

Release of rosmarinic acid from semisolid formulations and its penetration through human skin *ex vivo*

ADA STELMAKIENĖ
KRISTINA RAMANAUSKIENĖ
VITALIS BRIEDIS

Department of Clinical Pharmacy
Lithuanian University of Health
Sciences, Kaunas, Lithuania

The aim of this study was to evaluate the release of rosmarinic acid (RA) from the experimental topical formulations with the *Melissa officinalis* L. extract and to evaluate its penetration through undamaged human skin *ex vivo*. The results of the *in vitro* release study showed that higher amounts of RA were released from the emulsion vehicle when lemon balm extract was added in its dry form. An inverse correlation was detected between the released amount of RA and the consistency index of the formulation. Different penetration of RA into the skin may be influenced by the characteristics of the vehicle as well as by the form of the extract. The results of penetration assessment showed that the intensity of RA penetration was influenced by its lipophilic properties: RA was accumulating in the epidermis, while the dermis served as a barrier, impeding its deeper penetration.

Keywords: human skin penetration, rosmarinic acid, rheology, release

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Atopic dermatitis is one of the most common skin diseases. Since dermatologic products from natural raw materials are becoming increasingly popular, it is relevant to produce a semisolid transdermal preparation that would have not only natural active ingredients, but a natural base as well. A clinical study performed by Lee *et al.* (1) showed »the possible clinical use of rosmarinic acid (RA) as a therapeutic agent for atopic dermatitis«. Rosmarinic acid demonstrated cellular protective properties against the damaging radical species induced by UVB radiation (2). Scientific literature describes penetration studies with RA using Caco-2 cell monolayers (3), which reflects penetration of RA through the intestinal wall. Research has shown (3) that the botanical matrix had no effect on RA penetration through the Caco-2 cell monolayer. Lee *et al.* (1) performed a clinical study to evaluate the effect of a topical RA preparation. However, in our review of scientific literature, we failed to find any studies analyzing the penetration of RA through undamaged full-thickness human skin.

* Correspondence; e-mail: ada.stelmakiene@yahoo.com

RA is the key active ingredient of a *Melissa officinalis* L. extract (4). *European Pharmacopoeia*, monograph *Melissa officinalis* L. leaves, states that *Melissa officinalis* L. leaves should contain at least 4 % of hydroxycinnamic acids expressed as RA. RA is also known as an antiviral (5), anti-inflammatory (5), antioxidant (6), photoprotective (2) and anti-proliferative (6) component of *Melissa officinalis* L., which makes it relevant to apply the *Melissa officinalis* L. extract as a natural source of RA in the modeling of semisolid formulations.

The aim of this study was to evaluate the release of rosmarinic acid from the experimental topical formulations with the *Melissa officinalis* L. extract and to evaluate its penetration through undamaged human skin *ex vivo*.

EXPERIMENTAL

Materials

Rosmarinic acid, HPLC-grade acetonitrile, and acetic acid were purchased from Sigma Aldrich Chemie GmbH (Germany). Standardized dry lemon balm extract (*sicc. Extractum Melissa officinalis*) was purchased from Naturex (France).

Methods

Preparation of semisolid formulations. – The components of the formulation are listed in Table I. The choice of the components for the ointment and w/o cream semisolid vehicles were developed with reference to the earlier published research data (7). In accordance with the study of Lee *et al.* (1), the formulations were produced with 4 % of dry lemon balm extract containing 80 mg g⁻¹ RA. Formulations were prepared according to the general technological principles of semisolid preparation.

Quantification of rosmarinic acid by HPLC. – The amount of RA was evaluated by applying the capillary HPLC method, which was developed and validated, using the Agilent 1260 Infinity Capillary LC System (Agilent Technologies, USA) with an Agilent diode array detector. The separation was performed on a C18 ACE (5 μm) 150x0.5 mm i.d. column. The

Table I. Composition (g) of semisolid formulations with *Melissa officinalis* L. extract

	Beeswax	Paraffin	Vaseline	Honey	Cocoa butter	Cholesterol	Olive oil	Water	Aqueous lemon balm extract, 1:15	Dry extract
N 1	6.0	5.0	5.0	-	10.0	3.0	67.0	-	-	4.0
N 2	15.0	-	-	10.0	10.0	3.0	58.0	-	-	4.0
N 3	5.0	-	-	10.0	10.0	3.0	33.0	35.0	-	4.0
N 4	5.0	-	-	10.0	10.0	3.0	37.0	-	35.0	-

mobile phase consisted of solvents A (0.5 % aqueous solution of acetic acid, V/V) and B (acetonitrile) using the following gradient elution: 23 % of B at time 0, 40 % of B at 10 min, and 70 % of B at 11–15 min, and then returned to the initial conditions by 10-minute re-equilibration, with the total run time of 25 minutes. The analysis was carried out at a flow rate of 10 $\mu\text{L min}^{-1}$, with the detection wavelength set at 330 nm.

Rheological flow behavior test. – The test ($n = 5$) was performed using the Carri-med CSL² 500 rheometer (TA Instruments, Germany), applying the cone-and-plate geometry system (cone diameter 60 mm, angle 2°, sample thickness 150 μm). The shear rate was increased from 0 to 500 s^{-1} for 2 minutes. The rheological properties of the samples were determined according to the Oswald de Waele (8) equation:

$$\sigma = K \dot{\gamma}^n$$

where σ is shear stress (Pa), $\dot{\gamma}$ is shear rate (s^{-1}), and K is the consistency index [$(\text{Pa s})^n$] which is the indicator of viscosity. The flow behavior index (n) is a dimensionless number that indicates the closeness to the Newtonian flow. For a Newtonian liquid $n = 1$ for a dilatant fluid $n > 1$, and for a pseudoplastic fluid $n < 1$ (8).

In vitro release study. – *In vitro* release experiments were performed using modified Franz-type diffusion cells (9). The semisolid sample (1.00 ± 0.02 g) was placed into the cell with a dialysis membrane Cuprophan[®] (Medicell International Ltd., UK). The area of the diffusion was 1.77 cm^2 . Thermostated (37 ± 0.2 °C) purified water acted as the acceptor medium. The medium was stirred using a magnetic stirrer. Samples from the acceptor solution were taken at 1, 2, 4, and 6 h and were immediately replaced with the same volume of fresh acceptor solution.

Ex vivo penetration through the full-thickness undamaged human skin. – Abdominal skin of Caucasian women (age range 25–40 years) was obtained from the Department of Plastic and Reconstructive Surgery (the Hospital of the Lithuanian University of Health Sciences, Lithuania) after cosmetic surgery. It was stored at -20 °C for not longer than 6 months before use. Kaunas Regional Biomedical Research Ethics Committee had approved the use of human skin for transdermal penetration studies. A Bronaugh-type flow-through diffusion cell with full-thickness human skin was used for *ex vivo* skin penetration experiments ($n = 3$). The experiment was performed according to the methods proposed by Kezutyte *et al.* and Zilius *et al.* (9, 10).

Statistical data evaluation was performed using the SPSS software with one-way ANOVA. Tukey's *post hoc* test was performed for multiple comparisons.

RESULTS AND DISCUSSION

Stable vehicles were developed in our previous research (7). Vehicles contained all components indicated in Table I, except *Melissa officinalis* L. extract. Formulation N1 was a hydrophobic vehicle with solid paraffin and vaseline, formulation N2 a hydrophobic vehicle with beeswax instead of paraffin and honey instead of vaseline, formulation N3 was an emulsion vehicle with dry *Melissa officinalis* L. extract, and formulation N4 was an emulsion vehicle with aqueous *Melissa officinalis* L. extract. During the first stage of the study, we evaluated the influence of the semisolid bases and the addition of the extract on the rheological characteristics of the formulations by determining their consistency index and

flow behavior index. Modification of viscosity is an important part of the preparation of semisolid formulations and is regarded to be a critical quality attribute in the development stage of the product (11). To evaluate the possible effect of temperature on the structure of semisolid formulations during their storage period, we selected the temperature of 30 °C, which was approximate to the melting temperature of the experimental formulations as well as to the skin surface temperature. The obtained results showed that all the investigated formulations displayed pseudoplastic characteristics. The results of the experiments showed that addition of the *Melissa officinalis* L. extract significantly reduced consistency indices of the formulations and increased their flow behavior indices compared to the rheological characteristics of the vehicles (Fig. 1).

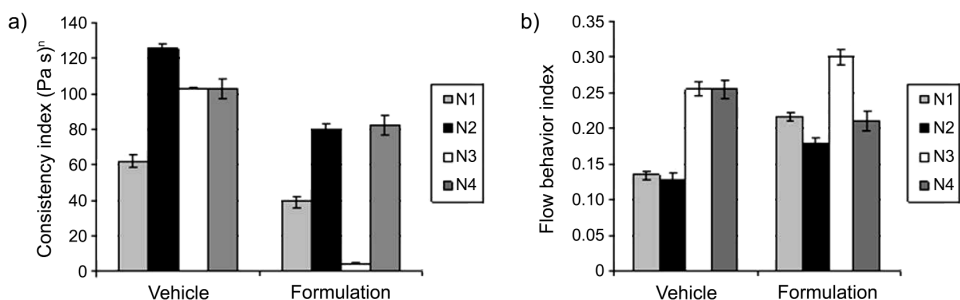


Fig. 1. Rheological properties of semisolid formulations with *Melissa officinalis* L. extract and vehicles at 30 °C: a) consistency index, b) flow behaviour index (mean \pm SD, $n = 5$).

The addition of dry *Melissa officinalis* L. extract had the greatest influence on the rheological properties of N3 as compared to those of hydrophobic formulations (N1 and N2). The results showed that the addition of the active substance in its dry form had a greater impact on the rheological properties of emulsion formulations. The calculated consistency and flow behavior indices reflected the effect of the *Melissa officinalis* L. extract on the weakening of the structure of semisolid formulations, which may affect the expiry date of formulations.

The results of the RA release studies were in line with the published data, demonstrating that the release of drug substances from semisolid vehicles is influenced not only by the properties of the drug molecule, but also by the properties of the vehicle (12). Data presented in Fig. 2 show that higher amounts of RA were released from the vehicles (N1, N2, and N3) when the *Melissa officinalis* L. extract was added in its dry form compared to formulation N4, which contained an aqueous lemon-balm extract. The results of the experiments demonstrated that formulation N4 released the lowest amounts (less than 1 %) of RA (Fig. 2) within 6 hours of testing. Hypothetically, it can be stated that the diffusion of RA from the aqueous phase of this emulsion was hampered by the inner lipophilic phase. The highest amounts (39.6 %) of RA were released from the emulsion vehicle N3 within 6 hours. Results of the statistical analysis revealed a statistically significant ($p < 0.01$) inverse correlation (Spearman's correlation coefficient of -0.82) between the consistency index and the amount of RA released from the formulations within 6 hours of the experiment. However, no statistically significant differences between N1, N2 or N4 were found.

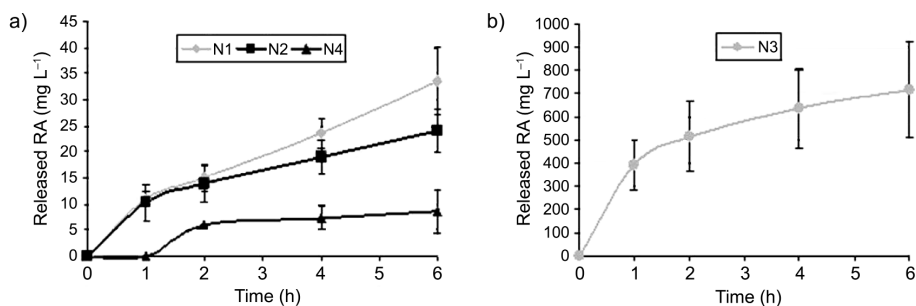


Fig. 2. Release profiles of rosmarinic acid from semisolid formulations with *Melissa officinalis* L. extract: a) N1, N2 and N4, b) N3 (mean \pm SD, $n = 3$).

A statistically significant ($p < 0.05$) difference of RA release from N3 could be determined by the consistency index, which was significantly ($p < 0.05$) different from that of the emulsion base. Since the consistency index indicates viscosity (8), and viscosity, in turn, has an inversely proportional influence on the diffusion process, a decrease in viscosity could lead to an increase in the substance release rate.

Penetration of RA from the aqueous *Melissa officinalis* L. extract through the skin was influenced only by the diffusion process and the properties of the active substance itself. It was therefore, used as a penetration reference. Penetration tests through undamaged human skin within 24 hours revealed that the aqueous *Melissa officinalis* L. extract penetrated the epidermis and the dermis; unfortunately, the amounts traced in the dermis could not be assessed quantitatively. The results confirmed the data presented in the literature, indicating that passive diffusion of a solute from its vehicle into the skin is determined by the unique molecular and physical properties of the diffusant, the vehicle and the skin (13).

The results of the study showed that RA from hydrophobic formulations N1 and N2 penetrated the epidermis within 24 hours of the experiment (Table II), and the amount penetrated (0.20 % and 0.15 %, respectively) was half that of RA from the reference. No RA was found in the dermis. The obtained results also showed that the penetration of RA was limited by the release of the active substance from the dosage form. It should be noted that hydrophobic formulations contain 15 % of substances that form a film on the surface of the skin (beeswax, paraffin, or petroleum jelly) (14), which may constitute an additional barrier to RA. RA that penetrated from N4 was also traced only in the epidermis (0.22 %). The results of the statistical analysis revealed that there was no statistically significant difference in the amounts of RA that penetrated the epidermis from formulations N1, N2, and N4. The results of the experiments demonstrated that the penetration of RA from formulation N3 into the epidermis was approximately 6-fold higher (1.08 %) compared to the results after the application of N1, N2 and N4. The quantities of RA were significantly higher in the epidermis when emulsion N3 was used as a vehicle for RA if compared to the application of the reference. The results of the penetration assessment showed that RA was accumulating in the epidermis, and the hydrophilic dermis acted as a barrier preventing deeper penetration and entry of RA into systemic blood flow. Data showed that the penetration of RA from N3 was enhanced because the penetration was greater than the refer-

Table II. Penetration of rosmarinic acid into the epidermis and dermis from semisolid formulations with *Melissa officinalis* L. extract

Rosmarinic acid (%)	Aqueous extract of <i>Melissa officinalis</i> L. (1:15)	Sample			
		N 1	N 2	N 3	N 4
Epidermis	0.42 ± 0.05	0.20 ± 0.02	0.15 ± 0.00	1.08 ± 0.18	0.22 ± 0.01
Dermis	tr	0	0	tr	0
Acceptor	0	0	0	0	0

tr – traces

^aAfter 24-hours application.

ence. The enhancer was probably olive oil, which was one of the components of the vehicle. Olive oil contains high amounts of oleic acid, which is known as a penetration enhancer (15). RA was not found in the acceptor phase of any sample. These results have show that RA used topically generates only local effects.

CONCLUSIONS

The results of the *in vitro* release study showed that higher amounts of RA were released from the emulsion vehicle when the *Melissa officinalis* L. extract was added in its dry form. An inverse correlation was detected between the amount of RA released and the consistency index of the vehicle, the decreasing viscosity of the formulations resulting in higher amounts of RA being released. Differences in penetration of RA into the skin may be influenced by the characteristics of the vehicle as well as by the form of the extract. The results have shown that RA used topically generates only local effects. Further studies are required for the development of more effective lemon balm extract-containing formulations that would ensure a better penetration of RA into the skin.

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