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Hydrodynamic injection for microchip electrophoresis: Development of an innovative passive system

Amandine Dispas [a,b], Paul Emonts [a], Denis Vandormael [c], Damien Bernier [d], Fabian Dortu [d], Eric Ziemons [b], Philippe Hubert [b], Jacques Crommen [a], Marianne Fillet [a,*]

[a] University of Liege, CIRM, Laboratory for the Analysis of Medicines, Liege, Belgium
[b] University of Liege, CIRM, Laboratory of Pharmaceutical Analytical Chemistry, Liege, Belgium
[c] Sirris, Microfabrication Application Lab, Liege, Belgium
[d] Multitel, Departement of Applied Photonics – Biophotonics, Mons, Belgium

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ABSTRACT

The world is facing challenges in terms of environmental sustainability and rising costs. In this context, miniaturized equipment such as microfluidic devices can be used to manipulate small volumes of fluids in micrometer-sized channels incorporating multiple components such as pumps, valves and mixers. Due to their high flexibility, these devices can provide sustainable alternatives to traditional platforms. The present study focuses on the design of a user-friendly and reliable microfluidic capillary electrophoresis chip for pharmaceutical applications. To meet pharmaceutical requirements in terms of quantification performance and overcome the injection variability usually observed with such microfluidic systems, a reliable and reproducible design for hydrodynamic injection using passive valves has been developed. It has been successfully used for synthetic cathinone analysis.

1. Introduction

Research in the analytical sciences is aimed at continuously improving analytical methods and instruments. In addition to the amelioration of the analytical performances, the common objective is to provide faster, greener, cheaper, and more sensitive techniques. In this context, the introduction of the concept and technology of microfluidics makes it possible to propose microfluidic separation techniques [1]. The miniaturization of liquid chromatography was widely described with different approaches such as nano- and micro-LC as well as chip-based systems [2-4]. The interest of these systems in a wide range of applications has been reported in the literature [5-8]. Nevertheless, as Haghighi et al. [9] have clearly summarized, several technical challenges must be overcome to integrate all the required elements (i.e. pump, injector, stationary phase and detector) into a single instrument. In addition, efforts must be made to ensure that these analytical systems are fully integrated and easy to use. In this context, the miniaturization of capillary electrophoresis (CE) into electrophoresis-on-a-chip (MCE) could be presented as an interesting alternative. MCE consists of a chip-based device comprising an injection channel and a separation channel. This separation technique offers high efficiency with a simpler configuration than chromatographic devices (no need for stationary phase and pumps, and less complex interfaces). In MCE systems, all separation modes available in CE can be used on a chip scale by easily adjusting the background electrolyte, offering a wide range of applications. The technical aspects of MCE systems have been described in several review articles [10,11]. One of the main technical challenges of MCE instrumentation is sample injection [12]. The simplest device involves electrokinetic injection using single transverse channels. The sample is injected by applying a voltage to the ends of the shorter channel to bring the analytes to the intersection of the two channels prior to separation. As generally observed in conventional CE, this injection mode is discriminating, subject to matrix effect and generally presents poor repeatability. To overcome these drawbacks, several hydrodynamic (HD) injection systems have been developed [13,14]. In MCE, HD injection is generally performed in two stages: sample loading followed by sample distribution. In the first stage, the sample is introduced into the injection intersection to form a sample plug. Then, during the dispensing stage, the sample plug is introduced into the separation channel to perform the separation. Most of prototypes feature

* Corresponding author at: University of Liege, CIRM, Laboratory for the Analysis of Medicines, Liege, Belgium.
E-mail address: Marianne.fillet@uliege.be (M. Fillet).

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hydrodynamic injection using complex chip designs. The first strategy relies on the use of external pumps to apply positive or negative pressure. Although syringe pumps are generally used because of their ability to drive tiny volumes at very low flow rates with great precision, other types of pumps, such as micro-vacuum pumps or peristaltic pumps, have also been employed [12,15]. To avoid the need for an external device, the valves used to control HD injection can be integrated directly into the chip [15]. Another complex design encompasses a multilayer microfluidic device with an externally actuated on-chip peristaltic pump and pneumatic microvalves [16]. The main drawbacks of these systems are the high production cost of chips with integrated injection valves (and pumps). To avoid the use of external outputs and/or sophisticated chip designs and interfaces, other driving forces have been employed to induce and control sample flow in chips. Indeed, hydrostatic pressure has been used in several MCE systems to drive the sample to the separation channel intersection. Generated by the difference in liquid level between the reservoirs, this driving force was also obtained by developing tilting microchips (in which hydrostatic pressure is applied by tilting the microchip) [17]. Despite the simplicity of the design, the lack of pressure stability and continuous sample pushing during separation strongly affect repeatability. It is also possible to use microscale fluid and material properties to achieve passive fluid manipulation. Ito et al. have shown how the permeability properties of PDMS can be used to autonomously fill the MCE device [18]. This system comprises two geometry-based passive valves to define a sample plug directly integrated into the separation device. In another system a passive hydrophobic valve was used to define a metering chamber [19,20]. These simpler approaches offer interesting prospects for developing easy-to-use, low-cost MCE systems, and merit further research. In this context, the aim of the present paper is to propose an innovative passive MCE prototype based on capillary valves. This system has been developed with a view to future use in pharmaceutical analysis. Efforts have been made to design a user-friendly, reliable and high-performance MCE chip.

2. Material and methods

2.1. Chemicals and reagents

All model compounds (HPLC grade >98.5 % and racemic mixtures) were acquired from Lipomed (Arlesheim, Switzerland): cathinone HCl (CAT) (2-amino-1-phenylpropan-1-one); methylone HCl (βk-MDMA) (1-(1,3-benzodioxol-5-yl)-2-(methylamino)propan-1-one); (amphetamine)$_2$ H_2SO_4 (AMP) (1-phenylpropan-2-amine); pentylone HCl (βk-MBDP) (1-(1,3-benzodioxol-5-yl)-2-(methylamino)pentan-1-one) and clephedrone HCl (4-CMC) (1-(4-chlorophenyl)-2-(methylamino)propan-1-one). FITC isomer I (>97 %) was purchased from Sigma Aldrich (Overijse, Belgium). DMSO was obtained from VWR (Leuven, Belgium). Methanol was acquired from JT. Baker (Deventer, The Netherlands).

Borax ($Na_2B_4O_7$. 10 H_2O) and sodium hydroxide (NaOH) were obtained from Merck and VWR Chemicals (Leuven, Belgium), respectively. Decaethylene glycol monododecyl ether (C12E10 or dodecyl-poly (ethylene oxide-10) ether) was purchased from Sigma Aldrich (Overijse, Belgium). Both Methocel® A15 LV (MC) and Hypromellose® (HPMC) were acquired from DuPont (Antwerp, Belgium). HPC was purchased from Sigma Aldrich (St Louis, MO, USA). MilIi-Q water was daily produced using Millipore system (Bedford, MA, USA).

PMMA Altuglas VS-UVT® clear 100 (Resinex Belgium NV, Arendonk) was used as chip material. 250 μm thick PMMA sheets were used for chip sealing (Goodfellow GmbH, Friedberg, Germany). Platinum wire (0.5 mm, 99.995 % purity) was also obtained from Goodfellow.

2.2. Chip manufacturing and sealing

The chip steel mold was manufactured by both ultra-high speed micro-milling technique using a FANUC Robodrill α-D21MiB5 equipment (FANUC Benelux BV, Mechelen, Belgium) and by micro-EDM milling process using a SARIX SX-100 HPM micro 3D-EDM milling equipment (SARIX SA, Sant'Antonino, Switzerland) to perform the fine tuning. The chips were manufactured by injection molding process using a BOY XSV equipment (BOY Machines, Exton, PA, USA) coupled to a TEMPRO plus D Vario temperature controller (Wittmann Battenfeld Benelux NV, Holsbeek, Belgium). The structure of the valves as well as the channels were embossed to the bottom plate. The chips were finally sealed with precut 250 μm thick PMMA sheets by solvent assisted thermal bonding. Both the unstructured PMMA sheet and the chip were exposed during 40 s in a saturated atmosphere of dichloromethane using a laboratory-made system. Then the two parts were brought together and placed on a laboratory-made silicon mounting tool before being pressed in a thermo-regulated press Collin LAB-Line P 200 S (Collin Lab & Pilot solutions GmbH, Maitenbeth, Germany) in which the inferior and superior plates were respectively heated at 75 °C and 85 °C. A low pressure of 0.1 bar during 5 s was firstly applied to avoid air trapping followed by a pressure of 7 bars during 3 min. The chips were stored at room temperature during a minimum period of 24 h before utilization.

2.3. Instrumentation

The laboratory-made laser-induced fluorescence (LIF) system was constructed by using a laser (488 nm, 20 mW, model QFLD-488-20S, Qphotonics, Ann Arbor, MI, USA) as light source and a 50 μm ID optical fiber (model: M42L02, Thorlabs, Newton, NJ, USA) coupled to an achromatic collimator lens (19 mm EFL, AC127-019-A, Thorlabs) and a 500 nm long-pass excitation filter (model: FESH0500, Thorlabs). Fluorescence was collected through an aspherical lens (25 mm Diameter x 25 mm EFL, Edmund Optics 49102, Barrington, NJ, USA), a dichroic mirror (DMPL505R, Thorlabs), a 535 ± 11 nm band-pass emission filter (model: MF535-22, Thorlabs) and a CMOS camera (model: UI-3260CP-M-GL from IDS Imaging Development Systems GmbH, Obersulm, Germany) with a 100 mm EFL achromatic tube lens (AC254-100-A, Thorlabs). The power supply was a model SHR 4060 (Iseg Spezialelektronik GmbH, Radeberg, Germany). The coaxial wire was split and soldered to the platinum wires. A laboratory-made chip cover was fabricated in polyamide. The latter helps implement the interface chip-detector and integrates the platinum electrodes and cables. A visual description of the system is available in the Supplementary Materials Figs. 1, 2 and 3.

2.4. Sample preparation and labeling

The labeling protocol was developed during a previous study [21]. FITC solutions were daily prepared by dissolving FITC in DMSO at the target concentration to obtain a FITC/analyte(s) ratio of 5. The labeling reaction mixture was prepared by adding 200 μL of a mixture containing 50 μM of each analyte in 50 μM borate buffer (reaction buffer), 400 μL of 50 mM borate buffer (adjusted to pH 9.3 with a 1 M NaOH solution) and 400 μL of FITC in DMSO solution in an amber Eppendorf microtube. An Eppendorf ThermoMixer C (Aarschot, Belgium) was used to carry out the labeling reaction under a medium agitation set at 700 rpm and 5 °C during 32 min. Next, the labeled mixtures were diluted in water/-methanol 90/10 v/v prior to analysis. The working condition was set at 100 nM for each analyte (considering 100 % labeling process).

3. Results and discussion

3.1. Chip design

Poly(methyl methacrylate) (PMMA) was chosen as the chip material because of its many advantages and its ability to develop a low-cost MCE system. PMMA offers high optical transparency and good electrophoretic properties (e.g. homogeneous surfaces and established changes in surface chemistries, exhibiting stable EOF, buffer compatibility, good thermal conductivity and suitable electrical insulating properties) [5,22,

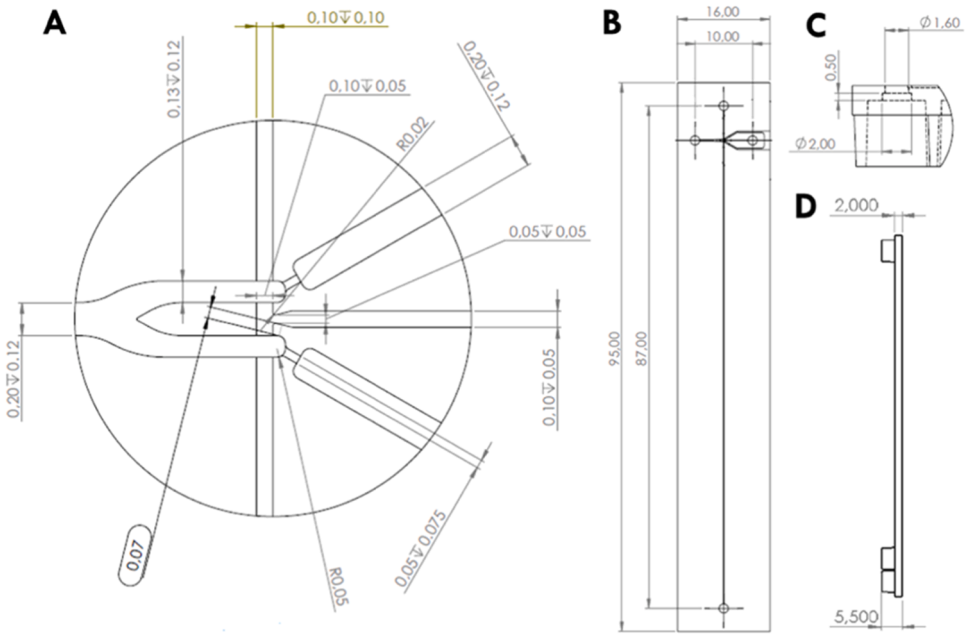

Fig. 1. Chip design and dimensions. (A) Zoom on the injection system. (B) and (C) Macroscopic chip dimensions: top and lateral views. (D) Luer dimensions.

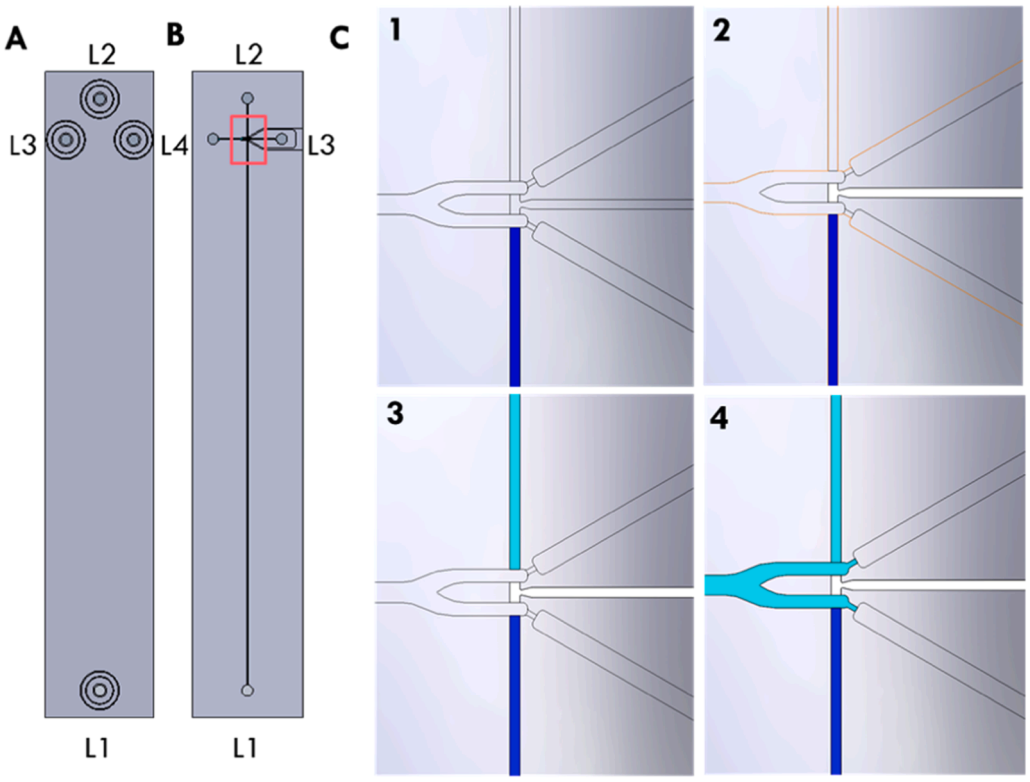

Fig. 2. Schemes of the top face (A) and the bottom face (B) of the MCE chip. The spontaneous filling of the system (C) was performed in four steps: (1) filling of the separation channel (outlet) with BGE and additive via L1; (2) filling of the sample plug with the sample via L3; (3) filling of the inlet channel with BGE (buffer) via L2; (4) Connection of the fluids by filling the fluid junction channel with BGE (buffer) via L4.

23]. Due to its relatively hydrophilic properties, PMMA is an attractive material for reducing non-specific adsorption of analytes, often observed due to larger surface-to-volume ratios at the microscopic scale [24]. In addition, PMMA has attractive mechanical and chemical properties that enable simple manufacturing procedures. In addition to its compatibility with mass production processes, PMMA is an affordable polymer with numerous advantages, including biological compatibility and gas impermeability. In order to offer a self-contained, passive injection system, a capillary force-driven PMMA microfluidic chip was designed. The system is based on passive filling of all channels (i.e. the channels with BGE and sample). The separation channel has a common cross-section of 100 μm square and a length of 82 mm. As shown in Fig. 2, a sample plug has been designed in the extension of the separation channel to inject a well-defined volume of sample. Geometry-based

Table 1
Current values obtained by applying voltages from −700 to −2000 V during 450 s. BGE: 25 mM borate buffer pH 9.3.

	Current values (μA)				
Voltage (V)	Min	Max	Δmin-max	Average	RSD (%)
−2000	−39.2	−42.4	3.2	−41.3	1.5
−1800	−35.8	−38.3	2.5	−37.7	1.3
−1500	−33.3	−35.5	2.2	−34.8	2.1
−1000	−25.0	−25.9	0.9	−25.6	0.8
−700	−15.3	−15.8	0.5	−15.6	0.5

capillary valves have been integrated on both sides of the sample plug to limit the volume of sample introduced. This mechanism is based on surface tension forces between the fluid and the microchannel walls, and a pressure barrier can be generated to form a valve by an abrupt change in channel depth and aspect ratio. Several capillary valve-based chips have been designed and fabricated (see Supplementary data Fig. 4). All prototypes have been experimentally tested to assess adequate passive channel filling, capillary valve efficiency and current stability when voltage is applied. Efforts were also made to reduce the sample length in order to minimize peak broadening. These experiments led to progressive improvements in the design and, finally, to the proposal and in-depth evaluation of the chip design shown in Fig. 1. This optimized chip design includes a 1 nL sample plug defined by two capillary valves. These valves are also used to stop the filling of the separation channel and prevent unwanted mixing between the sample and the BGE.

In practice, the chip is used as shown in Fig. 2. The separation channel is spontaneously filled by pipetting 45 μL of BGE into the outlet luer (L1). Next, sample injection is performed by introducing 5 μL of sample into the injection luer (L3). Next, the inlet channel is spontaneously plugged by adding 45 μL of BGE to the inlet luer (L2). When the sample plug, separation channel and inlet channel are correctly filled, as shown in Fig. 2 C3, 45 μL of BGE is introduced into L4. This last step is necessary to achieve fluid junction. This is synchronized with the application of voltage to start the MCE analysis.

3.2. Chip design assessment and MCE separation

To evaluate the separation performance of the MCE prototype, cathinones and synthetic amphetamines were chosen as model compounds. These small molecules with amino groups are well representative of small pharmaceutical compounds. In addition, these compounds have previously been studied in the laboratory using a conventional CE method [25]. The EOF obtained in PMMA microchannels is lower than that found in fused silica capillaries due to the lesser presence of ionizable functional groups and the lower voltages applied in MCE. In this context, the reverse polarity mode (negative voltage) was chosen to obtain sufficient migration of the labeled analytes, which are negatively charged at this pH due to the labeling with FITC. Initial experiments were carried out using borate buffer at a concentration of 75 mM, as previously done in CE [25]. This relatively high concentration generates a high current in the microfluidic channels, resulting in excessive Joule heat generation. As thermoregulation was not implemented in the system, the buffer concentration was reduced. The use of borate buffer at a concentration of 25 mM was evaluated for voltage from - 700 to −2000 V. As illustrated in Table 1, the measured currents were ranging from −15 to −42 μA, respectively. Current stability was also estimated for these voltages with RSD (%) values lower than 2 % for 7.5 min run time.

Although a relatively small impact on the calculated RSD values was observed, a decrease of current with time was obtained at higher voltages leading to wider current amplitudes (Δmin-max). Consequently, the use of a 25 mM borate buffer at a voltage of −1000 V, corresponding to a field strength of around 115 V.cm-1, was adopted for further development of the method. In CE, the composition of the BGE plays the main role in adjusting the selectivity of the method. In the present project, as PMMA induces a lower EOF than fused silica, the selection of an appropriate BGE composition is more critical. In the literature, the use of complex BGEs comprising additives (i.e. surfactants, cyclodextrin, organic modifiers, etc.) is often reported for the analysis of pharmaceuticals [26]. In the present study, the addition of cellulosic polymers was investigated [27-30]. Hydroxypropyl cellulose (HPC), hydroxypropyl methyl cellulose (HPMC) or methyl cellulose (MC) added to the borate BGE were selected. The three cellulose derivatives showed interesting separation capability and were therefore studied at concentrations ranging from 0.5 to 3 %. It is important to note that the use of such an additive increases the viscosity of BGE. This has a direct impact on channel filling. Despite a slight increase in viscosity, HPC- and MC-based BGEs exhibited good spontaneous filling properties, while HPMC concentrations above 0.5 % resulted in an increase in viscosity that limited spontaneous migration through the separation channel. In addition to passive channel filling, the chip design relies on passive capillary valves. The presence of additives weakened the vent valves, limiting their effectiveness over time. To overcome this drawback and guarantee the efficiency of the capillary valves throughout the analysis, the separation channel was filled with additive-containing BGE, while

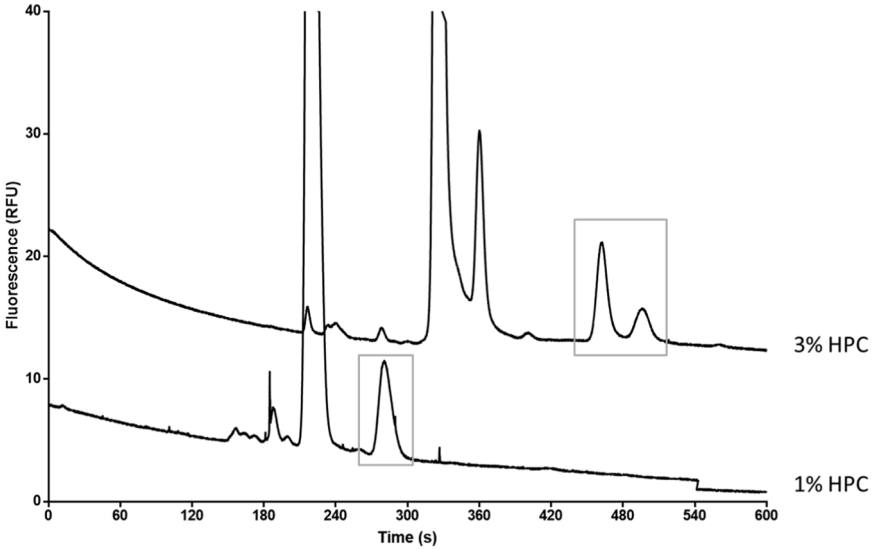

Fig. 3. Electropherograms obtained by adding 1 % or 3 % HPC to 25 mM borate buffer (pH 9.3). Voltage: −1000 V; Sample: 100 nM AMP and Bk-MDMA.

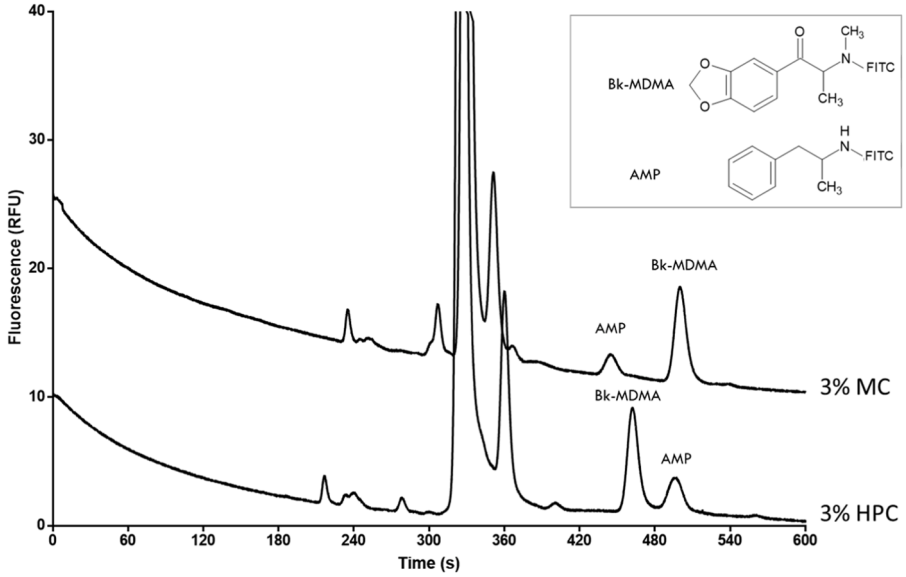

Fig. 4. Electropherograms obtained by adding 3 % MC or 3 % HPC to 25 mM borate buffer (pH 9.3). Voltage: −1000 V; Sample: 100 nM AMP and Bk-MDMA.

the inlet and fluid junction channels were filled with borate buffer (see Fig. 2). This protocol has no impact on the separation mechanism, as separation occurs only in the separation channel filled with BGE and additive(s). In addition, HPMC was subsequently eliminated due to its viscosity. The passive chip design results in a simpler, less costly MCE chip, but presents certain technical constraints with regard to BGE compatibility. By increasing HPC or MC concentration from 0.5 to 1 %, FITC peak migration times were not significantly affected, but FITC/analyte resolution was improved. These observations suggest that the pore size of the network structure formed by 1 % HPC or MC was not sufficient to discriminate closely migrating labeled analytes, but was only capable of separating FITC from these analytes. Then, as the concentration of HPC or MC in the separation buffer was further increased, an improvement in analyte selectivity and resolution was observed. For example, the BGE containing 1 % HPC led to a co-migration of AMP and Bk-MDMA, while a 3 % concentration of HPC gave rise to near-baseline separation, as shown in Fig. 3. Nevertheless, these separation improvements were also associated with an increase in migration times from ~ 215 s to ~ 320 s (considering the main FITC peak), indicating an impact of the sieving matrix on analyte migration velocity. Acting as a sieving material, this separation mode seems to be well suited to the MCE separation of analytes sharing the same charge and may avoid the potential adsorption of labeled analytes to PMMA walls.

The two BGEs containing 3 % of polymer showed the best separation, as shown in Fig. 4. Despite equivalent FITC migration times, the MC-based BGE was the most appropriate separation medium. In fact, it enabled a separation of the analytes with a resolution higher than 1.5 in a similar analysis time, compared to the HPC-based BGE. Separation efficiencies of the order of 5000 plates were achieved. Interestingly, a reversal in migration order was observed for the analytes with the HPC-based BGE, indicating potential additional interactions between the analytes and the polymer.

However, the migration time of FITC did not appear to be influenced by the nature of the cellulose polymer. Overall, the use of 25 mM borate buffer (pH 9.3) containing 3 % MCas BGE led to the baseline separation of the two model analytes in <9 min, while the viscosity was maintained low enough to ensure spontaneous filling of the chip.

3.3. Evaluation of analytical performance

As described in the previous section, an innovative passive MCE prototype based on capillary valves has been designed and successfully evaluated. The use of hydrodynamic injection has been previously presented as the key concept for proposing reliable MCE injection [26]. In this context, the performance of the MCE chip was evaluated under the optimized analytical conditions. The repeatability of migration times, peak areas and peak heights was evaluated in triplicate using three independent chips by analyzing a mixture containing 100 nM AMP and βk-MDMA. Estimated RSD values (%) are presented in Table 2. Migration time variability between chips is better than some results reported for commercial or laboratory-made MCE devices [31,32].

Table 2
Relative standard deviation (RSD values) (%) estimated from three analyses performed using three independent chips. Sample: 100 nM AMP and Bk-MDMA.

	RSD (%) values ($n = 3$)		
	AMP	Bk-MDMA	AMP/Bk-MDMA
Migration time	3.6	3.9	0.3
Peak area	28.9	26.1	8.4
Peak height	28.2	34.9	7.6

The use of an internal standard is generally required in CE because of the very small volumes of sample injected. Therefore, the implementation of an appropriate internal standard to normalize the results is necessary. It is important to note that the present MCE chip has an injection volume of 1 nL, which is even lower than the usual injection volume in CE (a few nL). In addition to the fluctuations observed for each analyte, normalization of each result using the second analyte as an internal standard ("AMP/Bk-MDMA" column in Table 2) yielded interesting results with RSD% values of 8.4 % and 7.6 % for peak area and peak height, respectively. These promising results highlighted the value of hydrodynamic injection for improving method reproducibility compared to electrokinetic injection [26]. Nevertheless, better precision performances have been described for complex systems including microvalves (RSD values below 2 %) [33,34]. However, these values were obtained using multiple injections on the same chip. The present study proposes a single-use chip, so that reproducibility results are representative of inter-chip precision. It is also important to bear in mind that the chip design developed is based on passive filling and injection, in order to offer a simple, low-cost device.

Finally, signal-to-noise ratios were calculated to estimate the sensitivity achieved using the MCE system. Analyzing the mixture containing 100 nM of each labeled analyte, the mean S/N ratios ($n = 3$) were 15 and 66 for MPA and Bk-MDMA respectively, indicating promising system

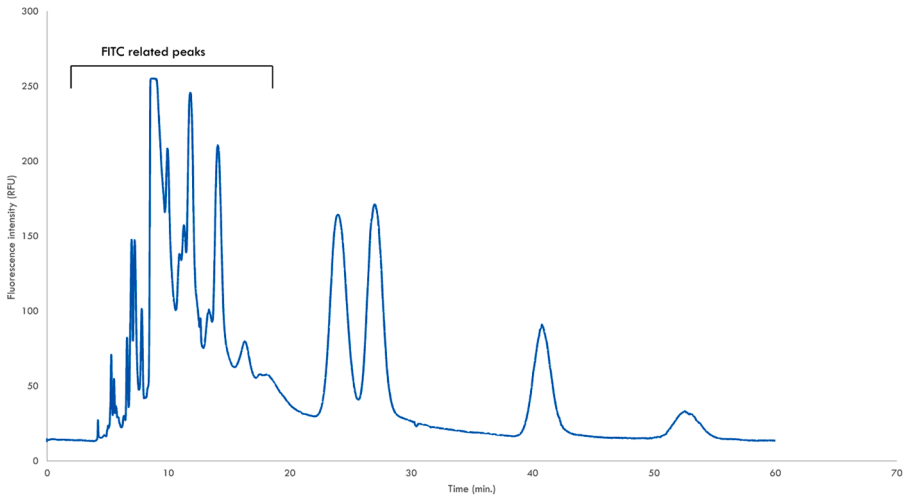

Fig. 5. Electropherograms using 25 mM borate buffer (pH 9.3) including 3 % MC and 2 mM C12E10 as BGE; Voltage: −1000 V; Sample: 100 nM AMP, Bk-MDMA, CAT, 4-CMC, βk-MBDP. FITC-related peaks were identified by means of blank FITC sample. No peak identification was made for the analytes.

sensitivity with LODs and LOQs in the nM range.

3.4. Perspectives for improving method selectivity

The use of a sieving matrix by adding a low concentration of polymers to the separation buffer offered interesting separation capability for developing a MCE method. However, this separation capability is counterbalanced by the short separation length. In this context, the combination of several separation mechanisms has been previously applied in MCE [35,36]. BGEs containing polymers and cyclodextrins, surfactants or additives have been successfully used to achieve difficult MCE separations. The interest of a neutral surfactant for separating cathinone and amphetamine derivatives has been already described in CE [25]. In this context, the potential of combining the two mechanisms (i.e. cellulose polymer and neutral surfactant) to increase the separation capability was evaluated. The combination of methylcellulose (3% w/v) and 2 mM C12E10 surfactant in 25 mM borate buffer was tested. The chip design incorporating two passive capillary valves performed well under these conditions, although the addition of surfactant can be a challenge for such valves. As shown in Fig. 5, this combination of additives increases the separation performance. The main drawback of this strategy is the increase in analysis time. These initial results offer interesting prospects for the development of MCE methods using an optimized chip design.

4. Conclusions

An innovative MCE prototype based on passive capillary valves has been developed. The proof-of-concept of the filling and injection mechanism of the passive channels was established for the chip design. Integrating the chip into a laboratory-built MCE-LIF instrumentation has enabled us to propose a prototype suitable for developing and evaluating analytical methods. The interest of using cellulosic polymers as a sieving matrix was demonstrated. In addition, the significant impact of the nature of the polymer on the selectivity and migration behavior of the analytes seems to offer promising flexibility for future method optimization. Interestingly, the benefit of combining separation mechanisms has been assessed. Finally, the quantitative performance of the MCE method was evaluated. The reproducibility of migration times was demonstrated, with RSD values below 4 % and even below 1 % for normalized migration times. The benefit of internal standardization was also demonstrated with RSD values of less than 10 % for normalized peak areas. These results demonstrate the value of MCE devices for pharmaceutical analysis.

CRediT authorship contribution statement

Amandine Dispas: Writing – original draft, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Paul Emonts:** Writing – original draft, Methodology, Investigation, Formal analysis. **Denis Vandormael:** Methodology, Funding acquisition, Conceptualization. **Damien Bernier:** Resources, Methodology. **Fabian Dortu:** Resources, Methodology. **Eric Ziemons:** Supervision. **Philippe Hubert:** Resources. **Jacques Crommen:** Writing – review & editing. **Marianne Fillet:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2024.100141.

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