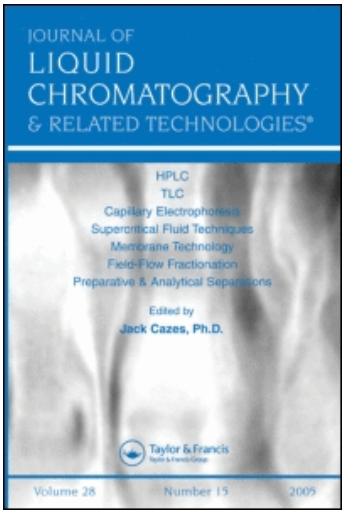

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Ascorbic Acid Determination by Hydrophobic Liquid Chromatography of the Osazone Derivative. Application to the Analysis of Aqueous Humor

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ASCORBIC ACID DETERMINATION BY HYDROPHOBIC LIQUID CHROMATOGRAPHY OF THE OSAZONE DERIVATIVE. APPLICATION TO THE ANALYSIS OF AQUEOUS HUMOR (*)

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ABSTRACT

The dinitrophenylhydrazine colorimetric method for the analysis of ascorbic acid has been converted to a high-performance liquid chromatography (HPLC) procedure. The bis-(dinitrophenyl) hydrazone resulting from the reaction of ascorbic acid and dinitrophenylhydrazine is separated on a reversed-phase system from other components in the reaction mixture and used for quantitation purposes. The specificity of the analysis is thus greatly improved and its sensitivity is brought down to the picomol level. The HPLC procedure described here is therefore specially suitable for the analysis of very small volume samples when high specificity and sensitivity are of prime importance. With this method, as in the original colorimetric procedure, it is also possible to specify

* A preliminary report of this work was presented at the 1980 Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), Orlando, Florida.

the contribution of the reduced and oxidized forms of ascorbic acid to the total amount present in the sample. Basically, the same approach could be advantageously used to improve other colorimetric methods of analysis. Human aqueous humor samples taken from senile cataract patients at the time of surgery show variable but significant amounts of oxidized ascorbic acid. This oxidized fraction, therefore, should not be neglected as is frequently done when the ascorbic acid system of the aqueous humor is studied.

INTRODUCTION

The potential role of ascorbic acid in biological systems has stimulated wide multidisciplinary interest in this compound over the years. It is not, then surprising that a variety of now classical analytical methods exist for its quantitation (1-3) and that improved procedures have recently been reported in the literature (5-7). Methods (4, 5, 8) based upon reactions that permit measurement of both reduced and oxidized forms of ascorbic acid are of special significance because of the ability of ascorbic acid to participate in biological oxidation-reduction systems. In order to better evaluate the possible role of ascorbic acid in such systems, it is important to define, when a particular system is analyzed, the fraction of total ascorbic acid present in the oxidized or in the reduced form and how these fractions might be affected under variations imposed upon the system.

These analytical requirements are fulfilled by the widely used original or modified method of J.H. Roe (4, 9, 10) based upon the reaction of 2,4-dinitrophenylhydrazine (DNPH) with oxidized as-

corbic acid to form the bis (dinitrophenyl) hydrazone (osazone) derivative. We have now adapted this colorimetric method to high-performance liquid chromatography (HPLC), the osazone product being isolated on a C^{18}-hydrophobic column.

The HPLC method has been applied to study the ascorbic acid system in samples of human aqueous humor from cataract patients.

MATERIALS AND METHODS

High Performance Liquid Chromatography (HPLC). A model ALC/GPC-204 Liquid Chromatograph with a fixed wavelength (254 nm) UV-monitor (Waters Associates, Inc., Milford, Mass.) was used for this work.

Solvents and Reagents. Acetonitrile (ACN) was obtained from Waters Associates. HPLC grade ethyl acetate (EtOAc) and spectranalyzed methanol (MeOH) were from J.T. Baker (Phillipsburg, NJ). Water for HPLC was prepared with a combined Milli-RO/destillator/Milli-Q/0.45 μm ultrafiltration membrane system (Millipore Corp., Bedford, Mass.). The aqueous part of the ACN buffer to be used in HPLC was filtered through a Millipore membrane (0.45 μm, HAWP 04700) before mixing it with the solvent.

Ascorbic acid was obtained from Sigma Chemical Co. (Saint Louis, Missouri). Meta-phosphoric acid (HPO_3); 2,4-dinitrophe-

nylhydrazine (DNPH); bromine (Br_2); thiourea and sulfuric acid were from J.T. Baker.

UV-Visible Spectrophotometry. A Perkin-Elmer model 552 Spectrophotometer (Norwalk, CT) was used for recording the UV-Vis absorption spectra shown in this report.

Preparation of samples. Ascorbic acid standard solutions were prepared in 5% meta-phosphoric acid and kept in the refrigerator for periods not greater than one week.

Aqueous humor samples were obtained by corneal puncture in patients ready to undergo cataract surgery. Aliquots of 20 µl were immediately added to 0.5 ml of 5% meta-phosphoric acid.

Derivatization of ascorbic acid. A. Common procedure. To 0.5 ml of 5% HPO_3, 20 µl of a standard solution of ascorbic acid or 20 µl of aqueous humor were added.

Ascorbic acid present in these samples was oxidized with 0.5 µl of Br_2. Excess Br_2 was removed by bubbling air through the solution until clear. Total (oxidized + reduced) ascorbic acid in the sample is obtained from this analysis.

Duplicate samples were also prepared which were not submitted to Br_2 oxidation. Therefore only oxidized forms of ascorbic acid already present in the sample will be detected in this analysis. Reduced ascorbic acid in the sample can be calculated by difference between those two determinations.

After adding to the tubes 0.5 ml of 1% thiourea in 5% HPO_3 and 20 µl of 2% DNPH in 9N H_2SO_4, the reaction between DNPH and oxidized ascorbic acid was allowed to proceed for 4 hrs. at 37°C. The acid solution of DNPH was prepared and filtered just before the analysis (Millipore FHLP01300 membrane, 0.45 µm, mounted on a plastic Swinnex).

The red osazone formed was extracted in 0.5 ml of EtOAc and aliquots of 5 µl of this extract were directly injected into the chromatograph. Alternatively, the EtOAc extract was separated from the aqueous phase underneath, kept at -25°C and HPLC analyzed next day. Unless otherwise stated, the common procedure just described was used throughout this work.

B. Microprocedure. Microassays were also performed in 1.0 ml Reacti-Vials (Pierce, Rockford, Ill.) containing 5 µl sample and 50 µl 5% HPO_3. Br_2 oxidation was carried out in this case by adding to the vial 5 µl of a freshly prepared solution of Br_2 (2 drops in 5 ml 5% HPO_3). After waiting for 10 min., 50 µl of 1% thiourea and 10 µl of 0.4% DNPH in 9N H_2SO_4 were added. Incubation conditions were as before. The mixture was extracted with 75 µl of EtOAc and 5 µl of this extract were injected into the column.

HPLC Conditions. A µ-Bondapak-C^{18} column (Waters Associates, 3.9mm × 30cm, 10µ) was used for the analytical separation of the osazone of ascorbic acid from other components in the EtOAc extract.

The eluant solvent was 50% acetonitrile containing 10mM sodium acetate buffer, pH 4.1. Flow rate was 1.0 ml/min. The recorder was set at a chart speed of 0.2 in/min. The effluent was monitored at 254nm. The sensitivity setting of the monitor depended on the amounts of ascorbic acid to be analyzed.

Purification of the Osazone of Ascorbic Acid. The osazone of ascorbic acid was prepared in mg amounts by scaling up the common procedure described above. The red precipitate was collected and briefly washed with water and EtOH over a Millipore 0.45 um filter HAWP 04700 and then was redissolved in EtOAc.

Recrystallization of DNPH. DNPH was dissolved to saturation in warm EtOH. The solution was allowed to slowly cool down. Crystals formed were recovered by filtration, washed with cold EtOH and dried.

RESULTS

The osazone of ascorbic acid prepared as indicated in the previous section was dissolved in EtOAc and used to establish the HPLC conditions to be followed in the determination of ascorbic acid.

Figure 1 shows representative HPLC profiles of EtOAc extracts of actual samples. Profile A was obtained when the sample was either 5% HPO_3 or reduced ascorbic acid with the Br_2 step omitted

from the procedure. Profile B represents the typical chromatogram obtained when oxidized ascorbic acid is produced in the sample by Br_2 or when it already exists as such in the sample. The elution time of peak 6 in profile B (about 9 min) corresponds to that of the purified osazone of ascorbic acid.

Profile A shows an almost flat baseline at the level of peak 6 when the monitor is set at a relatively low sensitivity (.02 Absorbance Units Full Scale, AUFS). A small background peak which coelutes with the osazone becomes apparent at higher sensitivities (profile D, dotted line). The elution time of the osazone in D is about 7.5 min.

Figure 1 also indicates that the osazone peak is the only evident product of the reaction between oxidized ascorbic acid and DNPH because all the other peaks in profile B are also present in profile A.

It was possible to identify the source and, in some cases, the nature of peaks 1-5 and 7 by successively excluding or adding components to the derivatizing mixture and by studying the UV-Visible spectra of the isolated HPLC peaks.

Thiourea and EtOAc contribute to form peak 1. Peak 2 and partially peaks 4 and 5 are due to impurities in EtOAc.

Peak 3 corresponds to unreacted DNPH. Peak 7 and most of peaks 4 and 5 are due to components present in the acidic solution

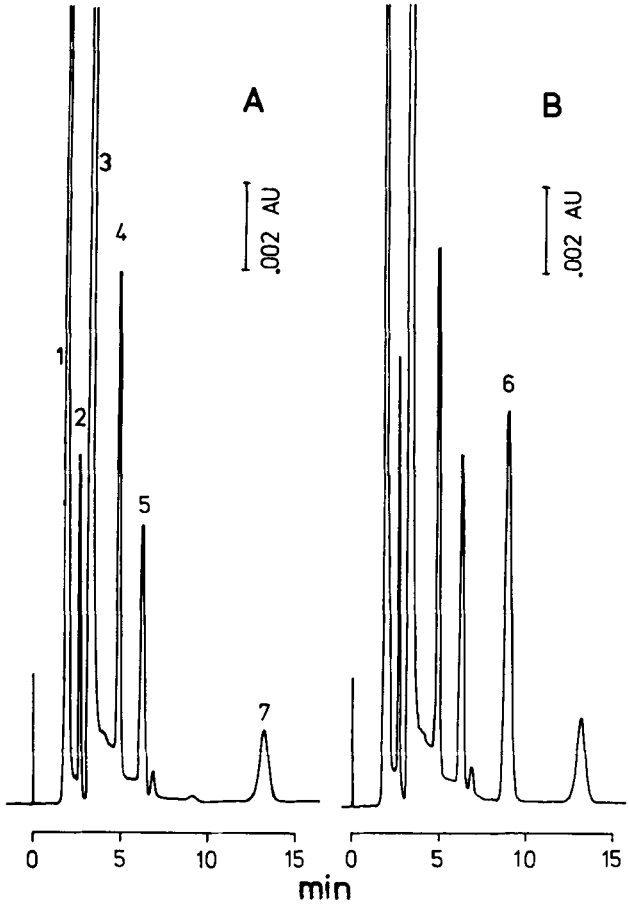


FIGURE 1A & B

of DNPH. Two commercial preparations of DNPH were tested and both showed these additional components. Although they represent a small fraction relative to DNPH (Fig.1C) peaks 4, 5 and 7 become very apparent at high sensitivities (Fig.1D). The recrystallization of DNPH, as described in the METHODS section, above, did not result in the long-term dissapearance of these extra peaks.

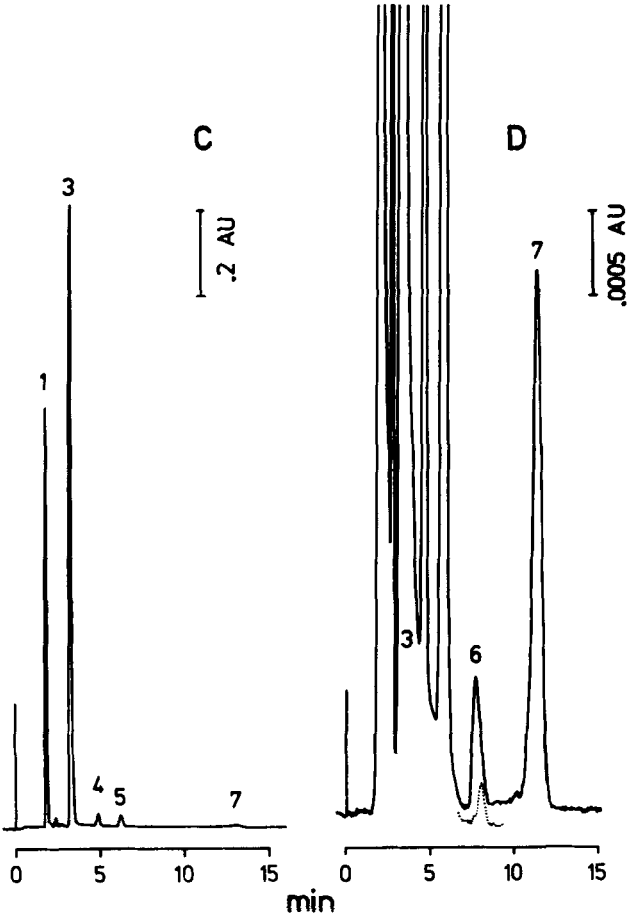


FIGURE 1

Representative HPLC profiles of the method described in the text. A and C are blank samples. B and D correspond respectively to 1.1 and 0.1 nanomols of ascorbic acid injected into the column.

Figure 2A (curve a) shows the absorption spectrum of the EtOAc-extracted derivatization mixture in the absence of ascorbic acid. This spectrum is dominated by DNPH (no osazone has been produ-

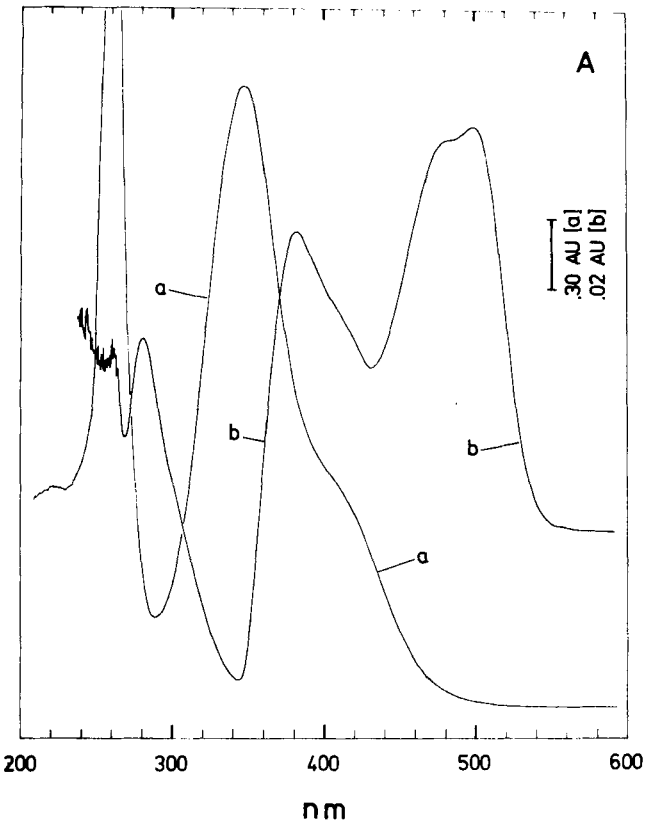


FIGURE 2

UV-Vis absorption spectra of the samples described in the text after properly diluted: EtOAc extracts (A), osazone of ascorbic acid (B) and HPLC peaks (C).

ced) with a peak of absorption at about 350 nm. When the osazone of ascorbic acid has been formed in the derivatizing mixture, a new component, absorbing at 480-500 nm appears which is best seen in the differential spectrum of the same figure (curve b). The

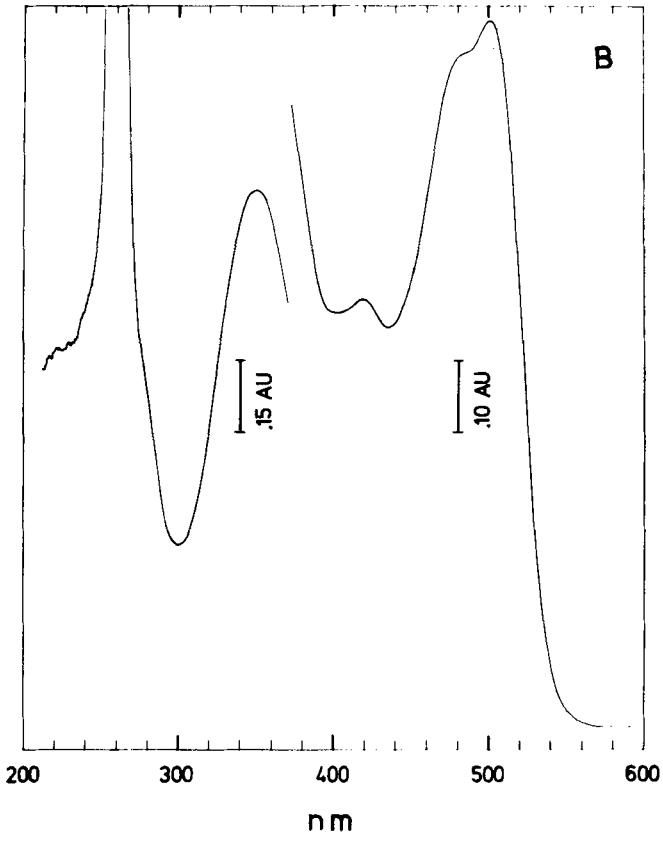


FIGURE 2B

double absorption band at 480 and 500 is characteristic of the osazone as shown by the absorption spectrum of the purified compound in Fig. 2B. The osazone also exhibits and absorption maximum at 350 nm. In the differential spectra of Fig. 2A (curve b) the latter has been distorted, however, probably because of the simultaneous dissappearance of DNPH to form the osazone.

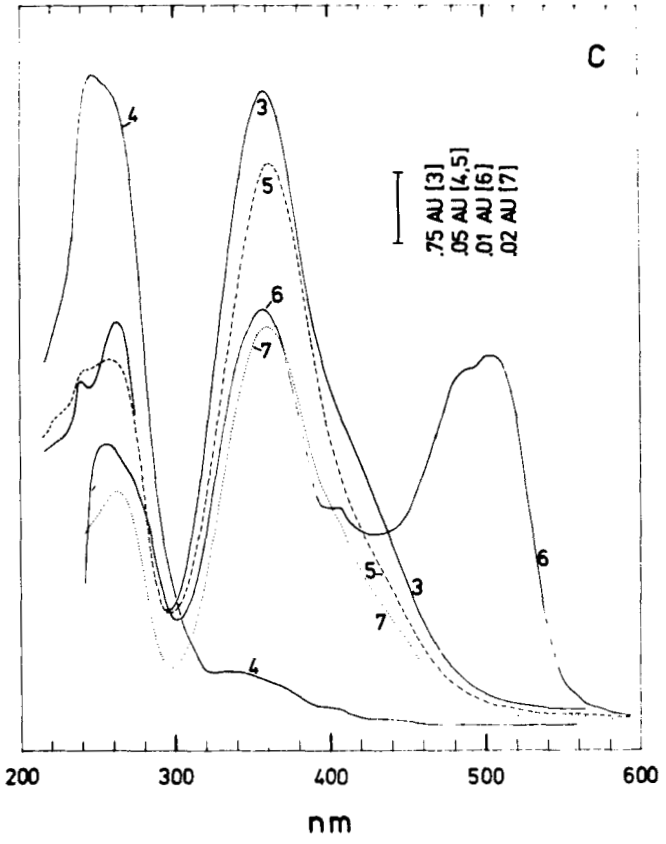


FIGURE 2C

Some of the peaks isolated by HPLC were also analyzed for light absorption. For this the chromatographic conditions were manipulated to obtain baseline peak resolution of the components eluted before peak 6. The UV-Vis spectra of peaks 3 to 7 are shown in Fig. 2C. We mentioned already that peaks 5 and 7 are closely associated with DNPH. They also show essentially the same spectra as DNPH (peak 3) with absorption maxima at about

360 nm. It should be noted the red-shift of about 10 nm in the absorption maxima of DNPH that has ocurred when the ACN buffer is used, instead of EtOAc, as the solvent.

Peak 6, as expected, shows both a maximum at about 360 nm and the double absorption maxima at 480 and 500 nm of the osazone. The latter maxima have not been affected by the change in the polarity of the environment.

Only the height of peak 6 is linearly related to the concentration of ascorbic acid originally present in the sample. Excellent linearity (correlation coefficient, 0.996) was found for sample concentrations of ascorbic acid up to 5.68 mM (Fig.3) which corresponds to 1.136 n moles injected into the chromatograph when the analysis is started with 20 µl samples. Most points in figure 3 are mean values of at least two independent determinations, and generally showed standard deviations (S.D.) of 7% of the mean.

Samples of aqueous humor taken from patients at the time of cataract surgery were analyzed for ascorbic acid with the present procedure. Table 1 shows the individual data obtained.

DISCUSSION

Roe's colorimetric procedure for the determination of ascorbic acid is based upon the reactions shown in Scheme 1.

Oxidized forms of ascorbic acid (DHA and DKG) are able to react with DNPH to form the same water-insoluble osazone (1),

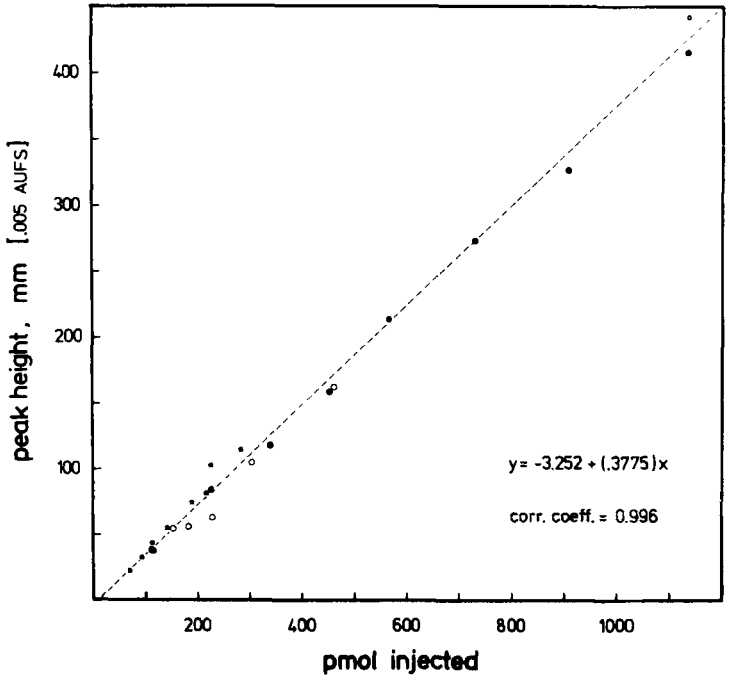


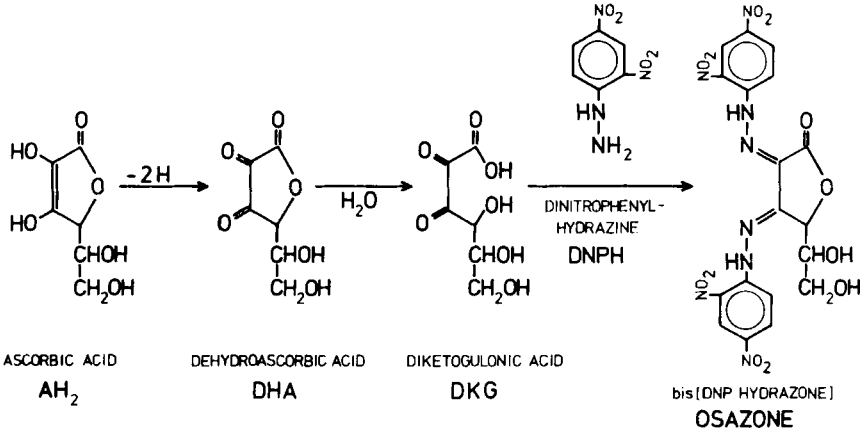
FIGURE 3

Correlation between the height of the osazone peak and the amount of ascorbic acid injected into the column. Squares and solid circles represent data obtained with the common HPLC procedure described in the text (20 µl samples). Empty circles represent preliminary data obtained with the microassay procedure (5 µl samples). Original peak heights were converted to a full scale sensitivity range of 0.005 absorbance units.

TABLE 1

Ascorbic Acid in the Aqueous Humor of Cataract Patients

Sample Identification	Ascorbic Acid		
	Total mM	Oxidized mM(%)	Reduced (%)
8036	1.020	.181 (17.7)	82.3
8037	.700	.133 (19.9)	81.0
8043	1.900	.088 (4.6)	95.4
8053	1.750	.297 (17.0)	83.0
8060	1.156	.130 (11.2)	88.8
8061	1.730	.369 (21.3)	78.7
8068	2.035	-	-
8076	1.464	.113 (7.7)	92.3
8084	1.960	.213 (10.9)	89.1
Averages	1.524	.190 (12.5)	87.5



SCHEME 1

Main reaction sequence in the dinitrophenylhydrazine (DNPH) based methods for the determination of ascorbic acid. Reduced ascorbic acid (AH_2) is oxidized under relatively mild conditions to dehydroascorbic acid (DHA). The latter, in an acidic aqueous medium is spontaneously converted to diketogulonic acid (DKG), the only species probably able to react with DNPH to form the osazone derivative (1).

while reduced ascorbic acid (AH_2) is not. If ascorbic acid in a sample is submitted to oxidation (Norit, Br_2, Cu^{2+}, etc.) a value for total ascorbic acid in that sample is obtained (AH_2+DHA+DKG). If oxidation of the sample is omitted, the amount of DHA + DKG in the sample can be determined. AH_2 is given by the difference between those two determinations.

Although, in this work, no attempt was made to obtain individual values for DHA and DKG, this is possible by choosing analytical conditions that specifically reduce DHA to AH_2 without DKG being affected (1,10).

The coupling of oxidized ascorbic acid and DNPH is carried out in Roe's based procedures under acidic conditions (about 1.5 N H_2SO_4) and in the presence of thiourea to avoid unwanted oxidation of ascorbic acid during the incubation time (4). A great molar excess (about 300 times minimum) of DNPH over ascorbic acid has been generally employed (1-3). An incubation temperature of 37°C is recommended to reduce interferences by sugars (11).

In the colorimetric method the final step consists of dissolving the osazone by adding H_2SO_4 to a final concentration of about 15 N (1-3). This results in a molecular rearrangement of the osazone, spectrally revealed by a red shift of 20 nm that occurs upon acidification. This step would also help to destroy small amounts of

sugar osazones formed during the derivatization (11). The colored solution is read at 520 nm.

In order to improve the specificity of the method when low levels of ascorbic acid are measured relative to blank values or to the levels of possible interferring substances (H_2O_2, sugars and their degradation products, other unidentified osazone-forming compounds) (2, 11), several modifications have been made to the original procedure, such as isolating the insoluble osazone by filtration and then dissolving it in a smaller volume (12); or introducing a thin layer or column chromatographic step aimed to specifically purify and then quantitate the osazone of ascorbic acid (2). These additional steps, added to the long incubation period required to form the osazone result in extremely time-consuming methodology. Loss of material may also occur.

We have adapted the colorimetric procedure to a HPLC method. The reaction mixture containing the osazone is extracted into EtOAc and the components are separated on a C^{18} μ-Bondapak hydrophobic column. This way of finishing Roe's procedure results in a great increase of its specificity and reduces the chances of interference because of the resolving power inherent to the HPLC step added to the method. Also considerable amount of time is saved in comparison to the use of other types of chromatography.

For the common HPLC procedure described here we used a lower concentration of H_2SO_4 during the derivatization step (about 0.17 N) as well as a lower molar ratio between the reactants. A minimum 20-fold excess of DNPH over ascorbic acid was used. The linear relationship between ascorbic acid concentration and the amount of osazone formed under those conditions was preserved and less acid and DNPH entered the column. With the procedure described for 20 µl samples the detection of about 50 picomol of ascorbic acid injected into the HPLC system is possible. This compares very well with the most sensitive methods available (6).

Because in our common procedure only 1/100 of the EtOAc extract is actually injected into the column we prepared a microassay requiring less volume of sample (5 µl) which, on the other hand, was extracted with proportionally less volume of EtOAc so as to increase the amount of osazone in the 5 µl aliquot injected into the column. The coupling mixture used in the microassay contained a DNPH/ascorbic acid molar ratio of about 28 and H_2SO_4 was 0.75 N. When the former was lowered to 15 and the normality of H_2SO_4 was reduced to 0.16 N the linear relationship (Fig.3) between ascorbic acid in the sample and the height of the osazone peak obtained with the microassay was lost.

We had no option with the instrumentation available to us but to monitor the eluate from the HPLC column at 254 nm when this work was done. However, the eluate should have been ideally monitored at 500 nm, based upon the absorption spectrum of the osazone (Fig. 2B). Waters Associates Product Department has just developed to our request a 500 nm conversion kit not available before that allows the Waters 440 Absorbance Monitor to be used at the wavelength required by this method.

When the HPLC procedure for ascorbic acid determination was preliminarily applied to human aqueous humor samples from cataract patients significant amounts of oxidized ascorbic acid (DHA+ DKG) were detected in most cases. Because care was taken to immediately protect reduced ascorbic acid in those samples with 5% HPO_3 it does not appear justifiable to neglect this fraction when aqueous humor samples are studied, at least if they proceed from cataract patients. The possible correlation that might exist between oxidized ascorbic acid and the intensity or type of cataract is being investigated.

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