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Trace analysis of D-tyrosine in biological samples by microchip electrophoresis with laser induced fluorescence detection☆

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ABSTRACT

A rapid and sensitive microchip electrophoresis (MCE) method with laser induced fluorescence (LIF) detection has been developed for the quantification of D-tyrosine (Tyr) in biological samples. The assay was performed using a MCE-LIF system with glass/poly(dimethylsiloxane) (PDMS) hybrid microchip after pre-column derivatization of amino acids with fluorescein isothiocyanate (FITC). Chiral separation of the derivatives was achieved by cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) using γ-CD as chiral selector in the running buffer. D/L-Tyr enantiomer was well separated in less than 140 s. The limit of detection ($S/N = 3$) was 3.3×10^{-8} M. Using the present method, D-Tyr level in human plasma was found to vary significantly from normal humans to patients suffering from renal failure.

1. Introduction

Amino acids are a group of bioactive molecules in organism, and most exist in an L- and a D-form. However, it had been long believed that only L-amino acids were present in animal and human bodies. Along with the progress in analytical technique, free D-amino acids have been reported to be widely present in tissues and body fluids of mammalian [1–7], and their biological functions and regulation mechanisms are matters of interest. Free D-tyrosine (D-Tyr) is one of the D-amino acids naturally occurring in tissues and body fluids of mammalian [8–10], and has been found in the human plasma [11]. The amount of D-Tyr in human body is reported to be closely related with some diseases. Young et al. [11] reported that the plasma D-Tyr levels were significantly greater in patients suffering from chronic renal failure than in normal humans.

In order to a better understanding of the physiological and pathological function of D-Tyr, the development of simple, sensitive and reliable analytical methods for the determination of D-Tyr in biological sample is highly necessitated. Historically, several methods based on gas chromatography (GC) [9,10,12], high-performance liquid chromatography (HPLC) [11,13,14] and capillary electrophoresis (CE) [15] have been reported for the determination of D-Tyr.

Microchip electrophoresis (MCE), which is considered as a miniaturized version of classical CE, has received as a powerful technique for the separation of chiral compounds [16,17]. It offers many advantages over conventional analytical technologies including high separation efficiency, extremely low consumption of sample and reagent, easy integration and automatization. MCE-based chiral separations have been applied to separate many chiral compounds such as drugs, catecholamines and amino acids [18–27]. Although previous works have given efficient separation for some chiral compounds, little attention is paid to its application in the analysis of real samples. Especially, the quantitative analysis of chiral compounds in complex biochemical mixtures by MCE remains challenging, and with many devices success has only been achieved with low-complexity samples [28].

In this work, our effort is focussed on the determination of D-Tyr in biological sample by a MCE procedure. High sensitivity of detection was achieved by laser induced fluorescence (LIF) detection using FITC as precolumn derivatization reagent. The conditions for chiral separation of Tyr enantiomers were systematically studied. And the quantification of D-Tyr in human plasma from healthy subjects and patients with renal failure was demonstrated.

2. Experimental

2.1. Chemicals and solutions

Amino acids, agmatine (Agm), epinephrine (E), dopamine (DA), glutathione (GSH), γ-amino-n-butyric acid (GABA), fluorescein isothiocyanate (FITC) and α-, β-, γ-cyclodextrin (α-, β-, γ-CD)

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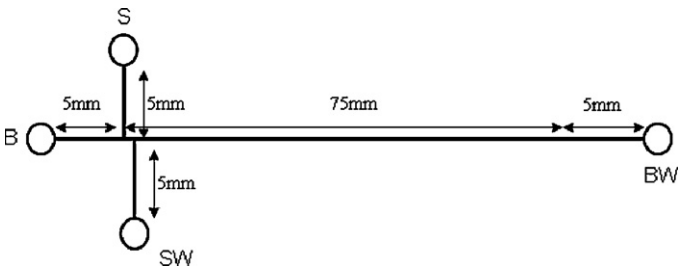

Fig. 1. The layout and dimensions of the glass/PDMS hybrid chip used in this work. S: sample reservoir; SW: sample waste reservoir; B: buffer reservoir; BW: buffer waste reservoir.

were obtained from Sigma (St. Louis, USA). Sodium dodecyl sulfate (SDS) and sodium borate were purchased from Shanghai Chemical Reagent (Shanghai, China). All other chemicals used in this work were of analytical grade. Water was purified by employing a Milli-Q plus 185 equip from Millipore (Bedford, MA, USA), and used throughout the work. The running buffer was 30 mM borate buffer (pH 9.6, adjusted with 1 M NaOH solution) containing 40 mM SDS and 14 mM γ-CD. Stock solutions of 1.0 mM amino acid were prepared in 0.1 M HCl and diluted with 30 mM borate buffer (pH 9.6) as needed. The 2.0 mM FITC solution was prepared in acetone and kept in dark at 4 °C. All solutions were filtered through 0.22 μm membrane filters before use.

2.2. Apparatus and microfluidic chip

The microchip electrophoresis-confocal laser induced fluorescence detection system with 473 nm semiconductor laser at 15 mW was built by the Shandong Normal University. A multi-terminal high voltage power supply, variable in the range of 0–6000 V, was used for sample injection and MCE separation. The output signal was recorded and processed with a computer using a chromatography data system (Zhejiang University Star Information Technology, Hangzhou, China). A home-made glass/PDMS hybrid microfluidic chip was used for the separation of sample. The fabrication of the chip was performed according to the procedure described previously [29]. Its schematic layout and dimensions are illustrated in Fig. 1. All channels etched in glass substrates were 20 μm deep and 60 μm wide. The distance between sampling channel and sample waste channel is 50 μm. All reservoirs were 4 mm in diameter and 2 mm deep. The present microchip is disposable when using same chip to analyze the plasma sample.

2.3. Human plasma sample preparation

The human blood samples were collected from healthy volunteers and patients suffering from renal failure. The blood samples were collected into vials with trisodium citrate, and centrifuged at 2000 × *g* for 15 min immediately to obtain the plasma, which was stored at −20 °C until analysis. A 100 μL volume of plasma sample was deproteinized by adding double amount of acetonitrile solution. After centrifuging at 12,000 × *g* for 20 min, the supernatant was transferred into a 1.0 ml vial and dried with an N_2 stream. The residue was dissolved in 100 μL of 30 mM borate buffer (pH 9.2). The solution was vortexed and kept at 4 °C.

2.4. Precolumn derivatization

Appropriate amounts of the amino acids solution or 10 μL of sample solution were first mixed in 80 μL of 20 mM borate buffer (pH 9.2). Then, FITC solution (in acetone) was added to give a final concentration 20 times greater than the concentration of amino acids or total concentration of analytes. Typically, the mixed solution was reacted at room temperature in the dark for 14 h. The derivatization solution was stored in the dark at 4 °C.

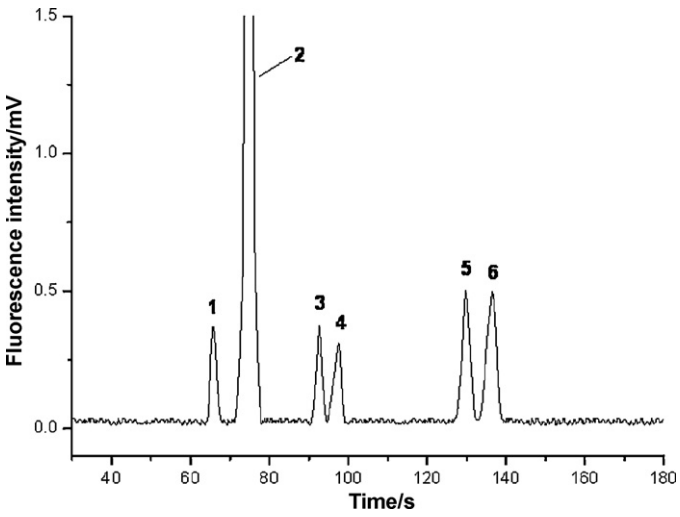

Fig. 2. Separation of FITC-D/L-Tyr enantiomers. Running buffer composition was 30 mM borate containing 14 mM γ-CD and 40 mM SDS (pH 9.6). D/L-Tyr enantiomers concentration was 6.0×10^{-7} M. The sample injection and separation voltage were shown in Section 2. Peak identification: 1, 3, 4: decomposition product of FITC; 2: FITC; 5: FITC-D-Tyr; 6: FITC-L-Tyr.

2.5. Microchip electrophoresis

Before repetitive runs, the microfluidic channel on the microchip was rinsed sequentially with 0.1 M NaOH, water and electrophoretic buffer for 5 min each. Prior to the MCE separation, all reservoirs were filled with the electrophoretic buffer. Vacuum was applied to the reservoir BW to fill all channels with the electrophoretic buffer. Then, the electrophoretic buffer solution in reservoir S was replaced by sample solution. For loading the sample solution, a set of electrical potentials were applied to four reservoirs: reservoir S at 900 V, reservoir B at 300 V, reservoir BW at 450 V, reservoir SW at grounded. The sample solution was transported from reservoir S to SW in pinched mode. After 15 s, potentials were switched to reservoir B, S and SW at 2600, 1600 and 1600 V, respectively, while reservoir BW was grounded for separation and detection.

3. Results and discussion

3.1. Separation of FITC-D/L-Tyr enantiomers

FITC reacts with Tyr, forming highly fluorescent derivative. The reaction proceeds readily in aqueous solution. FITC-Tyr derivatization can be sensitively detected using the 473 nm laser line from a semiconductor laser for fluorescence excitation. However, the separation of FITC-D/L-Tyr enantiomers had been a challenge. To achieve an efficient enantiomer separation, the separation conditions such as type of CD, CD concentration, SDS concentration, pH and concentration of running buffer, and separation voltage were examined. First, several chiral selectors including α-CD, β-CD and γ-CD were tested at concentration range of 6–16 mM. It was found that no enantioseparation was observed when α-CD and β-CD was added to running buffer. While γ-CD was added to running buffer, the enantioseparation of FITC-D/L-Tyr was achieved. The best resolution for FITC-D/L-Tyr enantiomers was obtained when 14 mM γ-CD was used. It was noted that SDS has great effects on the separation of FITC-D/L-Tyr enantiomers. Thus, the SDS concentration was tested in the range of 15–50 mM. The results indicated that

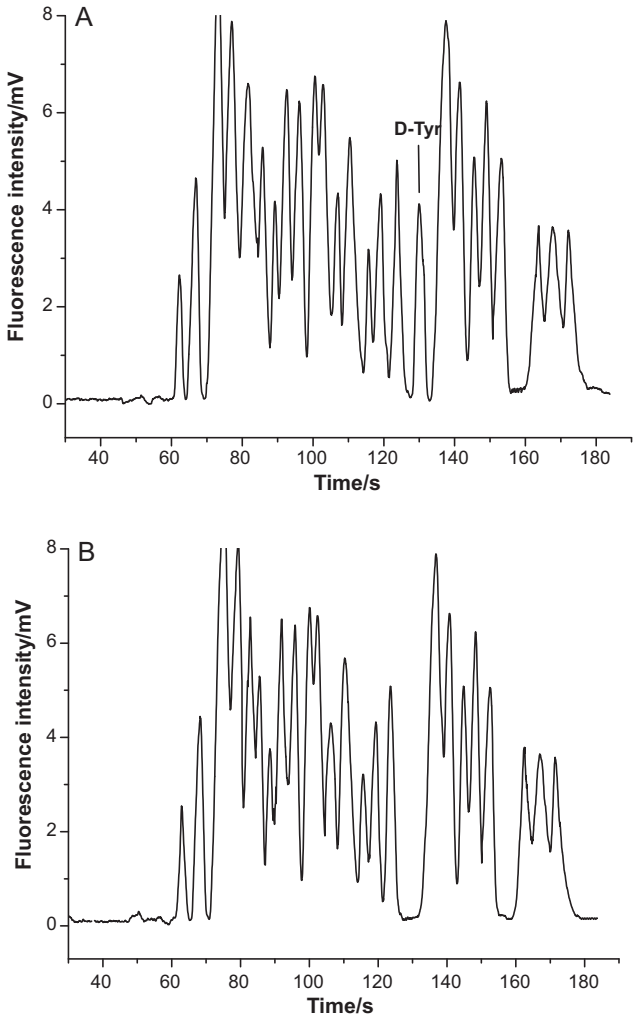

Fig. 3. Electropherograms obtained from the separation of a mixture containing 20 protein amino acids, GSH, DA, E, GABA, and Agm, with (A) and without D-Tyr (B). MCE conditions are as in Fig. 2.

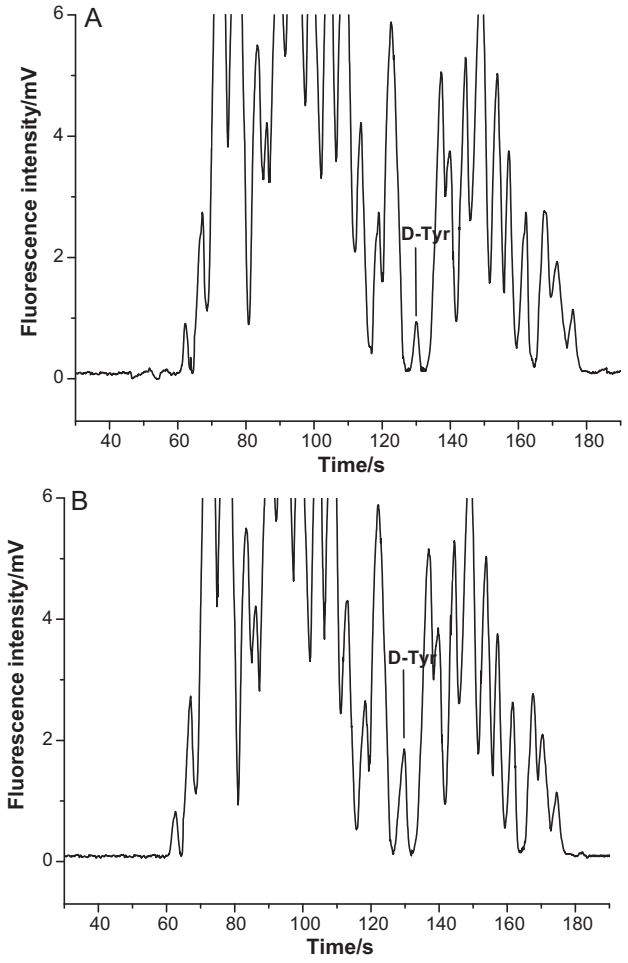

Fig. 4. Electropherograms obtained from the separation of a plasma sample from healthy volunteers (A) and the sample spiked with D-Tyr at 1.0×10^{-6} M (B). MCE conditions are as in Fig. 2.

the best resolution was obtained with running buffer containing 40 mM SDS. The effects of pH and concentration of running buffer were also examined using background electrolyte from borate in the concentration range of 10–35 mM and at different pH values. The optimum resolution was obtained with 30 mM borate buffer at pH 9.6. Separation voltage affected the migration rate and the resolution of FITC-D/L-Tyr enantiomers. It was examined in the range of 2000–3000 V. The results showed that migration times and the resolution decreased with the increase of separation voltage. By considering both the analysis time and the resolution, 2600 V separation voltage is considered optimal. According to the experimental results described above, optimal conditions for the separation of FITC-D/L-Tyr enantiomers were confirmed as following: 2600 V separation voltage and a running buffer containing 30 mM sodium borate, 14 mM γ-CD and 40 mM SDS at pH 9.6. Under the optimized conditions, the typical electrophorogram for the separation of FITC-D/L-Tyr enantiomers is shown in Fig. 2.

3.2. Interference study

Many endogenous amino acids, biogenic amines and small peptides exist in biological matrices. They can react with FITC forming fluorescent derivatives, which may interfere with the determination of D-Tyr. Therefore, the influence from co-existing compounds such as 20 protein amino acids, agmatine (Agm), epinephrine (E), dopamine (DA), glutathione (GSH), and γ-amino-n-butyric acid (GABA) was investigated. Fig. 3 shows the typical electropherograms obtained from the separation of a mixture containing the above-mentioned compounds with (Fig. 3A) and without D-Tyr (Fig. 3B). By comparison Fig. 3A to Fig. 3B, there is no peak observed across the migration time of D-Tyr in the electropherogram without D-Tyr. To evaluate the effect of the adsorption of endogenous components in human plasma onto the channel surface, the human plasma sample was repeatedly separated 5 times, and the RSDs of the migrating time and peak height were calculated to be less than 2.6% and 3.9%, respectively. These results indicated that the adsorption of endogenous components in human plasma onto the channel surface had no obvious effects on detection time and peak height. The results also suggested that none of these endogenous components would interfere with the determination of D-Tyr in biological samples.

3.3. Analytical figures of merit

The MCE-LIF detection method was evaluated in terms of response linearity, the limit of detection, and the precision. The validation was performed with standard solutions due to lack of blank plasma samples. Under the optimized conditions, seven-point calibration curve of D-Tyr was prepared by assaying the standard solutions of D-Tyr at concentrations ranging from 6.0×10^{-8} to

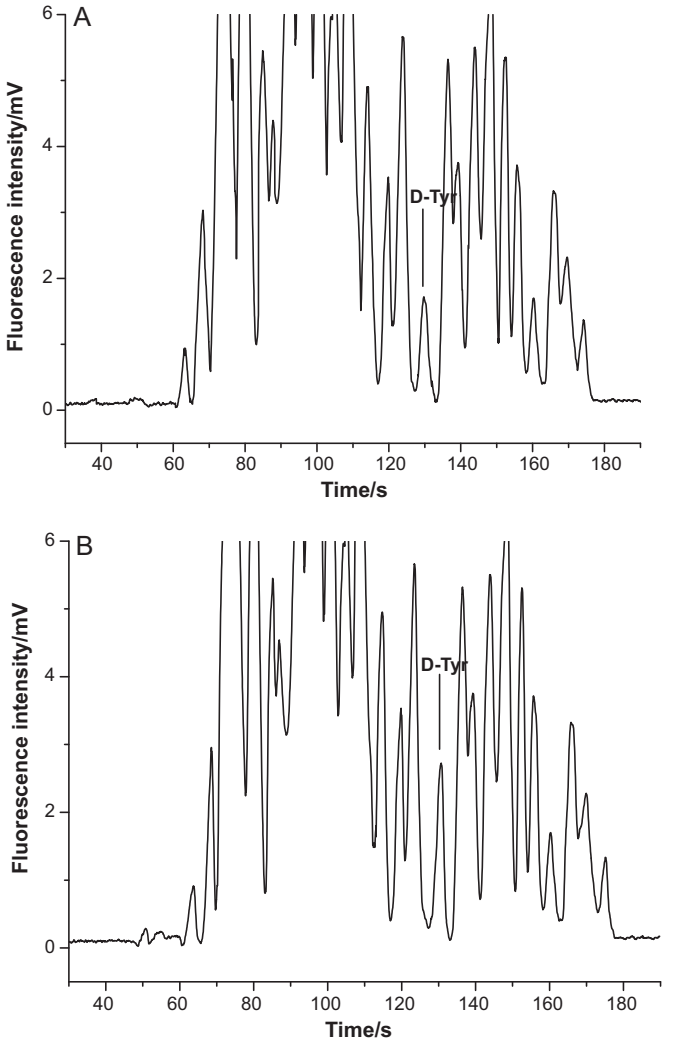

Fig. 5. Electropherograms obtained from the separation of a plasma sample from patients with renal failure (A) and the sample spiked with D-Tyr at 1.0×10^{-6} M (B). MCE conditions are as in Fig. 2.

6.0×10^{-6} M. Linear regression analysis of the results yielded the following equation:

$$H = 8.247C + 2.74 \quad r^2 = 0.9945$$

where H is the peak height (μV), and C is the concentration of D-Tyr in the derivative solution (10^{-8} M). Based on $S/N = 3$, the detection limit for D-Tyr was estimated to be 3.3×10^{-8} M. For precision study, three standard solutions of D-Tyr at 8×10^{-8} M, 8×10^{-7} M and 4×10^{-6} M were separated for 7 times each, and the peak heights and migration times were recorded. The precision was evaluated by the relative standard deviations (RSDs) of the peak height and migration time. The results obtained indicated that RSDs of peak height and migration time were less than 3.7% and 2.4%, respectively. RSDs% in terms of interday ($n = 6$) for the peak height and migration time were also evaluated, RSDs% were less than 5.1% and 3.5%, respectively.

3.4. Analysis of human plasma samples

The present MCE-LIF method was applied to the determination of D-Tyr in plasma samples from four adult healthy volunteers and three patients with renal failure. Figs. 4A and 5A show the typical electropherograms obtained from the separations of plasma from a healthy volunteer and a patient, respectively. The determination of D-Tyr in a plasma sample on a single MCE was achieved within 180 s. The peak corresponding to D-Tyr can be well identified based on the migration time. To further verify the peak identification, the samples were spiked with D-Tyr at 1.0×10^{-6} M and separated again. The electropherograms obtained are shown in Figs. 4B and 5B. By comparing the two traces shown in Figs. 4 and 5, the peak corresponding to D-Tyr increased in size without other major changes in the electropherograms. On the other hand, the peak corresponding to D-Tyr was also well identified when other separation conditions were adopted. The analytical results are summarized in Table 1. D-Tyr level in the serum samples from four healthy subjects was found be in the range of 0.79–1.02 μM. However, it was found in the range from 1.93 to 2.60 μM for three patients with renal disease. These results are in accordance with those obtained by HPLC method [10]. The precision of the detection results was evaluated by repeatedly analyzing each human plasma sample five times within one working day. The relative standard deviations (RSDs) were less than 4.3%. Recoveries of D-Tyr from human plasma samples were also studied by analyzing the samples spiked with standard D-Tyr solution. The results obtained are also summarized in Table 1. It was found that the recoveries are in the range of 94–101%.

Table 1
Analytical results of D-Tyr in human plasma.

Sample	Found (μM)	RSD (%, $n = 5$)	Added (μM)	Total found (μM)	Recovery (%)
1	0.81	3.5	1.00	1.76	95
2	0.94	2.8	1.00	1.95	101
3	0.79	4.2	1.00	1.75	96
4	1.02	3.3	1.00	2.00	98
5[a]	1.93	2.5	1.00	2.90	97
6[a]	2.60	4.0	1.00	3.54	94
7[a]	2.48	3.1	1.00	3.44	96

[a] Analytical results of D-Tyr in serum samples from patients with renal failure.

4. Conclusion

A new MCE-LIF method was developed for the separation and determination of D/L-Tyr enantiomers. The chiral MCE-LIF system established in the present investigation enables the sensitive and selective determination of trace amounts of D-Tyr in human plasma, and has been applied to the quantification of D-Tyr in the plasma samples from healthy subjects and patients with renal failure. The results have approved that the plasma D-Tyr level was significantly greater for patients than normals. This will help investigations on the physiological role and regulatory system of D-Tyr in mammals.

Acknowledgments

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