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Topical delivery of retinyl ascorbate: 4. Comparative anti-oxidant activity towards DPPH

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Abstract

The free radical scavenging properties of retinyl ascorbate (RA-AsA) were determined by monitoring the decomposition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a function of time and in comparison with ascorbic acid (AsA), ascorbic acid palmitate (AsA-Pal), retinoic acid (RA), retinol (ROL) and retinol palmitate (Rol-Pal). The rate constant of RA-AsA ($mean_3 \pm SD$) was $4.9 \pm 0.3\,M^{-1}\,s^{-1}$, and indicated greater potency as an antioxidant compared to the rest of the test compounds (AsA $3.4 \pm 0.4\,M^{-1}\,s^{-1}$, AsA-Pal, $2.9 \pm 0.2\,M^{-1}\,s^{-1}$, RA $1.4 \pm 0.3\,M^{-1}\,s^{-1}$, ROL $1.3 \pm 0.1\,M^{-1}\,s^{-1}$, Rol-Pal exhibited insignificant activity). The decomposition rate constant of DPPH, $5 \pm 0.6 \times 10^{-8}\,M^{-1}\,s^{-1}$, in ethanol and BHA, $154 \pm 3\,M^{-1}\,s^{-1}$ were both used as control. The compound RA-2-carboxy-2-hydroxy-ethanoate was isolated by prep-TLC and was identified, by ^{13}C and $^{1}HNMR$ spectroscopy, as the major by-product from the reaction of RA-AsA with DPPH, which was also found to be potent antioxidant, $2.1 \pm 0.2\,M^{-1}\,s^{-1}$. This suggests that oxidation of AsA moiety did not lead to the production of erythrulose species, which could cause deleterious modifications of cellular proteins.

Keywords: *Retinyl ascorbate, Anti-oxidant, Co-drug, Topical drug delivery*

Abbreviations: *RA-AsA, all-trans* retinyl ascorbate; AsA, L-ascorbic acid; AsA-Pal, L-ascorbic acid palmetate; BHA, butylated hydroxylanisol; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EC50, effective concentration to reduce absorbance by 50%; IP%, inhibited percentage; RA, all-*trans*-retinoic acid; ROL, all-*trans*-retinol; Rol-Pal, all-*trans* retinol palmetate; TLC, thin layer chromatography

Introduction

Reactive oxygen species (ROS) are implicated in UV-induced damage to skin [1]. This oxidative damage could be an initiator in the pathogenesis of skin cancer and photoaging [2,3]. Indigenous antioxidants that protect skin against ROS include various low molecular weight antioxidants (glutathione, tocopherols and ubiquinol) and antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase and thioredoxin reductase) [4–6]. Topical administration of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system and thus may be a successful strategy for diminishing ultraviolet radiation-mediated oxidative damage in the skin [6,7].

AsA is a ubiquitous cellular water-soluble antioxidant [8–10], which can easily undergo spontaneous auto-degradation in solution, especially in the presence of metal ions. In a physiological environment, oxidised AsA, dehydroascorbate (DHA), if not reduced back immediately [11], it undergoes further degradation to form ketones and aldehydes which are potent glycating agents with proteins [12–15]. Retinoids are also available in cells in the form of retinoid esters used mainly as cellular division regulators after conversion to the more active retinoic acid (all-*trans*-RA). RA undergoes configuration changes to form the 9-*cis* and 11-*cis* forms on exposure to light [7,16].

In our previous work, the co-drug retinyl ascorbate (RA-AsA, Figure 1) was shown to penetrate and partially decompose within the skin, thereby regenerating both species *in situ* and having overcome the stratum corneum [17]. Also, from previous solubility and

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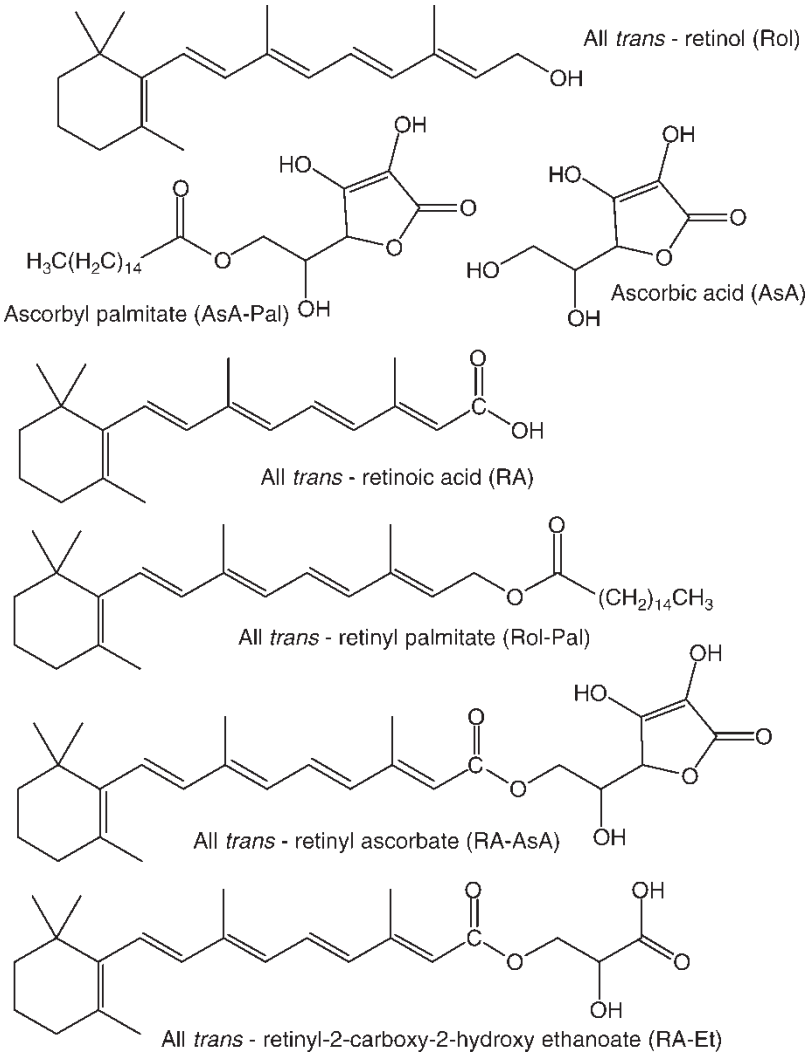

Figure 1. Structures of the test compounds and proposed structure of by-product, (CS ChemDraw Ultra).

stability studies, RA-AsA was found to be light sensitive and reactive to atmospheric oxygen in solution form. To determine the antioxidant activity of RA-AsA, a method based on the scavenging of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical that has been used extensively to predict the antioxidant activities of various natural and synthetic chemical compounds, was used [18–22]. Three experimental parameters were determined to assess the activity of RA-AsA in terms of reducing DPPH: (1) inhibited percentage (IP%) of DPPH, (2) concentration of antioxidant required to reduce absorbance by 50% of that shown by a blank test (EC_{50}), (3) the rate constant as measure of potency. The antioxidant activity of RA-AsA was compared to those of AsA, AsA-Pal (Figure 1), which are used commercially for the same purpose. Further comparisons of activities of DPPH with RA, ROL and Rol-Pal were also determined, since retinoids have been claimed to also possess antioxidant properties [23,24]. The rate constant for the decomposition of DPPH in ethanol was determined and used as control, as a reaction between DPPH and the compound would be indicated by changes to the constant. Comparisons with published data, IP% and EC_{50}, for AsA and AsA-Pal, along with the rate constant for butylated hydroxylanisol (BHA), were used to validate the experimental technique [18–22,25].

Materials

RA-AsA was synthesised in our laboratories [17]. All-*trans*-retinoic acid (RA), L-ascorbic acid (AsA), all-*trans*-retinol (Rol), all-*trans*-retinol palmitate (Rol-Pal), (all of purity >99%), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, (90%, were all obtained from Sigma-Aldrich, UK). Ultra free-CL 2 ml tubes, containing low-binding Durapore PVDF 0.22 μm filter inserts, were from Millipore, Bedford, MA, USA. Hydroxylamine hydrochloride, potassium hydroxide, ferric chloride, silver nitrite were of Analar grade. All solvents (HPLC grade) were obtained from Fisher (Loughborough, UK), except for CDCl3 (99.8 atom % D) which was obtained from Acros Organics, Loughborough, UK. Glass-backed thin layer

chromatography (TLC) plates (SIL G-25 UV254, 20 × 20 cm) were obtained from Machery-Nagel (Duren, Germany).

Methods

Free radical scavenging activity

The free radical scavenging activity of each compound was determined using the relatively stable free radical DPPH according to a widely followed procedure, with slight modification [18–22]. DPPH degradation in ethanol (as recommended by manufacturer, Calbiochem, UK) and BHA were used as control [18]. The rate of decomposition of DPPH in ethanol was monitored using a Perkin–Elmer Lambda 5 (UV–Vis) over 30 min at room temperature. The zero-order decomposition rate constant k ($M\,min^{-1}$) was calculated as the gradient of A_0–A_t vs. time. The same was determined for the test compounds. The data were then used to estimate the reaction time for the following procedures, for which loss of reactants would mainly be due to the reaction rather than due to auto degradation in the solvent. The decolourisation of DPPH solution was monitored at regular intervals using the Perkin–Elmer Lambda 5 spectrophotometer at 517 nm at room temperature over a period of 25 min (1 min for BHA) and under light-shield. Reaction mixtures containing 0.2 ml of 0.5 mM DPPH in ethanol and 0.8 ml of 1.25×10^{-3}, 1.25×10^{-4}, 6.25×10^{-5}, 1.25×10^{-5}, 1.25×10^{-6} M test compound in ethanol/water (1:1, v/v) were added in turn to a 1 ml quartz cuvette. The blank consisted of the reactants without DPPH.

Due to the volatility of the reactants and the possibility of the formation of new chemical species as by-products that could also be free radicals and/or antioxidants, the first-order rate constants (k) were determined for each concentration per antioxidant from the plot of $\ln[A_0/A_t]$ vs. time min. Potency was estimated from the slope of least squares fitting of plots of the rate constants k per min vs. [substrate] M, the values were then converted to seconds for convenience because the test compounds exhibited significant differences in activity [18].

The EC_{50} values, the concentration of each sample required to give 50% of the absorbance shown by a blank test, were taken as the point of interception from the plot of percentage of the remaining and scavenged DPPH vs. antioxidant concentration (M) [19]. Scavenging activity, in terms of the inhibited percentage of DPPH (% IP), was determined from $(A_0–A_t)/A_0 \times 100$ at an equimolar concentration of reactants, 10^{-4} M, at 20 min of reaction time. All data were presented as $n = 3 \pm$ SD.

Isolation of by-product from RA-AsA and DPPH reaction

The reduction of DPPH was observed to proceed faster when incubated with RA-AsA than that with any of the rest of the test compounds, hence, it was decided to investigate the structural integrity of RA-AsA after losing (one or more of) its protons to DPPH. A reaction mixture of 1 ml, 0.1 mM DPPH and 4 ml, 1 mM RA-AsA was left at room temperature for 15 min, under light-shield. Some of the solvent was evaporated at low temperature and under nitrogen and a band of the residue was added to the baseline of the TLC plate, which was then eluted with petroleum ether:ethanol:chloroform:water (40:30:29:1, v:v:v:v). Control samples of RA-AsA, AsA, RA and DPPH were also spotted. The band of an unknown compound was identified and removed from the plate, dissolved in ethanol, filtered and evaporated to dryness. The ester linkage was confirmed by placing 2 mg of the product in 1 ml of saturated ethanolic solution of hydroxylamine hydrochloride and 1 ml of 20% ethanolic potassium hydroxide were added. The mixture was heated to boiling point and acidified with 5% hydrochloric acid. Then, a 5% solution of ferric chloride was added dropwise until the colour of deep red-purple was formed [26]. The decomposition constant (k, $M\,s^{-1}$) was determined as above, IP% and potency ($M^{-1}\,s^{-1}$) were also determined but with concentrations of 1.25×10^{-3}, 1×10^{-3}, 8×10^{-4}, 4×10^{-4} and 1×10^{-4} M over 5 min of reaction time. A sample of 5 mg was then dissolved in $CDCl_3$ for ^{13}C and ^{1}H NMR analysis.

Results and discussion

The DPPH colorimetric method, originally developed by Blois [27], is widely used for the determination of free radical scavenging capacity in phytotechnology, food technology and pharmacology/toxicology. The antioxidant activity is based on the reduction of DPPH, which has a strong absorption maximum at 517 nm. As the odd electron of the radical forms pairs in the presence of hydrogen donor (namely the antioxidant) the absorption reduces stoichiometrically with respect to the number of electrons captured [28–33].

In an effort to quantify the free radical scavenging activity of RA-AsA the rate constant for the reduction of DPPH was measured in the presence of the ester, then compared with those of AsA, AsA-Pal, RA, ROL, and Rol-Pal (Table I). The zero-order decomposition of DPPH (K_{DPPH}) in ethanol after 30 min was calculated as $5 \times 10^{-8}\,M\,s^{-1}$, and those for RA-AsA and AsA were 1.7×10^{-8} and $3.3 \times 10^{-8}\,M\,s^{-1}$, respectively, in aqueous ethanol. No decomposition could be detected for the rest of the test compounds in the same solvent (RA-Et is discussed below), thus the absorbance was noted for the first 20–25 min of each reaction to limit the influence of such degradation on the final results. The rate constant was also used as control for the experiment. K_{DPPH} increased in a first-order process in the presence of antioxidants,

Table I. Free radical scavenging activity of RA-AsA against DPPH, compared to other test anti-oxidants.

Compound	Decomposition[a] constant (k) in vehicle, $M\,s^{-1}$	Decomposition [b] constant (k_{DPPH}) in presence of antioxidant, s^{-1}	Scavenging activity [c](%, @ 10^{-4} M)		EC50 [d] (10^{-5} M)		Rate constant [e] ($M^{-1}\,s^{-1}$)		R^2 [f]
			Observed	Literature value	Observed	Literature value	Observed	Literature value	
DPPH	$5 \pm 0.6 \times 10^{-8}$	–	–	–	–	–	–	–	0.992
RA-AsA	$2 \pm 0.7 \times 10^{-8}$	$7 \pm (0.9) \times 10^{-3}$	96 ± 2	–	0.52 ± 0.3	–	4.9 ± 0.3	–	0.996
AsA	$3 \pm 0.9 \times 10^{-8}$	$5 \pm (0.9) \times 10^{-3}$	95 ± 3	94.3 ± 1.7	2.3 ± 0.2	2.2	3.4 ± 0.4	–	0.996
AsA-Pal	0	$4 \pm (0.7) \times 10^{-3}$	97 ± 2	95.7 ± 0.1	3.8 ± 0.2	2.9	2.9 ± 0.2	–	0.999
RA	0	$2 \pm (0.5) \times 10^{-3}$	76 ± 4	–	19 ± 3	–	1.4 ± 0.3	–	0.985
ROL	0	$1.7 \pm (0.1) \times 10^{-3}$	7 1 ± 2	–	31 ± 4	–	1.3 ± 0.1	–	0.987
Rol-Pal	0	Insignificant	Insignificant	–	Insignificant	–	Insignificant	–	–
RA-Et	$7 \pm 2 \times 10^{-7}$	$3.3 \pm (0.9) \times 10^{-3}$	95 ± 4	–	Undetermined	–	2.1 ± 0.2	–	0.991
BHA	0	0.1	98 ± 2	–	0.02 ± 0.01	–	154 ± 3	150	0.993

[a] The figures indicate the k value as a measure of stability in ethanol for DPPH and ethanol:water, 50:50 (v/v) for the rest of the test compounds, and taken as the slope of the zero-order relationship between A_0 and A_t vs. t plot.

[b] The figures indicate the k value as a measure of reduction of DPPH in the presence of antioxidants, calculated from the slope of second-order rate plots.

[c] The scavenging activity (IP) was determined for the equimolar reaction of DPPH and a test compound over 20 min at room temperature in the dark calculated from $[A_0]$ to $[A_t]/[A_0] \times 100$.

[d] EC50 represents the amount of compound that will scavenge 50% of DPPH in 20 min at room temperature in the dark measured as the point of intercept in the plot of percentage remaining and scavenged vs. test compounds concentration.

[e] Rate constant is a measure of the potency of the test compounds in scavenging DPPH, was determined by plotting the slope values for the different concentrations from the first-order plot of ln $[A_0/A_t]$ vs. time against concentration.

[f] All values were determined by the least squares linear regression using Excel soft ware, the correlation coefficients (r) shown are the representative value. Three replicates were used for each experiment; the literature values are also presented for Ref. [17,18].

the order was determined from the best fit simple square linear regression with correlation coefficients (r) between 0.97 and 0.99, the values are listed in Table I. Expressing the scavenging activity in terms of IP% indicated similarity of such activities between RA-AsA (96%) and those for AsA (96%) and AsA-Pal (97%). Those of RA and ROL were 76 and 71%, respectively, in equimolar concentration with DPPH (Table I). Therefore, IP% is not a satisfactory experimental parameter for assessing the true activity for RA-AsA at different concentrations, neither does it reflect the activity rate. Hence, the EC_{50} values were determined (Figure 2), to provide a clearer indication as to the strength of activity of RA-AsA as an antioxidant. A concentration of 0.52×10^{-5} M was required to reduce 0.5 mM of DPPH by 50% in 20 min, which was substantially lower than the concentrations of ROL, RA, AsA-Pal and AsA required to achieve the same effect (31, 19, 3.8 and 2.3×10^{-5} M, respectively). However, in view of the difference of time during which DPPH was reduced per concentration per test compound, it became apparent that the test compounds reduced DPPH at different rates, suggesting different potencies for each compound. Hence, it became necessary to express the rate constants as the function of concentration of antioxidant. From this data (Figures 3 and 4; Table I) it became clear that RA-AsA reduced DPPH at faster rate, $4.9\,M^{-1}\,s^{-1}$, than AsA and AsA-Pal, 3.4 and $2.9\,M^{-1}\,s^{-1}$, respectively, and 1.4 and $1.3\,M^{-1}\,s^{-1}$ for RA and ROL, respectively, thus indicating greater anti-oxidant potency. No activity could be determined for Rol-Pal, which suggested the ester linkage was not hydrolysed in the reaction environment. It therefore follows that the antioxidant activity demonstrated by RA-AsA and AsA-Pal were influenced by the hydroxyl groups present in the AsA moiety, at least initially in the case of RA-AsA. Furthermore, since RA expressed antioxidant activity, the hydrolysis of the ester bond in RA-AsA would mean a continued free radical scavenging properties.

Free radical oxidation reactions have complex kinetic behaviour partly because reaction products can catalyse further reactions [18]. AsA serves as the primary water-soluble antioxidant in living cells [7–10] where it preferentially interacts with ROS generated during oxidative stress, thus protecting cell constituents from oxidative damage [7,8]. The recognised oxidation product of AsA is DHA [12]. Unlike AsA, DHA is both unstable in the absence of oxygen and is a reactive electrophile, which generates further reactive degradation products over time in solution [12,19–22]. The major resultant products of DHA are erythrulose and oxalate (Figure 2) [12]. These electrophilic products, erythrulose in particular, react with nucleophiles on proteins, specifically lysinyl and arginyl residues, resulting in structurally deleterious modifications of proteins [13]. Tissues maintain a low concentration of DHA due to its reduction back to AsA by glutathione, both enzymatically and non-enzymatically [11,12]. Increases in DHA degradation have been hypothesized to be involved in the aetiology of a variety of diseases, including senile cataract, aging, diabetes and Alzheimer's disease [12–15].

The reaction between RA-AsA and DPPH yielded was followed by TLC and indicated the presence of an additional band, hence major by-product. This was isolated from the plate and analysed by ^{1}H NMR (Bruker Avance DPX300 operating at 300 MHz and 27°C) and mass spectrometry (ThermoFinnigan LCQ Deca, ESI probe, methanolic infusion @ $3\,\mu l\,min^{-1}$).

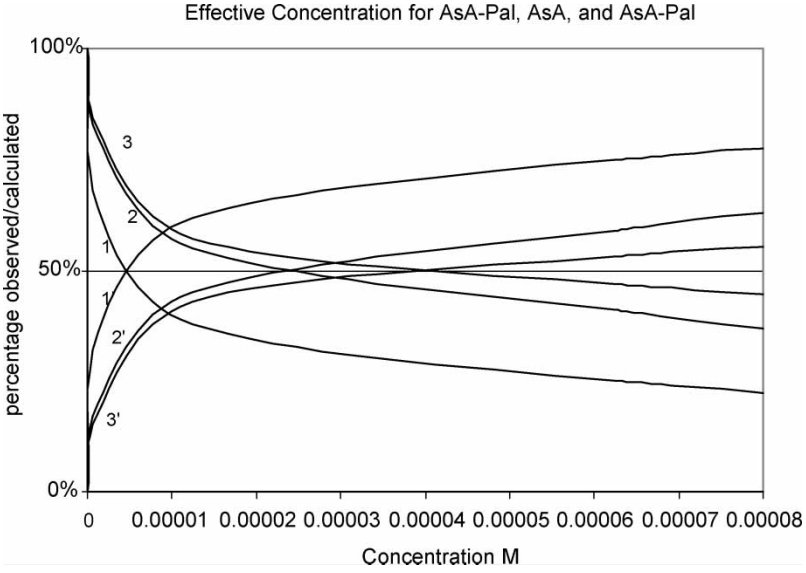

Figure 2. The EC_{50} plots for reduction of DPPH in the presence of antioxidants. 1,2,3,1′,2′, 3′, are the observed and calculated percentage of DPPH in the presence of RA-AsA, AsA and AsA-Pal, respectively. EC_{50} values for the retinoids were determined in a similar way.

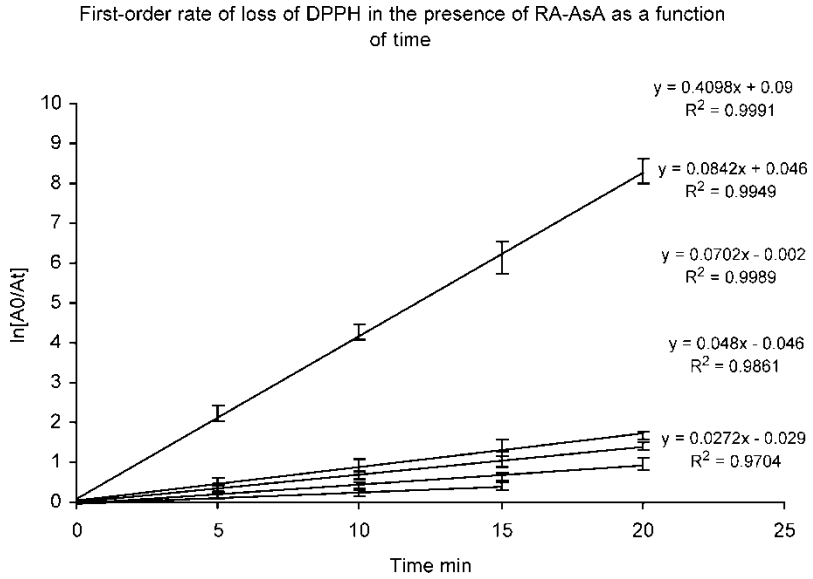

Figure 3. RA-AsA scavenging activity of the free radical DPPH. The slope of each best-fit regression line is taken as the decomposition constant of DPPH per concentration of RA-AsA as function of time, $n = 3$.

The NMR data was as follows: ^{1}H NMR (CD_3OD, 300 MHz), δ (in ppm) 1.09 (s, C16 and C17, 6H), 1.49 (m, C2, 2H), 1.62 (m, C3, 2H), 2.03 (m. C4, 2H), 1.72 (s, C18, 3H), 1.99 (s, C19, 3H), 2.35 (s, C20, 3H), 5.77 (s, C14, 1H), 6.29 (dd, C12, 1H), 6.96 (dd, C11, 1H), 6.17 (dd, C10, 1H), 6.15 (m, C8, 1H), 6.24 (m, C7, 1H), 4.5 (d, C3′a, 1H), 4.67 (d, C3′b, 1H), 4.7 (dd, C2′, 1H), 1.97 (m, C2′-OH), 10.88 (m, COOH); ^{13}CNMR; 33.7 (C1), 39.7 (C2), 18.8 (C3), 32.7 (C4), 130.4 (C5), 136.2 (C6), 137.6 (C9), 139.1 (C13), 137.3 (C7), 136.2 (C8), 130.3 (C10), 126.4 (C11), 125.9 (C12), 125.17 (C14), 28.9 (C16 and C17), 21.7 (C18), 12.8 (C19 and C20), 64.2 (C3′), 68.7 (C2′), 165.8 (C = O), 172.7 (COOH) (Figure 1); mass spectrum, M^+, *m/z* 388 base peak, *m/z* 311 (−OH). It can be hypothesised that the byproduct was a retinoic acid ester with a two carbon acid chain: RA-2-carboxy-2-hydroxy-ethanoate (RA-Et) and suggests that the AsA moiety did not separate completely on oxidation of its hydroxyl groups. This in turn suggests the degradation pathway of the AsA moiety in RA-AsA did not follow the previously proposed sequence illustrated in Figure 5, indicating that the formation of erythrulose was less likely as a consequence. It is not a surprise that RA-Et is not an erythrulose derivative as the latter is a major degradation product of DHA only under anaerobic conditions [12,15]. However, it is more of a surprise that a major derivative under aerobic conditions is a lactic acid derivative rather than a threonic acid derivative.

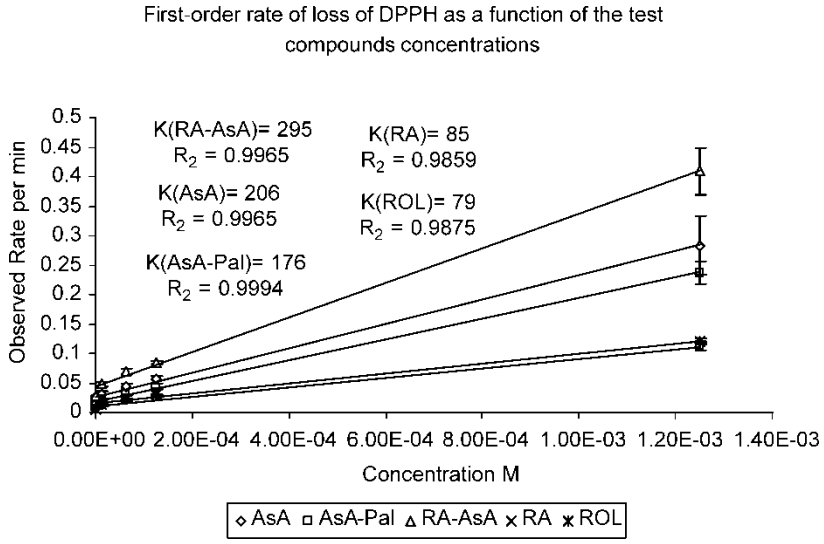

Figure 4. Comparison of DPPH decomposition rate constants as function of concentration of each compound Table 1. Radical scavenging activity of RA-AsA against DPPH, compared to other commercially available antioxidants (mean ± SD).

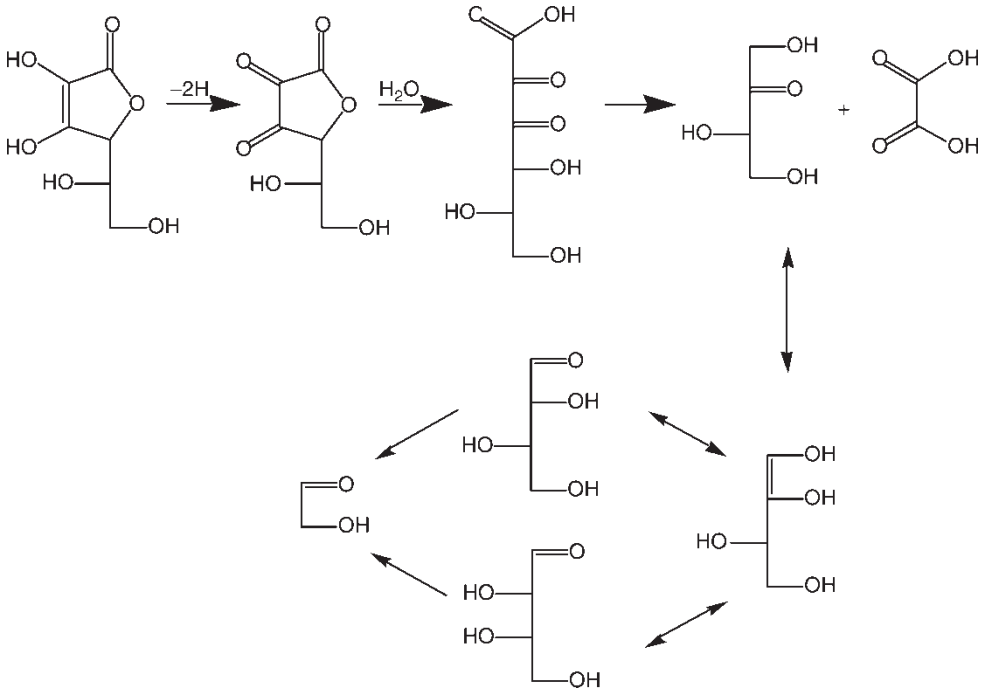

Figure 5. Scheme for the degradation of ascorbic acid in an aqueous environment *in vitro* [12].

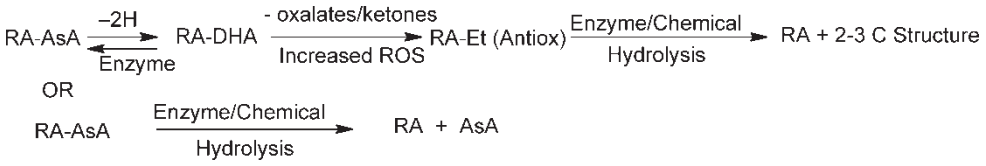

Figure 6. Hypothesised scheme for the metabolism of RA-AsA (antiox = antioxidant, 2-3 C = short chain moiety of 2 or 3 carbon units).

RA-Et exhibited potent antioxidant characteristics against DPPH, $k = 2.1\,M^{-1}\,s^{-1}$ over 5 min because of the instability of RA-Et, which had a decomposition constant of $6.7 \times 10^{-7}\,M\,s^{-1}$. K_{DPPH}, in the presence of RA-Et was $3.3 \pm 0.9 \times 10^{-3}\,s^{-1}$ and may explain the higher potency of RA-AsA as an antioxidant, whereby RA-AsA generates, upon oxidation and degradation, further antioxidants and thus provides greater activity than AsA. Thus, increased oxidative stress would lead AsA to generate more free radicals whereas the same is not likely to happen with RA-AsA, although, this scenario requires further detailed investigation. Based on these findings we hypothesise the series of reactions in Figures 3 and 6 as a plausible sequence of events.

BHA was used as control to monitor the accuracy of our experimental method [18,23]. Experimental rate constant of $154 \pm 3\,M^{-1}\,s^{-1}$ was consistent with the literature value of $150\,M^{-1}\,s^{-1}$ [18]. BHA reacts with DPPH several-fold faster than RA-AsA, indicating that BHA could be used as surrogate antioxidant and stabiliser in formulations containing RA-AsA. Literature values of the scavenging activities for the ascorbates, IP ~95% and EC_{50} of 2.2 and $2.9 \times 10^{-5}\,M$ for AsA and AsA-Pal, respectively [19], were also in agreement with our experimental values.

In conclusion, RA-AsA exhibited antioxidant properties some 30–40% greater potency than exhibited by the ascorbates and some 70% more than exhibited by the retinoids, apart from Rol-Pal, which had no discernible antioxidant properties. Furthermore, the degradation of the AsA moiety of RA-AsA is not likely to produce erythrulose as a by-product—the latter is implicated in a number of pathogenic reactions in cells. RA-AsA is likely to generate more antioxidant species under increased oxidative stress and enzyme hydrolysis, thus lower concentrations could be used in topical formulations (Figures 5 and 6).

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