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Permeation of bioactive constituents from *Arnica montana* preparations through human skin in-vitro

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Abstract

This study investigated and characterised transdermal permeation of bioactive agents from a topically applied *Arnica montana* tincture. Permeation experiments conducted over 48 h used polydimethylsiloxane (silastic) and human epidermal membranes mounted in Franz-type diffusion cells with a methanol–water (50:50 v/v) receptor fluid. A commercially available tincture of *A. montana* L. derived from dried Spanish flower heads was a donor solution. Further donor solutions prepared from this stock tincture concentrated the tincture constituents 1, 2 and 10 fold and its sesquiterpene lactones 10 fold. Permeants were assayed using a high-performance liquid chromatography method. Five components permeated through silastic membranes providing peaks with relative retention factors to an internal standard (santonin) of 0.28, 1.18, 1.45, 1.98 and 2.76, respectively. No permeant was detected within 12 h of applying the *Arnica* tincture onto human epidermal membranes. However, after 12 h, the first two of these components were detected. These were shown by Zimmermann reagent reaction to be sesquiterpene lactones and liquid chromatography/diode array detection/mass spectrometry indicated that these two permeants were 11,13-dihydrohelenalin (DH) analogues (methacrylate and tiglate esters). The same two components were also detected within 3 h of topical application of the 10-fold concentrated tincture and the concentrated sesquiterpene lactone extract.

Introduction

Arnica montana (Leopard's bane) is a plant of the Asteraceae family whose flower heads have a long history of use in traditional medicine to treat different ailments mainly related to injuries, accidents and inflammation in animals and man (Willuhn 1998), and which has recently generated considerable interest as a herbal topical anti-inflammatory medicament. Alcoholic extracts of the dried flower head of *A. montana* have been marketed and used alone or in combination with other ingredients to produce phytopharmaceutical preparations. Ointments, creams (containing typically 20–25% of the tincture), gels, oils and lotions have been used to treat mild to moderate inflammation (Sweetman 2005). Recently, a supercritical carbon dioxide extract of these flower heads has also been marketed for anti-inflammatory use (Bergonzi et al 2005).

The flower heads of two species, *Arnica montana* and *Arnica chamissonis* ssp *foliosa*, have been classified by the German Pharmacopoeia (DAB9) as a herbal drug (*Arnicae flos*) and approved by the German Commission E to be used externally to treat various conditions such as haematomas, dislocations, sprains, bruising, rheumatic muscle and joint complaints and inflamed insect bites (Nichterlein 1995). However, its internal use has been discouraged because of severe side effects, which were reported after oral administration of flower-head preparations (Willuhn 1998).

Investigations of the chemical constituents of *Arnicae flos* resulted in the isolation and identification of more than 150 secondary metabolites. Pharmacological and toxicological studies suggest that sesquiterpene lactones (SLs) of the pseudoguainolide type, notably helenalin, and 11α,13-dihydrohelenalin and their esters (Figure 1) are the main bioactive compounds that mediate the biological activity of *A. montana* preparations (Willuhn 1998). It is interesting that the SLs have other biological actions, such as antineoplastic, antibacterial and antifungal activity (Willuhn 1998).

The quantity and type of SLs in the flower heads of *Arnica* species vary considerably. Using traditional extraction and HPLC analysis, SLs ranged from 0.31 to 0.91% w/w in

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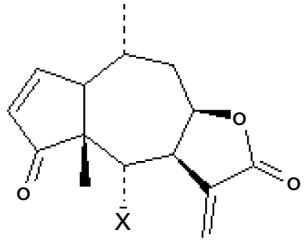

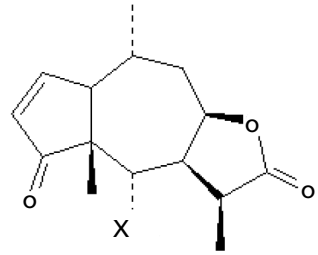

X	Helenalin and its esters	11α, 13- Dihydrohelenalin and its esters
OH	Helenalin (H)	Dihydrohelenalin (DH)
CH_3COO	H-acetate	DH-acetate
$H_2C=C(CH_3)COO$	H-methacrylate	DH-methacrylate
$(CH_3)_2CHCOO$	H-isobutyrate	DH-isobutyrate
$CH_3CH=C(CH_3)COO$	H-tiglate	DH-tiglate
$(CH_3)_2CHCH_2COO$	H-isovaleryate	DH-isovaleryate

Figure 1 Chemical structure of some sesquiterpene lactones of helenalin and 11α,13-dihydrohelenalin and their esters.

A. montana flower heads and between 0.63 and 1.74% w/w in *A. chamissonis* ssp *foliosa* (Leven and Willuhn, 1987), calculated with reference to the dried weight of the flower heads. Interestingly, the quantity of SLs increases up to 9.5% w/w in the supercritical carbon dioxide *A. montana* extract (Bergonzi et al 2005). For standardisation, the European Pharmacopoeia (2002) suggests a minimum SL content of 0.4% w/w in *A. montana* preparations used as herbal medications.

It has been shown that *Arnica* flower heads grown in different geographical areas give varying SL compositions. For example, mid-European and Spanish originating *A. montana* flowers are widely used but geographically isolated chemotypes of the plant. Qualitative SL pattern analysis has shown that helenalin esters are the dominant SLs in the middle European type, while dihydrohelenalin esters are nearly exclusively dominant in the Spanish type (Willuhn et al 1994). However, *A. chamissonis* ssp *foliosa* contains, in addition to the helenalin and dihydrohelenalin esters, arnifolins and chamissonolids (Leven & Willuhn 1987).

The efficacy of topically applied preparations of *A. montana* is controversial; some controlled clinical studies have shown that *A. montana* preparations were effective treatments for joint stiffness and acute muscle injuries compared with placebo (Schmidt & DiHom 1996; Knuesel et al 2002), but this was not the case in prevention or resolution of bruising (Alonso et al 2002). This discrepancy in clinical efficacy may be due to variability in the quantities and types of the endogenous bioactive SLs (Leven 1988), with consequent variation in their biological activity (Klaas et al 2002). A lack of quality control over potency and purity of these natural products and formulations produces variable performance of such preparations in clinical use. Therefore, to develop effective well-controlled topical *A. montana* formulations, percutaneous absorption of the bioactive agents from *A. montana* preparations merits investigation.

We have previously shown that two bioactive agents permeated through human skin following a topical application of *A. montana* tincture (Tekko et al 2003). Wagner et al (2004) attempted to investigate the penetration of SLs from tinctures derived from two different types of *A. montana*. However, these authors measured permeation of the SLs through porcine skin from the applied tincture by tape stripping with results reported as the sum of the SL compounds rather than the individual permeation of each SL. Other researchers investigated transdermal permeation of SLs from a supercritical carbon dioxide *Arnica* extract (Bergonzi et al 2005). However, dimethyl sulfoxide (DMSO) and oleic acid were used in formulating the applied *Arnica* extract and as receptor fluids in the permeation studies. Using DMSO at such high concentrations could damage the skin barrier (Barry 1983), while oleic acid is also a potent permeation enhancer and could interact with the lipid domains within the stratum corneum so enhancing skin permeability (Williams 2003).

In this study, we investigated permeation of SLs through polydimethylsiloxane membranes (silastic) and human epidermal membranes (HEMs) from topically applied tinctures of *A. montana*. Permeants were identified and quantified; their permeation profiles were elucidated and the effect of other tincture ingredients on their permeation rate was examined.

Materials and Methods

Materials

An *Arnica montana* stock tincture (ST, 10% w/v solution) in ethanol–water (45:55 v/v) was purchased from Herbal Apothecary (UK). Santonin was obtained from Sigma Chemical Company (St Louis, MO). Polydimethylsiloxane (silastic) membrane was obtained from Dow Corning, France. Methanol (HPLC grade), chloroform, n-hexane, toluene, dichloromethane and ethyl acetate (all of analytical grade) were obtained from Sigma-Aldrich (Germany). *m*-Dinitrobenzene (98%) was from Acros Organics (USA). Sodium hydroxide (98%) was obtained from BDH (UK). Tritiated water was purchased from Amersham Pharmacia Biotech (UK).

OptiPhase High Safe III scintillation cocktail (Wallac Scintillation Products) was purchased from Fisher Chemicals (UK). Human epidermal membranes (HEMs) were prepared from post-mortem Caucasian human abdominal skin specimens obtained from 12 female donors aged 55–90 years.

Preparation of donor solutions

Volumes (20, 40 and 200 mL) from the stock tincture were evaporated under vacuum at ambient temperature to dryness. The residue of each solution was reconstituted with 20 mL of methanol–water (50:50 v/v) resulting in donor systems DS1, DS2 and DS10 at concentrations of 1-, 2- and 10-fold of the stock solution, respectively.

Preparation of sesquiterpene lactones extract (SLE10)

A sample (200 mL) of stock tincture was concentrated under vacuum at ambient temperature to half its volume. This was extracted three times with 200 mL of dichloromethane–ethyl acetate (50:50 v/v) according to Leven & Willuhn (1987). The organic extracts were dried using anhydrous sodium sulfate, then evaporated under vacuum at ambient temperature to dryness. The residue was reconstituted with 20 mL of methanol–water (50:50 v/v) producing a crude 10-fold concentrated SL extract (SLE10).

Preparation of membranes

Silastic membrane was immersed in boiling de-ionised water for 5 min to remove any impurities. HEMs were prepared from full-thickness skin specimens by a heat separation technique (Kligman & Christophers 1963), and then hydrated for 24 h by immersion in an aqueous solution of 0.002% w/v sodium azide at room temperature.

In-vitro permeation studies design

Diffusion studies were performed using occluded Franz-type diffusion cells with an effective diffusional area of $2.15 \pm 0.10\,cm^2$ and a receptor compartment volume of 5 ± 0.5 mL. The receptor chambers of all diffusion cells were filled with methanol–water (50:50 v/v) to provide sink conditions for the permeants and stirred magnetically. The diffusion cells were equilibrated at 32 ± 1°C for 12 h before conducting permeation studies over 48 h. Blank diffusion cells (no donor solution loaded) were also utilised to avoid erroneous results arising from membrane components leaching into the receptor fluid during the permeation studies.

Diffusion studies were performed through silastic membrane by loading 2 mL of stock tincture (ST) into the donor compartments of each diffusion cell ($n \geq 3$). For HEMs, 2 mL of each solution (ST, DS1, DS2, DS10 and SLE10) was introduced into the donor compartment of each diffusion cell. Each donor solution was tested at least on four diffusion cells employing HEMs prepared from at least two different skin specimens. Samples (1 mL) were withdrawn from the receptor compartments at regular intervals over the diffusion study period and replenished with fresh temperature-equilibrated receptor fluid. Collected samples were kept at 4°C until analysis.

Assessment of the barrier integrity of HEMs

To investigate the effect of our protocol on HEM integrity, preliminary diffusion studies used tritiated water applied to HEMs in Franz-type diffusion cells for 87 h with aqueous sodium azide (0.002% w/v) as the receptor fluid. Tritiated water (2 mL, activity $0.4\,\mu Ci\,mL^{-1}$) was applied to the donor compartment of each diffusion cell ($n \geq 6$). Samples (1 mL) were withdrawn hourly for the first 6 h, then every 6 h thereafter.

The effects of methanol–water (50:50 v/v) on HEMs barrier integrity, after permeation studies using *A. montana* solutions, was likewise assessed by tritiated water permeation. After the *A. montana* permeation study, receptor and donor solutions were removed and cells were rinsed three times with 1 mL of fresh receptor solution. Both chambers of the diffusion cells were filled with 0.002% w/v sodium azide and equilibrated for 3 h before replacing the solution in the donor chamber of each diffusion cell with 2 mL of tritiated water. Samples from the receptor chamber were collected hourly for 6 h then mixed with 5 mL of scintillation fluid, vortexed and equilibrated before assay by scintillation counting. The tritiated water permeability coefficient (K_p) was calculated using Fick's first law:

$$J = K_p \times C_0 \quad (1)$$

where J is flux and C_0 is the concentration of the donor solution.

HPLC analysis

Analysis of the *Arnica* tincture permeants in receptor solutions was performed using a high-performance liquid chromatography method developed and validated from Leven & Willuhn (1987). The instrument (Hewlett Packard 1100; Palo Alto, California) was fitted with a UV/visible diode-array detector (DAD) and a column (250×4.6 mm i.d.) packed with 5 μm ODS (C_{18}) Hypersil particles (Jones Chromatography, UK) protected by a guard column (25×4.6 mm, i.d.) packed with the same materials. Injection was by an auto-sampler with a 100 μL loop and the solvent reservoirs were de-gassed by a steam of helium. Samples (100 μL) from permeation studies were spiked with 20 μL of 100 $\mu g\,mL^{-1}$ santonin methanolic solution (internal standard) and analysed for SL content using methanol–water (50:50 v/v) mobile phase pumped isocratically at $1\,mL\,min^{-1}$ through the column at 30°C with UV detection at 225 and 254 nm.

Zimmermann test

Zimmerman reagent was prepared as two separate solutions; solution A was prepared by dissolving *m*-dinitrobenzene (0.51 g) in toluene to 10 mL and solution B was prepared by dissolving sodium hydroxide (0.612 g) in methanol–water (4.5:2.5 v/v) to make 7 mL. Compounds were collected separately as they eluted from the HPLC column and evaporated

to dryness under vacuum at room temperature. The residue of each compound was dissolved in 100 μL of n-hexane (n = 3); 33 μL of solution A was added followed by 66 μL of solution B before shaking for 3 min. After 30 min, the colour of the lower layer was compared with a control (reagent and n-hexane mixture) to determine the presence of SLs (Bisset 1994).

Permeant identification by liquid chromatography/diode array detection/mass spectrometry (LC/DAD/MS)

The analyses were carried out on an HPLC system (Waters Alliance model 2695) equipped with a dual UV absorbance detector (Waters model 2487) and a mass spectrometer (Micromass Quattro Ultima Triple-quad). HPLC separation used the method above except the column temperature was ambient and the mobile phase was at 0.2 mL min^{-1}. Electrospray positive-ion mode (ES+) was used for ionisation. The mass spectrometer operated at a source temperature of 120°C, desolvation temperature 360°C, gas (nitrogen) flow 35 L h^{-1}, capillary voltage 5000 V and collision cell pressure was 1×10^{-4} mbar. Full scan spectra from *m/z* 20 to 400 were obtained (scan time 1 s).

Determination of principle sesquiterpene lactone permeants (C1 and C2) in stock tincture

Following permeation studies, two principle sesquiterpene lactone permeants (C1 and C2) were detected. Subsequently, their content in the stock tincture was determined. One millilitre of santonin (internal standard) in a methanolic solution (1 mg mL^{-1}) was added to 20 mL of *A. montana* stock tincture (ST) and the combined solution was then concentrated under vacuum at room temperature to 10 mL. The water layer was extracted three times with 100 mL of dichloromethane–ethyl acetate (50:50 v/v) according to Leven & Willuhn (1987). The combined organic extracts were dried using anhydrous sodium sulfate, before evaporation under vacuum at ambient temperature to dryness. The residue was dissolved in 40 mL dichloromethane and then filtered; the filter was also rinsed with an additional 20 mL of dichloromethane and added to the filtrate. The combined filtrate was evaporated under vacuum at room temperature to dryness and the residue was then dissolved in 20 mL of methanol and diluted with an equal volume of de-ionised water before adding 14 g of aluminium oxide (neutral). The suspension was shaken well for 5 min before it was centrifuged at 4500 rev min^{-1} for 20 min and then filtered. The filtrate was evaporated under vacuum at room temperature to dryness and the residue was then dissolved in 2 mL methanol and diluted with an equal volume of de-ionised water. The obtained extract was filtered through a PTFE syringe filter (0.45 μm, 13 mm) before HPLC analysis.

Calculations and data analysis

Arnica components permeation was calculated using equation 2 (Leven & Willuhn 1987).

$$C(sl) = [A(sl) \times C(st) \times f(sl)]/A(st) \quad (2)$$

where C(sl) is the amount of the sesquiterpene (μg) and A(sl) is its peak area (mAU*S), C(st) is the amount of santonin (μg) added to the receptor samples, f(sl) is the peak correlation factor between sesquiterpene lactones and santonin and A(st) is the peak area of the santonin (mAU*S). For f(sl), a value of 0.88 was taken for the dihydrohelenalin derivatives (methacrylate and tiglate esters) of the lactones after Willuhn & Leven (1991).

The relative retention factor (K_s) of each permeant with reference to the internal standard (santonin) was calculated using equation 3.

$$K_s = (t_x - t_s)/t_s \quad (3)$$

where t_s is the retention time of santonin and t_x is the retention time of the permeant.

Permeation profiles were constructed by plotting the cumulative amounts of the permeants as a function of time (h) and data are presented as mean ± standard deviation (s.d.). Results were analysed by using one-way analysis of variance/post-hoc Scheffé's tests (StatsDirect Ltd, Cheshire, UK) with significance at $P = 0.05$.

Results and Discussion

Diffusion of bioactive agents from *A. montana* stock tincture (ST) through silastic membranes

After applying ST onto silastic membranes, five well-separated components (C1, C2, C3, C4 and C5) permeated in detectable quantities, providing peaks with retention times of 10.23, 17.41, 19.55, 23.78 and 29.98 min, respectively (Figure 2A). The internal standard (santonin, S) had a retention time of 7.97 min. The relative retention factors (K_s) of C1, C2, C3, C4 and C5 were 0.28, 1.18, 1.45, 1.98 and 2.76, respectively.

Diffusion of bioactive agents from *A. montana* solutions through human epidermal membranes (HEMs)

No permeants were detected within the first 12 h of applying ST as a donor solution to human epidermal membranes. After that, two components were detected in the receptor phase, providing peaks with relative retention factors (K_s) of 0.28 and 1.18, but at very low amounts (Figure 2B). These components correspond to C1 and C2, which had permeated through silastic membranes. No further permeants were detected over the 48 h of the permeation study. Analysis of the stock tincture showed 23 ± 2.7 μg mL^{-1} of C1 and 15.1 ± 1.9 μg mL^{-1} of C2 were present (mean ± s.d., n = 3). Stability of C1 and C2 was evaluated in a skin extract using samples of receptor fluid over 48 h at room temperature. Results showed no significant difference in component C1 or C2 contents over 48 h, demonstrating that these SLs were appropriately stable throughout the experimental time-course.

Both C1 and C2 were also detected in the receptor solution after topical application of *A. montana* tincture constituents reformulated into a methanol–water (50:50 v/v) vehicle at the same concentration (DS1), 2-fold concentrated (DS2) and 10-fold concentrated donor solutions (DS10 and SLE10) with

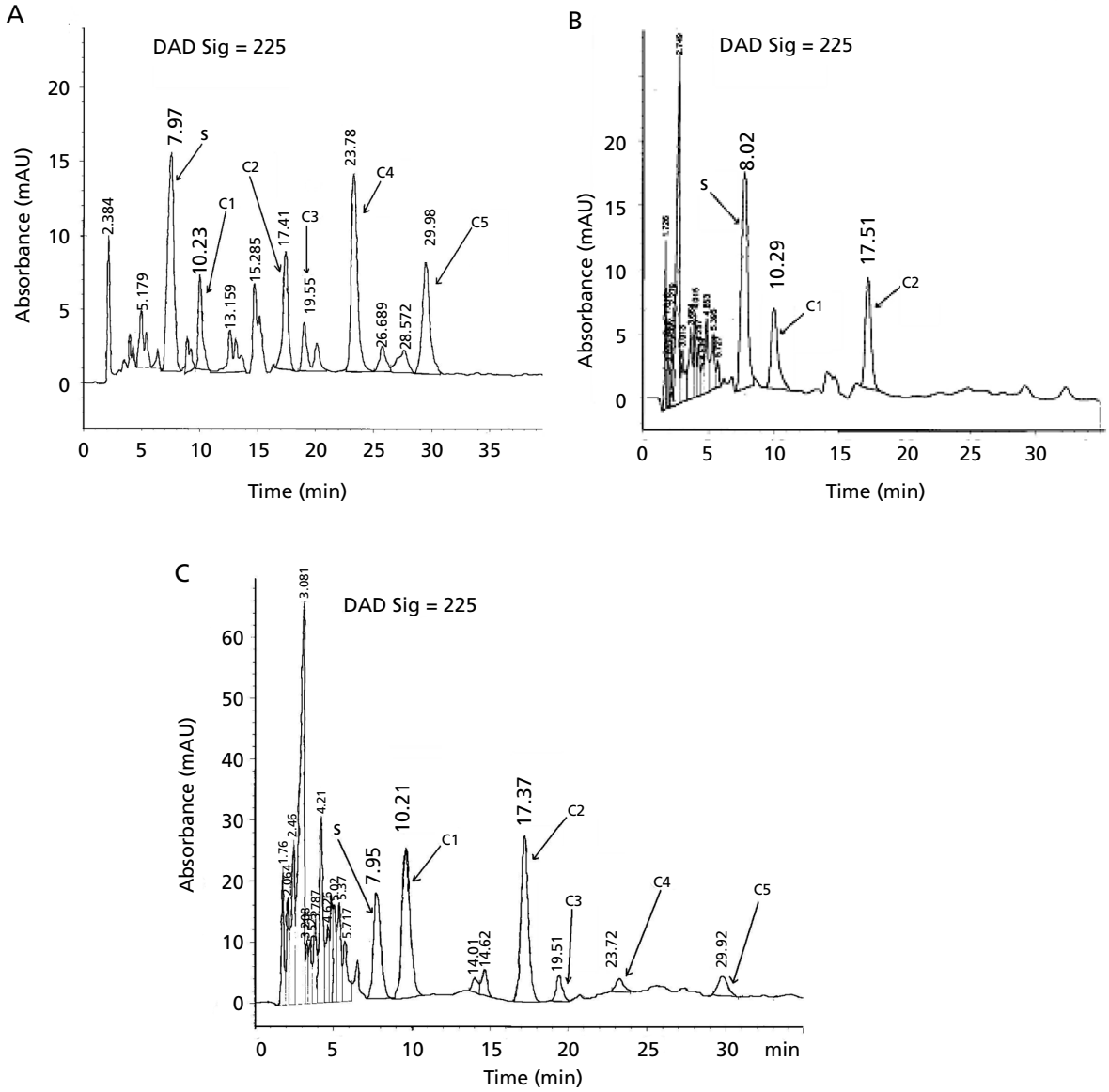

Figure 2 Chromatograms of permeants detected after applying various donor solutions of *A. montana* 12 h post application of stock tincture to silastic membranes (A), 36 h post application of stock tincture onto human epidermal membranes (B) or 12 h post application of the 10-fold concentrated sesquiterpene lactone extract (SLE10) onto human epidermal membranes (C). S indicates the santonin peak, C1, C2, C3, C4 and C5 represent peaks of the five permeants.

reference to the stock tincture. Over the 48-h study period, DS1 and DS2 only delivered detectable amounts of these two permeants. With the more concentrated DS10 and SLE10, both C1 and C2 were detected in the receptor solution 3 h post application. Additionally, other components (C3, C4 and C5), which permeated through silastic membranes, were also delivered from DS10 and SLE10 but at low levels and were first detected 9 h post dosing (Figure 2C).

Assessment of the barrier integrity of HEMs by tritiated water permeation

Tritiated water, with a typical permeability coefficient (K_p) through human skin between 0.5 and 1.5×10^{-3} cm h^{-1}, is extensively used in transdermal permeation studies to assess membrane permeability. Many workers accept HEMs with a permeability coefficient between 0.1 and 10×10^{-3} cm h^{-1} for in-vitro permeation studies (Williams 2003). Therefore, we used this compound to assess the barrier integrity of HEMs under our experimental conditions.

Diffusion studies using 0.002% w/v sodium azide solution as a receptor fluid and over a period of 87 h showed that the permeability coefficient of tritiated water was $1.49\pm0.11\times10^{-3}$ cm h^{-1}. This is in good agreement with that reported by Harrison et al (1984) and Bronaugh et al (1986), indicating that the barrier to water permeation was not compromised after 87 h of study.

After completing the permeation studies with *A. montana* systems, the K_p of tritiated water increased to $6.5\pm2.12\times10^{-3}$ cm

h^{-1}. Although this is approximately 5-fold higher than the typical value, it is still within the broadly accepted limits and showed that no gross damage to the skin membrane had occurred (Williams 2003). This finding indicates that the barrier integrity of HEMs was not greatly affected by the long period of permeation or by using methanol–water (50:50 v/v) as a receptor solution, thus giving confidence in the permeation data for components from *A. montana* preparations through HEMs.

Permeant identification by Zimmermann reagent reaction and LC/DAD/MS

SLs that contain cyclopenten-4-one moiety in their structure (e.g. helenalin and dihydrohelenalin, and their esters) or 2α-hydroxy-cyclopentan-4-one (e.g. arnifolins and dihydroarnifolins, and their esters) can react with *m*-dinitrobenzene (Zimmermann reagent) producing a dark brown coloured product (Leven 1988). Samples of C1 and C2, following permeation through HEMs, gave a dark brown colour in the lower layer of the reaction mixture in contrast to the control, which did not show any colour change, indicating that C1 and C2 are indeed SLs.

Mass spectra of C1 and C2 showed the characteristic fragmentation patterns of m/z ions (Table 1). The common ions (*m/z*) species using (ES+) are $[MH]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$.

The ion molecular weights in Table 1 suggest that C1 and C2 are probably methacrylate (DHM) and tiglate (DHT) esters of 11α,13-dihydrohelenalin, with molecular weights of 332.4 and 346.4, respectively. This may be expected since the tincture used flower heads of Spanish *A. montana*, which has been shown to contain 11α,13-dihydrohelenalin esters of which methacrylate and tiglate esters are the most abundant (Leven 1988).

To date, the literature does not describe the physicochemical properties of methacrylate (C1) and tiglate (C2) esters of 11α,13-dihydrohelenalin. However, calculated values for log partition coefficient (octanol/water) and aqueous solubility are log $P_{o/w} = 2.07$, solubility 0.131 mg mL^{-1} for C1 and log $P_{o/w} = 2.82$ and solubility 0.0476 mg mL^{-1} for C2 (Interactive Analysis log P predictor; ChemSilico LLC, USA). Clearly, both permeants are lipophilic with characteristics somewhat similar to established transdermally applied agents, such as estrogens. Thus both C1 and C2 appear to be good candidate molecules for topical application.

Table 1 Fragments species (m/z) of santonin and the two permeants C1 and C2, and their percentage in the mass spectrum

Compound	Fragments (m/z)	Fragment %	Fragment species	MW
Santonin	247	100	$[MH]^+$	246
	269	100	$[M+Na]^+$	
C1	333.4	60	$[MH]^+$	332.4
C2	347.4	20	$[MH]^+$	346.4
	364.4	25	$[M+NH_4]^+$	
	369	100	$[M+Na]^+$	

Influence of vehicle and concentration on C1 and C2 permeation

Figures 3 and 4 show permeation profiles of C1 and C2, respectively, after applying donor solutions ST, DS1, DS2, DS10 and SLE10 onto HEMs. Cumulative amounts (μg cm^{-2}) of C1 and C2 permeating silastic and HEMs 24 h after applying these donor solutions are summarised in Table 2. For comparison, the cumulative amounts of C1 and C2 that traversed the silastic membrane within 24 h of applying the stock tincture were 3.46 ± 0.22 and 3.61 ± 0.69 μg cm^{-2}, respectively. These levels are around 5-fold higher than those through HEMs and are as expected considering that silastic membrane is more permeable to lipophilic permeants than HEMs (Barry 1983). Likewise, pseudo-steady-state fluxes of these permeants through the artificial membrane were about 5-fold higher than through HEMs (Table 3).

An analysis of variance test on the cumulative amounts of both C1 and C2 permeating silastic and HEMs 24 h from these donor solutions shows that F (variance ratio) for both C1 and C2 are 37.63 and 297, respectively, which is >3.44 ($F_{critical}$) indicating that at least two means from each group are significantly different from one other. A post-hoc Scheffé's test was then performed to determine which specific mean pairs differed significantly at $P = 0.05$.

Permeation profiles of C1 from ST and DS1 were similar (Figure 3A, B), suggesting that changing the vehicle from ethanol–water (45:55 v/v) in ST donor solution to methanol–water (50:50 v/v) in DS1 donor solution had little effect on C1 permeation. Applying DS2 and DS10 resulted, as expected, in significant increases in the permeated amounts of C1 (Figure 3C, D). Table 2 shows that stock tincture and DS1 delivered low amounts of C1 within 24 h (0.55 and 0.63 μg cm^{-2}, respectively) whereas increasing the concentration of *A. montana* constituents 2 and 10 fold delivered 3.1 and 9.2 μg cm^{-2} of C1, respectively, approximately 6- and 16-fold higher than from stock tincture. This suggests that the increased C1 permeation was not simply due to increased thermodynamic activity in the donor solution (which provides the driving force for drug permeation), but a penetration enhancing effect of other *A. montana* constituents, such as essential oils and other components, may operate. Wagner et al (2004) reported that thymol derivatives, one of the major components of the essential oils in *A. montana* tincture, act as permeation enhancers when used at high concentrations.

Table 2 also shows no significant difference ($P > 0.999$) in the amounts of C2 permeating in 24 h from stock tincture and DS1, indicating that changing the vehicle from ethanol–water (45:55 v/v) to methanol–water (50:50 v/v) had no effect on permeation. However, from the permeation profile in Figure 4A, B, it appears that at extended time periods permeation from DS1 may be reduced compared with stock tincture. Applying DS2, unexpectedly, did not show a significant increase in C2 penetration ($P > 0.05$; Figure 4C, Table 2) and DS10 application resulted in around a 6-fold increase in C2 absorption (Figure 4D, Table 2).

As described above, *Arnica* constituents may act as permeation enhancers when present at sufficient thermodynamic activity as in DS10, particularly for C1, but these enhancers

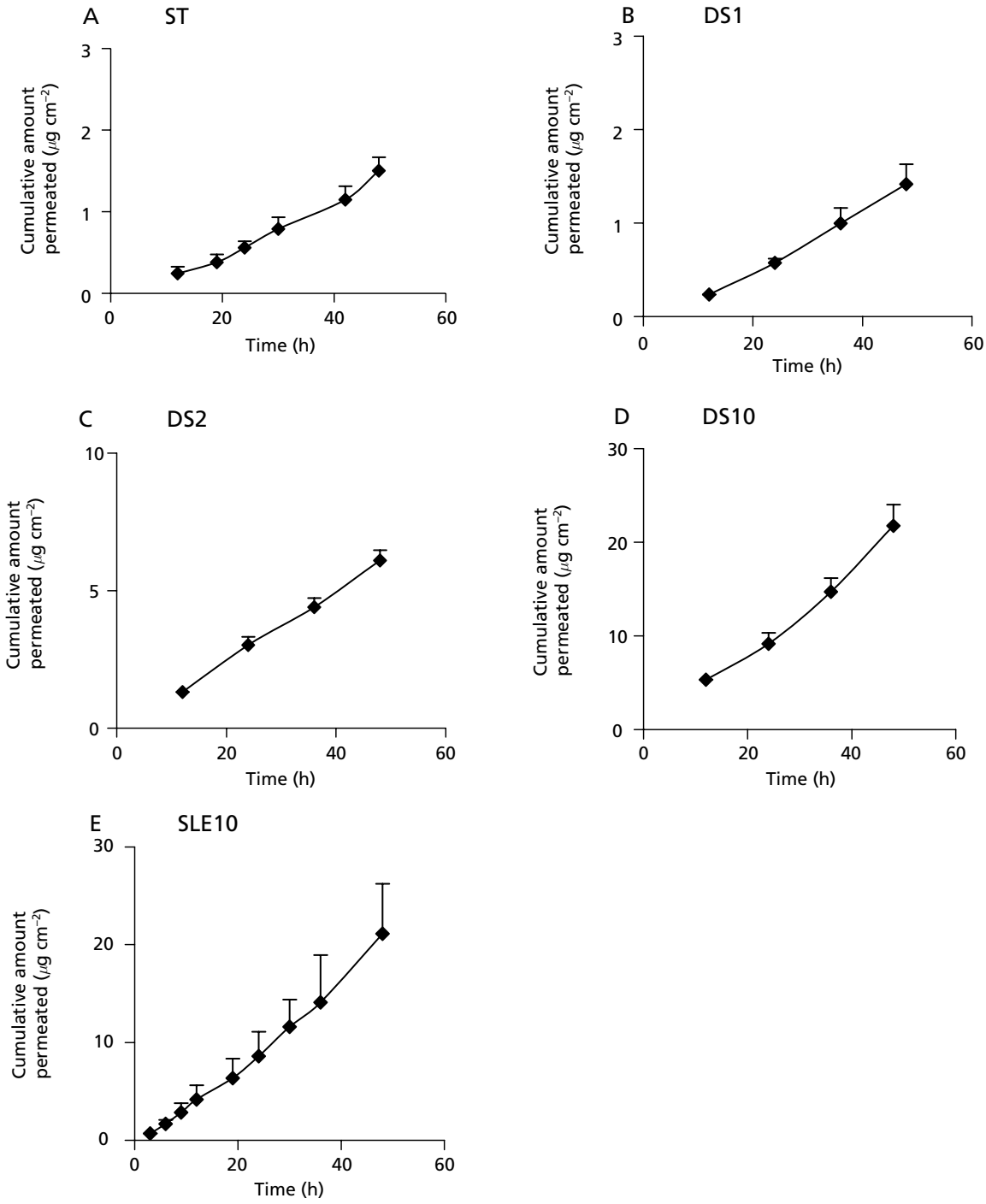

Figure 3 Permeation profiles of component C1 through human epidermal membranes from stock tincture (ST) (A), DS1 (B), DS2 (C), DS10 (D) or from SLE10 (E).

appear to have little effect on C2 permeation or may even retard its delivery. HPLC analysis showed that C2 retention time was considerably greater than that of C1; C2 is more lipophilic than C1 (C2 calculated log $P_{o/w}=2.82$, C1 calculated log $P_{o/w}=2.07$) and so may be retained in the stratum corneum thus hindering delivery through the epidermal tissue. As a complex naturally derived product, *A. montana* tincture is a mixture of many lipophilic and hydrophilic constituents at very low amounts and, after concentration, some of these components may interact with skin constituents causing changes in membrane permeability. An investigation into the effects of other endogenous *A. montana* compounds on permeation of C1 and C2 through human skin membranes would be of great interest.

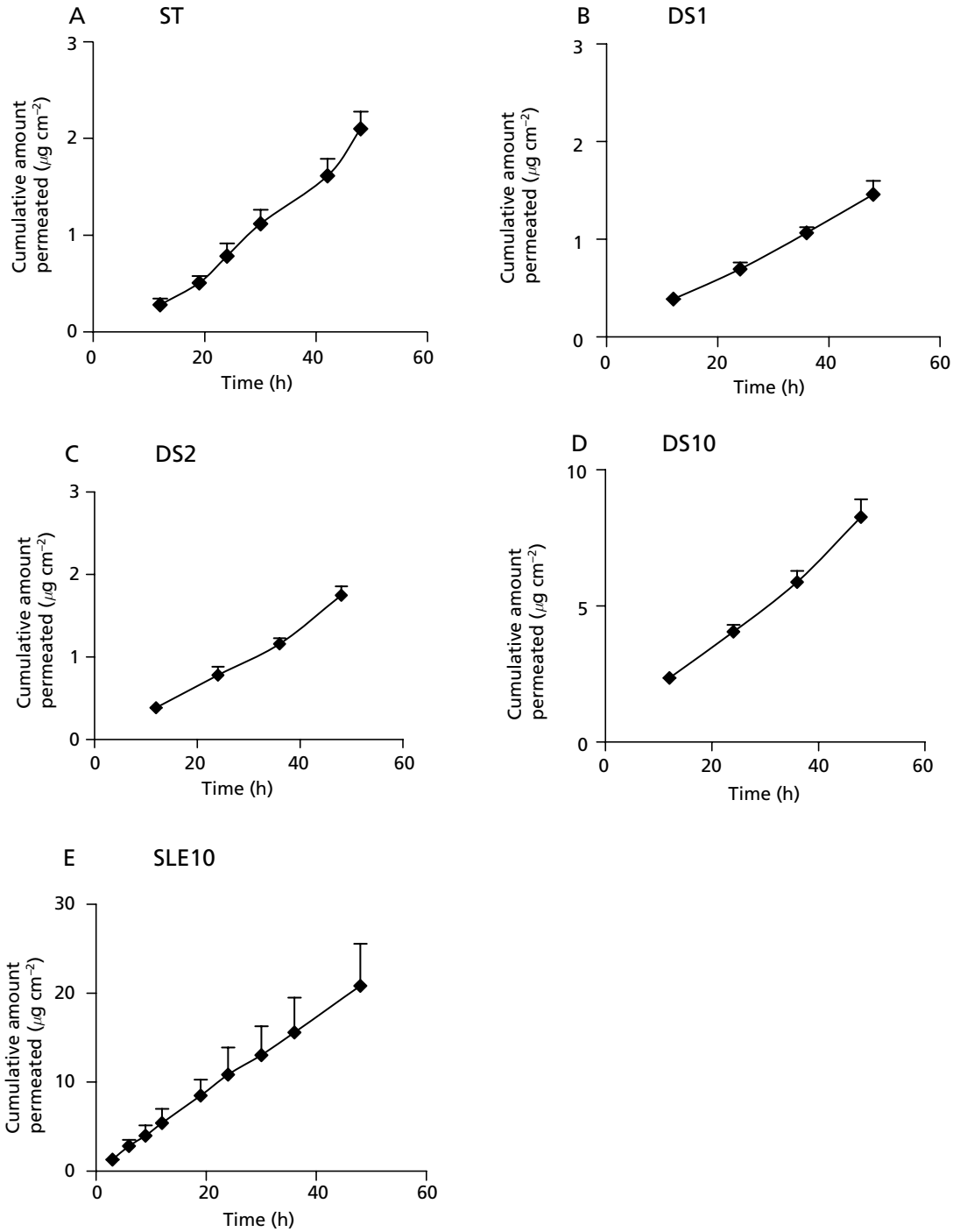

Figure 4 Permeation profiles of the component C2 through human epidermal membranes from stock tincture (ST) (A), DS1 (B), DS2 (C), DS10 (D) or from SLE10 (E).

Permeation of C1 and C2 from SLE10

Isolating SLs with other lipophilic constituents from *A. montana* tincture and concentrating them 10 fold also significantly increased the amount of C1 permeated in comparison with that from stock tincture, though permeation was not significantly different ($P > 0.99$) from that using DS10 (Figure 3E). In contrast, SL extraction produced a significant increase ($P < 0.05$) in the amount of C2 permeated in comparison with that from DS10 (Figure 4E, Table 2). The SL extraction process essentially removed many hydrophilic constituents from DS10. Increased delivery from the SL extract from which hydrophilic tincture components have been removed in comparison with the intact DS10 system suggests that the hydrophilic moieties may retard C2 permeation.

Table 2 Cumulative amounts of C1 and C2 permeated through human epidermal membranes 24 h after applying donor solutions (stock tincture (ST), DS1, DS2, DS10 and SLE10) and permeation enhancement ratios (ER, calculated relative to permeation from ST)

Donor solution	C1 (μg cm^{-2})	ER	C2 (μg cm^{-2})	ER
ST	0.55 ± 0.2	—	0.72 ± 0.2	—
DS1	0.63 ± 0.2	1.1	0.74 ± 0.1	1
DS2	3.1 ± 0.2	5.6	0.73 ± 0.2	1
DS10	9.2 ± 1.5	16.7	4.1 ± 0.4	5.7
SLE10	8.6 ± 2.8	15.6	11.7 ± 3.1	16.3
ST through silastic membrane	3.46 ± 0.22	—	3.61 ± 0.69	—

Data are mean ± s.d., n ≥ 4.

Table 3 Steady-state fluxes of C1 and C2 after applying *A. montana* donor solutions (stock tincture (ST), DS1, DS2, DS10 and SLE10) onto human epidermal membranes

Donor solution	C1 flux (μg cm^{-2} h^{-1})	C2 flux (μg cm^{-2} h^{-1})
ST	0.032 ± 0.004	0.044 ± 0.004
DS1	0.030 ± 0.006	0.030 ± 0.004
DS2	0.128 ± 0.015	0.036 ± 0.004
DS10	0.44 ± 0.043	0.167 ± 0.012
SLE10	0.43 ± 0.15	0.43 ± 0.11
ST through silastic membrane	0.16 ± 0.013	0.15 ± 0.013

Data are mean ± s.d., n ≥ 4.

However, SLE10 also concentrated other lipophilic constituents such as fatty acids and hydrocarbon monoterpenes, which are present in the original tincture (Bisset 1994) and which may then interact with lipid domains within stratum corneum (Williams & Barry 1989; Cornwell et al 1996), promoting permeation. These findings suggest that extraction of SLs along with other non-water soluble compounds improves transdermal delivery of C1 and C2 and potentially other SLs after topical application.

Efficacy of transdermal delivery of SLs in clinical therapy

Experimentally determined steady-state fluxes of methacrylate and tiglate esters of dihydrohelenalin (DHM and DHT) through HEMs after topical application of stock tincture and concentrated donor solutions are summarised in Table 3.

In-vitro studies showed DHM and DHT esters at 100 μM (corresponding to 33.2 and 34.6 μg mL^{-1}, respectively) gave complete inhibition of NF-AT and NF- $\kappa\beta$, proteins that are responsible for the transcription of genes encoding various inflammatory mediators (Klaas et al 2002). Furthermore, in vivo studies demonstrated that DHM showed strong activity as a topical anti-inflammatory agent and reduced croton oil-provoked ear oedema in mice at a dosage of 0.332 μg cm^{-2} (Klaas et al, 2002).

For systemic treatment using SLE10 from a typical patch size of 10 cm^2, the permeated amounts of DHM and DHT will be approximately 100 μg per day. Similar application of a stock tincture formulation would only deliver around 7.5 and 10 μg of DHM and DHT per day, respectively. To date, the pharmacokinetics of DHM or DHT have not been examined, so systemic efficacy of these dosages remains unclear. However, our findings show that detectable amounts of pharmacologically active agents from *A. montana* can be delivered through human skin in-vitro.

Conclusions

From an ethanolic tincture of *A. montana* flower head extract, five compounds permeated through silastic membranes in detectable quantities. HEM permeation studies showed that two of these traversed human skin from the tincture, though at very low levels. A chemical colour reaction (Zimmermann test) suggested that these two compounds were sesquiterpene lactones that contain either a cyclopenten-4-one (e.g. helenalin and dihydrohelenalin and their esters) or a 2α-hydroxycyclopentan-4-one moiety in their structure. LC/DAD/MS analysis indicated that these two compounds are probably dihydrohelenalin methacrylate and tiglate, respectively. Extraction of SLs and other lipophilic compounds from the tincture best delivered total amounts of DHM and DHT. Further studies are required to deliver these bioactive agents transdermally in a rapid and controlled manner for effective systemic treatment, as these two SLs compounds could be of use as anti-inflammatory agents after isolation from herbal sources.

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