Simultaneous Determination of Ascorbic Acid and Free Malondialdehyde in Human Serum by HPLC–UV

Ascorbic acid and malondialdehyde levels are two important parameters for the measurement of oxidative stress in biological systems. Ascorbic acid is an important antioxidant from dietary sources. Malondialdehyde, one of the major secondary oxidation products of peroxidized polyunsaturated fatty acids, has been shown to be of biological significance. A new method for measurement of ascorbic acid and malondialdehyde was tested with the same analysis conditions in human serum from diabetic patients and healthy individuals. Ascorbic acid and malondialdehyde levels of patients with diabetes mellitus were significantly different from the controls. The detection limits for ascorbic acid and malondialdehyde were $1.3 \times 10^{-8}$ mol/L and $1.02 \times 10^{-8}$ mol/L, respectively. This simple, rapid, and sensitive method is useful for clinical measurements.

R eactive oxygen species are formed and degraded by all aerobic organisms, leading to either physiological concentrations required for normal cell function or excessive quantities; this state is called oxidative stress (1). Oxidative stress has been associated with a number of diseases and conditions in humans such as cancer, aging, pulmonary fibrosis, diabetes mellitus, and ischemia, which can be induced by reactive oxygen species (3). There is considerable evidence that oxidative stress is implicated in the development of diabetic complications (4). Reactive oxygen species–mediated oxidation of membrane lipids results in the formation of lipid peroxidation products such as malondialdehyde (5) and isoprostanes (6). Malondialdehyde level still happens to be the most widely used index of lipid peroxidation (7,8).

The cellular antioxidant systems can be divided into two major groups, enzymatic and nonenzymatic (1). Some nonenzymatic low molecular weight antioxidant compounds such as ascorbic acid, a-tocopherol (vitamin E), and carotenoids are consumed and can fall below normal ranges. Plasma or serum antioxidant measurement in humans can be influenced by a variety of parameters related to disease, nutrition, and lifestyle (9).

Ascorbic acid not only is an essential vitamin required for the prevention of deficiency diseases such as scurvy, it also might be useful for reducing the risk of developing chronic diseases such as cancer, cardiovascular disorders, and cataracts. The protective mechanism probably is due to the antioxidant effect of ascorbic acid (10).

Therefore, applications of various detection systems and analytical procedures for the separation of ascorbic acid and malondialdehyde have been reviewed extensively (8,10–18). In a previous paper, Karatas and colleagues (19) gave a simple malondialdehyde determination method with high performance liquid chromatography (HPLC).

The procedure presented here was developed for the determination of ascorbic acid and malondialdehyde under the same analysis conditions. The present study reports a fast and simple method for quantification of those compounds in human serum by HPLC and applies the method to serum samples in diabetic patients and controls.

Materials and Methods

Instrumentation: Chromatographic determinations were performed on a Cecil 1100 series HPLC (Cecil Inst., Ltd., Cambridge, United Kingdom) equipped with an 1100 series pump and UV absorbance detector. An HP 3395 integrator (Agilent Technologies, Palo Alto, California) was used to...
Figure 1: HPLC chromatograms from separations obtained using a mobile phase of (a) 1%, (b) 5%, (c) 7.5%, (d) 10%, (e) 12.5%, (f) 15%, (g) 17.5%, (h) 20%, (i) 25%, and (j) 30% methanol in monobasic potassium phosphate buffer. UV detection wavelength: 250 nm. Peaks: 1: multiple peak, 2: ascorbic acid, 3: malondialdehyde, 4: unknown.
record retention times and chromatograms and evaluate peak heights. A stainless steel, 150 mm × 4.6 mm, 5-mm dp, Wakosil II 5C18 RS column (SGE, Inc., Australia) was used at room temperature.

**Solvent and reagent:** The reagent 1,1,3,3-tetraethoxypropane was purchased from Sigma (St. Louis, Missouri). Ascorbic acid, methanol, hydroxide, monobasic potassium phosphate, phosphoric acid, hydrochloric acid, and perchloric acid were purchased from Merck (Darmstadt, Germany).

**Preparation of ascorbic acid and malondialdehyde standards:** Standards from an ascorbic acid stock solution (300 mM) in the range of 2.5–25 mM were prepared by dilution with 5% perchloric acid.

A malondialdehyde standard solution was prepared by acid hydrolysis of 1,1,3,3-tetraethoxypropane according to the method of Tsaknis and colleagues with small modifications (20). 1,1,3,3-Tetraethoxypropane (10 mL) dissolved in 10 mL of 0.1 M hydrochloric acid was heated in a boiling water bath for 5 min. After cooling, the mixture was diluted to 100 mL with water. This stock-standard solution contained 2.92 mg/mL of malondialdehyde. The stock solution was diluted and used for calibrations. The intra- and interassay coefficients for ascorbic acid and malondialdehyde were 2.7% and 5.6%, and 1.0% and 2.3%, respectively.

**Sample collection:** Blood samples were taken from volunteers at the Laboratory of Biochemistry, Firat University Medical School, Elazig, Turkey. Blood samples were collected without anticoagulant from the healthy volunteers and from the patients with type II diabetes mellitus. Serums were obtained by centrifugation, then were stored at 2 °C 80 °C until used.

**Sample preparation and assay procedure:** 25 mL of 0.1 M perchloric acid and 55 mL of distilled water were added to a 20-mL aliquot portion of human serum. Addition of acid was necessary to precipitate proteins and release the malondialdehyde bound to the amino groups of proteins and other amino compounds (21). Acid addition also was needed to maintain the stability of ascorbic acid (22). The samples then were centrifuged at 4500 rpm for 5 min and used for HPLC analysis.

The optimum absorption wavelength was 250 nm and flow rate was 1.2 mL/min. Chromatograms were monitored at 250 nm, and the injection volume was 20 μL. The retention times of ascorbic acid and malondialdehyde were 1.85 min and 2.70 min, respectively.

**Statistical analysis:** Comparisons of ascorbic acid and malondialdehyde levels between control and patient samples were performed using the Mann-Whitney U test. Statistical analyses were performed with SPSS 10.0 for Windows software (SPSS, Inc., Chicago, Illinois). The limit of statistical significance was set at P = 0.05.

**Results and Discussion**

**Method development:** Determination of ascorbic acid has been described by Cerhata and colleagues (12) and Tavazzi and colleagues (23), and malondialdehyde has been described by Karatas and colleagues (19) in my laboratory. Ascorbic acid and malondialdehyde were determined by using the same supernatant, but the mobile phase, column, flow rate, and wavelength were different. Ascorbic acid was determined by using a Li-60 column, a 3.7 mM monobasic potassium phosphate buffer (pH 4) as mobile phase, a flow rate of 1.0 mL/min, and a wavelength of 245 nm. Malondialdehyde was determined using a 250 mm 3 3.9 mm, 10-mm dp, Techpak C18 column (HPLC Tech., Garden City, United Kingdom), 65:35 (v/v) 30 mM monobasic potassium phosphate–methanol (pH 4) as mobile phase, a flow rate of 1.5 mL/min, and a wavelength of 254 nm.

Hultqvist and colleagues (7) determined ascorbic acid using a Supelcosil LC-18 column (Supelco, Inc., Bellefonte, Pennsylvania), 2:98 (v/v) methanol–5 mM monobasic potassium phosphate (pH 3.2) as the mobile phase, and coulometric electrochemical detection.

In the analysis of ascorbic acid using electrochemical detection, some researchers have applied potassium phosphate–acetoniitrite (14) and phosphate buffers containing disodium EDTA at various pH values (10,15,17,18) as mobile phases. In this work, it was decided to determine the ascorbic acid and malondialdehyde using the same column, mobile phase, flow rate, and absorption wavelength. Therefore, different mobile phases, columns (Li-60, C18, ODS-2), flow rates (0.8–1.5 mL/min), and wavelengths (240–260 nm) were used to analyze the human serum sample. However, the best results were obtained using the Wakosil II 5C18 column with the potassium phosphate–methanol mobile phase. Then, to improve the separation, different percentages of methanol in 30 mM monobasic potassium phosphate (1, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25, and 30%) were tested.

The optimum absorption wavelength was 250 nm and flow rate was 1.2 mL/min. Typical chromatograms of ascorbic acid and malondialdehyde were obtained under the conditions described previously. The change of the retention times and peak heights of ascorbic acid and malondialdehyde at the various methanol percentages is shown in Figure 1.

As shown in Figure 1, the peak heights of ascorbic acid and malondialdehyde were low at low methanol percentages and increased with increasing percentages of methanol. The malondialdehyde peak was interfering with the unknown peak (peak 4) until the mobile phase was at least 15% methanol, and it overlaid with the unknown peak at 7.5% methanol. With methanol concentrations above 17.5%, the ascorbic acid peak (peak 2) began interfering with the multiple peaks (labeled peak 1). Optimum analysis conditions were used to obtain the chromatogram in Figure 1g, where no interference is seen.

Serum samples were analyzed at optimized conditions. Ascorbic acid and malondialdehyde concentrations from diabetics and control subjects are shown in Table I.

As seen from the table, the diabetic patients had lower ascorbic acid levels and higher malondialdehyde levels than those of healthy controls.
the controls ($P < 0.05$). It is well known that serum and plasma ascorbic acid levels in diabetics are lower than in healthy people (24), and malondialdehyde levels in diabetics are higher than in healthy people (25–27). The results in Table I confirm these studies.

It has been suggested that extraction of ascorbic acid using methanol–water–EDTA is straightforward for its stability for more than 12 h (28). In addition, Paulo and colleagues (29) used 100% methanol as mobile phase for the determination of ascorbic acid. These studies support the elution of the ascorbic acid peak using the mobile phase with methanol. Washko and colleagues (28) reported that there are disadvantages to HPLC with either amperometric or coulometric detectors. These systems have a high initial cost and require a trained operator. Both types of detectors also are sensitive to contaminants, an issue that can be troublesome at high sensitivity. In this study, sensitive and reproducible results were obtained in all cases using HPLC with UV detection. The proposed method is rapid, simple, and easy to perform and also minimizes chemical materials and time loss. It is preferable that the determination and quantification of ascorbic acid and malondialdehyde are performed using the same analysis conditions.

References
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