Ascorbic Acid Assay using Sequential Injection Analysis

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Abstract
A sequential injection analysis (SIA) method for ascorbic acid assay in pharmaceuticals utilizing crude zucchini (Cucurbita pepo) extract was compared with commercial horseradish peroxidase (HRP) assay. The peroxidase activity in the crude extract of zucchini was determined by the formation of a chromophore between guaiacol (0.05M) and hydrogen peroxide (10 mM) measured spectrophotometrically at 470 nm. The method adopted for assaying ascorbic acid is based on its inhibition of peroxidase catalyzing the formation of tetraguaiacol from guaiacol in the presence of hydrogen peroxide using a sequential injection analysis (SIA) manifold controlled by an in-house developed software. The percentage of inhibition of tetraguaiacol formation is proportional to the concentration of ascorbic acid. Km values for hydrogen peroxide using HRP and zucchini peroxidase were found to be 1.530mM and 0.868mM respectively. The conditions for the SIA system used to assay ascorbic acid in pharmaceutical and food samples were optimized at 0.9 ml/min flow rate, pH of 7 and hydrogen peroxide and guaiacol concentrations at 1.75 mM and 0.025M respectively as well as reaction time of 10 seconds. Linear range for the standards was found to be 0 to 120µg/ml (r = 0.9967 - 0.9998, n = 7). The recovery of ascorbic acid from samples ranged from 91.1 to 112.1% for the zucchini extract. Results were comparable with titrimetry using 2,6-dichlorophenolindophenol (USP method). The described SIA procedure presents a simple and rapid assay for ascorbic acid in pharmaceuticals where ingredients contained in the tablets do not interfere with the analytical method.

Keywords: Ascorbic acid, sequential injection analysis, peroxidase, inhibition

Introduction
Ascorbic acid is a vital dietary supplement involved in many important biological processes such as regeneration and formation of skin and muscles. It also plays an important role as an antioxidant and free radical scavenger. The various methods used for its determination in food and pharmaceutical preparations include titrimetry using 2,6-dichlorophenolindophenol [1], high performance liquid chromatography [2], voltammetry using carbon paste electrode [3], spectrophotometry [4-5], flow injection analysis using spectrophotometric detection [6-10] and chemiluminescence [11] as well as assays employing enzymes such as peroxidase [12] and ascorbate oxidase [13].

Flow Injection analysis (FIA) [14-16] is a low pressure analytical method used to detect the presence of an analyte where the measurement is made in a dynamic state at precise timing and controlled sample dispersion. Aliquots of sample and reagent(s) are aspirated into the FIA system and allowed to mix by using computer-controlled software. The coloured product formed from the reaction is thus monitored in the ultraviolet -visible detector. The peak height or peak area recorded is thus proportional to the concentration of the analyte in the sample. From the standards injected into the system, the concentration of the unknown can therefore be determined.

The advantages of FIA include automation, accuracy, speed of analysis, use of very small reagent and sample volumes and data capture by the personal computer through interfacing. A review of the different methods using flow injection for the determination of ascorbic acid was presented by Yebra-Biurrun [17]. Sequential injection analysis (SIA) [18,19], on the other hand, is a slightly modified form of FIA where the sample and reagent are aspirated in an orderly sequence into a holding loop where definite and discrete sample and reagent zones are formed in the loop before being propelled into the reactor coil and finally to the detector for sensing and analysis. By using an appropriate geometric configuration, radial mixing of sample and reagent will enable them to react to form the product.

Ascorbic acid interferes with the peroxidase-catalyzed reaction of hydrogen peroxide with hydrogen donors. The spectrophotometric method developed by Zhu et al [5] is based on the inhibition of peroxidase catalyzing the formation of a chromophore between p-chlorophenol and 4-aminoantipyrine by ascorbic acid at 25°C. Detection of the product formed is at a wavelength of 505nm.

The peroxidase enzyme catalyses four different types of reactions namely peroxidation, oxidation, catalytic reaction and hydroxylation. Under normal assay conditions where phenolic substrates are used, peroxidation reactions are the most important. The reaction mechanism of peroxidase in the peroxidative reaction is shown below:

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\[
\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{HRP-I} + \text{H}_2\text{O} \\
\text{HRP-I} + \text{AH}_2 \rightarrow \text{HRP-II} + \text{AH}^* \\
\text{HRP-II} + \text{AH}_2 \rightarrow \text{HRP} + \text{AH}^* + \text{H}_2\text{O}
\]

where \(\text{AH}_2\) and \(\text{AH}^*\) are hydrogen donor and free radical product respectively and \(\text{HRP}\) is horseradish peroxidase. The overall reaction for the above is

\[
\text{H}_2\text{O}_2 + 2\text{AH}_2 \rightarrow 2\text{AH}^* + 2\text{H}_2\text{O}
\]

\(\text{AH}^*\) thus formed will undergo further reaction depending on the presence of oxygen and characteristics of the phenolic compound.

If \(\text{AH}_2\) is guaiacol, the free radical will interact with one another to form the polymer product, \(\text{HAAH}\) which will act as a hydrogen donor to form tetraguaiacol as shown below:

![Reaction Diagram]

If \(\text{AH}_2\) is ascorbic acid, the free radical will interact with one another to yield a reduced compound (\(\text{AH}_2\)) and an oxidized compound (\(\text{A}\)).

The oxidative and hydroxylation activities do not arise from direct enzyme activity but from the secondary reaction caused by the \(\text{H}_2\text{O}^*\) free radical formation. Because both guaiacol and ascorbic acid can act as hydrogen donors, the presence of both compounds will result in their competition for reaction with \(\text{HRP-I}\) and \(\text{HRP-II}\) to form the \(\text{AH}^*\) free radical. As a result of this, when the concentration of ascorbic acid increases, the formation of free radical from ascorbic acid will correspondingly increase and thus the free radical formation from guaiacol will correspondingly decrease. Hence the formation of tetraguaiacol will decrease with the increase in ascorbic acid.

The aim of this study is to determine the ascorbic acid content in some pharmaceutical products by using SIA method. The results obtained were also compared with that obtained using the USP method.

Materials and Methods

Materials

Polyclar -AT, ascorbic acid, sodium bicarbonate and oxalic acid were all purchased from BDH Chemicals Ltd (Poole, England). Guaiacol, borax and acetone were obtained from E. Merck (Darmstadt, Germany), disodium hydrogen phosphate dihydrate, AR from Prolabo (Manchester, England) whereas horseradish peroxidase, (EC 1.11.1.7) and 2,6-dichlorophenolindophenol, sodium salt were from Sigma Chemical Co (St Louis, MO, USA). Hydrogen peroxide (30%), bromothymol blue and boric acid were from J.T. Baker (Phillipsburg, NJ, USA), Hayashi Pure Chemical Industries Ltd (Osaka, Japan) and Riedel-de-Haen (Seelze, Germany) respectively. Potassium permanganate, sodium dihydrogen phosphate dihydrate, AR and disodium hydrogen phosphate dihydrate, AR from R & M Marketing (Essex, England) were used as received. Vitamin C tablets (125mg) and Enervon-C multi-vitamin tablets (500mg) were from Upha Corp (M) Sdn Bhd (Selangor, Malaysia) and Unam Corporation (M) Sdn Bhd (Selangor, Malaysia) respectively. All reagents used were of analytical grade. Unless stated otherwise, distilled water was used throughout the experiment.

Methods

Preparation of zucchini extract

A 6-g sample of polyvinylpyrrolidone, Polyclar AT which functions as a protective and stabilizing agent was used to adsorb the phenolic compounds in the zucchini extract. It was boiled for 10 min in 10% hydrochloric acid and then washed with distilled water until free from chloride ions. Finally the Polyclar AT was washed with acetone and dried at 95-100°C for 2-3 hours. The washed and dried Polyclar-AT was mixed with 100ml of phosphate buffer (pH 6, 0.1M) cooled to 4°C.

Fifty grams of washed, hand-peeled zucchini were cut into small pieces and then homogenized in a mortar containing 6 g of washed and dried Polyclar AT in 100ml of phosphate buffer (pH6, 0.1M) on ice for 15-20min. The homogenized mixture was then filtered through four layers of muslin cloth and centrifuged in a Heraeus Sepatech (Germany) centrifuge at 11,200rpm, 4°C for 25 min. The supernatant was kept at 0°C.

Determination of peroxidase activity in zucchini extract

Horseradish peroxidase stock solution (7units/ml), 0.05M guaiacol and 10mM \(\text{H}_2\text{O}_2\) were prepared in phosphate buffer (pH7, 0.1M). For horseradish peroxidase activity assay, 0.014, 0.07, 0.14 and 0.21 unit/ml were prepared by diluting the horseradish peroxidase stock solution appropriately in phosphate buffer. Aliquots of
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concentrations of ascorbic acid. The difference in peak
then determined in the absence and presence of varying
measurements for tetraguaiacol formation were monitored
at 470nm. Peak height readings in absorbance units were
respectively. The zucchini extract was adjusted to contain
and H$_2$O$_2$ used were 0.175 unit/ml, 0.025M and 1.75 mM

Figure 1: Schematic representation of the SIA system for

The SIA assembly (Figure 1) consisted of a Gilson
Minipuls 3 peristaltic pump (Villiers-le-Bel, France), a
Valco multiposition injector (Schenkon, Switzerland), a
homemade amplifier and a Linear UVIS 204 detector
(Fremont, CA, USA) all interfaced with an IBM-
compatible personal computer and controlled by an in-
house written software using Quick Basic version 4.5.
Teflon tubings (0.5mm ID x 1.5mm OD and 0.8mm ID x
1.5mm OD ) purchased from Alltech Associates
(Deerfield, IL, USA) were used. Solutions of peroxidase,
ascorbic acid and guaiacol-cum-hydrogen peroxide were
placed in port positions 4, 5 and 6 of the multiposition
injector respectively. They were aspirated for a duration
of 5 seconds, 3 seconds and 5 seconds respectively into
the holding coil (200mm x 0.8mm I.D.) at a flow rate of
0.9ml/min. The mixture was then propelled into the two
reactor coils (540mm x 0.8mm I.D.) and subsequently
allowed to react for 10 seconds before channeling the
solution into the detector for data sampling. Each sample
was injected four times and an average peak height
reading was taken. The above procedure was carried out
for the prepared standards and samples. For blank
measurements ascorbic acid in port position 5 was
replaced with distilled water.

Distilled water

Figure 1: Schematic representation of the SIA system for
the assay of ascorbic acid. $P =$ peristaltic pump; $V =$ multiport injection valve. Ports 4,5,6 for the
aspiration of sample and reagent; $HC =$ holding
coil; $RC =$ reactor coil; $D =$ detector; $W =$ waste.

Analysis of ascorbic acid

The concentrations of horseradish peroxidase, guaiacol
and H$_2$O$_2$ used were 0.175 unit/ml, 0.025M and 1.75 mM
respectively. The zucchini extract was adjusted to contain
peroxidase activity of 0.175unit/ml before use. Absorbance
measurements for tetraguaiacol formation were monitored
at 470nm. Peak height readings in absorbance units were
then determined in the absence and presence of varying
concentrations of ascorbic acid. The difference in peak
height reflects the percentage inhibition of tetraguaiacol
formation by different concentrations of ascorbic acid. The
effect of interferences was studied by spiking urea,
uric acid, lactic acid, and glucose (all at 0.5mg/ml) as
well as glutamic acid and cinnamic acid (at 0.3mg/ml)
singly into the ascorbic acid standard and then injecting
the spiked samples into the SIA system as described earlier.

Determination of ascorbic acid in pharmaceuticals using
horseradish peroxidase and zucchini extract

The zucchini extract was diluted with phosphate buffer
to give an activity similar to that of the prepared
commercial horseradish peroxidase activity. Standard
solutions of ascorbic acid (20-120 µg/ml) were diluted
from the freshly prepared stock solution of 400 µg/ml. At
the same time about 0.0313-0.0320g samples of ten
crushed vitamin C tablets were weighed and dissolved in
distilled water as in the case of ascorbic acid standard
and then filtered through Whatman No 1 filter paper. It
was appropriately diluted to yield around 40 µg/ml of
ascorbic acid. This same procedure was repeated for five
multivitamin tablets where the weight of crushed sample
used was 0.0153-0.0158g. As for orange juice, 5ml of the
juice was measured and filtered through Whatman No 1
filter paper before adjusting to 50 ml using distilled water.
This was further diluted fivefold to yield a final
concentration of approximately 40 µg/ml before SIA
analysis.

For comparison the ascorbic acid content in some
pharmaceutical preparations was determined using
titrimetry with 2,6 dichlorophenolindophenol as indicator.
The results obtained for an orange juice and two
pharmaceutical preparations namely vitamin C tablets
and multivitamin tablets were compared.

Results and Discussion

The peroxidase enzyme which is found in many fruits
and vegetables is stable for at least five months when
stored at 4°C. It has an optimum activity at pH 6-7. The
catalytic oxidation of guaiacol to tetraguaiacol by zucchini
peroxidase, monitored spectrophotometrically at 470nm,
for the determination of hydrogen peroxide using FIA
has been reported [19]. In this study the peroxidase
extracted from zucchini gave a Km value of 0.868mM
with H$_2$O$_2$ as substrate as compared to 1.530mM for
commercial horseradish peroxidase. Polyclar AT which
functions as a protective and stabilizing agent, was used
to adsorb the phenolic compounds in the zucchini
peroxidase.

In the present study the inhibitory effect of ascorbic
acid on peroxidase was investigated using SIA. For
horseradish peroxidase and crude zucchini peroxidase,
100% inhibition of the enzyme on the catalytic
transformation of guaiacol to tetraguaiacol was achieved
at ascorbic acid concentration of 160 µg/ml and 360 µg/
ml respectively (Figure 2).
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Optimisation of the SIA system

The different components of the SIA system were software-controlled. The parameters optimized included physical and chemical variables. By using 0.02g/l bromothymol blue in borax buffer (pH 9, 0.01 M) and injecting into multi-port injector positions 4, 5 and 6 of the manifold for different durations of time, the extent of overlap of peaks and peak height using different geometric configurations could be determined (Table 1). Peroxidase at position 4 for 5seconds, ascorbic acid or distilled water at position 5 for 3 seconds and H2 O2  - cum - guaiacol at position 6 for another 5 seconds gave maximum band overlap and peak height. The flow rate was fixed at 0.9ml/min as shown by the maximum peak height obtained at this flow rate (Figure 3).

![Figure 2: Comparison of the inhibition of crude zucchini peroxidase (o—o) and horseradish peroxidase (♦—♦) by ascorbic acid at concentrations of hydrogen peroxide at 1.75mM, guaiacol at 0.025M and peroxidase at 0.175unit/ml.](image)

Table 1: Comparison of peak heights obtained using different configurations and aspiration times.

<table>
<thead>
<tr>
<th>Port positions</th>
<th>Zone overlap for port positions</th>
<th>Peak height (Absorbance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 5 6</td>
<td>4 &amp; 5</td>
<td>5 &amp; 6</td>
</tr>
<tr>
<td>Aspiration time (secs)</td>
<td>10 5 5</td>
<td>0.755 0.694 0.511 0.730</td>
</tr>
<tr>
<td></td>
<td>5 5 5</td>
<td>0.804 0.753 0.552 0.706</td>
</tr>
<tr>
<td></td>
<td>5 3 5</td>
<td>0.857 0.776 0.688 0.805</td>
</tr>
<tr>
<td></td>
<td>5 10 5</td>
<td>0.814 0.694 0.500 0.602</td>
</tr>
</tbody>
</table>

![Figure 3: Optimisation of flow rate for ascorbic acid assay using SIA manifold.](image)

The chemical variables studied were optimum concentrations of the reagents namely H2O2 and guaiacol, peroxidase activity and the duration of reaction time. Hydrogen peroxide concentrations at 0.5mM, 1.0mM, 1.75mM, 2.5mM and 5.0mM were investigated at peroxidase activity of 0.175unit/ml. Ascorbic acid concentrations used in the inhibition study ranged from 0-120µg/ml for horseradish peroxidase and 0-360µg/ml for zucchini peroxidase. Hydrogen peroxide concentration at 1.75 mM is optimal in terms of peak height, baseline stability and wide linear range of ascorbic acid concentration for horseradish peroxidase at 0.175unit/ml (Figure 4). At hydrogen peroxide concentration of 0.5mM and 1.0mM, the linear dynamic range was much narrower although a higher inhibition of the enzyme was obtained. On the other hand, at hydrogen peroxide concentrations of 2.5mM and 5.0mM a lower inhibition of the enzyme was observed for the same ascorbic acid concentration. In view of this, hydrogen peroxide concentration was fixed at 1.75mM. The formation of tetraguaiacol in the absence and presence of ascorbic acid was then determined at this concentration of hydrogen peroxide.

In order to determine the optimum guaiacol concentration, 0.0125M, 0.025M, 0.0375M and 0.05M solutions of guaiacol were used at a fixed hydrogen

![Figure 4: Effect of varying hydrogen peroxide and ascorbic acid concentrations on the inhibition of horseradish peroxidase (0.175unit/ml) at guaiacol concentration of 0.025M. Hydrogen peroxide concentrations used were 0.5mM (♦—♦), 1mM (■—■), 1.75mM (♦—♦), 2.5mM (x—x) and 5.00mM (o—o).](image)
peroxide concentration of 1.75 mM and HRP activity at 0.175 unit/ml. Guaiacol at 0.025M was found to be optimum (Figure 5). Peak height and linear range of ascorbic acid concentrations from 0-160 µg/ml were again determined. Horseradish peroxidase and zucchini peroxidase activity was fixed at 0.175 unit/ml concentration for all subsequent assays. The reaction time for maximum peak height to develop was investigated from 0 to 25 seconds at 5 second interval (Figure 6). A reaction time of 10 seconds was found to be optimum.

The effect of urea, uric acid, lactic acid, and glucose (all at 0.5mg/ml) as well as glutamic acid and cinnamic acid (at 0.3mg/ml) on the detection of ascorbic acid was studied by spiking these interferences, at the stated concentration, singly into the ascorbic acid preparation and then injecting the spiked samples into the SIA manifold as described earlier. These interferences were found not to interfere with the analytical method.

### Analysis of Vitamin C tablets and orange juice

The ascorbic acid determined in orange juice and two different pharmaceutical preparations with the SIA method as described utilizing horseradish peroxidase and zucchini peroxidase were compared with that assayed using the USP method (Table 2). The results obtained are in close agreement with that determined using titrimetry (USP method). For Vitamin C tablets, the correlation coefficients (r) for linear graph for ascorbic acid using zucchini extract and horseradish peroxidase are 0.9997 and 0.9998 respectively. Recovery tests with the proposed method for ascorbic acid added to the tablets using horseradish peroxidase and zucchini peroxidase gave values of 99.6% and 106.0% for Vitamin C tablets respectively (Table3). As for the Multivitamin tablets, recovery of 91.1% and 91.4% were obtained correspondingly for zucchini extract and horseradish peroxidase.

### Table 2: Comparison of ascorbic acid content in some samples determined using USP and SIA methods. (C* = Ascorbic acid concentration in µg/ml; P** = Percentage of inhibition in tetraguaiacol formation; r = Correlation coefficient for n = 7 and RE*** = Relative Error).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stated Content (mg)</th>
<th>Titrimetry (USP) (mg)</th>
<th>SIA (Horseradish peroxidase) (mg)</th>
<th>RE***</th>
<th>SIA (zucchini extract) (mg)</th>
<th>RE***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C tablet</td>
<td>125</td>
<td>124.9</td>
<td>123.2 ± 7.2 P** = 0.83 + 0.60C* (r = 0.9998)</td>
<td>-1.4</td>
<td>132.8 ± 3.7 P** = -0.24 + 0.53C* (r = 0.9997)</td>
<td>+6.3</td>
</tr>
<tr>
<td>Tablet multivitamin</td>
<td>500</td>
<td>466.0</td>
<td>422.9 ± 4.6 P** = 0.83 + 0.60C* (r = 0.9998)</td>
<td>-9.2</td>
<td>484.3 ± 12.9 P** = -1.26 + 0.56C* (r = 0.9991)</td>
<td>+3.9</td>
</tr>
<tr>
<td>Orange juice</td>
<td>-</td>
<td>192.7</td>
<td>209.4 ± 9.9 P** = -1.27 + 0.57C* (r = 0.9974)</td>
<td>+8.7</td>
<td>245.2 ± 5.4 P** = -3.02 + 0.62C* (r = 0.9967)</td>
<td>+27.2</td>
</tr>
</tbody>
</table>
Ascorbic acid Assay

Table 3: Recovery tests for ascorbic acid in the pharmaceutical preparations with SIA method utilizing horseradish peroxidase and crude zucchini extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of spiked ascorbic acid (mg)</th>
<th>Percentage of recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Vitamin C tablets</td>
<td>0</td>
<td>99.6</td>
</tr>
<tr>
<td>(mg/tablet)</td>
<td>120.0</td>
<td></td>
</tr>
<tr>
<td>Multivitamin tablets</td>
<td>0</td>
<td>91.4</td>
</tr>
<tr>
<td>(mg/tablet)</td>
<td>489.2</td>
<td></td>
</tr>
<tr>
<td>Orange juice</td>
<td>0</td>
<td>112.4</td>
</tr>
<tr>
<td>(mg/100ml)</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

Conclusion

The SIA peroxidase procedure described in this paper presents a simple and fast assay method for ascorbic acid in vitamin C tablets and orange juice. Ingredients such as lactose, glucose, glutamic acid, urea, cinnamic acid and uric acid do not interfere with the analytical method.

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References