Journal of Chromatography B, 875 (2008) 248–253

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Enantioseparation of pharmaceutical compounds by multiplexed capillary electrophoresis using highly sulphated α-, β- and γ-cyclodextrins☆

Luis Saavedra[a], Beverly Nickerson[b], Ricardo E. Borjas[b], Frédéric Lynen[a], Pat Sandra[a,*]

[a] Pfizer Analytical Research Centre, Ghent University, Krijgslaan 281 S4-bis, B-9000, Belgium
[b] Pfizer Global Research & Development, 445 Eastern Point Road, Groton, CT 06340, USA

ARTICLE INFO

Article history:
Received 14 April 2008
Accepted 9 July 2008
Available online 25 July 2008

Dedicated to Prof. Wolfgang Lindner on the occasion of his 65th birthday.

Keywords:
Multichannel capillary electrophoresis
Chiral separations
Pharmaceuticals
High-throughput
System performance

ABSTRACT

A multiplexed capillary electrophoresis (CE) system equipped with 96 channels was evaluated for high-throughput screening of enantiomers of solutes of pharmaceutical interest. Using highly (DS ~ 12) sulphated α-, β- and γ-cyclodextrins under acidic conditions (pH 2.5) only 48 channels could be used because of the high conductivity of the chiral selectors. Method transfer from a single channel to a 48 channel CE system is described. Under optimised conditions, the analysis time on the multiplexed 48 channel CE system is ca. five to eight times the analysis time on the single channel CE system. The figures of merit for the multiplexed system are presented as well as performance evaluation including throughput and productivity gain. Intra-day precision ($n = 6$) ranged from 2.0 to 16.5% and from 2.2 to 15.5% for migration time and resolution, respectively. These values increased with ca. 10% for intermediate precision.

1. Introduction

Chiral purity is a major issue in the pharmaceutical industry. Nowadays regulatory agencies require the fully enantiomeric separation and quantification of any chiral drug prior to commercialization because enantiomers can produce different therapeutic and even adverse effects [1]. The pharmaceutical industry strives to produce effective, safe and high-quality medicines and in this research-based industry one of the main challenges is shortening discovery time in order to push new drug candidates into the development pipeline as soon as possible. High-throughput technologies that speed up drug discovery in elucidating promising leads became, in the 1990s, mainstays in drug discovery programs. Combinatorial techniques allow synthesizing in short times large numbers of libraries that need analytical control on identity and purity. As a consequence, the number of samples submitted by drug discovery scientists for analysis has significantly increased over recent years. On the other hand, in the 2000s, the goal of discovery has shifted from a sole pursuit of high-throughput towards performing high-quality and innovative compounds. Under this view, the biggest challenges to analysts are to minimize assay time and maximize analytical information by utilizing newer technologies and approaches in order to perform rapid analytical method development.

Multiplexed capillary electrophoresis (CE) was developed in response to the high-analytical throughput needs required for DNA sequencing of the Human Genome Project [2]. In 1999, Gong and Yeung introduced a 96-capillary array instrument equipped with UV detection suitable for a wider range of applications [3]. The performance of the system has been demonstrated in several applications including micellar electrokinetic chromatography (MEKC) [4], comprehensive peptide mapping [5], enantiomeric separations [6], enzyme activity [7], organic reaction monitoring [8] and DNA sequencing [9]. A multiplexed CE instrument consisting of a 96-capillary array and with UV absorbance detection became recently commercially available. Main applications fields in pharmaceutical analysis are log P determinations [10,11] and pK_a determinations [12]. Marsh and Altria reported their experience in a pharmaceutical environment with a multiplex CE system for log P and pK_a determinations [13]. Reports on the use for chiral separations are rather scarce [14].

Our experience with a multiplexed CE system for high-throughput chiral screening is reported. CE is a powerful technique for separating chiral compounds and complementary to HPLC and SFC. Cyclodextrins (CDs) are the principal chiral selectors in enantioselective capillary separation techniques and readers are

☆ This paper is part of the Special Issue 'Enantioseparations', dedicated to W. Lindner, edited by B. Chankvetadze and E. Francotte.

* Corresponding author. Tel.: +32 56 204031; fax: +32 56 204859.
E-mail address: pat.sandra@richrom.com (P. Sandra).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.07.020

referred to recent reviews articles for in-depth information [15–21]. Highly sulphated α-, β- and γ-cyclodextrins (HS-CDs) with degree of substitution ~12 were chosen as chiral selectors because they can be applied to a large variety of enantiomeric compounds of pharmaceutical interest [22–24] and moreover, they are commercially available. The use of HS-CDs in a screening approach is well documented in the literature [25–28]. Although the characterization of these chiral selectors has been documented by the manufacturer and a good understanding of the separation mechanism as well as the recognition pattern has been reported [29], a screening strategy is still necessary to ensure a maximum degree of success. Advantages and drawbacks of the multiplexed systems are discussed in terms of performance, figures of merit, throughput and productivity gain, and this in comparison with a single channel system. Perrin et al. [26] introduced short-end injection to drastically reduce the analysis time in a screening method based on HS-CDs. Unfortunately this cannot be done on the multiplex CE system and therefore conventional long-end injection was performed on the single channel system.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated all chemicals were from Sigma–Aldrich (St. Louis, MO, USA). *o*-Phosphoric acid was from Merck (Darmstadt, Germany), DMSO from Janssen (Geel, Belgium), HS-α-, HS-β- and HS-γ-CDs from Analis/Beckman (Namur, Belgium) and ultra pure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Samples and buffers preparation

Stock solutions of the racemates were prepared at 5 mg/mL in DMSO. Sample solutions at 250 μg/mL in water were freshly prepared daily from the stock solutions and kept at 4 °C in total darkness until utilization. 1,3,6,8-Pyrentetrasulphonate (PTS) was added as system performance marker at a concentration of 250 μg/mL in water. Quantitative elution of the marker reflects channel and injection quality.

The separation buffer was prepared according to the guidelines given by the manufacturer. Phosphate (PO_4)/triethylamine (TEA) buffer was prepared at a concentration of 50 mM by weighing the corresponding amount of phosphoric acid and raising the pH up to the desired value with TEA. Commercially available stock solutions of HS-CDs at 20% (w/v) were used. The final background electrolyte (BGE) consisted of 25 mM of PO_4/TEA pH 2.5 with 5% of chiral selector, made by mixing 2 parts of phosphate buffer pH 2.5, 1 part of water and 1 part of HS-CD solution at 20%. A 5% (w/v) of S-β-CD was prepared in the same way from the commercially available powder (Sigma–Aldrich).

2.3. Instrumentation

A multiplexed CE instrument from Advanced Analytical Technologies (Aimes, IO, USA) equipped with a 96-channel capillary array cartridge (CAC) of 55 cm length (33 cm effective) and 50 μm ID was used for all experiments. UV detection was performed at 214 nm. The electric filed was −100 V/cm (−5.5 kV of applied voltage). Injection of the samples was performed hydrodynamically at −0.5 psi for 10 s. Run time was set at 100 min. Data were collected with the cePRO data manager suite from Advanced Analytical Technologies.

Single CE experiments were performed on a Beckman MDQ system (Analis, Namur, Belgium). The capillary was 40 cm in length (30 cm effective) and 50 μm ID. UV detection was at 214 nm. The electric field was −500 V/cm (−20 kV of applied voltage). The injection was at 0.5 psi for 5 s and the run time was set at 20 min. Data were collected with the 32 Karat 7.0 software from Beckman.

3. Results and discussion

Although our understanding of the mechanisms of enantioseparation are continuously increasing, trial and error experiments still have to be carried out to chose the best chiral selector for a given racemate separation. Based on our experience and data published in the literature, e.g. [22–24,26], it is known that HS-γ-CD and HS-β-CD are by far the best chiral selectors for acidic and basic pharmaceuticals and should be the first CDs to be tried out. But because ultimately the enantioselectivity depends on every particular chiral selector–analyte pair, screening approaches with the three cavity sizes is needed to ensure the maximum degree of success. This is important because the highest resolution guarantees the lowest detection limits in enantiomeric excess determinations.

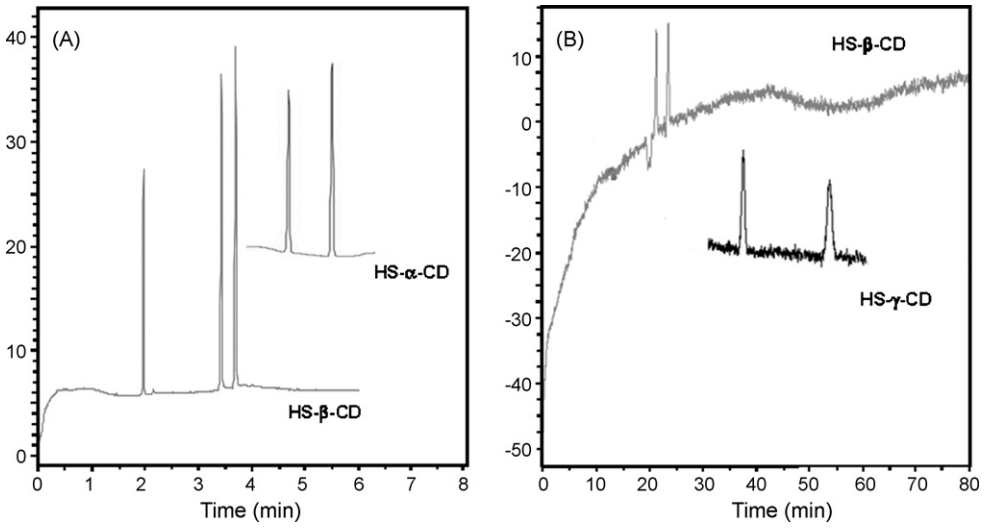

Fig. 1. Illustration of the enantioselectivity of HS-CDs: (A) verapamil in HS-β-CD 5% and HS-α-CD 5% analyzed on the single channel system, −20 kV (−500 V/cm), UV detection at 214 nm. (B) 3-phenylphtalide in HS-β-CD 5% and HS-γ-CD 5% analyzed on the multiplexed channel system, −5.5 kV (−100 V/cm), UV detection at 214 nm.

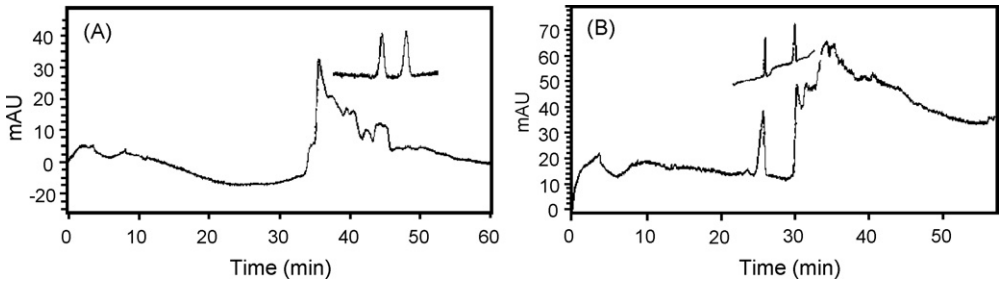

Fig. 2. Baseline stability due to the background electrolyte in the outlet vial: (A) 4-chloro-phenylalanine ethyl ester, inlet buffer HS-α-CD 5% in PO_4/TEA 25 mM pH 2.5; outlet buffer PO_4/TEA 25 mM pH 2.5. Overlaid, with outlet buffer containing 5% of S-β-CD. (B) Mepivacaine, inlet buffer HS-β-CD 5% in PO_4/TEA 25 mM pH 2.5; outlet buffer was PO_4/TEA 25 mM pH 2.5. Overlaid, with outlet buffer containing 5% of S-β-CD. Analyses at −5.5 kV (−100 V/cm). UV detection at 214 nm.

The complementary nature of the HS-CDs is illustrated in Fig. 1 for verapamil (Fig. 1A) analysed with HS-α-CD and HS-γ-CD on the single CE system and for 3-phenylphtalide (Fig. 1B) analysed with HS-β-CD and HS-γ-CD on the multiplex CE system.

It is therefore common practice in drug discovery to evaluate the α-, β- and γ-cyclodextrins. This means that in pre-screening experiments three analyses per sample have to be carried out. The use of multiplexed parallel separation techniques can contribute to reduce the total analysis time in the screening steps and later in enantiomeric excess determinations. An important aspect, however, is the translation of the generic conditions of a single channel CE analysis to a multi-array set-up. In the generic single capillary method, columns are typically 40 cm in length and the applied voltage is −20 kV (−500 V/cm) generating ca. 250 μA. The standard array cartridge is composed of 96 capillaries of 55 cm in length and the maximum allowable current for the whole system is 3 mA. This means that if all columns are used, each individual column should not produce more than 30 μA of current intensity. With sulphated CDs this is not realistic and therefore only 48 columns are used simultaneously (see further). The unused columns were filled with water during the rinsing step leaving the corresponding wells empty during the separation process. In addition, only −5.5 kV (−100 V/cm) voltage was applied because this generated ca. 2.4 mA. Because the electric field was reduced by a factor of 5 compared to the single capillary system the total run time was increased accordingly to 100 min. However, migration time shifts varied from 5 to 8+ fold depending on the degree of interaction with the cyclodextrins. For strong interactions (i.e. fast migration) the time shifts were close to fivefold, similar to the difference in the applied electric field. For weak interactions (i.e. slow migration) time shifts of eightfold were noted. Another important factor to be mentioned is the refrigeration efficiency of the system. Noticeable differences in the refrigeration efficiency of the multiplexed system have been measured compared to the single channel one [30]. It is well known that the temperature affects the interaction between cyclodextrins and analytes [31,32]. This non-linear behaviour makes method translation challenging in terms of analysis time. Solutes eluting on the single channel CE instrument in the 12–20 min range on a specific CD form, did not elute in 100 min on the multiplex system, e.g. indapamine on HS-α-CD, 2-phenoxypropionic acid on HS-β-CD and benzoin on HS-γ-CD. On the other hand, the obtained resolutions on the two systems were very similar (Table 1).

An important aspect is the buffer composition along the entire column as this may play an important role in the separation process. Due to the multiplex system configuration all the channels merge together into one reservoir at the outlet tip. This reservoir has to be filled up with buffer to close the electric circuit. In any CE screening set-up (chiral or achiral) that involves the use of different BGEs in the inlet, there will be a gradient throughout the capillary. When only PO_4/TEA buffer at pH 2.5 was used in the outlet reservoir baseline fluctuations and current instability were observed as a consequence of the strong difference of conductivity along the column. This is illustrated in Fig. 2 showing the analysis of the racemates of 4-chlorophenylalanine ethyl ester (Fig. 2A) using HS-α-CD and of mepivacaine (Fig. 2B) using HS-β-CD. The CDs were added in the inlet vial only and the buffer in the in- and outlet vials was PO_4/TEA buffer at pH 2.5. A hump in the baseline is observed after 30–35 min masking the solutes. The presence of this type of humps is documented in the HS-CD leaflet of Beckman (Beckman Instructions 725869-AB). By adding 5% (w/v) of S-β-CD (DS ∼ 4) to the outlet buffer, the conductivity was mostly equalized giving more stable baselines, cleaner peak shapes and a more stable current profile all along the run time. This is illustrated in Fig. 2A and B, showing the overlaid electropherograms with and without β-S-CD in the outlet vial. Note that the same effect was observed disregarding what type (α, β or γ) of CD is used in the separation capillary.

However, this procedure increases the cost per analysis due to the large amount of β-CD needed to fill the reservoir. This is the reason why the cheaper S-β-CD is used instead of the expensive HS-β-CD. Robustness is also an important aspect of the multiplex CE system and to avoid false negatives by injection problems, individual column performances, etc., it was decided to perform two analysis per sample. This reduced the throughput by a factor of two but increased the robustness drastically.

The filling of a well plate is illustrated in Fig. 3 and a typical data set, in this case for tryptophan butyl ester is shown in Fig. 4.

For this particular compound the resolution was the highest on α-CD (R_s, 4.2), followed by γ-CD (R_s, 2.8) and β-CD (R_s, 1.5).

Over the last year, hundreds of racemates were analysed on the multiplex CE system and 14.7% false negatives were detected. Most often false negatives were due to the fact that no signals were registered which is attributed to an injection problem or resolution was deteriorated which can be ascribed to insufficient conditioning of a particular column in the array. This stresses the need for two analyses per sample.

Table 1
Resolution on single channel and multiplex CE systems

Compound	HS-α-CD 5% Single/multiplex	HS-β-CD 5% Single/multiplex	HS-γ-CD 5% Single/multiplex
Mianserin	2.7/2.6	3.8/4.2	3.2/2.9
Ketoprofen	ND/ND	1.6/1.5	ND/ND
Alprenolol	2.9/2.3	2.6/2.4	2.2/2.7
Aminoglutethimide	1.6/1.4	2.8/3.6	1.8/1.1
Mepivacaine	2.4/2.0	2.7/2.5	1.2/1.2
Fluoxetine	4.9/4.6	1.9/2.0	11.2/9.1
Trihexyphenidyl	0.8/1.0	1.5/1.4	ND/ND
Troger's base	6.8/7.1	8.3/8.1	4.2/3.6

ND: not detected.

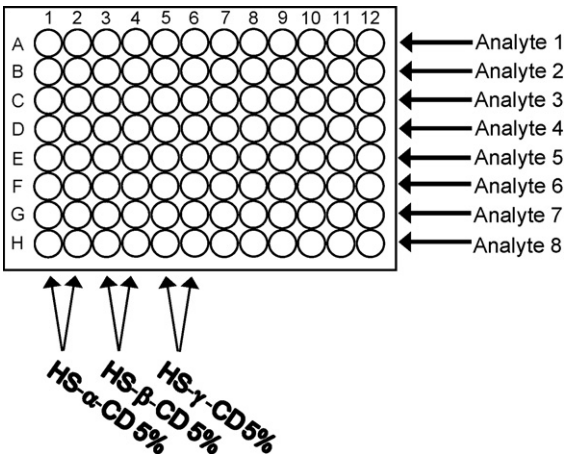

Fig. 3. Schematic representation of well plate for chiral screening. Each combination is analysed in duplicate. Compounds, from **1** to **8**, were: mianserin, ketoprofen, alprenolol, aminoglutethimide, mepivacaine, fluoxetine, trihehyphenidyl and Troger's base (see Fig. 5 for electropherograms).

Repeatability was studied by analysing the racemates shown in Fig. 5, six times (intra-day) and repeating the same sequence 10 days later (intermediate precision). The RSD% on migration time and resolution (R_s) are presented in Table 2. For most of the solutes the data are excellent for a high-throughput screening method but some outliers are noted both for migration time (17.8) and resolution (15.8).

Comparing productivity and throughput gain of the multiplex CE set-up over the single CE system gave the following results. Note, however, that as a rule of thumb, also two analysis per sample are performed on the single CE instrument and that conditioning and rinsing procedures on both systems are roughly done in the same time. For the analysis of 8 compounds (as illustrated in Fig. 5) 24 separation results (8 on 3 CDs) are obtained in 100 min. On the single CE instrument the generation of 8 data for one CD takes 320 min and for the 3 CDs 960 min. The throughput gain is thus a factor of 9.6. This gain is important but only correct for data needed within a working day, what is mostly the case in drug discovery. However, while on the single capillary system the analytical sequence can be programmed overnight with the only limitation the number of positions available in the sample tray, this cannot be performed with the present hardware of the multiplexed CE and every run needs to be set-up manually prior to analysis. If 24 h operation is

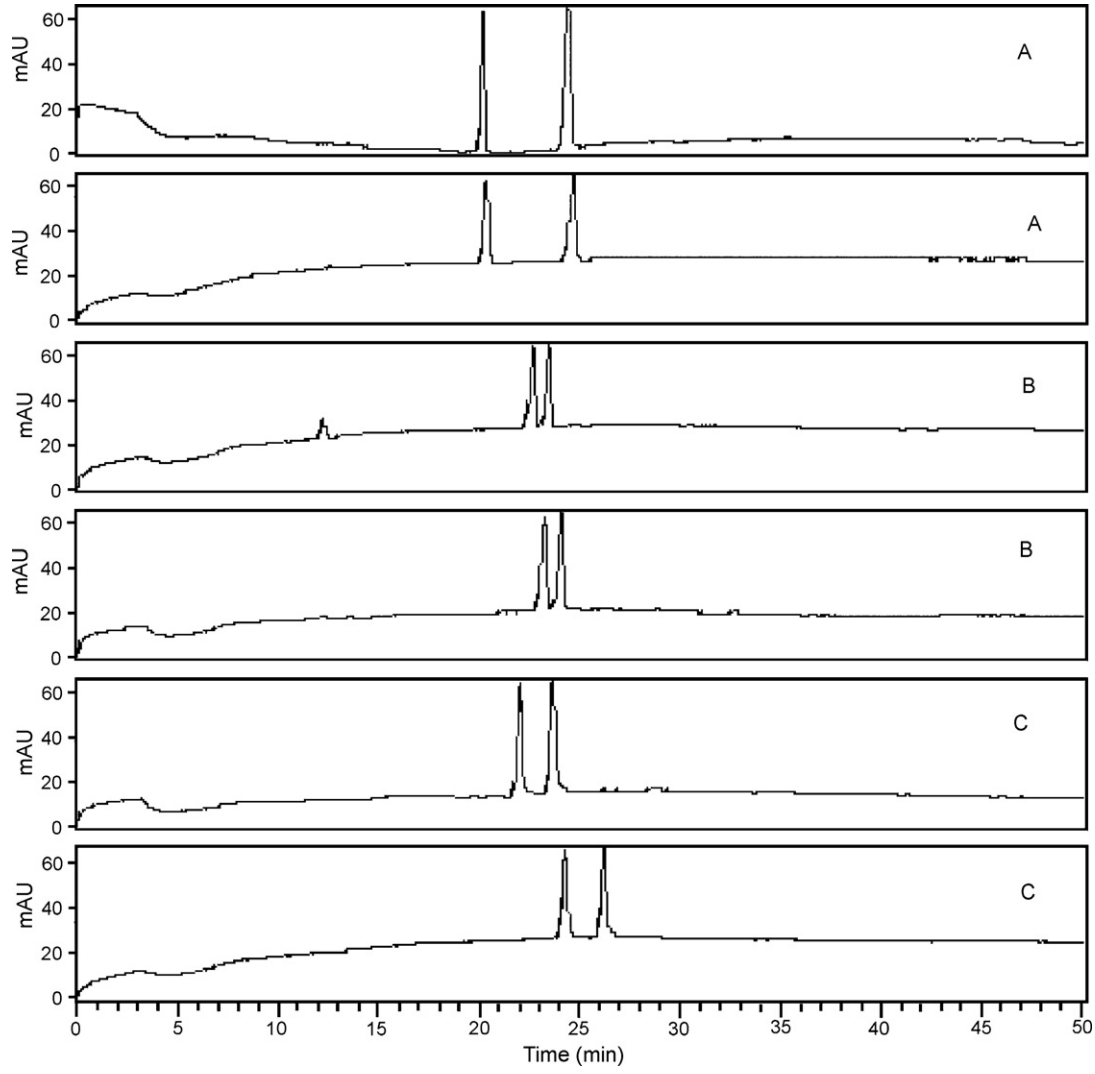

Fig. 4. Electropherograms of tryptophan butyl ester in (A) HS-α-CDs, (B) HS-β-CD and (C) HS-γ-CD 5%. Other conditions see Fig. 2.

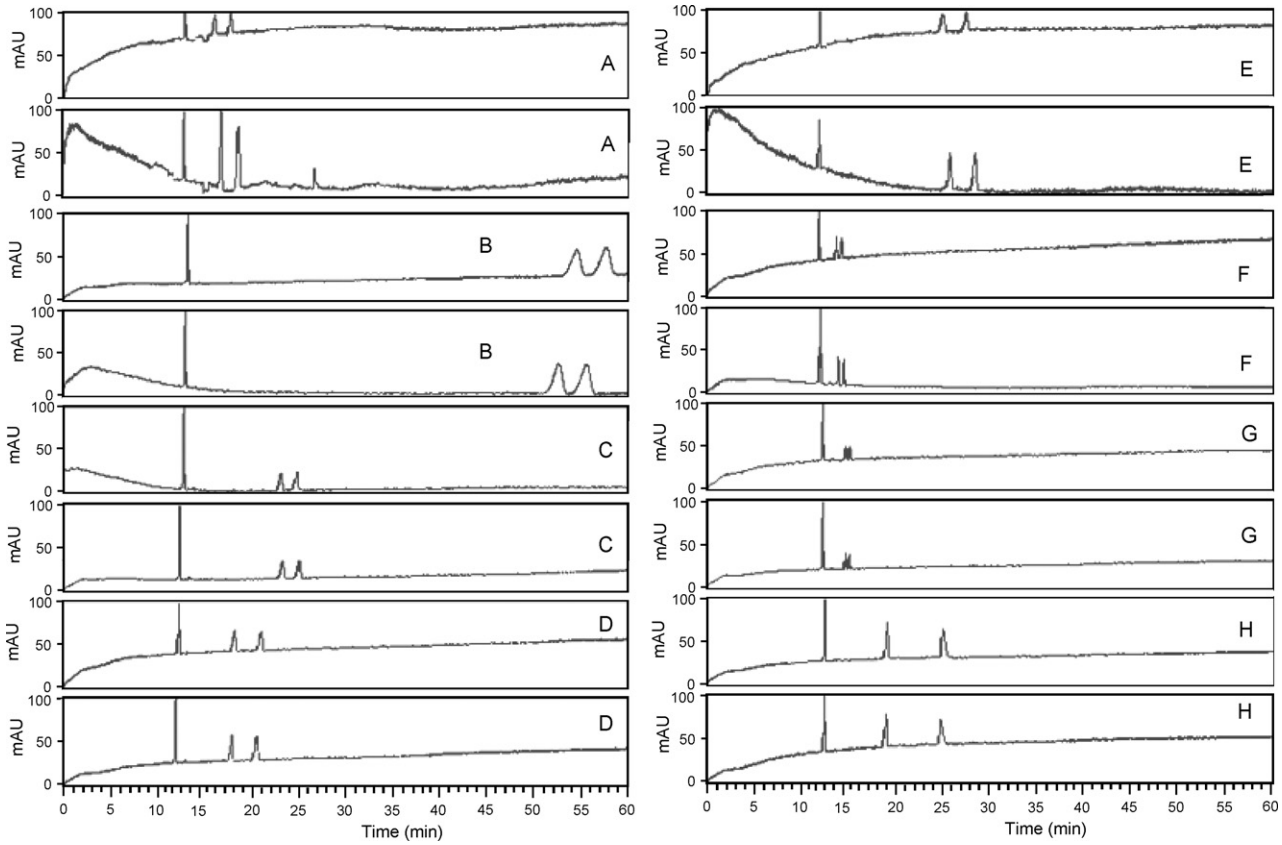

Fig. 5. Results for the separations on HS-β-CD in Fig. 3. (A) Mianserin, (B) ketoprofen, (C) alprenolol, (D) aminoglutethimide, (E) mepivacaine, (F) fluoxetine, (G) trihexyphenydil and (H) Troger's base. Buffer HS-β-CD 5% in PO_4/TEA 25 mM pH 2.5 buffer, outlet 5% of S-β-CD, −5.5 kV (−100 V/cm), UV detection at 214 nm.

important, the throughput gain of the multiplex system over the single capillary system drops to a factor of 3.2.

Concerning enantiomeric excess determinations, the multiplexed CE has proven to have enough sensitivity to determine enantiomeric excess up to 1:99 for resolutions of ca. 3. This is illustrated in Fig. 6 showing the determination of the enantiomeric excess of DL-tryptophan methyl ester at 5 (D):95 (L) and 1 (D):99 (L) ratios. For R_s-values larger than 5, 0.1% can easily be determined.

Table 2
Intra-day repeatability and intermediate precision (italic)

Compound	HS-α-CD 5%	HS-β-CD 5%	HS-γ-CD 5%
Mianserin	t_2 = 2.2% (3.5%) R_s = 2.9% (3.2%)	t_2 = 2.1% (2.5%) R_s = 4.2% (5.0%)	t_2 = 2.0% (3.1%) R_s = 3.6% (3.8%)
Ketoprofen	ND	t_2 = 5.3% (5.9%) R_s = 5.0% (5.6%)	ND
Alprenolol	t_2 = 2.5% (3.1%) R_s = 1.9% (2.2%)	t_2 = 2.3% (2.6%) R_s = 4.3% (4.9%)	t_2 = 4.0% (4.1%) R_s = 4.1% (4.6%)
Aminoglutethimide	t_2 = 8.8% (10.1%) R_s = 4.1% (4.5%)	t_2 = 7.4% (7.9%) R_s = 3.4% (4.3%)	t_2 = 4.5% (4.7%) R_s = 2.6% (3.8%)
Mepivacaine	t_2 = 3.9% (4.5%) R_s = 5.9% (6.1%)	t_2 = 2.4% (2.7%) R_s = 3.1% (3.4%)	t_2 = 2.8% (3.3%) R_s = 2.9% (3.5%)
Fluoxetine	t_2 = 2.0% (2.2%) R_s = 15.4% (18.1%)	t_2 = 2.7% (3.3%) R_s = 2.5% (2.9%)	t_2 = 16.5% (17.8%) R_s = 13.9% (15.5%)
Trihexyphenidyl	t_2 = 2.3% (2.5%) R_s = 15.5% (15.8%)	t_2 = 3.0% (3.3%) R_s = 6.9% (7.5%)	ND
Troger's base	t_2 = 1.8% (2.0%) R_s = 4.8% (6.3%)	t_2 = 2.1% (2.6%) R_s = 11.1% (12.0%)	t_2 = 2.9% (3.3%) R_s = 11.7% (12.2%)

ND: not detected.

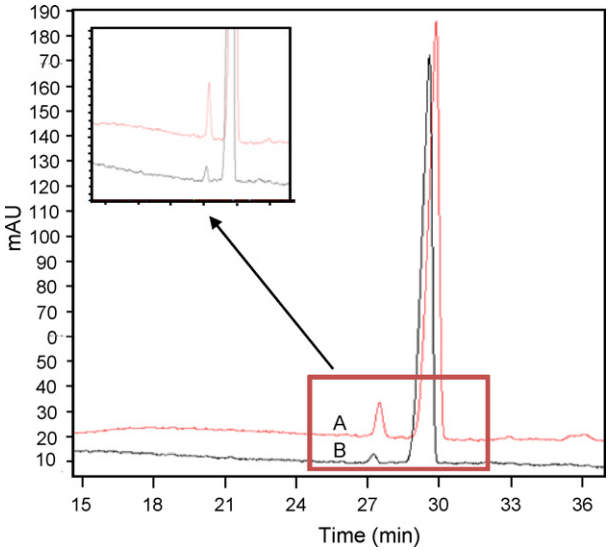

Fig. 6. Enantiomeric excess determination for tryptophan methyl ester at 5 (A) and 1 (B) % ratio. Conditions see Fig. 5.

4. Conclusions

A multiplexed CE instrument has been assessed for high-throughput chiral screening of pharmaceutical compounds. Because of voltage restrictions, only 48 channels out of 98 can be used due to the high conductivity of the chiral selectors. For within day operation, the throughput is increased 9.6-fold compared to a single channel instrument. For 24 h operation this drops to 3.2

because the multiplex CE instrument cannot be programmed for overnight operation. Intra-day repeatability and intermediate precision was acceptable for chiral screening purposes.

References

[1] S.K. Branch, in: G. Subramanian (Ed.), Chiral Separation Techniques: A Practical Approach, Wiley–VCH, Weinheim, 2001, p. 319.
[2] F.S. Collins, A. Patrinos, E. Jordan, A. Chakravarti, R. Gesteland, L. Walters, E. Fearon, L. Hartwelt, C.H. Langley, R.A. Mathies, M. Olson, A.J. Pawson, T. Pollard, A. Williamson, B. Wold, K. Buetow, E. Branscomb, M. Capecchi, G. Church, H. Garner, R.A. Gibbs, T. Hawkins, K. Hodgson, M. Knotek, M. Meisler, G.M. Rubin, L.M. Smith, R.F. Smith, M. Westerfield, E.W. Clayton, N.L. Fisher, C.E. Lerman, J.D. McInerney, W. Nebo, N. Press, D. Valle, D. Grp, N. Grp, Science 282 (1998) 682.
[3] X.Y. Gong, E.S. Yeung, Anal. Chem. 71 (1999) 4989.
[4] G. Xue, H.M. Pang, E.S. Yeung, Anal. Chem. 71 (1999) 2642.
[5] S.H. Kang, X.Y. Gong, E.S. Yeung, Anal. Chem. 72 (2000) 3014.
[6] W.W. Zhong, E.S. Yeung, Electrophoresis 23 (2002) 2996.
[7] L.J. Ma, X.Y. Gong, E.S. Yeung, Anal. Chem. 72 (2000) 3383.
[8] Y.H. Zhang, X.Y. Gong, H.M. Zhang, R.C. Larock, E.S. Yeung, J. Comb. Chem. 2 (2000) 450.
[9] W.W. Zhong, E.S. Yeung, J. Chromatogr. A 960 (2002) 229.
[10] K.R. Wehmeyer, J. Tu, Y.K. Jin, S. King, M. Stella, D.T. Stanton, J. Kenseth, K.S. Wong, LC GC North Am. (2004) 90.
[11] K.S. Wong, J. Kenseth, R. Strasburg, J. Pharm. Sci. 93 (2004) 916.
[12] C.X. Zhou, Y.K. Jin, J.R. Kenseth, M. Stella, K.R. Wehmeyer, W.R. Heineman, J. Pharm. Sci. 94 (2005) 576.
[13] A. Marsh, K. Altria, Chromatographia 64 (2006) 327.
[14] J. Kenseth, A. Bastin, LC GC North Am. 22 (2004) S28.
[15] K. Altria, A. Marsh, C. Sânger-van de Gried, Electrophoresis 27 (2006) 2263.
[16] A. Van Eeckhaut, Y. Michotte, Electrophoresis 27 (2006) 2880.
[17] B. Chankvetadze, J. Chromatogr. A 1168 (2007) 45.
[18] G. Gübitz, M.G. Schmid, Electrophoresis 28 (2007) 114.
[19] K.A. Kahle, J.P. Foley, Electrophoresis 28 (2007) 2503.
[20] G.K.E. Scriba, in: G. Subramanian (Ed.), Chiral Separation Techniques, 3rd ed., Wiley–VCH, Weinheim, 2007, p. 333.
[21] G.K.E. Scriba, J. Sep. Sci. 31 (2008) 1991.
[22] K. Verleysen, S. Sabah, G. Scriba, A. Chen, P. Sandra, J. Chromatogr. A 824 (1998) 91.
[23] K. Verleysen, T. Van den Bosch, P. Sandra, Electrophoresis 20 (1999) 2650.
[24] A. Fu-Tai, R.A. Evangelista, J. Chin. Chem. Soc. 46 (1999) 847.
[25] J. Chapman, F.-T.A. Chen, LC GC North Am. 2001 (2001) 4.
[26] C. Perrin, Y. Vander Heyden, M. Maftouh, D.L. Massart, Electrophoresis 22 (2001) 3203.
[27] N. Matthijs, C. Perrin, M. Maftouh, D.L. Massart, Y. Vander Heyden, J. Pharm. Biomed. Anal. 27 (2002) 515.
[28] F.-T.A. Chen, G. Shen, R.A. Evangelista, J. Chromatogr. A 924 (2001) 523.
[29] F.-T.A. Chen, R.A. Evangelista, Am. Lab. 34 (2002) 32.
[30] L. Saavedra, T. Bo, B. Nickerson, R.E. Borjas, P. Sandra, Electrophoresis (submitted for publication).
[31] K.M. Balss, W.N. Vreeland, K.W. Phinney, D. Ross, Anal. Chem. 76 (2004) 7243.
[32] X.P. Li, V.L. McGuffin, J. Liq. Chromatogr. Relat. Technol. 30 (2007) 965.