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Rapid chiral on-chip separation with simplified amperometric detection

Maria A. Schwarz, Peter C. Hauser*

The University of Basel, Department of Chemistry, Spitalstrasse 51, 4056 Basel, Switzerland

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Abstract

The enantiomers of adrenaline, noradrenaline, ephedrine and pseudoephedrine were separated by capillary electrophoresis on a micromachined device. Detection was carried out with a new two-electrode amperometric detector, eliminating the need for individual counter and reference electrodes. Separation of the isomers was achieved by employing carboxymethyl-β-cyclodextrin as chiral selector in the buffer, partly with the additional inclusion of the crown ether 18-crown-6. Plate numbers of up to 20,000, chiral resolutions of 2.5 and detection limits of the order of 10^{-7} *M* were achieved. All separations were completed in less then 3 min.

Keywords: On-chip separation; Enantiomer separation; Detection, electrophoresis; Adrenaline; Noradrenaline; Ephedrine; Pseudoephedrine

1. Introduction

The importance of the chiral analysis of natural and artificial β-phenylethylamine derivatives, such as adrenaline, noradrenaline, ephedrine and pseudoephedrine and others, in biological fluids and pharmaceutical preparations is well recognised as only the L-enantiomers show a physiological effect. HPLC is commonly used in chiral separations, but often suffers from poor selectivity, efficiency and long analysis times [1,2]. Capillary electrophoresis has also been successfully applied and has the advantages of faster analysis times and lower reagent consumption [2,3]. Generally, a drastic further reduction in analysis times can often be achieved by employing electrophoretic separation on micromachined separation devices [4–7]. Short analysis times for these compounds are highly desirable given the large number of samples which need to be processed in clinical analysis and the pharmaceutical industry.

Enantiomeric separations in capillary electrophoresis have been achieved by inclusion of a chiral selector in the background buffer. The separation is based on the formation of diastereoisomeric complexes between enantiomers and the complexing chiral agent. Besides chiral crown ethers, proteins and oligosaccharides, mainly native and modified α-, β-, or γ-cyclodextrins (CDs) have been employed. These compounds are cyclic oligosaccharides with the shape of a truncated cone and contain a hydrophobic cavity. The formation of inclusion complexes is governed by hydrophobic interaction in the cavity

*Corresponding author. Tel.: +41-6-1267-1003; fax: +41-6-1267-1013.

E-mail address: peter.hauser@unibas.ch (P.C. Hauser).

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and the formation of hydrogen bonds between hydroxyl or other groups (e.g. carboxymethyl, hydroxypropyl) on the rim of the cyclodextrin and substituents of the chiral centre. Examples in the literature also demonstrate the utility of using anionic cyclodextrin derivatives such as carboxymethyl-CD [8,9] or sulfated CDs [10,11], in particular for the separation of neutral or cationic analytes. A recent review on the use of cyclodextrins in capillary electrophoresis is available [12].

Enantiomeric separation on micromachined electrophoretic devices has been reported previously [13–15]. Both Hutt et al. [13] and Rodriguez et al. [14] have reported the chiral separation of individual amino acids in 2–4 min employing γ-cyclodextrin [14]. The amino acids had been derivatized with fluorescein isothiocyanate in order to render the analytes detectable by fluorescence. Wallenborg et al. have reported the chiral separation of amphetamine and related compounds (including ephedrine and pseudoephedrine, which were also investigated in this study) in 5–6 min by addition of a sulfated cyclodextrin to the background electrolyte solution [15]. Also in this case fluorescence detection was employed and the analytes had to be derivatized prior to injection into the separation device.

For conventional capillary electrophoresis, UV-absorption spectrometry is the dominant detection technique even though the detection sensitivity is limited because of the short optical pathlengths. For micromachined devices the pathlength is even shorter and absorbance detection has rarely been implemented, presumably also because of geometrical constraints. Commonly the more sensitive fluorescence technique is adopted for on-chip detection. However, as most species are not fluorescent, derivatization has to be employed. This is not possible for all species and in order to develop alternatives, a number of research groups have recently investigated amperometric detection for micromachined separation devices [16–23]. Amperometric detection is based on the oxidation (or in some cases reduction) of the analyte at a working electrode. The method is simple and has good detection limits, although the sensitivity of fluorescence measurements cannot be matched. Electrochemical detection methods are often considered incompatible with the high voltages applied for electrophoretic separation. With properly designed detector cells this is, however, not a problem.

A simplified amperometric detector arrangement, which consists of the working electrode and the electrophoretic ground electrode only, was recently reported by our group for conventional [24] and chip-based [23] electrophoretic separations. The electrophoretic ground electrode was found to provide a sufficiently stable potential to be used as pseudo-reference electrode. This allows the elimination of conventional reference electrodes, which are based on a flowing liquid junction and represent an obstacle in the overall miniaturization of the instrumentation. A simple electronic circuit is used for signal transduction rather than a conventional potentiostat. The application of this arrangement to the direct detection of the enantiomers of underivatized adrenaline, noradrenaline, ephedrine and pseudoephedrine, separated in the presence of cyclodextrins, is presented herein.

2. Experimental section

2.1. Apparatus

The glass microchip containing a manifold in the standard elongated cross configuration was purchased from Micralyne (model MC-BF4-TT100, Edmonton, Canada). The total length of the separation channel is 85 mm with a semicircular cross-section of 50 μm width and 20 μm depth. The joint formed by the injection and separation channels has the shape of a double T. At the detection end, the channel was widened by us to a diameter of about 130 μm by etching with 40% hydrofluoric acid in order to allow better access for the detector electrode. Pipette tips, serving as buffer reservoirs, were glued to the top of the chip with an epoxy adhesive (Epo-Tek OG 116, Polyscience, Baar, Switzerland). The modified chip was then mounted on an inverse microscope (model DM IL, Leica, Basel, Switzerland) equipped with an XYZ-micromanipulator as a working electrode holder. This electrode was connected to a laboratory-built amperometric detector circuitry, which allows the setting of the potential at the working electrode and converts the measured current into a voltage for data-acquisition [24].

Injection and separation voltages were produced by two high voltage power supplies (Model CZE1000R, Start-Spellman Ltd., Pulborough, UK), which were controlled by a purpose built interface connected to a multipurpose I/O-card (Model PCI-MIO-16XE-50, National Instruments, Austin, TX) located in a personal computer (Power-Macintosh 7300, Apple, Cupertino, CA). All parameters were set, and data acquisition was performed, with a program written in LabVIEW (National Instruments).

2.2. Working electrodes

A Teflon-coated platinum wire (platinum core diameter: 50 μm, outer diameter including Teflon coating: 75 μm; available from Advent Research Materials Ltd., Oxford, UK) was employed for detection. This electrode was gold plated by dipping the end for 10 min into a solution containing 20 m*M* AuCN, 23 m*M* KCN, 115 m*M* K_2HPO_4 and 110 m*M* KH_2PO_4 under application of a deposition current of 1.5 μA. The working electrodes were freshly prepared before every series of measurement.

2.3. Reagents and methods

The following reagents were obtained from Fluka (Buchs, Switzerland): 3-hydroxytyramine hydrochloride (dopamine hydrochloride, D), D,L-epinephrine hydrochloride (adrenaline, A), D,L-norepinephrine hydrochloride (noradrenaline, NA), D,L-ephedrine hydrochloride (E), D,L-pseudoephedrine hydrochloride (PE), 2-(4-morpholino)ethanesulfonic acid hydrate (MES hydrate), carboxymethyl-β-cyclodextrin (CM-β-CD), (2-hydroxypropyl)-γ-cyclodextrin (HP-γ-CD), methyl-β-cyclodextrin (M-β-CD), sulfated β-cyclodextrin (S-β-CD), 18-crown-6, potassium dihydrogen phosphate, sodium hydroxide, 2-(*N*-morpholino)ethanesulfonic acid (MES). All reagents were of analytical grade. The standard mixtures were prepared by dissolving the substances in deionized water. The separation channels were preconditioned daily for 3 min with a 1 *M* NaOH-solution and before every run for 3 min with the appropriate running buffer. Fresh sample and buffer solutions were prepared daily. The detection potentials for all measurements were optimized by acquiring hydrodynamic voltammograms. All values are quoted with respect to the electrophoretic ground electrode.

3. Results and discussion

For the chiral separation of catecholamines in capillary electrophoresis the use of different derivatives of β-cyclodextrin has been reported (carboxymethyl-β-cyclodextrin and heptakis-2,6-di-*O*-methyl-β-cyclodextrin [9], dimethyl-β-cyclodextrin [25], sulfated β-cyclodextrin [11]). In our study carboxymethyl-β-cyclodextrin (CM-β-CD) was mainly used and the buffer composition was optimized for best resolution and sensitivity of the amperometric detection mode. Chiral separations are often carried out in the acidic pH-range in order to minimize the electroosmotic flow. However, because the oxidation reaction of catecholamines is pH-dependent and therefore for amperometric detection higher sensitivity is obtained at higher pH-values, first separations were carried out at neutral pH-value. The normal electrophoretic polarity was employed with the injection end at positive voltage and the detection end at ground.

The chiral separation of adrenaline and noradrenaline are illustrated by the electropherograms of Fig. 1. For the separation of the catecholamines only small concentrations of CM-β-CD were required. At a concentration of 3.9 mg ml^{-1} CM-β-CD adrenaline could be completely separated (Fig. 1a); a baseline resolution of noradrenaline was achieved by using a concentration of 6.5 mg ml^{-1} CM-β-CD (Fig. 1b). Note the inclusion of the nonchiral dopamine in the electropherograms for adrenaline. As can be seen, there is an effect of the cyclodextrin concentration on the mobility, the resolution and the detector sensitivity. Better resolution is obtained at higher concentrations of the additive, in both cases, presumably because of a higher degree of complexation. The optimum concentration of cyclodextrins in chiral separations depends on both the selectivity and the stability of the host–guest complex formed. These aspects have been discussed in detail by Wren et al. [26]. It can also be seen that in the case of noradrenaline for concentrations of the cyclodextrin above about 5 mg ml^{-1}, the amperometric signal decreased significantly. This effect

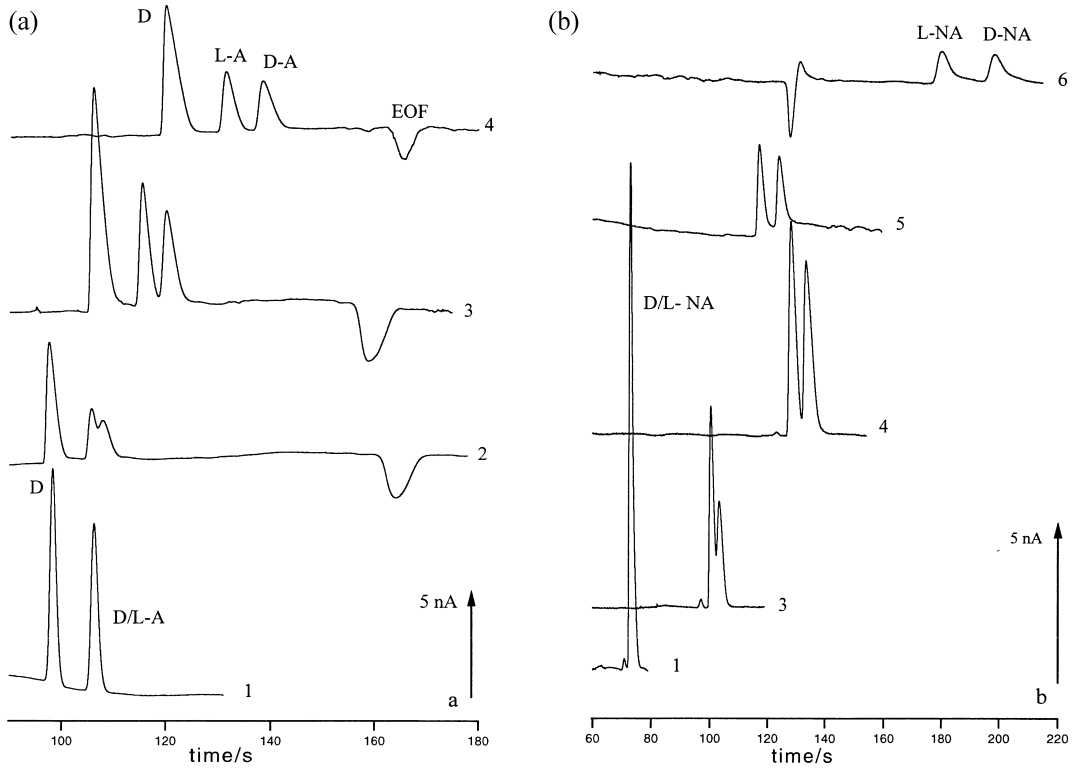

Fig. 1. Effect of CM-β-CD on the enantiomeric separation of (a) adrenaline and (b) noradrenaline on the microchip. Buffer: 50 m*M* MES, 20 m*M* phosphate, pH = 6.7. (1) 0, (2) 1.3 mg ml^{-1}, (3) 2.6 mg ml^{-1}, (4) 3.9 mg ml^{-1}, (5) 6.5 mg ml^{-1}, (6) 16 mg ml^{-1} CM-β-CD. Separation voltage, 2 kV; detection potential, 1200 mV; injection, 1 kV (2 s); concentration of standards, 10^{-4} *M*.

was previously reported for amperometric detection in conventional capillary electophoresis employing cyclodextrins by Fang et al. [27]. Presumably the complexed fraction of the analyte is not electroactive and the complex is highly stable. Plate numbers for D- and L-adrenaline were found to be 19,000 and 17,000 plates/m, respectively. The detection limits ($3 \times S/N$) for adrenaline were determined as 2.9×10^{-7} *M* and the chiral selectivity (L/D) as 5.25 at 3 mg ml^{-1} CM-β-CD.

Ephedrine and pseudoephedrine have, to our knowledge, so far only been detected with optical means in capillary electrophoresis. Direct amperometric detection of these substances is not considered readily possible and for this reason Cookeas et al. have recently described the use of a carbon paste electrode modified with cobalt phthalocyanine as an electrocatalyst for the determination in flow-injection analysis [28]. In this study, the direct electrochemical oxidation of the ephedrine and pseudoephedrine was achieved at a gold electrode at high pH-value. The results are given in Fig. 2. For pseudoephedrine, baseline resolution could be obtained for the two enantiomers (Fig. 2a), while for ephedrine a complete separation could not be achieved for the conditions studied (Fig. 2b). Also in this case, it was found that the sensitivity is influenced by the concentration of CM-β-CD. At concentrations above 20 mg ml^{-1}, only very small signals are obtained and it is therefore not possible to improve the separation by increasing the concentration of the cyclodextrin. The use of a neutral cyclodextrin, as often used in the chiral separation of these species [29–31], is also not possible as ephedrine and pseudoephedrine are also neutral at the high pH-value used for detection and therefore the complexes are not separable by electrophoresis under these conditions.

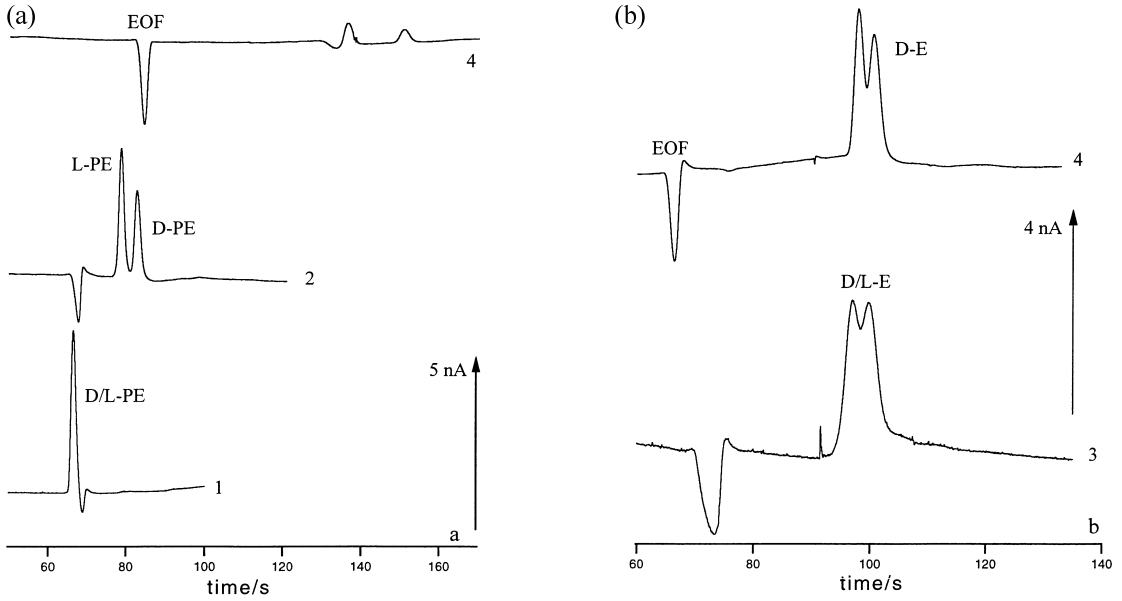

Fig. 2. Effect of CM-β-CD on the enatiomeric separation of (a) pseudoephedrine and (b) ephedrine. Buffer: 100 m*M* NaOH, pH = 12.9. (1) 0 mg ml^{-1}, (2) 3.9 mg ml^{-1}, (3) 10 mg ml^{-1}, (4) 16 mg ml^{-1} CM-β-CD. Separation voltage, 3 kV; detection potential, 1300 mV; injection, 1 kV (2 s); concentration of standards, 2×10^{-4} *M*.

As has been demonstrated above, rapid baseline separation of the optical isomers of adrenaline and noradrenaline could be achieved. However, when the isomeric mixtures of both of these compounds were present concurrently it was not possible to find conditions with respect to the concentration of CM-β-CD (varied between 3.9 and 6.5 mg ml^{-1}) at which all four species could be separated. In a simultaneous run, the L-form of adrenaline and the D-enantiomer of noradrenaline always coeluted (not shown). A further improvement in the already good separation was therefore required for complete resolution of all species.

A different host–guest complexation-based separation method employed in capillary electrophoresis is the use of crown ethers. Kuhn et al. demonstrated that a synergistic effect can be obtained by combining a crown ether with a cyclodextrin in the running buffer in capillary electrophoresis [32]. Huang et al. have expanded on this approach and postulated the formation of sandwich complexes of the form CE|A|CD (CE, crown ether; A, analyte; CD, cyclodextrin) [33,34].

The separation of the enantiomers of noradrenaline in the presence of 18-crown-6 is illustrated in Fig. 3. The crown ether on its own has a small effect on the migration, but on its own cannot lead to a chiral separation (Fig. 3b). In combination with the carboxymethylated cyclodextrin (Fig. 3c), a chiral separation which is better then the one reported in Fig. 1b is obtained. The resolution factors (*R*) were determined as 1.30 and 1.35 (5.2 mg ml^{-1} CM-β-CD, pH = 6.7) with and without the addition of 12 m*M* 18-crown-6, respectively. For adrenaline a similar, but more pronounced, improvement was obtained with the respective resolution factors determined as 1.66 and 1.99.

A further increase in the chiral separation could be obtained by optimizing the pH-value, as illustrated in the electropherogram given in Fig. 3d. For a buffer containing both the carboxymethylated cyclodextrin and the crown ether a lowering of the pH-value to 2.5 led to increased migration times and to improved separation. The increased migration times may have been caused by a reduction of the electroosmotic flow, an effect on complex stability or a combination of both. The resolution factor, *R*, for noradrenaline at the pH-value of 2.5 was determined as 1.71 (as opposed to 1.35 for a pH-value of 6.7). The corresponding data for adrenaline were 2.49 and 1.99,

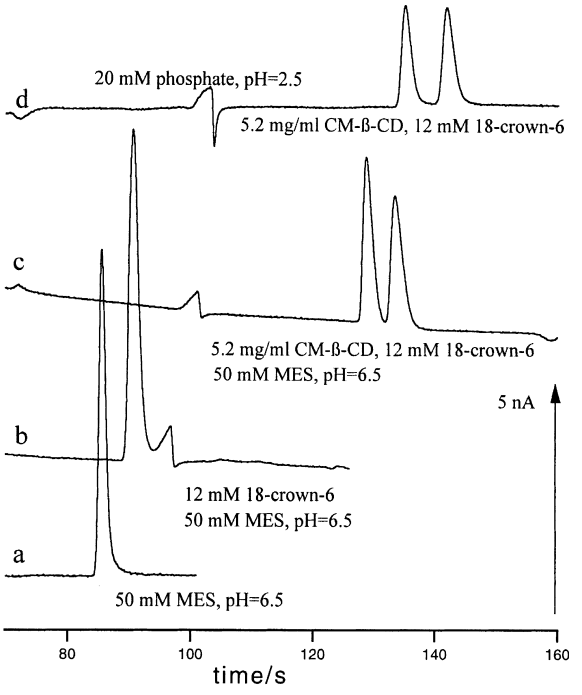

Fig. 3. Enantiomeric separation of noradrenaline with CM-β-CD by formation of sandwich complexes with 18-crown-6 at pH 6.5 and 2.5. Separation voltage, 3 kV; detection potential, 1500 mV; injection, 1 kV (3 s); concentration of standard, 10^{-4} *M*.

respectively. The effect of lowering the pH-value of the buffer on detector sensitivity was found to be negligible in this case.

In an attempt to further maximize chiral resolution, three other cyclodextrin derivatives, namely (2-hydroxypropyl)-γ-cyclodextrin (HP-γ-CD), methyl-β-cyclodextrin (M-β-CD) and sulfated β-cyclodextrin (S-β-CD), were tested for their efficiency to separate the enantiomers of adrenaline and noradrenaline. As illustrated in Fig. 4 for the example of noradrenaline, both methyl- and hydroxypropyl-cyclodextrin, led to at least a partial separation. The addition of the crown ether had a strong synergistic effect in the case of the hydroxypropyl-cyclodextrin, but showed little difference in the case of the methyl-compound. A reduction in sensitivity was found in both cases when using the two reagents concurrently in the buffer (note the different scales in the figure). Similar results were obtained on the separation and sensitivity of detection for adrenaline with methyl- and hydroxypropyl-cyclodextrin. Note, that higher concentrations of the additives (30 and 10 mg ml^{-1}) were required then in the case of carboxymethyl cyclodextrin (typically 5 mg ml^{-1}). Sulfated cyclodextrin led to good separations at a low concentration of 3 m*M*, however, the peak heights were significantly reduced and the migration times very long. This indicates the formation of complexes with high stability constants. Best performance in terms of chiral separation and amperometric detection sensitivity for adrenaline and noradrenaline could thus be obtained with the carboxymethyl-cyclodextrin.

As illustrated in Fig. 5, fast chiral separation of a mixture of the catecholamines dopamine, adrenaline and noradrenaline could indeed be achieved by exploiting the synergistic effect of the two complexing agents, the non chiral crown-18-ether-6 and carboxymethyl-cyclodextrin. To our knowledge, a chiral separation of this mixture in capillary electrophoresis has not been demonstrated previously and could be achieved here in less than 3 min. Note the strong shift of dopamine compared to the separation without the crown ether (Fig. 1a). Apparently the formation of a sandwich complex with the cyclodextrin and crown ether is most pronounced with this compound.

4. Conclusion

Unprecedented fast chiral separations of catecholamines could be demonstrated on a micromachined electrophoretic separation device utilizing a combination of cyclodextrin derivatives and crown-ethers. Good results were achieved with concentrations of the cyclodextrins which are about an order of magnitude lower than those usually employed [12]. This is of interest in view of the relatively high cost of these compounds. The utility of the simplified two-electrode detection scheme for amperometric detection was further demonstrated. Electrochemical detection has the advantage of not requiring chemical derivatization, unlike the fluorescence technique more commonly used for microfabricated analytical devices. On the other hand, conditions in terms of buffer composition have to be arrived at which satisfy the requirements for both separation and detection. The sensitivity of the amperometric detection may not be adequate for the direct detection

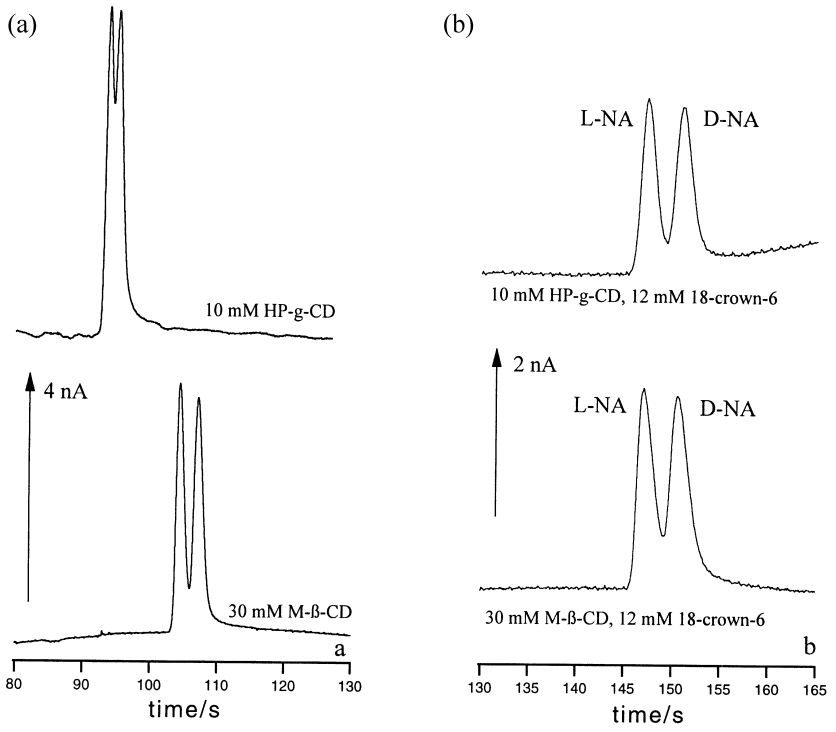

Fig. 4. Enantiomeric separation of noradrenaline with HP-γ-CD and M-β-CD. Buffer: 50 m*M* phosphate, pH = 2.5 (a) without, (b) with 12 m*M* 18-crown-6. Separation voltage, 3 kV; detection potential, 1500 mV; injection, 1 kV (2 s); concentration of standards, 2×10^{-4} m*M*.

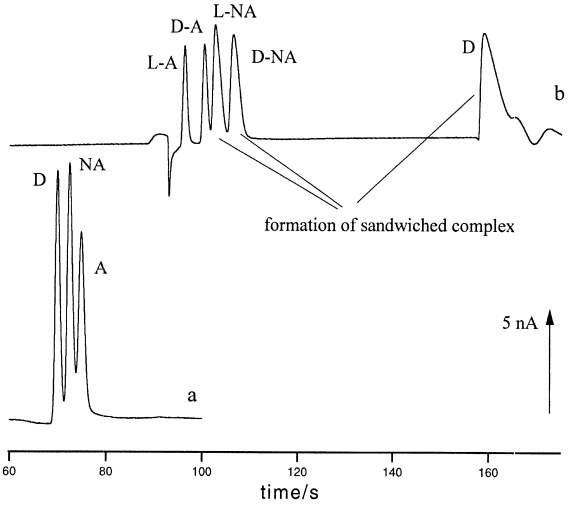

Fig. 5. Enantiomer separation of a mixture of adrenaline, noradrenaline and dopamine employing sandwich complexes. Buffer: (a) 50 m*M* MES, pH = 6.7; (b) 20 m*M* phosphate, pH = 3.0, 5.2 mg ml^{-1} CM-β-CD, 12 m*M* 18-crown-6. Separation voltage, 3 kV; detection potential, (a) 1300 mV, (b) 1500 mV; injection, 1 kV (3 s); concentration of standards, 10^{-4} *M*.

of the neurotransmitters in biological fluids. However, preconcentration is also routinely carried out for such samples when analysed by HPLC or conventional capillary electrophoresis. Pharmaceutical preparations may of course be determined without pretreatment. It was found that a benefit of the fast run times is not only the high sample throughput but also the reduction in time required for method development.

Acknowledgements

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References

[1] S.M. Han, Biomed. Chromatogr. 11 (1997) 259.

[2] F. Bressolle, M. Audran, T.N. Pham, J.J. Vallon, J. Chromatogr. B 687 (1996) 303.
[3] B. Chankvetadze, G. Blaschke, J. Chromatogr. A 906 (2001) 309.
[4] D.J. Harrison, K. Fluri, K. Seiler, Z. Fan, C.S. Effenhauser, A. Manz, Science 261 (1993) 895.
[5] C.S. Effenhauser, A. Manz, H.M. Widmer, Anal. Chem. 65 (1993) 2637.
[6] S.C. Jacobson, A.W. Moore, J.M. Ramsey, Anal. Chem. 67 (1995) 2059.
[7] I. Rodriguez, Y. Zhang, H.K. Lee, S.F.Y. Li, J. Chromatogr. A 781 (1997) 287.
[8] S. Sarac, B. Chankvetadze, G. Blaschke, J. Chromatogr. A 875 (2000) 379.
[9] S. Fanali, S. Furlanetto, Z. Aturki, S. Pinzauti, Chromatographia 48 (1998) 395.
[10] S.L. Tamasier-Karolak, M.A. Stenger, A. Bommart, Electrophoresis 20 (1999) 2656.
[11] K.H. Gahm, A.M. Stalcup, Chirality 8 (1996) 316.
[12] S. Fanali, J. Chromatogr. A 875 (2000) 89.
[13] L.D. Hutt, D.P. Glavin, J.L. Bada, R.A. Mathies, Anal. Chem. 71 (1999) 4000.
[14] I. Rodriguez, L.J. Jin, S.F.Y. Li, Electrophoresis 21 (2000) 211.
[15] S.R. Wallenborg, I.S. Lurie, D.W. Arnold, C.G. Bailey, Electrophoresis 21 (2000) 3257.
[16] J.S. Rossier, R. Ferrigno, H.H. Girault, J. Electroanal. Chem. 492 (2000) 15.
[17] D.C. Chen, F.L. Hsu, D.Z. Zhan, C.H. Chen, Anal. Chem. 73 (2001) 758.
[18] A.T. Woolley, K. Lao, A.N. Glazer, R.A. Mathies, Anal. Chem. 70 (1998) 684.
[19] Y. Liu, J.C. Fanguy, J.M. Bledsoe, C.S. Henry, Anal. Chem. 72 (2000) 5939.
[20] A.J. Gawron, R.S. Martin, S.M. Lunte, Electrophoresis 22 (2001) 242.
[21] J. Wang, M.P. Chatrathi, A. Mulchandani, W. Chen, Anal. Chem. 73 (2001) 1804.
[22] A. Hilmi, J.H.T. Luong, Anal. Chem. 72 (2000) 4677.
[23] M.A. Schwarz, B. Galliker, K. Fluri, T. Kappes, P.C. Hauser, Analyst 126 (2001) 147.
[24] T. Kappes, P.C. Hauser, Analyst 124 (1999) 1035.
[25] W. Wei, G. Luo, C. Yan, Z. Sun, J. High Res. Chromatogr. 21 (1998) 440.
[26] S.A.C. Wren, R.C. Rowe, J. Chromatogr. 603 (1992) 235.
[27] X. Fang, F. Gong, Y. Fang, Anal. Chem. (1998) 4030.
[28] E.G. Cookeas, E. Efastathiou, Analyst 125 (2000) 1147.
[29] M. Wedig, U. Holzgrabe, Electrophoresis 20 (1999) 2698.
[30] I.S. Lurie, R.F.X. Klein, T.A. Dalcason, M.J. Lebelle, R. Brenneisen, R.E. Weinberger, Anal. Chem. 66 (1994) 4019.
[31] M. Heuermann, G. Blaschke, J. Chromatogr. 648 (1993) 267.
[32] R. Kuhn, C. Steinmetz, T. Bereuter, P. Haas, F. Erni, J. Chromatogr. A 666 (1994) 367.
[33] W.X. Huang, S.D. Fazio, R.V. Vivilecchia, J. Chromatogr. A 781 (1997) 129.
[34] W.X. Huang, H. Xu, S.D. Fazio, R.V. Vivilecchia, J. Chromatogr. B 695 (1997) 157.