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RESEARCH PAPER

Skin Healing Preparations: Compared In Vitro Diffusion of the Active Ingredients

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ABSTRACT

We have developed skin healing preparations based on propolis extract:ointments, emulsions, and transparent oil-water (TOW) gels. The formulations were optimized in terms of macroscopic characteristics including spreadability and penetrability, and limpidity and isotropy for the TOW gels. We describe here the results of a study of in vitro diffusion of the active ingredients, flavonoids, and phenolic acids, from some of the preparations we developed. These results show the influence of the dosage form and the choice of excipients on the release of the active product. The least satisfactory results were obtained for hydrophobic ointments and o/w emulsions. The strongest diffusion was obtained with a TOW gel associating isopropyl palmitate, Eumulgin B_3®, and Cetiol HE®.

INTRODUCTION

Propolis is a resinous substance collected by bees on many plant species. It displays a wide range of pharmacological properties (1-3) that lend it therapeutic potential, but it has a complex and variable chemical composition that is difficult to control (4,5),

In previous work we standardized the preparation of an extract of propolis to ensure optimal reliable quality. Obtaining this extract required selecting the propolis batch and optimizing each stage of the manufacturing procedure (grinding, extraction, workup of the extract solution, etc.) (6).

The microbiological activity of propolis results from the combined action of a great number of constituents, though most of it is attributed to the phenolic acids and flavonoids, especially galangine. For this reason we developed a method of HPLC analysis which is able to rapidly identify the 14 main components of the standard propolis extract. Also, galangine was assayed using an external standard by a validated protocol. It accounted for 15% (w/w) of the total flavonoids in the extract (7).

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This extract was shown to be safe and nonirritant (8). In addition, pharmacological tests in the albino rabbit and hairless rat showed the extract had skin healing properties at a concentration of 5% (9).

Because a purified standardized extract displaying useful therapeutic properties was available, we undertook the development of dosage forms for several types of preparations designed for the treatment of bedsores (10–12).

We needed to evaluate how readily the active ingredients were released from these preparations. Two objectives were pursued in the work reported here: (i) To compare the in vitro release of the propolis extract from various administration forms: ointments, emulsions, and transparent oil-water (TOW) gels; and (ii) to study the effects of the excipients on the release of the active ingredients from these preparations.

MATERIALS AND METHODS

Raw Materials

The concentrated extract of propolis (8% w/v) was laboratory-prepared using a previously validated procedure (6).

As excipients for the hydrophilic ointments we tested several PEG 400–PEG 4000 mixtures (Prolabo, France), some associated with cetyl alcohol (Coopération Pharmaceutique Française, France).

The hydrophobic ointment contained a composite excipient, Hydrocerine SL® (Roc, France).

The oil base for the emulsions was thick paraffin oil (5% w/w) (Coopération Pharmaceutique Française) supplemented with glycerine as a moistener (5% w/w) (Coopération Pharmaceutique Française), isopropyl palmitate (6% w/w) (Unichema, France) as a skin penetrant, and Cetiol HE® (8% w/w) (Henkel, France) as a solubilizing oil.

The other excipients, classified according to their main function, are given in Table 1.

The TOW gels were made up mainly of water, oil, and emulsifiers. In the formulations developed, the oil phase was thick paraffin oil or isopropyl palmitate associated with Cetiol HE (Henkel).

As non-ionic emulsifiers we tested an ethoxylated cetylstearyl alcohol, Eumulgin B_3® (Sidobre Sinnova), and a polyethoxylated oleic ester, Brij 96® (ICI).

Methods

Preparation of Pharmaceutical Forms

For the hydrophilic ointments, the propolis extract was solubilized in PEG 400 on a water bath at 40°C. This solution was incorporated into a mixture of PEG 4000 and cetyl alcohol, and fluidized at the same temperature. Purified water was gradually added to the preceding phase at 40°C by means of an IKA RW20 helical stirrer, and the stirring was maintained until the mixture had completely cooled.

For the hydrophobic ointments, a Hobart-type mixer-blender was used to blend the composite excipient, previously fluidified on a water bath at 40°C, into the propolis extract softened at the same temperature.

For the emulsions, the propolis extract, previously fluidified on a water bath at 40°C was added to the lipid excipients on a water bath at 70°C. The aqueous phase, heated to the same temperature, was blended in using a helical stirrer. During dispersion, the stirring rate was

Table 1

Excipients Tested for Emulsion Formulation

Type of Excipient	Chemical Designation	Commercial Name (Supplier)
Consistency factors	Cetyl alcohol	(Coopération Pharmaceutique Française)
	Mono and diglycerides of stearic and palmitic acids	Cutina MD® (Sidobre Sinnova)
Non-ionic surfactants	Sorbitan mono-oleate	Arlacel 165® (ICI)
	Polyethoxylated sorbitan mono-oleate	Tween 85® (ICI)
	Polyethoxylated cetyl alcohol	Eumulgin B_1®
		Eumulgin B_2® (Sidobre Sinnova)
Self-emulsifying bases	Mixture of cetylstearyl alcohol and polyethoxylated cetylstearyl alcohol	Emulgade 1000 NI® (Sidobre Sinnova)

gradually increased to 500–800 rpm according to the consistency of the cream. After complete cooling, the emulsion was vacuum-degassed.

For the TOW gels, the propolis extract, fluidized beforehand, was blended into the lipid phase containing the emulsifiers on a water bath at 70°C. The aqueous phase, heated to the same temperature, was then stirred in. As above, stirring was maintained until the mixture had completely cooled (800–900 rpm).

Quality Testing

The following tests were carried out 24 hr after preparation.

Macroscopic Characteristics

The homogeneity of the various dosage forms was assessed by visual inspection.

Spreadability

The spreading diameter (SD) of exactly 1 ± 0.1 g of the preparation compressed between two 20-cm square glass plates, the upper one of which weighed 125 ± 1 g, was measured. The preparation sample was weighed directly on the center of the plate. The measurement was carried out on a support plate connected to a thermostat system that ensured a constant temperature of 22 ± 0.5°C (13).

We used the following classifications: stiff ($SD \leq 40$ mm), semistiff ($40 < SD \leq 50$ mm), and semifluid ($50 < SD \leq 70$ mm).

Penetrability

The penetrability of ointments and creams was measured at 21 ± 2°C using a 5-sec delayed action electric penetrometer (Prolabo, Mahler cone principle). The value of the depth to which the cone penetrates was read from a graduated dial to within 0.1 mm. This is the penetrability (P).

The following scale was used; stiff ($P \leq 270$ 10^{-1} mm), semistiff ($270 < P \leq 300$ 10^{-1} mm), semifluid ($P > 300$ 10^{-1} mm).

pH of hydrophilic ointments and emulsions was determined by means of a Schott CG 837 pH meter.

Rheology of emulsions was studied at 21 ± 2°C using a Brookfield RV TD V2 viscosimeter fitted with an SC4 28/13 R adaptor.

Limpidity of TOW gels was determined using the method described in the French Pharmacopoeia (14), extended by spectrophotometric measurement of the transmission at 610 nm ($T\%$) of the reference solutions and the experimental TOW gels.

The gels were classified into limpid ($T\% \leq 80$), slightly opalescent ($42 < T\% < 80$), opalescent ($22 < T\% \leq 42$), and very opalescent ($T\% \leq 22$).

Optical anisotropy of TOW gels was measured by means of a Zeiss 61687 polarized light microscope.

Physical Stability

Physical stability tests were carried out at ambient temperature (21 ± 2°C) and in the dark at 4°C. For each formula, the tests were repeated after 6, 12, and 24 months storage.

In Vitro Release Tests

Materials

We used a Plexiglass cell derived from that proposed by Bottari and prescribed by the French Pharmacopoeia for dissolution testing of transdermal devices (14). This cell was made of chemically inert Plexiglas, and comprised a support 2.6 cm deep and 4.5 cm in diameter, corresponding to a volume of 4.13 ml, and a lid with a central opening 4.0 cm in diameter bounding a diffusion surface of 12.56 cm^2. The separate components of the cell, the support and the lid, allowed the artificial membrane to be inserted to act as a barrier between the preparation contained in the cell (donor compartment) and the dissolving medium in the reactor tank (receptor compartment) of a Dissolutest apparatus (Prolabo).

The membrane used was a Fluoropore FHLP® artificial hydrophobic membrane of porosity 0.5 mm and diameter 47 mm. The diffusion medium was 600 ml of 70% (v/v) ethanol.

Operating Procedure

The test sample was weighed directly in the support after leveling. The cell used here contained approx. 12 g of preparation. The membrane was wetted with methanol by soaking for 1 min and then placed on the test sample. The lid was then fitted on and the cell immediately laid horizontally, lid up, in the tank containing 600 ml of dissolution medium maintained at 32°C. The paddle was positioned 2.5 cm above the cell and the stirring rate was set at 80 rpm. This rate provided adequate homogenization without turbulence.

A 2-ml sample of medium was taken every 30 min for 8 hr, and immediately made up by adding 2 ml of 70% (v/v) ethanol.

The activity of the propolis extract is due to its phenolic components, flavonoids, and phenolic acids, which were assayed by UV photometry. The absorption at 280 nm of each sample was measured using a Beckman DU 64 UV spectrophotometer.

The percentage of the active ingredients (flavonoids and phenolic acids) released per unit time was then calculated from a standard range, the highest point of which corresponded to 100% theoretical diffusion for 12 g of preparation (i.e., 1 mg/ml of receptor medium).

RESULTS AND DISCUSSION

Results

Ointments

The characteristics of the formulations developed are shown in Table 2. This table also indicates the results of tests of in vitro release of active ingredients from the different ointments.

Hydrophilic ointments suitable for the in situ treatment of bedsores were studied. The use of polyethylene glycols as anhydrous water-soluble excipients makes these similar to emulsions with continuous aqueous phases.

All of the formulations from 1 to 8 contain a PEG 400–PEG 4000 mixture associated in some cases with cetyl alcohol. Importantly, these excipients are nonirritant, and the antibacterial, and antifungal properties of propolis make preservatives unnecessary. All of the ointments tested were perfectly homogeneous and were stiff, as indicated for preventive trophic massages and curative treatment at the epidermis formation stage.

The pH values of the ointments lay between 5.25 and 5.55, a range perfectly compatible with skin application. After 24 months all of the ointments displayed satisfactory physical stability.

For the test of in vitro release of active ingredients from the different hydrophilic ointments, the choice of membrane was guided by a study of the literature and preliminary testing. We selected the hydrophobic polymer membrane Fluoropore FHLP® 0.5 mm, which is made of polytetrafluoroethylene welded to a high-density polyethylene screen. This porous structure, which contains over 70% empty space, minimizes interactions between the membrane and the active ingredients and acts here essentially as a dialysis membrane. Table 2 shows that for the formulations containing no cetyl alcohol, the diffusion of the active ingredient was very

Table 2

Characteristics of Ointments Containing 5% Propolis Extract

	Formulation No.								
	1	2	3	4	5	6	7	8	9
Raw materials (% w/w)									
Propolis extract	5	5	5	5	5	5	5	5	5
PEG 400	50	52	52	50	52	52	40	40	–
PEG 4000	35	33	29	30	28	24	30	26	–
Cetyl alcohol	–	–	4	–	–	4	–	4	–
Hydrocerine SL	–	–	–	–	–	–	–	–	95
Purified water	10	10	10	15	15	15	25	25	–
pH after 24 months	5.5	5.2	5.3	5.5	5.2	5.3	5.3	5.3	–
Initial value	5.5	5.2	5.3	5.5	5.2	5.3	5.3	5.3	
Spreadability after 24 months	25	32	31	26	33	31	37	36	41
SD after 1 min									
Initial value	26	32	31	26	33	30	35	36	41
Penetrability after 24 months	209	255	248	245	257	250	276	270	285
(1/10 mm)	212	253	247	245	255	249	270	270	290
% Diffused at 8 hr[a]	45.4	44.9	43.1	44	42.5	41.0	41.3	24.3	3.1
Standard deviation	0.5	0.4	0.4	0.5	0.6	0.6	0.5	0.3	0.2

[a]Mean of three measurements.

similar, irrespective of the percentage of water and the proportions of the PEG 400–PEG 4000 (between 41.3 and 45.4% diffused at 8 hr).

In formulations 3, 6, and 8 we replaced part of the PEG 4000 with cetyl alcohol. For formulations 3 and 6, containing respectively 10 and 15% water, the diffusion of the active ingredients was not modified. In contrast, formulation 8, containing 25% water, did not favor release, which fell by approx. 40%.

The hydrophobic ointment we developed (formulation 9) simulates the effect of an occlusive dressing and so is suitable for application to bedsores during the formation of erythematous or oedematous lesions or blistering. This formulation was based on Hydrocerine SL and was semi-stiff, physically stable, and afforded only a weak diffusion of the active ingredient (approx. 3% after 8 hr).

o/w Emulsions

For anti-bedsore therapy we tested emulsions displaying three consistencies: stiff creams applied directly in situ, semistiff creams designed for impregnation of compresses used in early stages, and semifluid creams suitable for preventive treatment during skin regrowth.

The emulsification of the lipid components required a consistency factor (cetyl alcohol or Cutina MD) associated with either non-ionic ester surfactants (Arlacel 165, Tween 85, Eumulgin B_1 or B_2) or a self-emulsifying base (Emulgade 1000 NI).

The characteristics of the different emulsions and the percentages of active ingredient diffused in vitro from these pharmaceutical forms are shown in Tables 3 and 4. The choice of excipients for these formulations has been discussed in previous papers (11,12).

The emulsions prepared were all perfectly homogeneous and had pH values compatible with skin application (pH 5.02–6.46). Physical stability studies on the emulsions over 24 months showed good stability.

The diffusion of the active ingredient from the emulsions was 2–5 times weaker than from hydrophilic ointments.

The results for the emulsions containing the mixture Arlacel 165 (6%)–Tween 85 (3%) showed that Cutina MD was preferable to cetyl alcohol as a consistency

Table 3

Characteristics of o/w Emulsions Containing 5% Propolis Extract and Arlacel 165 and Tween 85 as Surfactants

	Formulation No.						
	10	11	12	13	14	15	16
Raw materials (% w/w)							
Propolis extract	5	5	5	5	5	5	5
Paraffin oil	5	5	5	5	5	5	5
Glycerine	5	5	5	5	5	5	5
Isopropyl palmitate	6	6	6	6	6	6	6
Cétiol HE	8	8	8	8	8	8	8
Arlacel 165	6	6	6	6	3	9	–
Tween 85	3	3	3	3	6	–	9
Cetyl alcohol	–	–	5	10	5	5	10
Cutina MD	12	10	–	–	–	–	–
Purified water	50	52	57	52	57	57	52
pH after 24 months	5.0	5.3	5.5	5.1	5.5	5.3	5.6
Initial value	5.0	5.3	5.5	5.1	5.5	5.3	5.6
Spreadability after 24 months							
SD after 1 min	35	37	46	35	50	35	50
Initial value	34	37	45	33	50	35	49
Penetrability after 24 months (1/10 mm)	288	288	299	280	300	275	295
Initial value	286	286	299	278	300	274	296
% Diffused at 8 hr[a]	16.2	17.9	7.6	8.3	12.9	7.8	15.78
Standard deviation	0.6	0.7	2.1	2	0.8	0.6	3

[a]Mean of three measurements.

Table 4

Characteristics of Other o/w Emulsions Containing 5% Propolis Extract

	Formulation No.			
	17	18	19	20
Raw materials (% w/w)				
Propolis extract	5	5	5	5
Paraffin oil	5	5	5	5
Glycerine	5	5	5	5
Isopropyl palmitate	6	6	6	6
Cétiol HE	8	8	8	8
Eumulgin B_1	1.5	1.5	–	–
Eumulgin B_2	1.5	1.5	–	–
Emulgade 1000 NI	–	–	2	2
Cetyl alcohol	–	5	–	10
Cutina MD	12	–	12	–
Purified water	56	63	57	59
pH after 24 months	6.4	5.5	6.4	5.1
Initial value	6.4	5.5	6.4	5.1
Spreadability after 24 months				
SD after 1 min	50	55	50	52
Initial value	50	55	50	51
Penetrability after 24 months				
(1/10 mm)	299	320	300	310
Initial value	298	318	299	303
% Diffused at 8 hr[a]	18.6	17	19.5	20.9
Standard deviation	1.5	1.8	0.8	0.9

[a]Mean of three measurements.

agent, because the percentage of active ingredient released was twice as high for formulations 10 and 11 as for formulations 12 and 13.

Analysis of formulations 14, 15, and 16 showed that the association Arlacel–cetyl alcohol impeded diffusion, unlike the mixture Tween 85–cetyl alcohol. Formulation 16 required 10% of a consistency agent to ensure macroscopic stability.

Table 4 gives the results obtained for the other o/w emulsions. For creams containing the mixture Eumulgin B_1–Eumulgin B_2, the nature of the consistency factor had no significant influence on the release of the active ingredient (respectively, 18.6 and 17% for formulations 17 and 18). This was also the case for the emulsions containing the self-emulsifying base Emulgade 1000 NI. Stabilization of the formulation 20 required 10% cetyl alcohol.

Tow Gels

TOW gels are semisolid systems composed mainly of water, oil, and non-ionic emulsifiers. They have a typical jelly-like consistency, are transparent and optically isotropic, and will resonate when their container is lightly struck (15).

TOW gels have numerous advantages, both physicochemical and biopharmaceutical: they are nonocclusive and nonirritant, and can be washed off with water; they spread easily on the skin to form a thin, transparent, nongreasy film. These specific features make TOW gels useful for the treatment of skin pathologies, especially because they can improve the percutaneous absorption of active ingredients, particularly lipophilic substances (15). In addition, they are thermodynamically stable and appreciably more stable than emulsions.

Preliminary tests and a literature study (16–18) indicated two suitable formulations; their compositions and characteristics are shown in Table 5. Formulation 21 has the appearance of a stiff, limpid, isotropic jelly. Formulation 22 is semifluid, very slightly opalescent and is also isotropic. These TOW gels both display excellent macroscopic stabilities but differ considerably in their ability to release the active ingredients. The percentage of active ingredient diffused after 8 hr was about three

Table 5

Characteristics of TOW Gels Containing 5% Propolis Extract

	Formulation No.	
	21	22
Raw materials (% w/w)		
Propolis extract	5	5
Cétiol HE	18	–
Paraffin oil	–	5
Isopropyl palmitate	8	–
Eumulgin B_3	12	–
Brij 96	–	40
Purified water	57	50
Spreadability after 24 months SD after 1 min	35	55
Initial value	34	55
Penetrability after 24 months (1/10 mm)	183	315
Initial value	183	314
% Transmission	82	78
Behavior in polarized light	Isotropic	Isotropic
% Diffused at 8 hr	60.1	22.3
Standard deviation	4.2	1.0

times higher than that released from formulation 22 in the same time. Also, the rate of diffusion from this TOW gel was much greater than observed from the other dosage forms. Hence this form appears particularly promising and well-suited for dressing ulcerations and stimulating skin renewal.

Discussion

Figure 1 shows the influence of the dosage form, ointment, emulsion, and TOW gel, on the in vitro diffusion of the active ingredients. It also illustrates the importance of excipient choice in each of these forms.

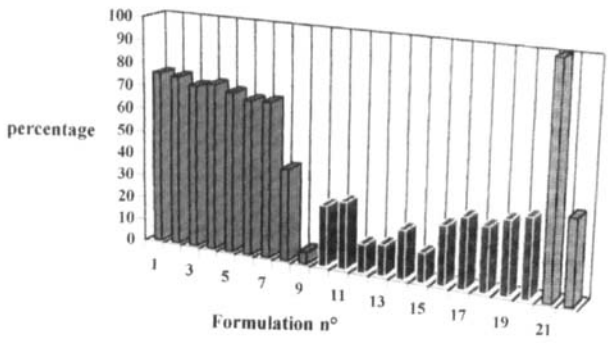

Figure 1. Compared diffusion of phenolic constituents of propolis extract from different pharmaceutical forms.

The formulations were scored on a scale from 0 to 100 relative to the preparation giving the greatest diffusion of active ingredients chosen as a reference (100% diffusion). This is the TOW gel associating isopropylpalmitate, Cetiol HE, and Eumulgin B_3 (formulation 21). This dosage form can be considered as an isotropic cubic liquid crystal system. It is obtained only for well-defined proportions of the different constituents (oil phase, emulsifier, and water) and requires a high concentration of non-ionic surfactant. The strong release of the liposoluble active ingredient can be explained by the high proportion of Eumulgin B_3 in the formulation; in addition, the liquid crystal structure of TOW gels may favor percutaneous absorption (15).

However, the diffusion of the active ingredients from the TOW gel based on paraffin oil and Brij 96 (formulation 22) fell by 63%, which suggests that the nature of the emulsifier is important.

For the ointments, the weakest diffusion was obtained for the hydrophobic ointment. The difference in diffusion rate between this ointment and the hydrophilic ointments is due to a preferential affinity of the propolis extract for the lipophilic excipient, Hydrocerine SL. For hydrophilic ointments, the vehicle–medium partition coefficient favors the medium. The realease of active ingredients remained in a narrow range (69–76% of the reference value), except for formulation 8.

The emulsions allowed only a weak diffusion of the active ingredient, 3 to 10 times weaker than that obtained with the reference TOW gel. The poorest results were obtained with the association Arlacel 165–cetyl alcohol (formulations 12, 13, and 15). The rate of release of active ingredient is linked to the coefficient of partition between the o/w emulsion and the diffusion medium. However, the problem is complicated here by the presence of a disperse phase that can act as a trap for the active ingredient.

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