Review

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Vitamin C measurement in critical illness: challenges, methodologies and quality improvements

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Abstract

Background: There is renewed interest in high-dose vitamin C interventions in clinical medicine due to its antioxidant properties, safe use and cost-effectiveness. Yet, randomised control trials (RCTs) employing these interventions are failing to include robust analytical methodology and proper sample handling and processing techniques. Consequently, comparisons between studies becomes impossible as there is no metrological traceability and results may be prone to pre-analytical errors.
Content: Through published vitamin C stability studies, method comparison papers and data from vitamin C external quality assurance programs, an assessment was made on the functionality of current methods for critically ill patient samples.
Summary: Data was obtained from two external quality assurance programs, two papers assessing sample stability and interlaboratory agreement and a publication on vitamin C method comparisons. A shift from spectrophotometric and enzymatic methodologies to high performance liquid chromatography (HPLC) greatly improved the variability and interlaboratory agreement. Therefore, the current analytical performance of vitamin C HPLC methodologies are acceptable for the requirements of a high-dose vitamin C RCTs.
Outlook: Recommendations across the total testing process of vitamin C have been provided to improve the quality of the results. The harmonisation of sample handling and processing procedures will further improve the reliability of current analytical methodologies.

Keywords: ascorbic acid; critically ill; intensive care; measurement; vitamin C.

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Introduction

Vitamin C, or ascorbic acid (literally "against scurvy"), is well described in the popular literature for its role as an antioxidant. Vitamin C is also involved in gene expression as well as various cellular metabolic processes in a non-antioxidant role [1]. It is a water-soluble vitamin that is naturally present in some foods and is added to others as well as being available as a dietary supplement. Humans, unlike most animals, are unable to synthesize vitamin C endogenously, so must obtain it from their diet. Anecdotal evidence regarding the effectiveness of high-dose vitamin C on health has led to renewed interest in clinical medicine, which is highlighted in an intervention strategy for critical care patients with septic shock [2].

We have identified 29 trials conducted over the last decade employing vitamin C interventions in critically ill patients, with many using doses of ≥2.0 g/day. These trials are composed of 23 randomised control trials (RCTs) [3–25], two prospective [26, 27] and four retrospective analyses [2, 28–30]. Of these, 21 reported a variety of clinical benefits when an antioxidant supplementation protocol was implemented [2–4, 7–9, 12–14, 16–18, 20–22, 24, 26–30]; six included an analytical method for vitamin C

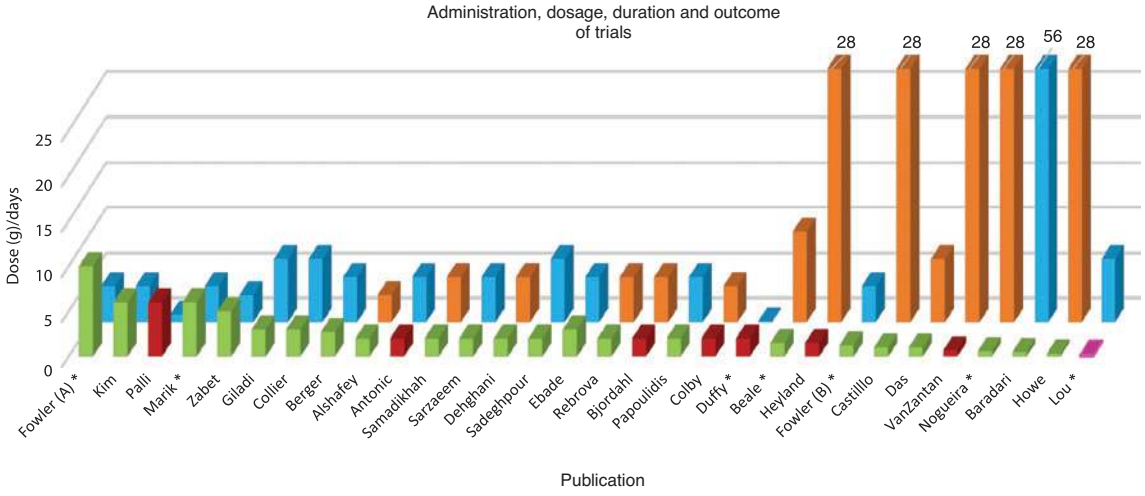

Figure 1: Administration route, dosage, duration and outcome of trials.
Front bars: Dosage, green colour indicates positive change in outcome measures; red indicates no change in outcome measures, pink indicates no data available. Back bars: Duration, blue indicates IV administration; orange indicates enteral/oral administration. Numbers above bars represent number of days for trials if >25 days. These trials were found through electronic searching of the Embase, Medline, PubMed and the Cochrane Library databases. Search keywords for all databases included vitamin C as well as its pseudonyms and terms related to critical illness. *Trials that included the analysis of vitamin C. Details of method information in Supplementary Material 2.

in their protocol [2, 3, 5, 12, 14, 19]; five included the analysis of vitamin C post dose [3, 5, 12, 14, 19] and a single RCT included a sample handling and processing protocol as well as the stability of the analyte [14]. A significant variation exists in the analytical design of these studies (Figure 1, Supplementary Material 1).

The inclusion of vitamin C analysis in these trials was to: (1) determine the prevalence of hypovitaminosis in their study cohort, and therefore justify supplementation; (2) ensure that the dosing regimen was sufficient to return vitamin C levels to normal [2, 5, 14]; (3) and/or to monitor the effects the concentrations had on the Sequential Organ Failure Assessment (SOFA) scores, endothelial function, soluble adhesion molecules, oxidative stress and/or inflammatory markers [14, 19]. However, the majority of the studies (79%) are missing a key component by not including the distribution and clearance of vitamin C in blood and tissue to correlate the clinical outcomes to the intervention.

Methodology is a key factor in producing reliable vitamin C results. With clinical medicine moving towards high-dose vitamin C therapy, the analytical side of vitaminology may need to accommodate for this trend. Considerations for vitamin C analysis span the total testing process from stability of the sample, understanding the measurand, through to interpretation against reference data. The harmonisation of these processes would ensure that all laboratories have an appropriate protocol in place and that interpretation of the data is consistent.

In this article, we evaluated the current vitamin C measurement methodologies and critically assessed their use for trials involving high-dose vitamin C administration in the critically ill patient population.

Literature search of methods

Original, full-text, articles published in peer-reviewed journals were compiled through electronic searching of the Embase, Medline and PubMed databases. The search keywords included ascorbic acid, ascorbate, vitamin C as well as terms related to the comparison of analytical methodologies such as analysis, method, measure or measurement, comparison, evaluation and assessment. To further refine the search, the terms serum and plasma were included as search keywords, study types were limited to human only and the date of publication was limited from 2008 to current. This period encompasses the beginnings of the introduction of vitamin C into the Royal College of Pathologists Australasia Quality Assurance Program (RCPAQAP). Through these search parameters only a single publication on direct vitamin C method comparisons was identified.

Clinical aspects of vitamin C

The physiology and pathophysiology of vitamin C has been extensively covered in both its antioxidant and non-antioxidant roles [31–34]. Thus, it will only be lightly discussed here. Vitamin C is a water-soluble vitamin, which has been estimated to have a plasma half-life of 7–14 days [35].

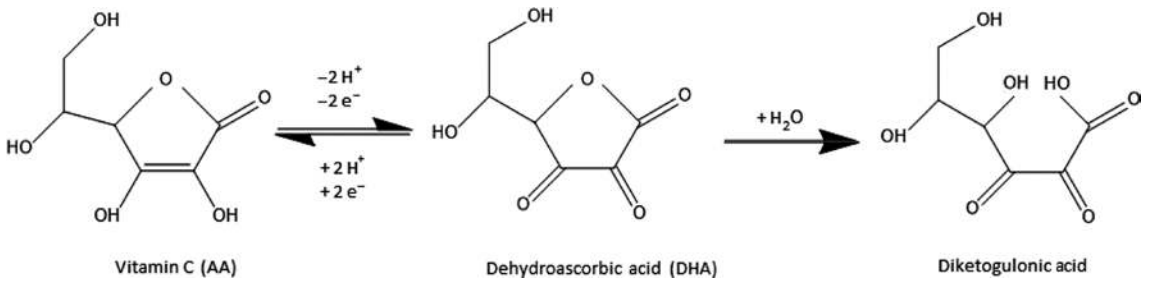

Figure 2: Metabolism of vitamin C.
The reversible reaction between ascorbic acid and ascorbate has been omitted for diagram simplicity. Image created using ChemDraw (PerkinElmer Informatics, Massachusetts, USA).

However, these values are based on healthy males subjected to depletion-repletion cycles and so may not reflect the half-life in critically ill populations [36–39]. In addition, vitamin C is absorbed through the intestines; therefore there are saturable absorption limits (~0.4 g) which will reduce the bioavailability of higher enteral doses [40, 41].

Low blood vitamin C levels are considered to be caused by either a reduced intake (e.g. deprivation of fresh fruits and vegetables) or an increased rate of depletion or intracellular uptake (or redistribution) which occurs following trauma or infection [42–45]. Increased distribution is associated with high leukocyte turnover (as there is a high intracellular vitamin C concentration in these cells), and oxidative stress, where the redox attributes of vitamin C reduces the impact of reactive species attenuating its antioxidant properties via hydrolysis to 2,3-diketogulonic acid (Figure 2) [31, 35].

Patients in the intensive care unit (ICU) have been shown to have a high prevalence of low vitamin C levels (11.0–23.0 μmol/L) and so are at risk of developing blood levels similar to those seen with vitamin C deficiency (<11.0 μmol/L) [14, 44, 46]. Low vitamin C levels are thought to be associated with higher SOFA scores and increased mortality [47]. Oxidative stress is believed to be one of the primary causes of vitamin C deficiency in ICU patients. This is also true for patients outside of the ICU setting [48]. Its prevalence is elevated in sepsis, trauma, infection, haemorrhage, multiple organ failure, stroke, cardiac disease and ischemic tissue injury from cardiac surgery [4, 8, 40, 44, 47, 49, 50]. Many of these illnesses have a high risk of morbidity and mortality as well as increased length of stay in the ICU and hospital [8, 51]. High-dose vitamin C supplementation may reduce oxidative damage through neutralising the reactive species and stopping or decreasing lipid peroxidation, improving tissue perfusion, tissue oxygenation (avoiding hypoxia) and alleviating potential organ dysfunction [42]. Additionally, a range of positive outcomes have been reported in trials which employed a vitamin C intervention strategy. These include faster organ function recovery, lower inflammatory response, reduced length of stay in the ICU, reduced length of stay in hospital, reduced mortality rate, increased ventilator-free days and reduced incidence of post-operative atrial fibrillation [2–4, 7–9, 12–14, 16–18, 20–22, 24, 26–30].

Although the current literature is yet to provide robust evidence on the benefits of high-dose vitamin C intervention in the critically ill, there is already an indication that only high doses are capable of correcting low plasma vitamin C levels [22, 32, 47]. The analysis of vitamin C in these patients is therefore imperative to monitor levels upon admission and during high-dose intervention to ensure that the dose and duration of the therapy is sufficient to maintain targeted blood levels of vitamin C [52]. It should be noted that vitamin C is suggested to have a pro-oxidant effect in the presence of freely dissolved metals, such as excess iron in haemodialysis patients [53]. However, more conclusive work in this area is required [54].

Methods for the measurement of vitamin C

Any testing procedure of vitamin C that is used to monitor current plasma levels, provides information on the pharmacokinetics of therapy or is used to calculate optimal dosages requires a protocol. This will reduce sample loss or degradation prior to analysis. It must also include a reliable, robust method of measurement, which covers the entire investigatory range of deficiency, hypovitaminosis C and high-dose supranormal levels.

Vitamin C has several measurands that can be analysed separately or in combination:

A) ascorbic acid only;
B) ascorbic acid and its oxidised form dehydroascorbic acid (DHA) separately;
C) total vitamin C (the sum of ascorbic acid + DHA) where DHA is reduced back to ascorbic acid prior to analysis.

Either methods B or C provides an overview of the total antioxidant availability. Measuring only ascorbic acid underestimates this by excluding DHA which has approximately the same biologically activity as ascorbic acid [55]. The term vitamin C will be used as a general term from this point on and will encompass all forms of vitamin C unless otherwise noted.

Pre-analytical

Pre-analytical variables principally stem from the patient's status, sample integrity (i.e. haemolysis) or sample handling and processing (i.e. type of anticoagulant, photosensitivity, temperature, pH and storage) and are likely to affect the accuracy of the measurements. Intra-individual variables such as the fasting state, smoking status, iron supplement intake or medications may affect the vitamin C status and are something to be aware of. However, in an ICU environment these become less pertinent, but this information should still be collected by the admissions team. *In vitro* haemolysis is another pre-analytical error which may occur despite careful attention to the necessary collection requirements. Haemolysis causes the release of free haemoglobin-iron which can rapidly reduce vitamin C. The effect can be minimised by the addition of tris(2-carboxyethyl) phosphine hydrochloride (TCEP), to the stabilised plasma. However, the dilution of the plasma with the reducing reagent must be corrected for before result reporting.

The analysis of vitamin C was included in the study design of several trials [2, 3, 5, 12, 14, 19]. However, details regarding sample handling and processing are typically lacking. Of the six trials which included vitamin C analysis the majority opted for plasma (67%) as the sample type whilst the remainder used serum (33%). Sample handling included cold centrifugation (33%) and storage was always frozen (50%) where mentioned. The addition of a stabiliser and/or reducing reagent was disclosed in some (50%) (Supplementary Material 2).

Sample type

Plasma is the most common matrix used for vitamin C analysis. However, tissue levels of vitamin C can be up to 100-fold more concentrated [31, 33, 56]. Plasma vitamin C concentrations rise and fall rapidly during supplementation/depletion unlike leukocytes, where changes in concentration levels are less dramatic [56, 57]. Therefore, plasma vitamin C may reflect rapid changes in the metabolic demands of the body whilst levels in leukocytes may be a better reflection of body stores [31, 58]. As vitamin C deficiency is largely unseen in the general population, the need to obtain information on tissue concentrations has never been of relevance. This is highlighted in only a handful of publications which have determined the tissue concentration of vitamin C in monocytes, neutrophils, lymphocytes, platelets, erythrocytes, skeletal muscle and several organ types [39, 55, 56, 59–61]. Supplementation with vitamin C does appear to increase tissue levels; however, there is currently no solid evidence to suggest whether there is a correlation between tissue and plasma vitamin C levels [55, 59, 61–63].

The most convenient way to measure tissue vitamin C levels would be through leukocytes. However, leukocyte vitamin C analysis requires a relatively large sample volume and is often plagued with poor precision and accuracy through contamination (e.g. platelets) during the matrix separation [57, 63, 64]. Additionally, there is little information on expected values of the body stores of vitamin C in healthy and diseased populations.

Plasma analysis of vitamin C is a faster and simpler method and can be readily separated from other contaminating sources. Commercial kits, quality controls and calibrators are available as are plasma vitamin C external quality assurance (EQA) programs streamlining method adoption and validation. This simplicity has driven plasma vitamin C analysis over tissue matrices.

Anti-coagulants

The choice of vacutainer was investigated by Lykkesfeldt who concluded that K_3-ethylenediaminetetraacetic acid (EDTA) tubes were superior in preventing *ex vivo* metabolism of vitamin C to DHA [65]. However, K_2-EDTA, lithium heparin and plain serum tubes were also found to be excellent for total vitamin C analysis, where the DHA is reduced back to ascorbic acid prior to measurement. DHA can be reduced to ascorbic acid through either the addition of a reducing reagent or by shifting the pH of the solution so that it is lower than the pKa of vitamin C (4.7) as the reaction between ascorbic acid and DHA is reversible (Figure 2) [66]. This has been backed by other studies which have successfully used either EDTA or lithium heparin in their stability studies [67–70].

Timing

Vitamin C is notoriously unstable with significant *ex vivo* metabolism to DHA and 2,3-diketogluonic acid within 2 h

of sample collection (Figure 2) [68, 70]. Separation of the serum or plasma should occur immediately followed by storage in an ultra-low-temperature environment (–70 and –80 °C). Investigations into delayed separation indicated significant loss after 2–6 h post collection in untreated samples kept at 4 °C or up to 16 h with the inclusion of diothreitol (DTT) [68, 70, 71]. Untreated samples, kept at room temperature, had a greatly reduced stability and vitamin C could not be recovered following the addition of a reducing reagent [70].

Reducing reagents, acids and preservatives

Lyophilised vitamin C material, preserved with the reducing reagent DTT, has been shown to be stable for up to 6 years [72]. A second reducing reagent, TCEP, has also been used with success to achieve stability of to up to 1 year when reduction is performed prior to analysis [70, 73]. Other studies incorporated an acid for sample deproteinisation, such as trichloroacetic acid (TCA), metaphosphoric acid (MPA) or perchloric acid (PCA) as well as a metal chelator such as EDTA or diethylene-triaminepentaacetic acid (DTPA) instead of a reducing reagent to stabilise the sample [67–70, 74]. These have also had success with recorded stability of up to 1.5 years.

Temperature

Ultra-low temperatures between –70 and –80 °C for long-term storage have been investigated with stability duration from 80 days to 5 years for samples that have been treated with a stabilising reagent [69, 74]. Non-stabilised plasma samples at these temperatures have a reduced shelf life of 2–52 weeks [69, 70]. Those stored at –20 °C or 4 °C, or room temperature were only deemed suitable for short-term storage [68 – 71).

Post sample preparation

Salminen and colleagues investigated the stability of vitamin C post sample preparation to mimic unforeseen delays of analysis [67]. A loss of <5% was determined in samples kept at 4 °C for up to 48 h. Additionally, if samples were frozen post sample preparation and re-analysed, losses >20% were observed. Each of the processes described above have had varying success in extending the stability and reducing the pre-analytical variables of vitamin C. Currently, the only universal agreement is that EDTA and lithium heparin tubes are an appropriate collection tube and that separation of the serum or plasma should be performed immediately after collection. The addition of a stabiliser or reducing reagent and storage conditions vary among these studies. Despite there being no collective agreement on a single acid or metal chelator, the inclusion of a stabilising reagent during sample processing is an appropriate procedure to accommodate sample instability and reduce pre-analytical errors.

Analytical

The imprecision and bias of a methodology can be one of the greatest contributors to the generation of erroneous results. The analytical performance of vitamin C measurement methodologies had been previously explored by Margolis and colleagues back in 1993 – 1994 and 2003 [72, 75]. The comparisons performed from 1993 to 1994 were designed by the National Institute of Standards and Technology (NIST). Four serum sample lots containing varying vitamin C concentrations were preserved in MPA and sent out to participating laboratories to be analysed in duplicate. These distributions occurred three times and were titled “Round robin IV, V and VI”.

Over the study period, 23 laboratories participated in this exercise, which included several methodologies. These were auto-analyser (n = 1), dichloro-phenolindophenol (DCIP, n = 2), o-phenylenediamine (OPD, n = 1), dinitrophenylhydrazine (DNPH, n = 3), enzymatic (n = 1), liquid chromatography (LC)-OPD detection (n = 1), LC-electrochemical detection (LC-ECD, n = 14) and LC with spectrophotometric detection (n = 1). This trial concluded that the measurement of ascorbic acid had a much higher repeatability estimate compared to that of total vitamin C.

The repeatability estimate was based on the within-sample and within-lot data from each participant. Additional findings from this study included the overestimation of total vitamin C in DNPH methodologies and the underestimation in the OPD method. These comparison studies resulted in poor interlaboratory agreement with an average coefficient of variance (CV) of 15.0% (min 12.0%, median 15.5%, max 17.0%). This was calculated from all four lots after the removal of the DNPH and OPD methods from the data. It was concluded that total vitamin C results cannot be compared among different methods in the absence of reference materials [72].

In 2003, Margolis and colleagues distributed samples with two levels of a certified reference material for vitamin C, developed by NIST, in a serum matrix stabilised in MPA to 17 laboratories [75, 76]. A CV of 21.9% for

Table 1: Overview of methodologies and their performance over the two certified reference materials for vitamin C.

Methodology	No. of labs	Low vitamin C level		High vitamin C level	
		Average, μmol/L	CV%	Average, μmol/L	CV%
HPLC-fluorescence	1	7.2	[a]	24.7	[a]
HPLC-ECD	7	9.8	19.8	26.6	24.3
HPLC-UV	3	8.9	18.0	27.5	6.7
Enzymatic	3	9.7	21.4	29.0	7.2
Spectrophotometric	3	9.6	30.3	30.6	24.2
All methods	17	9.5	21.9	27.8	19.2

Targets set by NIST: low vitamin C level 10.07 μmol/L, high vitamin C level 30.57 μmol/L. ECD, electrochemical detection; UV, ultraviolet.
[a]CV% was calculated only if the participation was ≥2.

low vitamin C concentration (10.07 ± 0.21 μmol/L) and 19.2% (30.57 ± 0.28 μmol/L) for high vitamin C concentration was observed indicating a lack of interlaboratory accuracy. There were several methodologies used in this study, which mainly included high performance liquid chromatography (HPLC) with differing detection methods. An overview of the methods and their accompanying performance can be seen in Table 1.

The six trials which included vitamin C analysis typically employed HPLC with various detection systems (83%) except one which employed an enzymatic methodology (17%). The measurand is total vitamin C for some (50%) and for the others it was unclear (50%). The lower limits of the reference indices varied from 20 to 50 μmol/L and the upper limits from 50 to 125 μmol/L. No trial indicated the assay imprecision or any enrolment in an EQA program (Supplementary Material 2).

Current analytical performance

Literature search

The literature search produced only one publication which directly compared two methods (HPLC with ECD and HPLC with ultraviolet [UV] detection), for total vitamin C quantification [77]. Regression and Bland-Altman analysis were performed on a total of 80 samples. The results between the two methods were determined to be equivalent for total vitamin C. Over the range measured (4–133 μmol/L) a small bias (3.7 μmol/L) was observed for the HPLC-UV method, which did not exist at concentrations of clinical interest, <28.4 μmol/L. It is unclear whether this bias would increase at levels >133 μmol/L as these concentrations are likely to be observed in high-dose vitamin C trials [14, 52, 78].

External quality assurance

For the outcomes of any trials to be comparable, the analytical techniques utilised must be traceable to a higher-order methodology or directly compared against known target values, typically through an EQA program. There is currently no higher-order reference method or procedure for vitamin C listed in the Joint Committee of Traceability in Laboratory Medicine (JCTLM) database [31]. Therefore, results generated from different studies can only be compared if their analytical methods participate in an EQA program and the reported results are equivalent.

Previous studies by Margolis and colleagues highlighted the need for improvement in vitamin C analysis [72, 75]. Since then, there has been the development of several EQA programs for serum and plasma vitamin C based in the US (NIST ceased the program in 2015), Germany (INSTAND e.V.) and Australasia (RCPAQAP).

The INSTAND e.V. program has over 30 years of experience in a plasma vitamin C EQA program, with the majority of the participants employing HPLC-based analytical methodologies [79]. The earliest data available online indicates that, in October 2014, this program had 45 participants (average 47.7, median 46.5, maximum 63 in 2018 and minimum 30 in 2015) giving it the largest number of participants for a plasma vitamin C EQA program. The program has four rounds each year with each round consisting of two lyophilised samples with a total of eight samples analysed annually.

Based on the available online data, target values are set by INSTAND e.V. to cover an approximate plasma vitamin C range of 30–163 μmol/L in which the program does not appear to utilise the same concentration sample twice [80]. This EQA program includes a generous ±36% allowable error over the entire concentration range. Since 2014, this program has achieved an average CV of 11.8% for each round (median 11.5%, low 8.8% and high 17.1%).

The RCPAQAP plasma vitamin C program was initiated in 2010 where 13 laboratories participated in the RCAPQAP's cycle 22, which included 12 lyophilised samples (six linearly related samples analysed in duplicate) over a 6-month period [81]. The allowable error for this program is based on biological variation data with a ±25% for concentrations >36 μmol/L and ±9.0 μmol/L for concentrations up to 36 μmol/L. According to the RCPAQAP's end of cycle 22 report, there were three analytical methods employed; HPLC –fluorescence (n = 9), colourmetric analysis (n = 2), 2,4 dinitrophenylhydrazine analysis (n = 1) [81]. One laboratory did not disclose the analytical principle. Overall, these laboratories produced an average CV of 18.9% (median 13.3%). This correlates with the two previous trials by Margolis and colleagues with calculated CVs of 15–21.9%.

In one of the recent end of cycle reports (cycle 39) there were 22 participants and four reported analytical methods; HPLC- fluorescence (n = 7), HPLC-ECD (n = 3), HPLC- UV (n = 9) and enzymatic endpoint (n = 2) with at least seven countries participating [82, 83]. Again, one laboratory did not disclose the analytical principle. This cycle showed a significant improvement from the original cycle with an overall average CV of 5.9% (median 6.9%). In fact, based on the average CV% from the end of cycle reports over the last decade, the vitamin C program is one of the most improved programs in the RCPAQAP's repertoire [84].

Overall, the statistics from both quality assurance programs indicate good interlaboratory agreement and imply that current analytical methodologies for vitamin C analysis provide harmonised results. These improvements in interlaboratory agreement may stem from a shift away from enzymatic, spectrophotometric and colorimetric procedures, which were prone to low sensitivity and specificity as well as interferences, towards HPLC-based methodologies [57]. Additionally, these often utilise commercially manufactured kits which include stabilising reagents and internal standards, further improving method standardisation [52]. Finally, the development of EQA programs has clearly aided in the improvement of interlaboratory agreement of vitamin C.

Post-analytical concepts

The origins of the clinical decision point for vitamin C deficiency appear to be founded from early works on depletion/repletion studies [61, 64, 85]. Very early studies suggested levels below 23 and 28 μmol/L were indicative of vitamin C depletion [86]. In 1973, the Nutrition Canada national survey recommended levels of

Table 2: Recommendations and rationale of vitamin C analysis across the total testing process.

Testing procedure	Recommendation	Rationale
Pre-analytical	1. Tube type: Samples should be collected into lithium heparin or EDTA tubes	Minimal sample oxidation has been observed in these tube types compared to others [67–70]
	2. Sample handling: Samples should be centrifuged at 4 °C after collection and the plasma should be separated immediately	Significant analyte loss has been associated with delayed sample separation [68, 70, 71]
	3. Stabilisation: An acid stabiliser with a metal chelator (e.g. PCA/DTPA) should be added in equal volumes to the separated plasma	Stability of samples recorded up to 1.5 years [67–70, 74]
	4. Storage: Separated, stabilised plasma samples should be stored between –70 and –80 °C	Higher temperatures suitable for only very short-term stability [68–71]
Analytical	5. Quantification: Plasma samples should be analysed using HPLC-based methodologies (e.g. ECD, UV or mass spectrometry)	Colorimetric, spectrophotometric and enzymatic methods are prone to low sensitivity and specificity and have known biological interferences [56]
	6. Quality assurance: The plasma vitamin C method must be enrolled in an EQA program	External unbiased monitoring of the performance of the methodology
	7. Delayed analysis: Once prepared, samples must be run within 48 h. Samples should not be frozen after preparation	Loss of analyte up to 5% when analysed at 48 h. Loss if >20% when prepared samples are frozen before analysis [67]
Post-analytical	8. Reference indices Deficiency: <11 μmol/L Hypovitaminosis C: 11–23 μmol/L Replete/sufficient: >23 μmol/L	These have large-scale national surveys which agreed upon these indices [56, 87]

11.4–22.8 μmol/L as indicative of a low vitamin C range [87]. This was re-enforced by the findings in the second National Health and Nutrition Examination Survey in the US (1976–1980) [57].

Currently there does not appear to be standardised normal, or expected, reference indices for serum/plasma vitamin C. Many of the published indices range from 23 to 100 μmol/L [2, 14, 44, 78, 88]. One epidemiological study on European populations reported a vitamin C reference range of 6.25–116.81 μmol/L across five countries [89]. However, there does appear to be some agreement at the diagnostic threshold of >23 μmol/L being considered sufficient. Therefore, vitamin C should be reported using the diagnostic threshold value. A list of all the recommendations across the total testing process and their rationale can be seen in Table 2.

Discussion

The efficacy of high-dose vitamin C therapy is still in question due to many of the published trials being single centre, unblinded, small in size, inclusive of post-hoc/retrospective analysis or affected by additional confounders. To determine whether high-dose vitamin C supplementation is clinically beneficial to ICU patients, more vigorous, well-planned and high-quality RCTs are still required and with them the need for a robust analytical method to help define the physiological role vitamin C plays in the critically ill.

The INSTAND e.V. and RCPAQAP EQA programs have indicated that the current methodologies for vitamin C analysis are fit for purpose and there is acceptable inter-laboratory agreement. However, both NIST and RCPAQAP integrate a stabiliser or reducing reagent into the medium of their EQA samples to stop the oxidation of vitamin C [72, 90]. In patient samples, there are often no stabilisers and oxidation will readily occur *ex vivo* and continue to do so up until analysis. Therefore, several factors need to be considered before implementing a vitamin C method for routine analysis or incorporation into a high-dose vitamin C RCT.

1. How should the samples be collected and stored?
   Laboratories must consider that pre-analytical error cannot be monitored through EQA programs. Therefore, incorporating appropriate sample handling and processing techniques into a method protocol is essential to reduce any loss before analysis minimising pre-analytical error. This includes following the recommendations set out in Table 2.
2. Is the method HPLC based?
   HPLC methodologies for vitamin C quantification have become increasingly popular over the years. Analysis by HPLC has increased by 53% since the inception of the RCPAQAP plasma vitamin C program in 2010 and now accounts for >90% of the participants. These methods are reliable and robust with some instrument formats accommodating commercially available reagent kits. These kits may include calibrators, mobile phase, light-protective centrifuge tubes, precipitation reagents and an internal standard. One commercially available kit was employed by 33% of the participants enrolled in the RCPAQAP cycle 39 for plasma vitamin C which achieved a CV of 6.4%. HPLC-ECD is considered the unofficial gold standard method for vitamin C quantification but HPLC-UV has been shown to perform equivalently [70, 77].
3. Does the method cover the expected range of deficiency and supranormal levels?
   HPLC-based methods are highly sensitive with a large dynamic range with published vitamin C methods reporting limit of quantitation levels of 1.0–5.6 μmol/L and linearity up to 5000 μmol/L [91, 92]. These ranges cover both vitamin C deficiency, <11.0 μmol/L, and high, >250 μmol/L, levels.
4. What should be reported (ascorbic acid, ascorbic acid and DHA or total vitamin C)?
   Measuring only ascorbic acid may result in the underestimation of bioavailability as it is likely that some of the supplemented vitamin C has been metabolised to DHA, which has approximately the same biological activity as ascorbic acid [57]. DHA is commonly calculated by subtracting the ascorbic acid result from the total vitamin C result, which requires the addition of a strong acid or reducing reagent and a second analysis [74]. However, it can be simultaneously quantified with ascorbic acid [93]. Measuring total vitamin C is a simpler method for monitoring patient vitamin C concentrations in the ICU. As the concentration of DHA is not pertinent and vitamin C is being supplemented intravenously, it would appear more beneficial to monitor the total vitamin C availability to ensure its blood levels remain high.

Conclusions

The current analytical performance of vitamin C methodologies are acceptable for the requirements of high-dose vitamin C RCTs. However, for vitamin C to be

correctly integrated into an interventional therapy with a robust reliable analytical methodology several factors are required. These include the establishment of proper sample handling and processing protocols including the addition of a stabiliser and ultra-low-temperature storage, the incorporation of HPLC-based methodology and the enrolment and participation in an EQA program for vitamin C. The harmonisation of these processes and protocols will also aid in ensuring that there is agreement in results between laboratories. Adherence to these recommendations for the procedures of collection, storage, analysis and interpretation of vitamin C in the clinical setting will support the outcomes of future clinical trial data.

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