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Development and stability of semisolid preparations based on a supercritical CO_2 Arnica extract

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Abstract

Conventional herbal drug preparations (HDP) based on *Arnica montana* L. have a low content of the active principles, sesquiterpene lactones, which show poor stability and low physical compatibility in semisolid formulations. Recently, an innovative supercritical carbon dioxide (CO_2) extract with high sesquiterpene content has been marketed. Development of six semisolid preparations (cetomacrogol, polysorbate 60, polawax, anphyphil, natrosol and sepigel) based on this innovative CO_2 extract is discussed. Stability of these preparations was investigated according to ICH guidelines. The evaluation of in vitro release of active constituents was performed using the cell method reported in the European Pharmacopoeia. Preliminary data on in vivo permeation of three selected formulations is demonstrated using the "skin stripping" test, according to the FDA, in healthy subjects. Analysis of sesquiterpene lactones within the extract and in vitro and in vivo studies was performed by RP–HPLC–DAD–MS method. The cetomacrogol showed the best release profile in the in vitro test, while in the in vivo test the best preparation resulted polysorbate 60 and polawax.

Keywords: *Arnica montana* L. (Asteracee) supercritical CO_2 extract; Sesquiterpenes; Semisolid preparations; In vitro and in vivo tests

1. Introduction

Arnica montana L. is a well known plant in popular medicine for its anti-inflammatory action [1,2]. The herbal drug "Arnica flower" contains several classes of constituents such as flavonoids, quinic acid derivatives, sesquiterpenes, acetylenes and essential oil (0.2–0.35%) [3]. According to the European Pharmacopoeia, active constituents of Arnica are represented by sesquiterpene lactones (Fig. 1) and the herbal drug should contain not <0.40% (w/w) of total sesquiterpene lactones calculated as helenalintiglinate with reference to the dried drug [3]. Many herbal drug preparations (HDPs) and herbal medicinal products (HMPs) are marketed in Europe [4] and contain glycolic extract, fluid extract, tincture or dried extract. Within these conventional extracts, sesquiterpenes, flavonoids and quinic acid derivatives represent the characteristic constituents [3]. Among these, the tincture is mainly employed as such or as a component of ointments, creams, gels or compresses containing 5–25% (v/v) tincture [4]. Tincture is also evaporated to dryness and the dried extract used as active ingredient of these HDPs. However, both tincture and the dried extract obtained from the evaporation of the solvent contain a low percentage of sesquiterpenes (1.1% (w/w)) and their stability and physical compatibility in semisolid formulations is very poor, as published for the first time in the present paper. Recently, an innovative supercritical carbon dioxide (CO_2) extract has been marketed. This innovative extract, characterized by the authors using both conventional HPLC analysis and NMR spectroscopy [5] contains about 9.5% (w/w) of sesquiterpenes, while neither flavonoids, nor caffeoyl quinic acid derivatives were present and it can be a good alternative for topical semisolid formulations.

In a previous paper, 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 [6].

In this paper the development of several semisolid preparations based on this innovative CO_2 extract is reported. The tested preparations were four creams: cetomacrogol emulsion

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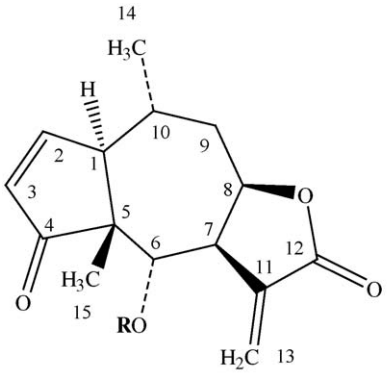
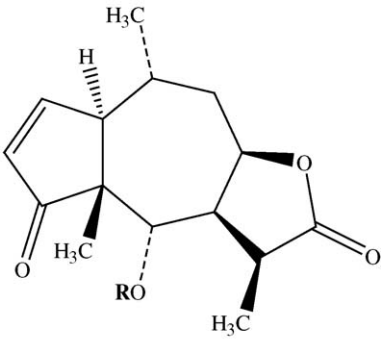


1: Helenalin



2: 11α,13-Dihydrohelenalin

R	a: CH_3CO-	-acetyl
	b: $CH_2=C(CH_3)CO$-	-α-methacryl
	c: $CH_3CH=C(CH_3)CO$-	-tigloyl
	d: $(CH_3)_2CHCO$-	-isobutyryl
	e: $CH_3CH_2CH(CH_3)CO$-	-2-metylbutyryl
	f: $(CH_3)_2CHCH_2CO$-	-isovaleryl

Fig. 1. Structure of sesquiterpene lactones.

W/O, anphyphil cream, polawax emulsion O/W, polysorbate 60 emulsion O/W and two gels: natrosol and sepigel. Stability of these preparations, compared with tincture and CO_2 extract alone, was investigated according to ICH guidelines [7] using a HPLC method developed by the authors for the analysis of sesquiterpenes [5]. The same analytical method was also used for the evaluation of the in vitro and in vivo tests. The in vitro dissolution of sesquiterpenes was performed using a cell method according to the European Pharmacopoeia for the evaluation of transdermal patches [8], and the preliminary evaluation of the in vivo absorption of active principles from the formulations was described by the stripping technique according to FDA [9,10]. This study was performed on three subjects using three preparations, i.e. polysorbate 60, cetomacrogol and polawax.

2. Experimental

2.1. Chemicals

Santonin (99%) was purchased from Sigma–Aldrich Division (Milano, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA). Methanol, acetonitrile and formic acid were HPLC grade from Chromasolv, Allied Signal (Milano, Italy). Silicone 18–350 mPa, sepigel 305, glycerine, polyethylene glycol monostearate, propylene glycol, isopropyl myristate, alcohol cetostearylic, cetomacrogol (1 0 0 0), alcohol stearylic, glyceryl monostearate, natrosol 250 MR, white vaseline, liquid paraffin, liquid semi synthetic triglycerides were from Galeno (Comeana, Prato, Italy). Polysorbate 60 and polawax were from Azienda Chimica Farmaceutica A.C.E.F S.p.A (Fiorenzuola D'Arda, Piacenza, Italy). Glucate DO was from Galenia s.a.s (Genova, Italy); PEG 4000 from Sigma–Aldrich Division (Milano, Italy). Adhesive discs D-SQUAME were a gift from Difa Cooper S.p.A. (Caronno P., Varese, Italy).

Cellulose nitrate membrane filter (2 μm) Type RS was from Sartorius GmbH (Goettingen, Germany).

2.2. Plant material

Flores Arnicae (ID 5932) was a gift from Kneipp Werke, Würzburg, Germany.

Supercritical CO_2 extract was a gift from Arkopharma (Nice, France).

Tincture of Arnica was prepared according to German Pharmacopea, 9th edition [4].

2.3. HPLC–DAD and HPLC–MS apparatus

The HPLC system consisted of a HP 1100L instrument with a DAD and managed by a HP 9000 workstation (Helwett & Packard, Palo Alto, CA, USA). Column: 201 TP 54 RP-18 (254 mm × 4.6 mm, 5 μm, 300 Å, Vydac Separation Group Hesperia, CA, USA); oven temperature: 26 °C. The eluents were: (A) water adjusted to pH 3.2 by formic acid; (B) methanol and (C) acetonitrile using a linear gradient of 35 min as reported in the literature [5]. Flow elution: 0.8 ml/min; injection volume: 10 μL. UV–vis spectra were recorded in the range 200–450 nm. Sesquiterpenes were detected at 220 nm. A chromatogram of the CO_2 Arnica extract is reported in Fig. 2.

The HPLC system was interfaced with a HP 1100 MSD API-Electrospray (Hewlett & Packard, Palo Alto, CA, USA). The

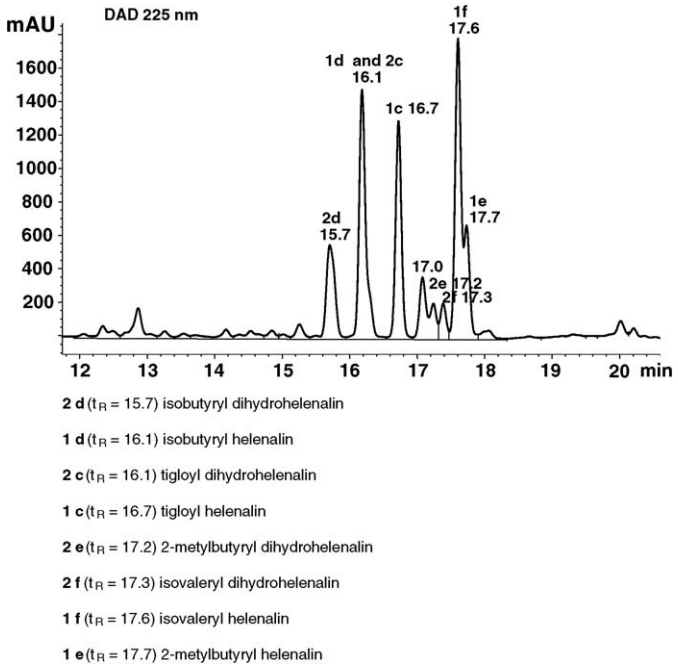


Fig. 2. HPLC chromatogram of the CO_2 Arnica extract.

interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the usage 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).

2.4. Preparation of the semisolid formulations

Several semisolid preparations were prepared and evaluated to test their physico-chemical stability after the addition of 10% CO_2 extract:

- *cetomacrogol* emulsion W/O according to National Formulary of the Italian Pharmacopoeia [11];
- *anphyphil* cream according to National Formulary of the Italian Pharmacopoeia [11];
- *polawax emulsion O/W*: polawax 10%, vaseline oil 14%, silicone (18–350 mPa) 1%, purified water 70%, glycerine 5%;
- *polysorbate 60 emulsion O/W*: polysorbate 60 (polyoxyethylene sorbitan monostearate, Tween 60) 3%, glucate DO (methylglucose dioleate) 3%, vaseline 15%, silicone (18–350 mPa) 1%, sepigel 305 (polyacrylamide and C-13-14 isoparaffin and laureth-7) 3%, purified water 70%, glycerine 5%;
- *natrosol gel*: purified water 97%, hydroxyethilcellulose (natrosol® 250 MR) 3%;
- *sepigel gel*: purified water 95%, sepigel 305 (polyacrylammide and C-13-14 isoparaffin and laureth-7) 5%.

2.5. Preparation of santonin solution

A standard stock solution of santonin was obtained by dissolving 1 mg in 1 ml of methanol. This solution was used to build a calibration curve.

2.6. Quantification of constituents

The percentage of total sesquiterpene lactones, expressed as santonin, was calculated using a calibration curve. This ($y = 0.0006x - 0.1018$, $R^2 = 0.9993$) shows a linear relationship in the range investigated (25–150% of the standard solution, 1 mg/ml).

2.7. Thermal stability testing

The samples were introduced into inert and transparent containers. All samples were exposed under two different temperature conditions: $+25 \pm 2$ °C with 60 ± 5% relative humidity (RH) for the long-term testing and at $+40 \pm 2$ °C with 75 ± 5% RH for accelerated testing. The constituent content was evaluated fortnightly. Climatic chambers (Angelantoni Industry S.p.A., Massa Martana, Perugia, Italy) of the Analytical Research division of the A. Menarini s.r.l., Florence, Italy, were employed: a climatic chamber at $+40 \pm 2$ °C and 75 ± 5% RH, mod. EOS 330°, n. 6134 and a CDZ walk-in chamber at $+25 \pm 2$ °C with 60 ± 5% RH, mod. mFIP, n. 00466.

2.8. In vitro release study

The in vitro evaluation was performed using a cell method to determine the dissolution rate of the active ingredients of transdermal patches, as reported in the European Pharmacopoeia [8]. All in vitro release experiments were performed at 37 °C (±2 °C). An amount of 300 mg of the semisolid preparations was deposited in a internal cavity (diameter 2 cm) and covered by a cellulose nitrate membrane.Myristate (50 ml) was used as acceptor phase. The analysis was performed in triplicate. An aliquot (5 ml) of acceptor phase was taken after 30, 60 and 180 min and replaced with the same amount of fresh isopropyl myristate. The sesquiterpenes were extracted from the aliquot with MeOH and analysed by HPLC–DAD–MS.

2.9. In vivo permeation study

A preliminary analysis of the in vivo penetration of the semisolid preparation was evaluated by the "skin stripping" test according to the FDA [9,10]. Three healthy women aged 27–36 were used in the pilot study. An amount of 250 mg of the semisolid preparations were applied on a 1.0 cm^2 portion of the inner forearm. The preparations were removed after 30, 60 and 180 min; each site was washed and the stratum corneum was removed by eight strippings with transparent adhesive tape (Adhesive discs D-SQUAME) [12–14]. Each disc was extracted with acetonitrile (1 ml) and 100 μl of the solution was analysed by HPLC–DAD–MS after filtration through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE)

Table 1
Residual percentage (±S.D., $n = 3$) of sesquiterpenes lactone in the long-term testing (+25 °C, 60% RH)

Sample	15 days (residual %)	30 days (residual %)	60 days (residual %)
CO_2 extract	99.7 ± 0.07	99.5 ± 0.03	97.9 ± 0.3
Tincture	99.8 ± 0.01	97.8 ± 0.02	96.3 ± 0.2
Cetomacrogol	94.9 ± 0.07	87.7 ± 0.04	44.1 ± 1.0
Anphyphil	99.3 ± 0.05	98.7 ± 1.2	95.7 ± 1.0
Polawax	99.5 ± 0.09	99.4 ± 0.4	90.0 ± 0.95
Polysorbate 60	99.5 ± 0.5	98.8 ± 0.03	89.9 ± 0.06
Natrosol	78.1 ± 1.0	64.2 ± 0.9	n.d.
Sepigel	99.8 ± 0.2	99.7 ± 0.08	92.3 ± 1.2

membrane ($d = 13$ mm, porosity 0.45 μm, Lida manufacturing Corp.).

3. Results and discussion

3.1. Thermal stability testing of the extracts

As the first step, we evaluated the thermal stability of both the lyophilised tincture and supercritical CO_2 extract of *A. montana* L. in order to use them as active ingredients in the semisolid preparations. Stability was assessed according to ICH guidelines as described in Section 2. The residual percentages of sesquiterpene lactones after 15, 30 and 60 days of testing were evaluated by HPLC and are reported in Tables 1 and 2. Both the lyophilised tincture and the CO_2 extract showed a residual percentage of more than 97% in both the long-term and accelerated testings during the investigated period. These stability data were favourable and suggested that these extracts may be good candidates as active ingredients of the semisolid preparations.

3.2. Preparation of the semisolid formulations and evaluation of their thermal stability

Six semisolid formulations, namely four emulsions (cetomacrogol, amphyphil cream, polawax, polysorbate 60) and two gels (natrosol and sepigel) were prepared. Their compatibility with the two extracts was assessed by adding increasing amounts of the extracts to reach 10% (w/w) of each semisolid formulation. In the case of the dried extract obtained by the lyophilisation of the tincture, however, no phase separations or other physical variations (consistency, etc.) were noted until 3% (w/w). If CO_2 extract was used as active substance, it was possibile to reach a concentration up to 10% (w/w) (corresponding to about 1% of the active principles sesquiterpenes) without phase separation to all the six preparations.

Thus, due to the very low content of active constituents (1.1% (w/w)), the preparations containing the lyophilised tincture were not evaluated for in vitro and in vivo studies, while six semisolid preparations containing 10% of supercritical CO_2 extract of *A. montana* L. were prepared and evaluated for stability over a 3-month period according to ICH guidelines [7]. In the thermal stability testing, the samples were introduced into inert and transparent containers. For the HPLC analysis, the sesquiterpenes were extracted from the semisolid formulations with acetonitrile. The results of stability at +25 and +40 °C, obtained by HPLC–DAD–MS analysis, are shown in Tables 1 and 2, respectively. As evidenced from the long-term stability data, sepigel was the most stable gel preparation with the residual percentage of sesquiterpenes over 90% (92.3%) of the initial value content. Instead, natrosol gel was highly unstable and after only 15 days the residual percentage of sesquiterpenes decreased to 78.1%. After 2 months, the active principles were no longer detectable and there was a phase separation. In the case of the accelerated test, natrosol preparation degraded completely after 15 days.

Anphyphil cream was the most stable emulsion with a residual content of sesquiterpenes over 90% after 2 months both in long-term (95.7%) and accelerated (98.3%) testing. This emulsion was the only preparation with a percentage of sesquiterpenes more than 90% at +40 °C after 2 months. Instead, also polawax, sepigel and polawax 60 emulsions at +25 °C showed residual percentages of 90.0%, 92.3% and 89.9%, respectively.

3.3. In vitro release study

In this study, the diffusion of sesquiterpenes through a cellulose nitrate membrane and isopropyl myristate as receiver

Table 2
Residual percentage (±S.D., $n = 3$) of sesquiterpenes lactone in the accelerated testing (+40 °C, 75% RH)

Semisolid preparation	15 days (residual %)	30 days (residual %)	60 days (residual %)
CO_2 extract	99.1 ± 0.05	98.9 ± 0.03	97.6 ± 0.06
Tincture	98.2 ± 0.09	95.4 ± 0.07	91.8 ± 1.0
Cetomacrogol	79.0 ± 0.5	78.1 ± 0.7	48.7 ± 1.03
Anphyphil	99.7 ± 0.2	99.2 ± 0.4	98.3 ± 0.03
Polawax	99.3 ± 0.08	92.7 ± 0.4	83.8 ± 0.6
Polysorbate 60	99.2 ± 0.2	85.7 ± 0.08	58.4 ± 1.3
Natrosol	68.8 ± 1.0	n.d.	n.d.
Sepigel	94.6 ± 1.2	82.9 ± 1.2	70.0 ± 0.06

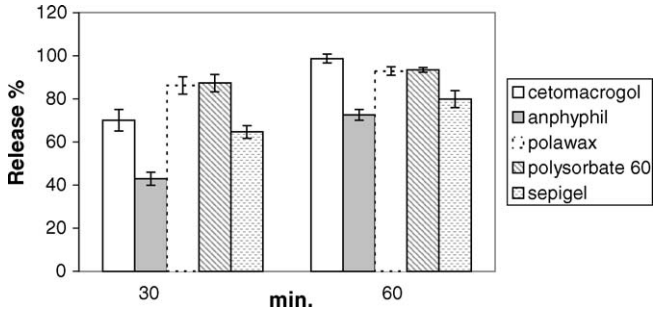


Fig. 3. Permeated amounts (data are means ± S.D., $n = 3$) of the sesquiterpenes lactone from different semisolid preparations in the in vitro test (using as a cellulose nitrate membrane and isopropyl myristate as recipe phase).

medium was investigated during 60 min. This compound showed a pronounced enhancing effect, regarding the permeation flux, permeability coefficient and diffusion coefficient. This was attributed to solubility parameter of isopropyl myristate being closer to the solubility parameter of human skin, and such a pronounced enhancing effect was probably caused by its passage across the skin barrier through the lipid pathway [15].

Each sample was analysed in triplicate. The in vitro evaluation was obtained using a cell method according to the European Pharmacopoeia [8]. All the preparations were evaluated, with the exception of natrosol formulation due to its poor stability during storage in the long-term and accelerated conditions.

The release profiles of each tested formulation containing 10% of CO_2 extract are reported in Fig. 3. The amounts of released sesquiterpenes quantified by HPLC–DAD–MS after 30 min revealed that polawax and polysorbate 60 preparations showed the highest amount of released sesquiterpenes: 86.2% and 87.4%, respectively. The lowest released amount was found with the amphiphil cream.

However, after 60 min, the best release profiles of sesquiterpenes were noted for cetomacrogol (98.7%), polawax (92.9%) and polisorbate 60 (93.5%). The amount of sesquiterpenes released after 60 min from amphiphil cream and sepigel were 72.5% and 79.9%, respectively.

3.4. In vivo release study

A preliminary evaluation of the in vivo skin permeation of active principles from the preparations was also performed according to the "stripping" test [9,10], to determine the quantity of sesquiterpenes in the stripped layers of the stratum corneum. This evaluation was performed using the formulations having the best in vitro release profiles, i.e. polysorbate 60, cetomacrogol and polawax. The test was carried out as described in Section 2 and the quantification of sesquiterpenes in the skin was performed by HPLC–DAD–MS. The lowest content of sesquiterpenes in the treated skin was found with cetomacrogol (0.97 μg after 30 min, 8.17 μg after 60 min and 6.90 μg after 180 min). Polawax preparation showed a better profile with values of 2.44, 6.69 and 12.93 μg after 30, 60 and 180 min, respectively. The highest amounts of sesquiterpenes were obtained by polysorbate 60: 5.33 μg after 30 min, 10.16 μg after 30 min and 28.8 μg after 180 min.

4. Conclusions

In this study several technological and biopharmaceutical characteristics of some semisolid formulations containing 10% (w/w) of an innovative supercritical CO_2 extract of *A. montana* L. were evaluated. This innovative extract results very high content of the active constituents sesquiterpenes and, at the same time, it is very stable in semisolid preparations, by comparison with the creams containing lyophilised tincture. Moreover the fatty acids and essential oils present in the extract can act as penetration enhancers, improving the penetration of sesquiterpenes [9,10].

In vitro and in vivo tests were also performed on the semisolid preparations in order to study the release profiles of the phytocomplex from the different formulations.

The formulation cetomacrogol, which showed the best release profile of sesquiterpene in the in vitro test, however, showed the lowest penetration rate of sesquiterpenes in the skin. Polysorbate 60 and polawax, which had similar behaviour in the in vitro test, were very different in the in vivo test: the content of sesquiterpenes in the skin treated with the polawax preparation was two times greater than that released from polysorbate 60.

Despite a similar content of excipients in the formulations, the release and penetration of the active constituents was quite different, probably related to the different tensides. In particular, polysorbate 60 contained glucate DO and sepigel 305, modern emulgators generally used in emulsions to increase stability. Both are derivatives of highly hydrophylic molecules (glucose and polyacrilamide), modified to obtain an adequate lipophilic level. They have both emulsifying and viscosing effects. The different in vivo release of the active constituents from the semisolid preparations should be due to the presence of glucate DO and sepigel 305. From the analysis of the structure of glucate DO, it can be assumed that it interferes with the structure of the epidermis and as a consequence it improves the absorption of sesquiterpenes.

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