High-Performance Liquid Chromatographic Determination of Pyrroloquinolone Quinone as Acetone Adduct

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A high performance liquid chromatographic method for the determination of pyrroloquinoline quinone (PQQ) is described. PQQ reacts readily with acetone in a weakly alkaline medium, giving a stable adduct, 5-acetonyl PQQ. The adduct was separated on a Develosil C$_{18}$-5 column using a mixture of methanol and 0.06 M phosphoric acid, and was detected at 254 nm. The response was linear over the range (1-20)$\times10^{-6}$ M of PQQ and the detection limit was 2 pmol for a 20-$\mu$L injection volume.

Keywords Pyrroloquinoline quinone, high performance liquid chromatography, acetone adduct, ultraviolet detection

Pyrroloquinolone quinone (PQQ) functions as a prosthetic group in enzymes such as methanol dehydrogenase$^{1,2}$, copper-containing amine oxidase$^{3}$ and methylamine dehydrogenase$^{4}$, and has been found to react with various compounds such as acetone$^{1,5,7}$, butyraldehyde$^{6}$, hydrazines$^{3,4,8}$, cyclopropanol$^{9}$, amino acids$^{10}$ and aminoguanidine$^{11}$. Some of these reactions have been applied to derivatization in the analysis of PQQ by high performance liquid chromatography (HPLC)$^{1,3-6}$. On the other hand, HPLC analyses of PQQ without derivatization have also been reported using UV$^2$ or electrochemical detection$^{12}$. However, a more selective detection method becomes necessary when complex samples are to be tested. Of the above-mentioned reactions of PQQ, acetone gave an easily preparable adduct, which appeared to be quite stable. In this study, the reaction of PQQ with acetone was examined in order to establish a simple and selective method for the determination of PQQ.

Experimental

Chemicals and solutions

PQQ was obtained from Ube Industries Ltd. (Tokyo, Japan). All other reagents were of the highest purity available. A 0.1 M sodium carbonate buffer (pH 9.2) was prepared by mixing 0.1 M disodium carbonate and 0.1 M nitric acid. A PQQ stock solution (1 mM) prepared in twice-distilled water was kept at 4$^\circ$C in the dark and an appropriate standard solution was made by dilution with twice-distilled water.

HPLC

A Model 5A high performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a UV spectrophotometric detector set at 254 nm and a Model 7125 syringe-loading sample injector (Rheodyne, Cotati, CA, USA) was used. The HPLC separations were performed on a Develosil C$_{18}$-5 column (250$\times$4.6 mm i.d.) (Nomura Chemical, Aichi, Japan) with a mobile phase consisting of methanol and 0.06 M phosphoric acid (3:7, v/v) at a flow rate of 1.0 ml min$^{-1}$ at room temperature.

Procedure

A 0.2-$\mu$L aliquot of PQQ standard solution was mixed with 0.1 ml of a 0.1 M sodium carbonate buffer (pH 9.2) and was allowed to react with 0.1 ml of aqueous 16% (v/v) acetone containing p-cresol (0.5 mg ml$^{-1}$) as an internal standard (IS) at 35$^\circ$C for 30 min. The reaction mixture (20-$\mu$L) was injected into the HPLC apparatus.

Results and Discussion

A typical separation of the PQQ-acetone adduct is illustrated using p-cresol as an IS in Fig. 1(B). The adduct has its $\lambda_{\max}$ at 254 nm$^{1,5,7}$, the optimum HPLC conditions were established on the basis of a series of preliminary investigations, as shown in Fig. 1. Under the HPLC conditions, the retention times of the PQQ-acetone adduct and the IS were 8.8 and 16 min, respectively.

The effect of the acetone concentration in the reaction mixture on the reaction yield of the PQQ-acetone adduct was examined. A PQQ solution (3$\times$10$^{-5}$ M) was used to prepare sample solutions containing various concentrations of acetone according to Procedure. As can be seen in Fig. 2, the formation of the adduct was low below a 0.1% (v/v) concentration of acetone, and some amount of PQQ remained unreacted (Fig. 1(C)). Above 10% (v/v) adduct formation also showed a drop, probably due...
to the formation of the 5,6-diacetonyl adduct of PQQ (Figs. 1(A) and 2). The peak ratio to the IS was held constant over the range 0.1 - 10% (v/v) of acetone.

The effect of the pH of the reaction mixture was also examined (Fig. 3); PQQ readily reacted with acetone in a weakly alkaline (pH 8.0 - 10.0) solution. Under acidic conditions, the reaction yield was very low, while above pH 11.0 the adduct became unstable.

Next, the reaction behavior was examined by performing repetitive injections of a constant amount of the reaction mixture, but varying the reaction temperature (20 - 60°C) and period (Fig. 4). Although at 20°C the reaction proceeded very slowly, incubation above 30°C was found to be sufficient for a quantitative reaction. The reaction yield at 60°C showed a tendency to be slightly lower. A constant conversion into the adduct was achieved within only 10 min; 30-min was selected for reproducible results. Forrest et al. reported that the acetone adduct was very base labile. In our experiment, however, the adduct was relatively stable in a weakly alkaline medium as well as under acidic conditions; the resulting product thus remained completely unchanged in the peak height ratio to the IS up to 2 h at pH 9.2, which decreased to about 60% after 2 d. At pH 12 the adduct was labile and decomposed noticeably within only 1 h. The reaction of PQQ with some other ketones such as 2-butanone, 2-pentanone and 3-pentanone was examined. Since these ketones were not freely mixible with water, aqueous ethanol was used as the reaction solvent. The reaction yields were much lower compared to that with acetone.

Good linearity was obtained between the peak-height ratio to the IS and the amount of sample injected over the range (2 - 40)X10^-11 mol of PQQ (r=0.998) and the detection limit at 0.005 AUFS was 2 pmol within a 20 µl injection volume.

The influence of some coexisting substances (glycine, urea, guanidine, guanidoacetic acid, phenylhydrazine, benzamidine, ethanol and ammonia) on the PQQ-acetone adduct formation was examined as follows: to 0.3 ml of a solution containing an equal molar concentration (0.1 M) of acetone and the compound, 0.2 ml of a 3X10^-5 M PQQ solution was added; the solution was allowed to stand at 35°C for 30 min. Glycine and ammonia decreased the peak height ratio to 65%. Phenylhydrazine reacted with acetone without giving the acetone adduct peak. The other compounds tested had no influence. p-Cresol used in
low concentrations as IS also did not show any reaction with PQQ. Although free PQQ has been chromatographed in this condition, the peak was broad (Fig. 1(C)). The sensitivity and selectivity of the analysis were enhanced by the present derivatization, since a much sharper peak was obtained for the PQQ-acetone adduct. It is also known that the acetone adduct is strongly fluorescent. Therefore, a great improvement in sensitivity is expected by using fluorescence detection.

The present procedure is much simpler and more convenient compared with those presented in previous papers, and may be useful for enzymatic studies of PQQ.

References


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