The Estimation of 'True Ascorbic Acid' in Blood

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(Received 8 November 1943)

Recent work has suggested that the blood ascorbic acid concentration may provide a satisfactory index of the nutritional level of vitamin C. The concentration in whole blood is preferable for this purpose to the plasma concentration, which shows greater fluctuation; but the chief disadvantage of the blood ascorbic acid concentration as a nutritional index is the technical difficulty of estimating it accurately. The normal values obtained by different sets of workers show wide variation, and there is considerable doubt as to how much of the reducing substances estimated by these methods represents true ascorbic acid. For this reason Butler & Cushman [1940] suggest the use of the term 'apparent ascorbic acid' for the blood estimates, since they conclude that 'satisfactory criteria for the validity of whole blood analyses are lacking'.

In the present investigation the simple indophenol titration method of Deeny, Murdock & Rogan [1942] was chosen for special study. Preliminary experiments showed that a significant quantity of non-ascorbic acid reducing substance could be removed by treating the blood filtrates with lead acetate and so obtaining a precipitate of lead phosphate. This procedure removes certain SH derivatives, but not ascorbic acid [Richter & Croft, 1943]. Blood ascorbic acid figures obtained by this lead precipitation method were then compared with figures obtained on the same samples by the ascorbic oxidase method. Good agreement between estimates obtained by these two distinct methods is considered to be satisfactory evidence of the validity of the blood ascorbic acid determination.

The lead precipitation method described is very simple, and as it requires no special apparatus it appears very suitable for use in clinical investigation.

METHODS

Venous blood containing sodium oxalate (0.30 g./100 ml.) or sodium citrate (0.05 ml. saturated solution to 5 ml. blood) was used. Special care was taken to avoid haemolysis.

Lead precipitation procedure. The oxalated blood (4 ml.) was put in a 15 ml. centrifuge tube containing 0.05 ml. *sec.*-octanol, and coal gas was bubbled through. Distilled water (4 ml.) was added

and the gassing continued for 10 min. 2 ml. of freshly prepared 32% HPO_3 were added, followed after mixing by 1 ml. 25% $\text{Pb}(\text{Ac})_2 \cdot 3\text{H}_2\text{O}$ and 2 ml. saturated sodium acetate solution. After thorough mixing, the suspension was centrifuged. Any cloudiness in the supernatant fluid indicated that the HPO_3 solution was not sufficiently fresh. 2 ml. portions of the supernatant solution were taken for titration.

The dye solution (2 mg. 2:6-dichlorophenolindophenol in 100 ml. warm water) was standardized by titration against a freshly made solution containing 2 mg. ascorbic acid in 400 ml. 2% HPO_3 . On one occasion it was found that erroneous results were due to a rapid fading of the dye solution (30% in 24 hr.). This was traced to an impurity in the particular specimen of dye. Other specimens supplied by the same firm gave satisfactorily stable solutions, but the stability of the dye solutions should be checked before use.

The dye solution was added from a 2 ml. microburette to 2.0 ml. ascorbic acid solution in a small conical vessel made from the end of a conical centrifuge tube. The solution was stirred with a glass rod after each addition and the titration was continued until a faint pink colour persisted for 10 sec. and matched a solution made by adding 0.05 ml. of dye to 1 ml. of 1% HPO_3 in a similar conical vessel: the amount of dye solution added in the blank was subtracted from the final burette reading. As the pink colour at the end-point was very faint, it was essential that the titration should be done against a white background and in a good light. After some practice, duplicate estimations showed good agreement.

In calculation of the result a small correction was applied for the ascorbic acid contained in the protein precipitate obtained in deproteinization. The precipitate was found to contain 65% of water, by drying to constant weight. With the volumes of blood and reagents given the total volume was 13 ml., of which about 2.2 ml. were occupied by the moist precipitate. The effective volume of the aqueous solution was therefore taken as 12.2 ml. The ascorbic acid concentration in whole blood (mg./100 ml.) was obtained by multiplying the mg. ascorbic acid found in 2 ml. of the supernatant solution by the factor $\frac{1}{2} (12.2) \times \frac{1}{4} (100) = 152.5$.

RESULTS

Removal of non-ascorbic acid reducing substances with lead acetate. Ascorbic acid estimations were carried out on a series of blood specimens by the method of Deeny *et al.* [1942]. It was then found that when the blood filtrates obtained after deproteinization with HPO_3 and sodium acetate were treated with 1 ml. 25 % lead acetate solution, and the lead phosphate precipitate was removed by centrifuging, titration with the indophenol dye generally gave a significantly lower result, owing to the removal of non-ascorbic acid reducing substances (Table 1). Allowance has been made for the dilution by the lead acetate solution.

Table 1. Ascorbic acid content of fresh and of stored blood

Time of storage (days)	Total dye reducing substance, estimated as ascorbic acid (mg./100 ml.)	'True ascorbic acid' by lead precipitation (mg./100 ml.)	Non-ascorbic acid reducing substance (%)
(1) Oxalated blood			
0	0.78	0.70	11
0	0.60	0.54	11
(2) Citrated blood			
0	0.58	0.54	7
2	0.57	0.43	33
6	0.63	0.57	10
6	0.46	0.38	21
6	0.46	0.38	21
6	0.25	0.23	9
6	0.67	0.50	34

The stored specimens were kept at 3°.

Estimation by ascorbic acid oxidase. That the lower ascorbic acid figures obtained after lead acetate treatment were due to the removal of non-ascorbic acid reducing substances was confirmed by estimating the ascorbic acid by an independent method. The ascorbic oxidase was used for the quantitative oxidation of the ascorbic acid. The oxidase was prepared from cauliflower and the experimental procedure was that of Richter & Croft [1943]. The use of this method was made difficult by the relatively low concentration of ascorbic acid in the blood filtrates and for this reason the method cannot be recommended for blood; but with great care satisfactory agreement of duplicates was obtained. Each figure given in Table 2 represents the means of two separate estimations, i.e. of six burette readings.

It was clear that the figures obtained by the ascorbic oxidase method agreed very much more closely with those obtained by the lead precipitation method procedure (correlation coefficient 1.00) than

Table 2. Estimation of ascorbic acid in blood by (a) ascorbic acid oxidase and by (b) lead methods

Total reducing substance as ascorbic acid (mg./100 ml.)	'True ascorbic acid'	
	(a) By oxidase method (mg./100 ml.)	(b) By lead method (mg./100 ml.)
	(1) Oxalated blood	
0.50	0.37	0.36
0.36	0.31	0.30
0.22	0.21	0.21
0.62	0.56	0.56
(2) Citrated blood		
0.51	0.42	0.44
0.31	0.27	0.26
0.40	0.30	0.32

The correlation coefficient between the total reducing substance and the figures obtained by the ascorbic oxidase method was 0.96: between figures by the lead precipitation method and the ascorbic oxidase method, 1.00. The blood specimens were used within 3 hr. of collection.

with the figures for the total reducing substance given by the method of Deeny *et al.* [1942] (correlation coefficient 0.96). The closeness of the agreement gave evidence of the reliability of the lead precipitation method.

Subsequent work showed that it was unnecessary to carry out the two precipitations separately and to precipitate with lead acetate after a preliminary deproteinization: identical figures were obtained when these two stages were combined as described under 'Methods'.

The stability of ascorbic acid in citrated whole blood

Heinemann [1938] and Kassan & Roe [1940] reported that the level of ascorbic acid in whole blood remained constant for 16-24 hr. at room temperature and for 52 hr. at 0°. As it was desired to analyze specimens sent by rail and by post, this point was further investigated. Estimations were made on specimens of citrated and oxalated blood immediately after collection and after storage for 24 and 48 hr. at various temperatures, with or without occasional shaking (Table 3).

There was no significant loss of ascorbic acid under these conditions for 24 hr., and even in 48 hr. the maximum loss observed in 10 specimens was only 22 %. The range of blood ascorbic acid concentrations is wide—from about 1.5 mg./100 ml. in the fully saturated individual to below 0.15 mg./100 ml. in a person with scurvy. In clinical work only the approximate level is usually required in order to demonstrate or to exclude the existence of gross deficiency. A loss of even 22 % would not render the result entirely useless for this purpose.

Table 3. *Stability of ascorbic acid in citrated and oxalated whole blood*

Anti-coagulant	Percentage of initial ascorbic acid remaining after (hr. of storage)			Initial ascorbic acid content (mg./100 ml.)	Temp. (°)
	0	24	48		
Saturated citrate	100	100	—	0.30	16
	100	96	—	0.27	16
	100	98	91	0.55	14
	100	100	84	0.48	14
3.8% citrate	100	100	80	0.27	21
	100	—	100	0.19	20
	100	—	90	0.15	20
	100	—	78	0.30	20
	100	—	84	0.26	20
	100	97	91	0.32	19
Oxalate	100	97	85	0.34	20
	100	100	89	0.30	20

Blood preserved with saturated citrate contained 0.05 ml. saturated sodium citrate solution to 5 ml. blood; with 3.8% citrate, 2.5 ml. 3.8% sodium citrate solution to 10 ml. blood; with oxalate, 0.03 g. sodium oxalate to 10 ml. blood.

DISCUSSION

In attempts to assess the nutritional level of vitamin C in a subject, either the saturation test [Harris, 1942] or the blood ascorbic acid concentration can give useful information. The saturation test is simple to carry out, but its usefulness is limited in that it requires the accurate collection of urine, dosage with ascorbic acid and repeated estimations for a period of several days. The blood ascorbic acid concentration, on the other hand, is so constant that a single estimation is usually adequate, and it has the great advantage that the estimation need not be carried out immediately after collection of the blood specimens. Ascorbic acid is stabilized by the reducing systems present in citrated or oxalated whole blood, so that there is no significant loss on storage for 24 hr. at ordinary temperature. Estimations can thus be carried out satisfactorily even on specimens sent by post. The chief disadvantage of the blood concentration as a nutritional index is the difficulty of estimating it accurately. There is, however, no standard accepted method for the estimation of blood ascorbic acid, and some of the methods in current use are subject to serious criticism [Butler & Cushman, 1940]. Several photoelectric absorptiometric and other

methods have been described which have given good results in the hands of experienced research workers, but they are too difficult for routine use by the average clinical laboratory technician and we must agree with Bessey [1938] that their accuracy has generally been overrated.

It is evident from the present investigation that the blood ascorbic acid figures obtained by methods such as that of Deeny *et al.* [1942] are too high, owing to the inclusion of a variable amount of extraneous reducing substances. The lead precipitation method described corrects this source of error and gives somewhat lower figures. In order to obtain the greatest possible accuracy a relatively large volume of blood (4 ml.) was used and the volume of the other additions was kept small. After some practice in recognition of the end-point of the titration, duplicate estimations showed very close agreement, and the reliability of the method was confirmed by comparison with the ascorbic oxidase method for estimating 'true ascorbic acid'.

The method has been applied satisfactorily to estimation of the blood ascorbic acid in factory workers in the Birmingham area: the blood specimens were sent by rail and titrated within 24 hr. at Mill Hill. The blood ascorbic acid concentration of 350 normal individuals varied from 0.15 to 0.85 mg./100 ml., with a mean level of 0.25 during the months of May, June and July 1943. Fruit and vegetables were scarce in the Birmingham area during this period.

SUMMARY

1. Figures obtained for the blood ascorbic acid concentration by certain current methods are too high owing to the inclusion of a variable amount of extraneous reducing substance.
2. A simple lead precipitation method is described which eliminates this source of error.
3. The validity of the estimates by the lead precipitation method was demonstrated by comparison with the ascorbic oxidase method for 'true ascorbic acid'.
4. There is no significant loss of ascorbic acid in specimens of citrated whole blood kept for 24 hr. at 20°.

The authors thank Dr Russell Fraser and Dr Maxwell Jones for collecting most of the blood samples, Dr Maclay for his interest and the Rockefeller Foundation for supporting this investigation.

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