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Note

High-performance liquid chromatographic determination of ascorbic acid in human tears

RICHARD R. HOWARD, II, TIM PETERSON and PETER R. KASTL*

Department of Ophthalmology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112 (U.S.A.)

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Several high-performance liquid chromatographic (HPLC) assays for ascorbic acid have been described in the literature. Floridi et al. [1] have developed an HPLC method for the determination of ascorbic acid in foods. Rose and Nahrwold showed that accurate quantitative analysis of ascorbic acid could be achieved in various samples of guinea pig tissues and serum [2], and used HPLC to separate and quantify ascorbic acid and dehydroascorbic acid in biological samples, foods and pharmaceutical vitamin preparations [3]. Bigler and Kelly [4] developed a rapid, quantitative technique for the separation of ascorbate and ascorbate-2-sulfate for the purpose of examining the metabolism, hydrolysis and chemical purity of ascorbic-2-sulfate. A method for the analysis of ascorbic acid (reduced form) in human aqueous humor has been established by Lam and Lee [5]. No report has discussed the use of HPLC for the qualitative and quantitative analysis of ascorbic acid in tears.

In this paper, we present an HPLC asay for quantifying vitamin C in tears collected with Schirmer strips. The aim of this investigtion was to develop a fast and reliable HPLC method for ascorbate determination in a small tear volume (5–10 μl) without sample pretreatment.

EXPERIMENTAL

Apparatus

We used a Perkin-Elmer Series 4 liquid chromatograph equipped with a Rheodyne 1725 injector valve, an ISS-100 autosampler and a LC-85B spectrophotometric detector set at 245 nm (Perkin-Elmer, Norwalk, CT, U.S.A).

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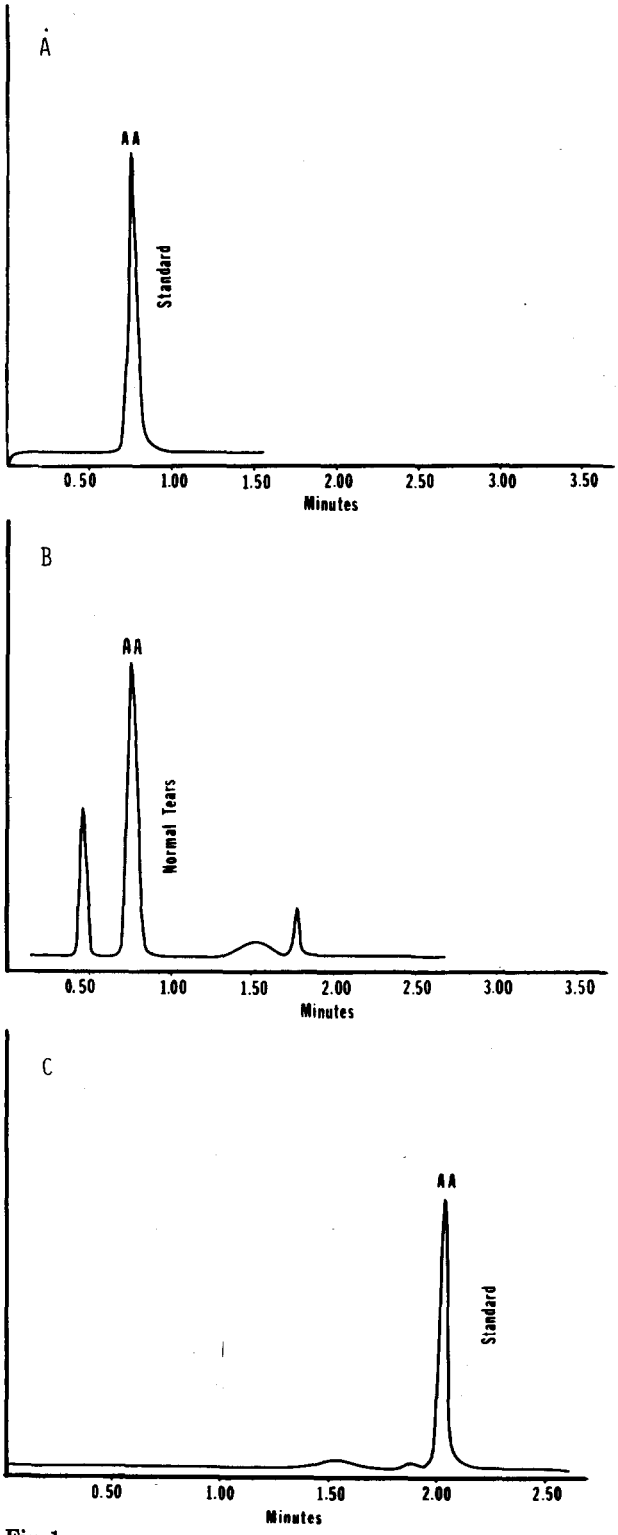

Fig. 1.

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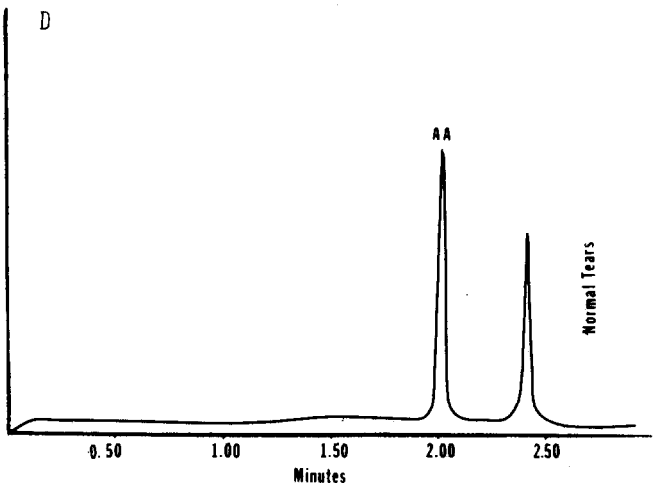

Fig. 1. Chromatograms of ascorbic acid (AA) from standard solutions and normal human tear samples. (A) Standard solution with 17.6 mg/dl AA; (B) normal human tear sample; (C) standard solution with 0.7 mg/dl AA; (D) normal human tear sample. Chromatographic conditions: (A, B) column, C_{18}; injection volume, 90 μl; mobile phase, 0.1% phosphoric acid (pH 3.0)-methanol (50:50); flow-rate, 1.5 ml/min; (C, D) column, C_8; injection volume, 90 μl; mobile phase, 0.1% phosphoric acid (pH 3.0)-methanol (90:10); flow-rate, 0.6 ml/min.

A Perkin-Elmer 3600 data station was employed to calculate peak areas. Separation was achieved with a 3-μm C_{18} column (10 cm$\times$4.6 mm) (Rainin Instruments, Woburn, MA, U.S.A), a 3-μm C_8 column (10 cm$\times$4.6 mm) (Rainin) and a 10-μm C_8 (25 cm$\times$4.6 mm) LiChrosorb column (Alltech Applied Science, State College, PA, U.S.A.). Each column was fit with a guard column packed with the appropriate pellicular C_{18} or C_8 packing material. The mobile phase was methanol–water (10:90) buffered with 0.1% phosphoric acid. An isocratic elution at flow-rates of 0.5–2 ml/min was used.

Reagents and materials

Ascorbic acid, homocysteine and phosphoric acid were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade solvent were degassed by sparging with helium before use. Standardized Schirmer papers (35 mm$\times$5 mm) (developed and standardized by Halberg and Berens, Coopervision Pharmaceuticals, c/o Theatres Service, College Park, GA, U.S.A.) were placed in polyethylene sleeves sealed at one end to prevent evaporation of vitamin C standards and tears [6].

Sample preparation

A standard calibration curve was made in the following way: stock solutions of ascorbic acid were diluted in aqueous homocysteine (2 m*M*), a known anti-oxidant, to concentrations ranging from 0.2 to 2 mg/dl [5]. Schirmer strips were prepared by bending the strips at the notch and securing them at the edge of a counter with tape. The larger portion of the strip hung vertically over the edge of the counter. Volumes (10 μl) of the above solutions were applied to the smaller

bent portion of the strips with a microliter pipette (1 μl at a time) at time intervals approximating the normal human tear secretion rate (0.5–2.0 μl/min) [6, 7]. The strips were nitrogen-dried and cut at the notch into precise 10-mm segments, then placed into the ISS 100 autosampler microvials. Each microvial was filled with 100-μl volumes of aqueous homocysteine (2 m*M*) and vortexed for 25 s. The vials were placed into the ISS 100 autosampler and 90 μl of each sample were injected. Peak areas were plotted against concentration.

Human tear samples were obtained on Schirmer strips which had been saturated with 0.1 *M* homocysteine and air-dried. Each strip was folded 5 mm from the rounded end notch and placed into the lateral third of the lower cul-de-sac of the eye [6, 8]. At the time of the insertion of the tip, the subject was instructed to keep the eyes closed for the duration of the test. The strips remained in the eyes for 5 min and were then removed. In all cases, the wet length exceeded 15 mm. The strips were then air-dried and processed as above.

RESULTS AND DISCUSSION

Identification and purity

Fig. 1A–D illustrate the detection of ascorbic acid in tears. The ascorbate in standard solutions analyzed using C_8 and C_{18} columns demonstrated the same retention time as the suspected ascorbate peak in tears. Fig. 2 shows representative spectra of an ascorbic acid standard solution and a normal tear sample taken at the peak crest, using stopped-flow technique. In both cases the maximum wavelength was the same (245 nm) as was the general shape of each spectrum.

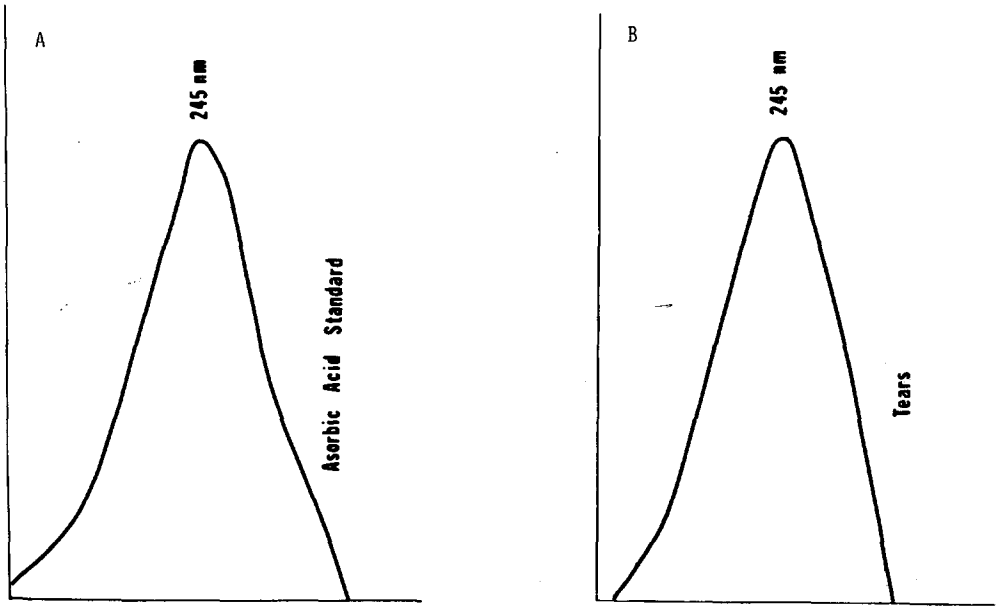

Fig. 2. UV spectrum of ascorbic cid from (A) standard solution and (B) normal human tears taken at peak crest, using stopped-flow technique.

In addition, the purity of the ascorbic acid peak in tears was confirmed by calculating the absorbance ratios of the leading edge, crest and trailing edge of the ascorbate standard peak and the confirmed ascorbate peak in tears. No difference in purity between the ascorbate standard and tear ascorbate was observed. Thus, ascorbic acid in tears was identified on the basis of retention time, spectral data and absorbance ratios.

Potential interference from other endogenous compounds in tears was ruled out by adding a known amount of ascorbic acid to a sample of tears. The retention time was unaltered and the peak area increased linearly with the amount added.

Precision and accuracy

Precision studies were accomplished in order to improve the chromatographic assay. Under the chromatographic conditions described in Fig. 1A, a coefficient of variation (C.V.) of 1.2% ($n=5$) was obtained for a 1.5 mg/dl solution. Interassay precision was determined by measuring reduced ascorbic acid (9.3 mg/dl) on different days over one week ($n=5$), giving a C.V. of 2.9%.

The accuracy of this method was demonstrated by a linear calibration curve, plotted as the peak area of ascorbic acid versus the concentration of ascorbic acid, in the concentration range 0.2–2 mg/dl.

Human tear ascorbic acid

The concentration of ascorbic acid in human tears was found to be 1.14 ± 0.6 mg/dl (mean $\pm$ S.D., $n=50$). This chromatographic method measures only the reduced form of ascorbic acid. Determination of total vitamin C is achieved by reduction of dehydroascorbic acid to ascorbic acid by homocysteine [5]. Detection of the reduced form at 245 nm under acidic conditions (pH 3) allows accurate analysis of as little as 0.2 μg of total ascorbic acid [5].

In conclusion, this chromatographic method appears particularly suitable for rapid evaluation of ascorbic acid in tears at μmol levels. Tear samples can be collected on commonly available Schirmer strips, dried and transported to the laboratory at leisure. This technique, in addition to being accurate and sensitive, is simple, fast and sufficiently economical to serve clinical needs and can be used to determine ascorbic acid more rapidly and with fewer sample manipulations than standard colorimetric techniques [1, 5]. This assay has potential utility for quantitative measurement of both stores of vitamin C, allowing for the evalution of the extent of vitamin C deficiency.

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