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New Aqueous Gel Based on Soluble Cyclodextrin/Vitamin A Inclusion Complex

S. WEISSE[1], B. PERLY[1]*, J.-P. DALBIEZ[1], F. BARATON-OUVRARD[2], J.-C. ARCHAMBAULT[2], P. ANDRÉ[2], P. ROLLIN[3] and F. DJEDAÏNI-PILARD[4]

[1]*Service de Chimie Moléculaire, Bat 125, CEA Saclay F-91191 Gif sur Yvette Cedex, France;* [2]*Parfums Christian Dior, 45800 St Jean de Braye, France;* [3]*ICOA – University of Orléans; 45065 Orleans, France;* [4]*University of Picardie Jules Verne, Laboratoire des Glucides, 33 rue St. Leu, F-80039 Amiens, France*

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Abstract

Rameb (randomized dimethyl-β-cyclodextrin) was mixed with vitamin A propionate (PVA) (molar ratio 10/1 in water) and a water soluble complex was formed and studied by HPLC and NMR (structure, concentration and stability of PVA). Then solution was used to form an aqueous gel. The skin absorption of PVA through stratum corneum, epidermis and dermis of human skin (on modified Franz cells) was assayed by HPLC and was compared to that of a reference gel (or oil) without cyclodextrin. The solution obtained contains a maximum of 10 mg/mL PVA (if saturated with Rameb) and the PVA can remain stable up to 90 days in solution, and up to 1 year if freeze-dried (storage at 4 °C, in the dark). The results of the different experiments of skin distribution were statistically analyzed and show that when complexed with Rameb, the amount of PVA that penetrates each skin layer is significantly higher than pure PVA. The results also show that PVA cannot pass through the dermis and enter the circulation.

Introduction

Retinol (vitamin A) has important functions related to vision, reproduction, growth and epithelium proliferation [1] and therefore represents a high interest for pharmaceutical and cosmetic industries, but it is not water soluble and very unstable in the presence of light and oxygen, which limit its use. We propose here to use cyclodextrins and in this case per-(2,6-*O*-dimethyl)-β-cyclodextrin (Rameb) to improve the stability, the aqueous solubility and the dermal bioavailability of an ester of vitamin A and form an aqueous gel containing vitamin A propionate (PVA) to be tested on human skin.

Experimental

Materials

Vitamin A propionate A or PVA (2.5 Mio I.E/g) was obtained from BASF (Germany) and per-(2,6-*O*-dimethyl-β-cyclodextrin (Rameb) from WACKER (Germany). It is randomized dimethyl-β-cyclodextrin, but for the NMR experiments we used highly purified homogeneous per-(2,6-*O*-dimethyl)-β-cyclodextrin [2] in order to visualize precisely the different protons of the CD. All chemicals were purchased from Fluka and used without further treatments. Deuterated solvents were purchased from Euriso-Top (Saclay-France).

Preparation of inclusion complexes

The CD/drug mixtures were prepared by mixing accurately weighted quantities in water. The mixtures were stirred during a few hours then centrifuged 10 min at 6000 rpm. The aqueous solutions were filtered on Millex-SG 0.22 μm then freeze-dried before redissolution of 10 mg in 0.4 mL D_2O for NMR study. The precipitates, if present, were washed many times by centrifugation with water and ether to eliminate the free species, then dried slowly under a stream of nitrogen and freeze-dried before redissolution of 10 mg in 0.4 mL d6-dmso.

NMR Studies

^{1}H-NMR experiments were performed at 500.13 MHz using a Bruker DRX500 spectrometer using the pulse programs available from the Bruker library. All measurements were performed at 25 °C under careful temperature regulation ($\pm$0.1 K). Chemical shifts are given relative to external tetramethylsilane (TMS = 0 ppm) and calibration was performed using the signal of the residual protons of the solvent as a secondary reference. All NMR data were processed and plotted using the UXNMR program and an INDY work station (Silicon graphics). Scalar correlation (COSY, Relay experiments) were processed in the absolute value mode after zero-filling resulting in a 1 K $\times$ 1 K data matrix. For dipolar correlation (ROESY experiment), the phase sensitive (TPPI) sequence was used and processing resulted in a 1 K $\times$ 1 K matrix.

* Author for correspondence: E-mail: bruno@drecam.saclay.cea.fr

HPLC

For the stability study of the Rameb/PVA solution, analytical HPLC was carried out with a Waters Delta Prep 3000 chromatograph equipped with an LSED detector and a Nova-Pack® C18 WATERS column, by elution with methanol (Chromasolv® filtered on Millicup PTFE 0.5 μm) at 1 mL/mn. An UV detector was used (WATERS Lambda Max at 340 nm) in the case where the interesting peaks were covered by those of the CD with LSED (since CD is not detectable by UV). For the assays following the skin absorption experiments, we used a BECKMAN system Gold chromatograph with a Merck Lichrosher 125-4 RP 18 (5 μM) column and a Beckman led array detector, mobile phase was acetonitrile/water 90/10 v/v at 1 mL/mn^{-1}.

Mass spectrometry

The integrity of molecular structure was confirmed by Mass Spectrometry (MS) using electrospray infusion mode performed in positive mode on a Q-TOF Spectrometer (Micromass UK).

Preparation of aqueous gels

We prepared an aqueous solution of the complex, with initial concentrations being 40 and 400 mM respectively, then 1.8% w/w Natrosol® (hydroxyethylcellulose, Hercules Inc., USA) was added to form the gel. A reference aqueous gel without CD was prepared with water, Natrosol® (1.8% w/w), PVA and polysorbate 60 (2.5% w/w). The final concentration of PVA and Rameb in both gels was determined precisely for each experiment (Table I). In one case (S5) we used as reference an oil (carnation oil, Witco-Rewo) containing 0.75% PVA. In one case (S7) we used all the skin layers including the dermis, to check if the CD or the PVA can pass in the blood. In one case (S8) we used a physical mixture of PVA and Rameb in an aqueous gel (no complex) as reference.

Skin penetration study

This study, performed on fresh human skin pieces, must determine the amount of vitamin A propionate which penetrate into the different layers of the skin, from our CD made aqueous gel and a reference gel or oil. We used a device containing 15 Franz-type cells (surface 2 cm^2, receptor liquid volume 4.2 mL) in a 37 °C thermostatic bath. For each experiment a different skin batch was used. The receptor liquid contains phosphate buffer 10 mM, NaCl 120 mM, KCL 2.7 mM, pH 7.4 (Sigma) sodium azide 0.1% w/w, ethanol (20% v/v) and a surfactant (polyoxyethylene polyoxypropylene butyl ether, polyethylated hydrogenated castor oil, purified water: Wackherr colorants) 4% v/v. On each cell, exactly 1 g of gel was deposited (always 8 cells for the Rameb/PVA gel and 7 cells for the reference) and covered with Parafilm®. After 24 h, the cells were dismantled, the excess gel on the surface was removed and the skins were treated as such: the stratum corneum was collected by the stripping method (7 strippings, pressure 300 g/cm^2, time 12 sec, D'Squam®, Eviderm Corporation, Dallas) and the PVA extracted by solubilization in 2 mL methanol and after 2 h agitation, filtered (0.22 μm) then assayed by HPLC. For the epidermis, the PVA was extracted by solubilization in 2 mL methanol and after 2 h agitation, filtered (0.22 μm) then assayed by HPLC. The receptor liquid was assayed directly by HPLC. The gels were submitted to the following treatments before PVA assays: 250 μL were sampled and sonicated in 25 mL isopropanol to release PVA from the cellulose matrix, then filtered on 0.22 μm filter. A sample of each gel was kept to be assayed in time and determine long term stability of PVA in the gels. The percentage of initial amount of PVA that has passed is calculated from the concentration found in each layer. The results of the PVA assays were statistically analyzed using the StatAdvisor Software. This procedure uses a multifactor analysis of variance. The F-Test in the Anova table allows to identify the significant factors and for each one, the Multiple Range Test determines which means are significantly different from which other and which Confidence Interval must be applied to this conclusion.

Results and discussion

Determination of the inclusion phenomena by NMR study

The solution obtained after 24 h mixing is clear, yellow and no precipitate can be seen. After centrifugation there is no change. Therefore two assumptions can be proposed: there is no inclusion complex, or there is a water soluble inclusion complex. The ^{1}H-NMR study of the aqueous phase in D_2O (after filtration and freeze-drying) shows that vitamin A propionate, like Rameb is present in the aqueous phase. When comparing this spectrum with that of Rameb alone, registered in the same conditions, we noticed that the inner protons of the CD (H3 and H5) experience a chemical shift (0.04 ppm) which is significant of the inclusion phenomenon [3] (data not shown).

The Rameb/PVA complex being water soluble, it is possible to perform a bidimensional NMR experiment (T-Roesy) to identify the protons of the vitamin A propionate molecule which are implicated in the inclusion phenomenon i.e. those showing cross-peaks with the inner protons of the CD. Indeed, the cross-peaks indicate a spatial proximity (<4 Å) of those protons. The 2D spectrum (Figure 1) shows intense cross-peaks between the aliphatic protons of vitamin A propionate and the H3, H5 protons of the CD. Therefore, we can assume that the aliphatic cycle is involved in the inclusion process, while the vinyl moiety appears to stay outside the cavity. This hypothesis agreed with the results obtained by Guo et al. [4].

We also noticed that the ^{1}H-NMR spectrum of vitamin A propionate in the complex (in D_2O) is different from that of vitamin A propionate alone (in d6-dmso).The different solvents and the interactions with the CD explain those differences (chemical shift, shape) at some point but not entirely. First of all, it is necessary to check that vitamin A propionate does not experience any major chemical modification due to its inclusion in the CD. In order to do so we

Table 1. Composition of the batches for each skin penetration experiment

	S2	S4	S5	S7	S8
%PVA w/w	0.72	0.76	0.96	0.86	0.45
%Rameb w/w	47.3	50	53.3	48	25
Rameb/PVA gel					
% PVA w/w	0.72	0.72	0.75	0.81	0.45
% Rameb w/w	No	No	No	No	30
Reference	Aqueous gel	Aqueous gel	oil	Aqueous gel	Aqueous gel

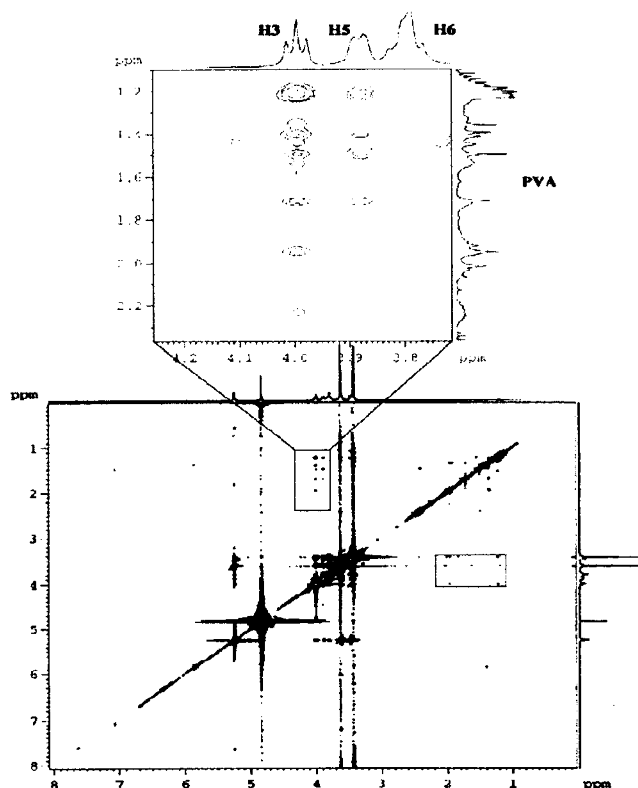
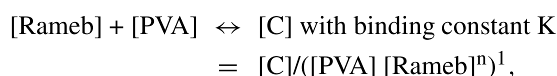


Figure 1. T-Roesy (spin lock 300 ms, attenuation 22 dB) of the RameB/PVA complex in D_2O at 500 MHz (20 mM).

performed a HPLC analysis: the chromatogram confirmed the chemical integrity of the molecule in the complex.

Then, the following assumption can be made: the complexation process follows the equation:



$$[\text{Rameb}] + [\text{PVA}] \leftrightarrow [\text{C}] \text{ with binding constant K} = [\text{C}]/([\text{PVA}]\,[\text{Rameb}]^n)^1,$$

where [Rameb] and [PVA] represent the concentrations of the free forms and [C] the concentration of the complex. In solution there is always a part of free vitamin A propionate, the amount of which depends on the initial concentrations of [PVA]t and [Rameb]t. And this free vitamin A propionate is not protected by the cavity of the CD and therefore experiences a quick degradation process in aqueous solution, which might explain the differences on the ^{1}H-NMR spectrum. So it is important to determine the optimized conditions for the preparation i.e. the [Rameb]t/[PVA]t ratio where the amount of free [PVA] is as small as possible, to check if the batches studied previously contain a certain amount of free PVA that would be unstable. Therefore, the influence of the concentration of Rameb on the final concentration of complex was studied.

Determination of the influence of the concentration of Rameb

When K is known or can be estimated, it is possible, for any mixing with known initial concentrations, to determine [C], and then the free amount of [PVA] and [Rameb] in solution by resolving the following:

$$\text{K} = [\text{C}]/([\text{PVA}]\,[\text{Rameb}]^n)^1 \text{ with } [\text{PVA}] = [\text{PVA}]\text{t} - [\text{C}] \text{ and } [\text{Rameb}] = [\text{Rameb}]\text{t} - [\text{C}],$$

The solution is written:

$$[\text{C}] = (\text{D} - (\text{D}^2 - 4.\text{K}^2.[\text{PVA}]\text{t}.[\text{Rameb}]\text{t})^{0,5}).2\text{K}^{-1},$$

with

$$\text{D} = \text{K}.[\text{PVA}]\text{t} + \text{K}.[\text{Rameb}]\text{t} + 1.$$

Based on previous studies concerning water soluble complexes poorly stable in solution, it is possible to estimate that: $500 < K < 1000$ [5]. The value of n will be estimated as 1 and confirmed later.

For [Rameb]t = [PVA]t = 20 mM and K = 500, we have in solution 27% free vitamin A propionate, which then experienced oxidation in the aqueous medium and as a consequence signs of degradation appear on the ^{1}H-NMR spectrum. On the other side when [Rameb]t $>$ 10 [PVA]t, only less than 1% free vitamin A propionate is found, whatever value we use for K. The complexes which correspond to the following concentrations have been prepared and studied by HPLC and ^{1}H-NMR: [PVA]t = 0.02 M and [Rameb]t = 0.02, 0.05, 0.1, 0.2, 0.3, 0.4 M. The results (data not shown) clearly show that for [Rameb]t = [PVA]t = 20 mM the complex is highly degraded whereas it is not for [Rameb]t = 10 [PVA]t: we can conclude that the whole vitamin A propionate is, at any given time, protected in the inclusion complex. In those conditions, using a solution saturated with Rameb, the maximal solubility reached for PVA is 10 mg/mL.

Determination of the stoichiometry

The determination of the stoichiometry in the case of a water soluble complex can be done using the method of Job [6] which is based on the measurement of the chemical shifts of the protons of the CD and those of the guest molecule for a ratio r = [Rameb]/([Rameb]+[PVA]) going from 0 to 1. In our case, this method cannot be used because it is necessary to maintain r close to 1 to obtain a stable complex. Hence, two other methods were used: Mass Spectrometry and the Higuchi and Connors method [7].

Mass Spectrometry was carried out in Electrospray mode (ESI-MS), because it is the most accurate technique for non covalent associations. This technique detects complexes according to their molecular weight which allows the stoichiometry to be found simultaneously. The sample we used for this analysis is the same as the one used for the ^{1}H-NMR in D_2O. The 1/1 stoichiometry of the complex is clearly established by the presence of peaks m/z = 1665 for $[Rameb + PVA + Na]^+$ and m/z = 845 for $[Rameb + PVA + 2Na]^2$ (data not shown). The Higuchi and Connors method uses solubility phase diagrams and in this case we can see that the concentration of solubilized vitamin A propionate grows almost with a linear way (water soluble complex) with a slope close to 1 (0.98 for the last three points) which indicates a 1/1 stoichiometry. This results are in accordance with those obtained by Mass Spectrometry and with the geometry of the complex described by the T-Roesy experiment.

This result can be justified by the larger cavity of the Rameb, due to its methyl groups on each face, which allow a bigger part of the vitamin A propionate to be included. Furthermore, it has been shown that in a 1/2 inclusion complex with β-CD the dimer were most of the time stabilized by hydrogen bonds between the secondary hydroxyls, which is no longer possible with Rameb.

Stability study

We have previously demonstrated that the mass action law requires the presence of 10 equivalents of Rameb as initial concentration to obtain a stable complex. Once this is established, all complexes were prepared according to this law and the influence of the environment (light, heat, oxygen) was studied on several batches by HPLC and ^{1}H-NMR.

The results show that in aqueous solution the vitamin A propionate remains stable for 60 days if protected from light and at 4 °C, but only 15 days if kept at 25 °C under natural light. We also studied the complex in its freeze-dried form: the vitamin A propionate can remain stable 6 months even without any protection (kept at 25 °C under natural light) and more than a year when protected from light, at 4 °C (Figure 2). As post freeze-drying solubilization is very easy, this results can provide a new way for vitamin A propionate storage. It should be noted that the vitamin A propionate alone is stable less than 10 days at 25 °C under natural light.

Skin penetration study

The results of PVA assays for each experiment are presented in Table II.

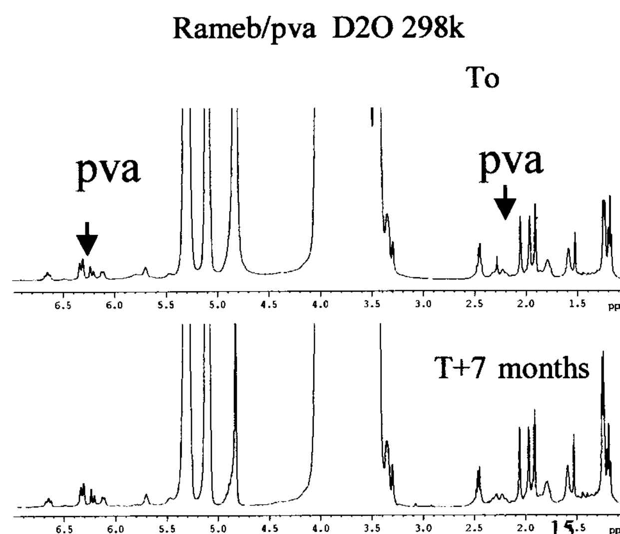


Figure 2. ^{1}H-NMR spectra (500 MHz, 298 K, D_2O) of the Rameb/PVA complex (20 mM) in solution at T_0 and after 7 months (freeze-dried form stored at 4 °C in the dark).

It must be emphasized here that the different experiments can not be compared to one another. Indeed, each concerns a different skin batch and the diversity of human skin precludes comparison. So, the later can only be made within the results of one experiment.

Some fairly outstanding observations can be made from those results. A statistical multifactors analysis of variance was performed on each experiments (all cells) and shows that in each case, except the S7 experiment, the amount of vitamin A propionate that penetrates in the skin (all layers considered) is significantly higher when the molecule is included in the Rameb/PVA complex (Confidence Interval 95%). If the different compartments are analyzed individually the 95% Confidence Interval cannot always be maintained and sometimes drops to 86% (for significant difference between the reference and the Rameb/PVA gel) as the number of data is smaller and therefore the Confidence Interval is higher. However, there is no doubt concerning the effects on inclusion in the Rameb: it leads to greater skin penetration.

In the case of the S7 experiment, no meaningful difference was noted between the reference gel and the Rameb/PVA gel in term of quantitative penetration of vitamin A propionate. The reason is we kept the dermis but could not find a way to extract the PVA from this very thick layer. Therefore the dermis was not assayed and the only conclusion that can be drawn is that the PVA do not penetrate farther than dermis, since no PVA was recovered in the receptor liquid. Most of the PVA is probably stuck in the dermis where, like in epidermis, it is converted in active retinoids i.e. the form that will bound with the cellular receptor [8]. The important point of this experiment is that PVA can not reach the circulation after topical application of the gel.

Table 2. Amount of PVA found in the stratum corneum, the epidermis and the receptor phase (as % of initial amount of PVA and using 8 values for the Rameb/PVA gel and 7 values for the reference gel for each experiment)

Experiment		Stratum corneum	Epidermis	Receptor liquid
S2 Reference	mean	0.378	0.139	0
	standard deviation	*0.086*	*0.154*	*0*
S2 Rameb/PVA	mean	0.612	0.516	0.003
	standard deviation	*0.427*	*0.374*	*0.004*
S4 Reference	mean	0.618	0.051	0.017
	standard deviation	*0.41*	*0.025*	*0.032*
S4 Rameb/PVA	mean	0.864	0.145	0.066
	standard deviation	*0.317*	*0.181*	*0.08*
S5 Reference	mean	0.034	0.113	0.003
	standard deviation	*0.037*	*0.151*	*0.009*
S5 Rameb/PVA	mean	0.36	0.132	0.019
	standard deviation	*0.224*	*0.235*	*0.022*
S7 Reference	mean	0.174	0.112	0
	standard deviation	*0.189*	*0.052*	*0*
S7 Rameb/PVA	mean	0.221	0.062	0
	standard deviation	*0.118*	*0.044*	*0*

Conclusion

A very stable and soluble complex can be formed between Rameb-CD and the vitamin A propionate. An aqueous gel based on this solution significantly improves the amount of vitamin A that penetrates the different layers of human skin and it was proven not to reach the circulation.

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