ASCORBIC ACID IN CEREBROSPINAL FLUID

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The determination of ascorbic acid in biological fluids has already received considerable attention. The actual assay of this vitamin by the use of 2,6-dichlorophenol indophenol in which molar solutions of the dye are reduced by equivalent molar solutions of this particular organic acid is dependent on the following:

(*a*) Elimination of interfering reducing substances such as cysteine, glutathione, and other compounds containing sulphhydryl groups, and (*b*) inhibition of the oxidation of ascorbic acid present in samples and filtrate systems.

Tillmans, Hirsch and Hirsch (1) first developed the method of determining the presence of ascorbic acid in serum. This and subsequent methods of assaying ascorbic acid in plasma have been discussed by Pijoan and Klemperer (2) who have shown that in plasma the rôle of the interfering R–S–H compounds is negligible and that the important feature is to prevent the oxidation of ascorbic acid.

However, this finding is not true for most biological fluids, and the presence of R–S–H compounds becomes an important factor. Emmerie and van Eekelen (3, 4) demonstrated that in the urine and tissue extracts the indophenol reducing substance was not ascorbic acid but rather the end products of protein metabolism, and direct titration of urine with dye yielded errors of considerable magnitude. Plaut and Bülow (5) and Tatsumi, Nagao, Okamura and Gamo (6), in determining the ascorbic acid content of cerebrospinal fluid, used the method of direct titration and in some instances the method of van Eekelen and Emmerie in which interfering substances were removed.

In this laboratory (7) the experiments of van Eekelen and his coworkers have been repeated and while their method is the only one which attempts to determine the quantity of ascorbic acid in biological fluids there were certain factors which had to be reconsidered. The use of mercuric acetate to precipitate out the R–S–H compounds results in a slight loss of ascorbic acid. This occurs chiefly when the mercuric acetate reacts with H_2S to form mercuric sulphide. Furthermore, van Eekelen and his coworkers realized that in collection and deproteinization there was an oxidation of ascorbic acid. To overcome this loss they attempted to reduce the oxidized acid into its original state by the use of H_2S, and to obviate the reduction of 2, 6-dichlorophenol indophenol by H_2S they aerated the filtrates with nitrogen. Oxidation-reduction experiments performed in this laboratory by the use of the foregoing systems never gave quantitative results although the method appeared to be the best available for the study of urine and tissue ascorbic acid. We therefore were led in our studies to eliminate the effects of the oxidation of ascorbic acid and of the rôle of the interfering reducing substances in colorimetry. Almost complete inhibition of the oxidation of ascorbic acid in biological systems can be carried out by KCN. However, the elimination of interfering substances is not an easy performance. Patients on a high protein diet will, as Heinemann (8) has shown, eliminate considerable reducing substances in the urine, and this is proportionable to the intake of protein or compounds containing the S–H group. On reconsideration of the problem we were impressed by two factors that have been developed by Mindlin (9).

(1) The dye, 2,6-dichlorophenol indophenol, is fairly stable in a buffer acetate system at a pH of 4.63 and does not undergo reduction by any of the reagents used for deproteinization.

(2) The reduction of the dye by compounds containing sulphhydryl groups occurs at a much slower rate than its reduction by ascorbic acid.

By this we mean that the addition of cysteine and thiosulphate will require between 10 seconds to several minutes to completely reduce a known volume of dye in a buffer acetate system, whereas

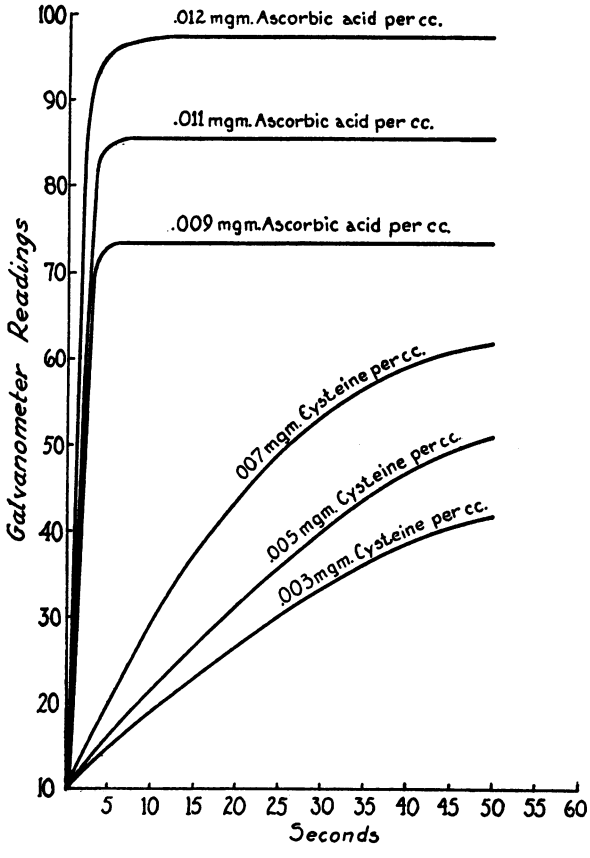

FIG. 1. RATE OF REDUCTION OF 2,6-DICHLOROPHENOL INDOPHENOL BY ASCORBIC ACID AND CYSTEINE

all of the reduction of the dye by ascorbic acid hydroxyl groups occurs within the first minute after mixing. The additional experiments and studies carried out on individuals with high cysteine diets have been performed in our laboratory and were controlled by the recrystallization of ascorbic acid from certain biological fluids. It can be seen from the following data that, at a pH of 4.63, the rate of reduction of 2,6-dichlorophenol indophenol by minute amounts of ascorbic acid and cysteine is demonstrated (Figure 1).

GENERAL PRINCIPLES AND METHODS

The spectrophotometric curve of the rose-violet color of 2,6-dichlorophenol indophenol in an acetate buffer system at pH 4.63 has a single absorption band at 520 mμ. A glass filter of the type used by Evelyn was therefore chosen which transmits a narrow spectral band in the vicinity of 520 mμ so that light passing through the filter can be absorbed by 2,6-dichlorophenol indophenol in the acetate system described.[1] Thus a beam of light at such a wavelength falls on a photoelectric cell which produces a deflection in the galvanometer to which it is connected. The entire process is carried out by the use of the Evelyn photoelectric colorimeter (10).

Nine cc. of a buffer acetate solution are placed in an Evelyn tube. To this is added 1 cc. of 1/2000 M. 2,6-dichlorophenol indophenol (29 mgm. dye dissolved in 200 cc. of water). In preparing the dye solution it is convenient to dissolve the dye in water heated at 85° C.; shake for 15 minutes and filter if necessary. The dye is standardized against ascorbic acid (Hoffmann-La Roche), the equivalent molar concentration of ascorbic acid being 17.6 mgm. in a 200 cc. water KCN mixture (10 mgm. KCN to volumes of 200 cc. distilled water).

To the dye-acetate buffer system are added 2 cc. volumes of the solution to be tested or of protein free filtrates. Readings should be done at once after mixing. Cerebrospinal fluid must be collected by dry syringes and transferred to tubes containing 10 mgm. of KCN. Deproteinization with metaphosphoric acid is carried out by the method described by Pijoan and Klemperer (2). Since the entire process is based on the decolorization of the dye, and since the dye buffer, acetate buffer system causes a deflection of the galvanometer because of the color present, this reading must be subtracted as a blank from the final reading. Using the glass filter, transmitting light at 520 mμ, the galvanometer string is set at 64. With this center setting (determined by the use of the reagents without dye set at 100) the 12 cc. blank of the dye acetate system causes the string to move to 32.5. On the addition of ascorbic acid to this system a new reading is immediately obtained and a calibration curve can be developed with varying quantities of ascorbic acid or other reducing substances. The ascorbic acid content can be obtained in mgm. per 100 cc. from the following equations.

L is the difference in optical density between a given amount of dye plus A cc. of water and the same amount of dye plus A cc. of sample solution thus

$$(1) \quad C = \frac{L}{K_1}$$

where C is the concentration of ascorbic acid in the final solution, and K_1 is a constant which can be determined by measuring L values for known quantities of ascorbic acid. Equation 1 expresses the fact that the reaction between dye and ascorbic acid is stoichiometric.

If a standard solution of ascorbic acid (with KCN) containing 1 mgm. per cent is prepared and if 2 cc. of this solution is made up to 10 cc. with metaphosphoric acid and an aliquot of 2 cc. is taken (as would be the case in cerebrospinal fluid), the aliquot would contain 0.004 mgm. of ascorbic acid. In 12 cc., which is the final volume of dye, buffer acetate, and aliquot, the concen-

[1] The type of single glass filter used is that made by The Corning Glass Works for the Rubicon Company of Philadelphia known as 520 M with transmission limits from 470 to 580 millimicrons.

tration of ascorbic acid would be 0.000335 mgm. per cc. The L value for such a system has been found by experiment to be 0.051 (log galvanometer-log blank).

Thus substituting in Equation 1 we find

$$0.000335 = \frac{0.051}{K_1} \quad \text{or} \quad K_1 = 235.$$

If A = cc. volume of original aliquot used and V = final volume then the concentration of ascorbic acid in the sample is

(2) $X = \frac{100}{A} \times V \times C$ = mgm. ascorbic acid in 100 cc. aliquot or filtrate

or

(3) $X = \frac{100 \times V \times L}{A \times K_1}$ = mgm. ascorbic acid in 100 cc. aliquot or filtrate.

Solving either of Equations 2 or 3 the yield would be 0.2 mgm. per cent filtrate. And as 100 cc. of filtrate contains 20 cc. of cerebrospinal fluid, the yield of ascorbic acid would be 0.2 mgm. × 5 or 1 mgm. per cent. These equations are submitted in case different volumes or different aliquots are used and can be substituted for the volumes used in this investigation. To simplify the calculation for routine work, a new constant is introduced, K_2, providing all determinations are carried out using the volumes mentioned in the procedure presented. Thus

(4) $K_2 = \frac{A}{V} \times K_1 = 39.2$

or

(5) Mgm. ascorbic acid per 100 cc. cerebrospinal fluid

$$= \frac{100L}{K_2} \times 5.$$

Assay of cerebrospinal fluid in cerebrospinal fluid as contrasted to plasma

A 2 cc. aliquot of cerebrospinal fluid filtrate as compared to a 2 cc. aliquot of a plasma filtrate is compared in Figure 2. It can be seen that there are present other reducing substances in spinal fluid as compared in blood plasma and that the values taken at a 5 seconds reading are identical. Both samples were taken simultaneously from the same subject.

Plaut and Bülow (5), by the use of direct titration which measured total reducing substance or by the use of mercuric acetate filtrate, obtained variable results which in most instances yielded values for cerebrospinal fluid surprisingly higher than those found in plasma. We were unable to obtain such findings, and in most cases, as shown in Table I, the cerebrospinal fluid

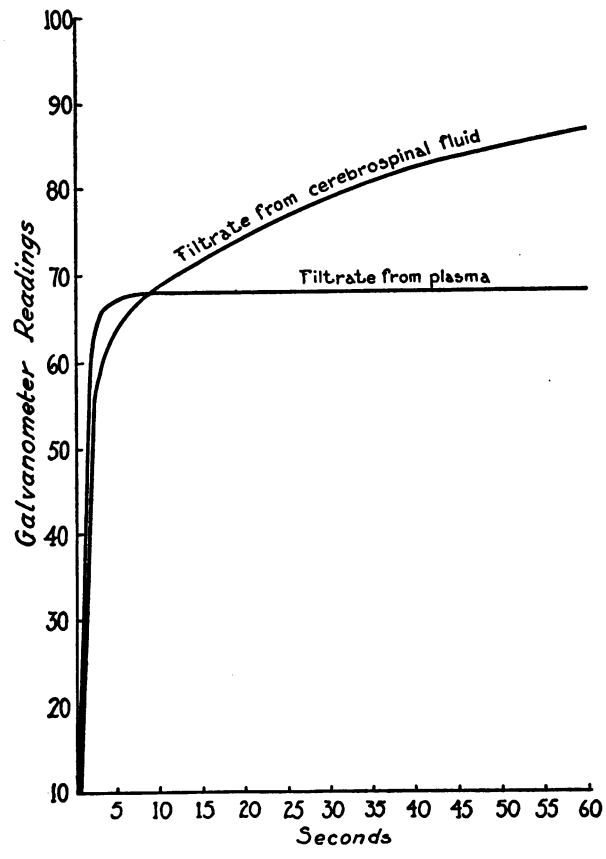

FIG. 2. RELATIONSHIP OF REDUCING SUBSTANCES IN CEREBROSPINAL FLUID AND PLASMA

TABLE I

Comparison of values of ascorbic acid in plasma and cerebrospinal fluid

Subject	Plasma *mgm. per cent*	Cerebrospinal fluid *mgm. per cent*
G. R.	1.132	1.130
S. E.	0.684	0.685
C. R.	0.802	0.800
O. B.	0.813	0.814
R. D.	0.716	0.716
M. R.	0.882	0.884
A. S.	0.915	0.968
R. H.	0.940	0.859
T. C.	1.143	1.165
M. S.	0.940	0.968
A. P.	0.691	0.706

content of ascorbic acid was similar to that in plasma. The discrepancies observed are well within experimental error. Addition of ascorbic acid to cerebrospinal fluid is further protected by KCN against oxidation by copper or other oxidizing substances present in this fluid (Table II).

In light of the recent work of Barron and his associates (11) and of King and his coworkers

TABLE II

Addition of ascorbic acid to cerebrospinal fluid in KCN systems

Initial value	Added amount of ascorbic acid	Theoretical recovery	Actual recovery in 1 hour
mgm. per cc.	*mgm. per cc.*	*mgm. per cc.*	*mgm. per cc.*
0.008.....	0.001	0.009	0.0086
0.008.....	0.001	0.009	0.0084
0.008.....	0.001	0.009	0.0088
0.008.....	0.0025	0.0105	0.0100
0.008.....	0.0025	0.0105	0.0102
0.008.....	0.0050	0.0130	0.0126
0.008.....	0.0050	0.0130	0.0128
0.008.....	0.0050	0.0130	0.0131

(12) it appears that " oxidases," enzymes involved in the oxidation of ascorbic acid, are unlikely and that the oxidation of organic acid must be chiefly attributed to minute amounts of copper; a process which can be inhibited by KCN.

The addition of known amounts of cysteine to the ascorbic acid-cerebrospinal fluid system yields an almost complete reduction of the dye at the end of 5 minutes. In short, it would appear

TABLE III

Addition of cysteine hydrochloride to ascorbic acid system in cerebrospinal fluid

Initial value of ascorbic acid	Added amount of cysteine	Assay 1 minute after mixing with dye-acetate buffer system	Reading 2 minutes after mixing with dye-acetate buffer system
mgm. per cc.	*mgm. per cc.*	*mgm. per cc.*	*mgm. per cc.*
0.008.....	0.002	0.00802	0.0098
0.008.....	0.003	0.00804	0.0112
0.008.....	0.003	0.00804	0.0110
0.008.....	0.004	0.00802	0.0120
0.008.....	0.004	0.00806	0.0118
0.008.....	0.005	0.00804	0.0130
0.008.....	0.005	0.00806	0.0128
0.008.....	0.006	0.00804	0.0138
0.008.....	0.006	0.00806	0.0142

from the readings presented in the figure and tables that the ideal time for noting the galvanometer deflection is at the end of 10 seconds.

CONCLUSIONS

1. A method is presented for the assay of ascorbic acid in cerebrospinal fluid. The method differs from that of plasma where titrations can be carried out in that the effects of interfering R–S–H substances must be eliminated.

2. The values for ascorbic acid in the cerebrospinal fluid are similar to those found in plasma.

The relationship between the velocity of the reduction of the dye due to ascorbic acid and R–S–H compounds was elaborated previous to our work in a study on blood by Dr. Butler and Dr. Mindlin (*vide* reference 9). We express our thanks for their advice and criticism.

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