Journal of Chromatography B, 877 (2009) 3753–3756

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short communication

# Rapid analysis and sensitive detection of DL-tryptophan by using shorter capillary column coupled with deep-UV fluorescence detector

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ARTICLE INFO

*Article history:*
Received 16 March 2009
Accepted 12 September 2009
Available online 17 September 2009

*Keywords:*
Capillary electrophoresis
Chiral separation
DL-Tryptophan
Cyclodextrin
Microfluidics

ABSTRACT

A laboratory made capillary electrophoresis (CE) system using shorter capillary column was designed and constructed in our research group. This promising CE system was then connected with confocal fluorescence microscopy which was also developed in our research group. In order to show the applicability of this new system on chiral separation of DL-tryptophan (DL-Trp), studies were undertaken using cyclodextrins (CDs) and their derivatives; sulfated (S-CDs) and highly sulfated cyclodextrins (HS-CDs) as chiral selectors. Different selector concentrations were tested at various pH levels (pH 2.5, 4.0, 6.0, 9.0) to optimize the separation conditions. The best results were achieved at lower pH values (e.g. pH 2.5 and pH 4.0). HS-β-CD and HS-γ-CD were found to be the most effective chiral selectors with a shorter retention time at low selector concentrations.



## 1. Introduction

Microfluidic systems have proven to be an advantageous platform for analytical and biological assays. They benefit from reduced requirements for expensive and toxic reagents, short analysis time, automation and portability, thereby reducing the amount of hands-on work and enabling more on-field analytical testing. However, the impact of microfluidics on science is still an open subject for researchers. After the first demonstration by Gassmann et al. [1], capillary electrophoresis (CE) has become a powerful separation technique for the chiral analyses, which are very important for the detection of chiral isomers separately because of their completely different pharmacokinetic, pharmacodynamic and possible toxic effects in the human body [2–5].

The goal of this work was to develop a laboratory made CE system in which the shorter capillary columns might be used because of its mentioned advantages above and to show the applicability of this new system to the chiral separations. For these reasons at first a new CE system was designed and built up in our research group. After completing all system suitability test measurements, DL-tryptophan (DL-Trp) was chosen as model chiral amino acid in order to show the applicability of the system on enantiomer separation. In most cases only one enantiomer exhibits the desired pharmacological effect and therefore an efficient separation of stereoisomers from their racemic mixture becomes an important issue. In the literature L-Trp was reported as being used exclusively as antidepressant [6,7] and hypnotist [8].

In order to obtain chiral isomers separately in CE, the addition of a chiral selector into the running electrolyte is necessary. Native cyclodextrins (CDs) [9–12] and derivatized CDs [13–17], crown ethers [18,19], and bile salts [20] are widely used as chiral selectors but CDs are preferred since they resolve a variety of drug substances. Furthermore, CDs are commercially available, UV transparent at short wave length usually used to monitor the enantiomers, cheap, and not toxic. In particular, successful applications of sulfated CDs to a wide range of analytes, including weak and strong acids and bases as well as zwitterions, have been reported by Evans and Stalcup [21]. The separation mechanism is mainly based on diastereomeric inclusion or incorporation of the analytes in the hydrophobic core of the CDs.

Studies related with chiral separation of Trp and Trp derivatives by using commercial CE systems have been reported extensively in the literature [15,22,23]. However, to our knowledge only two papers cover the chiral separation of DL-Trp by using chip electrophoresis with electrochemical detection [24,25] and no study was reported on miniaturized CE system.

The new and promising analysis and detection techniques are always a challenge. Native detection is particularly important when the derivatization is undesirable because of nonquantitative derivatization or multi-labeled product forming and when the low detection limits are necessary. Laser-induced fluorescence (LIF) detection is one of the most sensitive techniques used in CE. It is extensively used for fluorescently labeled biomolecules such as proteins, peptides and amino acids [5,26,27]. Mostly visible laser light is used for excitation source but nowadays UV, especially

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doi:10.1016/j.jchromb.2009.09.010

deep-UV lasers became an attractive source [28–32]. The most restrictive parameters for using UV-LIF detectors are that their high costs and the specific requirements of their set-up.

In this study, confocal fluorescence microscopy was used operating solid-state laser at 266 nm for native detection of Trp ($\lambda_{exc} \sim 280$ nm). Our research group has extensive experiences on the design, construction and the application of fluorescence detectors especially for confocal imaging and spectroscopy of nanoparticles and single molecules [33], detection and counting of fluorescent polystyrene spheres [34,35]. In this work, the applicability of deep-UV-LIF to laboratory made CE instrument was shown. CE studies were undertaken using CDs and their derivatives, sulfated cyclodextrins (S-CDs) and highly sulfated cyclodextrins (HS-CDs) as chiral selectors. In order to optimize the separation conditions, effect of selector and electrolyte nature, their concentration range at various pH levels and separation voltage were investigated. Several parameters controlling on the detection limits including focusing effect and laser power were tested and optimized. Finally, LOD and LOQ values for Trp enantiomers were found much lower than those obtained from traditional detection methods. Additionally, analysis time observed from this new system is in the range of chip electrophoresis applications.

## 2. Materials and methods

### 2.1. Chemicals

Sodiumphosphate monobasic ($NaH_2PO_4$), DL-tryptophan and D-Trp were from Fluka (Buchs, Switzerland). HCl and MeOH were from Merck (Darmstadt, Germany). NaOH was obtained from Erne AG (Dällikon, Switzerland). TRIS, MES, acetic acid, L-Trp, neutral and S-CDs were from Sigma–Aldrich (Buchs, Switzerland). HS-CDs were obtained from Beckman Coulter (Monheim, Germany). Deionised water was obtained from a GFL Glass Water Still double distillation system (GFL, Germany). Standards were freshly prepared in deionised water from their stock solutions.

### 2.2. Instrumentation

The details on miniaturized CE system and confocal fluorescence detection are explained in the following parts.

### 2.3. CE system

The miniaturized CE instrument used in this study was designed and built in our laboratory. As can be seen in Fig. 1, the main plate (15 cm × 11 cm × 1 cm, l × w × h) was made of PVC and another small PVC part was used for capillary window fixation. Two small holes were drilled for inlet and outlet of capillary column with a 6 cm distance from each other and platinum electrode wires were fixed near by. Bending radius of capillary column from horizontal to vertical position was 10 mm. Fused silica capillary column ($L_t$ = 10 cm, $L_d$ = 5 cm, I.D. = 50 μm, O.D. = 375 μm) was obtained from Agilent, Switzerland. The capillary window was burned indirectly for 7 s by using a spring filament based homemade instrument constructed for this purpose. Six chambered sample and buffer vial holder was made of polyoxymethylene (POM) and a round metal piece was used in order to fit the sample halls in the correct position enabling fast manual sample switching. All metal parts including screws and springs were of stainless steel. Silicon and teflon cables were used for grounding and applying high voltage, respectively. High voltage power supplier was from Spellman High Voltage Electronics Corporation (West Sussex, UK), model CZE100R with dual polarity for ±30 kV.

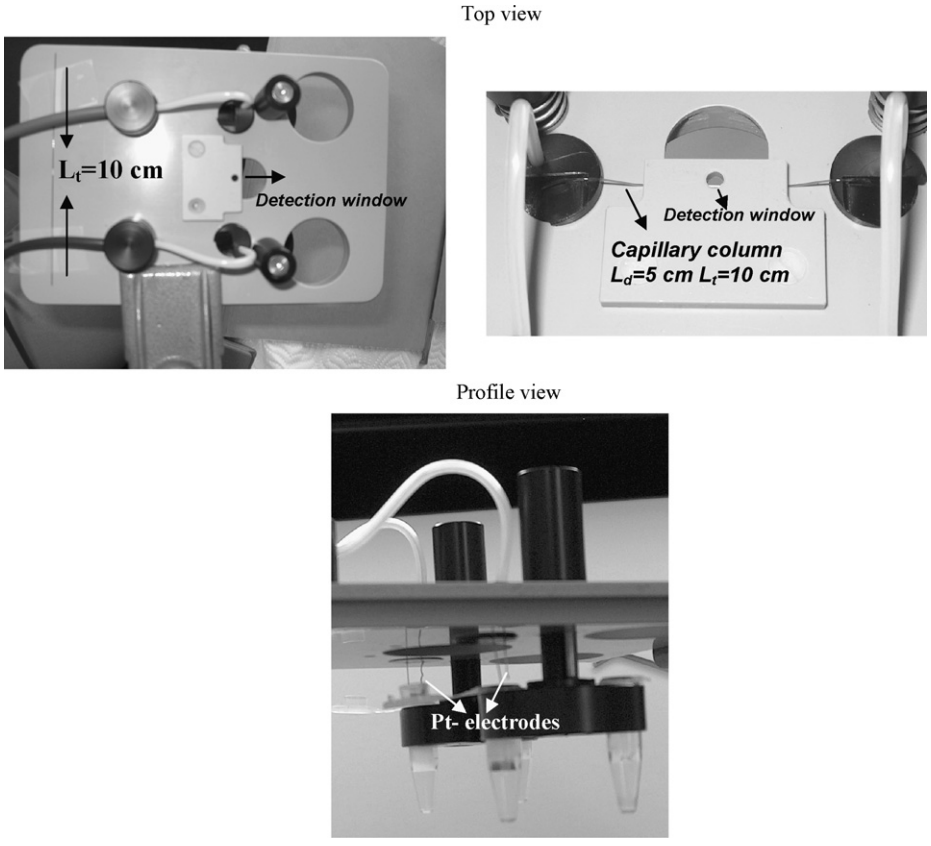


**Fig. 1.** CE system (top and profile view).

### 2.4. Detection system

It consists of a 266-nm UV mode-locked diode-pumped picosecond laser (modelGE-100-XHP-FHG, Time-Bandwidth Products, Inc., Switzerland) [36,37]. The laser system provides pulses with the duration of <10 ps and the repetition rate of 40 MHz. Maximum laser power output of 30 mW is adjusted by inserting different neutral density filters (Melles Griot). The fluorescence light was collected by the same objective and transmitted through the dichronic mirror. A lens focuses the light onto a capillary window for detection. Fluorescence intensity was recorded by means of time-correlated single photon counting (TCSPC) computer interface card (SPC-630, Becker&Hickl GmbH, Germany). Software written in C++ was developed for recording the acquisitions.

## 3. Results and discussion

In this work three types of CDs were investigated on the chiral separation of Trp; neutral CDs (α and γ), sulfated CDs (α and β) and highly sulfated CDs (α, β and γ). A 0.0–6.0% concentration range of S- and HS-CDs; 0.0–80 mM concentration range of neutral CDs were prepared in four different buffer solutions at four different pH levels; phosphate buffer at pH 2.5, acetate buffer at pH 4.0, MES buffer at pH 6.0 and finally in Tris buffer at pH 9.0. Preliminary investigations showed that no separation occurred with neutral CDs at all pH levels and poor interaction occurred with S-CDs only at pH 6.0 with MES buffer. The best results were obtained at lower pH values (e.g. pHs 2.5 and 4.0) by the addition of HS-β-CD and HS-γ-CD. HS-β-CD was able to resolve the tryptophan enantiomers (Fig. 2A) but HS-γ-CD was found to be the most effective chiral selector for this separation (Fig. 2B). HS-CDs have negative effective electrophoretic mobilities because of their multiply negative charges while the EOF at pH 2.5 and 4.0 was directed toward the cathode. Tryptophan enantiomers (p*I* = 5.89) interacted differently with the chiral selector while moving in the same direction of EOF and the opposite direction of HS-CDs at acidic pHs. At pH 4.0, EOF is not as strong as in neutral and basic conditions, furthermore at pH 2.5, it is almost suppressed therefore interactions between isomers and chiral selectors take place in longer time resulting in higher resolution. Since no effective separation was observed with neutral and S-CDs for the chiral separation of Trp, electrostatic interactions between positively charged Trp enantiomers and negatively charged HS-CDs may also be the possible effects on the separation beside cavity size of the selectors. Results showed that almost complete separation of Trp enantiomers could be achieved within 90 s with the addition of 0.1% HS-γ-CD at pH 2.5 and the complete separation with the addition of 0.15% HS-γ-CD at pH 2.5 within 2.5 min (Fig. 2B). Weng et al. [24] reported the analysis time as 70 s for the baseline separation of Trp enantiomers by using PMMA microfluidic chip with the effective length of 32 mm. Bi et al. [25] indicated the analysis time for baseline separation as around 60 s by using alumina gel-derived protein network on PMMA microchannel with the effective length of 35 cm. In both studies preparation of microchannels and chip station need laborious techniques. Comparing with these results, in our system better resolutions could be obtained with compatible analysis time and without any tedious column and/or buffer preparation step by using fused silica capillary column with the effective length of 5 cm. Increasing chiral selector concentration resulted in better resolution according to the charged resolving agent migration (CHARM) model [38] but with longer analysis time. Pure L-Trp was used to confirm the enantiomeric separation order. Applicability of the method for purity tests is demonstrated in Fig. 3. 1:400% (m/m) concentration ratio of D-Trp to L-Trp in a sample containing both enantiomers could be detected even under the higher separation voltage (6 kV). The detection and quantitation limits (S/N ratios are equal to 3 and 10, respectively) for L- and D-Trp were investigated under the conditions where the best results were obtained (Fig. 2B). LOD and LOQ values of Trp enantiomers were found as low as 13 and 40 fg/μl,

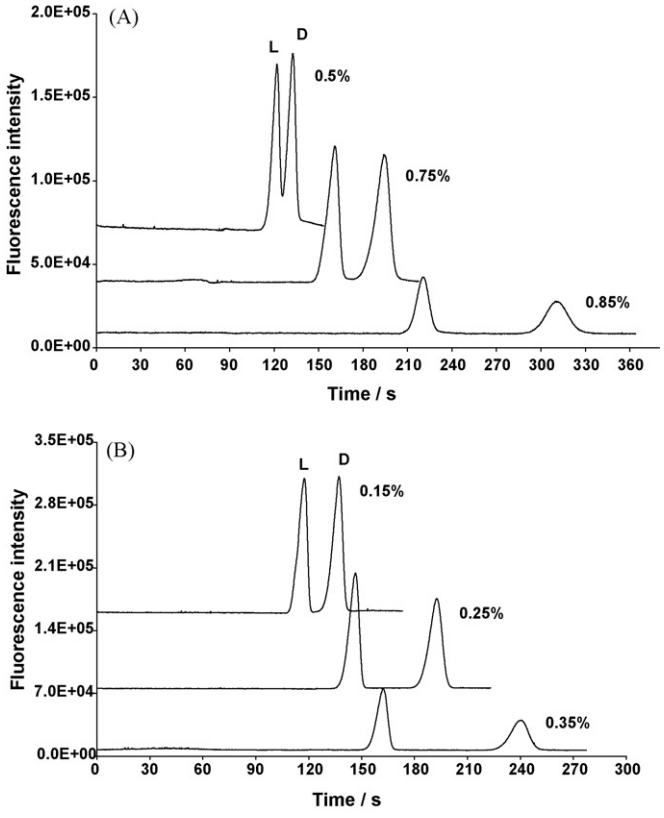


**Fig. 2.** Chiral separation of Trp with the increasing amount of (A) HS-β-CD and (B) HS-γ-CD. Separation conditions: buffer: 1.0 mM phosphate pH 2.5; injection: electrokinetically at 2 kV for 9 s; applied voltage: 4 kV, solid state UV laser at 266 nm.

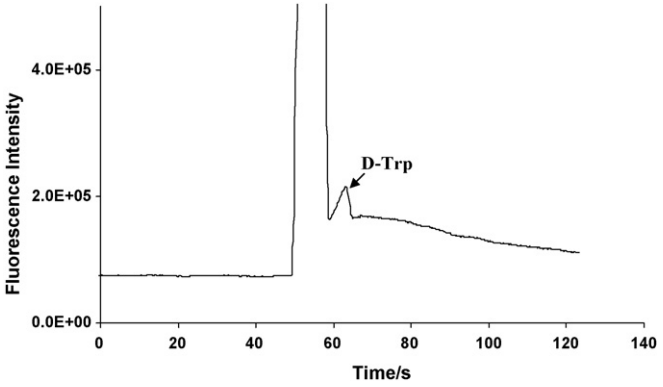


**Fig. 3.** Trace level determination of D-Trp in L-Trp (1:400%, m/m). Buffer: 1.0 mM phosphate with 0.15% HS-γ-CD at 6 kV. Other conditions are same as in Fig. 2.

**Table 1**
Precision results for chiral separation of DL-Trp in phosphate buffer containing 0.15% HS-γ-CD at pH 2.5.

| | Repeatability (RSD%, $n = 5$) | | | |
|---|---|---|---|---|
| | Interday | | Interday | |
| | *t* | *A* | *t* | *A* |
| D-Trp | 0.21 | 0.45 | 0.26 | 0.68 |
| L-Trp | 0.18 | 0.36 | 0.20 | 0.55 |

*t*: retention time; *A*: peak area.

**Table 2**
Separation parameters for DL-Trp (applied voltage = 4 kV).

| pH 2.5 | | | | | pH 4.0 | | | | |
|---|---|---|---|---|---|---|---|---|---|
| HS-β-CD% | $t_1$ (s) | $t_2$ (s) | $\alpha$ | $R_s$ | HS-β-CD% | $t_1$ (s) | $t_2$ (s) | $\alpha$ | $R_s$ |
| 0.5 | 122.4 | 133.2 | 1.09 | 0.78 | 0.5 | 63.4 | 64.8 | 1.02 | 0.09 |
| 0.75 | 160.8 | 195 | 1.21 | 1.52 | 0.75 | 77.6 | 80.6 | 1.04 | 0.17 |
| 0.85 | 516 | 679.8 | 1.32 | 1.72 | 1.0 | 102.4 | 112.2 | 1.10 | 0.44 |
| pH 2.5 | | | | | pH 4.0 | | | | |
| HS-γ-CD% | $t_1$ (s) | $t_2$ (s) | $\alpha$ | $R_s$ | HS-γ-CD% | $t_1$ (s) | $t_2$ (s) | $\alpha$ | $R_s$ |
| 0.15 | 117.6 | 137.4 | 1.17 | 1.40 | 0.5 | 73 | 77.4 | 1.06 | 0.57 |
| 0.25 | 142.2 | 177.6 | 1.25 | 2.41 | 1.0 | 102 | 114.8 | 1.12 | 1.11 |
| 0.35 | 162 | 240 | 1.48 | 3.61 | 1.5 | 120 | 142 | 1.18 | 1.87 |

$R_s = \frac{2(t_2-t_1)}{w_1+w_2}$ $\alpha = \frac{t_2}{t_1}$.
$t$: retention time; $w_{1/2}$: peak width at half of peak height.

respectively. Repeatability studies were reported as RSD% for retention time and peak areas observed from same day and different days measurements (Table 1). The effects of lens focusing and laser power on the detection sensitivity were previously investigated in a simple buffer (10 mM Tris at pH 9.0) and seen that the objective focusing and laser power are important factors in order to improve detection sensitivity. Increasing laser output power from 20 kW to 30 kW (focusing power changes from 4 kW to 6 kW) increases the sensitivity 10 times more [39]. Therefore, 30 kW output laser power was used for this study. System suitability tests are commonly used to verify resolution, column efficiency, and repeatability of a chromatographic system to ensure its adequacy for a particular analysis. Therefore, these analyses were completed by calculating resolution ($R_s$) and selectivity ($\alpha$) factors obtained by increasing HS-γ- and β-CD content in the acidic buffer electrolytes (acetate and phosphate) and the results were reported in Table 2. Separation voltage was tested for 2 and 4 kV. 4 kV was found to be optimum for efficient resolution and faster retention time.

## 4. Conclusion

In this study, CE, which is a modern analytical technique, was miniaturized and the efficiency of the system on chiral separations was demonstrated. Different selector systems containing neutral CDs, S-CDs and HS-CDs in different electrolyte solutions at various pH levels were studied for the enantioselective separation of Trp. Our results demonstrated that the analysis time is in competition with chip electrophoresis applications and the obtained LOD and LOQ limits from confocal fluorescence microscopy are the lowest limits which have been reported before. Therefore we believe that the combination of shortened capillary with confocal fluorescence microscopy will be very promising for trace chiral analysis which is very important especially in pharmaceutical and clinical investigations.

## Acknowledgements

We would like to thank Roland Zehnder (PCI institute, University of Zurich, Switzerland) for his mechanical support during the construction of miniaturized CE system. We also would like to thank Prof. Peter C. Hauser for his collaboration. Finally Dr. Gamze Kavran Belin thanks Werner Kloeti (University of Geneva, Switzerland) for the instrument that he built up specifically for capillary window.

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