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Quantitative analysis of pyoluteorin in anti-fungal fermentation liquor of *Pseudomonas* species by capillary zone electrophoresis with UV–vis detector

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Abstract

This paper investigated potential utility of capillary zone electrophoresis (CZE) for very succinct but robust quantitative analysis of pyoluteorin (Plt) in anti-fungal fermentation liquor of *Pseudomonas* species. The experimental conditions for the separation and quantification of Plt were optimized at first. The optimized conditions are: 80 mmol/L pH 8.40 Gly-NaOH buffer, 51 cm total length (42 cm effective) and 75 μm I.D. capillary, 230 nm wavelength, 25 kV, 13 mbar 10 s pressure sample injection and 24 °C air-cooling. Under the optimized conditions, the migration times of Plt and the internal standard phenobarbital are 2.09 and 2.49 min, respectively, the linear response of Plt concentration ranges from 5.0 to 1000 μg/mL with high correlation coefficient ($r = 0.99977$, $n = 9$), the limits of detection (LOD) and quantification (LOQ) for Plt are 0.66 and 2.2 μg/mL, the precision values (expressed as R.S.D.) of intra- and inter-day are 1.19–1.94% and 1.55–6.21%, respectively, the recoveries of Plt at three concentration levels of 750, 250 and 50 μg/mL range from 90.31% to 97.85% and to 98.96%, respectively. The developed method can be well used for the quantification of Plt in the fermentation liquor.

Keywords: Capillary zone electrophoresis; Fermentation; *Pseudomonas* species; Pyoluteorin

1. Introduction

Pyoluteorin (Plt) is an antibiotic substance produced by certain strains of *Pseudomonas* species, such as *Pseudomonas fluorescens* strain Pf-5, CHA0 and fluorescent *Pseudomonas* sp. *M18* [1,2]. It is composed of a bichlorinated pyrrole linked to a resorcinol moiety (see Fig. 1), which can inhibit phytopathogen fungi, including the plant pathogen *Pythium ultimum* effectively [3] and suppress plant disease caused by this fungus and in some instances contributes to the ecological competence of the producing strain within the rhizosphere [4–6].

The quantification of Plt in fermentation liquor of *Pseudomonas* strain CHA0, Pf-5 and M18 could be performed with some methods. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were used for the quantitative analysis of fractionate antibiotics recovered from natural materials [7], De Souza and Raaijmakers applied HPLC and TCL for the detection of Plt [8]. Besides, liquid chromatography–mass spectrometric (LC–MS) method [9] were also used for determining antibiotic metabolites. However, these methods hold some connatural disadvantages, such as complex of procedure, expensive consume of chromatographic column and of organic reagents with chromatographic grade, pollution of organic reagents in laboratory, etc. [10–12]. These disadvantages can be overcome by high performance capillary electrophoresis (HPCE).

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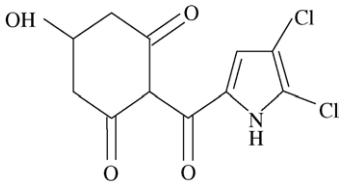

Fig. 1. Chemical structure of pyoluteorin.

HPCE is one of the most powerful analytical and separative techniques [10–12]. With simple online sample stacking [13–15], its determination sensitivity can reach at the degree of ng/mL, even at the level of pg/mL [16] with a normal UV–vis detector. However, no electrophoretic method has been reported for the determination of Plt so far.

Therefore, in this work, we developed CE method for the quantification of Plt in fermentation liquor of *Pseudomonas* sp. *M18*, optimized the parameters affecting the separation and sensitivity of Plt, and conducted the investigations on reliability, including specificity, selectivity, linearity, LOD, LOQ, accuracy and precision, etc. Herein, we reported the very succinct method and results.

2. Materials and methods

2.1. Apparatus

Experiments were carried out with an ACS 2000 HPCE apparatus (Beijing Cailu Scientific Inc., Beijing, China). The apparatus were comprised of a digital electric power supply (up to voltage 30 kV), a HW-2000 chromatography workstation and an UV–vis detector that could perform wavelength scanning from 190 to 740 nm. A fused-silica capillary was used (Factory of Yongnian Optical Fiber, Hebei, China). The capillary was 75 μm I.D. × 51 cm (42 cm effective length). I.D. 50 mm capillary was not chosen. Since in the investigation of Plt determination, the separation of Plt from others, such as MeOH and internal standard, could be well performed as shown in Fig. 3. Thus, we chose I.D. 75 μm capillary with better sensitivity, but not I.D. 50 μm one with poorer sensitivity. An Ultra-pure Water System (SG Ultra Clear system, Wasseraufbereitung und Regenerierstation Gmbh, Germany) was used to produce ultra pure water with specific conductivity down to 0.055 μS/cm for the analysis of HPCE.

2.2. Chemicals

Glycine (biological grade reagent) was purchased from the Shanghai Shisheng Cell and Bio-technology Company (Shanghai, China). Sodium hydroxide, Hydrochloric acid and methanol (guarantee reagent grade, GR) were purchased from the Shanghai Chemistry Reagent Company (Shanghai, China). Phenobarbital was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Plt used as standard reagent was purified from fermentation liquor of *Pseudomonas* species in-house. The purified Plt was analyzed and its purity reaches the chroma pure grade.

2.3. Fermentation conditions

The strain studied in this work was sp. *M18*, which was pre-cultured in a 150 mL conical flask containing 15 mL aliquot of King's B liquid broth at 28 °C for 12 h. A portion (8 mL) of this culture was used to inoculate 150 mL l aliquots King's B medium in 500 mL conical flasks at 28 °C for 60 h for production of Plt.

2.4. Extraction procedure

Plt, as well as phenobarbital, can be well purified from fermentation liquor of the strain sp. *M18* suspension with ethyl acetate [17]. 600 μL ethyl acetate and 50 μL internal standard (phenobarbital, 500 μg/mL) were added into 600 μL culture in one Eppendorf tube, then the tube was strongly vibrated for 2 min. After that, the mixture in the tube was centrifuged at 5590 × *g* for 5 min and Plt was extracted from the culture to the organic phase. The organic phase was transferred to another Eppendorf tube. Then the 600 μL culture was extracted by 300 μL ethyl acetate again. The two organic phases were combined together and dried by vacuum dryness. The residue after dryness in the tube was dissolved with MeOH and diluted to 500 μL with buffer for CE analysis. All solutions were stored at 4 °C in a refrigerator until use.

2.5. Preparation of buffer and standard solution

A series of Gly-NaOH buffers with different pH values and concentrations were prepared. The pH values of the buffers ranged from pH 8.0 to 11.5 and the concentrations changed from 20 to 200 mmol/L. The stock solution of internal standard was prepared by dissolving 1.0 mg phenobarbital in 2.0 mL MeOH and the concentration was 500 μg/mL.

The sample standard solutions were prepared in accordance with the following procedure. A series of stock solutions with different concentrations of Plt (0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5, 7.5 and 10 mg/mL) were made ahead of schedule, adding different concentration solutions of Plt 50 μL and phenobarbital 50 μL into blank fermentation liquor, then conducted in accordance with the procedure in Section 2.4. The final concentrations of dilution were 5, 10, 20, 50,100, 250, 500, 750 and 1000 μg/mL.

2.6. Analytical procedures

HPCE was conducted as these described below. Before use, the new capillary was conditioned by rinsing with 1 mol/L NaOH for 20 min, ultra-pure water for 20 min, 1 mol/L HCl for 20 min, ultra-pure water for 20 min, and running buffer for 30 min, in order. Between injections the capillary was rinsed with the running buffer for 3.0 min.

Detection wavelength was set at the maximum absorbance wavelength of 230 nm detected by an automatic wavelength scanning of Plt in the UV–vis detector in the CE apparatus. Gly-NaOH buffer (80 mmol/L), pH 8.40 was used as background electrolyte. Pressure injection of sample was chosen due to bias of electrokinetic sample injection to Plt as well as phenobarbital. The pressure was set at 13 mbar and the injection time was controlled at 10 s. The ultimate work voltage was 25 kV. Temperature control of capillary was carried out with 24 °C air-cooling. Detection data were collected and processed with the HW-2000 Chromatography Workstation Software.

3. Results and discussions

We firstly optimized the experimental conditions. After than, we investigated the validity of developed method. The followings are the results of optimization and investigations.

3.1. Optimization of conditions

3.1.1. Influence of applied voltage

Applied voltage has very important influence on migration time, current strength and resolution (see Fig. S1, available at doi:10.1016/j.jchromb.2005.07.047). The experiments show that the migration time of Plt decreased with the increase of the used voltage. Lower voltage produced longer migration time and poorer peak shape. At 30 kV, the migration time decrease greatly, but the resolution of peaks reduced possibly due to much high Joule's heating within the capillary and fast electromigration of analytes. Considering fast determination of Plt and maintain of good resolution, 25 kV was chosen as the work voltage as used in the following experiments.

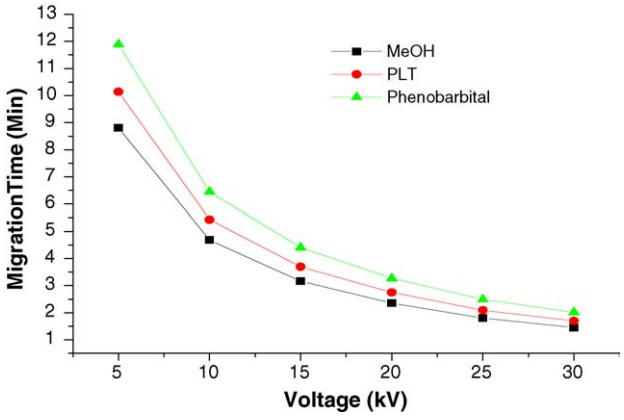

Fig. S1. Influence of applied voltage on migration times of Plt, internal standard and MeOH. Conditions: 80 mmol/L pH 8.40 Gly-NaOH buffer, 51 cm total length (42 cm effective length) and 75 μm I.D. capillary, 230 nm wavelength, 13 mbar 10 s pressure sample injection, 24 °C air-cooling.

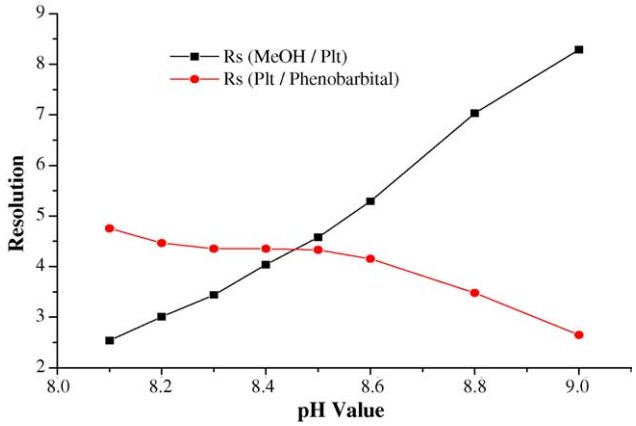

Fig. 2. Resolution between MeOH, Plt and phenobarbital as a function of pH value. Conditions: 25 kV, pH 8.10–9.00 80 mmol/L Gly-NaOH buffer, 51 cm total length (42 cm effective length) and 75 μm I.D. capillary, 230 nm wavelength, 13 mbar 10 s pressure sample injection, 24 °C air-cooling.

3.1.2. Effect of pH values of buffer

pH value of Gly-NaOH buffer has key importance on migration time and separation of Plt and internal standard, as evidently shown in Fig. 2, the increase of pH value from pH 8.10 to 9.00 led to the decrease of resolution between Plt and phenobarbital, but the increase of resolution between MeOH and analyte, over pH 8.80 the peak of Plt and internal standard can not be separated on baseline. The best resolution of Plt and internal standard acquired at pH 8.10, however, under this pH value, the resolution of MeOH and Plt was bad, which was not allowed in the determination analysis. Considering good separation among the MeOH, Plt and internal standard peaks, as well as short migration times of these peaks (Fig. S2, available at doi:10.1016/j.jchromb.2005.07.047), we finally chose the pH 8.40 as the optimized pH value. Under this condition, the resolutions are quite well (Fig. 2) and the migration times of Plt and phenobarbital peaks were 2.09 and 2.49 min, respectively (as shown in Fig. S2).

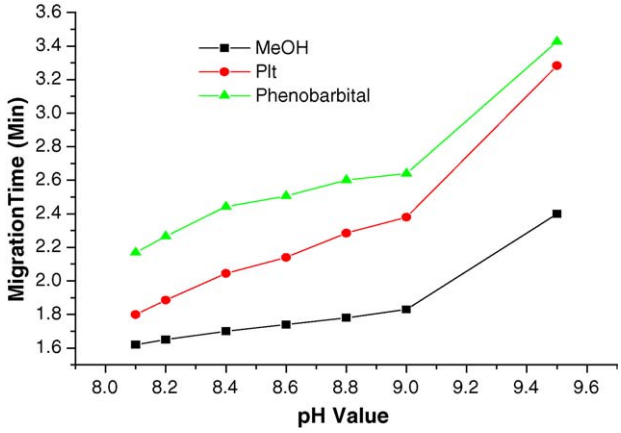

Fig. S2. Effect of pH value on the migration times of MeOH, Plt and phenobarbital. Conditions: pH 8.10–9.00 Gly-NaOH, 25 kV, other conditions are the same as those in Fig. 2.

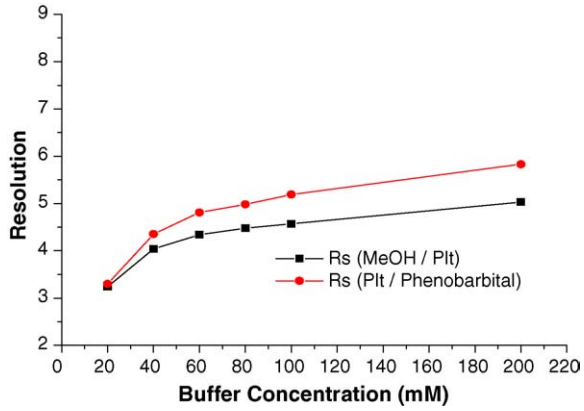

Fig. S3. Effect of buffer concentration on resolution between MeOH, Plt and internal standard. Conditions: 20–200 mol/L pH 8.40 Gly-NaOH buffer, 25 kV. Other conditions are the same as those in Fig. 2.

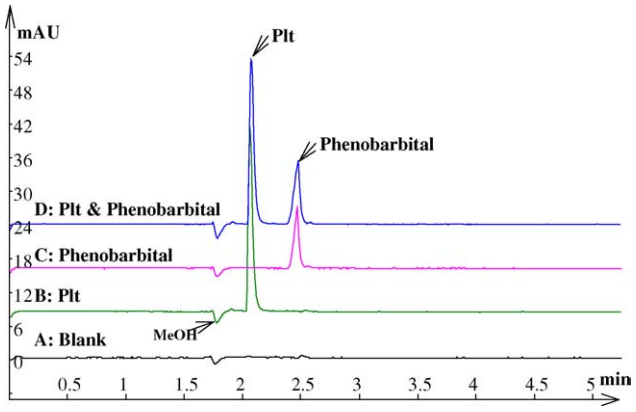

Fig. 3. Electrophoregrams of CZE: (A) blank sample of fermentation; (B) Plt extracted from fermentation liquor without internal standard in buffer; (C) internal standard phenobarbital extracted from fermentation liquor in buffer; and (D) Plt and internal standard extracted from the fermentation liquor in buffer. Conditions: 25 kV, 80 mmol/L pH 8.40 Gly-NaOH buffer. Other conditions are the same as those in Fig. 2.

3.1.3. Effect of buffer concentration

Buffer concentration has evident influence on resolution, peak shape, sensitivity and detection noise, especially electric current (see Fig. S3, available at doi:10.1016/j.jchromb.2005.07.047). The results indicate that the resolution among MeOH, Plt and internal standard were increased with the increasing of buffer concentration, at the same time high concentration of buffer led to much higher current. With the 80 mmol/L buffer, the resolution of Plt and phenobarbital were acceptable, the current was at a lower level, and the peak width of Plt was narrow. Finally, 80 mmol/L was chose as the optimized concentration. Our comparative studies showed that 80 mmol/L buffer could supply a quite stable background buffer for the determination of Plt for at least 1 month.

3.1.4. Optimized conditions

Finally, we achieved the following optimized conditions: 80 mmol/L pH 8.40 Gly-NaOH buffer, 51 cm total length (42 cm effective) and 75 μm I.D. capillary, 230 nm wavelength, 25 kV, 13 mbar 10 s pressure sample injection and 24 °C air-cooling.

3.2. Method validation

Validity of a newly developed analytical technique needs to be strictly demonstrated before its application to actual determination of antibiotics. In this work, various validation criteria of the developed method of capillary zone electrophoresis (CZE), such as specificity, precision, linearity of response, accuracy, detection limit (LOD) and quantification limit (LOQ), etc. were assessed as described below.

3.2.1. Specificity of method

Specificity of a newly developed analytic method should be firstly demonstrated due to its key importance and priority. In the demonstration of this paper, numerous samples of Plt extracted from the fermentation liquor with ethyl acetate were analyzed repeatedly under different conditions. The purpose of the analyses was to make sure whether or not the peak of target analyte in the electrophoregrams was exclusive under the given experimental conditions optimized above. We found no interferential peaks appeared in fermentation in different buffer pH value, concentration, and longer separation time.

Fig. 3 shows the demonstration of exclusive peak of target analyte. Panel A is the electrophoregram of a blank sample of fermentation, only MeOH peak can be seen. Panel B unveils the Plt extracted from fermentation liquor without internal standard in buffer. Panel C proves the symmetrical peak of internal standard phenobarbital extracted from fermentation liquor in buffer. Panel D displays the peaks of MeOH, Plt and internal standard extracted from the fermentation liquor. All of the experiments discussed above evidently indicate the specificity of the developed CZE method for the determination of targeted analyte.

3.2.2. Linearity

Linearity of detector response versus concentration of analyte was determined by constructing a calibration curve from a set of standard solutions of Plt extracted from blank fermentation liquor with nine different concentrations (see Fig. S4, available at doi:10.1016/j.jchromb.2005.07.047). The linear ranged from 5 to 1000 μg/mL, the correlation coefficient for Plt standards was 0.99977 ($n = 9$).

3.2.3. Precision

Precision of a new method was evaluated by measuring intra- and inter-day relative standard deviations (R.S.D.) of peak areas ratio between analyte and internal standard extracted from the fermentation liquor. The intra-day values of R.S.D. were calculated based on six replicate runs every 2 h of three different concentrations of 50, 250 and 750 μg/mL in 1 day. The inter-day values of R.S.D. were

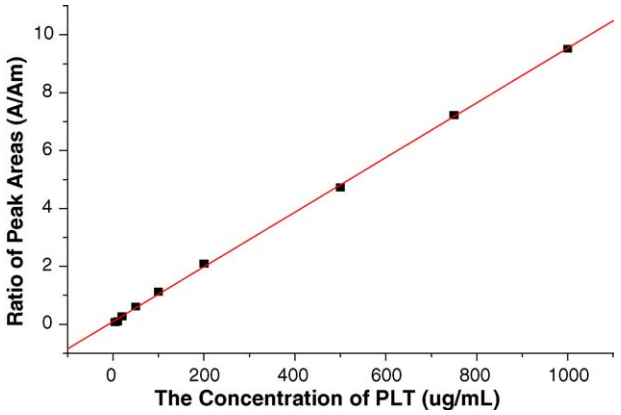

Fig. S4. Linearity of peak area ratio of Plt and phenobarbital vs. the concentration of Plt. pH 8.40; the conditions are the same as those in Fig. 2.

Table 1
The intra-day variations of peak area ratios between Plt and phenobarbital ($r = 3$, $n = 6$)

Sample concentration (μg/mL)	Peak area ratio	S.D.	R.S.D. (%)
50	0.62	0.01	1.94
250	3.19	0.04	1.19
750	7.58	0.12	1.64

evaluated using five date sets from the same three standard solutions obtained on five different days. The two precision results were given in Tables 1 and 2, respectively. The values of R.S.D. of the intra-day runs ranged from 1.19% to 1.94%. The inter-day precision or repeatability, expressed as the R.S.D. was 1.44–6.88%. The results implied that the operating conditions selected above could provide a good stable electrophoretic system with quite good repeatability (see Tables 1 and 2).

3.2.4. Accuracy

The accuracy was evaluated from a set of recovery experiments performed on samples with three different concentrations. A blank fermentation liquor of *Pseudomonas* species without Plt was spiked with standard solution of Plt and internal standard of Phenobarbital. The concentrations of Plt in the spiked fermentation liquor were controlled at 50, 250, 750 μg/mL, and the concentration of phenobarbital was 50 μg/mL. The samples used for the recovery runs were prepared as described above (see Section 2.4). The recovery was calculated by the ratio value of Plt and phenobarbital which extracted from the fermentation liquor to the ratio value of them resolving in the run buffer directly. The recovery values of Plt in the spiked samples of fermentation liquor were 98.96 ± 2.81% at 50 μg/mL, 97.85 ± 6.70% at 250 μg/mL, and 90.31 ± 1.00% at 750 μg/mL (see Table 3), these values were all within the acceptable range.

Table 2
The inter-day variations of peak area ratios between Plt and phenobarbital ($r = 3$, $n = 5$)

Sample concentration (μg/mL)	Peak area ratio	S.D.	R.S.D. (%)
50	0.58	0.04	6.88
250	3.12	0.05	1.44
750	7.38	0.14	1.84

Table 3
The recovery of Plt under different concentrations ($r = 3$, $n = 3$)

Added Plt (μg/mL)	Peak areas ratio of Plt and phenobarbital		Recovery (%)
	Extraction from fermentation liquor	Resolving in buffer directly	
50	0.59	0.60	98.96 ± 2.81
250	3.17	3.21	97.85 ± 6.70
750	7.39	8.18	90.31 ± 1.00

3.2.5. Limits of detection and quantification

It was calculated by setting the signal-to-noise ratio of 3:1 that the limit of detection (LOD) of the developed method was 0.66 μg/mL under 10 s, 13 mbar pressure sample injection. The limit of quantification (LOQ) was computed by use of signal-to-noise ratios of 10:1 that of the developed method was 2.20 μg/mL. A further reduction of LOD and LOQ could be achieved by using long sample injection time, but the peaks of Plt and phenobarbital became wide.

4. Conclusions

Plt is a new anti-fungal metabolite. It is essential to have a appropriate method to analyze its production in fermentation liquor. In this paper, a simple and robust method for the separation and quantification of Plt in fermentation liquor was developed based on CZE. Owing to the high precision, good accuracy and much wide linear range of determination and low detection limit, the new method described allows rapid monitoring of antibiotic production by *Pseudomonas* species in different culture conditions in laboratories or factories. Recent studies in our laboratory were to enhance the output of Plt in cultures of *Pseudomonas* species, this method is a good helper for the purpose.

Acknowledgments

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