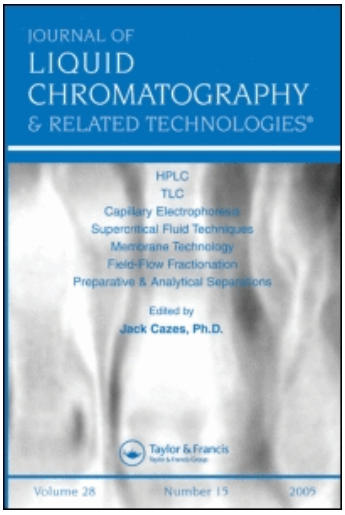

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Determination of Harpagide, 8-Para-Coumaroyl Harpagide, and Harpagoside by High Performance Liquid Chromatography in Harpagophytum Procumbens Drugs and in a Commercial Extract

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DETERMINATION OF HARPAGIDE, 8-PARA-COUMAROYL HARPAGIDE, AND HARPAGOSIDE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN HARPAGOPHYTUM PROCUMBENS DRUGS AND IN A COMMERCIAL EXTRACT

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ABSTRACT

A selective method for the determination of HARPAGIDE (HG), 8-para-coumaroyl HARPAGIDE (8PCHG) and HARPAGOSIDE (HS) is described. HS and 8PCHG are the two main compounds extracted from commercial dry extract of Harpagophytum secondary roots.

Analysis of iridoids was performed using a linear gradient system of methanol / water as mobile phase, on RP 18 column. For HG Light-Scattering and UV detection were used for the quantification. Assays were linear in the concentration ranges studied. Statistic evaluation of the method was also realised to improve its security. In a total run time of 60 mn this procedure permitted to quantify simultaneously these three iridoids in the drug including HG for the first time in HPLC.

INTRODUCTION

Harpagophytum procumbens is a medicinal plant [1] which grows in Kalaharia desert (Namibia, South Africa) and presents anti-inflammatory properties [2].
Main active compounds have been isolated and identified: They are glucoiridoids [3](fig. 1),

HARPAGOSIDE (HS) and 8 PARA-COUMAROYL HARPAGIDE (8PCHG) were found in large proportion in the drug and HARPAGIDE (HG) is not a negligible

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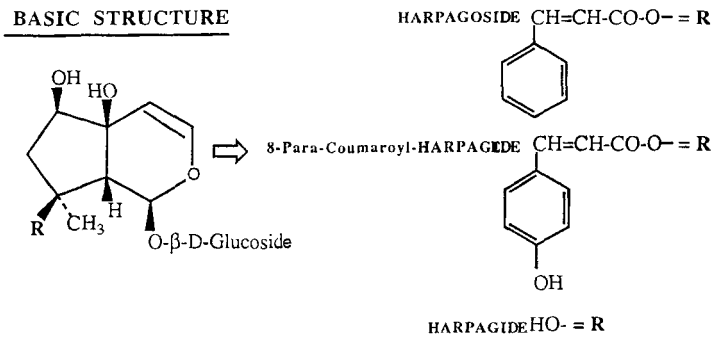

FIGURE 1 : Glucoiridoïds of Harpagophytum procumbens [3]

iridoid, so its quantification is very important because it is a direct metabolite of HS and 8 PCHG.

In order to use Harpagophytum for new pharmaceutical applications, it was necessary to know a sensitive and selective method for the quantification of drug's active components and also their main metabolite : HG.

A few methods using HPLC are described [4] [5] for the assay of HS but until now, there was not any procedure reported for the analysis of 8PCHG and HG.The very low UV absorbtion of HG did not permit quantitative assay by HPLC. The use of Light-Scattering detector and UV detector allows the simultaneous assay of all the components.

The aim of our study was to establish a sensitive and rapid method to appreciate quality and stability of the drug.

MATERIALS

Standards :

8 PCHG and HS (for preparation of HG) were obtained by preparative liquid chromatography from dry commercial extract of Harpagophytum secondary roots (RES PHARMA ®).
HG for HPLC was prepared by hydrolysis of HS isolated by preparative liquid chromatography.
HS was purchased from Extrasynthèse ® Genay France

Samples

Drug :

Drugs were purchased from Alban Muller ® (Marseille, France) (little discs) and Technic Arome ® (Allauch, France)(peaces 1 cm3). Both were grinded and placed into a dessicator before preparation.

Extract :
Extract was purchased from RES PHARMA ® (Paris, France) (fine water soluble powder)

Solvents for isolation :

Solvents were from Farmitalia Carlo Erba ® Chemicals (Milano, Italy) Puro grade for isolation.

Solvents for HPLC :

We used special solvents grade for residute analysis to prepare HPLC mobile phases.
Methylic Alcohol RS 414917 RSE for electronic use ERBATRON, Farmitalia Carlo Erba ® Milano, Italy.
Purified and desionised Water was from Milli-Ro system Millipore (USA).
All solvents were filtered throught a Millipore filter (0,45 µm for methanol, and 0,2 µm for water). A solvent in line filter 0,2 µm Wathman ® (Maidstone, England) was installed before HPLC pump for the water.

Stationary preparative phases :

- Aluminium-oxyd, activity I, II Merck (Paris, France)
- Silica-gel, kieselgel 60 Merck
- Preparative HPLC RP 18 column Merck Lichroprep 15 - 25 µm, 5 X 14 cm.
- Preparative HPLC RP 18 pre-column Jones Chromatography (Hengoed, Austria), 15 - 35 µm, 5 X 5 cm.
- Sep-pak ® Waters C18 cartridges for solid phase extraction (Milford, Massachusetts, USA)

Apparatus :

Preparative HPLC system consisted of one C.E.D.I. (Lannemesan, France) equipped with two pumps.

Analytic HPLC system consisted of :
2 pumps : Waters ® Model 501.
1 20 µl rheodyne injector.
1 Waters ® programmable multiwavelenght detector Model 490.
1 Light-Scattering detector (Cunow DDL 21, Waters, St Quentin en Yveline France)
All the system was monitored by a Waters 840 data and chromatographic control station installed on a digital professional 380 computer.

As stationary phase, a Phenomenex ® RP 18 (Interchim France) Bondclone 10 µm 300 X 3,9 mm column was used with a precolumn Guard pak ® µBondapak C18 Waters.

Composition of the mobile phase as well as the UV detector wavelenght and Light Scattering Detector settings are described in the following.

METHOD

Isolation of standards :

Dry extract was extracted with methanol, then, after evaporation under vacuo, the residue was chromatographied on Aluminium-oxyd (Ethanol 80v : H2O 20v) and on Silica-gel (CHCl3 : Methanol) preparative open column. A final purification was realised by HPLC (preparative column RP 18 with solvent Methanol : H2O). Hydrolysis of HS was performed with NaOH 0,2% and isolation of HG was achieved by HPLC (preparative column RP 18, solvent Methanol : H2O).
Identification was performed by NMR C^{13} spectroscopy by comparison of experimental data with references [3].

Chromatographic conditions

The analytical column was maintained at room temperature, the mobile phase consisted of Methanol / Water gradient (fig 2) delivered at a flow rate of 1 ml/min, the UV multichanel detector was set at 278, 305 and 312 nm to monitor HS and 8PCHG respectively on a large range of concentrations.

Under these conditions, the retention times for HS and 8PCHG were 36,3 min and 30,8 min respectively. The total run time was 60 min.

In serie after UV detector with a 500 nm lenght capillar, was connected a Light Scattering Detector (DDL). For evaporative, the nebulization of the eluent was provided by a stream of pressurized air at 1,6 bars. The nebulized solvent was evapored at 63 ± 3°C. The pressurized air was filtered through a millex FG 50 (0,2 µm, Millipore).

Under these conditions the retention times for HG, 8PCHG and HS were 8 min, 31 min and 36,5 min, respectively.

Preparation of standards :

Solutions of HS, 8PCHG, HG were prepared at 10 mg/ml in water and cold stored at -20°C until use.
Appropriate dilutions of the stock were made just before analysis with water to prepare standards. Solutions containing HS and 8PCHG were stable for one week. HG in solution at ambient temperature was stable during no more one hour. Peak areas were processed to establish a relation with concentrations.

Preparation of assays :

Drugs :
5 g of drug were placed into 70 ml of methylic alcohol and warmed during 15 minutes. Preparation was filtered and completed to 100 ml of methylic alcohol and evaporated in vacuo. The residue was dissolved into 50 ml of water, 2 ml of this preparation were concentred on SEP PACK ®. Elution was performed with 10 ml of water and 10 ml of methanol, the methanolic fraction was evapored under vacuo and dissolved with 500 µl of water. The final solution was filtered (0,2 µm Dynagard ® Merck, Paris) and injected.

FIGURE 2a

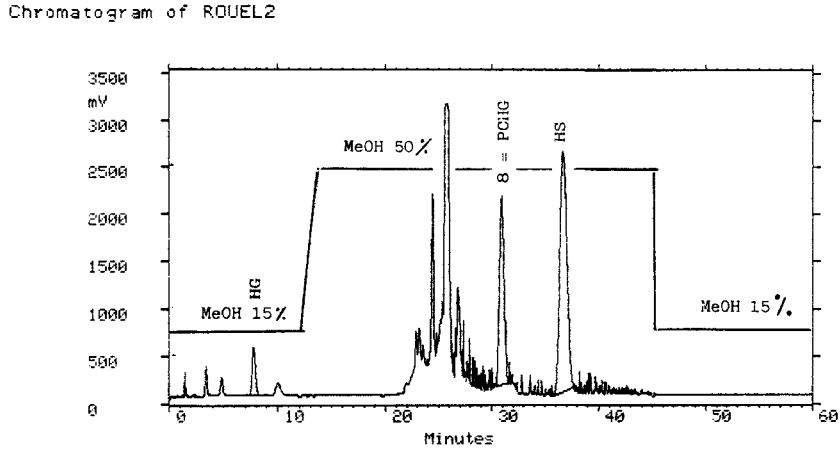

FIGURE 2b

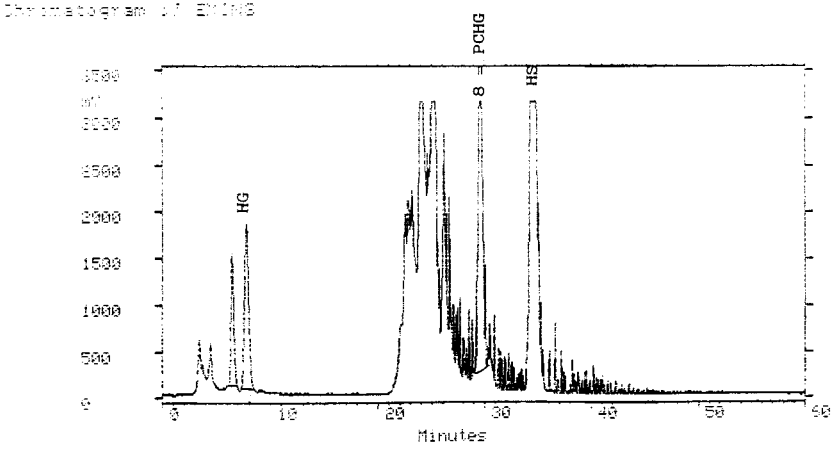

FIGURE 2 : Chromatogram of a drug (a)
Chromatogram of an extract (b)

Extract :
5 g of dry extract were dispersed in 100 ml of water. 2 ml of the solution were concentrated on SEP PACK ®, with the same method than described previously.

RESULTS

Assay of standards

Linearity
Calibration curves were plotted by correlating the peak areas against the corresponding concentrations, the responses were linear in the concentration range studied; the square regression lines and the corresponding coefficients of correlation are described in the following table.

With light scattering detection the relation between Concentrations and Areas is an exponentiel curve. We took [Log (Areas) - 5] and [Log (Concentrations) + 2] in order to propose a linear curve of calibration. So, coefficients a and b were calculated with the Log method above described.

Y = a X + b.
Y = Response (Area or Log (Area)); X = Concentration (mg/ml).
r = Coefficient of correlation.
n = number of calibration curves from which the data were calculated

Corresponding calibration curves are described by Fig 3.

Injection reproducibility of references samples

One synthetic solution of the 3 references samples was injected 10 times. Concentration of each sample and coefficient of variation observed are described in the following TABLE 2.

Preparation reproducibility of references samples

Five solutions of HS, 8PCHG were prepared and injected twice. Coefficient of variation observed for the five analysis and correspondent concentrations are displayed in the TABLE 3.

TABLE 1

Compounds	Detection	a	b	r	n
HS	UV 278 nm	50407674	-89452	0,9998	3
	UV 305 nm	18801766	-1154236	0,9993	3
	DDL	1,9468	-1,1398	0,9981	3
8PCHG	UV 278 nm	28193498	-724201	0,9992	3
	UV 312 nm	42225630	22390	0,9994	3
	DDL	1,8879	-0,831564	0,9995	3
HG	DDL	1,9404	-1,44357	0,9982	3

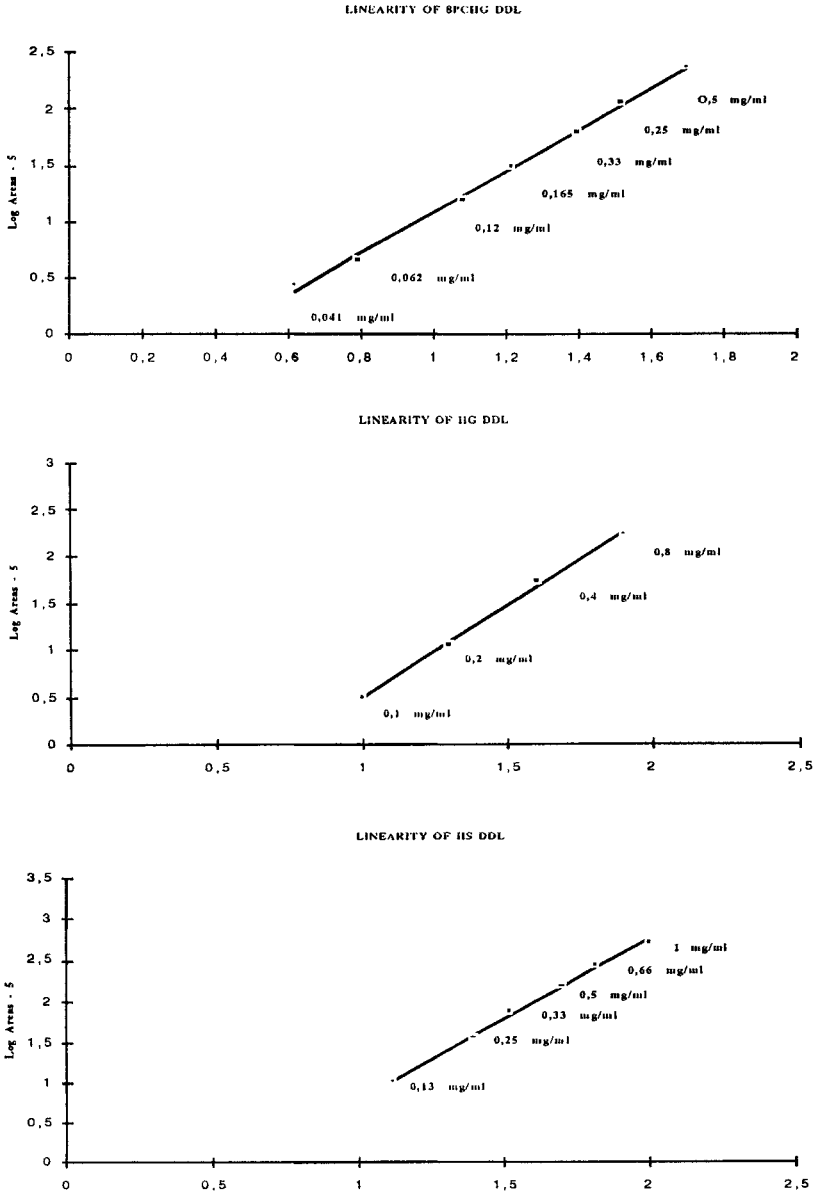

FIGURE 3 : Calibration curves with DDL

TABLE 2

Compounds	HG (0,8 mg/ml) DDL	8PCHG (1 mg/ml) DDL	8PCHG (1 mg/ml) UV 278 nm	HS (2 mg/ml) DDL	HS (2 mg/ml) UV 305 nm
Average	17747330	28215629	27284507	90517456	36178026
$\sigma 2$	461209	1931394	430856	1948255	605520
Coef of Variation	2,60%	6,85%	1,58%	2,15%	1,67%

Compounds	HS 0,2 mg/ml UV 278 nm	8 PCHG 0,06 mg/ml UV 312 nm
Average	10828267	2747117
$\sigma 2$	166323	40121
Coef of variation	1,54%	1,46%

TABLE 3

Compounds	HG (0,8 mg/ml) DDL	8PCHG (1 mg/ml) DDL	8PCHG (1 mg/ml) UV 278 nm	HS (2 mg/ml) DDL	HS (2 mg/ml) UV 305 nm
Areas' average	18225698	33338323	25698286	91077167	36288233
$\sigma 2$	760229	894877	243775	4016324	349475
Coef of Variation	4,17%	2,68%	0,95%	4,41%	0,96%

Compounds	HS 0,2 mg/ml UV 278 nm	8PCHG 0,06 mg/ml UV 312 nm
Average	*10655909*	1737040,4
$\sigma 2$	247851,17	22831,64
Coef of Variation	*1,54%*	1,31%

Detection limits

Limits of detection were observed with a minimum signal-to-noise ratio of 2:1.

8PCHG with : UV 312 nm detection = 2 µg/ml,
DDL = 50 µg/ml,
HS with : UV 278 nm detection = 4 µg/ml,
DDL = 50 µg/ml,
HG with DDL detection = 60 µg/ml.

Quantification limits

Quantification limits were for HS : 8 µg/ml and for 8PCHG : 4 µg/ml. For ten injections, coefficients of variation were 2,44 % and 3,05 % respectively. These results were obtained with UV detection (respectively : 278 and 312 nm). With the DDL we found, in order to titrate simultaneously the three compounds, 100 µg/ml and the following coefficients of variation : HG = 7,54 %, 8PCHG = 4,98 %, HS = 7,56 % for ten consecutive injections.

Assay of samples

Assays were realised on two samples of drugs (Alban Muller ® and Technic Aromes ®) and a commercial extract : RES PHARMA ®.
A 1/20 dilution has been performed for injections realised at 278 nm and 312 nm wavelenghts to avoid saturation of the detector.
Injections and methods of preparation are reproducible.
A part of results are summarized in the TABLES 4 and 5

Quantification of samples :

The final concentration in drugs and extract was (in g /100 g of sample).

The difference between each detection mode was 5 % max.

DISCUSSION

Our method enables quantification of the main iridoids of Harpagophytum. Even if Light-Scattering Detection is less sensitive than UV detection for the HS and 8PCHG, it permits to titrate drugs or extracts with a quantification limit of 0,05 %.
The concentration of the iridoids are similar in the different samples studied.
This method presents a great interest for Harpagide. It was until now impossible to

TABLE 4
Injection reproducibility of extracts

Compounds n=10	HG DDL	8PCHG UV 278 nm	8 PCHG UV 312 nm	HS UV 278 nm	HS UV 305 nm
Areas' average	26161233	38532201	2747117	9840648	58428109
σ2	945422	443739	40121	153932	581160
Coef of variation	3,61%	1,15%	1,46%	1,56%	0,99%

TABLE 5
Preparation reproducibility of extracts

Compounds n=10	HG DDL	8 PCHG UV 312 nm	8PCHG DDL	HS UV 278 nm	HS DDL
Areas' average	24942936	3600447	37339706	10105709	57372130
σ2	307501	55650	755635	295357	820691
Coef of variation	1,23%	1,46%	2,02%	2,92%	1,43%

TABLE 6

Compounds	HG	8PCHG	HS
Drugs			
Technic Aromes	0,3	0,98	1,64
Alban Muller	0,37	1,07	1,64
Extract			
Res Pharma	0,56	0,7	1,61

titrate it by traditionnal HPLC detection method, combination of the two detection modes allows the assay of the three compounds simultaneously.

For UV detection the different wavelenghts employed permit quantification on a large range of concentrations :
- For low concentrations, 278 nm for Harpagoside and 312 nm for 8 Para Coumaroyl Harpagide, will be used (with a quantification limit in extracts of 0,004 % for HS and 0,002 % for 8 PCHG).
- For high concentrations, 305 nm for HS and 278 nm for 8PCHG will be chosen to ovoid a dilution and so titrate the three products simultaneously. (Run time is 60 min for each analysis).
- For medium concentrations (no more than 0,8 %) it is possible to use the Light-Scattering Detector for HS, 8PCHG and HG without dilution of the sample prepared with the method described previously.

Comparison between both detections was very important to do an evalution of Light Scattering Detector performances. For the HG there is not any solution with UV detection, so this detector is interesting. But for the other compounds, it is less sensitive and less reproducible than UV detector.

Therefore, the complete separation and the simultaneous determination of Harpagoside, 8 para-coumaroyl Harpagide and Harpagide is possible by the proposed method in one hour. It is the first HPLC method described for 8PCHG and HG using an original kind of detection : the Light Scattering Detector for HG.

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