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Journal of Pharmaceutical and Biomedical Analysis
25 (2001) 605–611

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Degradation of dehydroascorbic acid in parenteral nutrition mixtures

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Received 11 October 2000; received in revised form 10 November 2000; accepted 12 November 2000

Abstract

The degradation of ascorbic acid (AA) stored in parenteral nutrition (PN) regimens is initially by oxidation, catalysed by trace elements, in particular copper. After prolonged storage the concentration of AA remains relatively constant, with little variation, due to the lack of available oxygen. The initial degradation product is dehydroascorbic acid (DHAA). This is generated in an anaerobic environment, and is hypothesised to degrade by hydrolysis. It is the purpose of this investigation to ascertain the effect of temperature and trace elements on the anaerobic degradation of DHAA, and to identify the kinetics of the reaction. A stability-indicating reversed-phase HPLC assay was used. The column contained C_{18} reverse-phase packing (Luna), mean diameter 5 μm. The column dimensions were 15 cm long with an internal diameter of 0.4 cm. The mobile phase consisted of methanol: phosphate buffer (pH 7.8: 0.067 mol dm^{-3}) at a ratio of 40: 60 (v/v) and also included Cetrimide (mixed alkyltrimethylammonium bromide) (0.05 mol dm^{-3}) as an ion pair reagent. The flow rate was 0.7 ml min^{-1} and detection was by ultra-violet light absorption at 278 nm. This assay was used to monitor the degradation rate of DHAA in PN mixtures with and without trace elements over a range of temperatures (5–35°C). Results indicated a first order reaction that was temperature-dependent but trace elements independent.

Keywords: Dehydroascorbic acid; Kinetics; Parenteral nutrition; Reversed-phase HPLC; Trace elements; Stability

1. Introduction

Parenteral nutrition (PN) is a medical technique to provide nutrition for those whose intestines are unable to absorb nutrients by the normal oral route. It is infused through a catheter directly into a peripheral or central vein. In many cases where this method of nutrition is used it is preferable for patients to have one 'big bag' containing all the required nutrients to meet their daily needs. However, mixing the nutrients, which include glucose, lipids, amino acids, electrolytes, vitamins and trace elements, provides a chemically unstable mixture with many possible reactions occurring.

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0731-7085/01/$ - see front matter
PII: S0731-7085(00)00589-6

The most important examples include oxidation of ascorbic acid [1], reduction of thiamine [2] and photo-degradation of vitamin A [3] and particular amino acids [4].

Ascorbic acid (AA) is a water-soluble vitamin, and, as it is easily excreted from the body, it is an essential daily component of our diet for a healthy existence. AA is structurally one of the simplest of the vitamins [5]. Even though it is a simple molecule, the enediol structure dictates that it participates in complex redox chemical reactions [6]. The reactions most relevant to AA redox reactions in PN mixtures are described in Fig. 1 [6]. It has been known for many years that AA is easily oxidised by reaction with dissolved oxygen. The first degradation product is dehydroascorbic acid (DHAA) (Fig. 1). This reaction can be reversed by a strong reducing agent, for example dithiothreitol (DTT). DHAA degrades by way of hydrolysis or oxidation to 2,3-diketogulonic acid. Both AA and DHAA are biologically active, although the other degradation products lack the biological activity necessary to prevent scurvy [6]. Thus, in order to have an accurate measure of antiscorbutic activity, the most appropriate property to use is total AA (TAA) concentration, which is the sum of AA and DHAA concentrations. Therefore in order to determine total antiscorbutic activity, DHAA is reacted with an appropriate reducing agent [7], for example DTT [8], to convert DHAA to AA prior to AA analysis.

The principle cause of AA loss in compounded PN mixtures is reaction with oxygen. This originates from particular components, for example glucose infusion, as well as from the compounding process and oxygen transmission through the plastic bag wall, leading to oxygen dissolved in the mixture. This last source is minimised by using low permeability multi-layered bags [9]. If these bags are used then the losses of AA can be at least partially predicted [1].

Oxygen in the bag will react with AA catalysed by trace elements, in particular copper [10], producing DHAA and creating essentially anaerobic conditions. Consequently, loss of antiscorbutic activity results from DHAA degradation, which is a reaction that occurs largely in an anaerobic environment. The kinetics of DHAA degradation are poorly understood and there is no data relating to the kinetics of this process in PN mixtures. Since AA is the least stable component in PN mixtures [9], data to describe the kinetics of AA/DHAA degradation is essential in determining the effect of PN composition and storage conditions on the shelf life of individual PN mixture.

It was therefore the purpose of this study to determine the kinetics of DHAA degradation in PN mixtures, and in particular to evaluate the influence of temperature and trace elements on the degradation of DHAA, thus providing kinetic data to enable estimates of likely losses of antiscorbutic activity in PN mixtures during storage and administration.

2. Experimental

2.1. Materials and reagents

The following commercial infusions and additives were employed: Synthamin 14®, a mixture of 15 amino acids (leucine, isoleucine, lysine, valine, phenylalanine, histidine, threonine, methionine, tryptophan, alanine, arginine, glycine, proline, serine and tyrosine)(Baxter Healthcare Ltd., Thet-

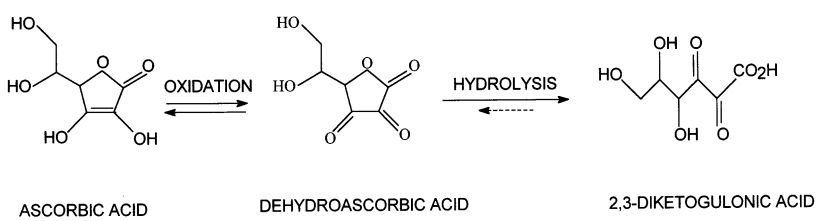

Fig. 1. Degradation pathway for ascorbic acid in aqueous solution.

ford, UK), glucose 20% w/v (Viaflex®, Baxter Healthcare Ltd.) and Additrace®, a trace element preparation containing ferric chloride, zinc chloride, manganese chloride, copper chloride, chromic chloride, sodium selenite, sodium molybdate, sodium fluoride and potassium iodide (Fresenius Kabi Ltd., Birchwood, Warrington, UK).

The following chemicals were used: dehydroascorbic acid (no quoted purity statement) (Aldrich Chemical Company, Dorset, UK), L-ascorbic acid (> 99% purity ACS reagent) (Sigma Chemical Company Ltd., Dorset, UK), methanol (99.8% purity, HPLC grade) (Fisher Scientific UK Ltd, Loughborough, UK), DL-dithiothreitol (> 99% purity) (Sigma Chemical Company Ltd.), disodium hydrogen orthophosphate 12-hydrate ($Na_2HPO_4.12H_2O$) (99% purity) (BDH Laboratory Supplies, Poole, UK), potassium dihydrogen orthophosphate (KH_2PO_4) (99.7% purity) (Fisher Scientific UK Ltd.), mixed alkyltrimethylammonium bromide (Cetrimide) (no quoted purity statement) (Sigma Chemical Company Ltd.) and water, deionised-distilled and stored in glass containers.

2.2. Analysis of DHAA in PN mixtures

Because DHAA fails to ion pair effectively, and possesses low UV-absorbing properties, the assay design is based on the conversion of DHAA to AA, which can then be determined by an ion-pairing HPLC method with detection by UV absorption, which has been previously described [9]. HPLC equipment comprised a Spectra Series P100 LC Pump (Thermo Separations Products, Stone, UK) connected to a Linear UVIS200 UV Detector (HPLC Technology Ltd., Macclesfield, UK) and a Spectra Series AS300 Autosampler (Thermo Separations Products). These were used alongside an in-line degasser DEG103 (Kontron Instruments Ltd., Watford, UK). The column contained C_{18} reverse-phase packing (Luna), mean diameter 5 μm (Phenomenex Ltd., Macclesfield, UK). The column dimensions were 15 cm long with an internal diameter of 0.4 cm. The mobile phase consisted of methanol–phosphate buffer (pH 7.8; 0.067 mol dm^{-3}) at a ratio of 40:60 (v/v). The mobile phase also contained Cetrimide (mixed alkyltrimethylammonium bromide) (0.05 mol dm^{-3}) as an ion pair reagent. The flow rate was 0.7 ml min^{-1} [9]. Detection was by ultra-violet light absorption at 278 nm and quantification was by electronic integration using computer-based software, 'Winner on Windows' (Thermo Separations Products). This method provided resolution of the AA peak from other components in the PN mixture. A sample chromatogram and a three dimensional spectral analysis (Fig. 2a and b) demonstrates the resolution of the method.. Peak purity was determined by comparing overlayed plots of the apex of the chromatogram with points corresponding to 50% of ascending and descending slopes respectively, over the range 200–350 nm. Comparison of the UV scans indicated that the ascending peak had a comparative purity of 95.4%,and the descending peak 99.2% of the apex,respectively.

2.3. Experimental design

A 'standard' PN mixture representing a typical adult regimen was employed for all experiments. This comprised Synthamin 14®: glucose 20% w/v (2:3, v/v), to which was added 0.4% v/v Additrace, as required. Asceptic techniques were used to minimise microbial contamination of the nutrient-rich mixtures. PN mixtures were stored at the appropriate temperature, protected from daylight, to allow pre-equilibration prior to the addition of DHAA. DHAA solutions containing 50 mg ml^{-1} in water were freshly prepared. 1.25 ml of this solution was accurately added to a 25-ml volumetric flask. The volume was made up to 25 ml by adding PN mixture. This mixture was then added by syringe to a sealed tube that had been previously flushed with nitrogen. The mixture and headspace were further flushed with nitrogen to remove any residual air and the filled tubes returned to the appropriate storage temperature. Samples (approximately 1.5 ml) were withdrawn at appropriate intervals through the rubber septum of the tube cap, using a syringe with attached needle, for analysis by the stability-indicating HPLC method. Injections from two external standards (prepared to contain 30 and 70 μg ml^{-1}) and each sample dilution were made on to the chromatograph in triplicate using the bracketing technique.

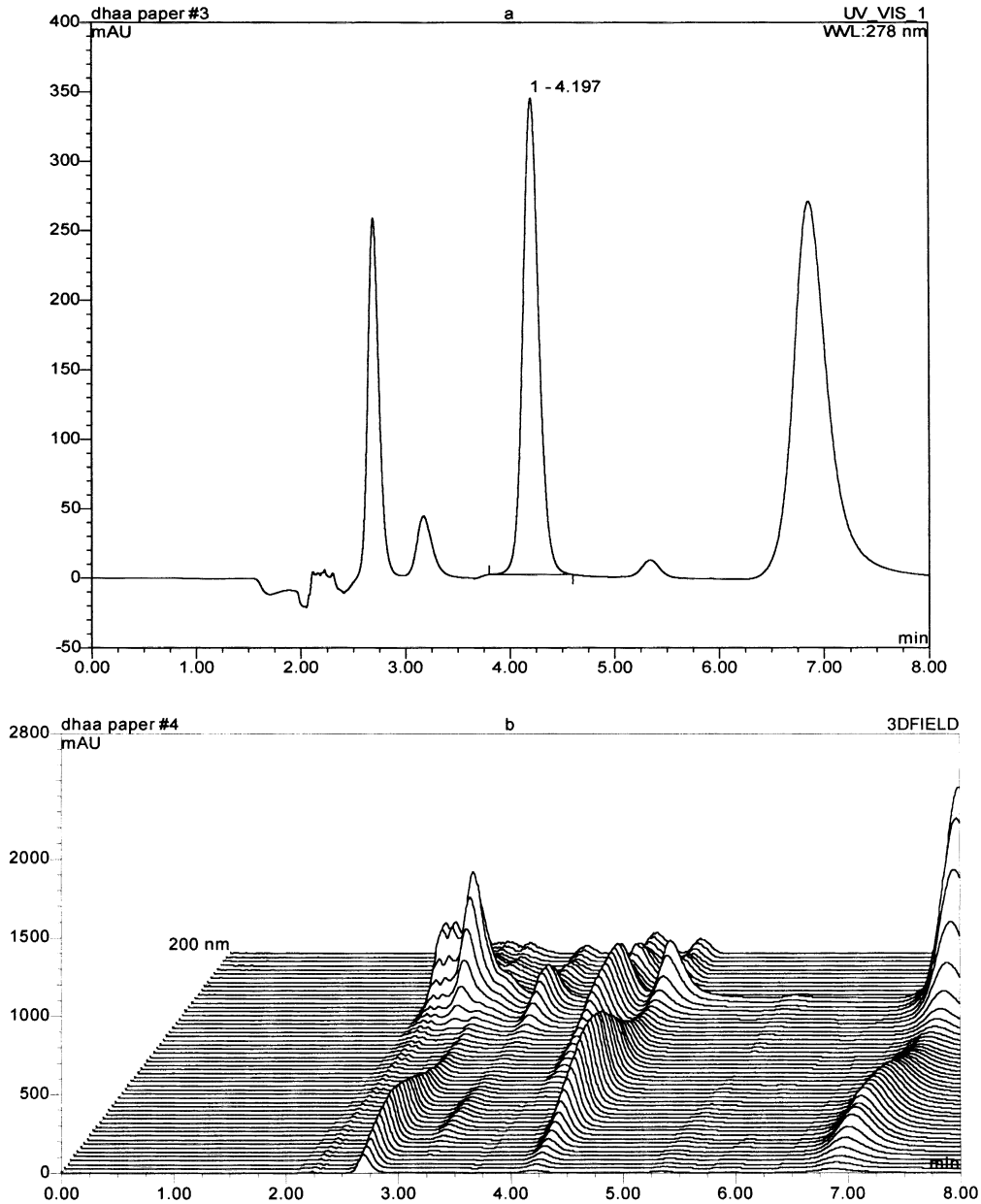

Fig. 2. HPLC analysis of dehydroascorbic acid (100 μg ml^{-1}) in a PN mixture. (a) Chromatogram: peak: 1, ascorbic acid. Vertical axis, absorbance at 278 nm (mAU); horizontal axis, retention time (min). (b) Diode array spectrum of the chromatogram: vertical axis, absorbance (mAU); horizontal axis, retention time (min); third axis, wavelength 200–330 nm.

2.4. Preparation of samples for analysis

Samples were assayed for AA and TAA concentrations. Amber-glass vials were used throughout experimentation. For AA analysis the amber glass vials were capped and flushed with nitrogen to remove residual air. The sample was then injected into the vial and the sample and vial-

headspace were flushed with nitrogen. For TAA analysis 0.5 ml of the solution, accurately measured, was added to 0.5 ml of 0.008 mol dm^{-3} DTT in the vial, to reduce DHAA to AA [6,9]. Mixtures were allowed to stand for 15 min prior to analysis, which was shown to allow adequate time for maximum conversion [8]. The recovery rate of DHAA in a simple aqueous solution was $92 \pm 1\%$ ($n = 3$). This assumes 100% purity of the DHAA sample, which the purity statement does not provide.

2.5. Validation of the analytical HPLC method

2.5.1. AA method for analysis

Solutions of AA dissolved in standard PN mixture over a range of concentrations (approximately 20–100 μg ml^{-1}, accurately prepared) were assayed by triplicate injections using the bracketing technique, to confirm the reliability of the assay. The results assessing the relationship between AA concentration and peak area, repeated on two occasions, indicated acceptable linearity (r^2 greater than 0.9999). The mean calculated intercept was -1.6 μg ml^{-1}, and inter-batch variability was less than 1% (average R.S.D. for triplicate repeat injections).

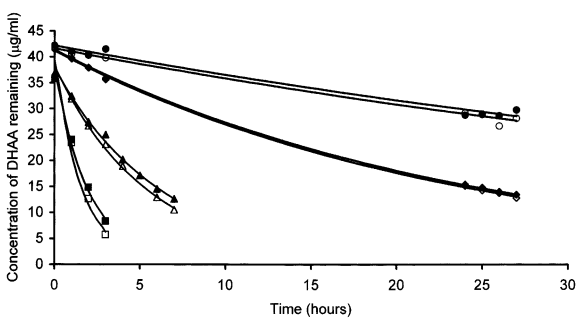

Fig. 3. Degradation of dehydroascorbic acid in a PN mixture at different temperatures without (open symbols) and with (solid symbols) the addition of trace elements. Circles, 35°C, diamonds, 25°C, triangles, 15°C squares, 5°C. Vertical axis, concentration of DHAA remaining (μg ml^{-1}); horizontal axis, time (h).

2.5.2. DHAA method for analysis

Linearity of peak area response against DHAA concentration in a standard PN mixture, over a range of concentrations from approximately 20–100 μg ml^{-1} (accurately prepared) was determined. The correlation coefficient (r^2) was 0.9936 and the mean calculated intercept was $+1.6$ μg ml^{-1} and inter-batch variability was less than 2.2% (average R.S.D. for triplicate repeat injections). The limit of detection in PN mixtures is approx. 1 μg ml^{-1} in PN mixtures [9].

2.5.3. Confirmation of the method as stability-indicating

The method is stability-indicating with respect to the determination of anti-scurvitic activity because DHAA degrades in PN mixtures by hydrolysis to 2,3-diketo-L-gulonic acid [6]. This primary degradation product will not react with DTT to produce AA [8]. The method was evaluated to confirm that it was stability-indicating. Solutions of DHAA (100 μg ml^{-1}) were heat-stressed (50°C for 1 h) in the PN mixture to promote hydrolytic degradation in situ. Samples were then chromatographed following the addition of DTT to convert any residual DHAA to AA. An approximately 50% reduction in peak area was observed and assessment of peak purity by diode array confirmed that the AA peak exhibited levels of purity greater than 95%.

3. Results

3.1. Effect of temperature on DHAA degradation

The extent of degradation of DHAA was measured and compared at different temperatures (range 5–35°C), including and excluding trace elements. Fig. 3 shows the degradation at four different temperatures. Degradation appears to follow an exponential decay curve at all temperatures investigated. The presence of trace elements does not appear to affect degradation rates. A logarithmic plot of ln(concentration) against time was shown to be linear at all temperatures (r^2 greater than 0.9961), confirming the exponential decay.

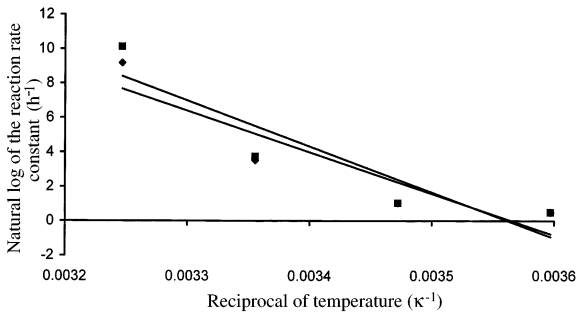

Fig. 4. An Arrhenius-type plot of the degradation of DHAA over the temperature range 5–35°C. Vertical axis, concentration of DHAA remaining (μg ml^{-1}); horizontal axis, time (h).

The reaction is confirmed to be first order by the linear logarithmic plot. A second order plot, the reciprocal of concentration against time, was plotted as confirmation. A linear response is obtained if the reaction is second order. However, the result gave a non-linear response suggesting that the reaction is not second order.

The temperature dependence of the degradation of DHAA can be examined using an Arrhenius-type plot. The rate constants are determined at each temperature, and the effect of temperature related to the rate constant by

$$\kappa = a \exp(-b/T) \quad (1)$$

where κ is the reaction rate constant (h^{-1}), and a and b are constants (b is the 'activation energy parameter').

In linear form this becomes

$$\ln \kappa = \ln a - b/T \quad (2)$$

and a plot of $\ln \kappa$ against $1/T$ is linear (Fig. 4). When PN mixtures are administered, changes in temperature can significantly affect the rate of degradation of DHAA. It is considered important to quantify the effect of temperature change on the stability of DHAA. This is achieved using the form of the Arrhenius equation (Eq. (1)). Rate data were collected over the temperature range 5–35°C and rate constants were obtained for each temperature. These rate constants were then fitted to the Arrhenius equation in it's linearised form (Eq. (2)). Regression data analysis (95% confidence level) gives the best-fit values for a and b to be:

With trace elements

$a = 30.8 \pm 22.6$

$b = 8832.1 \pm 6613.6$

Without trace elements

$a = 30.9 \pm 11.1$

$b = 8814.0 \pm 3249.2$

According to the Arrhenius equation (Eq. (1)) the units for temperature that should be used are Kelvins (as used here). However, if degrees Celsius is used then the numerical values will be different, and so will the calculated value for b. This equation also allows prediction of the rate of degradation and hence a remaining concentration for any time at any temperature within the range studied (5–35°C).

The values for b obtained with and without any trace elements (potential catalyst) are similar. The closeness of these values suggests that dissolved trace elements have no catalytic effect.

4. Discussion

The degradation of DHAA under the study conditions appeared, as expected, to be influenced by changes in temperature. At higher temperatures the rate of degradation was increased compared with the rate at lower temperatures (Fig. 3). The temperature range was chosen to ascertain it's influence after compounding the bag, during storage in a refrigerator, through to infusion at room temperature (at home or on a hospital ward). However, the inclusion of trace elements had no effect on the rate of degradation of DHAA, in contrast to AA. In Fig. 3 it can be seen that the rate of degradation of the solutions of DHAA containing trace elements is very similar to the rate of degradation without trace elements.

The order of reaction was confirmed as first order. Consequently it is possible to estimate the

extent of DHAA degradation in PN mixtures during storage and administration. From the data it is possible to estimate that approximately 90% of the DHAA generated will have degraded within 7 days storage at 5°C. It can also be estimated that during infusion, say over a 12-h period, which is typical for parenteral nutrition administration, the proportion of residual DHAA likely to be degraded will approach 85%. It should be noted that the temperature profile of the PN infusion will rise relatively slowly in the bag itself and therefore losses in practice are likely to be less than would be calculated assuming the solution commenced at room temperature.

In conclusion, the degradation reaction of DHAA in anaerobic PN mixtures is temperature-dependent but trace element-independent. The trace elements contained in the additive used were iron, zinc, manganese, copper, chromium, selenium, molybdenum and fluorine. Therefore, considering the wider aspect of antiscorbutic activity of PN mixtures delivered to patients, the AA which is added to the regimen will degrade to DHAA by oxidation caused by dissolved oxygen. This reaction is catalysed by trace elements, in particular copper [10]. Once all of the molecular oxygen has reacted, the AA oxidation pathway will be halted. However, the DHAA degradation pathway will continue. This will be accelerated if the bag is stored at room temperature. This further highlights the necessity of maximum removal of oxygen and minimal aeration of solutions during compounding, as, without considerable AA degradation, DHAA cannot be produced, therefore providing maximum antiscorbutic activity to the patient. It also indicates that DHAA in PN mixtures is more unstable than was originally thought [9], and therefore biological activity in PN mixtures stored in multi-layered bags is largely dependent on residual AA concentration rather than DHAA concentration, since AA is stable in PN mixtures in the absence of oxygen.

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