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Simultaneous determination of δ-aminolevulinic acid, porphobilinogen, levulinic acid and glycine in culture broth by capillary electrophoresis

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Abstract

Capillary electrophoretic simultaneous determination of a mixture containing δ-aminolevulinic acid, porphobilinogen, levulinic acid and glycine was investigated. With increases in the sodium tetraborate buffer concentration (5–70 m*M*), resolution of the four components was improved, but the migration time was increased. Alternatively, with increases in the applied voltage (5–22.5 kV), a shortened migration time was seen but this adversely affected resolution. The components were separated with high resolution by using a fused-silica capillary column (75 cm×75 μm I.D.) filled with 30 m*M* sodium tetraborate buffer (pH 9.3–9.4) under the applied voltage of 20 kV (constant voltage mode). When the established method was applied to the culture broth of *Rhodopseudomonas sphaeroides*, a photosynthetic bacterium, the four components mentioned above were separated with good resolution. Furthermore, the use of this method would provide a fast, sensitive and specific method for monitoring the administration of δ-aminolevulinic acid in photodynamic cancer therapy, for the measurement of δ-aminolevulinic acid dehydratase activity in erythrocytes, and for testing the δ-aminolevulinic acid assay and for impurities in drug formulation.

Keywords: Capillary electrophoresis; δ-Aminolevulinic acid; Porphobilinogen; Levulinic acid; Glycine

1. Introduction

δ-Aminolevulinic acid (ALA) has been well known as an intermediate for the biosynthesis of tetrapyrroles such as porphyrin, heme and vitamin B_{12} analogues [1,2]. Recently, ALA has received great attention as a new biodegradable herbicide and insecticide that is inhibitory to weeds, but is not harmful to crops, animals and humans [3–5], and as a prodrug for photodynamic diagnosis and therapy of cancer [6–9]. In addition, ALA has been reported to promote the growth and yield of several crops and vegetables [10]. ALA is very expensive because it is usually synthesized chemically via complex processes. Therefore, biological production using microorganisms has been suggested as an inexpensive way to produce ALA. Several photosynthetic Rhodospirillaceae, such as *Rhodospirillum*, *Rhodobacter* and *Rhodopseudomonas* species, excrete endogenous ALA, which is synthesized intra-cellularly from glycine and succinate by ALA synthetase. However, ALA dehydratase (ALAD) catalyzes the condensation of two molecules of ALA into porphobilinogen (PBG) as shown in Fig. 1 [11–13]. To enhance the accumulation of ALA in the biological production, levulinic acid (LA), a competitive inhibitor of ALAD

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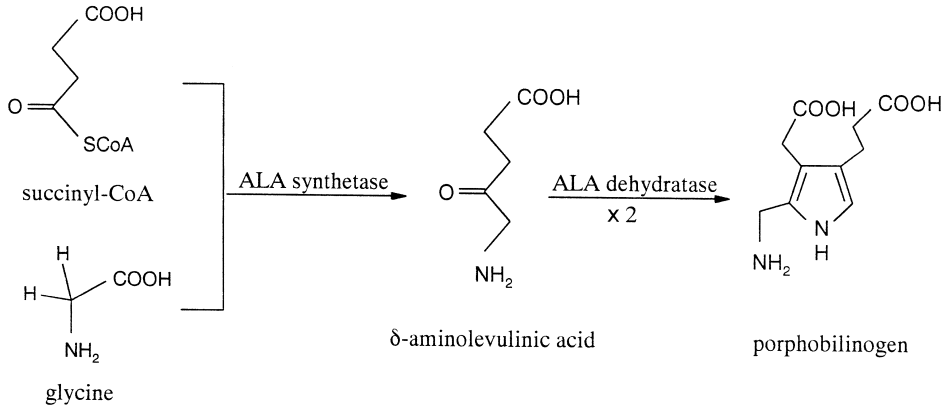

Fig. 1. Biosynthetic pathway of δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) from succinyl-CoA and glycine by photosynthetic Rhodospirillaceae, such as *Rhodospirillum*, *Rhodobacter* and *Rhodopseudomonas* species.

in tetrapyrrole biosynthetic pathway [14,15], should be added to the culture medium.

In several clinical studies, ALAD is very sensitive to lead, and the measurement of ALAD activity in erythrocytes is one of the best methods to confirm lead poisoning [16,17]. The excretion of excessive PBG in urine is usually an indication of acute porphyria [13,18].

ALA and PBG have been generally determined by the Mauzerall and Granick's method [19], but not simultaneously. They should be separated by ion-exchange chromatography and then colorized by Ehrlich's reagent prior to spectrophotometric measurement. However, this method is complex and time-consuming. They have also been quantified simultaneously by high-performance liquid chromatography (HPLC) equipped with ion-exchange chromatography [20] or ion-pairing chromatography [21]. LA has been determined by gas chromatography [22], HPLC [23] and capillary electrophoresis (CE) [24].

Recently, CE has been shown to be a powerful separation tool for the determination of biological samples [25]. In comparison to liquid chromatographic methods, CE affords rapid, automated, and reproducible separation and high-resolution with small sample volumes without sample pretreatment [26]. Bunke et al. reported the quantification of ALA and its two degradation products by CE, but did not PBG [27]. Furthermore, the simultaneous determination of ALA and PBG in urine by using micellar electrokinetic chromatography was reported by Luo et al. [18], but the sensitivity of this method was lower for ALA. However, they developed a new method for separation and detection of ALA and PBG using capillary zone electrophoresis coupled to electro-spray ionization mass spectrometry to overcome this [28]. There have been no reports regarding the simultaneous determination of ALA, PBG, LA and glycine in fermentation broth of microorganism through CE.

In the present article, we determined the optimum operation conditions of CE method for the simultaneous quantification of ALA, PBG, LA and glycine in a biological sample obtained from the fermentation broth of a photosynthetic bacterium, *Rhodopseudomonas sphaeroides*.

2. Experimental

2.1. Materials and reagents

ALA and PBG were purchased from Sigma Chemical Co. (St. Louis, MO, USA). LA was from Fluka (Buchs, Switzerland), glycine and sodium tetraborate were from Yakuri Pure Chemicals Co. Ltd. (Osaka, Japan). All solutions were prepared with deionized water. ALA, PBG, LA and glycine were dissolved at concentrations of 2, 0.25, 2 and 10 m*M*, respectively, and used as stock solutions. Sodium tetraborate was used as running buffer for

CE, and its concentration was in the range of 10 to 70 m*M* with pH 9.30~9.40 without pH adjustment.

2.2. Instrumentation

A Model 3850 Capillary Electropherograph (ISCO, Inc., USA) equipped with an on-column UV detector was used for CE. Peaks were integrated using DSCHROM (Donam, Inc., Seoul, Korea), a data acquisition software package. The untreated fused-silica capillary column (ISCO, Inc., USA) was 75 cm in length (40 cm to the detector) and had an inner diameter of 75 μm. The samples were introduced into the column using split-flow injection method.

2.3. CE conditions and calibration

The fused-silica capillary column was initially conditioned by rinsing with 0.1 *M* HCl for 30 min and with deionized water for 5 min. Then, 0.1 *M* NaOH was used to rinse the column for 30 min, followed by rinsing with water for 5 min before filling with the running buffer. Between each run, the column was rinsed with water for 1 min and then filled with running buffer. Detection was performed by direct UV absorbance at 205 nm. All experiments were conducted at 26±1°C. The two operation modes of constant current and constant voltage were used in this study. Voltage supplied was in the range of 10 to 22.5 kV. A series of standard solutions of ALA, PBG, LA and glycine were injected into the capillary column to test the linearity of response to various concentrations of the components under optimum separation conditions.

2.4. Microorganism, culture conditions and biological sample preparation

The bacterial strain, *Rhodopseudomonas* (*Rp.*) *sphaeroides* KCTC 12203, was used in this study. Strain stock was stored in culture medium with 50% (v/v) glycerol at −20°C. The culture medium consisted of glucose (60 m*M*), sodium succinate (40 m*M*), glycine (30 m*M*), corn steep liquor (3 g/l), $(NH_4)_2HPO_4$ (20 m*M*), $MgSO_4 \cdot 7H_2O$ (0.1 m*M*), $MnSO_4 \cdot H_2O$ (5 μg/l), iron(III) citrate·$5H_2O$ (3 μ*M*), nicotinic acid (1 mg/l), thiamine hydrochloride (1 mg/l) and biotin (10 μg/l). For seed culture of *Rp. sphaeroides*, 0.5 ml of the glycerol stock was transferred into a 20 ml serum bottle containing 15 ml of sterile culture medium and cultivated in shaking incubator (30°C, 200 rpm) under light illumination (5 klux) for 24 h. The main culture was conducted by transferring 3 ml of the seed culture into a 50 ml serum bottle containing 40 ml of sterile culture medium, and incubated for 48 h with the same conditions. After 24 h of cultivation, LA was added to a concentration of 15 m*M* since it is a known inhibitor of ALAD, allowing ALA to accumulate in the medium.

A total of 1 ml of the main culture broth was centrifuged at 14 000 *g* for 15 min at 4°C. Authentic solution containing ALA, PBG, LA and glycine was spiked in the supernatant diluted approximately, and the spiked supernatant was injected into the capillary column to identify the components.

3. Results and discussion

3.1. Constant current mode

In the preliminary experiments, an investigation into the effect of sodium tetraborate buffer concentration on the steady-state current and voltage (setting voltage: 20 kV and current: 45 μA) was conducted. When the concentration of sodium tetraborate buffer was from 5 to 15 m*M*, the applied voltage was maintained at 20 kV. However, the applied voltage decreased gradually with higher buffer concentrations. Therefore, a current of above 45 μA was required to maintain the voltage of 20 kV when using buffer concentration that were greater than 20 m*M*. The maximum allowable voltage and current were 20 kV and 45 μA, respectively (data not shown). Figs. 2 and 3 show the migration time (at 5–40 m*M*) and the electropherograms (at 5, 25 and 40 m*M*) dependent upon the sodium tetraborate buffer concentration, respectively. The four components were not sufficiently separated when the lower buffer concentrations were used. With higher buffer concentration, longer migration time was seen and the resolution was improved, but the peak of each component was broadened. The peaks of ALA and PBG, which overlapped at a buffer concentration

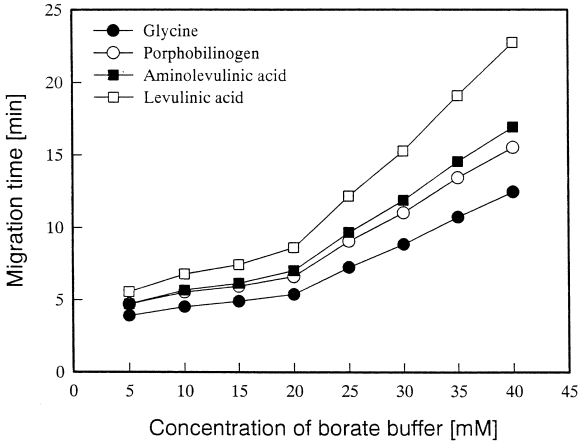

Fig. 2. Effect of sodium tetraborate buffer concentration on migration time under constant current mode (set current: 45 μA and set voltage: 20 kV). Analytical conditions; capillary column (75 cm×75 μm I.D.), detection wavelength: 205 nm, temperature: 26°C.

of 5 m*M*, were resolved completely when the buffer concentration was above 20 m*M*.

3.2. Constant voltage mode

The applied current increased steadily with the corresponding increase in the buffer concentration when run at a constant voltage of 20 kV (data not shown). Thus, the effect of sodium tetraborate buffer concentration on migration time and resolution of the four components under constant voltage mode (setting voltage: 20 kV and current: 150 μA) was investigated. As found earlier, longer migration time was seen along with a better resolution when the buffer concentration was increased, but baseline noise intensified with concentrations greater than 50 m*M* (Figs. 4 and 5). Using 30 m*M* buffer, the effect of applied constant voltage of varying intensities on separation of the four compounds was studied. The results are shown in Fig. 6. The migration time was decreased but the resolution was reduced as well with increases in the applied voltage. In addition, although a significant change in time was seen for each increment in the voltage, there was not much difference between the migration time for voltage of 20 and 22.5 kV.

We selected the voltage of 20 kV and the buffer concentration of 30 m*M* under constant voltage

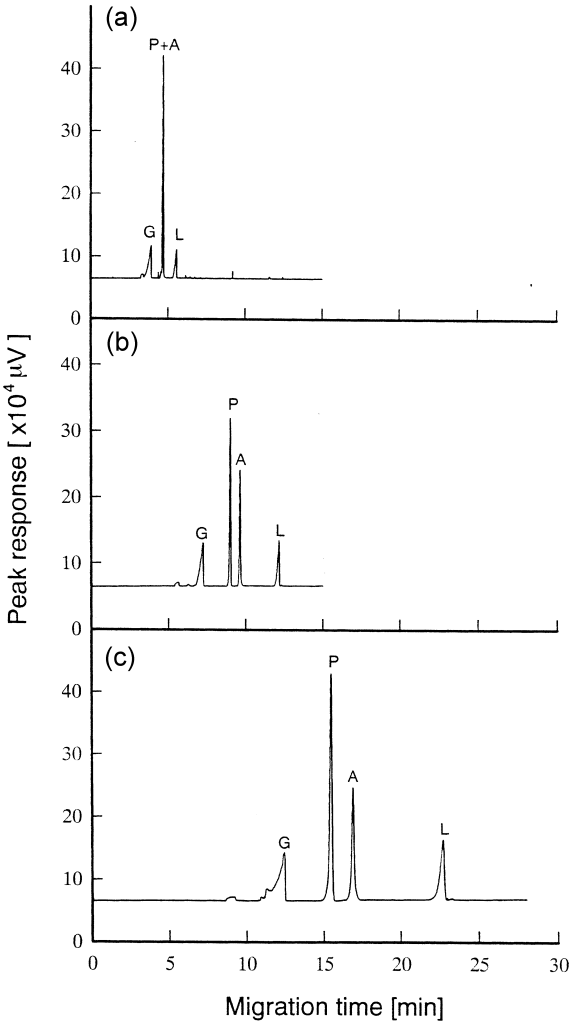

Fig. 3. Electropherograms of a mixture of ALA, PBG, LA and glycine in sodium tetraborate buffer of 5 m*M* (a), 25 m*M* (b), and 40 m*M* (c). A: ALA, P: PBG, L: LA, and G: glycine. Analytical conditions: as specified in Fig. 2.

mode as the optimum conditions for the separation of the four components for the following reasons: first, ALA and PBG were poorly separated at low buffer concentration (Figs. 2–5); second, baseline noise was seen when the buffer concentration was higher than 30 m*M* (Fig. 5c); third, migration time under constant voltage and buffer concentration of 30 m*M* (9.8 min) was shorter than those with a constant current and buffer concentration of 25 m*M* (12.2 min) (Figs. 2 and 4).

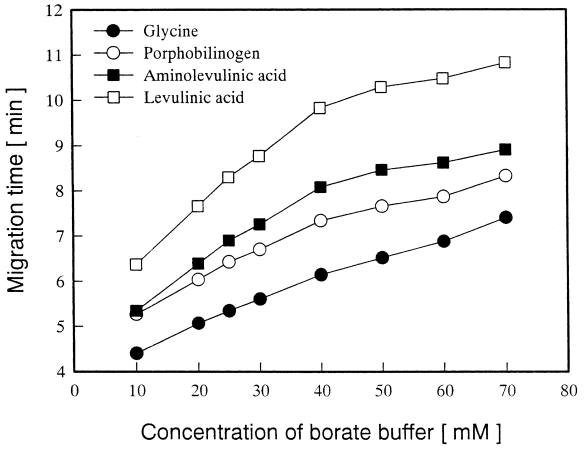

Fig. 4. Effect of sodium tetraborate buffer concentration on migration time under constant voltage mode (set current: 150 μA and set voltage: 20 kV). Analytical conditions: as specified in Fig. 2.

3.3. Calibration of ALA, PBG, LA and glycine

The linearity of response with respect to peak-height of ALA, PBG, LA and glycine was evaluated with various concentrations of each component (ALA, 0.4–2.0; PBG, 0.05–0.25; LA, 0.40–2.0; and glycine, 2.0–10.0 m*M*) under optimum separation conditions. The relationship between the peak height and the concentrations of the four components showed very linear responses, with correlation coefficient of 0.9825, 0.9999, 0.9673 and 0.9901, respectively. The relative standard deviations (R.S.D) for the migration time of ALA, PBG, LA and glycine were 7.5, 5.9, 6.1 and 3.5%, respectively, and R.S.D for the peak-height were 5.8, 4.9, 3.3 and 3.6%, respectively.

3.4. Determination of ALA, PBG, LA and glycine in biological sample

The optimum conditions for CE were applied to the determination of ALA, PBG, LA and glycine in the culture broth of a photosynthetic bacterium, *Rp. sphaeroides*. Fig. 7 shows the electropherogram of the culture broth diluted 5-fold after centrifugation. The peaks of each component were identified through the addition of authentic standard solution

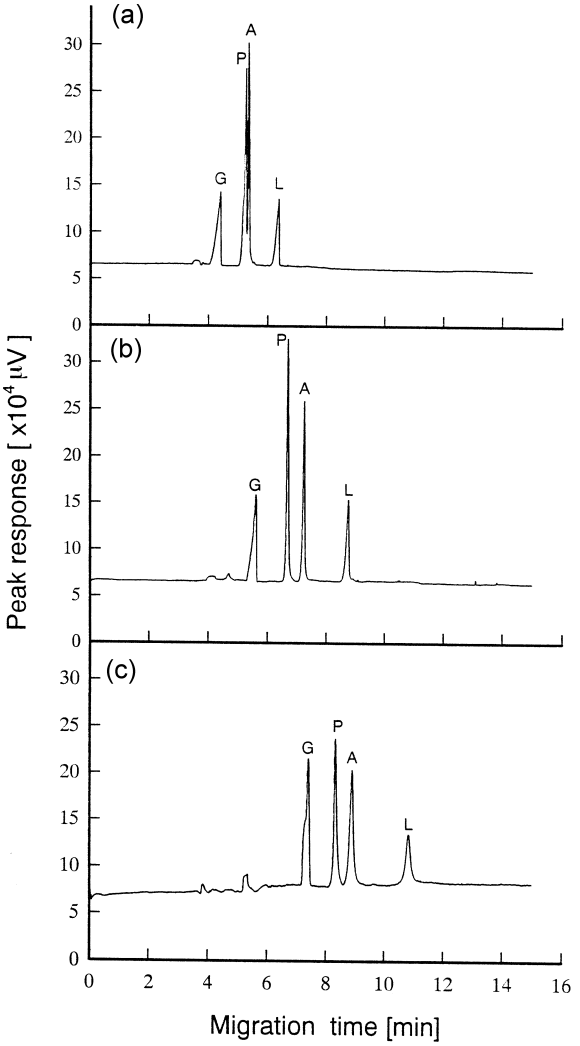

Fig. 5. Electropherograms of mixture of ALA, PBG, LA, and glycine in sodium tetraborate buffer of 10 m*M* (a), 30 m*M* (b), and 70 m*M* (c). A: ALA, P: PBG, L: LA, G: glycine. Analytical conditions: as specified in Fig. 2.

into the broth. The other peaks were components of the media, such as organic acids, vitamins, and carbohydrates. Each migration time of ALA, PBG, LA and glycine was similar to that of the standard in Fig. 5B. Therefore, CE was found to be an efficient method to simultaneously determine the presence of ALA, PBG, LA and glycine within biological samples for fermentative, medical, and clinical research.

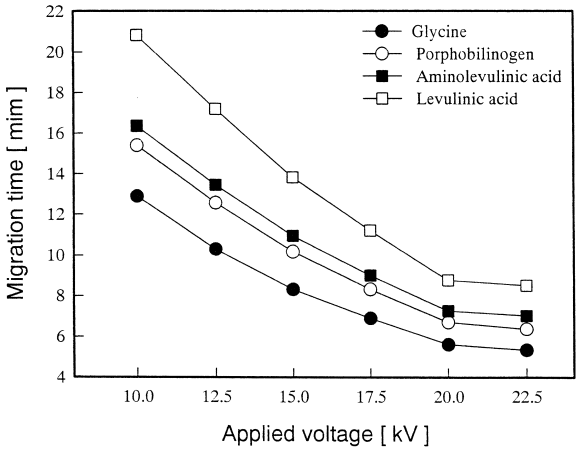

Fig. 6. Effect of applied voltage on migration time under constant voltage mode (set current: 150 μA). Analytical conditions: as specified in Fig. 2, with 30 m*M* sodium tetraborate buffer.

4. Conclusions

We established separation and quantification conditions of ALA, PBG, LA and glycine by CE. Under constant current or constant voltage, increases in the sodium tetraborate buffer concentrations gave an improved resolution of the four components, but required a longer migration time. The migration time was shortened with increases in the applied voltage, but this also adversely affected the resolution. Each component was separated well using 30 m*M* sodium tetraborate buffer with a constant voltage of 20 kV. The simultaneous determination of ALA, PBG, LA and glycine present in the culture broth of a photosynthetic bacterium, *Rp. sphaeroides*, was possible. Furthermore, it would be available to provide a fast, sensitive and specific method for monitoring the administration of ALA in photodynamic cancer therapy, measuring the activity of ALAD in erythrocytes, and testing the ALA assay and impurity in drug formulation.

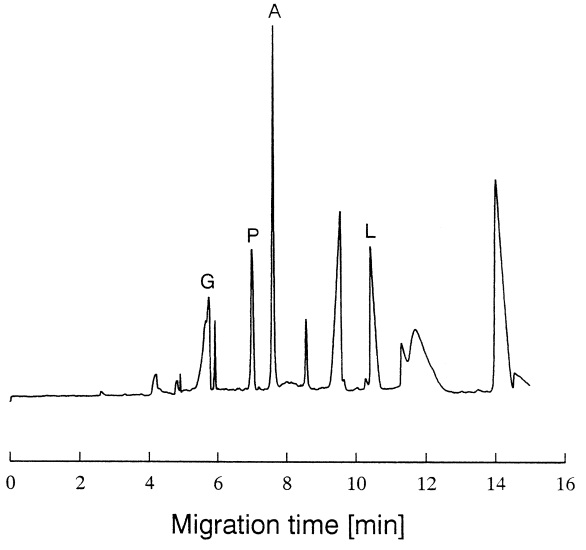

Fig. 7. Electropherogram of culture broth of a photosynthetic bacterium, *Rhodopseudomonas sphaeroides* (set current: 150 μA and voltage: 20 kV). A: ALA, P: PBG, L: LA, G: glycine. Analytical conditions: as specified in Fig. 2, with 30 m*M* sodium tetraborate buffer.

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