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SHORT COMMUNICATION

Ion-Pair LC Analysis of Pyrroloquinoline Quinone in Neurotransmitter Amino Acid Incubations: Determination of Chemical Kinetics

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Abstract Neurotransmitters are the chemical messengers of the brain. Many neurodegenerative diseases of the central nervous system are related to abnormal neurotransmitter activity. Pyrroloquinoline quinine (PQQ) has previously been shown to be a promising candidate for preventing cognitive deficit in neurodegeneration. To investigate whether PQQ can modulate the levels of brain neurotransmitter amino acids, a rapid and reliable ion-pair liquid chromatographic method was established and validated for the analysis of PQQ in reaction mixtures containing specific neurotransmitter amino acids. The reaction mixtures were separated on an amethyst C18-P reverse-phase column with 35:65 (v/v) acetonitrile:20 mM potassium dihydrogen phosphate, pH 5.5, containing 20 mM tetrabutyl ammonium bromide as mobile phase at a flow rate of 0.8 mL min^{-1}. The validated method was applied successfully to study the chemical kinetics of PQQ reactions with five neurotransmitter amino acids. Order of reaction n, rate constant k, and activation energy E_a values for the reactions were calculated. This work provides important information for studying the possible protective mechanisms of PQQ in neurodegenerative diseases. Furthermore, the simplicity of this method combined with its sensitivity and reliability make it a novel contribution in the field of neurotransmitter research.

Keywords Ion-pair liquid chromatography · Pyrroloquinoline quinine · Neurotransmitter amino acid · Chemical kinetics

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Introduction

Pyrroloquinoline quinine (PQQ, 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid) exists in a wide variety of foods and animal tissues [1] and has been identified as an enzyme cofactor in bacteria [2]. PQQ has been shown to be very important to physiological function and has the potential to be classified as a new member of the vitamin B family [3]. Previous studies reported that PQQ shows promise in the treatment of neurological and psychical disorders. For example, PQQ inhibits the amyloid fibril formation and cytotoxicity of C-truncated alphasynuclein variants to prevent Parkinson's disease [4, 5]. Moreover, PQQ was shown to be effective in preventing cognitive deficits in oxidative stress-induced neurodegeneration [6].

It is well established that certain amino acids play an important role in neurotransmission. The functional balance between excitatory amino acids (Glu, Asp) and inhibitory amino acids (Gly, GABA) regulates the central nervous system (CNS) [7]. Any change in the functional balance of neurotransmitters can result in the failure of cellular function and lead to disease [8]. For example, if the cerebrospinal fluid (CSF) Glu:GABA ratio increases, seizure activity can result [9]. Similarly, an extracellular release of both excitatory amino acids Glu and Asp may play an important role in the occurrence of seizure activity [10]. Other conditions, including anxiety, insomnia, and panic disorder have been attributed to imbalances between glutamate and GABA [11]. Aspartate and glycine form an excitatory:inhibitory pair in the spinal cord that can be compared to Glu and GABA in brain. "Human startle disease," a rare but potentially fatal disorder, is caused by mutation of glycine receptors [11]. D-Serine (D-Ser) has been studied most extensively and shown to play a role in

excitatory amino acid metabolism, as a co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor [12]. As such, D-Ser is important to a number of different processes in the CNS, ranging from synaptic plasticity to disease states, including schizophrenia [13].

In recent years, accumulating evidence has indicated that the neuroprotective function of PQQ is extraordinary and worthy of further exploration. PQQ undergoes condensation reactions with amino acids to produce derivatives that have strong free radical scavenging capabilities beneficial to several physiological processes [14]. Interestingly, PQQ has also been shown to bind NMDA receptors (NMDAR). ^{99m}Tc-PQQ has been described as a potential marker for the molecular imaging of NMDAR in psychical disorders [15]. In addition, binding studies between PQQ and DNA have provided information for research into the disease-resistant mechanisms of PQQ [16]. To investigate the effects of PQQ in regulating neurotransmitters, our laboratory established an improved ion-pair HPLC method for determining the levels of PQQ in reaction mixtures with neurotransmitter amino acids including Gly, D-Ser, Glu, Asp, and GABA. The method was fully validated for specificity, precision, and accuracy, and then successfully used to calculate chemical kinetic parameters.

Experimental

Materials and Reagents

Gly, D-Ser, Glu, Asp, and GABA were purchased from ACROS (Belgium, USA). Acetonitrile was of HPLC grade and obtained from CNW Technologies GmbH (Hanau, Germany). Potassium dihydrogen phosphate for HPLC was obtained from Sigma-Aldrich (St. Louis, MO, USA). Tetrabutyl ammonium bromide (TBAB) was purchased from Aladdin (Shanghai, China). PQQ was obtained from ShanghaiMed Co. (Shanghai, China). Double deionized water was purified using a water purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade and used without further purification.

Instrumentation and Chromatographic Conditions

All experiments were performed with a Waters 600 HPLC system equipped with a 2487 UV/VIS detector (Waters Corp., Otsego, MI, USA). Samples were run on a reversed-phase column (Amethyst C18-P, 5 μm, 4.6 × 250 mm, SEPAX, USA) using 35:65 (v/v) acetonitrile:20 mM potassium dihydrogen phosphate, pH 5.5, containing 20 mM TBAB as mobile phase. The flow rate was 0.8 mL min^{-1}, and the UV detection wavelength was 249 nm. Data were collected and processed with Waters Empower software. Water was purified in-house by a Milli-Q and Q-POD® system (Millipore). An ultrasonic cleaner (Huanan Ultrasonic Equipment Co., Guangzhou, China), pH Ø72 meter (Beckman, Brea, USA), thermostat water bath (Jintan Honghua Instrument Co., Jintan, China), and syringe filter (0.22 μm, Ø13, Hangzhou Anow Micro Filtration Co. Hangzhou, China) were used for sample preparation.

Preparation of Standard Solutions and Quality-Control Samples

A series of standard PQQ solutions ranging in concentration from 0.25 to 2.5 mM was prepared in 50 mM sodium dihydrogen phosphate buffer, pH 7.0 ($n = 9$) to determine linearity between concentration and detector response. Five neurotransmitter amino acids were dissolved in 50 mM sodium dihydrogen phosphate buffer (pH 7.0) (10 mM). Quality-control (QC) samples were prepared at low (0.5 mM), medium (1.0 mM), and high (2.0 mM) concentrations in the same manner as the five neurotransmitter amino acids used for calibration. All solutions were stored in the dark at 4 °C before analysis.

Stability

To ensure that PQQ was stable at reaction conditions, QC samples were incubated in a water bath at 37 and 50 °C for 24 h. The same procedure was done at room temperature (25 °C) as a control. The content of PQQ was determined over the reaction time course at 0, 2, 4, 8, 10, and 24 h.

Reactions of PQQ with Neurotransmitter Amino Acids

In the reactions, PQQ (1 mM) and the respective amino acid (10 mM) were mixed in 50 mM sodium dihydrogen phosphate buffer, pH 7.0 and incubated in separate reactions at 37 and 50 °C. Controls were carried out by incubating PQQ solution with the reaction buffer alone in the same incubation conditions. The amino acids were incubated separately in the same reaction buffer for use as blanks. Incubation samples of 10 μL were injected and analyzed by HPLC at reaction times of 0.5, 1, 2.5, 5, 7.5, 10, and 24 h. The content of free PQQ in reaction solutions was determined by standard curve analysis.

Results and Discussion

Development and Optimization of the HPLC Analysis Method

Different acetonitrile concentrations (25, 30, 35, 40, 50, 55, 60, 65, and 70 %) were investigated as an organic modifier

in mobile phase. The results showed that when the concentration of acetonitrile was greater than 35 %, PQQ and the reaction product oxazole (Fig. 1) were not completely separated. Therefore, 35 % acetonitrile was considered acceptable regarding resolution and appropriate retention time.

pH is an important factor in ion-pair HPLC [17]. For this reason, the PQQ retention time was studied over a wide pH range (2.0–10.0). The retention time of PQQ was gradually increased with increasing pH and reached a maximum at approximately pH 5.5. When the pH was continually increased, however, the retention time gradually decreased. The effects of different mobile phase pH values on the resolutions between PQQ and oxazole are shown in Fig. 2. The resolution was optimal between pH 4.0–6.0. Therefore, pH 5.5 was selected for our experiments.

To obtain suitable retention time and good separation of PQQ and oxazole, we paid much attention to different ion-pair reagents. In our trials, several ion-pair reagents, such as SDS, 1-pentyltriethyl-ammonium phosphate (Q5), tetrabutyl ammonium phosphate/tetrabutylammonium dihydrogen phosphate (TABP) and TBAB, were considered. The peak of PQQ showed tailing in Q5 ion-pair mobile phase. When using SDS, PQQ was not retained and eluted at 1.3 min. The response of PQQ in TABP was lower than in TABA. After several experiments with different

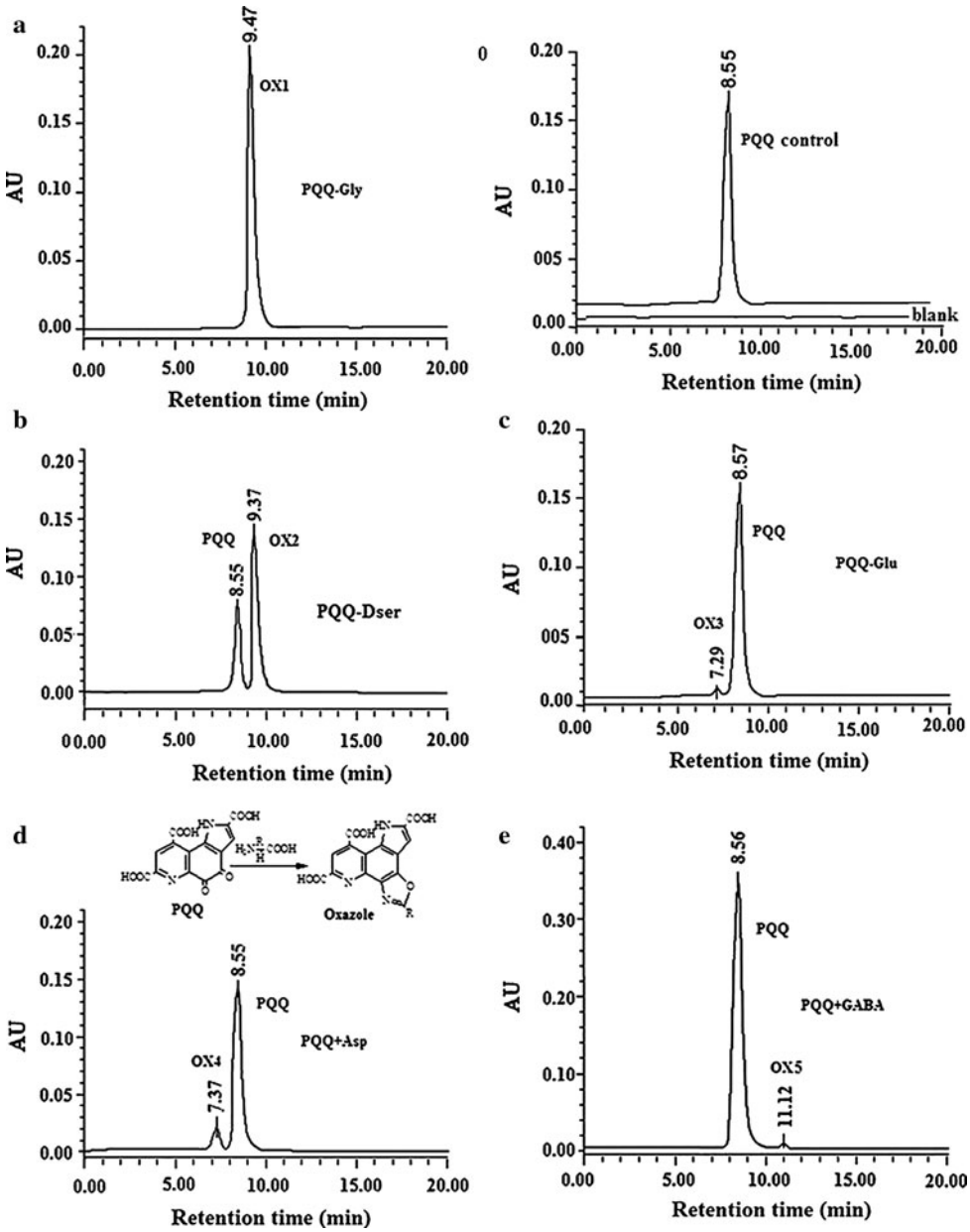

Fig. 1 HPLC chromatograms of reaction mixtures of PQQ with **a** Gly, **b** D-Ser, **c** Glu, **d** Asp, and **e** GABA for 24 h (37 °C). (0) PQQ standard (control) and five amino acids (blank) in phosphate buffer (pH 7.0)

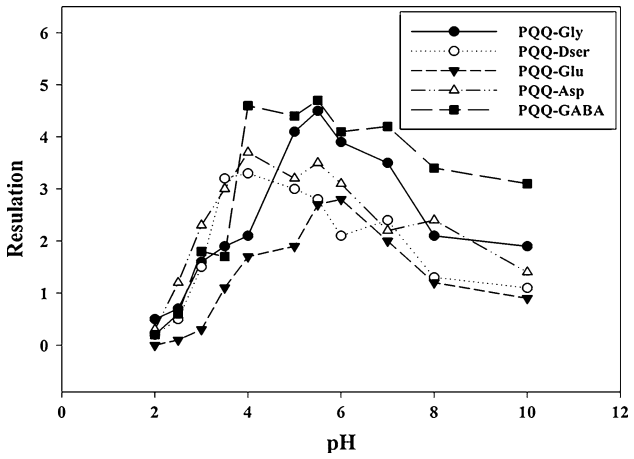

Fig. 2 The effects of different mobile phase pH values on the resolution between PQQ and oxazole

concentrations of TABA (10, 20, or 30 mM), 20 mM TABA was eventually preferred because it not only reached baseline separation compared with 10 mM TABA but also caused less damage to the column than 30 mM TABA. Moreover, to obtain the best peak shapes for the samples, we separated the reaction mixtures on different models of C18 columns (Lichrospher, Hypersil, SepaxHP-C18, AmethystC18-P, and Amethyst C18-H). The Amethyst C18-P column in combination with the 20 mM TABA assay produced good peak shapes and baseline separation (Fig. 1).

Method Validation

Selectivity

Representative chromatograms of blank, control, and spiked preparations of Gly, D-Ser, Glu, Asp, and GABA are shown in Fig. 1. The chromatograms demonstrated that the assay was selective for PQQ with a single peak of PQQ ($t_R = 8.5$ min), which was separated from the mixture of binding samples and not found in the blank.

Linearity and Precision

The linearity of PQQ was tested for concentrations ranging from 0.25 to 2.5 mM. The equation of the standard curves with the peak areas against the concentration of standards was $A = 475836C + 524678$ ($R^2 = 0.9998$). The relative standard deviations (RSD %) of the peak area responses and retention time were determined by six injections of the standard solution. The method precision was validated with RSDs of 1.22 and 0.58 % for peak areas and retention times, respectively. The accuracy and precision were assessed using QC samples of three concentrations (Table 1).

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

By using PQQ standards in phosphate buffer and on the basis of a signal-to-noise ratio (S/N) of 3:1, the limit of

Table 1 Accuracy, precision, and kinetic parameters

Sample	Accuracy and precision				Chemical kinetic parameters		
	Added (μM)	Found (μM)	Accuracy (%)	RSD (%)	k_1 (μM h^{-1})		Mean E_a (kJ mol^{-1})
					37 °C	50 °C	
Gly	2,000	1,863.4 ± 53.4	93.17	6.58	292.2	623.7	48.6
	1,000	964.3 ± 12.1	96.43	3.49			
	500	426.5 ± 16.3	85.3	5.72			
D-Ser	2,000	1,907.5 ± 18.4	95.38	4.84	54.6	131.3	56.2
	1,000	1,026.7 ± 39.6	102.67	7.19			
	500	495.9 ± 23.2	99.18	6.33			
Glu	2,000	1,802.5 ± 18.5	90.13	3.81	1.32	5.94	97.4
	1,000	923.8 ± 20.7	92.38	2.96			
	500	512.6 ± 15.9	102.52	5.65			
Asp	2,000	2,115.4 ± 33.1	105.77	4.27	2.7	15.2	111.2
	1,000	885.3 ± 16.2	88.53	6.36			
	500	426.2 ± 14.7	85.24	3.55			
GABA	2,000	1,812.3 ± 22.8	90.62	2.84	0.12	0.84	134.1
	1,000	1,062.5 ± 27.6	106.25	5.76			
	500	508.6 ± 19.6	101.72	4.15			

detection for PQQ was found to be 0.05 μM. The LOQ was evaluated with the corresponding standard solution at a signal-to-noise ratio of at least 10:1 to be approximately 0.2 μM. The precision and accuracy for LOQ were RE within 12 % and RSD less than 15 % ($n = 6$).

Stability

The concentration of PQQ was 95–101 % of control after incubation at 37 and 50 °C for more than 24 h. Therefore, PQQ was stable in the reaction conditions.

Reaction of PQQ with Amino acid Neurotransmitters

The reaction of PQQ with Gly produced oxazole 1 ($t_R = 9.47$ min) exclusively after incubating 24 h at 37 °C (Fig. 1a). The reaction of PQQ with D-Ser, Glu, Asp, and GABA, produced oxazole 2 ($t_R = 9.37$ min, Fig. 1b), oxazole 3($t_R = 7.29$ min, Fig. 1c), oxazole 4 ($t_R = 7.37$ min, Fig. 1d), and oxazole 5($t_R = 11.12$ min, Fig. 1e), respectively, with some free PQQ remaining.

To compare the effects of different neurotransmitter amino acids on free PQQ, a time course experiment was conducted using the developed and validated method. As shown in Fig. 3, after incubation with D-Ser, Glu, Asp, and GABA for 24 h at 37 °C, free PQQ was reduced to 31, 95.5, 89.5, and 98.6 %, respectively. However, free PQQ was reduced to zero and was completely converted into oxazole 1 after incubation with Gly for 24 h (Fig. 1a).

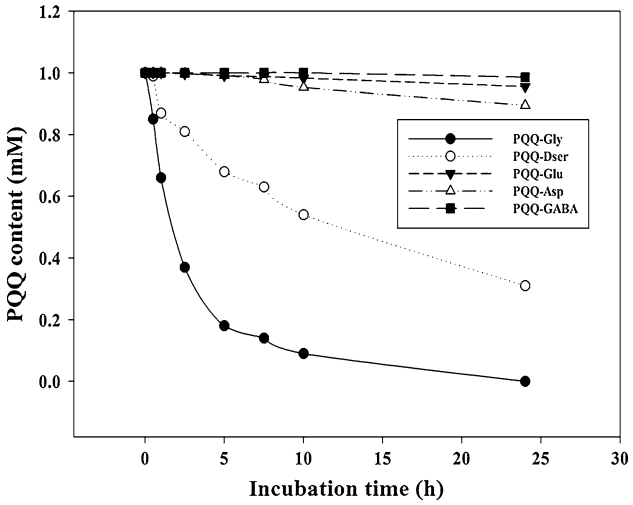

Fig. 3 Time-dependent content of PQQ incubated with different neurotransmitter amino acids at 37 °C. The initial concentration of PQQ was 1.0 mM

Chemical Kinetics of the Reactions Between PQQ and Amino acid Neurotransmitters

Chemical reactions can be classified based on their reaction kinetics. The general reaction form of PQQ with amino acid neurotransmitters is

$$A + B \rightarrow \text{products} \quad (1)$$

where A is PQQ and B is an amino acid neurotransmitter. Reactions are categorized as zero order, first order, second order, or third order. Because the concentrations of amino acids were much greater than that of PQQ, the order of reaction n of this reaction was apparently first order. Rate constant k was calculated using the following formula:

$$\text{First-order reaction } (n = 1): (A \rightarrow \text{products}) \ln \frac{a}{a - x} = k_1 t \quad (1)$$

where k is the rate constant of reaction, a is the initial concentration of the reactant A (PQQ), b is initial concentration of the reactant B, n is the order of the reaction with respect to A, and x is the concentration of product at a time t. Activation energy E_a was evaluated according to Arrhenius' equation (2), where k is the rate constant of reaction, T is temperature in Kelvin, and R is Rydberg constant (8.314 J mol^{-1} K).

$$\ln \frac{k_2}{k_1} = \frac{E_a}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \quad (2)$$

Rate constant k and activation energy E_a for our reactions were calculated and are listed in Table 1. From Table 1, it can be seen that mean rate constants increased with increasing temperature. The fact that k increased with increasing temperature indicated that higher temperatures accelerated the reaction rate. As shown in Table 1, the order of decreasing activation energy between PQQ and five amino acids was GABA > Asp > Glu > D-Ser > Gly. The results showed that the rate of the formation of oxazole 1 from Gly was much faster than that of oxazoles from the other amino acids. These results are in close agreement with those obtained by Glatz et al. [18]. This result is interesting because Gly acts as an inhibitory neurotransmitter in spinal cord and as a NMDAR co-agonist [19]. If it is easily converted into a derivative, its role as a free radical scavenger can be emphasized alongside its role as a neurotransmitter. The same can be said for NMDAR co-agonist D-Ser. However, the high activation energies and low rate constants we obtained for GABA, Glu, and Asp indicated that the reactions of PQQ with these amino acids were more difficult to achieve. These results are significant, given the roles these neurotransmitters have in biological function.

GABA and Glu are the most prevalent neurotransmitters in brain [11, 19]. The fact that PQQ cannot easily convert them into derivatives attests to the critical roles these amino acids play in the functional balance of brain chemistry. A similar conclusion applies to Asp, an important excitatory neurotransmitter in the spinal cord [19]. Taken together, these results strongly suggest that PQQ plays an important role in regulating amino acid neurotransmitter activity and provide insight for future research in the field of central nervous system drug discovery.

Comparison with Other Methods

The detection of free PQQ in biological samples is very difficult because of its high reactivity. Several methods have been developed for the determination of PQQ such as capillary electrophoresis (CE) with diode array or amperometric electrochemical detection [18, 20] and HPLC with fluorescence detection [21]. Noticeable shortcomings of these methods, including poor separation, peak tailing, and specific detection, have been found in the literature. PQQ is negatively charged and can form an ion-pair with a positive charge in mobile phase. In view of the unique advantage of ion-pair binding to alkaloids, ion-pair chromatography has been widely used to assay many types of charged compounds. In this study, we established an improved ion-pair HPLC method for analyzing PQQ levels in reaction mixtures with five different neurotransmitters amino acids. This method offers good analytical performance in terms of sensitivity, linearity, and precision. Previous reports have described detection limits at 0.1 μM using isotachophoresis and potential gradient with UV detection and 0.01 μM using HPLC and amperometric electrochemical detection [20]. In our HPLC ion-pair system, the detection limit is 0.05 μM, which is in very good agreement with these established standards. The final pH and acetonitrile concentration described in this assay achieved baseline separation and resulted in good peak shapes for PQQ and the five oxazoles. Furthermore, chemical kinetic parameters were successfully obtained using our method at first. This new assay, which combines simplicity, speed, and reliability has the potential to significantly aid research efforts in the field of neurotransmitter research.

Conclusion

We describe a new sensitive ion-pair HPLC method for analyzing levels of PQQ in neurotransmitter amino acid reaction mixtures. Our results show that this method can separate PQQ from the product oxazoles with good resolution using an amethyst C18-P reverse-phase column. This method is simple, accurate, and reproducible, and it can be also applied to study chemical kinetics. The results described here provide important information for future research into the neuroprotective mechanisms of PQQ.

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