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Short communication

Sensitive determination of pheomelanin after 5-carboxyfluorescein succinimidyl ester precapillary derivatization and micellar electrokinetic capillary chromatography with laser-induced fluorescence detection

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Abstract

The suitability of micellar electrokinetic capillary chromatography (MEKC) with laser-induced fluorescence (LIF) detection for the determination of pheomelanin in biological materials has been investigated. 5-Carboxyfluorescein succinimidyl ester was chosen as the labeling reagent to precapillary derivatize the two marker aminohydroxyphenylalanine (AHP) isomers produced after reductive hydrolysis of pheomelanin with hydriodic acid (HI). Various parameters affecting derivatization and separation were systematically studied. Under optimal conditions, the analytes could be separated within 18 min, and the relative standard deviations (R.S.D.) of migration time and corrected peak areas were less than 5.5%. Compared with the conventional high-performance liquid chromatography (HPLC) method with electrochemical detection, the 100-fold improvements in sensitivity were achieved by applying LIF detection. As a preliminary application, this method has been successfully applied to the determination of pheomelanin in two human melanoma cell cultures, black hair, melanoma tissue and urine samples of human melanoma patients with the spiked recoveries in the range of 88–96%.

Keywords: Pheomelanin; Aminohydroxyphenylalanine; Laser-induced fluorescence; Micellar electrokinetic capillary chromatography; Derivatization

1. Introduction

Pheomelanin is a yellow-to-reddish heterogeneous biopolymer mainly consisting of sulphur-containing benzothiazine derivatives, which is of great social, clinical and cosmetic significance [1]. In many pigment cells, pheomelanin may act as a powerful antioxidant [2,3], an efficient photoprotective pigment and free radical scavenger for the oxidizing and reducing radicals that participate in many physiology/pathology process in the body [4]. Moreover, there is much epidemiological evidence that the group of people with a higher pheomelanin/eumelanin ratio in their skin is at higher risk of getting melanoma skin cancer, in which metastatic melanoma is the most therapeutically refractory of all cancers [5].

Current analytical method commonly employed for pheomelanin quantitative analysis is high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [6–8]. However, the low sensitivity ascribed to electrochemical detection prevents the applicability of HPLC–ED method for the determination of trace levels of pheomelanin in real samples. Thus, quantification of femtomolar level of pheomelanin in biological materials is still a big challenge. Capillary electrophoresis (CE) with laser-induced fluorescence detection is a widely used technique in separation science with high separation efficiency, low operation costs, small amounts of reagents or samples required, and provides extremely high sensitivity, with detection limits approaching the single molecule level [9]. So far, this technique has been widely applied to

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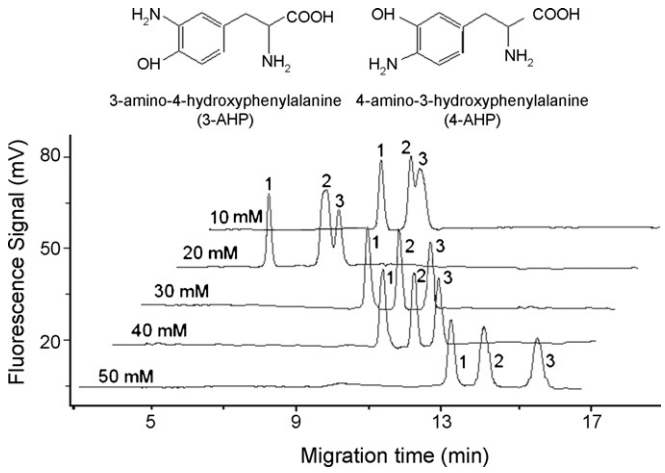

Fig. 1. Electropherograms of the standard mixture solution containing two CFSE-labeled marker AHP isomers (1 μM each) and 5 μM PPA (I.S.) with different SDS concentrations in 25 mM borate buffer at pH 9.0. Peak identifications: (1) I.S.; (2) 3-AHP; (3) 4-AHP. Conditions are described in Section 2.

analyze complex samples such as serum plasma and proteins [10,11].

The aim of this work is to explore the suitability of MEKC coupled with highly sensitive LIF detection for the determination of pheomelanin in biological materials. An excellent dye to label aliphatic amines with high selectivity for CE-LIF [12,13], 5-carboxyfluorescein succinimidyl ester (CFSE), is chosen as the fluorescent labeling reagent to precapillary derivatize the two marker aminohydroxyphenylalanine (AHP) isomers for pheomelanin (Fig. 1). To the best of our knowledge, no study has been done on the determination of pheomelanin in biological samples using CE-LIF method.

2. Experimental

2.1. Chemicals and reagents

Unless otherwise specified, all reagents were commercially available and analytical reagent grade. CSFE was purchased from Molecular Probes (Eugene, OR, USA) and prepared in dimethylformamide. Sodium tetraborate, boric acid, sodium hydroxide, phosphate-buffered saline (PBS) buffer, sodium dodecyl sulphate (SDS), hypophosphorous acid (H_3PO_2) and hydriodic acid were purchased from Sigma (St. Louis, MO, USA). 3-Amino-4-hydroxyphenylalanine (3-AHP) was obtained from Acros Organics (Geel, Belgium). Phenylpropanolamine (PPA) was purchased from Sigma and was used as the internal standard (I.S.). 4-Amino-3-hydroxyphenylalanine (4-AHP) was a gift from Professor S. Ito (Fujita University, Japan). Ultrapure water obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare all buffers and samples solutions. All buffers were degassed by sonication and filtered through 0.22 μm Millipore filters prior to use.

2.2. Sample preparation

Human melanoma cell lines (HS-6, HSCI) obtained from American Center for Type Culture Collection (ATCC, Manassas, VA, USA) were used in this study. The pretreatment of the cell cultures and the determination of the concentration of the protein in the cell suspensions were measured as described previously [14]. Urines samples were collected randomly during the day from melanoma patients and healthy subjects other than requesting collection for a 36-h period. Approximately 10 mL of urines were taken to glass tubes and then stored at −60 °C until analysis. No change of melanin concentrations was observed for at least 4 weeks at these storage conditions. The pretreatment of the hair samples were performed as described previously [15]. Finally, the hair samples were added by ultrapure water to make up the final concentration to 1.0 mg mL^{-1}. Melanoma tissue (10 mg) was added to a 10 mL lysis buffer [100 mM Tris–HCl, pH 7.6, 300 mM LiCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol and 1% SDS] to give a final concentration of 1.0 mg mL^{-1}. The tissue was homogenized using an Eppendorf Mixmate vibrator at 2000 rpm for 2 min. The dispersed melanoma cells were resuspended in PBS buffer (pH 7.2) in a similar way at a final cell density of 1×10^6 cells mL^{-1}.

For the analysis of two marker AHPs, chemical degradations of all the samples (10 μL urine or the homogenate corresponding to 0.01 mg hair, 0.01 mg melanoma tissue, or 10^4 melanoma cells) was mixed with 20 μL of 50% H_3PO_2, and 200 μL of 57% HI and then hydrolyzed according to Ito and Fujita [6]. After hydrolysis, extraction of the interested compounds in the samples was performed by a solid-phase extraction procedure according to Kolb et al. [7]. Then the two final reductive products of AHPs were precapillary derivatized with CFSE.

2.3. Labeling procedure

Precapillary derivatization reactions were performed manually as follows unless otherwise noted. Reactions were carried out in 100 μL plastic centrifuge tubes. Five microliters amino acid (1 μM), 5 μL PPA (5 μM) were added into a 10 μL borate buffer (50 mM, pH 9.0). After addition of 5 μL CFSE (1 mM), the resulting solution was homogenized and left to equilibrate at room temperature for 30 min for the completeness of the reaction. In the case of LIF detection, the reaction mixture was diluted 10- to 50-fold with ultrapure water prior to injection.

2.4. Electrophoresis procedure

All the separations were carried out on a Beckman Coulter P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) equipped with a LIF detector (Beckman P/ACE System Laser Module 488; 488 nm excitation, 520 nm emission). Samples were injected hydrodynamically at 50 mbar for 5 s into an uncoated fused-silica capillary (360 μm o.d. × 50 μm i.d × 60 cm total length with the effective length of 40 cm) from Yongnian Optic (Yongnian, Hebei, China) and performed at 25.0 ± 0.2 °C using a running voltage of 25 kV. The running buffer consisted of an aqueous solution with 25 mM

borate buffer containing 30 mM SDS at pH 9.0. Each day before starting analysis, the capillary was rinsed with 0.1 M NaOH (20 min), followed by ultrapure water (5 min) and finally by the running buffer for 2 min. This above flushing cycle was repeated to ensure the separation reproducibility for each injection. Between each run, the capillary was flushed with 0.1 M NaOH for 2 min, ultrapure water for 2 min, finally by rinsing with the running buffer for 5 min.

3. Results and discussion

In our previous study, naphthalene-2,3-dicarboxaldehyde (NDA) was used to label two AHPs for the determination of pheomelanin [16]. Although NDA shows good sensitivity and high stability for primary amines, its main drawbacks are the need to use the serious toxic CN^- solution to provide the necessary nucleophile for NDA derivatization, and multiple labeling may occurred in the derivatization reaction as AHPs contain two amino groups. Moreover, NDA derivatives require the use of an unordinary laser source of He–Cd laser (442 nm) for the efficient excitation. CFSE is one of the most widely used florescent reagents to label aliphatic amines due to its high absorptivity, near unity fluorescence quantum yield and good water solubility. Besides, CFSE has an excitation maximum ($\varepsilon = 88{,}000\ cm^{-1}\ M^{-1}$ at 490 nm, pH > 6.0 [17]) that very closely matches the spectral line of the easily obtainable argon-ion laser (488 nm). Moreover, the use of CFSE involves no serious toxic component and can be regarded as being relatively benign.

In this study, micellar electrokinetic capillary chromatography using a popular surfactant, SDS, was selected for the separation of two CFSE-labeled AHP derivatives. In MEKC, the main key factor affecting their separation was SDS concentration. The effects of different SDS concentrations (0–50 mM) on the migration times of CFSE-labeled AHPs were compared. From the results (Fig. 1), 30 mM was selected as the optimal SDS concentration in the borate buffer (25 mM, pH 9.0).

The yields of CFSE-AHP derivatives were found to be pH dependent. In a 50 mM borate buffer, the optimal pH for CFSE derivatization was pH 9.0. In order to ensure a complete derivatization, the concentration ratio of CFSE to AHPs in the reaction mixture was 10:1 or higher. Excess CFSE is hydrolyzed and the hydrolysis products produced some extra peaks in the electropherograms, however, under the optimal separation conditions, they did not interfere with the determination of CFSE-AHP derivatives. The maximum yield of derivatization was achieved after 30 min reaction in a 50 mM borate buffer (pH 9.0) at room temperature. We found that no significant degradation was observed for any CFSE-AHP derivative that was stored at room temperature in the dark over a period of 5 days. Since automation of a method requires sufficiently stable conjugation products, this finding is of great significance, which is in accordance with the results of Banks and Paquette [18].

Quantification was performed using the internal standard method for calibration under optimized conditions. The linear regression analysis, using ratio of corrected peak area of AHP/PPA as the function of the concentration of each AHP, was constructed from six levels of concentration and three repetitions per level, and PPA as internal standard. The linear regression equations were $y = 1.24 \times 10^5 \times -0.011$ (correlation coefficient, $r = 0.999$) and $y = 1.56 \times 10^5 \times -0.039$ ($r = 0.998$) with the linear range 0.001–1 μM for 3-AHP and 4-AHP, respectively, showing that the method was strictly linear and highly reproducible. The relative standard deviation (R.S.D.) values for migration times and corrected peak areas ($n = 10$) were 0.8–1.7%, 2.6–4.1% (within run), and 0.9–1.9%, 3.7–5.4% (between run, for a 5-day period), respectively. Based on a signal-to-noise ratio of 3 (S/N = 3), the lowest detectable concentrations for the two labeled AHPs in the analyzed samples were $0.9–1.2 \times 10^{-10}$ M for 3-AHP and $0.9–1.1 \times 10^{-10}$ M for 4-AHP, respectively, and the limits of quantification (S/N = 10) in the real samples were $2.6–4.5 \times 10^{-9}$ and $2.7–3.1 \times 10^{-9}$ M, which were caused by a 10-fold dilutions in the sample preparation procedure for two AHPs. The signal-to-noise ratio of the MEKC-LIF method was approximately 100 times higher than that of conventional HPLC–ED method for pheomelanin quantitative analysis [7–9], and similar to that of obtained in our previous work by HPLC method with fluorescent detection [16].

The applicability of the proposed method for biological analyses was evaluated by using diverse samples as the test samples where the analytes are in highly dispersed concentrations. Fig. 2 shows the typical electropherograms including the I.S. obtained from the samples using the described method. The peaks of analyses were identified by comparing the migration times of the analyses in the real samples with those of standards and spiking the standards to the real samples. Under the optimal conditions, the analytes in the samples could be completely separated within 18 min. It was found that the other endogenous co-existing amino compounds in the real samples, such as amino acids, ammonia, etc., produced many extraneous peaks in the electropherograms, however, under the optimized derivatization and separation conditions, they did not interfere with the determination of two marker AHPs. The quantitative results of two AHPs in those samples are summarized in Table 1. These values are similar to the ones obtained by conventional HPLC–ED

Table 1
Quantitative results of pheomelanin in biological samples with the proposed method

Samples	3-AHP[a]	4-AHP[a]
Cell culture (ng $10^6 \times cells^{-1}$)		
Human melanoma cell line (HS-6)	138.2 ± 2.27	367.9 ± 1.12
Human melanoma cell line (HSCI)	159.1 ± 2.12	710.4 ± 2.17
Hair (ng mg^{-1})		
Black hair	47.9 ± 2.65	107.2 ± 2.33
Melanoma tissue (ng mg^{-1})		
Melanoma tissue	200.5 ± 2.16	778.9 ± 3.08
Urine (μM)		
Human melanoma patient	432.6 ± 2.43	1807.3 ± 1.56
Healthy subjects	37.9 ± 1.45	87.2 ± 2.21

Pretreatments of the real samples were described in Section 2.

[a] The values are expressed as mean ± relative standard deviation (R.S.D.) ($n = 6$).

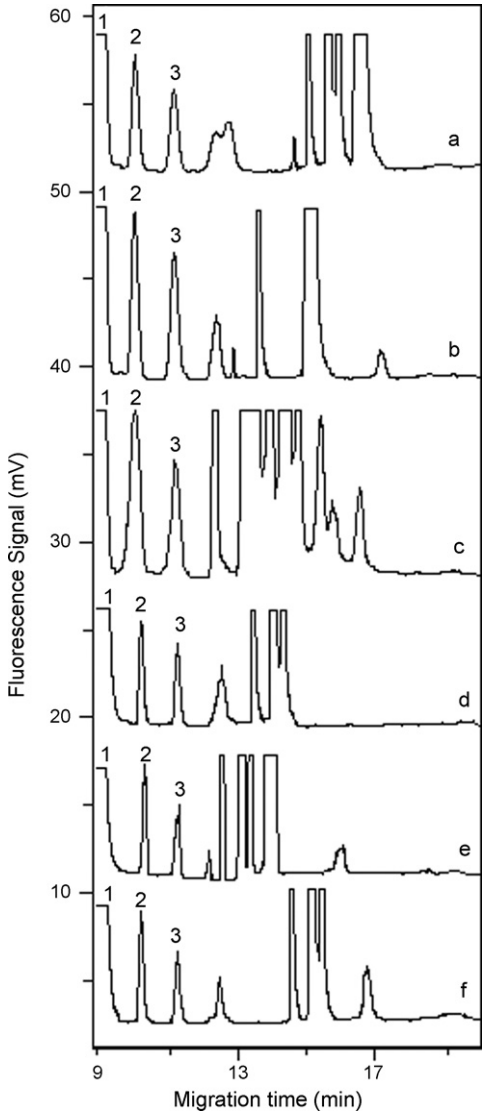

Fig. 2. The typical electropherograms of the CFSE-labeled AHPs in biological samples from (a) human HS-6 melanoma cell culture (b) human HSCI melanoma cell culture (c) black hair (d) human melanoma tissue (e) urine of melanoma patient (f) urine of healthy subject. Peak identifications: (1) I.S.; (2) 3-AHP; (3) 4-AHP. Conditions as in Fig. 1.

method [6–8] and obtained by HPLC method with fluorescent detection reported in our previous work [16]. Recoveries were estimated by triplicate analysis of the real samples spiked with concentrations of selected analytes at levels similar to the levels found in samples previously analyzed. Appropriate dilutions were performed in order to obtain a signal within the linear range. Obtained recoveries ranged 88–95% and 90–96% for 3-AHP and 4-AHP ($n = 6$ for each AHP), respectively, with the R.S.D. lower than 3.1%.

4. Conclusions

In this study, we have demonstrated an application of MEKC-LIF method that based on CFSE precapillary derivatization for the sensitive and reliable determination of pheomelanin in biological materials. The reliability of the proposed method was established by satisfactory calibration linearity and repeatability of corrected peak areas. The good resolution and detection limits achieved allow the direct determination of trace pheomelanin in diverse samples including human melanoma cell cultures, hairs, melanoma tissue and urine samples of human melanoma patients. Our results demonstrate that this proposed method seems to be an attractive choice for the determination of pheomelanin in diverse samples and a promising alterative for monitoring the development of melanomas.

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