New method for the estimation of platelet ascorbic acid

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SYNOPSIS Present techniques for the estimation of platelet ascorbic acid allow interference by other substances in the sample. A new and more specific method of analysis is presented. The proposed method owes its increased specificity to resolution of the extract by thin-layer chromatography. By this means ascorbic acid is separated from other reducing substances present. The separated ascorbic acid is eluted from the thin layer and estimated by a new and very sensitive procedure: ascorbic acid is made to react with ferric chloride and the ferrous ions so formed are estimated spectrophotometrically by the coloured derivative which they form with tripyridyl-S-triazine. Results obtained with normal blood platelets were consistently lower than simultaneous determinations by the dinitrophenylhydrazine (DNPH) method.

It has been demonstrated that the ascorbic acid content of platelets is high (Barkhan and Howard, 1958) and that platelet adhesiveness is reduced in scurvy (Born and Wright, 1967; Wilson, McNicol, and Douglas, 1967). These studies suggest that ascorbic acid is important in platelet metabolism and that the haemostatic defect which occurs in scurvy may be due, at least in part, to a platelet defect.

An essential tool in the further elucidation of the function of ascorbic acid in platelets is an accurate method for its estimation. Current methods are not specific for they allow interference from other substances present in the sample. In the method proposed here, specificity is obtained by thin-layer chromatography, and greater sensitivity by a new procedure dependent upon the reduction of ferric ions to ferrous ions by ascorbic acid.

MATERIALS

In principle, an extract of platelets is resolved by chromatography on a cellulose thin layer. By this means ascorbic acid is separated from other reducing substances present in the extract. It is then located with the aid of ultraviolet light, eluted from the thin layer and estimated by allowing it to react with excess ferric chloride. The ferrous ions formed by reduction of ferric ions react with the iron chromogen, tripyridyl-S-triazine (TPTZ) (Fischer and Price, 1964; Caraway, 1961) to form an intense blue derivative which is estimated spectrophotometrically to provide a measure of the amount of ascorbic acid present.

REAGENTS The following reagents are required.

1. FeCl3 stock solution (0.01 M FeCl3 in 0.2 M HCl)
2. 2, 4, 6-tripyridyl-S-triazine, C5N3(C8H4N)3 (0.004 M stock solution) This was prepared by dissolving 124.8 mg of tripyridyl-S-triazine in 1 ml of 1.0 M HCl and diluting to 100 ml with deionized water. The solution is stable indefinitely if kept in a well-stoppered bottle in the dark (Fischer and Price, 1964).
3. FeCl3-TPTZ reagent Of the FeCl3 stock solution, 5.0 ml was added to 25 ml of the tripyridyl-S-triazine stock solution and the volume made up to 500 ml with 0.05 M HCl. The solution thus contained 0.1 mM FeCl3 and 0.2 mM tripyridyl-S-triazine in 0.05 M HCl.
4. Ammonium acetate AR 30% w/v
5. Trichloracetic acid AR 8% w/v
6. Ascorbic acid standard solution (0.15 mg/ml in 8% trichloracetic acid) This should be prepared freshly and used within two hours.
7. Solvent for thin-layer chromatography Of redistilled n-propanol, 170 ml was added to 30 ml of deionized water and titrated to pH 3 with 1% oxalic acid. At this pH ascorbic acid is most stable (Uprety and Revis, 1964).
8. Acetic acid (AR) 1% w/v

To reduce the ‘blank’ value, reagents which had a minimal degree of iron contamination were used and all water was either double glass distilled or deionized. Glassware was acid cleaned to ensure freedom from iron contamination.

APPARATUS

TEFLON TISSUE GRINDER The capacity of the lower (grinding) chamber used in these studies was 1.0 ml and the total capacity (upper and lower chamber) was 10 ml. The teflon rod was rotated at 4,400 revolutions per minute by an electric motor.
THIN-LAYER CHROMATOGRAPHY PLATES

Cellulose (Whatman CC41) prewashed in n-propanol, was suspended in 1% acetic acid and applied to the glass plates to produce a layer 0.6 to 0.7 mm thick. The cellulose had sufficient fluorescence when irradiated at 254 mλ to render the ascorbic acid clearly visible. Thus it was unnecessary to use a fluorescent indicator in the adsorbent.

ELUTING PIPETTES

These were constructed from 10 cm lengths of 7 mm internal diameter glass tubing which were constricted and plugged at one end with glass wool (Malinek, 1965). The area of cellulose containing the ascorbic acid was sucked into the tube and allowed to form a layer over the glass wool plug. The ascorbic acid was eluted by passing 1% acetic acid through the cellulose layer.

PROCEDURE

PREPARATION OF PLATELETS

Blood, 30 ml, was collected into 3 ml of 3% disodium ethylene-diaminetetraacetic acid (EDTA). Platelet-rich plasma was then obtained by centrifugation at 300 g for 20 minutes at 4°C. Equal amounts of platelet-rich plasma were decanted into two tared tissue grinder chambers which were centrifuged at 1,200 g for 30 minutes at 4°C to form a platelet button. After discarding the platelet-poor plasma, the chamber with its platelet button was weighed. The number of platelets in the button was determined by performing platelet counts on both the platelet-rich and platelet-poor plasma. Of cold 8% trichloracetic acid, 0.1 ml was added to one platelet button (the sample) and 0.1 ml of the ascorbic acid standard solution was added to the other to provide an internal standard. The tissue grinder was immersed in ice and its contents ground for two minutes. Microscopy at this time showed that the platelets were completely disintegrated. The homogenate was centrifuged and 20 μl aliquots from the sample and from the internal standard were chromatographed side by side.

THIN-LAYER CHROMATOGRAPHY

Of each sample to be tested, 20 μl was spotted on to the thin layer. Ten such samples could be applied to one 20 × 20 cm plate. The chromatogram was developed in a tank surrounded by a black cardboard screen which minimized the breakdown of ascorbic acid by light. After the solvent had travelled 10 cm (about one hour) the plate was partially dried in a cold air stream. The ascorbic acid appeared as a dark spot under ultraviolet illumination (254 mλ) at about Rf 0.6. Elution of the ascorbic acid from the cellulose with 2 ml of 1% acetic acid was carried out as described above. The amount of ascorbic acid in the eluate was then determined by the Fe-tripryidyl-S-triazine reaction.

It is essential that both the developing solvent and the cellulose thin layer are iron free. In our earliest studies when this precaution was not taken, very low recoveries were obtained. This was probably due to breakdown of the ascorbic acid by oxidation, a process catalyzed by trace amounts of iron (Roe, 1954).

IRON-TRIPYRIDYL-S-TRIAZINE REACTION

Of the unknown sample, 2 ml and 1 ml of the FeCl₃-tripryidyl-S-triazine reagent were pooled in a beaker. The pH was momentarily adjusted to 1-7 to 1-9 with 30 M HCl and then brought to 4-0 to 4-5 with 30% ammonium acetate. This reduction in pH to less than 2 was required for complete colour development. Ferric ions at a pH greater than 2 aggregate to form multinuclear complexes which are chemically less reactive than free ferric ions which exist in acid solution (Eichorn, 1964). The final volume was adjusted to 40 ml by the addition of distilled water and the absorbance measured in a spectrophotometer at 593 mμ using a glass cell with a 4 cm light path. The whole procedure took approximately two minutes per sample. Two ml containing 1.25 μg/ml ascorbic acid in 1% acetic acid and 2 ml of 1% acetic acid were treated in the same manner as the unknown sample to determine the control (100%) and blank values.

CALCULATIONS

The amount of ascorbic acid in the platelet button was calculated from the formula:

\[ W = \frac{15A_s}{A_R - A_s} \]

where \( W \) = weight (μg) of ascorbic acid in platelet button,
\( A_s \) = absorbance generated (less the blank value) by ascorbic acid from the sample,
\( A_R \) = absorbance generated (less the blank value) by ascorbic acid from the internal standard.

RESULTS WITH THE IRON-TRIPYRIDYL-S-TRIAZINE REACTION

Sample solutions containing 0 to 1.8 μg/ml of ascorbic acid prepared in 1% acetic acid were tested by the Fe-tripryidyl-S-triazine action. Beer's law was followed over the concentration range 0 to 1.25 μg/ml (0-625 μg/ml of the final solution) (Fig. 1). For concentrations of ascorbic acid greater than 1.25 μg/ml, direct proportionality no longer occurred despite an excess of the reagents.

The reproducibility of the technique was shown on 10 ml samples from a solution containing 0-625 μg/ml of ascorbic acid. The mean increase in absorbance above the blank was 0.323 ± 0.008, with a standard deviation of 2.5%. Eleven blank determinations gave a mean absorbance of 0.043 ± 0.008.

RESULTS WITH THIN-LAYER CHROMATOGRAPHY

PURE SOLUTIONS OF ASCORBIC ACID

Samples each of 10 μl of 1% acetic acid containing known amounts of ascorbic acid were subjected to chromatography. The ascorbic acid from each sample was eluted and estimated with the Fe-tripryidyl-S-triazine reagent. The increase in absorbance above the blank was directly proportional to the amount of ascorbic acid.
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FIG. 1. Relationship of absorbance to the known concentration of ascorbic acid in 20 ml samples. Each point represents the mean from two samples.

in the original sample (Fig. 2). The mean recovery was 83% of that which was originally applied.

Nine 10 µl samples each containing 2.5 µg of ascorbic acid were subjected to thin-layer chromatography on the same plate. The ascorbic acid was eluted and the eluate estimated by the Fe-tripyridyl-S-triazine reaction. The increase in absorbance above the blank was 0.429 ± 0.024, a standard deviation of 5.6%. Nine blank spots (1% acetic acid) treated in the same manner yielded a mean absorbance of 0.051 ± 0.013. These results represented a recovery of 71%.

FIG. 2. Relationship between absorbance and known amounts of ascorbic acid in samples applied to the origin of the chromatogram. Each point represents the mean from two samples.

choice of a solvent system. The solvent, n-propanol: water = 85:15 buffered to pH 3 with 1% oxalic acid, produced a separation of ascorbic acid from cysteine, glutathione, and ferrous ions (Table I). These substances could be demonstrated as blue spots on a yellow background by spraying the developed chromatogram with 5% phosphomolybdic acid in methanol. Separation was also obtained from sulphite ions which in high concentration (50 mg/ml anhydrous sodium sulphate) were detected at Rf 25.

<table>
<thead>
<tr>
<th>Substances Applied to Origin</th>
<th>Ascorbic Acid</th>
<th>Cysteine</th>
<th>Glutathione</th>
<th>Ferrous Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>0.54</td>
<td>0.33</td>
<td>0.33</td>
<td>0.23</td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td></td>
<td>0.33</td>
<td>0.23</td>
</tr>
<tr>
<td>Ferrous iron</td>
<td>0.53</td>
<td>0.33</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid + cysteine</td>
<td></td>
<td>0.49</td>
<td>0.33</td>
<td>0.23</td>
</tr>
<tr>
<td>Ascorbic acid + glutathione</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid + ferrous iron</td>
<td></td>
<td>0.50</td>
<td>0.23</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as the ratio of the distance travelled by the substance from its origin to the distance travelled by the solvent front.

Platelets were homogenized in 8% trichloracetic acid and the precipitated protein was removed by centrifugation. Samples of the supernatant, each of 20 µl, were resolved by chromatography. Spraying the developed chromatogram with 5% phosphomolybdic acid revealed an oval spot at Rf 0.6 which appeared to be uncontaminated by other reducing substances. When 14C-labelled ascorbic acid was added to a platelet extract before chromatography, scanning for beta activity with a thin-layer chromatogram scanner showed that this spot at Rf 0.6 contained the label.

Chromatography and estimation of platelet extracts. Equal amounts of platelet-rich plasma were centrifuged in three tissue grinder chambers to obtain identical platelet buttons. To each platelet button was added 150 µl of 8% trichloracetic acid containing 0, 12.5, and 25 µg of ascorbic acid respectively. The platelet buttons were homogenized, the precipitated protein removed by centrifugation, and 20 µl samples from the supernatant were chromatographed and estimated by the Fe-tripyridyl S-triazine reaction. From the absorbance of the samples containing added ascorbic acid, the ascorbic
acid content of the sample to which no ascorbic acid had been added was calculated. The linear relationship between the absorbance generated by the eluate and the amount of ascorbic acid in the 20 μl sample is shown in Figure 3. The mean recovery of added ascorbic acid in this experiment was 42%.

As the recovery of ascorbic acid during thin-layer chromatography of pure ascorbic acid solutions has been shown to be much higher (70% to 80%), the following experiment was performed to elucidate the reason for the lower recovery from platelet extracts. 14C-labelled ascorbic acid was added to a platelet button. After homogenization and centrifugation, only 60% of the radioactivity was recovered in the supernatant. This suggests that the lower recovery obtained when platelet extracts were used may have been due to the adsorption of ascorbic acid on to the precipitate. This was confirmed and to allow for this loss of ascorbic acid which occurs during the
estimation of platelet ascorbic acid content, the use of internal standards is essential (see Methods).

HOMOGENIZATION OF SAMPLES The amount of ascorbic acid liberated from platelets by homogenization in a tissue grinder compared to that released by simply stirring with a glass rod was determined on four duplicate platelet buttons. The amount liberated by stirring was only 80% of that liberated by homogenization with a tissue grinder.

COMPARISON WITH THE DNPH PROCEDURE The determinations were made both by the proposed method and the dinitrophenylhydrazine (DNPH) procedure (Roe, 1954) on six different platelet samples. In the DNPH procedure, no attempt was made to separate ascorbic acid from interfering substances. With both methods internal standards were used. For comparison, external standards were also included for the DNPH procedure. The mean value obtained by the DNPH method (using internal standards) was more than twice that obtained by the proposed method (Table II) suggesting that substances other than ascorbic acid contribute to the result when the DNPH method is used.

If the results obtained using the DNPH procedure were calculated from external standards, they were much lower than when internal standards were used. Thus with this procedure also, some ascorbic acid is lost.

DISCUSSION

The methods commonly used for the estimation of ascorbic acid make use of one of two main principles. Some measure the coloured 2, 4-dinitrophenylhydrazine derivative of dehydroascorbic acid; others, such as the 2, 6-dichlorophenolindophenol method, measure the reducing power of the sample (György and Rubin, 1950; Roe, 1954). Methods which use 2, 4-dinitrophenylhydrazine are subject to interference from other substances in the sample which

**TABLE II**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Thin-layer Chromatography Method (A) (μg/10^6 platelets)</th>
<th>Dinitrophenylhydrazine Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Using Internal Standards (B) (μg/10^6 platelets)</td>
</tr>
<tr>
<td>1</td>
<td>1.7</td>
<td>7.2</td>
</tr>
<tr>
<td>2</td>
<td>2.7</td>
<td>11.5</td>
</tr>
<tr>
<td>3</td>
<td>6.0</td>
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<tr>
<td>4</td>
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<td>5</td>
<td>6.4</td>
<td>7.3</td>
</tr>
<tr>
<td>6</td>
<td>6.5</td>
<td>14.7</td>
</tr>
<tr>
<td>Mean</td>
<td>4.3</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Probability 0.02 > P > 0.01 0.025 > P > 0.1
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also form hydrazine derivatives (György and Rubin, 1950; Lu, 1939; Penrose and Quastel, 1937); methods which depend on the reducing power of the sample also measure other reducing substances which may be present.

Attempts have been made to increase the specificity of these procedures by paper chromatography (Prochazka, 1963) but the possibility of using the quicker and more versatile technique of thin-layer chromatography has not been adequately explored (Bolliger, 1965). The method proposed in this paper demonstrates that thin-layer chromatography provides a convenient method for the more specific estimation of ascorbic acid and the increased specificity of the procedure is demonstrated by the much lower values obtained compared with the DNPH method.

Since the volume of sample which can be applied to a thin-layer chromatogram is small, a very sensitive method was required for the estimation of the eluted ascorbic acid. The method proposed here appears to be more sensitive than other published methods. The molar extinction coefficient, calculated per mole of ascorbic acid, is 42,800 compared with 23,200 for the DNPH reaction and 9,770 for the ultraviolet absorbance of ascorbic acid at pH 3 (all these values as determined in this laboratory). It is more difficult to compare the sensitivity of other methods for ascorbic acid estimation as values for the extinction coefficients could not be found in the literature. The Fe-tripyridyl-S-triazine procedure requires a sample ascorbic acid concentration of 0 to 1.25 μg/ml. The 2, 6-dichlorophenolindophenol method requires 2 to 10 μg/ml and has about the same sensitivity as the DNPH procedure (Roe, 1954). The lower limit of sensitivity of the method of Kum-Tatt and Leong (1964), which utilizes the reduction of mercuric to mercurous ions, is 10 μg/ml.

Thus, by combining thin-layer chromatography with a sensitive method for ascorbic acid estimation, a more specific method for the estimation of ascorbic acid in platelets has been devised. It should be possible to apply this method to other biological samples.

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REFERENCES

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