Our experiments in this and the previous investigation with controlled tonography show that the facility of outflow can be determined by the way postulated by Grant and Friedenwald. Our controlled experiments have included the same assumptions regarding unchanged intraocular blood volume and stable production of aqueous as put forward by those authors more than 20 years ago. It is evident that these factors will influence the results of tonography in vivo. We are, however, concluding that the theoretical basis of the controlled tonography is in accordance with our practical experiments and, therefore, in principle is considered correct.

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Key words: tonography, experimental tonography, ocular volume displacement, ocular rigidity, fluid loss by tonography, enucleated eyes.

REFERENCES


Analysis of ascorbate concentration in the aqueous humor by high-pressure liquid chromatography. Kwok-Wai Lam and Pei-Fei Lee.

A high-pressure liquid chromatography instrument has been used to establish a method for the analyses of ascorbate concentration in aqueous humor. The method detects only the reduced form of ascorbate. The values obtained by this method are very similar to those of the colorimetric method. The ascorbate value in a group of random samples obtained from human glaucoma eyes varied between 3.5 to 31 mg. per cent, independent to blood ascorbate concentration.

Investigations of the biochemical changes associated with eye disease have been a difficult task. A major difficulty is due to the small amounts of pathologic samples, which can be obtained from the eye for biochemical analyses. Therefore, it is important to fully exploit modern technology of microanalysis in this area of investigation.

High-pressure liquid chromatography has become increasingly important for microanalyses of a wide variety of biological compounds. Recently, this laboratory explored the possible application of this instrument for microanalyses of the biochemical content of aqueous humor. During the initial study it was found that the modern liquid chromatography instrument can be used to develop a very sensitive and rapid procedure for the analysis of ascorbate concentration in aqueous humor. The detailed procedure established for the analysis of ascorbate and the preliminary analyses of eight aqueous humor samples obtained from glaucoma eyes are discussed in this report.

Methods.

Aqueous humor collection. Aqueous humor samples were obtained under an operating microscope at the time of glaucoma surgery in all eyes. No eye drop medication was given for at least 14 hours before the operation. The routine preparation, a sliding peripheral corneal incision was made with a Wheeler knife. A No. 30 gauge or No. 27 gauge needle cannula attached to a disposable tuberculin syringe was used to aspirate aqueous humor without totally collapsing the anterior chamber. Usually, about two-thirds the amount of total aqueous humor with withdrawn without injury to the iris or corneal endothelium by the tip of the needle cannula. Repeated withdrawal of aqueous humor was not attempted in any case in order to avoid the contamination of the secondary aqueous humor. If the anterior chamber was lost following the corneal incision, or during the cannula insertion into the anterior chamber, the aqueous sample collection was abandoned. The anterior chamber was then re-
Fig. 1. The chromatogram of a standard solution: a mixture of 0.4 micrograms epinephrine, 0.2 micrograms ascorbate, 0.1 microgram AMP, and 0.1 microgram 3,5-cyclic AMP, dissolved in 10 microliters of 1 mM KH₂PO₄, was injected into the column and eluted by the same buffer. Full scale of recorder, 0.04 O.D.

Ascorbate concentration in aqueous humor in all samples was analyzed by the chromatographic method and colorimetric method in order to confirm the variation of different samples. Only the colorimetric method was used for serum samples.

Chromatographic method. The Tracor's High-Pressure Liquid Chromatography equipment (Model 2300) was used in this study. The column was packed with a pellicular polyamide anion exchanger (Chromasep RPP). The dimension of the column was 2 mm. in diameter and 2 meters in length. It was washed with excessive amounts of 0.1 N HCl, then equilibrated overnight in 1 mM phosphate buffer (pH 3.8). The elution speed was adjusted to 0.38 ml. per minute. The eluate was analyzed in a ultraviolet (UV) monitor, 254 nm, with the recorder full scale set at an optical density value of 0.04. The analyses were performed at room temperature.

Two microliters of aqueous humor were drawn into a Hamilton Syringe containing eight microliters of 1 mM KH₂PO₄. The entire mixture was injected into the column. The ascorbate concentration in the sample was estimated by the height of the UV absorption peak. The linear proportional relationship of ascorbate concentration to peak height was determined previously by injecting known amounts of standard ascorbate solution into the column.

Colorimetric method. The method of Roe and Kuether² for the determination of ascorbic acid in whole blood and urine was modified by reducing all reagent volumes proportionally. An amount of 50 microliters of aqueous humor was diluted to 1 ml. with 5 per cent trichloroacetic acid and centrifuged for 10 minutes at 1,000 g to sediment the coagulated protein. Acid-washed Norit (50 mg.) was added to the supernatant to oxidize the ascorbate to dehydroascorbate. The sample was subjected to centrifugation at 1,000 g for 10 minutes. An aliquot of 0.4 ml. of the supernatant fraction was mixed with 0.01 ml. of thiourea (10 per cent solution prepared in 50 per cent ethanol), 0.1 ml. of dinitrophenylhydrazine (2 per cent in 9N H₂SO₄) and incubated at 37° C. for three hours. The colored product was dissolved by the addition of 0.5 ml. sulfuric acid (85 per cent), and the absorbance determined at 540 nm.

Results. The chromatographic separation of a mixture of epinephrine, ascorbate, AMP, and cyclic-AMP is shown in Fig. 1. The retention time of the column was 10.1 minutes. Epinephrine, ascorbate, AMP, and cyclic-AMP had retention time values of 12.6, 14.8, 27.0, and 42.0 minutes, respectively. The epinephrine and ascorbate peak were only partially separated under the present experimental conditions. One can achieve complete separation by simply reducing the elution speed to 0.15 ml. per minute. The relationship of elution speed and resolution has been explained by Snyder.¹ However, the small amounts of aromatic amines in the aqueous humor have negligible effect on the height of the ascorbate peak. Therefore, the elution speed of 0.38 ml. per minute was used in routine analyses. The peak is directly proportional to the sample injected as shown in Fig. 2.

Only the reduced form of ascorbate was detected by the chromatographic method, because deoxyascorbate does not absorb UV light. The reversible oxidation, reduction of ascorbate was demonstrated in Fig. 3. When a diluted solution
Table I. Comparison of chromatographic and colorimetric analyses of ascorbate concentration in human aqueous humor

<table>
<thead>
<tr>
<th>Type of glaucoma</th>
<th>Chromatographic method</th>
<th>Colorimetric method</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open angle</td>
<td>31.0</td>
<td>29.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Neovascular</td>
<td>20.0</td>
<td>20.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Angle closure</td>
<td>16.9</td>
<td>14.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Angle closure</td>
<td>15.0</td>
<td>17.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Congenital</td>
<td>13.0</td>
<td>13.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Congenital</td>
<td>10.0</td>
<td>9.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Neovascular</td>
<td>5.0</td>
<td>6.0</td>
<td>0.75</td>
</tr>
<tr>
<td>Serum</td>
<td>3.5</td>
<td>2.7</td>
<td>0.88</td>
</tr>
</tbody>
</table>

of ascorbate was shaken in air before applying to the column, the height of the ascorbate peak was greatly reduced (see Figs. 3, A and B). The ascorbate peak was recovered by mixing the oxidized sample with dithiothreitol (Fig. 3, C).

When a sample of aqueous humor was applied to the column (Fig. 4), the major UV absorption peak was observed in the location of ascorbate. A small peak, having a retention time of 12.6 minutes, was observed before the ascorbate peak. There was not enough AMP or cyclic-AMP to be detected in the present experimental procedure.

The ascorbate concentration in the aqueous humor of eight different individuals was analyzed by the chromatographic method and the results were compared with those obtained by the colorimetric method. The results obtained by both methods were very similar.

The mean of the differences (D) among the eight pairs of analyses for ascorbate concentration in aqueous humor is 0.34. The standard error of the differences (S_e) observed is 0.54. The t-distribution calculated according to the equation t = D/S_e is 0.63. Based on a t-value of 0.63 obtained from the eight pairs of analyses, the probability that the two methods gave identical results exceeds the 50 per cent level.

Discussion. The high-pressure liquid chromatography method for ascorbate analysis is accurate and sensitive. Only one to two microliters of aqueous humor is sufficient for the analysis by this method. The entire procedure involves only one step, that is the delivery of the sample to the column by a Hamilton syringe. Therefore, this method has a minimal manipulation error.

The chromatographic method detects only the compounds which absorb UV light (254 nm). The type of compounds which absorb UV light, expected to be present in the aqueous humor, includes ascorbate, nucleotides, and aromatic amines. A typical chromatogram, representing the resolution of these three types of compounds, is shown in Fig. 1. Aromatic amino acids and their metabolites, such as tyrosin, tryptophan, dopamine, norepinephrine, and epinephrine, have very low affinity to the resin, and are eluted out of the column in the same position as epinephrine. There is very little of these compounds in the aqueous to interfere with the ascorbate analysis.
in aqueous humor. The nucleotides, having more complex structures than AMP, such as ADP, ATP, and NAD, have a high affinity to the resins and are not eluted from the column by the 1 mM buffer used in this study. Therefore, the experimental procedure described in this report for ascorbate analysis is not affected by other compounds in the aqueous humor. The identical results, obtained by the colorimetric and chromatographic methods confirmed that the interference contributed by other compounds in the ascorbate peak is negligible.

The chromatographic method measures only the reduced form of ascorbate. Deoxyascorbate is not detected by the UV monitor. On the other hand, the colorimetric method involves a step which oxidizes ascorbate before reacting with the color reagent. Therefore, the value obtained by the colorimetric method is the summation of ascorbate and dehydroascorbate concentration. Since both methods gave similar results, ascorbate in the glaucoma eyes is dominated by the reduced form. Even the last three samples, having very low ascorbate concentration, are not due to a difference in the ratio of reduced and oxidized forms. The amount of deoxyascorbate in these samples is too low to be estimated by the difference of the two methods.

The preliminary analysis, described in this report, was planned for comparing the analytic methods. Much more work is needed to evaluate the significance of ascorbate concentration and the types of glaucoma. However, the data does indicate that the broad variation of ascorbate concentrations confirmed by two methods, in the aqueous humor of these glaucoma eyes is not related to the variation of concentration in serum (see Table I). It is more likely related to the pathologic conditions of the eye. The sensitive methods described in this communication will be useful in the future follow-up studies of ascorbate concentration in different types of glaucoma eyes and will spare the major portion of a sample for detailed investigations concerning the relationship of ascorbate concentration to the other constituents such as protein, ions and glutathione, in the pathologic samples.

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Key words: ascorbate, aqueous humor, glaucoma, liquid chromatography.

REFERENCES
2. Roe, J. H., and Kuether, C. A.: The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid, J. Biol. Chem. 147: 399, 1943.

Electrolyte and acid-base status of aqueous humor in perinatal and adult goats.
T. L. Tyler and S. Cassin.

These experiments were undertaken to determine electrolyte concentration and acid-base status of aqueous humor and arterial plasma H2O in perinatal and adult goats. The distribution of potassium, sodium, chloride, magnesium, and calcium between aqueous humor and plasma H2O in fetal, neonatal, and adult goats changed independently with age and could not be explained by passive Gibbs-Donnan equilibria. Anterior chamber aqueous humor has a reduced pH, Po2, HCO3-, and an elevated PCO2 compared to arterial blood in the neonate and adult.

Composition of aqueous humor (AH) is carefully regulated in the intracellular environment. Electrolyte concentrations and acid-base status of AH must be maintained within limits for optimal physiologic function of surrounding non-neural and neural tissue. The composition and formation of AH appears analogous to that of cerebrospinal fluid.

Investigation of the ontogeny of electrolyte composition in cerebrospinal fluid has revealed differences in distribution of electrolytes compared to plasma in animals of different ages.

Knowledge of the distribution of electrolytes between AH and plasma in fetal, neonatal, and adult animals is important in understanding normal transcellular ocular development and function, as well as congenital and adult anomalies.

This investigation compares the distribution of electrolytes between AH and arterial plasma.