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Skin penetration behaviour of sesquiterpene lactones from different *Arnica* preparations using a validated GC-MSD method

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Abstract

Preparations of *Arnica montana* L. are widely used for the topical treatment of inflammatory diseases. The anti-inflammatory activity is mainly attributed to their sesquiterpene lactones (SLs) from the helenalin and 11α,13-dihydrohelenalin type. To study the penetration kinetics of SLs in *Arnica* preparations, a stripping method with adhesive tape and pig skin as a model was used. For the determination of SLs in the stripped layers of the stratum corneum (SC), a gas chromatography/mass spectrometry method was developed and validated. Thereby the amount of helenalin derivatives was calculated as helenalin isobutyrate, and 11α,13-dihydrohelenalin derivatives as 11α,13-dihydrohelenalin methacrylate. This GC-MSD method is suitable also to determine low amounts of SLs in *Arnica* preparations.

The penetration behaviour of one gel preparation and two ointment preparations was investigated. The SLs of all preparations show a comparable penetration in and a permeation through the stratum corneum, the uppermost part of the skin. Interestingly, the gel preparation showed a decrease of the penetration rate over 4 h, whereas the penetration rate of ointments kept constant over time. Moreover, we could demonstrate that the totally penetrated amount of SLs only depends on the kind of the formulation and of the SLs-content in the formulation but not on the SLs composition or on the used extraction agent.

Keywords: *Arnica montana*; Herbal medicines; Sesquiterpene lactones; Skin penetration; Gas chromatography; Mass spectrometry; Validated method

1. Introduction

Preparations of *Arnica montana* flowers have been used in traditional medicine for topical treatment of post-trauma effects and inflammatory diseases [1]. Sesquiterpene lactones (SLs) of the 10α-methylpseudoguaianolide-type, such as helenalin and 11α,13-dihydrohelenalin esters, are the active components which mainly mediate the anti-inflammatory effects [1]. Two chemotypes of *A. montana* can be differentiated: the central European chemotype with dominance in helenalin esters and the Spanish chemotype with almost 11α,13-dihydrohelenalin esters [2]. Both chemotypes are used in commercial products. Alcoholic as well as oily extracts are prepared and used in gels, cremes, ointments or as Arnica oil.

Using a stripping method with adhesive tape and pig ear skin as a model [3–5], we have recently investigated the penetration kinetics of SLs in alcoholic Arnica preparations and in an Arnica gel [6]. We could show a sufficient penetration of SLs into and a permeation through the upper layer of the skin, the stratum corneum (SC), which is regarded as the main barrier and the greatest obstacle to transdermal diffusion [7]. Moreover, we demonstrated that isolated SLs being dissolved in an alcoholic solution permeated to a lower rate through the SC than SLs being in an alcoholic extract, indicating the advantageous use of plant preparations. Pig skin is the most suitable model for human skin because of similarities in the epidermal composition, dermal structure, lipid content, histochemistry and general morphology [3,8] as well as permeability behaviour [9].

In commercial products, oily extracts from Arnica flowers are also used in the form of ointments. Continuing our penetration studies, we therefore compared the penetration behaviour of SLs from a gel with that of an ointment. For a better comparison, we studied in both cases Arnica products prepared from flowerheads of the Spanish type. Two different incubation times were considered. Additonally, the correlation between the SLs

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content and the penetration rate was investigated using an oily extract enriched with SLs.

For the quantitative determination of SLs occurring in low amounts a selective and sensitive analytical method is necessary. Based on the established GC-FID method [2] which is not sufficient enough, a GC-MS analytical method was developed and validated enabling quantification of helenalin and 11α,13-dihydrohelenalin esters at low concentrations.

2. Experimental

2.1. Materials

Three different Arnica preparations were investigated: the gel preparation "Kneipp Arnika KühlGel®" (Ch.-B.: 0119656; preparation B), the ointment "Arnica-Kneipp Venensalbe®" (Ch.-B.: 0305895; preparation A) as well as an ointment enriched with additional SLs (Ch-B.: ASS37; preparation C). Preparation C is identical with A, but an Arnica CO_2-extract was finally added resulting in a 10-fold SL-content compared to preparation A. The composition of the preparations is given in Table 1. All preparations were manufactured by Kneipp Werke (Würzburg, Germany). Preparation B is a commercially available product, preparation A is now commercially available under the designation "Kneipp Arnika Salbe S".

11α,13-Dihydrohelenalin acetate and helenalin isobutyrate were isolated from flowerheads of *A. montana* as previously described [2]. Identity was confirmed by NMR and MS analysis, and purity was evaluated by GC and TLC analysis.

The used organic solvents and sodium sulphate (anhydrous) were of analytical grade and obtained from Merck (Darmstadt, Germany). Sephadex LH-20 was purchased from Pharmacia Biotech (Uppsala, Sweden) and Santonin from Sigma (St. Louis, USA).

2.2. GC-MSD system

GC analyses were carried out with an HP6890 series GC-system (Hewlett–Packard, Wilmington, USA) using helium 5.0 as carrier gas. A fused silica capillary column (25 m × 0.25 mm i.d.) coated with 0.25 μm dimethylsiloxan was used (Optima 1, Machery-Nagel, Düren, Germany). The flow rate was set to 1.0 mL/min. The temperature profile started at 120 °C followed by a rate of 10 °C/min to 270 °C, which was held for 20 min. The injector and detector temperatures were 290 °C, the injection volume was 1.0 μL and was not split.

An Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Palo Alto, USA) at ionisation energy of 70 eV was used. Identification of SLs in the quantitative analyses was achieved by holding the EI mass spectra between 40 and 400 amu. A sim mode was used at 246 amu (santonin and dihydrohelenalin derivatives) and 244 amu (helenalin derivatives) for quantification.

2.3. Validation parameters and procedures

Validation included tests on specificity, calibration curve, precision, accuracy and stability. Two matrices were used: one matrix obtained from an ointment according to the processing mentioned below and one matrix obtained from strips loaded with horny-layer according to the processing described below in the "skin penetration study" chapter. The composition of the used placebo ointment was identical to preparation A, but contained pure vegetable oil instead of an Arnica oil extract.

2.4. Determination of the SLs content in Arnica preparations

Extraction of SLs from ointment: 5.0 g ointment and 0.20 mg santonin (internal standard) were suspended in 25.0 mL acetone. After addition of 10 g sodium sulphate (anhydrous), extraction was done for 5 min. After a period of further 5 min, the solution was filtered. The residue was extracted again with 15 mL acetone and filtered. This extraction was repeated four times. The combined solutions were reduced to a volume of about 20 mL, 10 g sodium sulphate (anhydrous) was added, stirred for 5 min and filtered. Flask and filter were rinsed with acetone. The combined solution was evaporated. The clear, yellow oily residue was dissolved in 10 mL petroleum benzine (boiling range 40–60 °C) and extracted four times with 15 mL ethanol (65%, v/v). A waiting period of 10 min followed after each extraction period. The combined ethanolic solutions were evaporated and dissolved in 4 mL acetone, and put on a conditioned (2 mL acetone) solid phase extraction column (Chromabond® NH_2 6 mL/500 mg

Table 1
Composition (%, m/m) of the Arnica preparations A (Arnika Kneipp VenenSalbe®) and B (Kneipp Arnika Kühlgel®)

Preparation A Arnika Kneipp VenenSalbe®		Preparation B Kneipp Arnika Kühlgel®	
Water, demineralized	68.00	Water, demineralized	50.32
Arnica oil extract (1:3.5–4.5)	10.00	Arnica tincture (1:10)	25.00
Glycerol 85%	n.s.	Ethanol 96.5% (v/v)	22.30
Stearic and palmitic acid	n.s.	Polyacrylic acid (Carbopol 980 NF)	n.s.
Soya lecithine	n.s.	Tris amino ultra pure	n.s.
Emulsifying cetylic-stearic-alcohol (Lanette N®)	n.s.	Perfume oil	n.s.
Cetylic alcohol (Lanette 16)	n.s.	Camphor	n.s.
Glycerol monooleat	n.s.		
Benzyl alcohol	1.00		

n.s.: Not specified.

Art.-Nr. 730180, Machery-Nagel, Düren, Germany). The column was eluted with 2 mL of a mixture of acetone and ethyl acetate (1:1, v:v) and the eluate was evaporated to dryness. The residue was dissolved in 1 mL acetone and used as test solution for GC-MSD.

Extraction of SLs from gel: 0.20 mg santonin (internal standard) in ethanolic solution was added to 10.0 g gel. After concentration under reduced pressure, further extraction and measurement was carried out as described by Willuhn and Leven [2]. A 10.0 mL of the obtained solution were evaporated to dryness, the residue dissolved in 0.5 mL methanol and used as test solution.

2.5. Skin penetration studies

Fresh pig ears were kindly provided by a slaughterhouse. These ears were not treated with boiling water and were used within 7 h. Up to three application areas (20 mm × 50 mm) were marked on the shaved inner side of each ear.

A 200 μL of the sample was applied and distributed on the marked area. The incubation was done by a constant skin temperature of 32 °C. Subsequently, the horny-layer was removed by 23 strippings with adhesive tape. For each stripping, a tared adhesive tape was applied to the marked area and pressed on with a roller (500 g) by rolling 10 times to and fro without any additional pressure. After this procedure, the tape was stripped off quickly and firmly. The SC is normally removed after 16–20 strippings [4,10] indicated by the appearance of areas with a watery sheen, which comes from the underlying layer, the stratum granulosum [11].

The strips were weighed and the amount of the SC was determined by subtracting the tare. The depth of penetration was calculated by considering the area (10 cm^2), assuming uniform coverage of the tapes and an approximate stratum corneum density of 1 g/cm^3 [12].

The SLs were redissolved by treating the strips with acetone. A 2.62 μg Santonin was added as an internal standard and the resulting samples dried under reduced pressure. Each sample was redissolved in 200 μL methanol and centrifuged at 15,000 × *g* for 10 min. The supernatant was applied to a Sephadex column (2 cm^3) and eluted with 2 mL methanol. The eluate was dried again under reduced pressure, redissolved in 80 μL methanol and analysed by GC-MSD.

3. Results and discussion

3.1. Validation procedure

For quantification of small amounts of SLs in a complex matrix, a GC method with MS detection by a special mass was developed. The method was validated for the use in both fields: skin penetration behaviour and determination of SLs content in *Arnica* preparations. As specific masses, 246 amu for 11α,13-dihydrohelenalin derivatives as well as santonin and 244 amu for helenalin derivatives were selected. In the mass spectra of the individual SLs, the abundance of the specific masses show a constant proportion to the total ion abundance (Table 2) by which a rational quantification of the 11α,13-dihydrohelenalin derivatives as 11α,13-dihydrohelenalin methacrylate and of helenalin derivatives as helenalin isobutyrate was made possible. Only the abundance proportion of mass 244 of helenalin strongly differs to that one of helenalin isobutyrate. But this derivative could be found only in small amounts in Arnica flowers [13] and the resulting error is marginal. The use of an internal standard was necessary, because of the complex sample processing and the injection inaccuracy by GC analysis. As internal standard santonin was selected because of the chemical similarity to the SLs in Arnica flowers resulting in similar behaviour and properties, the good separation of santonin from Arnica SLs in the GC-chromatogram as well as because of the good availability.

3.1.1. Selectivity

Chromatograms of different blank samples were compared with those derived from blank samples spiked with 8 μM (2 × LOQ) 11α,13-dihydrohelenalin methacrylate, 0.9 μM (2 × LOQ) helenalin isobutyrate or 800 μM santonin and tested for peak interferences. All three substances were compared with a blank sample originating from the horny-layer according to the sample processing performed during penetration studies (horny-layer matrix) as well as with a blank sample originating from an ointment according to the processing used for the deter-

Table 2
Abundance proportions $P_{SL;\,246}$ of mass 246 amu (left) and $P_{SL;\,244}$ of mass 244 amu (right) on the total ion abundance of the individual SLs

11α,13-Dihydrohelenalin (DH) derivative	Abundance proportion $P_{SL;\,246}$		Helenalin (H) derivative	Abundance proportion $P_{SL;\,244}$	
	(%)	(%) Applied to $P_{DH\text{-}methacrylate;\,246}$		(%)	(%) Applied to $P_{H\text{-}isobutyrate;\,244}$
DH methacrylate	5.5	100	H isobutyrate	5.7	100
DH isovalerianate	5.7	104	H isovalerianate	5.3	93
DH tiglinate	6.2	112	H methacrylate	5.7	100
DH acetate	5.5	100	H tiglinate	4.7	82
DH isobutyrate	5.3	96	H acetate	5.5	96
DH	5.6	102	H	3.3	58

The proportions are given in (%) (column two and five) and applied to the abundance proportion of 11α,13-dihydrohelenalin methacrylate $P_{DH\text{-}methacrylate;\,246}$ (%) (column three) and of helenalin isobutyrate $P_{H\text{-}isobuytrate;\,244}$ (%) (column six), respectively.

mination of SLs in ointments (ointment matrix). Additionally, Arnica tincture from type "Arbo" (which has a comparable SLs pattern as the central European chemotype) as well as tincture from the Spanish chemotype were used as blank samples by proving selectivity concerning the internal standard santonin. No significant interferences of a matrix compound peak with the added SLs was observed.

3.1.2. Limit of quantification (LOQ)

To determine LOQ those concentrations of SLs meeting the requirements for precision and accuracy generally accepted in bioanalytical analysis were chosen. A LOQ of 4.4 μM or 1.5 ng on column for 11α,13-dihydrohelenalin methacrylate in ointment and horny-layer matrix, was validated. Precision ($n = 9$ on 3 different days) was determined as relative standard deviation (R.S.D.) of 5.3% (horny-layer matrix) or 3.8% (ointment matrix). Accuracy ($n = 9$) was determined to be in the range of −11.9 to −5.9% (ointment matrix) and −4.2 to +4.3% (horny-layer matrix) deviation from the nominal value. The same procedure was performed for helenalin isobutyrate at a concentration of 0.46 μM (0.16 ng on column) resulting in a precision of 8.1% R.S.D. (horny-layer matrix) and 4.2% R.S.D. (ointment matrix). Accuracy expressed as a deviation from nominal value was between −14.1 and −8.0 % (ointment matrix) and between −17.2 and −2.7% (horny-layer matrix).

LOQ of both SLs met the requirements for precision and accuracy generally accepted in bioanalytical analysis [14].

3.1.3. Limit of detection (LOD)

Determination of LOD based on the signal-to-noise ratio. A ratio of 3:1 is considered to be acceptable for the estimation of the detection limit [15]. 11α,13-Dihydrohelenalin methacrylate exhibited an LOD of 0.44 μM (0.15 ng on column) with a signal-to-noise ratio of about 3.4:1. With a signal-to-noise ratio of 4:1, a LOD of 0.09 μM (0.03 ng on column) was determined for helenalin isobutyrate.

3.1.4. Calibration curve

Linear correlation between the peak area and the SL concentration was shown at eight concentration levels. Due to the use of an internal standard, the peak area was corrected by the following equation:

$$A_{\mathrm{SL,corr}} = \frac{A_{\mathrm{SL}}}{A_{\mathrm{IST}}} c_{\mathrm{IST}} \quad (1)$$

Solutions were analysed in triplicate at each concentration level. The concentrations of SLs differ a lot between the main and secondary components in Arnica preparations as well as between the first and the last horny-layer strips. Therefore, calibration curves have to cover a wide range of SLs concentrations. For 11α,13-dihydrohelenalin methacrylate, linearity was shown between 549 and 4.39 μM, using concentrations of 549, 329, 220, 165, 110, 43.9 and 4.39 μM. The following calibration function was calculated:

$$c_{\mathrm{SL1}}[\mu\mathrm{M}] = 0.680 \times A_{\mathrm{SL1,corr}}[\mu\mathrm{M}]; \quad R^2 = 0.999 \quad (2)$$

For helenalin isobutyrate, linearity was shown between 581 and 0.46 μM, using concentrations of 581, 384, 232, 174, 116, 46, 4.6 and 0.46 μM and the following calibration function was calculated:

$$c_{\mathrm{SL2}}[\mu\mathrm{M}] = 0.521 \times A_{\mathrm{SL2,corr}}[\mu\mathrm{M}]; \quad R^2 = 0.999 \quad (3)$$

3.1.5. Precision

Precision data were subdivided into within-day precision and reproducibility. Within-day precision was determined by injecting three samples on 1 day. This procedure was repeated on 3 different days. To calculate reproducibility, data from the 3 validation days had to be corrected for weighting differences. Testing of reproducibility included differences in analyses, solvent batches and days. Precision was demonstrated for 11α,13-dihydrohelenalin methacrylate by injecting this SL in ointment and horny-layer matrix at concentration levels of 549, 220 and 4.39 μM (LOQ), covering the range of the calibration curve. For helenalin isobutyrate concentration levels of 581, 232 and 0.46 μM (LOQ) were used.

All data are presented in Table 3 and meet the requirements as proposed by the draft document of the FDA [14].

3.1.6. Accuracy

Mean area values of the within-day precision at the different concentration steps were used as "actual values", according to the FDA [14]. "True values" of areas could be calculated by inserting the actual SL concentration value into the calibration

Table 3
Validation data of precision, reproducibility and accuracy for 11α,13-dihydrohelenalin methacrylate and helenalin isobutyrate

Concentration level (μM)	R.S.D. of within-day precision (%)		R.S.D. of reproducibility (%)		Accuracy: deviation from true value (%)	
	Ointment matrix	Horny-layer matrix	Ointment matrix	Horny-layer matrix	Ointment matrix	Horny-layer matrix
11α,13-Dihydrohelenalin methacrylate						
549	1.9–3.4	0.8–2.0	2.3	2.0	−5.2 to −3.3	−2.4 to +0.6
220	0.4–2.7	2.8–3.7	2.0	4.0	−6.8 to −4.8	−6.3 to −0.5
4.4	2.0–3.2	1.8–7.6	3.8	5.3	−11.9 to −5.9	−4.2 to +4.3
Helenalin isobutyrate						
581	0.9–4.4	0.6–2.3	3.0	2.2	+8.0 to +3.8	−0.4 to +3.4
232	0.8–4.3	1.3–3.8	2.9	3.7	−4.0 to −1.5	−3.2 to +1.1
0.46	0.7–5.0	1.2–4.8	4.2	8.1	−14.1 to −8.0	−17.3 to −2.7

Table 4
Recovery (%) of 11α,13-dihydrohelenalin methacrylate and of helenalin isobutyrate at a concentration level of 220 μM in two different matrices after 24 and 48 h at 20 °C

Tested time (h)	Recovery of 11α,13-dihydrohelenalin methacrylate (%)		Recovery of helenalin isobutyrate (%)	
	Ointment matrix	Horny-layer matrix	Ointment matrix	Horny-layer matrix
24	100.2	105.2	102.4	100.2
48	98.1	106.2	99.7	104.4

curve. Accuracy was described by the percentage of relative deviation of the "actual value" from the "true value". Accuracy is presented in Table 3. The accuracy values are generally accepted in bioanalytical analysis [14].

3.1.7. Stability

Stability testing was carried out as autosampler stability (20 °C) for 24 and 48 h in both ointment and horny-layer matrix. The values were determined by triplicate injection. Both SLs were stable for 24 and 48 h at a concentration of 220 μM (11,13α-dihydrohelenalin methacrylate) and of 232 μM (helenalin isobutyrate) (Table 4).

3.1.8. Recovery

To determine the extraction efficiency of the strip processing method, 11α,13-dihydrohelenalin methacrylate solution was applied to strips, which had been loaded before with horny-layer by stripping. A recovery of 91.2 ± 1.1% ($n = 6$) was found.

Validation shows the usefulness of the method for determining small amounts of 11α,13-dihydrohelenalin and helenalin derivatives in a complex matrix. To our knwowledge [16], this method is the first one which uses GC-MSD for the quantification of SLs combining the sensitivity and selectivity of MSD and of GC analysis.

3.1.9. Determination of the SLs content in the used Arnica preparations

The validated method was used for the determination of the SLs content in the Arnica preparations. The results are summarized in Table 3. As expected, dihydrohelenalin derivatives dominated indicating the Spanish origin of the used *A. montana* flowers [2].

3.2. Comparison of the penetration behaviour of SLs from a gel and an ointment preparation

Penetration profiles of preparation B (gel) including a 1 and 4 h incubation time were studied and are summarized in Fig. 1. Each point of the figure represents the percentage amount of SLs, which has penetrated at least to the indicated depth of SC. In a semi-logarithmic scale, the first part is a straight line to a depth of about 12 μm, representing penetration into the SC and can be described by an exponential function [6]:

$$P = a^{b \times d} \quad (4)$$

where P is the penetration (%) and d is the depth of horny-layer (μm). This linear curve in the semi-logarithmic plot implies that each stripped layer offers the same resistance to penetration [7]. The exponential coefficient b (Eq. (4)) characterizes the extent of penetration: the more negative the coefficient b (the more the curve decrease), the worse is the penetration into the horny-layer. The second part indicates the subsequent stratum granulosum layer with a different penetration behaviour.

The exponential coefficient b of Arnica gel preparation B is almost identical to the coefficient of a gel preparation which was recently investigated and which was prepared from Arnica flowers type Arbo (preparation D) [6] indicating an equal penetration rate of both helenalin and 11α,13-dihydrohelenalin derivatives from the gel preparation. It has to be considered that the total content of SLs differs in both preparations: preparation B shows a content of 0.25 mM (calculated as 11α,13-dihydrohelenalin methacrylate, Table 5) whereas preparation D has a content of 0.47 mM [6] (calculated as helenalin isobutyrate). Therefore, higher amounts penetrate from preparation D into the SC.

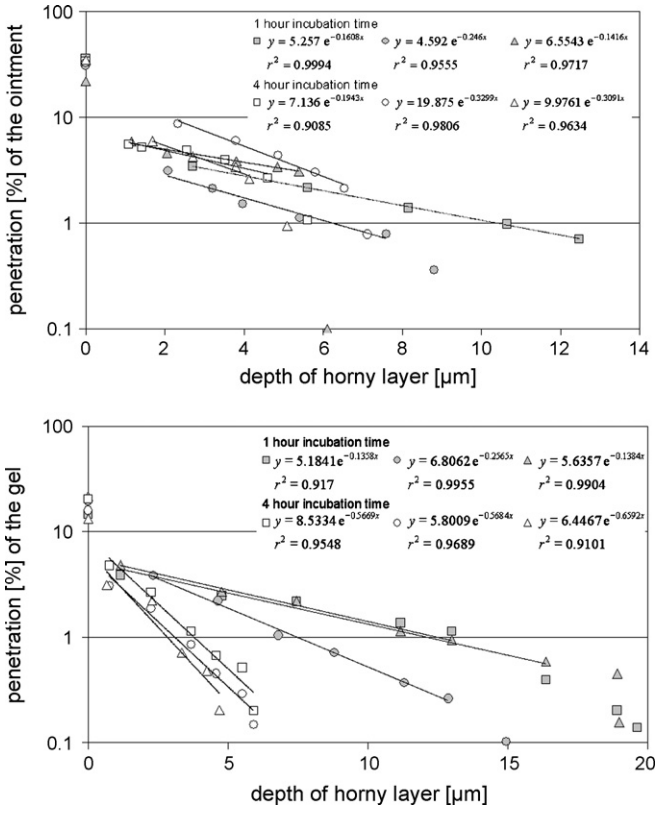

Fig. 1. Penetration profiles of Arnica ointment preparation A (top) and of Arnica gel preparation B (bottom) in three different experiments after an incubation time of 1 h (grey symbols) and 4 h (white symbols). Each point represents the percentage of SL, which has penetrated at least to the indicated depth of SC.

Table 5
SLs content in preparations A, B and C

	Preparation A	Preparation B	Preparation C
11α,13-Dihydrohelenalin methacrylate	5.71 ± 0.111 (172)	4.01 ± 0.012 (121)	56.64 ± 2.320 (1706)
11α,13-Dihydrohelenalin isovalerianate	2.68 ± 0.113 (81)	1.77 ± 0.022 (52)	25.96 ± 1.877 (782)
11α,13-Dihydrohelenalin tiglinate	1.54 ± 0.006 (46)	1.17 ± 0.010 (35)	14.88 ± 0.199 (448)
11α,13-Dihydrohelenalin acetate	0.81 ± 0.008 (24)	0.69 ± 0.035 (21)	9.01 ± 0.226 (271)
11α,13-Dihydrohelenalin isobutyrate	0.47 ± 0.038 (14)	0.33 ± 0.010 (10)	4.21 ± 0.103 (127)
11α,13-Dihydrohelenalin	not detected	0.13 ± 0.001 (4)	Not detected
Helenalin methacrylate	0.36 ± 0.263 (11)	0.07 ± 0.001 (2)	3.50 ± 0.428 (105)
Helenalin isovalerianate	Not detected	0.01 ± 0.001 (0.3)	Not detected
Helenalin tiglinate	0.04 ± 0.003 (1)	0.02 ± 0.000 (0.6)	0.72 ± 0.091 (22)
Total content of SL	11.61 ± 0.103 (349)	8.20 ± 0.045 (247)	114.92 ± 0.738 (3461)

Values are mean values of two independent measurements and given in SL mg/g preparation (in parenthesis concentrations in micromoles).

Penetration profile of Arnica preparation A containing 10% of an oily Arnica extract is shown in Fig. 1. The exponential coefficient obtained after a 1 h incubation time is similar to that one of preparation B (gel) indicating that the alcoholic as well as the oily extract may contain enhancers which increase the penetration rate of SLs. Moreover, the ointment itself also contains ingredients, such as fatty acids (more than 4.5%), which were previously shown to possess enhancing properties at concentrations between 0.25 and 10% [17]. However, an optimum in the lipid concentration seems to exist, as we demonstrated that higher concentrations of lipids (such as 10%) may form a kind of depot for the lipophilic SLs, resulting in a poorer penetration, whereas a lower lipid concentration (such as 2.5%) may act as enhancer [6]. Thus, the lipid concentrations seem to be an important factor which has to be considered when Arnica formulations are prepared for external application.

When comparing penetration profiles of both preparations after an incubation time of 4 h, a difference could be observed. Preparation B showed a significant reduction of the penetration rate after 4 h compared to 1 h (Fig. 1) and confirmed previous studies [6]. This effect may be explained by the drying of the gel over time, resulting in less movement of SLs from the gel into the SC. In contrast, ointment preparation A exhibited an almost constant penetration (Fig. 1). Accordingly, the different behaviour of preparation A and B is reflected in the exponential coefficients which is much more negative at 4 h for gel preparation B (Fig. 2). These differences have to be considered in the therapy. Using the ointment A, 2.3 ± 0.9% (after a 1 h incubation time) and 2.8 ± 0.8% (after 4 h of incubation) of the recovered SLs have penetrated at least to a SC depth of 5 μm. Concerning the gel preparation B, the amount of the recovered SLs which has penetrated at least to the same depth decrease from 2.4 ± 0.5% (after 1 h incubation time) to 0.3 ± 0.1% (after 4 h of incubation). Consequently, ointment preparations with a certain lipid content seem to be advantageous for a longer exposure compared to gel preparations. Gel preparations should be applied more frequently, but the former residues should be removed from the skin [6]. However, before final recommendations for applications can be made, it has to be kept in mind that we used healthy pig skin as a model which does not consider factors which influence penetration, such as the production of sweat and inflammation.

3.3. Influence of the SLs content and the individual 11α,13-dihydrohelenalin derivatives on penetration from an ointment preparation

The aim of most therapies is the enhancement of the effective compounds at the required location. Apart from pharmaceutical formulations or enhancers, it is mostly the concentration of the effective drug that is the deciding factor. Therefore, we studied preparation C containing a 10-fold higher content of 11α,13-dihydrohelenalin derivatives than preparation A, under the same conditions. The penetration behaviour of preparation C (Fig. 3) is similar to that one of ointment preparation A (Fig. 1). Like preparation A, preparation C shows no differences in the penetration between an incubation time of one and 4 h. The exponential coefficient of preparation C indicates a slightly worse percentage penetration compared with preparation A, but the difference is not statistically significant (Fig. 2). An almost 10-fold amount of SLs could be found in the SC applying preparation C (SLs content of 3461 μM) instead of preparation A (SLs content of 349 μM). Using an incubation time of 1 h, 5.2 μg SLs penetrated at least to the depth of 5 μm by applying preparation C, whereas only 0.54 μg SLs penetrate by applying preparation A.

Considering the penetration profiles of the five most common 11α,13-dihydrohelenalin derivatives within preparation C (see

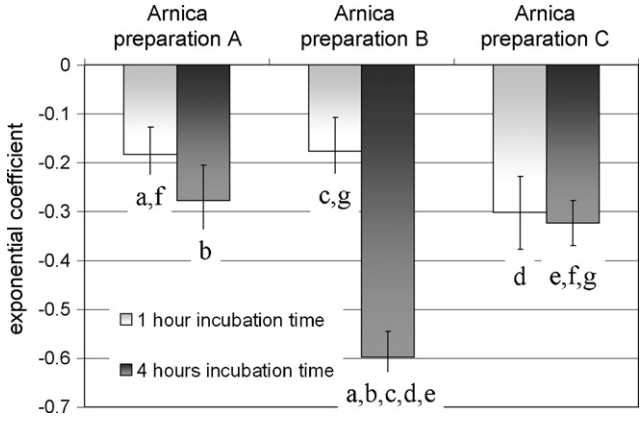

Fig. 2. Exponential coefficients (means of three different experiments) of the penetration curves of the ointment preparation A, the gel preparation B and the SLs-enriched ointments preparation C after an incubation time of 1 and 4 h. An equal letter indicates statistical significance ($p < 0.05$), respectively.

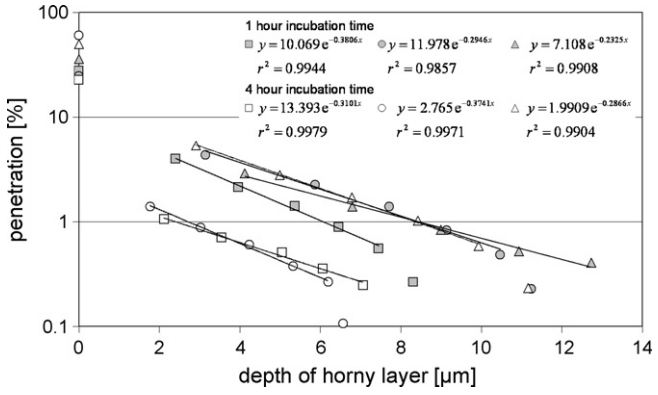

Fig. 3. Penetration profiles of Arnica ointment preparation C in three different experiments after an incubation time of 1 h (grey symbols) and 4 h (white symbols). Each point represents the percentage of SLs, which has penetrated at least to the indicated depth of SC.

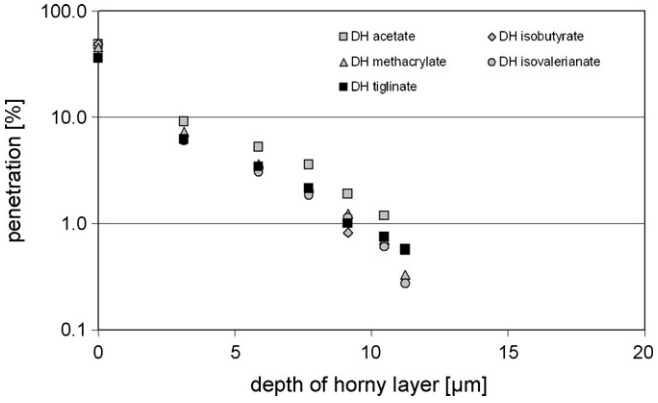

Fig. 4. Penetration profiles of the five most common 11α,13-dihydrohelenalin (DH) derivatives of Arnica preparation C after an incubation time of 1 h. Each point represents the percentage of SL, which has penetrated at least to the indicated depth of SC.

Fig. 4) all derivatives showed the same penetration behaviour. Consequently, no significant differences between their exponential coefficients were observed (Fig. 5). Therefore, neither the total amount of 11α,13-dihydrohelenalin derivatives (within limits) nor the percentage of the individual SLs influence the penetration rate.

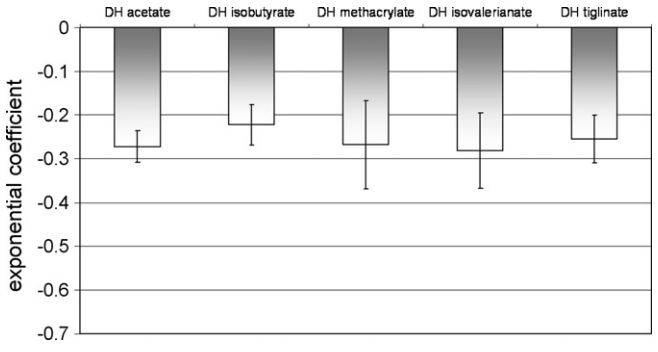

Fig. 5. Exponential coefficients of the penetration curves of the five most common 11α,13-dihydrohelenalin (DH) derivatives of preparation C. The coefficients are means of three independent experiments and did not differ significantly ($p < 0.05$) from one another.

4. Conclusion

By using a new validated GC-MS method, we could show that the penetration behaviour of SLs is independent of the type of SL, helenalin or 11α,13-dihydrohelenalin, of their acyl moieties or of the used extraction agents. Moreover, the penetration rate is also independent of the SLs content. Interestingly, the used gel shows a decrease of the penetration rate over time, whereas the penetration rate of ointments stays constant. Our results provided evidence that the total penetrated amount of SLs, which mediate the anti-inflammatory effect, depends only on the kind of formulation and the SLs content in the formulation, and not on the composition of the SLs fraction.

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