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ABSTRACT

Ascorbic acid (AA) is involved in important metabolic processes in the human body. However, its chemical instability requires the assessment of products containing AA. The aim of this study was to develop systems that improve AA stability and to evaluate its release profile, permeation, and skin retention *in vitro*. For this purpose, we prepared binary systems consisting of propylene glycol and water, microemulsions, liquid crystalline systems, and an emulsion. The AA content in these systems was evaluated over time by measuring the inhibition of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Our results demonstrated that the binary systems and molecular aggregates were less stable and effective than the emulsion. Thus, *in vitro* AA release, skin permeation, and retention were evaluated using the emulsion. Our results indicate that AA exhibits low release and permeation levels and a high retention rate in the skin, characteristics desirable in cosmetic products.

GRAPHICAL ABSTRACT

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KEYWORDS
Ascorbic acid; skin permeation and retention; stability; vitamin C

1. Introduction

Ascorbic Acid (AA), also known as vitamin C, is involved in important metabolic processes in the human body. Furthermore, it acts as an enzyme cofactor that plays a role in oxidation–reduction processes that increase iron absorption and neutralize free radicals.[1] When applied topically, AA can minimize the inflammatory response of the skin following exposure to sunlight; it has also been used for skin lightening because it is a tyrosinase inhibitor. However, this vitamin cannot be synthesized by the human body and, therefore, must

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be obtained from external sources.[2] Product characteristics, processing, packaging and storage conditions, oxygen, light, metal catalysts, enzymes, and pH are factors that may affect AA degradation.[3,4]

The chemical instability of AA necessitates that the efficacy, safety, and chemical stability of the finished products be examined.[5,6]

Emulsions are semisolid preparations that are widely used in formulations intended for topical application because they provide many pharmaceutical and cosmetic advantages. Therefore, it is important to examine the AA when incorporated in these systems in order to predict its stability in the final product.[7] Emulsions and gels tend to be more acceptable for users, as they are easy to apply and spread on the skin in addition to their refreshing effect.[8,9]

However, emulsions are thermodynamically unstable systems consisting of two immiscible liquids, which may be altered by temperature, storage time, shipping, and handling during the production process.[10,11]

Studies of transdermal drug delivery systems are extremely important for the preparation of dermal and transdermal pharmaceutical products.[12] The skin has many functions of which its protective function is the most important one related to skin permeation.[13] The skin, known as a "barrier zone," comprises three distinct layers: the *stratum corneum*, viable epidermis, and dermis in addition to a network of blood capillaries, hair follicles, sebaceous glands, and sweat glands. [14,15]

Percutaneous permeation of drugs from transdermal patches or dermatological preparations is the process by which drugs pass through the skin layers and reach the bloodstream. Although it is important that the drugs in dermatological preparations penetrate beyond the surface, it is not usually desirable for the drug to reach systemic circulation.[16,17]

The methods used to quantify the release and absorption of drugs through the skin can be divided into two categories: *in vivo* and *in vitro* methods. *In vitro* methods are relatively easy to perform and can be repeated several times and with various test conditions. However, they are limited because of the difficulty to obtain membranes (using human skin), storage requirements, high costs, and the variability of blood flow conditions, which may prevent reproducibility. In addition, *in vitro* release studies do not take into account the effect of degradation and the possible separation of the *stratum corneum* from the rest of the epidermis or the occurrence of inevitable physiological and anatomical changes due to the absence of blood flow.[14,18,19]

The quantitative determination of AA in formulations has proven challenging for pharmaceutical companies. This difficulty has raised an interest in the development of new methodologies that are easy to perform, specific, sensitive, and accurate.

Because many variables can interfere with AA concentration including handling and storage conditions of products containing AA, it is necessary to identify specific techniques for the quantification of AA in systems.

Most of the established methods for assaying of AA use high-performance liquid chromatography (HPLC). However, HPLC requires expensive equipment, columns with a short half-life, organic solvents for the preparation of solutions and/or mobile phases, and a longer analysis time than that needed for UV spectrophotometry. This leads to longer working hours for the analyst and reduces the availability of equipment. Moreover, the use of organic solvents in drug analysis is not environment-friendly because they generate toxic residues. Currently, HPLC is widely used for pharmaceutical analyses, however, its cost is substantially higher than that of UV spectrophotometry.[20]

A novel trend is to incorporate AA itself in the formulations using various encapsulation techniques rather than use stable derivatives of AA. The reasons for this include the benefits of AA for human health, which make AA the first choice for food, pharmaceutical, and cosmetic industries. In addition, AA is a relatively abundant raw material with a much lower cost than its stable derivatives. Thus, the aims of this study were to find a system that helps maintain AA in its active form and to evaluate the *in vitro* release, permeation, and skin retention of AA.

The aim of this study was to develop AA-containing pharmaceutical preparations for dermal application and to evaluate AA stability using an indirect quantification methodology with UV spectrophotometry. The strong antioxidant activity of AA and the linear correlation between its concentration and antioxidant activity make this a rapid, inexpensive, and easy to conduct assay.

2. Methods

2.1. Preparation of solution systems

Three solutions containing 0.01 g/mL of AA and different proportions of propylene glycol and water as a solvent system were prepared as shown in Table 1.

Propylene glycol was selected as a good solvent for this study because it is able to facilitate the permeation of AA through the skin.[21]

Water was added first, and then AA was immediately added to induce solubilization; propylene glycol was added last. However, in formulation S3, AA was solubilized with difficulty since the concentration of propylene glycol in this solution was 100%. This system required magnetic stirring for a period of approximately 2 hours to achieve complete solubilization.

Spectrophotometric quantification was performed following AA solubilization.

The solutions were stored in hermetically sealed amber glass bottles and the AA content was assayed for 90 days.

2.2. Preparation of the emulsion system

The emulsion constituents initially proposed for this study are described in Table 2. The hydrophobic phase of the emulsion consisted of cetostearyl alcohol, a fatty alcohol used in cosmetics as a thickener (especially in O/W systems), and octyl

Table 1. Proportions of water and propylene glycol in AA-containing solution systems.

	Water	Propylene glycol
S1	100%	0
S2	75%	25%
S3	0	100%

Table 2. Percentage composition of the proposed emulsion system.

INCI name	Percentage composition (w/w)	
	F1	F2
Cetearyl alcohol	9.00	9.00
Ceteareth–20	1.00	1.00
Ethylhexyl stearate	1.50	1.50
Propylene glycol	3.00	3.00
Dissodium EDTA	0.05	0.05
Methylparaben	0.18	0.18
Propylparaben	0.02	0.02
Imidazolidinyl Urea (50% solution)	0.20	0.20
Ascorbic acid	–	1.00
Purified water	q.s.p. 100.00%	q.s.p. 100.00%

stearate, a non-comedogenic emollient with high dermal compatibility, an average coefficient of spreadability, and a broad application spectrum.[22]

The antimicrobial preservatives, methylparaben and propylparaben, were added to the aqueous phase. In addition, the aqueous phase contained propylene glycol as a humectant and a solvent for the parabens, ethylenediaminetetraacetic acid (EDTA-Na2), an organic chelating agent, which forms very stable complexes with various metal ions, and imidazolidinyl urea, a highly effective preservative against gram-positive and gram-negative bacteria including *Pseudomonas aeruginosa*. Imidazolidinyl urea has an extensive compatibility range with cosmetic ingredients, including non-ionic, cationic, and anionic surfactants and proteins. When combined with paraben preservatives, they offer complete resistance against bacteria, fungi, and yeast.[22] AA was incorporated into the emulsion 24 hours after its preparation because the measurements were always carried out on the following day.

The emulsion was stored in tightly closed opaque bottles and protected from light and heat. Determination of AA was carried out for 90 days.

2.3. Preparation of systems containing supramolecular aggregates

A ternary phase diagram was constructed to represent the constituents and their concentrations. Thirty-six formulations were prepared using polysorbate 80 (surfactant), dicaprylyl carbonate (oily phase), and water (aqueous phase). Five grams of each formulation were prepared and stored in closed amber bottles in the dark.

Following manipulation, the structure of the formulations was evaluated by polarized light microscopy. The samples were placed on microscope slides and covered with a coverslip. The slides were observed using a polarized light microscope (Leica Leitz DM RXE) coupled to an image capture system (Moticam 2000).

2.4. AA quantification

An indirect AA quantification method was used because of the linear relationship between the antioxidant activity of AA and its concentration in the formulation. This method was based on the evaluation of antioxidant potential using the radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH).[23]

Determination of AA content was performed every 7 days for 90 days and all measurements were performed in triplicate.

Data were expressed as mean ± standard deviation and analyzed by analysis of variance (ANOVA) followed by Tukey's test (p-value <0.05 was considered significant), when appropriate. Statistical analysis and graph plotting were performed using the software Origin 7.

To construct the calibration curve, 1 mL of the different concentration AA aqueous solutions (from 0.5 to 5 µg/mL) was added to 2.5 mL of a DPPH solution in methanol (0.004%). The solutions were kept in dark and the absorbance was determined after 30 minutes at 515 nm.

Control solutions were prepared using only 1 mL of water and 2.5 mL of the DPPH solution in methanol (0.004%). The absorbance of these samples was used as the maximum absorbance to calculate the DPPH radical inhibition percentage (% inhibition).[24]

% Inhibition of DPPH = $[(A_{max} - A_{test})/A_{max}] \times 100$where:

A_{max} is the absorbance of DPPH at 515 nm in the absence of the sample (control).

A_{test} is the absorbance of DPPH at 515 nm in the presence of the sample.

To quantify AA in the formulations over time, the same procedure for determining antioxidant activity was performed as described above. To quantify AA in the solution systems, 2 mL of each solution was dissolved in 100 mL of water. For the emulsion, microemulsion, and liquid crystalline systems, 1 g of the sample was solubilized in 100 mL of deionized water. The determination of AA content was carried out every seven days and all measurements were performed in triplicate. The % inhibition of DPPH obtained for each formulation was applied to the Equation obtained from the linear regression of the calibration curve and the AA concentration was thus determined.

Data were expressed as mean ± standard deviation and analyzed by ANOVA followed by Tukey's test (p-value <0.05 is considered significant), when appropriate. Statistical analysis and graph plotting was performed using Origin software 7.

2.5. In vitro AA permeation, retention, and release

The release, permeation, and skin retention were examined under "sink conditions" for the systems in which AA is more stable at a concentration of 1%. The experiments were conducted in a covered Franz cell chamber to reduce the incidence of light exposure and thus minimize the possible degradation of the released or permeated AA.

2.5.1. Evaluation of AA release from emulsion systems

The release assays were performed with modified Franz diffusion cells with an area of 1.77 cm^2 using Microette equipment (Hanson Research) and a cellulose membrane (Sigma-Aldrich).[25–27]

The receptor compartment of the modified Franz cells was filled with 7.0 mL of 0.1 M phosphate buffer (pH 7.4). Samples (250 mg) of the cosmetic preparations were placed on the membrane. In one of the cells, 250 mg of an emulsion without AA was placed and the collected solution was used as the blank in the spectrophotometric readings.

The receptor solution was continuously stirred at 300 rpm using the magnetic agitator present in Franz cells chamber.

The reaction temperature was maintained at 37 ± 5°C using a circulating water bath in the jacketed cell.

The amount of AA released from the formulation was evaluated after 0.5, 1, 2, 4, and 8 hours. The experiment was repeated five times.

2.5.2. Evaluation of AA permeation

This test was conducted using a technique similar to that described above for the evaluation of AA release from cosmetic preparations, except that a biological membrane was used. The membrane selected for this study was biological skin dissected from non-scalded pig ears acquired from the Fridge Olhos d'Água (Ipuã - SP). Prior to starting the experiment, the skin was cut to obtain a thickness that corresponds to the epidermis and dermis (500 µm) using a dermatometer (Nouvag TCM300).

2.5.3. Evaluation of AA retention in the stratum corneum, epidermis, and dermis

The skin used for the permeation study was also used to evaluate AA retention in the epidermis and dermis.

The pig ear skin was removed from the diffusion apparatus after 2 hours of *in vitro* permeation. The area of the skin exposed to permeation was cleaned with distilled water and cropped. The obtained fragments were immersed in 4 mL of methanol and the resulting suspension was subjected to sonication for 30 minutes to disrupt the cells and then vortexed for 5 minutes. The skin fragments were removed and the solution was analyzed for antioxidant activity. This procedure was repeated after 4 and 8 hours of *in vitro* permeation.[28,29]

3. Results and discussion

3.1. Systems containing supramolecular aggregates

The ternary phase diagram (Figure 1) represents 36 formulations; three of these formulations, P2, P5 and P15, which correspond to a liquid crystal with lamellar phase, a liquid crystal with hexagonal phase, and a microemulsion, respectively (in accordance with the composition shown in Table 3), were selected for further analysis.

Table 3. Percentage composition of the proposed formulations.

	P2	P5	P15
Dicaprylyl carbonate	10%	20%	50%
Polysorbate 80	70%	60%	40%
Water	20%	20%	10%

To analyze the systems obtained through the phase diagram, we used polarized light microscopy to classify the liquid crystalline phases and to determine their optical behavior.

Under polarized light, a sample is considered anisotropic if it is able to change the plane of the incident light, whereas it is considered isotropic if it does not change the plane of the incident light.

The polarized light microscope is a common microscope in which light passes through a polarized system, which polarizes light waves in one plane. The use of a polarized light allows the study of structures known as anisotropic birefringent structures, which exhibit two different refractive indices during the incidence of light and isotropic structures that do not change the plane of polarization.[30–32]

Microemulsions are viewed as transparent liquid isotropic systems with micellar structures represented as a dark field. Liquid crystals can be categorized as anisotropic viscous systems, which can be either transparent or semi-transparent with a lamellar or hexagonal organized structure, represented by Maltese crosses and striations, respectively, or isotropic systems, such as cubic mesophases, which have high viscosity and exhibit the dark field feature when analyzed by polarized light microscopy.[30–32]

Emulsified systems also exhibit isotropic behavior and therefore, show dark field under polarized light. Microemulsions, emulsions, and cubic liquid crystal phases can be distinguished visually because microemulsions are transparent systems, emulsions are opaque, and cubic structure systems are transparent or translucent and viscous.[33]

Following system classification and visualization by polarized light microscopy, three formulations with different characteristics were selected for AA incorporation: the lamellar phase P2 formulation, the hexagonal phase P5 formulation, and the microemulsion P15 formulation.

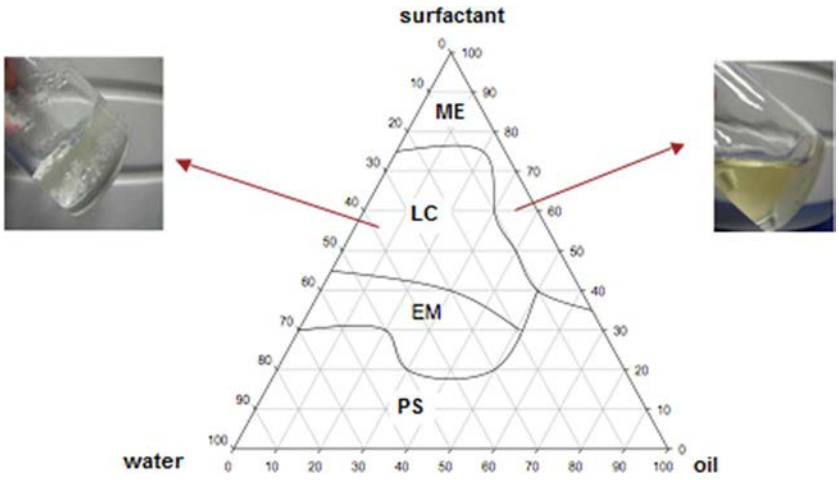

Figure 1. Ternary phase diagram showing a microemulsion (ME), a liquid crystal (LC), an emulsion (EM) and phase separation (PS) after polarized light microscopy. Polysorbate 80 (surfactant), water and dicaprylyl carbonate (oil).

3.2. Quantification of AA using the DPPH method

The AA concentration in each formulation was determined using the calculated DPPH radical inhibition percentage and the calibration curve. The correlation coefficient (R) of the calibration curveis 0.9978, the Y-axis intercept is −1.3358, and the slope equals 19.033. This calibration curve was subsequently used to quantify the AA concentration in the different preparations based on antioxidant activity.

Two additional standard curves were constructed using methanol ($y = 11.5515x - 1.79039$, $R = 0.99694$) and phosphate buffer pH 7.4 ($y = 6.20095x - 0.74841$, $R = 0.99905$) as solvents because these were used to evaluate AA release, skin permeation, and retention in cosmetic preparations.

3.2.1. Quantification of AA in the solution systems

As shown in Figure 2, all formulations exhibited a statistically similar AA concentration until day 14; however, after day 28, system S2 contained the highest AA concentration. Moreover, the final AA concentration (at day 90) in this system was 51% of the initial concentration.

Yuan *et al.* (1998) evaluated AA degradation in aqueous systems.[34] The concentration of AA decreased dramatically with time, which is in agreement with the results obtained for system S1 (100% water), which showed a residual AA concentration of only 29% on day 90, confirming the instability of AA in aqueous solutions.[34]

System S6 showed a final residual AA concentration of 45.70%, an intermediate value between those of systems S1 and S2. This suggests that AA stability is not directly proportional to the decrease in water content in the system or to the increase in propylene glycol content. Thus, the most appropriate proportions of water and organic solvents need to be selected in order to have an efficient system that can maintain AA activity.[35]

Many factors can affect AA activity; a 1:4 proportion of propylene glycol:water was previously found to be the most appropriate.[36] Thus, system S2 showed the greatest stability and ability to maintain AA activity.

3.2.2. Quantification of AA in emulsion systems

As shown in Figure 3, the fluctuations in AA concentration were lower in this system compared with those in solution systems. Moreover, the AA concentration in the emulsion on day 90 was higher than in all of the solutions; the emulsion showed a residual AA concentration of 82% of the initial concentration compared to 51% in system S2.

The results of the assay were in accordance with previously published studies reporting that the effectiveness of AA might be reduced because of its physico-chemical instability, especially under aerobic conditions and when exposed to light and high temperatures during storage.[38,39] In addition, in aqueous solutions, AA is easily oxidized to dehydro-L-ascorbic acid as well as other degradation products.[39]

3.2.3. Quantification of AA in systems containing supramolecular aggregates

As shown in Figure 4, there was a significant decrease in AA concentration in systems P2, P5, and P15. The formulation

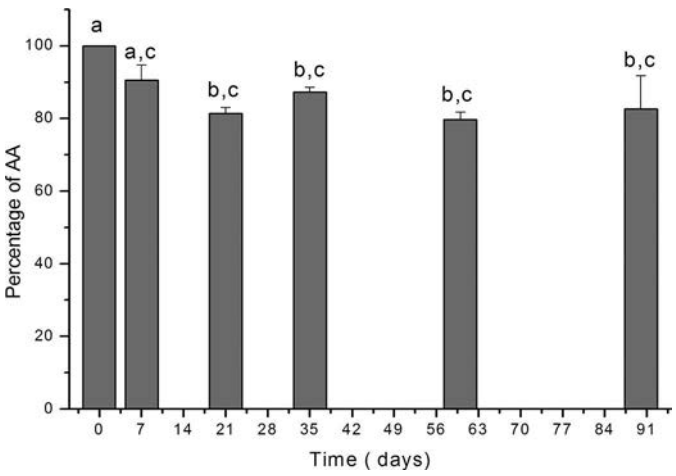

Figure 3. A graph showing the percentage of AA present in the emulsion for a period of 90 days (a, b, c: different letters to indicate statistically different values $p < 0.05$).

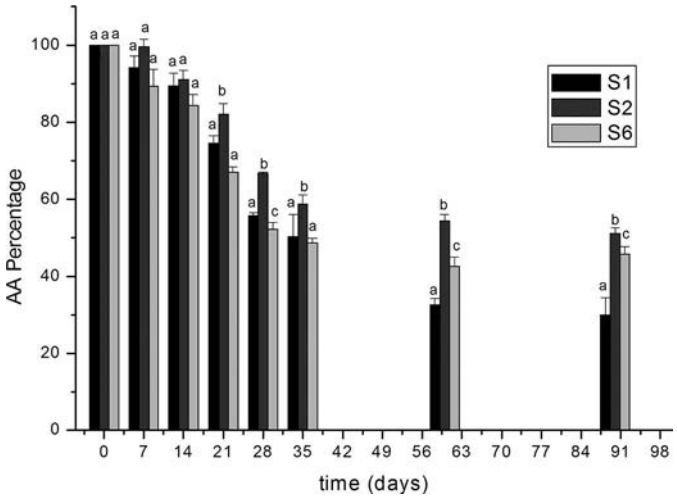

Figure 2. A graph showing the percentage of AA in the solutions of propylene glycol/water on the same day of preparation and during a period of 90 days (a, b, c: different letters to indicate statistically different values p < 0.05).

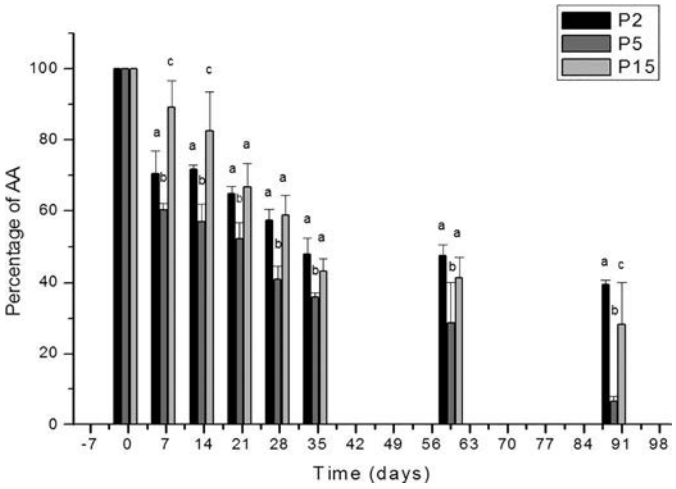

Figure 4. A comparative graph of the percentage of AA present in the systems P2, P5, and P15 for a period of 90 days.

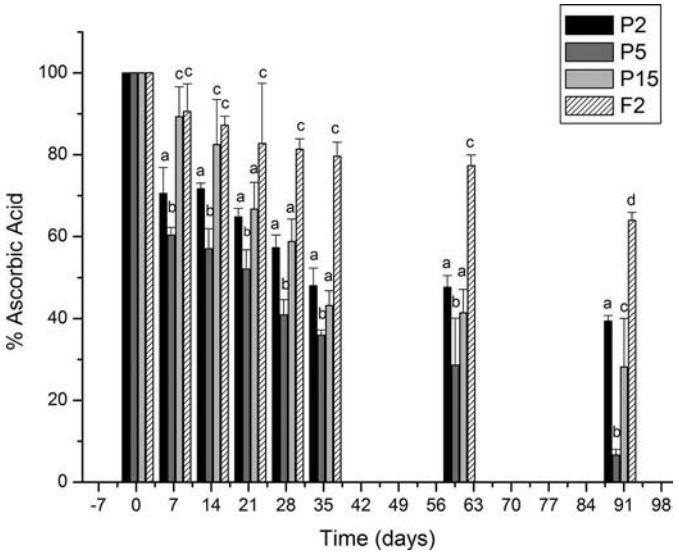

Figure 5. A comparative graph of the percentage of AA present in the systems P2, P5 and P15 compared to the emulsion (F2) for a period of 90 days. (a, b, c, d: Different letters to indicate statistically different values ($p < 0.05$). The samples were compared only in each study day. This does not indicate that the amount of AA on day 0 is statistically similar to Day 90).

containing the highest residual AA concentration on day 90 was system P2, (39.31%) compared to system P15 (28.14%) and system P5 (only 6.64%). The sharp drop in AA concentration in these systems can be attributed to their transparency.

Figure 5 shows a comparison between systems P2, P5, and P15 and the emulsion (F2). An analysis of Figure 5 clearly demonstrates that the emulsion was more stable than the other systems. The AA concentration in the emulsion was 63.98% compared to the initial concentration. This result might be due to the opacity of the emulsion system. Based on these results, only the F2 emulsion system was used for evaluation of AA release, permeation, and skin retention.

3.3. In vitro *AA release, permeation, and skin retention*

The emulsion system was selected to assay AA release, permeation, and skin (epidermis/dermis) retention because it showed the best results in terms of the chemical stability of AA over time.

The DPPH methodology was used to quantify AA in the release and transdermal permeation experiments. Thus, we used the calibration curves constructed using methanol and phosphate buffer (pH 7.4) as solvents. Phosphate buffer was used as a recipient environment for evaluation of AA skin permeation and methanol was used for AA extraction from the biological membrane to evaluate its retention in the epidermis/dermis.

3.3.1. *Evaluation of AA release from the emulsion system*

The results presented in Figure 6 suggest that there is a gradual increase in the concentration of released AA over time. The concentration of released AA is sufficient to exert an antioxidant activity.

The kinetic profile of emulsion-released AA was also evaluated. For this purpose, three models were applied to interpret

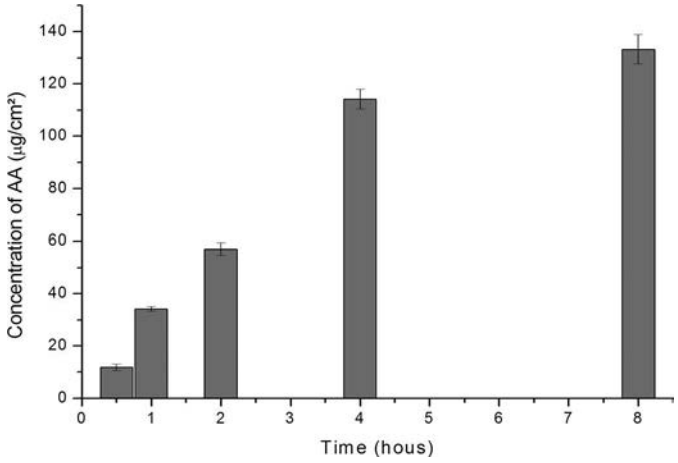

Figure 6. Concentration of AA released through the synthetic membrane at 1/2, 1, 2, 4, and 8 hours.

the release kinetics: zero order, first order, and the Higuchi model. The kinetic models were evaluated by linearization of the release data in accordance with the time and concentration of released AA. The zero order curve shows the relationship between the amount of AA released *versus* time, the first order model shows the relationship between the log concentration of released AA *versus* time, and the Higuchi model shows the relationship between the amount released *versus* the square root of time. The models were plotted and the correlation coefficient (R^2) was used to identify the most suitable model. As shown in Table 4, the zero order model demonstrated the highest R^2 value.

Based on these results, the emulsion system follows the zero order release kinetics model; release occurs at a constant rate, independent of AA concentration.[40]

3.3.2. *Evaluation of AA skin permeation in the emulsion system*

To evaluate skin permeation, the acceptor compartment was collected and the AA content was measured (Figure 7).

The results indicate that the amount of permeated AA increased over time, but the concentration was too low for AA to exhibit systemic pharmacological activity, which is a desirable characteristic for a topical anti-aging product.

3.3.3. *Evaluation of AA retained in the stratum corneum, epidermis, and dermis*

The concentration of AA retained in the epidermis, dermis, and *stratum corneum* was determined (Figure 8). Our results indicate that the AA concentration decreased over time, in accordance with the permeation test results, which showed increasing concentrations over time. In addition, it is noteworthy that the retained AA concentration was much higher than the permeated concentration;

Table 4. Linear correlation coefficients of the release kinetics profile of from the emulsion.

Models	Emulsion with AA
Zero-order model	**0.99363**
First-order model	0.98619
Higuchi model	0.99022

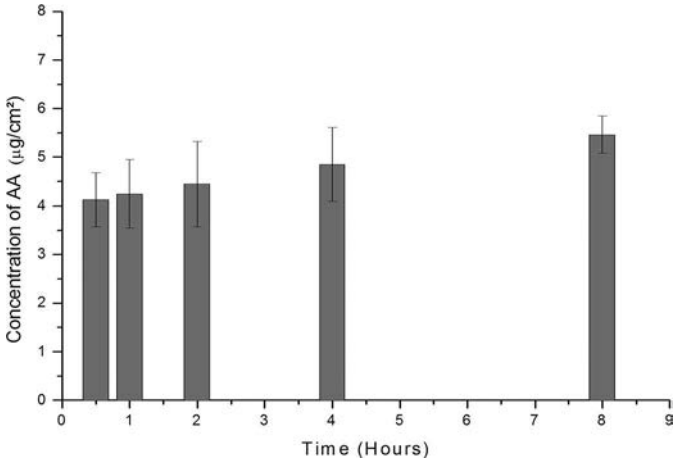

Figure 7. Concentration of AA permeated through the biological membrane from the emulsion system after 2, 4, and 8 hours.

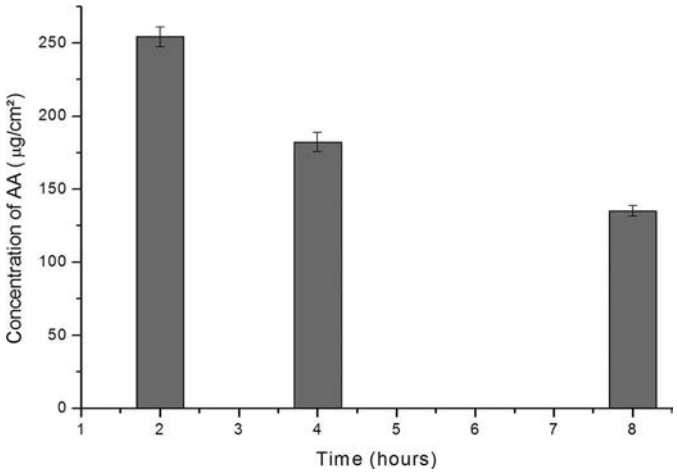

Figure 8. Concentration of AA retained in the stratum corneum, epidermis and dermis of the biological membrane. Measurements were made at 2, 4, and 8 hours.

this is interesting because AA is an important factor for collagen synthesis.[35]

4. Conclusion

The development of systems that can help maintain the stability of chemicals is of great importance for enhancing product effectiveness. In this study, we incorporated AA as an active ingredient in different systems.

The method used for AA quantification is effective. The F2 emulsion system was able to maintain the highest AA stability over 90 days, compared to the solutions, microemulsion, and crystalline liquid systems. This can be explained by the opacity of the emulsion system, which can protect AA from oxidation by sunlight.

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