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Evaluation of skin permeability of sesquiterpenes of an innovative supercritical carbon dioxide *Arnica* extract by HPLC/DAD/MS

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Recently, a supercritical carbon dioxide dried extract of *Arnica* flower, with a very high sesquiterpene content was developed. In view of using this extract in formulations for cutaneous application, the ability of sesquiterpenes to permeate the skin was evaluated by HPLC/DAD/MS using the following permeation enhancers: oleic acid (OA), dimethylsulfoxide (DMSO), lauroglycol, isopropyl myristate and Tween 80. A skin permeation study was performed using a modified Franz diffusion cell and the human stratum corneum and epidermis as membrane. Solutions of the enhancers were directly analysed after dilution with methanol or DMSO. A simple RP-HPLC-DAD-MS method for the quantification of the sesquiterpenes was developed and the method showed no interference with the other substances extracted from the skin and the permeation enhancers. The study evidenced that among the selected skin permeation enhancers, DMSO and OA can be considered as good candidates to be used in preparations for cutaneous application.

1. Introduction

Arnica (*Arnica montana* L.) is a well known herbal drug with anti-inflammatory activity and many herbal drug preparations can be found on the market (Pinchon and Pinkas 1988). Different constituents are present in the plant, i.e. lactone sesquiterpenes (LTS), flavonoids, phenylcarboxylic acids (such as chlorogenic acid and its derivatives) and coumarins.

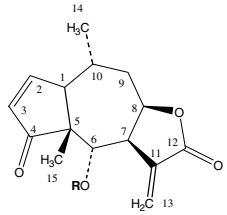
However, among them, LTS represented by esters of helenalin and dihydrohelenalin are the main active principles and their effects seem to be due to the inhibition of the activity of some enzymes and other functional proteins in living cells (Picman 1986; Schmidt 1999). Due to these constituents, *Arnica* is used in external preparations to treat bruises, sprains and inflammation caused by insect bites and for the symptomatic treatment of rheumatic complaints (Willuhn 1989). Thus, in the development of pharmaceutical preparations it appears attractive to have extracts with a high content of LTS.

Arnica tincture can be evaporated to dryness and the conventional dried extract can be used as the active ingredient of herbal drug preparations. However, both marketed tinctures and dry extracts have a small content of LTS although other constituents are also present (Willuhn 1989). Recently, a supercritical carbon dioxide (CO_2) extract of *Arnica* flowers (CO_2AE) was developed with a high content of LTS (Bilia et al. 2002).

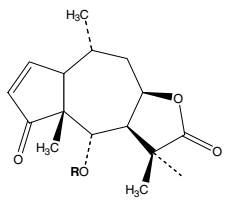
The aim of this study was to evaluate the permeability of LTS induced by different skin permeation enhancers using a simple, fast, direct and sensitive HPLC analytical method. Five well-known skin permeation enhancers were tested: oleic acid (OA), dimethylsulfoxide (DMSO), Lauroglycol® (LG), isopropyl myristate (IPM), and Tween® 80 (TW).

The *ex vivo* human skin permeation study was performed in view of using the supercritical CO_2 extract (CO_2AE) in formulations intended for cutaneous application.

The skin permeation study was performed using a modified Franz diffusion cell and human stratum corneum and epidermis as membranes (Minghetti 1999).



1: Helenalin



2: Dihydrohelenalin

R		
	a: CH_3CO-	-acetyl
	b: CH_2=C(CH_3)CO-	-α-metacryl
	c: CH_3CH=C(CH_3)CO-	-tiglioyl
	d: $(CH_3)_2CHCO$-	-isobutirryl
	e: $CH_3CH_2CH(CH_3)CO$-	-2-metylbutirryl
	f: $(CH_3)_2CHCH_2CO$-	-isovalerionyl

2. Investigations, results and discussion

The aim of this study was to evaluate by simple HPLC analysis the effects induced by different skin permeation enhancers (oleic acid, dimethylsulfoxide, lauroglycol, isopro-

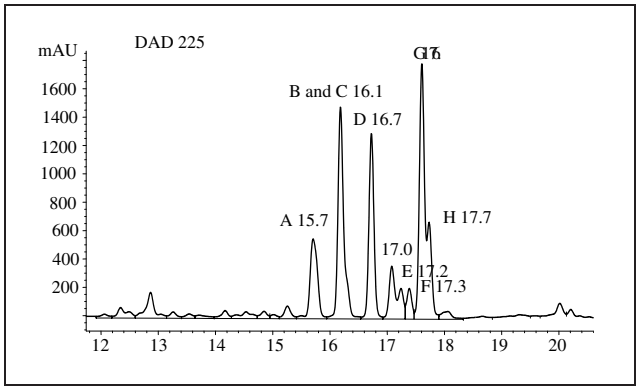


Fig. 1: Chromatogram of CO_2AE; A ($t_R = 15.5$) dihydrohelenalin isobutirryl, $[M + H^+ + Na^+] = m/z$ 357. B ($t_R = 16.1$) helenalin isobutyryl, $[M + H^+ + Na^+] = m/z$ 355. C ($t_R = 16.1$) dihydrohelenalin tiglioyl $[M + H^+ + Na^+] = m/z$ 369. D ($t_R = 16.7$) helenalin tiglioyl $[M + H^+ + Na^+]$ di m/z 367. E ($t_R = 17.2$) dihydrohelenalin-2-metylbutyryl $[M + H^+ + Na^+]$ di m/z 371. F ($t_R = 17.3$) dihydrohelenalin isovalerianyl $[M + H^+ + Na^+] = m/z$ 371. G ($t_R = 17.6$) helenalin isovalerianyl $[M + H^+ + Na^+] = m/z$ 369. H ($t_R = 17.7$) helenalin-2-metylbutyryl $[M + H^+ + Na^+] = m/z$ 369

pylmiristate, and Tween 80) on the percutaneous absorption of sesquiterpene lactones of *Arnica* in order to prepare herbal drug preparations (HDPs) with high content of LTS.

Recently, an innovative supercritical carbon dioxide (CO_2) extract (CO_2AE) with a high sesquiterpene content has been marketed (Bilia et al. 2002).

A typical chromatogram of the CO_2AE is shown in Fig. 1. Separation of constituents was obtained in a reasonable analysis time (28 min). The CO_2AE showed a high content of sesquiterpenes (about 9.5% w/w), helenalin and dihydroelenalin derivatives, but neither flavonoids nor quinic acid derivatives were detected.

The European Pharmacopoeia (4th edition, 2002) reports an HPLC method for the quantification of LTS in the herbal drug (*Arnica* flower) expressed as helenalin tiglinate and calculated using santonin as reference compound (European Pharmacopoeia 2002). However this method, i. e. RP-18 column and water-methanol as mobile phase, was not able to evaluate the investigated matrices, so other stationary phases with different granulometry were tested using different organic aqueous-based mobile phases, including water-acetonitrile, water-methanol and water-acetonitrile-methanol in combination with-phosphoric acid or formic acid.

The best separation conditions were obtained using a Hibar® Purospher STAR RP-18e (5 µm, 250 × 4.6 mm) column as stationary phase and the three-step gradient as the mobile phase reported in Table 1. This HPLC method was simple, direct and gave good accuracy, specificity and reproducibility, together with a good separation of all the LTS of the CO_2AE. In addition no interference with the other substances extracted from the skin and the permeation enhancers was observed.

The method has satisfactory been applied to the quantitative analysis of these constituents directly in the extract but also

Table 1: Mobile phase of HPLC analysis

Time (min)	H_2O (%)	CH_3OH (%)	CH_3CN%
0.10	68	10	22
20.00	5	15	80
28.00	68	10	22
35.00	68	10	22

Table 2: Solubility (± S.D., Standard deviation) of LTS in different vehicles

LTS amount (µg/ml) ± S.D.		Receiver medium (v/v)	
	Time zero	After 3 days	After 7 days
H_2O/EtOH 50/50	1.04 ± 0.03	1.20 ± 0.04	1.24 ± 0.02
H_2O/EtOH 30/70	1.87 ± 0.05	1.95 ± 0.03	2.27 ± 0.01
H_2O/EtOH 10/90	2.35 ± 0.09	2.89 ± 0.07	2.94 ± 0.06
H_2O/PG 50/50	0.43 ± 0.02	1.19 ± 0.03	1.12 ± 0.03
H_2O/PG 30/70	0.76 ± 0.02	1.07 ± 0.03	1.05 ± 0.05
H_2O/PG 10/90	1.44 ± 0.04	1.98 ± 0.05	2.0 ± 0.02

Table 3: Skin permeated amounts of the LTS of the CO_2AE extract

	LTS permeated amounts (µg/cm² ± d.s.)		
Enhancer	After 4 h	After 7 h	After 24 h
LG	n.d.	n.d.	n.d.
IPM	n.d.	n.d.	n.d.
TW	n.d.	n.d.	n.d.
DMSO	55 ± 21	467 ± 161	1010 ± 181
OA	2367 ± 166	2443 ± 195	2520 ± 277

Legend: LG Lauroglycol®; IPM Isopropylmyristate; TW Tween® 80; DMSO Dimethylsulfoxide; OA Oleic acid

in the receiver medium of a Franz diffusion cell and did not show any interference with other constituents of the skin that could be extracted during the permeation studies.

Due to the low solubility of CO_2AE both in aqueous, EtOH or propylene glycol (Table 2) mixtures (all the samples were analyzed in triplicate), generally used as receiver medium, the same permeation enhancer of the donor solution was used as receiver medium to obtain an efficient collection of the LTS during the permeation study. Results concerning the skin permeation study of LTS obtained by HPLC analysis are reported in Table 3.

In the case of LTS solutions obtained solubilizing the CO_2AE in LG, IPM and TW, the permeated amounts were not detected. In the case of DMSO, the sesquiterpene permeated amounts increased gradually. This skin permeation profile is reported in Fig. 2 as an example.

Considering that 2% w/v is the maximum solubility of the extract in this solvent, no further enhancement of LTS permeation could be obtained, as the maximum thermodynamic activity was reached. OA increased the sesquiter-

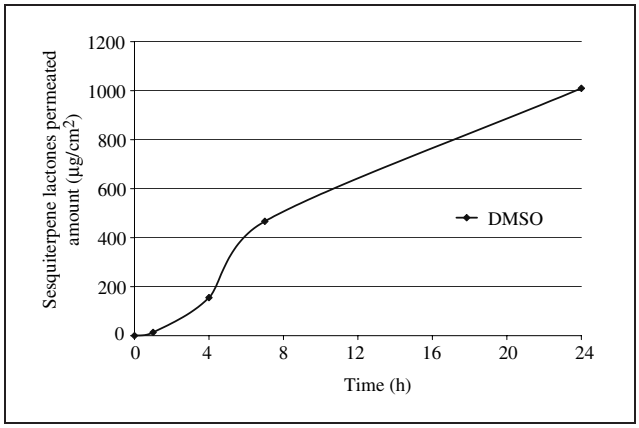


Fig. 2: *Ex vivo* skin permeation profile of sesquiterpene lactones through SCE (DMSO solution)

pene flux in a very significant way so that the equilibrium between the donor and the receptor phase was reached within the first hours (Table 3). The very high permeation flux is probably due to a disorganization of the intercellular lipid network causing a high LTS diffusion through the SCE. As OA and the extract are freely mixable, it would be possible to further increase LTS concentration in the vehicle, obtaining a higher concentration gradient across the skin that could improve the LTS permeated amount.
Among the investigated skin permeation enhancers, DMSO and OA can be considered good candidates as penetration enhancers for cutaneous application containing *Arnica* extracts as they were able to significantly enhance LTS skin permeation.

3. Experimental

3.1. Chemicals, samples and standards

A commercial CO_2 extract was kindly given by Arkopharma (Carros Cedex, France), (CO_2AE). Santonin was purchased (99.0%) from Sigma-Aldrich (Milan, Italy).
Dimethylsulfoxide-d_6 (99.8%) was purchased from Euriso-top (Gif-Sur-Yvette, France). Acetonitrile and methanol were HPLC grade from Merck (Darmstadt, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA). Propylene glycol, PG (ACEF, Italy). Ethanol, EtOH (Carlo Erba, Italy)
Oleic acid, OA (Polichimica, Italy). Dimethylsulfoxide, DMSO (Gaylord Chemical Corporation, USA). Lauroglycol®, LG (Gattefossé, France). Isopropyl myristate, IPM (ACEF, Italy), and Tween® 80, TW (Bregaglio, Italy).

3.2. HPLC-DAD-MS analysis

The HPLC system consisted of a HP 1100L instrument with a Diode Array Detector and managed by a HP 9000 workstation (Helwett & Packard, Palo Alto, CA, USA).
Different columns were used: 201 TP 54 RP-18 (5 μm, 254 × 4.4 mm, 300 Å, Vydac Separation Group Hesperia, CA, USA), ODS Hypersil (5 μm, 200 mm, 4.6 i.d.), ODS Spherisorb 5 (25 cm × 4 mm), Hibar® Purospher STAR RP-18e (5 μm, 250 × 4.6 mm, Merk, Darmstadt, Germany). Different organic aqueous-based mobile phases, including water-acetonitrile, water-methanol and water-acetonitrile-methanol in combination with phosphoric acid or formic acid were also tested.
The injected volume was 20 μl. The chromatograms were acquired at 225, 254, 280 and 350 nm. The LTS were evaluated at 225 nm. The LTS present in the CO_2AE and quantified were: dihydrohelenalin isobutyryl, helenalin isobutyryl, dihydrohelenalin tiglioyl, helenalin tiglioyl, dihydrohelenalin-2-metylbutyryl, dihydrohelenalin isovalerianyl, helenalin isovalerianyl and helenalin-2-metylbutyryl.
The limit of santonin quantification was 0.03 μg (sample concentration 0.01 mg/ml in CH_3CN, injected 3 μl, SD ± 0.28, n = 5).
The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Hewlett & Packard, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of HPLC-DAD analysis. Mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values: gas temperature 350 °C at a flow rate of 10 L/min, nebulizer pressure 30 p.s.i., quadrupole temperature 30 °C, and capillary voltage 3500 V. Full scan spectra from m/z 100 to 800 in the positive ion mode were obtained (scan time 1 s).

3.3. Preparation of samples

A standard stock solution of santonin was obtained by dissolving 1 mg in 1 ml of methanol.
The CO_2AE solution for each permeation enhancer was prepared at a concentration of 2% w/v. The permeated solutions were diluted 1 : 1 with methanol or DMSO. The solution was analyzed directly by HPLC-DAD-MS.

3.4. Quantification of constituents

The percentage of total LTS, expressed as santonin, was calculated using a calibration curve. The calibration curve ($y = 0{,}0006x - 0{,}1018$, $R^2 = 0{,}9993$) shows a linear relationship in the range investigated (25–150% of the standard solution, 1 mg/ml).
The cumulative amount permeated through the SCE per unit area was calculated from the concentration of each substance in the receiving medium and plotted as a function of time. Each data point on the plot represents a mean of three permeation experiments.

3.5. In vitro skin permeation study

Human abdomen skin was used for the skin permeation studies. Skin samples, obtained by surgical operation, were used within 48 h of removal and the epidermal layer (comprising the stratum corneum and viable epidermis, SCE) were separated by immersing the skin in distilled water at 60 ± 1 °C for 1 min and peeling it from the dermis. The heat-separated SCE membranes were dried in a desiccator at approximately 25% RH, wrapped in aluminium foil and stored at −20 ± 1 °C until use. Dried SCE samples were rehydrated at room temperature by immersion in saline solution for about 16 h before use. Each membrane was carefully mounted on a modified Franz-type diffusion cell of approximately 5 ml receiver capacity and fastened with a rigid clamp.
These cells, with respect to the original Franz-type diffusion cell, had a wider vertical column and the bowl shape was removed. Cells had a diffusion area of 0.636 cm^2. Each cell was individually calibrated with respect to its receiver volume and diffusion area.
At the start of the experiment, 500 μl of the CO_2AE solution in DMSO or 500 μl of each permeation enhancer containing the CO_2AE was applied to the diffusion cell as donor phase. Considering the low solubility of the extract in DMSO, all the solutions were prepared at the 2% w/v concentration.
The receiver medium was selected on the basis of the solubility study. The receiver medium was continuously stirred, with a small magnetic bar, and thermostated at 37 ± 1 °C, so that the skin surface temperature was 32 ± 1 °C. At predetermined times (1, 4, 7, and 24 h) 0.2 ml samples were withdrawn from the receiver compartment and replaced with fresh receiver medium. Each sample was diluted with methanol or DMSO (1 : 1 v/v). Samples were analyzed by HPLC following the method described below.
The recovery percentage in the different permeation enhancers, i.e. oleic acid (OA), dimethylsulfoxide (DMSO), lauroglycol (LG), isopropylmiristate (IPM), and Tween 80 (TW) was determined at four santonin concentration levels i.e. 25%, 50%, 100% and 150% relative to 1.5 mg/ml santonin standard. This was achieved by adding increasing amounts of santonin to the permeation enhancers and by diluting 1 : 1 with MeOH or DMSO. For each concentration the sample was analysed four times. In all determinations recoveries were more than 95%.

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