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HPLC Analysis of Ascorbic Acid (Vitamin C)

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Introduction

Although several indirect methods are available for measurement of ascorbic acid (AA, Vitamin C), the specificity is generally poor. In addition, for accurate evaluation of vitamin C status, dehydroascorbic acid (DHAA), the oxidation product of AA should also be measured simultaneously. Although direct measurement of DHAA is possible, the indirect method [1] described uses highly sensitive HPLC-electrochemical detector (ECD) equipment [2-6]. ECD is quite sensitive with relative lower oxidation-reduction potential compounds, i.e., less than 1000 mV. Based on these reported methods [2-6], total AA is estimated after the reduction of DHAA to AA and DHAA is then calculated from the difference between total AA and free AA (reduced form) originally present in the sample.

Protocol

Sample preparation

1. Mix plasma (25 µL) with metaphosphoric acid (5%, 225 µL) containing desferrioxamine mesylate (Sigma; 20 mM)
2. Centrifuge at 3000 g for 10 min.
3. Isolate supernatant for measurement of reduced and total AA.

Measurement of reduced AA

1. Mix supernatant (75 µL), distilled water (50 µL), and metaphosphoric acid (50%, 25 µL).
2. Centrifuge at 3000 g for 10 min.
3. Isolate supernatant (5 µL) for direct HPLC injection.

Measurement of total AA

1. Mix supernatant (75 µL), dithiothreitol (DTT; 10 mM, 25 µM, and K_2HPO_4 (40 mM, 25 µL; to maintain the pH of the mixture at 6.8).
2. React for 20 min at room temperature in the dark.
3. Add metaphosphoric acid (50%, 25 µL).
4. Centrifuge at 3000 g for 10 min.
5. Isolate supernatant (5 µL) for direct HPLC injection.

HPLC was performed with an lrika Instruments (Kyoto, Japan) Σ-871 chromatograph equipped with a 250 mm × 4 mm i.d. lrica RP-18T (ODS) column and lrika Amperometric S-875 ECD with glossy carbon working electrode set at 700 mV relative to Ag/AgCI. The mobile phase was 0.2 M KH_2PO_4-H_2PO_4 (pH 3.0) containing 50 µM EDT A.

The amount of DHAA is total AA minus reduced AA.

Discussion

Although the ratio of DHAA to AA is reported to be constant for whole blood stored at 4°C for 6 h, AA in plasma or serum is highly labile and readily oxidized to DHAA and subsequently to diketogulonic acid. To prevent oxidation of AA in plasma samples, add desferrioxamine. In the presence of 20 mM desferrioxamine, more than 98% of the AA content is preserved when plasma is stored at 4°C in the dark for 6 h. Dithiothreitol is used as the reductant for DHAA, because it is the most suitable at a neutral pH [7,8]. The recovery of DHAA added to plasma is 98.9% in the presence of 10 mM dithiothreitol at pH 6.8. This reaction was complete within 20 min at 25°C in the dark.

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