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Determination of pyrroloquinoline quinone by capillary zone electrophoresis

Zdeněk Glatz*, Martina Moravcová, Oldřich Janiczek

Department of Biochemistry, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

Abstract

A new method for the determination of pyrroloquinoline quinone by capillary zone electrophoresis has been developed. Separation conditions have been optimised with the respect to different parameters including pH and ionic strength of the background electrolyte, separation voltage and temperature of the capillary. A buffer consisting of 50 m*M* β-alanine–HCl pH 3.0 was found to be the most suitable electrolyte for this separation. An applied voltage of 25 kV (negative polarity) and a temperature of 25°C gave the best analysis of pyrroloquinoline quinone. The linear detection range for concentration versus peak area for the assay is from 5 to 500 μ*M* (correlation coefficient 0.9998) with a detection limit of 0.1–0.2 μ*M*. The inter-day reproducibility of the peak area was 2.5% and the inter-day reproducibility of the migration time was below 0.18%.

Keywords: Pyrroloquinoline quinone; Vitamins

1. Introduction

In 1979 pyrroloquinoline quinone-PQQ (4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid, Fig. 1) was identified as a coenzyme of methanol dehydrogenase [1]. It was subsequently detected in several bacterial enzymes [2]. Later, it was reported that PQQ is nutritionally important as a vitamin or growth factor in mammals [3] and trace levels of PQQ were detected in human and rat tissues [4]. Coenzyme PQQ, therefore seems to play an important role in mammals. The following pharmacological activities of PQQ have so far been reported: a protective effect against liver [5], heart [6] and brain injury [7], an inhibitory activity on the formation of hydrocortisone-induced cataract in embryos [8], a radical scavenger-like activity [9,10], enhanced effect on the activity of DNA synthesis in human fibroblasts [11] and a nerve growth factor-induced activity [12]. Consequently, the determination of PQQ in biological samples is of interest to many scientists.

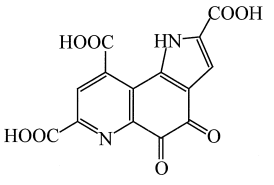

Fig. 1. The structure of PQQ.

Several methods such as bioassays based on the reconstitution of apoenzyme of glucose dehydrogenase [13,14], immunological assay [15], HPLC [13,16–18] and isotachophoresis [19] have been

*Corresponding author. Tel.: +42-5-4112-9401; fax: 42-5-4121-12149.
E-mail address: glatz@chemi.muni.cz (Z. Glatz)

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described for the determination of PQQ. A highly sensitive gas chromatography–mass spectrometry method has also been reported [4]. Recently Esaka et al. [20] have introduced a capillary electrophoretic method for the separation of PQQ and amino acid derivatives of PQQ. The method has been applied mainly to study mechanism and kinetics of the reaction of PQQ with amino acids and no quantitative parameters of this method have been evaluated.

In this study we developed a simple and sensitive method for the determination of PQQ by capillary zone electrophoresis. The method was successfully used to quantify PQQ in a growth medium of methanol-utilising bacteria and to study the reaction between PQQ and some amino acids.

2. Experimental

2.1. Materials and reagents

PQQ was obtained from Fluka (Buchs, Switzerland). All other chemicals and solvents were of analytical reagent grade, supplied from Sigma (St. Louis, MO, USA). All solutions were prepared with Milli Q Academic water (Millipore, Milford, WA, USA) and filtrated through a 0.2 μm membrane filter.

2.2. Capillary electrophoresis conditions

A Hewlett-Packard ^{3D}Capillary Electrophoresis System (Hewlett-Packard, Waldbronn, Germany) with a diode-array detector was used to carry out all CZE separations. Data were collected on a HP Vectra VL5 166 MHz personal computer using the Hewlett-Packard ^{3D}CE Chemstation Software. A Hewlett-Packard extended light path capillary (50 μm I.D., 363 μm O.D., 64.5 cm total length, 56.0 cm effective length) was used for all CZE separations. Injection was accomplished by an application of 50.0 mbar pressure to the inlet vial for 6.0 s. Separations were performed at 25 kV (negative polarity). Samples were detected using a diode-array detector at 249 nm with a bandwidth 10 nm. The capillary was washed with the background electrolyte for 3 mm before each run.

2.3. Sample preparation

Paracoccus denitrificans (CCM 982) obtained from the Czech Collection of Microorganisms, (Brno, Czech Republic) was grown aerobically at 30°C in a medium containing 1% methanol, 15 m*M* Na_2HPO_4, 30 m*M* KH_2PO_4, 50 m*M* NH_4Cl, 1 m*M* $MgSO_4$, 30 m*M* $FeC_6H_5O_2$ and minerals mixture [21]. For estimation of the production of PQQ in the supernatant, the culture broth was centrifuged at 10 000 *g* for 10 min.

The Accu Bond 200 mg C_{18} cartridge (J&W Scientific, Folsom, CA, USA) was washed with 10 ml of methanol and subsequently with 10 ml of water. The supematant was acidified with HCl to pH 2.0 and 1 ml portion of the clear supematant was applied to the cartridge. After washing with 10 ml of 2 m*M* HCl, PQQ was eluted with 1 ml of 70% methanol [22]. The sample was evaporated to dryness under vacuum. The residue was dissolved in 200 μl water for CZE analysis.

2.4. Reactions of PQQ with amino acids

Derivatives of amino acids with PQQ were prepared by incubation of PQQ (1 m*M*) and respective amino acid (25 m*M*) in 50 m*M* phosphate buffer pH 7 for 24 h at a room temperature [20]. The reaction mixtures were diluted 10 times with deionized water and analysed by CZE.

3. Results and discussion

3.1. Optimisation of CZE analysis

Buffer solution pH is one of the key parameters in CZE separation. Electroosmotic flow, solute charge state and analysis time are all affected when the pH buffer changes. Esaka et al. [20] used 10 m*M* phosphate buffer pH 8 containing 5% DMSO as a background electrolyte for the separation of PQQ and PQQ derivatives. At this pH PQQ has a highly anionic character due to pK values of 1.60, 2.20 and 3.30 for carboxyl groups [23] and migrates against the electroosmotic flow. It results in longer migration time, around 10 min. To obtain faster analysis phosphate, citrate and β-alanine–HCl separation

buffers pH 2.5–3.5 were evaluated in order to find an appropriate buffer for determination of PQQ. The silanol groups are hardly ionized at low pH and, consequently, the electroosmotic flow is thus minimised. Using these electrolyte systems, we investigated the effects of pH and ionic strength of background electrolyte, separation voltage and capillary temperature on separation efficiency and analysis time. Among the three, β-alanine–HCI gave the best overall performance: a sharp peak of PQQ with a short migration time and a stable baseline. Fig. 2 shows an electropherogram of PQQ standard under optimal conditions of 50 m*M* β-alanine–HCl (pH 3.0), 25 kV separation voltage (negative polarity) and 25°C temperature of capillary.

Reproducibility, linearity and sensitivity of the method were tested (Table 1). Reproducibility of migration time and peak area was evaluated for a 100 μ*M* standard of PQQ. The relative standard deviations were calculated for 10 analyses of the standard. Table 1 shows good reproducibility obtained for peak area and excellent reproducibility obtained for migration time, which is probably a result of the suppressed electroosmotic flow. Calibration graph was measured in the range 5–500 μ*M* of PQQ. The calibration graph for peak area was linear in this range with correlation coefficient better then 0.999 while the relationship between peak height and concentration was non-linear. The detection limit, determined by consecutive dilution of the standard, was in the range 0.1–0.2 μ*M* (1–2 fmols for 6 nl injection) at a signal-to-noise ratio of 3.

Table 1
Parameters of the developed method

Parameter	Value
Run time[a]	7 min.
Separation efficiency (*N*)	164 000
Migration time reproducibility ($n=10$)	0.18%
Peak area reproducibility ($n=10$)	2.5%
Linearity	5–500 μ*M*
Correlation coefficient ($n=10$)	0.9998
Limit of detection	0.1–0.2 μ*M*

[a] Including a 3 min. flush cycle. Samples: standards of PQQ in deionized water; Separation conditions: 50 m*M* M β-alanine–HCl (pH 3.00), separation voltage 25 kV (negative polarity), separation current 85 μA, 50 μm extended light path capillary (64.5 cm total length, 56.0 cm effective length), detection at 249 nm, temperature of capillary 25°C, injection 50 mbar for 6 s.

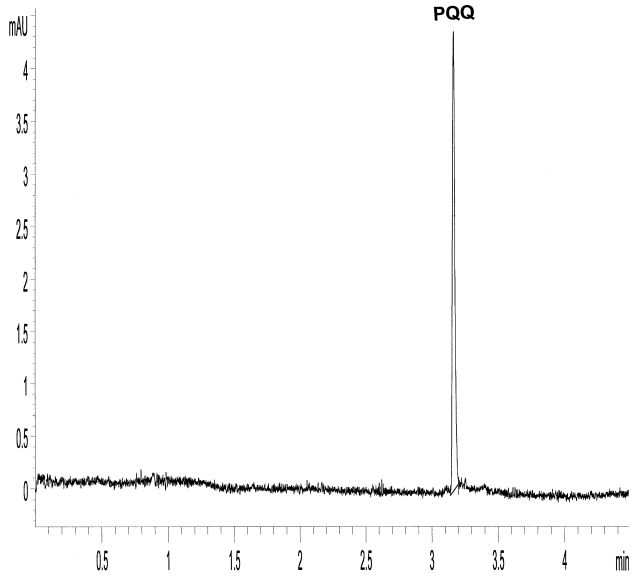

Fig. 2. CZE analysis of 10 μ*M* PQQ standard in deionized water. Separation conditions: 50 m*M* M β-alanine–HCl (pH 3.00), separation voltage 25 kV (negative polarity), 50 μm extended light path capillary (64.5 cm total length, 56.0 cm effective length), detection at 249 nm, temperature of capillary 25°C, injection 50 mbar for 6 s.

3.2. Biological sample analysis

The method was applied to determine PQQ in the culture medium of methanol-growth bacterium *P. denitrificans*. The samples were simply pretreated using C_{18} reversed-phase cartridge. The identification of PQQ was performed by spiking with standard (Fig. 3A and B). Furthermore, diode-array spectroscopy measurements, made on standard and sample peaks confirmed the identity of PQQ (match factors better than 900). The concentration of 0.2 μM PQQ in culture medium was determined. The estimated value agreed well with that published by van Kleef and Duine [24].

3.3. Reaction of PQQ with amino acids

The detection of free PQQ in biological samples is very difficult not only due to its low concentration, but also because of its high reactivity. PQQ as a quinone is electrophilic and thus it can react with nucleophilic reagents: alcohols, aldehydes, ketones, thiols, phenylhydrazines, amines, amino acids etc. Amino acids can be considered to be the most important and widely distributed nucleophilic components in biological sample. PQQ reacts with amino acids under aerobic conditions to form the corresponding oxazole derivatives (Fig. 4) that are proba-

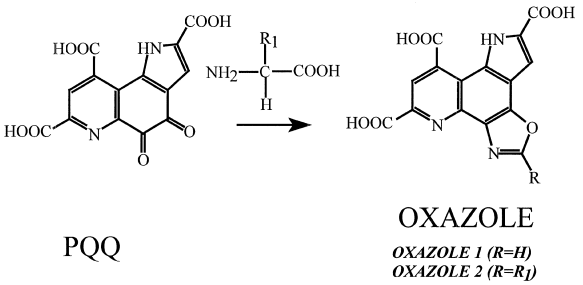

Fig. 4. Reaction of amino acids with PQQ.

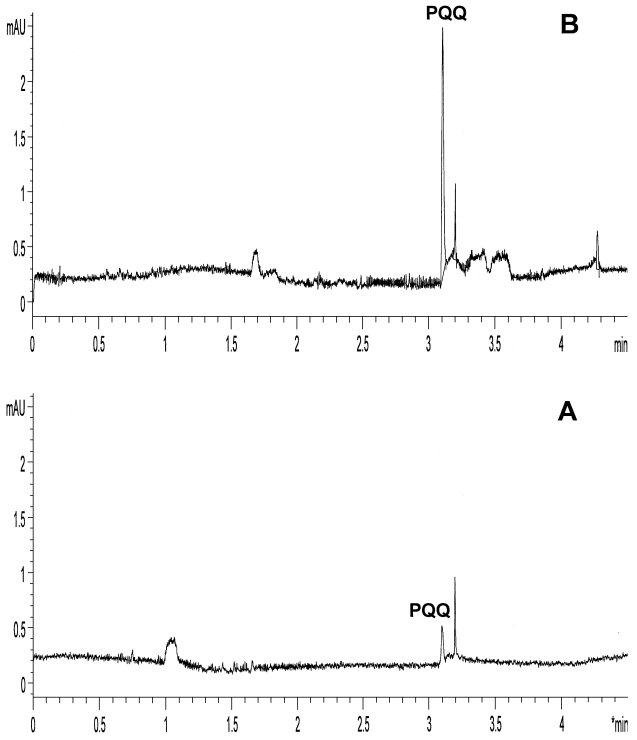

Fig. 3. Electropherograms of culture medium obtained from *Paracoccus denitrificans* after pretreatment with Accu Bond C_{18} cartridge, for conditions see Experimental. (A) Culture medium. (B) Culture medium spiked with PQQ standard. Separation conditions as in Fig. 2.

bly the major products from PQQ in reactions in biological fluids. Moreover, Esaka et al. [20] found that PQQ spiked in bovine serum is converted into unsubstituted oxazole 1 because the rate of the formation of oxazole 1 from glycine is much faster than that of oxazole 2 from other amino acids. The baseline resolution between PQQ and oxazole 1 is thus important with respect to the possibility of using this method for the determination of free PQQ in biological samples.

Therefore, the developed method was used for the analysis of the reactions of PQQ with typical examples of amino acids: glycine and valine. The reaction of PQQ with glycine gave oxazole 1 exclusively (Fig. 5A). In the reaction of PQQ with valine, oxazole 2 was generated with some PQQ remaining unreacted (Fig. 5B). As can be seen from Fig. 5, more than adequate resolution between PQQ and oxazole 1 would be achieved in one sample. The absorption spectra of both oxazoles obtained by diode-array detector exhibited the characteristic absorption maxima around 255 nm and 420 nm (Fig. 6). These results are in close agreement with those obtained by Esaka et al. [20] and Urakami et al. [25].

3.4. Comparison with other methods

Several methods have been described for the determination of PQQ. The method described in this paper has several advantages over those currently used. PQQ is negatively charged and UV absorbing at the pH used, and it can therefore be separated and detected directly, without the addition of substances for complex binding. The method is simple and easy to be used and can be readily automated. The migration time of PQQ 3.2 min. allows running many samples in a short time. Furthermore small amounts of samples and buffers are necessary.

The usually low concentration sensitivity typical for CZE separations with UV detection is in this method closer to the HPLC method than expected. Reports have described detection limits between 0.05

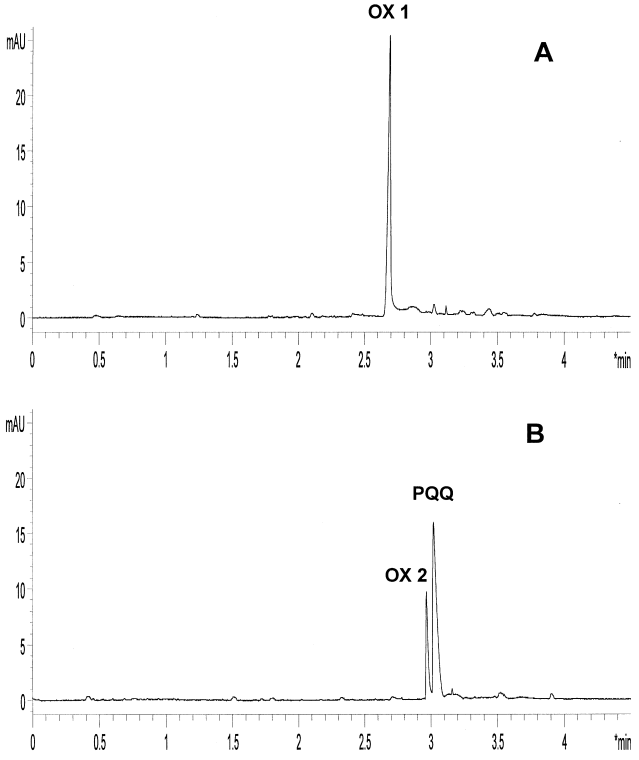

Fig. 5. Electrophoreograms of reaction mixtures of PQQ with (A) glycine and (B) valine at pH 7.0 for 24 h at a room temperature.

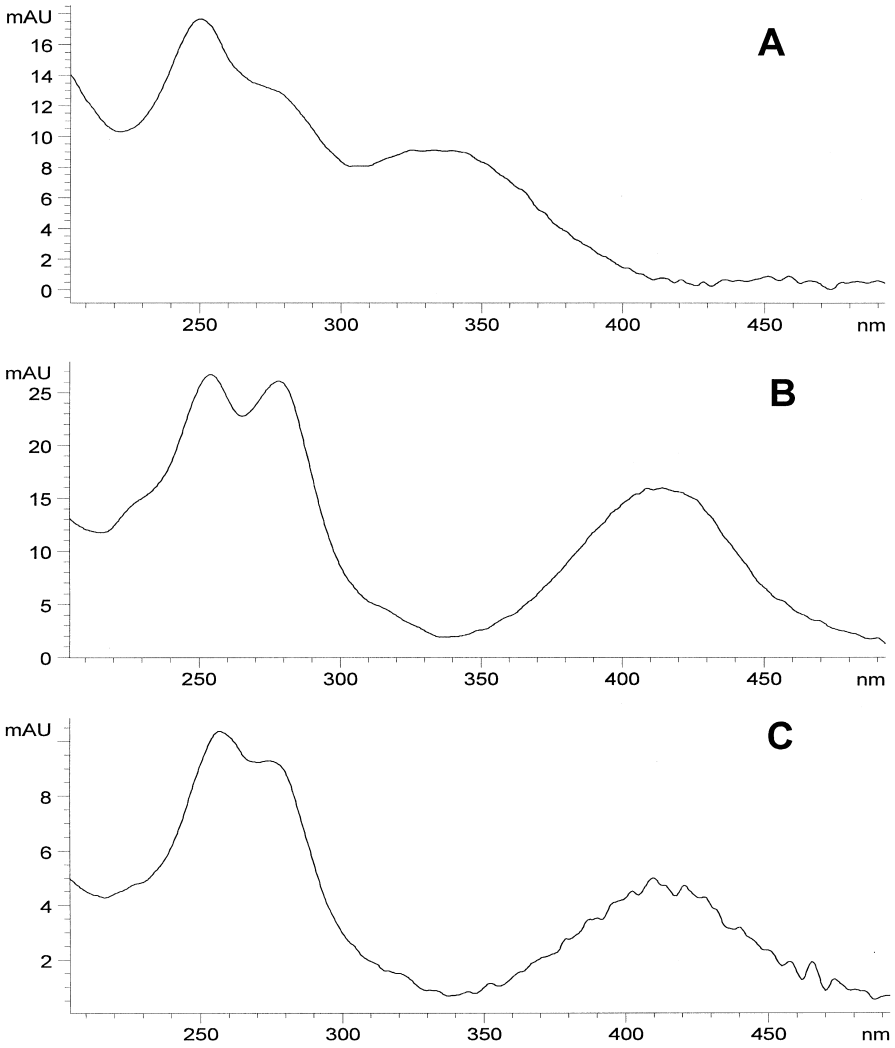

Fig. 6. Absorption spectra of the peaks: (A) PQQ, (B) oxazole 1-OX1 and (C) oxazole 2-OX2.

and 0.08 μ*M* using HPLC and fluorescence detection [13,16], 0.01 μ*M* using HPLC and amperometric electrochemical detection [17] and 0.1 μ*M* using isotachophoresis and potential gradient and UV detection [19]. In our CZE system the detection limit is 0.1–0.2 μ*M*. This low limit of detection was due to the high separation efficiency ($N=164\,000$) in comparison with HPLC ($N=4210$). The mass detection limit is many times lower than that one of HPLC because the calculated injection volume of CZE is much smaller than the injection volume of HPLC. The mass sensitivity is even comparable to the bioassay sensitivities [13,14]. The CZE determination of PQQ is thus excellent alternative to the bioassay techniques.

4. Conclusion

A new sensitive method for the determination of PQQ by CZE has been developed. The method is simple and rapid and can be used to estimate PQQ in biological samples. Moreover, it can be also applied to study the reaction of PQQ with various com-

pounds. That is very important in considering the occurrence and function of PQQ in live organisms.

Acknowledgements

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