobarbital displays a different permeability profile, as it is characterized by higher lipid solubility and penetrates the brain very quickly [59]. In the case of this drug, log k and log P_e values correlate well. Another outlier, zolpidem, also penetrates rapidly across the membrane [80] and, in turn, is better assessed by the PAMPA-BBB assay. The last one of the excluded drugs, fexofenadine, undergoes carrier-mediated influx and efflux transport, so it is hard to asses drug's permeability, using both “passive methods”. The PAMPA-BBB assay classifies fexofenadine as a CNS(+) compound, while the determined log k value indicates its low affinity to the biological membrane and in fact, the drug's CNS penetration is very limited. The results obtained for the excluded drugs well demonstrate the limitations of *in vitro* techniques for BBB penetration estimation.

As the calculated correlation coefficients for the presented linear regressions of log k and log BB/log P_e parameters are based on experimental data of a small set of 25 drugs, it is hard and too early to indicate clear ranges of log k values for CNS(+) and CNS(−) drugs, similar to those for log P_e in the PAMPA-BBB assay. Nevertheless, even at the early stage of method development, CEC proved to have the potential to be a quick and useful tool for permeability prediction of compounds. References with log k < -1.84 or log k > -1.61 (analysis under 20 kV voltage, with POPC/PS coating) are predominantly correctly assessed as CNS(−) or CNS(+) agents, respectively (Table 2). From a qualitative point of view, the percentage of correct predictions is comparable to the PAMPA-BBB assay data, as well as to some other *in vitro* separation methods proposed for BBB permeability assessment [11,12,17].

7. Conclusions

25 marketed drugs were used as references for the new, permeability prediction CEC method development. The best parameters for separation of reference compounds were obtained in the capillary of 50 μm internal diameter, coated with a POPC/PS liposome (80:20 mol %) layer, under the applied voltage of 20 kV. Based on drug migration times in the capillary, their log k parameters were calculated and then correlated with experimental log BB and log P_e values. The final log k

and log BB linear regression was based on 21 reference data with $R^2 = 0.4258$, for 20 kV voltage. The obtained R^2 value was better than that calculated from the corresponding reference correlation of PAMPA-BBB log P_e parameters and log BB *in vivo* data ($R^2 = 0.3678$, n = 21). The received correlation coefficients indicate obvious limitations of non-cell based *in vitro* methods. Due to their simplifications, no such technique will ever correlate very well with *in vivo* conditions. Nevertheless, for screening purposes, the presented CEC tool is comparable to the PAMPA-BBB method widely used in early drug discovery. For most references, CNS(+)/CNS(−) preliminary predictions are similar in the case of both methods and also in agreement with drugs' permeability literature classification. The presented model may be further validated with the extent number of compounds, including molecules predominantly charged in pH 7.4.

To conclude, the new method proves to have the potential to work well as a simple tool for early BBB permeability assessment of research compounds. It is an innovative, fast and relatively cheap alternative to the PAMPA-BBB technique. Also, it utilizes a liposomal coating of bilayer structure, closely mimicking natural phospholipid bilayers of BBB. Moreover, it preserves the main advantages of the other electrophoretic strategies, as well as the chromatographic separation models based on LC. In our opinion, it is worth following up on this method based on its potential application at the early drug discovery stage.

CRediT authorship contribution statement

Justyna Godyń: Conceptualization, Investigation, Writing - original draft, Writing - review & editing. **Dominika Gucwa:** Investigation. **Tereza Kobrlova:** Investigation. **Martin Novak:** Investigation. **Ondrej Soukup:** Investigation, Writing - original draft. **Barbara Malawska:** Writing - review & editing. **Marek Bajda:** Conceptualization, Investigation, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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