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Strategies for rapid chiral analysis by capillary electrophoresis

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Abstract

The aim of this study was to investigate four strategies to decrease chiral CE analysis time: (1) short-end injection technique, (2) high electric field through a capillary length reduction, (3) external pressure application and (4) capillary dynamically coated to generate an important electroosmotic flow. These approaches were applied for a simultaneous enantiomeric separation of amphetamine and four related compounds using a neutral derivatised cyclodextrin (hydroxypropyl-β-cyclodextrin) as chiral selector. Analysis time and CE performances, in terms of peak efficiency and resolution, were examined. Among the investigated strategies, the dynamic coating procedure appeared to be the most suitable approach to decrease analysis time (inferior to 7 min) and improve sensitivity. Furthermore, it exhibited very good migration time repeatability (0.1%). This benefit is of utmost interest in chiral analysis for an unambiguous peak identification, especially for a complex mixture such as reported in this study.
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1. Introduction

In the pharmaceutical field, analysis of chiral drugs is of utmost importance given the pharmacological and toxicological differences existing between enantiomers. Capillary electrophoresis (CE) is recognized as a technique of choice for enantiomer separation thanks to its high efficiency, low sample and reagent consumption and versatility [1]. By adding a chiral selector to the background electrolyte (BGE), it is possible to modify enantiomer mobility by forming labile diastereoisomeric complexes. Among the great number of chiral selectors, cyclodextrins (CDs) are undoubtedly the most popular and widely used [2–4]. Nowadays, a wide range of CDs with several functional groups inducing different enantioselectivities are commercially available. They allow the enantiomer resolution of a wide range of pharmaceutical compounds with analysis times between 10 and 30 min [4,5]. Because of the continuously increasing number of new substances in drug discovery and development processes, there is an extended demand for simple, rapid, efficient and economical analytical methods. For this reason, different strategies have been developed with a special attention to time reduction.

In capillary electrophoresis, t_m is the migration time (s) of a given compound and is expressed by:

$$t_m = \frac{L_e L_t}{\mu_{app} U} \quad (1)$$

where L_t and L_e are the capillary total and effective lengths (cm), respectively, U is the applied voltage (V) and μ_{app} is the apparent mobility ($cm^2\,V^{-1}\,s^{-1}$) defined as the sum of the effective (μ_{eff}) and electroosmotic (μ_{EOF}) mobilities.

Eq. (1) exhibits different possibilities to decrease migration times, either by decreasing L_t or L_e, or by increasing U or μ_{app}. Therefore, several approaches have been reported to achieve a fast separation on commercially available instruments, such as increasing the electric field through a reduction of the total capillary length at a high applied voltage [6–11]

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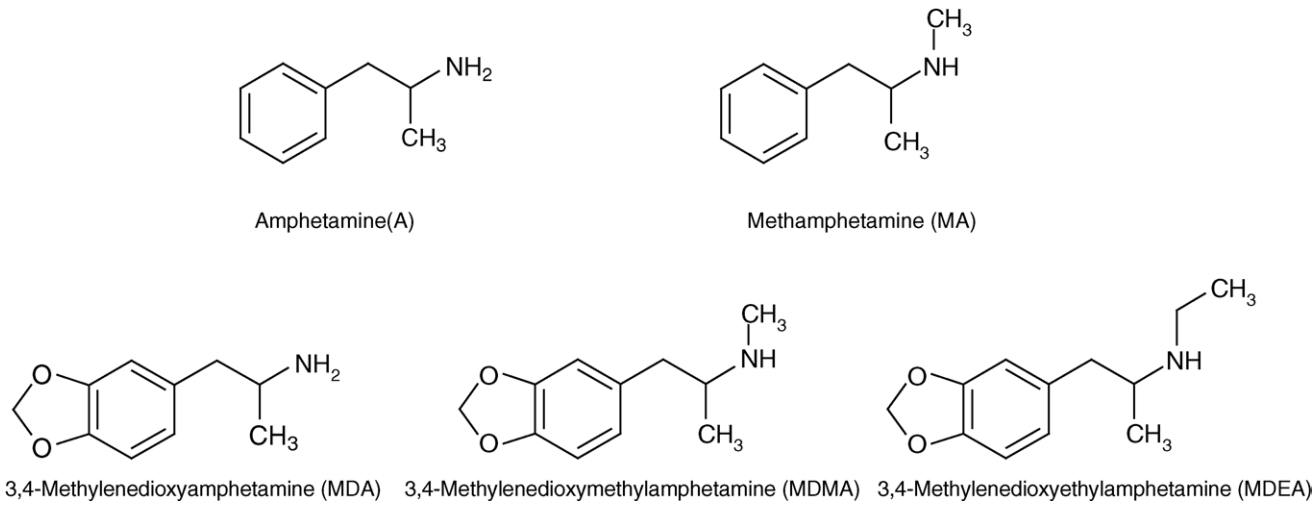

Fig. 1. Structures of amphetamine and related compounds.

or decreasing L_e by using the short-end injection technique [12–14]. More recently, another approach was developed which significantly increases μ_{app}, by increasing the electroosmotic mobility through a dynamical coating procedure of the capillary [15–18]. Finally, one can decrease analysis time by applying external pressure during the electrophoretic separation, often at the expense of efficiency [19]. This technique was successfully applied to reduce the running time of several CE analyses such as pK_a determination of pharmaceuticals [20] or protein-drug constant measurement [21].

Amphetamine and its analogues are substances which have a potent central nervous system stimulating effect. The amphetamine analogues most commonly consumed illegally in Europe are amphetamine (A) itself, methamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA) (Fig. 1). All of these compounds possess a chiral centre and it is well known that the *d*- and *l*-enantiomers exhibit different pharmacological activities. Hence, it is necessary to have at disposal an analytical procedure which separates all these enantiomers [22,23]. In this paper, the different strategies commonly used in achiral CE analysis to decrease analysis time were investigated for the simultaneous enantiomeric separation of amphetamine and four related compounds. The impact of the studied approaches on migration time, efficiency (*N*) and resolution (Rs) were discussed.

2. Materials and methods

2.1. Chemicals

The racemic amphetamine compounds, namely amphetamine (A), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) and 3,4-methylenedioxyethylamphetamine (MDEA), were purchased from Lipomed AG (Arlesheim, Switzerland). (2-hydroxy)Propyl-β-cyclodextrin was provided by Wacker-Chemie (Liestal, Switzerland). Tris(hydroxymethyl)-aminoethane (Tris) and 85% *ortho*-phosphoric acid were purchased from Sigma (Buchs, Switzerland). The Ceofix® pH 2.5 solutions were obtained from Analis (Namur, Belgium). Ultra-pure water was supplied by a Milli-Q RG unit from Millipore (Bedford, MA, USA).

2.2. Background electrolyte and sample preparation

The BGE was a 100 mM Tris–phosphate buffer set at pH 2.5. For this purpose, a solution of phosphoric acid 100 mM was prepared by an adequate dilution of the concentrated acid solution and a solution of Tris at 1 M was added to adjust the solution at pH 2.5.

A (2-hydroxy)propyl-β-cyclodextrin solution at 25 mg mL^{-1} was daily prepared by dissolving the corresponding amount of chiral selector in BGE.

The racemic amphetamines were stock solutions of 1 mg mL^{-1} in methanol stored at 4 °C. These solutions were diluted in water by a factor 50 to obtain an aqueous sample containing the five amphetamine compounds at 20 μg mL^{-1}.

2.3. Instrumentation and capillaries

CE experiments were carried out with a HP^{3D}CE system (Agilent, Waldbronn, Germany) equipped with a diode-array detector, an autosampler, a power supply able to deliver up to 30 kV and an external pressure system. A CE Chemstation (Agilent) was used for CE control, data acquisition and data handling. Separations were performed in bare fused silica capillaries from Polymicro (Phoenix, AZ, USA). Capillary dimensions were 50 and 375 μm for internal and external

diameters, respectively, with different total lengths, either 32.5 or 64.5 cm. All experiments were performed in the cathodic mode. The capillary was thermostated at 25 °C by a high velocity air stream, and a voltage of 30 kV was applied with a 12-s ramp (2500 V s^{-1}). The generated current was of 45 and 135 μA for the capillary of 64.5 and 32.5 cm, respectively. Samples were kept at ambient temperature in the autosampler and injected in hydrodynamic mode to fill approximately 1% of the effective capillary length. UV detection was carried out at 200 nm with a bandwidth (bw) of 10 nm and a reference signal set at 350 nm (bw 50 nm). The detector response time (Rt) was between 0.1 and 0.5 s and adapted in function of the tested conditions to obtain about 30–60 points per peak.

Before first use, fused silica (FS) capillaries were sequentially rinsed with methanol, NaOH 0.1 M, water, HCl 1 M, phosphoric acid 0.1 M and BGE for 1–5 min, depending on the capillary length (corresponding to about five rinsed capillary volumes). Prior to each sample injection, the capillary was rinsed by pressure (940 mbar) for 3 min with fresh BGE. When not in use, the capillary was rinsed with water and then dry stored. As the electrophoresis process alters the running buffer pH by electrolysis and subsequently changes migration times, the separation buffer was refreshed every four runs.

3. Results and discussion

The aim of this work was to carry out a rapid chiral separation of racemic drugs by capillary zone electrophoresis (CZE). For this purpose, a CZE method developed in our laboratory for the simultaneous enantiomeric analysis of amphetamine and four related compounds was selected [22] and different strategies were evaluated to reduce analysis time. By conventional method with a 64.5 cm capillary (L_e = 56 cm), the baseline separations of these enantiomers were achieved using (2-hydroxy)propyl-β-CD as chiral selector in a BGE made up of Tris–phosphate 100 mM set at pH 2.5. Samples were introduced by applying 50 mbar for 10 s (1% effective capillary length). Under these conditions, the running time and the generated current were 17.5 min and 45 μA, respectively. A typical electropherogram is reported in Fig. 2A.

The four tested approaches to decrease the run time were: (1) short-end injection technique, (2) high electric field through a capillary length reduction, (3) pressure application during analysis and (4) capillary dynamically coated to generate an important electroosmotic flow. The electropherograms are reported in Fig. 2 and CZE performances are summarized in Tables 1 and 2. Results in terms of migration time (t_m), efficiency (N), resolution (Rs) and signal-to-noise ratio (S/N), as well as experimental conditions are discussed in details for each strategy in the following sections.

Considering longitudinal diffusion as the only contribution to peak broadening, N and Rs were evaluated theoretically by:

$$N = \frac{\mu_{app} U}{2D} \frac{L_e}{L_t} \quad (2)$$

$$Rs = \frac{1}{4}\sqrt{\overline{N}} \frac{\Delta\mu}{\overline{\mu_{eff}} + \mu_{EOF}} \quad (3)$$

where D is the diffusion coefficient of the analyte (cm^2 s^{-1}), $\overline{N}$ and $\overline{\mu_{eff}}$ are the average values for efficiency and effective mobility, and $\Delta\mu$ is the mobility difference.

3.1. Short-end injection technique

The short-end injection technique was carried out by reducing the effective capillary length (L_e) by performing the injection on the detector side (L_e = 8.5 cm) instead of the conventional injection on its opposite side (L_e = 56 cm) with the same 64.5 cm capillary. With this technique, L_e is mainly dependent on the equipment at disposal. A sample volume corresponding to about 1% of the effective capillary length was injected (−15 mbar for 5 s).

A typical electropherogram is shown in Fig. 2B and CZE parameters are reported in Table 1. In these conditions, the running time was achieved in less than 3.5 min, i.e. five times less than for the conventional chiral analysis. However, enantiomeric resolutions inferior to 1.5 were recorded for four out of the five compounds due to low efficiencies with the effective capillary length reduction (Eqs. (2) and (3)). Nevertheless, considering the N/t_m ratio, no significant reduction was observed. Furthermore, the resolution for MDMA per time unit was increased, showing the high potential of short-end injection for easily separated compounds.

Although the injected amount was reduced, a good sensitivity was maintained because the longitudinal diffusion was minimized. Indeed, the signal-to-noise ratio values of the short-end injection technique were similar to those of the conventional method (Table 1). Thus, the short-end injection approach is particularly interesting for applications where the resolution is not critical, but should be avoided for difficult chiral separations.

3.2. High electric field

A second approach was carried out by applying a high electric field. For this purpose, the capillary length was reduced from 64.5 to 32.5 cm and the applied voltage of 30 kV was maintained. Thus, the electric field (E) was increased by a factor 2 (from 465 to 923 V cm^{-1}). To keep the capillary filling percentage of 1%, samples were injected by applying 25 mbar for 5 s.

The analysis of amphetamine and four related compound enantiomers was achieved in 2.7 min (Fig. 2C). According to Eq. (1), a time saving factor of about 4 was expected. However, a larger time decrease (factor 6.5) occurred because BGE viscosity was reduced due to a significant Joule effect (high electric current of 135 μA). Under these conditions,

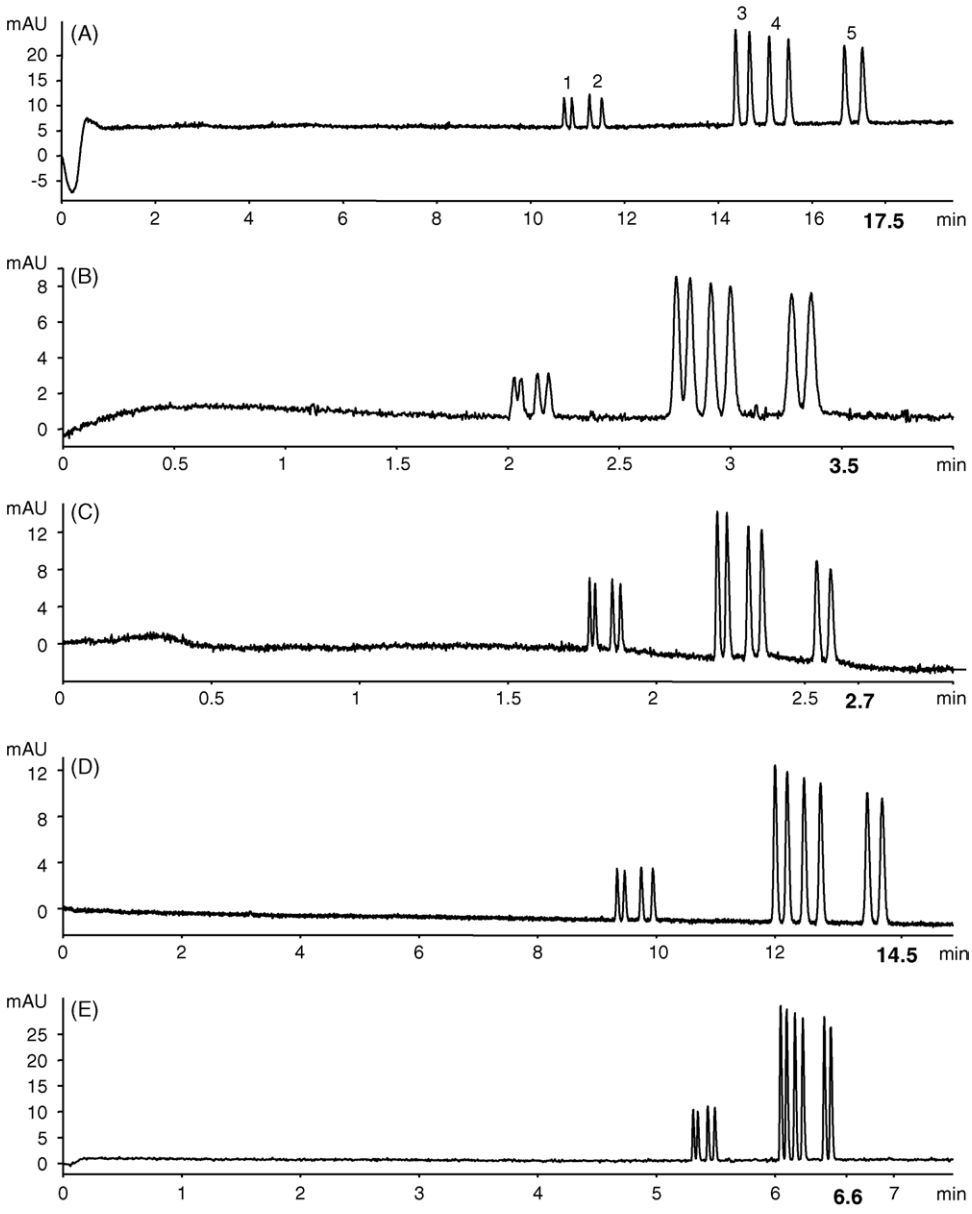

Fig. 2. Electropherograms of chiral analysis of A, MA, MDA, MDMA and MDEA enantiomers at 20 μg mL^{-1} by different approaches: (A) conventional mode; (B) short-end injection technique; (C) high electric field; (D) low pressure (10 mbar) application and (E) dynamic coating. Peaks are: 1, amphetamine; 2, methamphetamine; 3, MDA; 4, MDMA; 5, MDEA with the *l*-enantiomer always migrating first. Experimental conditions are described in text.

efficiencies were approximately constant as the diffusion coefficient increased simultaneously with μ_{app} (Eq. (2)). However, in terms of N/t_m ratio, these were the best results among all the investigated approaches. As reported by Eq. (3), an increase of μ_{app} induces a Rs reduction. Nevertheless, baseline resolution was achieved (except for amphetamine enantiomers with a Rs of 1.3) and the Rs/t_m ratio had the highest values, as reported in Table 1.

The high electric field technique was applied, but presented important drawbacks due to Joule effect. For example, capillary failure regularly occurred, and the repeatability of migration times was the worst, even if it remained acceptable (Table 2).

3.3. External pressure application

In order to evaluate the effect of external pressure on amphetamine chiral separation, three constant pressures were applied during the run: 10, 25 and 50 mbar. Analysis time decreased from 17.5, 14.5, 13 to 9 min for 0 (conventional chiral separation), 10, 25 and 50 mbar pressure was applied, respectively (data not shown). However, as expected [19] this time saving was to the detriment of efficiency and consequently of the enantiomeric resolution. Actually, the application of external pressure induced a parabolic flow profile due to the shear force on the capillary wall. Moreover, the signal-to-noise ratio was reduced with a high applied pressure.

Table 1
CE performances for enantioseparation of amphetamine and related compounds at 20 mg L^{-1} with conventional, short-end injection, high electric field, low pressure application and dynamic coating techniques

	Conventional mode						Short-end injection						High electric field					
	t_m (min)	N	N/t_m (s^{-1})	Rs	Rs/t_m (10^{-3} s^{-1})	S/N	t_m (min)	N	N/t_m (s^{-1})	Rs	Rs/t_m (10^{-3} s^{-1})	S/N	t_m (min)	N	N/t_m (s^{-1})	Rs	Rs/t_m (s^{-1})	S/N
A-1	10.77	300,000	464			5	2.04	41,000	332			4	1.76	277,000	2623			5
A-2	10.94	280,000	427	2.06	3.16	5	2.08	41,000	327	0.96	n.d.	4	1.78	216,000	2028	1.28	n.d.	5
MA-1	11.31	284,000	419			5	2.15	55,000	425			4	1.83	226,000	2052			6
MA-2	11.57	248,000	358	2.94	4.28	5	2.20	51,000	388	1.36	n.d.	4	1.86	226,000	2021	1.73	15.60	6
MDA-1	14.44	252,000	291			16	2.78	49,000	294			12	2.18	223,000	1701			13
MDA-2	14.74	240,000	271	2.52	2.88	16	2.85	45,000	261	1.34	n.d.	12	2.22	195,000	1467	1.71	183.00	13
MDMA-1	15.16	232,000	256			15	2.95	45,000	256			12	2.29	205,000	1493			12
MDMA-2	15.58	222,000	237	3.21	3.48	15	3.04	45,000	247	1.58	8.78	12	2.33	185,000	1324	2.13	15.4	12
MDEA-1	16.78	227,000	226			13	3.31	43,000	219			11	2.51	184,000	1249			9
MDEA-2	17.17	210,000	204	2.66	2.61	13	3.40	42,000	208	1.39	n.d.	11	2.56	175,000	1141	1.94	12.70	9

	Low pressure application (10 mbar)						Dynamic coating					
	t_m (min)	N	N/t_m (s^{-1})	Rs	Rs/t_m (10^{-3} s^{-1})	S/N	t_m (min)	N	N/t_m (s^{-1})	Rs	Rs/t_m (10^{-3} s^{-1})	S/N
A-1	8.97	292,000	543			4	5.31	590,000	1851			15
A-2	9.08	267,000	489	1.72	3.18	4	5.35	540,000	1684	1.42	n.d.	15
MA-1	9.35	258,000	461			5	5.43	547,000	1678			16
MA-2	9.53	236,000	413	2.42	4.27	5	5.49	515,000	1564	1.97	6.00	16
MDA-1	11.46	212,000	309			15	6.05	615,000	1694			49
MDA-2	11.65	199,000	285	1.87	2.70	15	6.10	576,000	1574	1.52	4.18	49
MDMA-1	11.92	173,000	241			14	6.17	600,000	1622			46
MDMA-2	12.18	181,000	248	2.34	3.24	14	6.23	581,000	1555	2.06	5.53	46
MDEA-1	12.94	182,000	234			12	6.41	633,000	1646			44
MDEA-2	13.17	166,000	210	1.89	2.41	12	6.47	603,000	1555	1.66	4.30	44

n.d.: not determined for Rs inferior to 1.5.

Table 2
Migration time repeatability obtained with strategies tested for the chiral separation of amphetamine and related compounds in term of coefficient of variation (CV) with $n = 10$

	Conventional mode (%)	Short-end injection (%)	High electric field (%)	Low pressure application (%)	Dynamic coating (%)
A-1	1.7	1.3	1.6	0.6	0.1
A-2	1.7	1.3	1.5	0.7	0.1
MA-1	1.7	1.3	1.6	0.7	0.1
MA-2	1.7	1.4	1.6	0.7	0.1
MDA-1	2.0	1.7	1.9	0.7	0.1
MDA-2	2.1	1.7	1.9	0.7	0.1
MDMA-1	2.1	1.7	1.9	0.8	0.1
MDMA-2	2.2	1.8	2.0	0.8	0.1
MDEA-1	2.4	1.9	2.0	0.8	0.2
MDEA-2	2.5	1.9	2.0	0.8	0.1

Thus, the low pressure of 10 mbar was a good compromise in terms of analysis time, resolution and sensitivity (Fig. 2D). Under these conditions, Rs were superior to 1.5. Other parameters were similar to those of conventional mode. However, migration time reduction was only of about 20%.

3.4. Dynamic coating procedure

Another approach to increase compound velocity was carried out by inducing a strong electroosmotic flow (EOF), which consequently increased μ_{app}. In fact, with the acidic BGE, no EOF was measured (within 1 h, acetone as marker), and μ_{app} of MDA-1 corresponded to 1.4×10^{-4} cm^2 V^{-1} s^{-1}. In order to increase EOF, a dynamic coating procedure was performed with a CEofix® solution kit. This procedure was applied in two steps. Firstly, a polycationic polymer (initiator) was introduced by pressure (930 mbar) for 1 min into the capillary (approximately 1 capillary volume) to form a stable coating with the silanol groups of the fused-silica surface. Secondly, a strong polyacidic polymer (accelerator) rinsed the capillary for 2 min (approximately 2 capillary volumes). It was associated with the initiator and therefore presented a negatively charged capillary surface, whatever the pH of the BGE [15,24]. In addition, this technique is also described as beneficial to stabilize migration times and prevent interactions between compounds and capillary walls [11,16,17]. More than 10 injections were carried out with one dynamic coating procedure. All other experimental conditions were similar to conventional methods. EOF mobility was of 2.2×10^{-4} cm^2 V^{-1} s^{-1} and μ_{app} of MDA-1 increased up to 3.3×10^{-4} cm^2 V^{-1} s^{-1}. Migration times were reduced by a factor 2.5, as illustrated in Fig. 2E and the analysis time decreased from 17.5 to 6.6 min.

As predicted by Eq. (2) and shown in Table 1, N increased by a factor 2, corresponding to the highest values among the investigated conditions. Furthermore, N/t_m ratio increased by a factor 4. It is noteworthy that both values remained almost constant for all the tested compounds whatever the time spent in the capillary. Secondary interaction with the capillary was reduced by dynamic coating. Moreover, a strong EOF improves efficiency in case of chiral separation when randomly CDs are used. Dynamic coating had no detrimental effects on the stereoselective interaction between the neutral derivatised CD and the cationic analytes. As a result, the signal-to-noise ratio increased by a factor 3 for the same injected amount, leading to the best sensitivity. Despite high N values, Rs were lower than those in conventional mode due to μ_{app} increase (Eq. (3)). However, chiral resolution remained superior to 1.5 for all the tested compounds (except for amphetamine) and Rs/t_m increased.

The dynamic coating procedure appeared to be the most suitable approach to decrease the running time of the chiral separation of amphetamine and related compound enantiomers. Another important benefit of this approach is that migration times are stabilized, as reported in Table 2. Indeed, coefficient of variation (CV, $n = 10$) of uncorrected t_m were 0.1% for each enantiomer, except for MDEA-1 (0.2%).

4. Concluding remarks

This paper investigated four CE approaches, generally used for achiral CE, to achieve a fast stereoselective separation of amphetamine and related compounds. All the studied methods exhibited migration time reductions at the cost of enantiomeric resolution decreases. Despite a sensitivity enhancement, short-end injection exhibited low efficiency and resolution due to the effective capillary length reduction. The shortest analysis time (2.7 min) was achieved with the high electric field procedure which main limitation is the important Joule effect. A low external pressure application could only reduce the analysis time by a factor of 1.2, keeping CE performances similar to the conventional mode. The most suitable strategy to speed-up the chiral analysis of amphetamine and related compounds was to use a dynamic coating procedure. Actually, the running time was reduced by a factor 2.5 and the analytical performances were excellent. Furthermore, dynamic coating exhibited sufficient

resolutions, as well as better sensitivity, and the most stable migration times compared to any other studied approach.

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