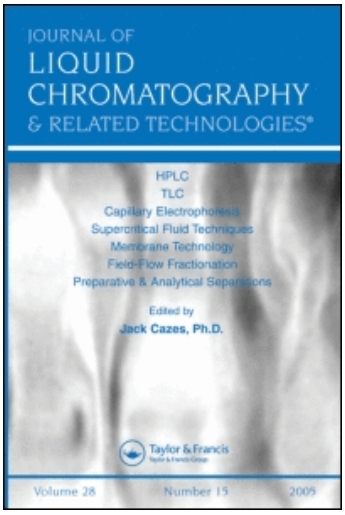

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Validation of A Fast-HPLC Method for the Separation of Iridoid Glycosides to Distinguish Between the *Harpagophytum* Species

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Abstract: A fast high-performance liquid chromatography (HPLC) method was developed and validated for the simultaneous determination of the iridoid glycosides harpagoside (HS) and 8-p-coumaroyl-harpagide (8pCHG) in extracts and preparations of *Harpagophytum procumbens* and *H. zeyheri.* The ratio between 8pCHG and the sum of HS and 8pCHG can be used to distinguish between both species.

Quantitation was accomplished with the internal standard (IS) method. The separation was performed on a monolithic silica column (Chromolith Performance RP-18e), under gradient conditions using a mobile phase of water (pH 2.0 adjusted with phosphoric acid) and acetonitrile. The elution of the analytes was monitored at 278 nm and conducted at a column temperature of 30°C. Because of the high porosity of the monolithic column the mobile phase was able to be pumped at a flow rate of 5.0 mL/min. The retention time of 8pCHG, HS, and the IS was 1.9 min, 2.1 min, and 3.0 min, respectively, and the total run time of the assay was 5 min.

The method was validated by specificity, linearity, accuracy, and precision. For the determination of method robustness a number of chromatographic parameters were varied.

Keywords: HPLC, Monolithic silica column, Method development and validation, *Harpagophytum procumbens* and *H. zeyheri*, Harpagoside, 8-p-Coumaroyl-harpagide

INTRODUCTION

Harpagophytum procumbens is a plant found in dry and sandy Kalahari regions of Namibia, Botswana, and South Africa. The common name of

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Devil's claw is derived from the plant's unusual claw-like fruits, which seem to be covered with numerous small hooks.

Extracts and preparations from the secondary tubers of *Harpagophytum procumbens* have become the focus of research as a potential therapeutic agent, and the ESCOP (European Scientific Cooperative on Phytotherapy) monograph on Devil's claw recommends preparations from the secondary tubers for treatment against rheumatic diseases like arthrosis and low back pain.[1–3] The clinical section of the ESCOP monograph includes several published clinical trials and in vitro studies in peer-reviewed journals,[2] and has added proof of this plant's pharmacological qualities.

The secondary tubers of *Harpagophytum procumbens* yield a variety of ingredients, mainly iridoid glycosides such as harpagoside and 8-p-coumaroyl-harpagide, and phenylethanol derivatives including acteoside (verbascoside) and isoacteoside.[4] The pharmacological active ingredients are still unknown but the main iridoid glycoside of harpagoside is involved and therefore, is used as a marker compound to assess the quality of *Harpagophytum procumbens.*

In accordance to the ESCOP monograph, only preparations from the *H. procumbens* may be used for therapeutic purposes. However, various amounts of the species *Harpagophytum zeyheri* can be found in commercial raw materials.

In comparison to the species *H. procumbens*, the constituent 8-p-coumaroyl-harpagide is present in the *H. zeyheri* species in much higher quantities. Therefore, the specific ratio between the percentage of 8-p-coumaroyl-harpagide and the sum of harpagoside and 8-p-coumaroyl-harpagide is a helpful measurement for the quality control to distinguish between the two species.[5] The ratio is below 10 in *H. procumbens* and above 31 in *H. zeyheri*,[6] and between 10 and 31 in extracts and preparations derived from mixtures of the two species.

A variety of methods for the determination of the iridoid glycosides harpagoside and 8-p-coumaroyl-harpagide in the *Harpagophytum* species have been published, and HPLC separation on reversed phase materials is the common method used.[7–9] On conventional particle-based RP18-silica columns, the pressure limitation of commercially available HPLC systems was found to be the limiting factor for fast separations, and run-times of 30-60 min are not uncommon in order to separate this complex mixture.[8]

In comparison to particle packed columns, monolithic columns consist of a silica rod instead of particles. The very high porosity of the stationary phase allows chromatography with much lower backpressure; higher flow rates become possible, resulting in shorter run times.[10,11]

In this study, the separation of the iridoid glycosides in extracts and preparations of the *Harpagophytum* species was developed using a monolithic silica-based C_{18} column in order to speed up analysis. The resulting fast-HPLC method was validated in accordance to the ICH guidelines Q2A and Q2B.[12]

EXPERIMENTAL

Chemicals

Acetonitrile, HPLC-gradient grade, and ortho-phosphoric acid (85%), Suprapur grade, were obtained from Merck (Darmstadt, Germany). Water used was purified by a Milli-Q water purification system (Milipore, Eschborn, Germany).

HPLC Equipment

The separation was performed using an Alliance HPLC-system (Waters, Eschborn, Germany). The instrument consisted of a 2695XE separation module with solvent degasser, temperature-controlled sample compartment and column heater, and a 2996 photo-diode array detector. The UV detection of the compounds of interest was carried out at 278 nm and the UV spectra were taken in the range of 210–400 nm. The injection volume was 10 μL.

For system control, data acquisition, and data processing, the Empower client/server software (Waters, Eschborn, Germany) was used. Statistical analysis was calculated using the MVA (Novia, Saarbrücken, Germany) statistical software (version 1.0).

Chromatographic Conditions

LC separation was carried out using two Chromolith Performance RP-18e columns with the dimension 100 x 4.6 mm, coupled in series (Merck, Darmstadt, Germany). The mobile phase A was water adjusted to pH = 2.0 with ortho-phosphoric acid. The mobile phase B was acetonitrile. The gradient mode was as follows: A linear gradient was started with 1% solvent B and was increased within 2 min to 50% B. After an isocratic elution with 50% B for 1 min, the gradient was returned to the initial conditions within 1 min to re-equilibrate the column. The flow-rate was 5.0 mL/min and the column temperature was 30°C.

Standards and Sample Preparation

The reference standard of harpagoside was from HWI-Analytik (Rheinzabern, Germany), 8-p-coumaroyl-harpagide was from Phytolab (Hamburg, Germany), and the internal standard cinnamic acid methyl ester was from Merck (Darmstadt, Germany).

A stock solution of cinnamic acid methyl ester (IS) was prepared by dissolving appropriate amounts in methanol.

The dried hydroalcoholic extracts of *Harpagophytum procumbens* (SteiHap69) and the commercial product (Sogoon tablets) used in this study were from Steiner and Co. (Berlin, Germany). A sample of *Harpagophytum zeyheri* was a gift of extract chemie (Stadthagen, Germany) and was used to make a hydroalcoholic extract. The weight ratio drug/extract was 4.7 : 1 in all samples.

The extraction of the iridoid glycosides was carried out by using methanol; 215 mg of the extract or powdered samples of the pharmaceutical tablets (equivalent to about 215 mg of the extract) were extracted with 25 mL methanol by use of a ultrasonic bath (Sonorex RK1029, Bandelin, Berlin, Germany) for 20 min. The internal standard solution of 1 mL was added. An aliquot of the obtained samples was filtered through a 0.45 μm-PTFE membrane filtration cartridge (Gelman Sciences, Dreieich, Germany) and transferred into the HPLC autosampler vial for analysis.

Quantitative determination was carried out using the internal standard method with cinnamic acid methyl ester as the internal standard. Values given for HS were calculated using a correction factor of 3.0488 in accordance with the monograph *Harpagophyti radix* of the European Pharmacopoeia.[13] Values given for 8pCHG were calculated using a correction factor of 5.4574. This correction factor is a combination of the factor of the monograph (to calculate 8pCHG as HS using the internal standard) and the correction factor 1.79 as given in the literature.[5]

Validation Study

In accordance with the ICH-Guidelines Q2A and Q2B the following validation characteristics were examined: Specificity, linearity, precision, accuracy, and robustness.

Specificity

To demonstrate the specificity of the method, the peaks of the analytes and the internal standard should be separated in a clear manner from other peaks.

Linearity

The linearity of the method was established by injection of standard solutions of the internal standard at seven concentration levels over the range of 0.005–0.105 mg/mL. The peak area versus concentration data was treated by linear regression analysis.

Precision and Accuracy

Precision was performed at two different levels–repeatability and intermediate precision. The repeatability ("intra-assay precision") was determined by

six repeated assays of the same lot of the *Harpagophytum* extract. For the intermediate precision, six determinations were repeated on a different day by another analyst on different equipment.

The accuracy of the method was assessed by nine determinations of the spiked analytes in blank matrices samples over three concentrations levels with three replicates each. The per cent recovery was then calculated.

Robustness

The robustness was considered during the development phase of the method by varying parameters such as influence of pH in the mobile phase, column temperature, flow rate, and different lots of HPLC columns. These method parameters were evaluated one factor at a time.

The stabilities of the analytical solution and of the internal standard solution were measured over 48 h.

RESULTS AND DISCUSSION

The chromatographic conditions were optimised to obtain good separations between the peaks of the iridoid glycosides, the internal standard, and their closest peaks. A fast linear gradient at a flow rate of 5 mL/min was found to adequately separate the compounds of interest with a reasonable short run time of only 5 min. Typical chromatograms for *H. procumbens* and *H. zeyheri* are given in Figures 1 and 2.

In accordance with the ICH-guidelines Q2A and Q2B, the following validation characteristics were examined: Specificity, linearity, precision, and accuracy.

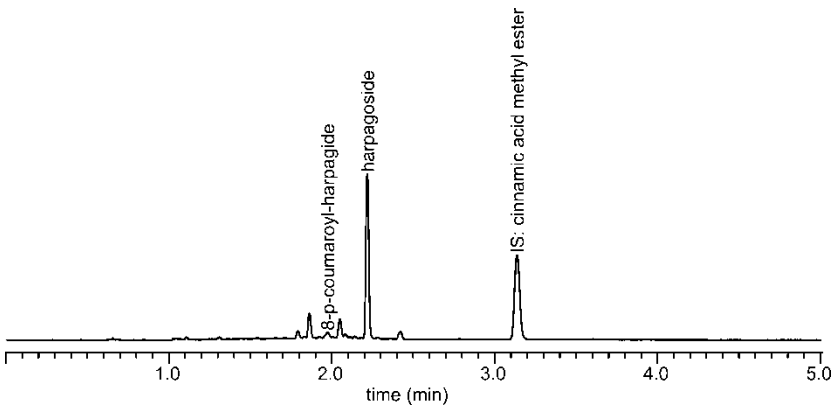

Figure 1. Representative chromatogram at 278 nm of *Harpagophytum procumbens*.

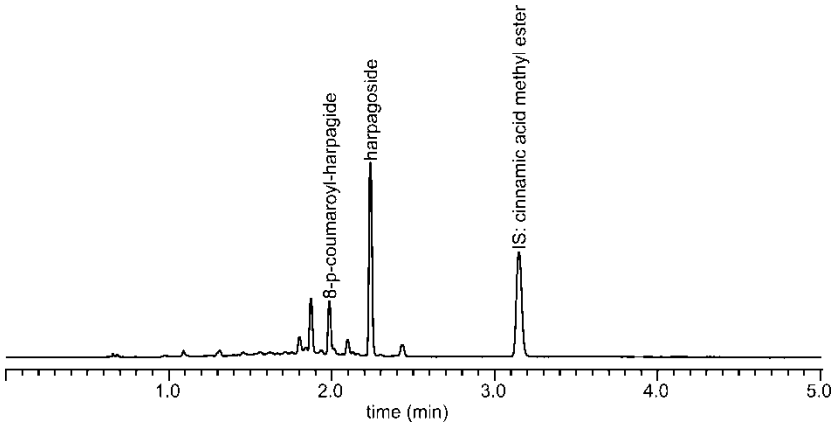

Figure 2. Representative chromatogram at 278 nm of *Harpagophytum zeyheri*.

Specificity

The ability of this method to separate the peaks of the analytes in conjunction with the internal standard in the extracts and preparations of the *Harpagophytum* species, indicates the specificity of the method. Using the online UV-spectra of the PDA-detector the peak purity was confirmed. The peak identification was performed by comparing the UV-spectra with those of the reference substances.

Linearity

The linearity of the internal standard, cinnamic acid methyl ester was measured over the concentration range 0.005–0.105 mg/mL and the following regression equation was found by plotting the peak area (y) against the concentration of the internal standard (x):

$$y = 2500 + 16440081 * x$$

The 95% confidence interval of the y-intercept showed no significant difference to 0. The coefficient of correlation r = 0.99995 demonstrated the excellent relationship between peak area and concentration.

Precision and Accuracy

The relative standard deviation of the repeatability (intra-day precision) was 0.6% for harpagoside and 0.5% for 8-p-coumaroyl-harpagide. The RSD

of the intermediate precision was 0.5% for harpagoside and 0.6% for 8-p-coumaroyl-harpagide. The very low RSD values show that the proposed method is precise.

The accuracy of the method was evaluated by recovery assays. The recovery of harpagoside and 8-p-coumaroyl-harpagide were very high with $>99\%$ for HS and 8pCHG, respectively, and the RSD was $<1\%$.

Robustness

There was no important effect on the peak shape and assay results using intentional variations in parameters such as pH of the mobile phase (± 0.5 pH units), column temperature ($\pm 5^{\circ}C$), flow rate ($\pm 0.5\,mL/min$), and different lots of HPLC columns. These method parameters were evaluated one factor at a time.

The compounds in the sample solutions were stable for at least 48 h after preparation.

The internal standard solution was found to be stable over the investigated period of 4 weeks at 4°C.

Application

After method validation, different samples of extracts and tablets were analyzed by the present method. Harpagoside and 8-p-coumaroyl-harpagide

Table 1. Results of the determination of iridoid glycosides in different batches of *Harpagophytum* extracts and a commercial product using the validated Fast-HPLC method on a monolithic column

		Amount of harpagoside (HS)		Amount of 8-p-coumaryoyl-harpagide (8pCHG)		Ratio %8pCHG/ (HS + 8pCHG)
	Batch no.	(%)	(mg/tbl.)	(%)	(mg/tbl.)	
Harpagophytum procumberns extracts						
Sample 1	WL29422	2.01		0.15		7
Sample 2	WL11831	1.92		0.16		8
Sample 3	WL12231	1.84		0.14		7
Harpagophytum zeyheri extracts						
Sample 4	260600/01	3.07		1.26		29
Sample 5	WL00221	1.90		0.94		33
Commercial product						
Sample 6	6911841		11.85		0.94	7
Sample 7	6912241		11.43		0.85	7

were detected from all samples, and the percentage of 8pCHG from the sum of HS and 8pCHG was calculated.

The ratio was between 7 and 8 in *H. procumbens* extracts and in different batches of a commercial product, indicating relatively pure *H. procumbens* raw material.

The ratio is above 29 in *H. zeyheri* extracts. The results are shown in Table 1.

CONCLUSION

In conclusion, the developed Fast-HPLC method for the determination of the iridoid glycosides harpagoside and 8-p-coumaroyl-harpagide is specific, precise, accurate, and robust. It was possible to separate and determine these compounds in a single run of only a 5 min run time. In addition, it was possible to distinguish between raw materials derived from *Harpagophytum procumbens* and *H. zeyheri.* The method may be used for quality control of extracts and its pharmaceutical preparations of the *Harpagophytum* species.

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