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Measurement of ascorbic acid in human aqueous humour and plasma and bovine aqueous humour by high-performance liquid chromatography with electrochemical detection

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Abstract: High-performance liquid chromatography (HPLC) on ODS silica with cetyltrimethylammonium bromide as an ion-pairing reagent and electrochemical detection (ECD) was used to determine ascorbic acid in bovine and human aqueous humour and human plasma. Hydroquinone was used as the internal standard. A calibration curve plotted with ascorbic acid concentrations in the range 0.5–5 μg ml^{-1} for peak height versus internal standard peak height had a correlation coefficient of 0.998. The RSD (precision) between analyses of the same diluted sample was 1.5% and the RSD (reproducibility) between analyses of separate aliquots of the same sample of aqueous humour was 1.6%.

Keywords: *Ascorbic acid; ion-pair HPLC; electrochemical detection; aqueous humour; plasma.*

Introduction

The high concentration of ascorbic acid in human and rabbit aqueous humour is well established [1, 2]. The exact role of ascorbic acid in aqueous humour is not clear but it has been postulated that it may serve as an antioxidant that protects the lens against free radical attack [3]; dietary intake of ascorbic acid and other antioxidants may delay or prevent cataract formation by inhibiting oxidative damage of lens proteins [4–6]. There is evidence that ascorbic acid may cause depolymerization of hyaluronic acid *in vitro* [7, 8] and this property is of interest in our present study *in vivo*.

A wide range of methods is available for the analysis of ascorbic acid. Colorometric procedures involving the reaction of ascorbic acid with 2,4-dichlorophenol-indophenol [9] or dinitrophenylhydrazine have been used [10]. HPLC on aminopropylsilyl columns with UV detection has been used for the analysis of ascorbic acid in food and biological fluids [11–13]. Methods with UV detection are of limited sensitivity and other substances in the biological matrix may give rise to interfering peaks in the chromatogram [14]. An improvement in selectivity was achieved by the reduction of ascorbic acid followed by reaction with dinitrophenylhydrazine and analysis by HPLC with colorimetric detection [15]. HPLC with ion-pairing and ECD or the use of ion-exchange columns with ECD is more sensitive than HPLC with UV detection for the determination of ascorbic acid [16–18] and is more suitable for the determination of ascorbic acid in biological samples such as plasma where concentrations are low.

In the present paper a method using reversed-phase HPLC with ion-pairing and electrochemical detection is described for the determination of ascorbic acid in human aqueous humour and plasma and in bovine aqueous humour.

Experimental

Chemicals

Ascorbic acid, hydroquinone, cetyltrimethylammonium bromide, metaphosphoric acid, sodium acetate and glacial acetic acid were obtained from Aldrich Chemical Co. (Gillingham, Dorset, UK). HPLC grade aceto-

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nitrile and water were obtained from Rathburn Chemical Co. (Walkerburn, Peebleshire, UK).

Bovine aqueous humour

Eyes from freshly killed cattle were obtained from the local abattoir. The aqueous humour was collected within 1 h of killing the animals. The surface of the eye was excised with a scalpel; the aqueous humour (≈0.5 ml) was collected and mixed with 0.5 ml of 0.1 M metaphosphoric acid and the samples were stored at −20°C until analysis.

Human aqueous humour

Human aqueous humour was collected from volunteer patients undergoing surgery for cataracts. The samples (30–200 μl) were collected as described previously [19] and transferred to sample tubes containing 200 μl of 0.1 M metaphosphoric acid and stored at −20°C. Blood samples were also collected from volunteer patients and centrifuged to obtain plasma; a 500-μl aliquot of the plasma was diluted by addition of 500 μl of metaphosphoric acid. The samples were stored at −20°C until analysis.

Preparation of samples

Stock solutions of hydroquinone and ascorbic acid (each 1 mg ml^{-1} in water) were freshly prepared each day.

A 200 μl volume of 30% trichloroacetic acid was added to 50-μl samples of bovine aqueous humour or human aqueous humour which had been preserved with metaphosphoric acid. The samples were then centrifuged; 1 μg of hydroquinone was added to 100 μl of the supernatant which was then diluted to 1 ml with the mobile phase. A 50 μl volume of the sample was then injected into the chromatograph using an autosampler.

A 200 μl volume of 30% trichloroacetic acid was added to the human plasma samples that had been preserved with metaphosphoric acid. The samples were then centrifuged to remove precipitated protein; 1 μg of hydroquinone was added to 500 μl of the supernatant which was then diluted to 1 ml with the mobile phase. A 50 μl volume of the sample was injected into the chromatograph using an autosampler.

Instrumentation

A Hewlett–Packard 1082 HPLC system with an autosampler was used in the analyses. The instrument was fitted with 10 × 4.6 mm i.d. column packed with 5-μm Spherisorb ODS-1 and with a SGE ODS-1 guard cartridge system (Burke Analytical, Glasgow, UK). The column eluent was monitored with a LC-4A amperometric detector (Bioanalytical Systems). The potential of the detector was set at 0.6 V versus an Ag–AgCl reference electrode.

The mobile phase was prepared by dissolving sodium acetate (0.08 M) and cetyltrimethylammonium bromide (CTMAB 0.001 M) in acetonitrile–water (5:95, v/v); the pH of the solution was adjusted to pH 4.2 with glacial acetic acid. The flow rate was adjusted to 1 ml min^{-1}.

Calibration curve

Solutions containing 1 μg of hydroquinone and 0.5–5 μg of ascorbic acid in 1 ml of the mobile phase were prepared. The solutions were then injected into the chromatograph.

Results and Discussion

The use of CTMAB as an ion-pairing reagent enabled the column to retain ascorbic acid while permitting a high water content in the mobile phase. The retention time of ascorbic acid was approximately 4 min. Cetylpyridinium chloride (CPC), cetrimide (CT) and tricaprylmethylammonium chloride (TCMC) were also tried as ion-pairing reagents. CPC and CT gave insufficient retention of the ascorbic acid which was eluted too close to the solvent front. TCMC, after initially appearing to be useful, eventually accumulated on the column so that ascorbic acid formed an ion pair which was no longer eluted. CTMAB caused retention of ascorbic acid where it was well resolved from the solvent front but without producing a long retention time.

Initially, D-isoascorbic acid was tested as an internal standard but its retention time was very close to that of ascorbic acid. The retention time of hydroquinone was about 6 min and this compound gave an instrumental response on a weight-for-weight basis about four times that of ascorbic acid.

Initially, 0.03 M ion-pairing reagent was used and it was found that the retention time of ascorbic acid gradually increased with time; when an ODS 2 column was used with a higher percentage loading of the reversed phase, the ion-pairing reagent rapidly built up on the column and eventually the ascorbic acid was no

longer eluted by the mobile phase with its low content of organic solvent. The retention time of ascorbic acid obtained with an ODS 1 column and 0.01 M ion-pairing reagent was stable to within ±0.1 min on a particular day.

A standard curve was constructed using a fixed amount of hydroquinone as the internal standard and varying the amount of ascorbic acid over the range 0.5–5 μg ml^{-1}. The curve was linear ($r = 0.998$). The precision was determined by injecting five replicates of the same sample of diluted bovine aqueous humour containing 246.4 μg ml^{-1} of ascorbic acid; the RSD was 1.49%. The reproducibility was determined on five separate analyses of the same sample of bovine aqueous humour containing 311.8 μg ml^{-1} of ascorbic acid; the RSD was 1.63%.

Figure 1(A) shows a HPLC–ECD trace for ascorbic acid in bovine aqueous humour in comparison with a trace [Fig. 1(B)] obtained by injection of a standard mixture containing 2 μg of ascorbic acid and 1 μg of hydroquinone. Ascorbic acid was quantified in 22 samples of bovine aqueous humour and the mean concentration was 524.9 ± 113.4 μg ml^{-1} (range 318.7–708.5 μg ml^{-1}).

Figure 1(C) and 1(D) shows HPLC–ECD traces for ascorbic acid in human aqueous humour and plasma; differences in retention times between traces A/B and C/D are due to removal of the HPLC column which, although obtained from the same manufacturer, obviously had a lower loading of stationary phase in the case of traces C/D. Ascorbic acid was quantified in 40 samples of human aqueous humour and the mean concentration was 260.8 ± 94.1 μg ml^{-1}. Previous work using HPLC with UV detection determined the ascorbic acid concentration in 10 samples of aqueous humour from 10 cataract patients; the mean level was 1378 ± 310 μM (242 ± 55 μg ml^{-1}) [12]. Another study found that the concentration of ascorbic acid in human aqueous humour was 200–250 μg ml^{-1} [15]: consequently the present results are in good agreement with those of earlier workers. The concentration of ascorbic acid in human aqueous humour is about 20 times that in plasma. Ascorbic acid was also quantified in 16 samples of human plasma; the mean level was 4.2 ± 3.6 μg ml^{-1} (range 0.3–11.1 μg ml^{-1}).

The study will be extended to examine the effect of oral administration of ascorbic acid on the aqueous humour concentration of ascorbic acid both in rabbits and humans. These findings will be related to the effects of ascorbic acid administration on the degree of polymerization of Healon® and on intra-ocular pressure.

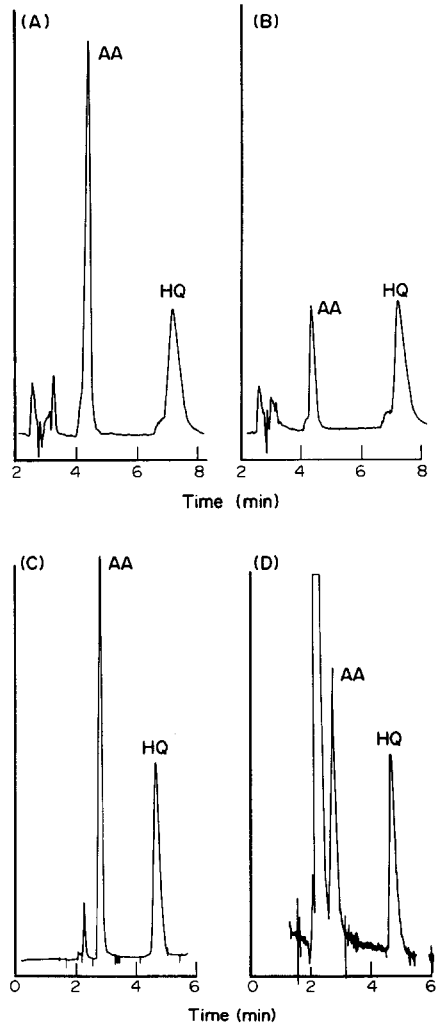

Figure 1
(A) HPLC trace of ascorbic acid (AA) in a 50-μl aliquot of bovine aqueous humour after addition of 1 μg of hydroquinone (HQ), dilution to 1 ml and injection of 50 μl into the chromatograph. (B) HPLC trace for a standard mixture of ascorbic acid (AA, 2 μg ml^{-1}) and hydroquinone (HQ, 1 μg ml^{-1}). (C) HPLC trace of ascorbic acid (AA) in a 50-μl aliquot of human aqueous humour after addition of 1 μg of hydroquinone (HQ), dilution to 1 ml and injection of 50 μl into the chromatograph. (D) HPLC trace of a 208-μl aliquot of human plasma containing 7.6 μg ml^{-1} of ascorbic acid after addition of 1 μg of hydroquinone (HQ).

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