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Separation of the enzyme cofactor pyrroloquinoline quinone and three isomeric analogues by capillary electrophoresis with ion-pairing media

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Abstract

The enzyme cofactor pyrroloquinoline quinone (PQQ) was successfully separated from three closely related isomeric analogues by capillary electrophoresis with ultraviolet detection. Rapid and efficient separation of all four negatively charged isomers with baseline resolution was achieved by the addition of low concentrations (1–5 m*M*) of short chain tetraalkylammonium (TAA) salts to the capillary buffer. The TAA cations act as ion-pairing agents and promote differential migration of the isomers with only a minimal reduction in the electroosmotic flow. The effects of the TAA salt concentration and the alkyl chain length were examined. Detection limits of PQQ and its isomers were in the range of 7–15 μ*M* with mass detection limits of 98–210 fmol.

Keywords: Pyrroloquinoline quinone; Enzymes

1. Introduction

In 1979 pyrroloquinoline quinone (PQQ, methoxatin, 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid) was shown to be a novel coenzyme in methanol dehydrogenase from methylotrophic bacteria [1,2]. Subsequent studies identified PQQ as a cofactor in other prokaryotic enzymes catalyzing oxidation reactions of alcohols, sugars and amines [1–4]. Since the discovery of PQQ, additional quinonoid cofactors have been characterized [5–10]. In some initial studies, they were mistakenly identified as covalently bound PQQ [11–13]. Given the low levels of PQQ previously observed [14,15] and their structural similarity to related quinonoid molecules, highly sensitive and selective analytical methods for the separation of PQQ from closely related isomers are crucial for investigating the role of PQQ in enzymatic systems. To this end, we have taken advantage of the benefits of microcolumn separation techniques to develop a capillary electrophoretic separation for PQQ (**1**) and three synthetic isomers (**2**–**4**) [16] (Fig. 1). The synthetic isomers are important since some may be

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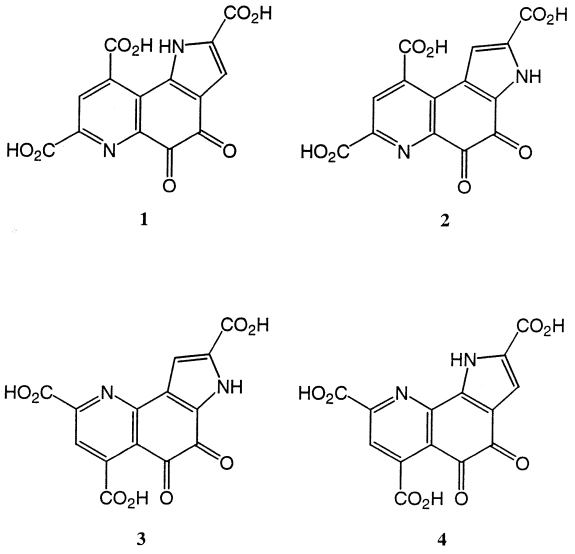

Fig. 1. Structures of PQQ and isomers **2**–**4**. PQQ (**1**), 4,5-dihydro-4,5-dioxo-3H-pyrrolo[3,2-f]quinoline-2,7,9-tricarboxylic acid (**2**), 5,6-dihydro-5,6-dioxo-7H-pyrrolo[2,3-h]quinoline-2,4,8-tricarboxylic acid (**3**) and 4,5-dihydro-4,5-dioxo-1H-pyrrolo[3,2-h]quinoline-2,6,8-tricarboxylic acid (**4**).

formed nonenzymatically in a sequence of still uncharacterized reactions [9] during the normal turnover of amine oxidases in nature [10].

When capillary zone electrophoresis (CZE) is used for the separation of structurally similar molecules, a lack of selectivity can result due to the similar charge-to-size ratios of the migrating species. To overcome this limitation, the addition of specific agents to the capillary buffer has been useful to modulate selectivity and enhance separation. Examples of compounds previously used as additives include cyclodextrins, surfactants, alkylsulfonic acids, short chain alkylammonium salts, and various chelating agents. A detailed review, which describes the use of additives in CZE, has recently been published [17]. Several studies have utilized short chain tetraalkylammonium (TAA) salts as additives for CZE separations [17–22]. TAA salts are useful due to the availability of a variety of alkyl chain lengths, ease of purification, solubility in both aqueous and nonaqueous media and the ability to effect separations of both neutral and ionic substances. For example, TAA salts have been used for the separation of ionic compounds in micellar electrokinetic chromatography [18], neutral compounds in nonaqueous media by solvophobic interactions [19], phenolic acids by ion-pairing interactions [20], oligosaccharide fragments from α_1-acid glycoprotein [21] and in the chiral separation of pharmaceutical compounds [22].

Previously, separation of PQQ and isomers **2**–**4** was attempted by HPLC on a reversed-phase C_{18} column in water–methanol–85% H_2PO_4 (80:20:0.1) with UV detection, which was not able to adequately resolve all four isomers [16]. Here, we have taken advantage of the high efficiency of CE and the enhanced selectivity afforded by the addition of low concentrations of TAA salts to the capillary buffer for the separation and detection of PQQ and isomers **2**–**4**.

2. Experimental

2.1. Materials

PQQ was purchased from Fluka (purity 99%, Ronkonkoma, NY, USA). Isomeric analogues of PQQ (**2**–**4**) were synthesized by the method of Zhang et al. [23] and were available from a previous study. Tetrapropylammonium perchlorate (TPAP) was purchased from Acros Organics (Fisher Scientific, Fairlawn, NJ, USA), while tetramethylammonium perchlorate (TMAP), tetraethylammonium perchlorate (TEAP) and tetrabutylammonium perchlorate (TBAP) were purchased from GFS Chemical (Columbus, OH, USA). 2,2′-Bipyridine was obtained from Matheson, Coleman and Bell (Cincinnati, OH, USA). Sodium phosphate buffer (10 m*M*, pH 7.4) was prepared in distilled deionized water purified by a Barnstead B-pure water system (Dubuque, IA, USA) and passed through a 0.2-μm Gelman Acrodisc filter (Fisher Scientific). All other chemicals were HPLC-grade.

2.2. Instrumentation

Electrophoretic separations were performed on a

P/ACE MDQ capillary electrophoresis system (Beckman Scientific Instruments, Fullerton, CA, USA) and were analyzed with P/ACE MDQ software on an IBM pentium computer. A 30-cm fused-silica capillary with an inner diameter of 50 μm, an outer diameter of 360 μm and a length of 20 cm to the detector was employed (Polymicro Technologies, Phoenix, AZ, USA).

2.3. Capillary electrophoretic separations

Stock solutions (300–400 μ*M*) of each isomer were prepared in phosphate buffer (10 m*M*, pH 7.4) and were appropriately diluted for separation and limit of detection studies. Solutions were filtered with a 0.2-μm Gelman Acrodisc filter and pressure injected for 3 s at 0.5 p.s.i. (1 p.s.i.=6894.76 Pa). The injection volume was calculated to be 14 nl based on the pressure of the injection and the length of time that the pressure was applied. The standard capillary buffer was phosphate buffer (10 m*M*, pH 7.4), which was modified with different TAA salts to achieve separation of the isomers. The separation voltage was 25 kV with a corresponding current between 22 and 25 μA. Although the absorption maxima for the isomers varied between 249 and 274 nm [16], a common wavelength of 254 nm was chosen for the analysis of the mixture of isomers. At this wavelength, the molar absorptivities for the four isomers varied by less than a factor of two. Prior to use, new capillaries were treated with hydrochloric acid (1 *M*), distilled deionized water, sodium hydroxide (1 *M*) and water again. All rinses were pressure rinses at 25 p.s.i. for 15 min. Between each analysis the capillary was rinsed with acetonitrile for 1.5 min, water for 2 min, sodium hydroxide (100 m*M*) for 2 min, followed by distilled deionized water for 2 min and the appropriate capillary buffer for 2 min. Inter-run rinses were also conducted at 25 p.s.i. All separations were performed at 25±0.2°C.

Calibration curves were obtained by plotting the average of four replicate measurements of the corrected peak area at five different isomer concentrations between 10 and 100 μ*M*. Migration order was established by injection of the individual isomers and spiking studies.

3. Results and discussion

3.1. Separation of PQQ and isomers **2**–**4**

Similar to the results obtained by HPLC [16], a mixture containing all four isomers was only partially separated by CZE in 10 m*M*, pH 7.4 phosphate buffer due to the identical charge-to-size ratios at pH 7.4. Isomer **2** was easily resolved, followed by isomers **1** and **3** which co-migrated and isomer **4** which migrated last and was only partially resolved from isomers **1** and **3**. Injection of each individual isomer confirmed the migration order of **2** (2.20 min), **1** (2.34 min), **3** (2.34 min) and **4** (2.38 min).

In order to gain selectivity for the separation of PQQ and isomers **2**–**4**, the short chain TAA cations TMAP, TEAP, TPAP and TBAP were examined for their ability to modulate the migration patterns of the negatively charged PQQ isomers. Each salt was added to the capillary buffer in 1 m*M* increments up to a concentration of 5 m*M*. At these low additive concentrations reasonable separation currents were observed in 10 m*M* phosphate buffer. As a result, the amount of joule heating through the capillary remained low and allowed the use of a shorter (30 cm) capillary combined with a high electric field strength (25 kV). Thus, fast and efficient separations were observed.

Separation was achieved with baseline or near baseline resolution for each TAA cation at one or more salt concentration. The effect of concentration of the TAA additives is clearly demonstrated in Fig. 2 for TEAP. As the concentration was increased from 1 to 5 m*M*, a gradual improvement in resolution was observed until baseline resolution was achieved at 5 m*M*. In general, as the length of the alkyl chain increased the concentration necessary for baseline resolution decreased, i.e., TMAP (5 m*M*), TEAP (5 m*M*), TPAP (2 m*M*) and TBAP (1 m*M*). Interestingly, higher concentrations of TPAP (5 m*M*) resulted in comigration of isomers **1** and **2**, while at 3 m*M* TBAP, isomers **1** and **2** comigrated and even changed order at a concentration of 4 m*M*.

Migration times were always less than 7 min, but shifted to longer times as the concentration of TAA increased. Reproducibility of the migration times for each isomer as a function of the TAA additive was

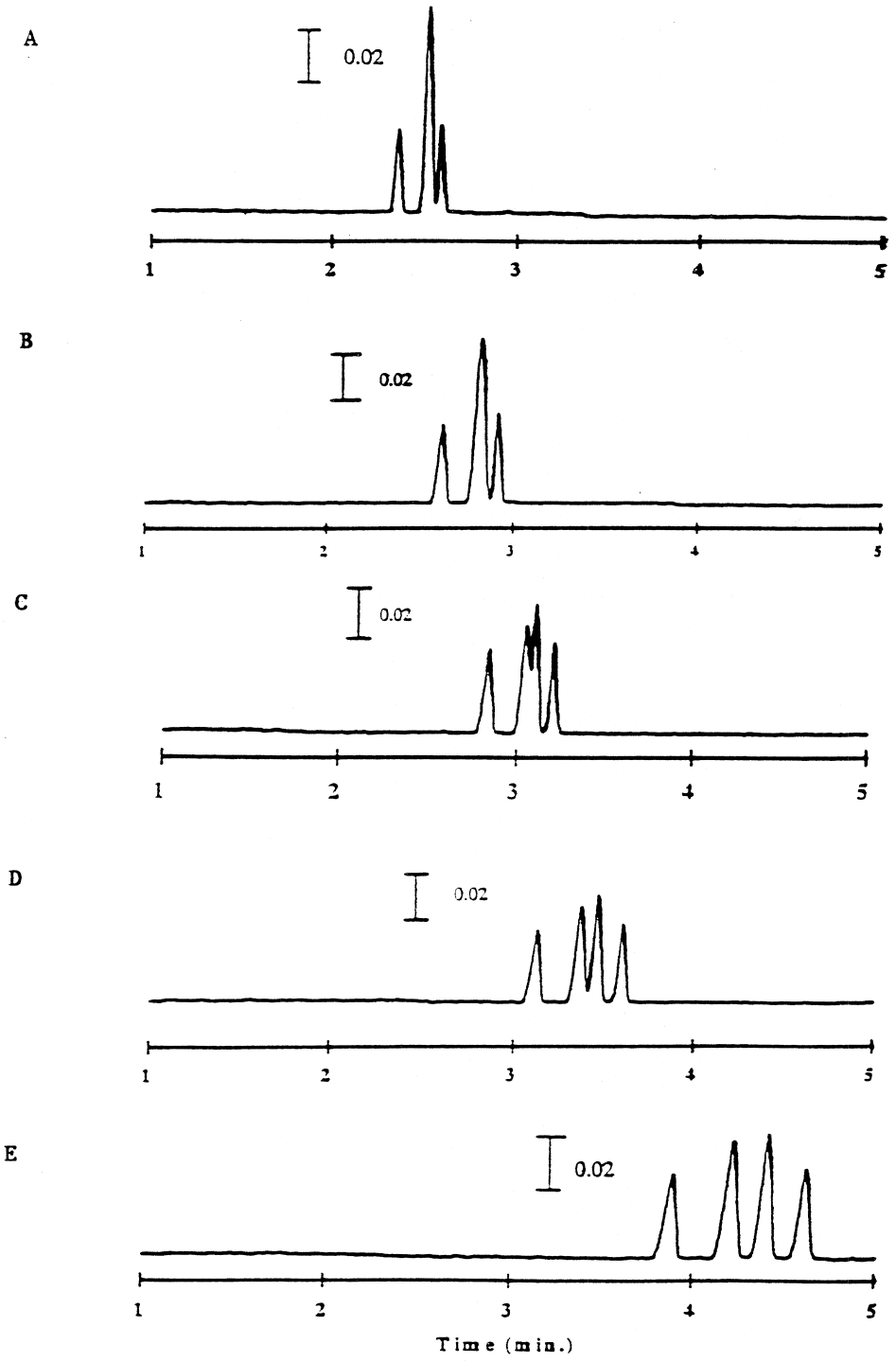

Fig. 2. Electropherograms of PQQ (**1**) and isomers (**2**–**4**) in phosphate buffer (10 m*M*, pH 7.4) as a function of TEAP concentration. (A) 1 m*M*, (B) 2 m*M*, (C) 3 m*M*, (D) 4 m*M* and (E) 5 m*M*. Applied voltage: 25 kV; detection: 254 nm; temperature: 25°C. The neutral marker, 2,2′-bipyridine, is observed at 0.8 min.

within a relative standard deviation of 0.03%. Frequent rinsing of the capillary was necessary to achieve consistent migration times due to adsorption of both the TAA cations and the quinones.

3.2. Effect of TAA additives on electroosmotic and electrophoretic mobility

The electroosmostic mobility (μ_{eo}), which was determined based on the migration of a neutral marker, and the electrophoretic mobility (μ_{ep}) were calculated from the following equations [19]:

$$\mu_{eo} = Ll/t_{eo}V \quad (1)$$

$$\mu_{obs} = \mu_{eo} + \mu_{ep} = Ll/t_{obs}V \quad (2)$$

where L is the total length of the capillary in cm, l is the effective length from the inlet to the detector in cm, μ_{obs} is the observed mobility, t_{obs} is the observed migration time in s, t_{eo} is the migration time of the 2,2′-bipyridine marker in s and V is the applied voltage in V. The results are summarized in Table 1. As the concentration of the TAA salt increased, μ_{eo} gradually decreased. This effect is consistent with previous studies that utilized short chain TAA salts in CZE [19,20] and is attributed to reduced double layer effects at the capillary wall [24]. μ_{eo} was essentially insensitive to the alkyl chain length.

As is also shown in Table 1, a negative value of μ_{ep} was obtained for all conditions studied. This result, in combination with the observation that the migration times for the isomers are longer than the 2,2′-bipyridine marker, indicates the isomers migrated as negatively charged species. With the exception of the addition of TBAP to the capillary buffer,

Table 1
Summary of μ_{eo} and μ_{ep} for PQQ and isomers **2–4** in 10 m*M* phosphate buffer, pH 7.4, as a function of concentration and type of TAA additive[a]

[TAA] (m*M*)	TAA	$\mu_{eo} \times 10^{-4}$ (cm^2/V s)	(**1**) $-\mu_{ep} \times 10^{-4}$ (cm^2/V s)	(**2**) $-\mu_{ep} \times 10^{-4}$ (cm^2/V s)	(**3**) $-\mu_{ep} \times 10^{-4}$ (cm^2/V s)	(**4**) $-\mu_{ep} \times 10^{-4}$ (cm^2/V s)
0		5.82(<0.01)	4.10(0.02)	3.99(0.03)	4.10(0.02)	4.10(0.02)
1	TMAP	5.71(<0.01)	4.30(0.05)	4.17(0.05)	4.32(0.03)	4.36(0.03)
	TEAP	5.80(<0.01)	4.24(0.01)	4.13(0.01)	4.24(0.01)	4.28(0.01)
	TPAP	5.71(<0.01)	4.27(0.01)	4.17(0.02)	4.32(0.01)	4.36(0.01)
	TBAP	5.63(0.02)	4.07(0.03)	3.98(0.04)	4.11(0.03)	4.16(0.03)
2	TMAP	5.48(<0.01)	4.30(0.01)	4.17(0.02)	4.32(0.01)	4.36(0.02)
	TEAP	5.65(0.08)	4.22(0.01)	4.11(0.01)	4.22(0.01)	4.26(0.01)
	TPAP	5.56(<0.01)	4.22(0.01)	4.15(0.01)	4.29(0.01)	4.33(0.01)
	TBAP	5.59(<0.01)	4.12(0.04)	4.06(0.04)	4.22(0.04)	4.25(0.03)
3	TMAP	5.33(<0.01)	4.30(0.03)	4.17(0.03)	4.32(0.02)	4.37(0.02)
	TEAP	5.48(<0.01)	4.17(0.08)	4.07(0.08)	4.19(0.05)	4.23(0.08)
	TPAP	5.42(0.08)	4.19(0.01)	4.13(0.01)	4.29(0.01)	4.33(0.01)
	TBAP	5.06(<0.01)	4.09(0.02)	4.06(0.02)	4.21(0.02)	4.25(0.02)
4	TMAP	5.14(0.08)	4.31(0.01)	4.18(0.01)	4.33(0.01)	4.38(<0.01)
	TEAP	5.39(0.08)	4.21(0.02)	4.12(0.03)	4.24(0.02)	4.28(0.02)
	TPAP	5.32(0.01)	4.29(<0.01)	4.25(<0.01)	4.41(<0.01)	4.44(<0.01)
	TBAP	5.39(0.08)	4.04(0.03)	4.06(0.03)	4.20(0.03)	4.23(0.03)
5	TMAP	5.03(0.07)	4.37(<0.01)	4.24(<0.01)	4.40(<0.01)	4.44(<0.01)
	TEAP	5.16(0.07)	4.25(<0.01)	4.17(<0.01)	4.29(<0.01)	4.33(<0.01)
	TPAP	5.39(<0.01)	4.14(0.01)	4.12(0.01)	4.27(<0.01)	4.30(<0.01)
	TBAP	5.32(0.08)	4.05(0.03)	4.09(0.02)	4.23(0.01)	4.25(0.01)

[a] Values are the average of four replicate measurements. Standard deviations are given in parentheses.

μ_{ep} was enhanced relative to the value when 10 m*M* phosphate was used as the capillary buffer. Migration towards the cathode was thus accomplished by the greater effect of μ_{eo} relative to μ_{ep}.

Weldon et al. [20] suggested enhanced selectivity in the presence of capillary buffer additives can be traced to the interaction of the additive with the analytes as well as with the wall of the capillary. The data in Table 1 indicate that enhanced selectivity was gained through subtle changes in the relative μ_{ep} of the isomers given that the decrease in μ_{eo} was the same for all isomers in any given separation attempt. This was accomplished by relatively small changes in the concentration of TAA cations in the capillary buffer. Therefore, separation is due to differences in the ion-pairing ability of the TAA cations with each isomer. It is reasonable to assume that in the capillary a rapid equilibrium, which is sensitive to the nature and concentration of the additive, was established to form an ion-pair complex. The sharp peaks and the clear trend that as the size of the alkyl substituent increased, the concentration required for baseline resolution decreased supports subtle differences in equilibrium interactions with the isomers. Clearly, μ_{ep} is sensitive to the reduced effective charge and an increased size of the migrating species. In general, baseline resolution occurred for this separation when the difference in μ_{ep} was as small as $3.0 \cdot 10^{-6}$ cm^2/V s.

The migration order of the isomers under the varying separation conditions generally correlates with the pyrrole ring pK_a values for the four isomers. The pyrrole ring pK_a values for the isomers in the order **2**, **1**, **3**, **4** are 9.85, 10.30, 10.71 and 10.88, respectively [16,25]. Thus, the position as well as the electronic properties of the pyrrole ring may be an important influence for ion-pair formation.

3.3. Performance characteristics

In the absence of the TAA cations, an average value for the number of theoretical plates (*N*) was found to be $9.6 \cdot 10^5$. In contrast, *N* increased slightly in the presence of the TAA cations in the order TMAP>TEAP>TPAP>TBAP.

Selectivity of the method was demonstrated by the baseline resolution of the isomers. A measure of the accuracy for CE quantitation was obtained by a comparison of the analytical concentration to the concentration determined from the corrected peak areas and by linear regression analysis in limit of detection studies. The percent relative error was typically less than 10% except for the lowest standard concentration (15 μ*M*), which was greater than 20%. The corresponding average value for the relative standard deviation was typically under 15% at higher standard concentrations and increased to greater than 25% at the lowest standard concentration. Plots of the corrected peak area versus concentration were linear over the range of analyte concentrations.

Detection limits ($S/N=3$) for isomers **1**–**4** were 7, 13, 15 and 11 μ*M*, respectively. The corresponding mass detection limits were calculated to be 98, 180, 210 and 150 fmol, respectively, based on an injection volume of 14 nl. Although not always easily comparable, the mass detection limits appear at least similar or lower with CE–UV than those reported for other direct analysis methods. These include the direct detection of PQQ by LC methods with amperometric [26] (10 pmol), amperometric with redox cycling [27] (0.2 pmol) and coulometric [28] (<1 pmol) detection methods. Detection limits for enzymatic methods range from 30 pmol [29] to approximately 0.1 pmol [30] and <10 pmol for a redox cycling bioassay [31]. PQQ has also been determined in various foods [14], bodily fluids and rat tissue samples [15] at the pmol level by gas chromatography with mass spectral detection following derivatization. Additional derivatization techniques followed by HPLC with UV [32] or fluorescence [33] detection have resulted in detection limits of 2 pmol for PQQ.

4. Conclusions

A simple, fast and sensitive CE method for the separation of PQQ from closely related quinone isomers has been developed by the use of short chain TAA additives in the capillary buffer. Differential ion-pairing between the positively charged TAA cations and the quinone anions selectively modulates μ_{ep} such that baseline resolution of all four isomers is observed. Separation is accomplished at low additive concentrations, which clearly minimizes the

reduction [20] and avoids reversal [34,35] of the electroosmotic flow typical in CE separations at higher additive concentrations.

Detection limits on the order of 100–200 fmol are comparable to the lowest detection limits obtained for PQQ by other more involved methods. Thus, the low detection limits in combination with the high separation efficiency, small sample volumes and ease of modification of separation conditions available with CE will benefit the identification and determination of PQQ in biological systems.

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