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Short communication

Comparison between HPLC and HPTLC-densitometry for the determination of harpagoside from *Harpagophytum procumbens* CO_2-extracts

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Abstract

Carbon dioxide (CO_2) extracts of the secondary roots of *Harpagophytum procumbens* were quantified by high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC). An isocratic HPLC method was used for the quantification of the iridoid glucoside harpagoside at 278 nm. A HPTLC assay was developed for the determination of harpagoside after coloration at 509 nm. The diode array detection of both analytical assays were used to examine the purity of harpagoside peaks and compared with the standards, respectively. The assays provide good accuracy, reproducibility and selectivity for the quantitative analysis of harpagoside.

The harpagoside contents of 15 different CO_2-extracts were compared by HPLC and HPTLC-densitometry. The quantitative results of both analytical methods did not show any statistical significance between each other, although a trend to slightly lower mean values could be found for the HPTLC method.
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Keywords: *Harpagophytum procumbens*; HPTLC; Densitometry; HPLC; Carbon dioxide

1. Introduction

Extracts of the secondary roots of *Harpagophytum procumbens* DC (Pedialaceae) are applied for the treatment of degenerative disorders of the musculoskeletal system like arthrosis or arthritis because of its anti-inflammatory activity. Carbohydrates (stachyose, raffinose, monosaccharides), iridoid glucosides (harpagide, harpagoside), 2-phenyl-ethyl derivates (acteoside, isoacteoside) and lipophilic substances are the main components. The active principle is still unknown, but harpagoisde (Fig. 1) designates as a marker.

Conventional extracts of *H. procumbens* are usually prepared by maceration using ethanol–water (6:4, w/w) and contain about 2.5% harpagoside. To enrich this harpagoside content, super- and subcritical carbon dioxide (CO_2) extractions have been investigated [1]. Supercritical fluid extraction (SFE) is an alternative technique to classical extraction methods and shows the advantages of shorter extraction time and reduction of the amount of organic solvents. Furthermore, the extracts can be enriched with substances of interest by varying the composition of the extraction fluid. Pure CO_2 is an excellent solvent for non-polar substances. The polarity of CO_2 can be increased by the addition of organic volatile solvents, such as methanol or ethanol [2]. The extraction of *H. procumbens* was performed with CO_2 and 25% (w/w) ethanol as modifier in the supercritical and the subcritical state. Under these conditions the harpagoside content of the extract could be increased to 20–30% [1]. The disadvantage of these CO_2-extraction fluids is the extraction of non-polar substances due to their lipophilic character. High amounts of these lipophilic substances are inconvenient and interfere with the existing analytical assays.

The quantitative determination of harpagoside has been usually carried out by high performance liquid

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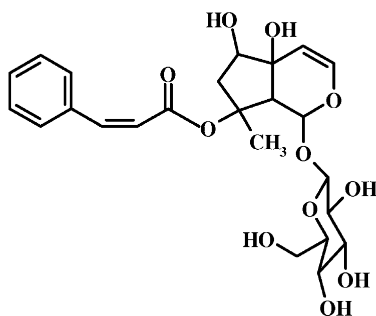

Fig. 1. Structural formula of harpagoside.

chromatography (HPLC). The European Pharmacopoeia [3] and Sticher et al. [4] describe an isocratic HPLC method using a mixture of methanol–water (1:1, v/v) as mobile phase. Optimizations of the existing isocratic HPLC methods have been conducted by introducing a mobile phase with a gradient profile [5]. This profile was chosen to analyse coumaroyl–harpagide and harpagide beneath harpagoside in *Harpagophytum* extracts, which was not possible under isocratic conditions.

A method of high performance thin layer chromatography (HPTLC) has been described [6]. The mobile phase consisted of dichlormethane–methanol (4:1, v/v) and the quantification of harpagoside was performed by UV detection at one fixed wavelength (286 nm).

The purpose of this work is to develop a reliable HPTLC procedure for the quantification of harpagoside in lipids containing CO_2-extracts of *H. procumbens* and to compare the results with the established HPLC method. To achieve high reproducible HPTLC results a TLC-scanner with a newly developed technique was used. The scanner was equipped with 50 identical optical fibres transporting light of different wavelengths from the lamp to the HPTLC plate and back to the diode array detector [7,8].

2. Experimental

2.1. Materials

Roots of *H. procumbens* were donated from Dr. Willmar Schwabe Pharmaceuticals GmbH and Co (Karlsruhe, Germany). Carbon dioxide (type 3.5) and helium (type 4.6) were supplied by Messer Griesheim GmbH (Krefeld, Germany). Absolute ethanol was from Bundesmonopolverwaltung (Verwaltungsstelle für Agraralkohol, Offenbach am Main, Germany). Methanol was supplied by Fisher Scientific (Loughborough, Great Britain). Ethyl acetate and methylcinnamate were purchased from Fluka Chemistry GmbH (Buchs, Switzerland). Nucleosil 60 F_{254} HPTLC plates (No. 1.05642), anisaldehyde, glacial acetic acid, concentrated sulphuric acid and *n*-propanol were from Merck KGaA (Darmstadt, Germany). The reference substance harpagoside was purchased from Extrasynthése (Genay, France). Cellulose acetate filters (0.2 μm) were obtained from Sartorius AG (Göttingen, Germany). All solvents were of HPLC grade.

2.2. CO_2 extraction of the crude drug

All extractions were performed using a pilot-scale batch extraction plant (SITEC Sieber Engineering, Maur, Switzerland). The supercritical fluid extraction apparatus has been described in details by Römpp et al. [9]. An amount of 200 g of granulated root powder of *H. procumbens* were extracted with CO_2/*n*-propanol (5%, w/w) to remove most of the lipophilc substances. After this pre-extraction step, the main-extraction was followed by extracting the same crude drug either in the supercritical or in the subcritical state with CO_2/ethanol (25%, w/w). Detailed extraction procedures are described by Günther et al. [1].

2.3. Sample solutions

Internal standard (HPLC): 600 mg methylcinnamate were dissolved in 100 ml methanol. Reference standards: 0.0859, 0.1718, 0.2148, 0.2578 and 0.3437 mg ml^{-1} harpagoside were dissolved in methanol. HPLC samples: 100–250 mg CO_2-extract were dissolved in methanol–water (1:1, v/v), 1 ml of internal standard was added and filled up to 25.0 ml in a volumetric flask. The samples were filtered through a 0.2 μm cellulose acetate filter prior to analysis. HPTLC samples: 30–50 mg CO_2-extract was dissolved in 50.0 ml methanol.

2.4. HPLC analysis

HPLC analyses were performed at room temperature using the following system: an isocratic pump system (Waters, model 616, Millipore, Milford, USA) with a diode-array detector hp 1040A (Hewlett-Packard, Boeblingen, Germany) and a Rheodyne (7125, Cotati, CA, USA) sample injector with an external loop (20 μl) under the following conditions: Nucleosil 100-5 RP 18 (8 × 4 mm i.d.) guard column (Macherey and Nagel, Düren, Germany), Nucleosil 100-5 RP 18 (125 × 4 mm i.d.) separation column (Macherey and Nagel, Düren, Germany). The method was performed according to European Pharmacopoeia [3] with the following changes: the mobile phase was degassed with helium and the flow rate was adjusted to 1 ml min^{-1}. Absorption spectra were measured in the range of 200–600 nm, while the harpagoside content was analysed at 278 nm. All samples were conducted in triplicate and the data were evaluated on HPLC ChemStation HP 79994A (Hewlett-Packard, Boeblingen, Germany).

2.5. Quantitative HPTLC analysis

Samples of 5 μl were applied on Nucleosil 60 F_{254} HPTLC plates (10 cm × 20 cm) as 10 mm bands using a Linomat IV applicator (15 s μl^{-1}, Camag, Muttenz, Switzerland). The

distance between the sample bands was set to be 5 mm and the distance from the lower plate edge was 10 mm (12 bands per plate). Plates were developed in a saturated vertical automated developing chamber ADC (Camag, Muttenz, Switzerland) using ethyl acetate–methanol–water (77:15:8, v/v/v). The solvent front was allowed to develop to a distance of 75 mm (25 min). After drying the plates with a stream of cold air for 1 min, the spots were visualised by immersion (DC-Tauchfix II, Lothar Baron Laborgeräte, Reichenau, Germany) in anisaldehyde reagent (anisaldehyde–glacial acetic acid–methanol–96% sulphuric acid, 0.5:10:85:5, v/v/v/v) for 2 s. After drying the HPTLC plates at room temperature, they were heated in an oven (type SI 5042, Heraeus, Hanau, Germany) at 120 °C for 5 min. Colour stabilization of the bands was achieved by cooling the plates at room temperature for 15 min. Quantification of the spots was performed by linear scanning in the reflectance mode using a diode-array scanner (PDA-Densitometer 2010, J&M GmbH, Aalen, Germany). Measurements were taken in the range of 400–1000 nm using a halogen lamp. Harpagoside quantification was done at the absorption maximum of 509 nm. Each determination was carried out in triplicate. All data were analysed by Spectralys 1.82 (J&M, Aalen, Germany).

3. Results and discussion

Conventional extracts of *H. procumbens* are produced using ethanol/water mixtures as extraction fluids. Due to the polarity of these solvents they are free from lipophilic substances. In contrast, solvent modified CO_2-extracts contain higher amounts of lipophilic compounds even after a pre-extraction step. The analytical methods for the quantification of harpagoside, mostly HPLC methods, are developed for conventional extracts. The undesired lipophilic substances from CO_2-extracts will deposit on the HPLC columns. The sample preparation becomes more time-consuming. Therefore, the HPLC method has to be optimized. A HPTLC determination using a modern scanner with diode array detection (DAD) makes it possible, to apply the extract containing solution directly on the HPTLC plates without prior preparation steps. The optimum wavelength for quantification can easily be chosen via DAD spectrum. The full spectrum is taken at once and allows judging the resulting densitogram. Therefore, both methods were compared to show the equality of the results.

3.1. HPLC assay

The pre-treatment of the HPLC samples was optimized by dissolving the extracts in the mobile phase (methanol–water (1:1, v/v)) instead of methanol. Substances of interest, like harpagoside dissolved completely in 50% methanol, while almost all undesired lipophilic substances remained as an insoluble residue, which was removed by filtering the samples through a 0.2 μm filter. The quantification of harpagoside was carried out using the external standard calibration method. Linearity was observed in the range of 18.91–403.37 mg/100 ml. The regression equation was $y = 371.138x - 406.56$, where y is the peak area at 278 nm and x is the harpagoside concentration in mg/100 ml, with a correlation coefficient r^2 of 0.9999. The relative standard deviation of the method was 0.42%. Validation data in comparison to the HPTLC method are presented in Table 1.

3.2. HPTLC assay

HPTLC-densitometry was developed for the quantification of harpagoside in *H. procumbens* CO_2-extracts. The best separation of all interesting constituents showed the mobile phase ethyl acetate–methanol–water (77:15:8, v/v/v). The CO_2-extract samples were applied directly onto the HPTLC plates without any filtration step. Lipophilic constituents of the extracts moved with the solvent front. Hence, there is no interference from the lipophilic substances on the separation of harpagoside. A typical HPTLC densitogram with a baseline separation of harpagoside and other constituents of a CO_2-extract is shown in Fig. 2.

Visualisation of the spots was performed using anisaldehyde reagent. The quantification at a visible wavelength of 509 nm showed a better baseline separation compared to the direct UV-measurement. By immersion of the plate, the

Table 1
Validation data of the HPLC and the HPTLC-densitometric assay

Parameter	HPLC	HPTLC
Repeatability R.S.D.[a] (%)	0.91	1.91
Intermediate precision R.S.D.[a] (%)	1.82	0.46
Limit of quantitation (μg ml^{-1})	30.75	87.48
Limit of detection (μg ml^{-1})	8.65	25.93

Accuracy

Reference value (%)	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)
90.00	91.72	1.80	91.52	1.94
111.08	110.93	0.32	112.74	1.29
120.23	122.86	0.28	124.56	1.70

[a] Relative standard deviation.

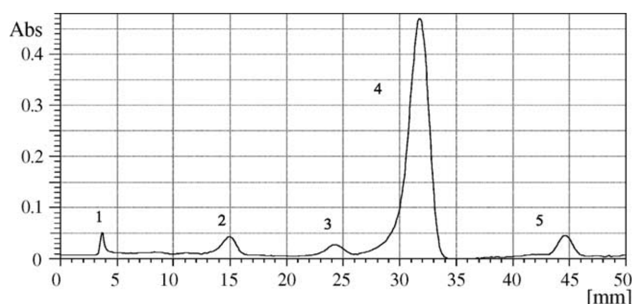

Fig. 2. HPTLC-densitogram at 509 nm of a CO_2-extract: (1) sugars, (2) harpagide, (3) 2-phenyl-ethyl derivate, (4) harpagoside, (5) lipophilic substances.

spots get a sharper contour and therefore a better baseline separation. As a consequence, the correlation between HPTLC and HPLC results was better when using the visible wavelength for quantification. The use of a diode-array detector in the quantification of harpagoside showed the following advantages. The spectra of the harpagoside peak in extracts were in good agreement with the spectrum of pure harpagoside with respect to the absorption maximum and the peak purity, which resulted in a match factor of 968. The calibration showed a second-order polynomial function: $y = -0.432x^2 + 48.930x + 33.819$ ($r^2 = 0.9955$) in the concentration range of 4.296–42.96 mg/100 ml where y is the peak area at 509 nm and x is the concentration in mg/100 ml. The coefficient of variation of the method was 4.27%. The validation data are represented in Table 1.

3.3. Comparison of the HPLC and the HPTLC method for 15 CO_2 extracts

The quantitative results of 15 different CO_2-extracts, prepared under different extraction conditions, using the HPLC and HPTLC determination, are shown in Fig. 3. There was no statistically significant difference between the mean values for all 15 extracts, although the HPTLC method showed slightly lower mean values compared with HPLC.

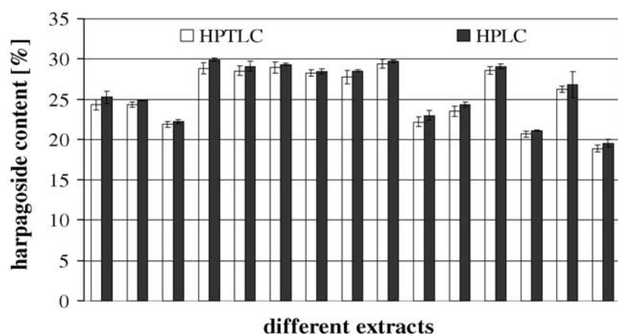

Fig. 3. Harpagoside content (%) of 15 different CO_2-extracts, produced under different extraction conditions, were determined by HPTLC and HPLC respectively ($n = 3$, mean ± 95% confidence interval).

Therefore, both analytical methods, HPTLC densitometry and HPLC, were found to be equal and could be used for the routine determination of harpagoside in *H. procumbens* extracts.

The equivalence of the HPLC and HPTLC densitometric assay in this study has been confirmed by other investigations. Extracts of *Caulophyllum thalictroides* [10], *Malva Silvestris* [11] and *Aesculus hippocastanum* [12] have been determined using both analytical methods and do not show any significant difference. Furthermore, HPLC as well as HPTLC showed equivalent results for synthetic products. Four different parabens [13] as well as acebutolol HCl in the presence of its acid-induced degradation product [14] could be determined by HPTLC as well as HPLC with the same results. In further investigations the determination of azidothymidine and its degradation product thymine in pharmaceutical oral dosage forms [15] as well as the determination of their impurities [16] were described. Both studies have shown the equivalence of HPLC and HPTLC densitometry.

Especially for herbal drug preparations, being multi-component systems, HPTLC seems to have some advantages over HPLC: the sample preparation is simple, the detection by dipping reagents enables specific colour reactions and the consumption of organic solvents as well as the analysis time is lower. Therefore HPTLC with diode-array detection should be considered as a powerful quantitative method.

4. Conclusion

The HPTLC densitometry provides similar reproducibility, accuracy and selectivity for the quantitative determination of harpagoside in CO_2-extracts of *H. procumbens* compared with an established HPLC method. The advantage of the HPTLC method compared with the HPLC method is the less time-consuming sample pre-treatment. A statistical comparison of the quantitative harpagoside determinations of 15 different CO_2-extracts did not show any statistical significance between HPLC and HPTLC.

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