ELSEVIER

Journal of Chromatography B, 702 (1997) 240–244

JOURNAL OF CHROMATOGRAPHY B

Short communication

Contribution to the quantitative and enantioselective determination of kavapyrones by high-performance liquid chromatography on ChiraSpher NT material

Georg Boonen[a], Mona-Antonia Beck[a], Hanns Häberlein[a,*]

[a]*Institut für Pharmazeutische Biologie der Philipps-Universität, Deutschhausstraße.17 A, D-35032 Marburg, Germany*

Received 30 May 1997; received in revised form 16 July 1997; accepted 17 July 1997

Abstract

A simultaneous HPLC separation of the enantiomers of kavain, dihydrokavain, methysticin and dihydromethysticin, as well as the achiral dienolides yangonin and desmethoxyyangonin was carried out on a ChiraSpher NT column. For quantitative determinations, calibration curves with correlation coefficients between 0.9982 and 0.9996 were established for the genuine kavapyrones. Detection limits between 0.25 μg and 0.5 μg per injection were measured at 240 nm. The defined scopes of work corresponded with the different kavapyrone amounts, depending on growth factors of distinct plant locations. The precision of the method was verified by analysing a phytopharmacon with a nominal value of 40 mg kavapyrones per tablet. The evaluation revealed 39.62 mg per tablet by the sum of single calculated kavapyrones. Relative standard deviations between 1.06% and 2.39% were found for the compounds under investigation. The accuracy of the method was proved by a recovery of 99.7%. To simplify the determination of the total kavapyrone amount, response factors and correlation factors for (+)-dihydrokavain, (+)-methysticin, (+)-dihydromethysticin, yangonin and desmethoxyyangonin were calculated relative to (+)-kavain. © 1997 Elsevier Science B.V.

Keywords: Kavapyrones; Kavain; Dihydrokavain; Methysticin; Dihydromethysticin; Yangonin; Desmethoxyyangonin

1. Introduction

Preparations of *Piper methysticum* Forst. as herbal medicine are used for nervous states of anxiety, uptightness and restlessness [1–5]. The kavapyrones are considered to be the active substances and are used for the standardization of herbal medicines. The six major kavapyrones of the plant extract can be classified into enolides and dienolides (Fig. 1).

In contrast to the dienolides, yangonin and desmethoxyyangonin, which are achiral α-pyrones, the enolides (+)-kavain, (+)-dihydrokavain, (+)-methysticin and (+)-dihydromethysticin represent 5,6-dihydro-α-pyrones with an asymmetric carbon atom.

Recently, the separation of chemically synthesized enolide racemates into pure enantiomers by preparative HPLC was described [6]. Thus, a time consuming and expensive isolation of the natural enolides from plant material is no longer necessary. With regard to enantioselective pharmacological activities, pure enantiomers should be tested in clinical trials and pharmacological investigations. Evidence of contamination with the wrong enantiomer requires

*Corresponding author.

0378-4347/97/$17.00
PII S0378-4347(97)00389-7

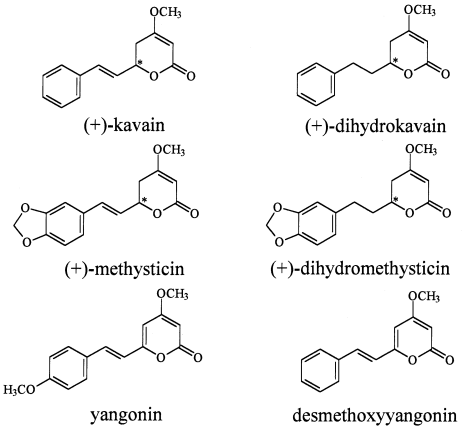

Fig. 1. Kavapyrones of *Piper methysticum* Forst.

enantioselective analytical methods. This paper describes a HPLC method to quantitatively determine kavapyrones using the chiral ChiraSpher NT material.

2. Experimental

2.1. Chemicals

1,4-Dioxane (LiChrosolv), *n*-hexane (SupraSolv), *n*-pentane (SupraSolv), dichloromethane (LiChrosolv) and ethanol (LiChrosolv) were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

The HPLC pump MSDS 600 E, diode array detector 996 and autosampler WISP 712 were from Waters (Eschborn, Germany). The HPLC column thermostat 5–85°C and the 4-channel-online degasser were from Knauer (Berlin, Germany) and the software MILLENNIUM V2.1 was from Waters.

2.3. Chromatography

Running conditions: analytical column: ChiraSpher NT (4×250 mm, 5 μm, a generous gift from Merck); eluent: 1,4-dioxane–*n*-hexane (18:82, w/w); flow-rate: 0.5 ml/min; temperature: 15°C; absorbance: 240 nm.

2.4. Sample preparation

2.4.1. Plant material

A percolate of *Piper methysticum* Forst. roots was prepared with ethanol using a ratio of 1 g of the drug to 16.7 ml of the extractant. The solvent was removed under reduced pressure. The remaining extract was dissolved in a ten-fold amount of 1,4-dioxane–*n*-hexane (18:82, w/w). After filtration (0.45 μm), 20 μl of the solution were investigated by HPLC.

2.4.2. Phytopharmacon

Approximately 1.5 g of powdered tablets were exhaustively extracted with dichloromethane. The organic solvent was evaporated and the remaining residue was dissolved in 50 ml of 1,4-dioxane–*n*-hexane (18:82, w/w). After filtration (0.45 μm), 25 μl of the solution were investigated by HPLC.

2.5. Reference substances

The pure enantiomeres (+)-kavain, (+)-dihydrokavain, (+)-methysticin and (+)-dihydromethysticin were obtained by preparative HPLC of the corresponding racemates on a ChiraSpher NT preparative column [6]. (±)-kavain, (±)-dihydrokavain, (±)-methysticin and (±)-dihydromethysticin, yangonin and desmethoxyyangonin were obtained from Krewel-Meuselbach (Eitorf, Germany). The kavapyrones were identified by NMR and mass spectroscopy [7].

2.6. Peak identification

Retention times and UV spectra of reference substances were used to identify the peaks in the chromatograms (for k' values see Table 1). Using a refractive index detector, the retention time of unretained compound (t_0) was determined by injecting *n*-pentane and measuring the retention time of the first peak in the chromatogram.

2.7. Calculation of factors

Response factors were calculated by the kavapyrone amounts in μg/ml divided by the corresponding peak areas in Vs. Correlation factors were

Table 1
Regression data and analytical parameters of kavapyrones

Kavapyrone	Capacity factor (k')	Rel. retention time (α)	Chrom. resolution (R)	Equation of the calibration curve (n=6)	Correlation coefficient	Scope of work (mg/ml)	Detection limit (μg/injection)	Response factor (μg/ml×V×s)	Correlation factor
Desmethoxyyangonin$_1$	4.48			$f_{(x)}$=0.241x+0.59	0.9983	0.25–0.75	0.0250	12.28	2.70
(−)-Dihydrokavain$_2$	5.24	$\alpha_{1/2}$=1.170	$R_{1/2}$=1.71						
(+)-Dihydrokavain$_3$	5.56	$\alpha_{2/3}$=1.061	$R_{2/3}$=1.14	$f_{(x)}$=0.486x+0.58	0.9989	0.25–1.00	0.0250	20.92	4.60
Yangonin$_4$	6.89	$\alpha_{3/4}$=1.239	$R_{3/4}$=1.88	$f_{(x)}$=0.425x+1.88	0.9993	0.50–2.50	0.0125	20.74	4.56
(−)-Kavain$_5$	9.04	$\alpha_{4/5}$=1.312	$R_{4/5}$=3.77						
(+)-Kavain$_6$	9.30	$\alpha_{5/6}$=1.029	$R_{5/6}$=0.50	$f_{(x)}$=0.128x−0.80	0.9982	1.25–2.25	0.0125	4.55	1.00
(−)-Dihydromethysticin$_7$	10.74	$\alpha_{6/7}$=1.155	$R_{6/7}$=1.00						
(+)-Dihydromethysticin$_8$	11.30	$\alpha_{7/8}$=1.052	$R_{7/8}$=1.17	$f_{(x)}$=0.367x+0.58	0.9993	0.25–1.25	0.0125	15.97	3.51
(−)-Methysticin$_9$	17.31	$\alpha_{8/9}$=1.532	$R_{8/9}$=7.41						
(+)-Methysticin$_{10}$	18.19	$\alpha_{9/10}$=1.051	$R_{9/10}$=0.93	$f_{(x)}$=0.262x+0.54	0.9996	0.25–0.75	0.0125	11.38	2.50

calculated by the response factor of each kavapyrone divided by the response factor of kavain.

3. Results and discussion

The HPLC fingerprint of the genuine kavapyrones from an ethanolic extract of *Piper methysticum* Forst. in comparison to the simultaneous separation of both the enolide racemates and the achiral dienolides of a test mixture is given in Fig. 2. The HPLC chromatogram of the plant extract shows only the genuine (+)-enantiomeres, because the natural synthesis of the enolides is enantioselective. Our previous investigations have shown that even minor flow-rate and temperature variations had an unusually large influence on the chromatographic resolution (R) of the kavapyrones [6]. The genuine kavapyrones were eluted in the following order: desmethoxyyangonin, (+)-dihydrokavain, yangonin, (+)-kavain, (+)-dihydromethysticin and (+)-methysticin. The HPLC chromatogram of the racemic kavapyrones consistently revealed longer retention times for the genuine dextrorotatary enantiomers in comparison to the corresponding laevorotatary enantiomers. Although the separation of enantiomers takes 120 min by isocratic elution, using a gradient procedure with shorter retention times is not an alternative. Particularly when conducting series of investigations, the reproduction of the initial conditions led to a loss of time. Values for the relative retention times (α), capacity factors (k') and chromatographic resolutions (R), calculated from the HPLC analysis of the

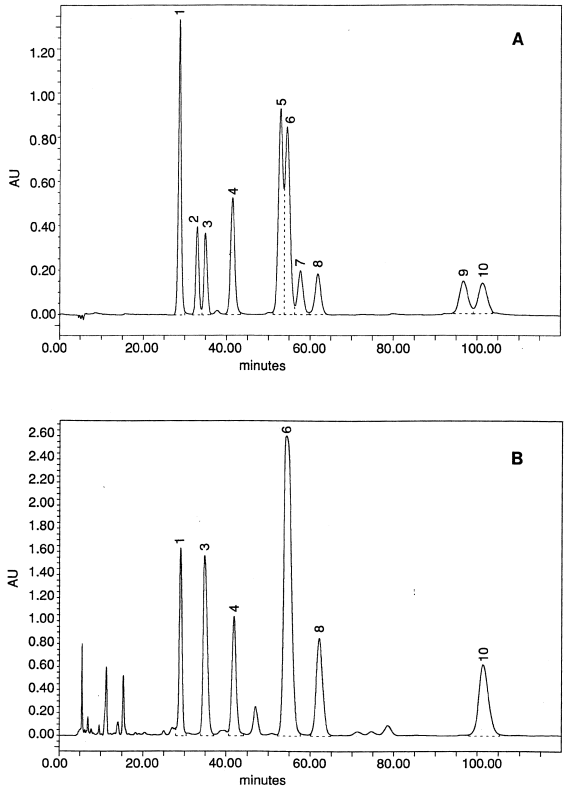

Fig. 2. HPLC chromatogram of synthetic kavapyrones (A) and a *Piper methysticum* Forst. extract (B): 1=desmethoxyyangonin, 2=(−)-dihydrokavain, 3=(+)-dihydrokavain, 4=yangonin, 5=(−)-kavain, 6=(+)-kavain, 7=(−)-dihydromethysticin, 8=(+)-dihydromethysticin, 9=(−)-methysticin, 10=(+)-methysticin.

kavapyrone test mixture (Fig. 2), are summarized in Table 1.

For quantitative determinations using external standards, calibration curves were established for every genuine kavapyrone. Therefore, no less than six different concentrations per kavapyrone were analysed by HPLC in triplicate. Correlation coefficients between 0.9982 and 0.9996 were found for the calibration curves (Table 1). With regard to the differences in the relative kavapyrone amounts, which depend on the growth factors of different plant locations, suitable linear realm of activities were selected [8]. Equations of standard curves, correlation coefficients, linear realm of activities and detection limits for the investigated kavapyrones are given in Table 1. The detection limit was defined by the kavapyrone concentration, which yielded a threefold peak height in comparison to the random noise of the baseline. Values between 0.25 μg and 0.5 μg per injection were found using a detection wavelength of 240 nm.

For the determination of the total kavapyrone amount in the crude drug of *Piper methysticum* Forst. as well as in solid and liquid formulations, response factors and correlation factors for (+)-dihydrokavain, (+)-methysticin, (+)-dihydromethysticin, yangonin and desmethoxyyangonin were calculated relative to (+)-kavain (Table 1). Kavain is the only kavapyrone which is available on the market, but only in the racemic form. Since the enantiomers did not show any difference in their absorption behaviour, racemic kavain is also applicable as a reference substance.

The accuracy of the quantitative kavapyrone determination was verified by analysing a phytopharmacon. From powdered tablets, the kavapyrones were exhaustively extracted with dichloromethane. The organic solvent was evaporated and the remaining residue was resolved in a defined portion of a 1,4-dioxane–*n*-hexane mixture (18:82, w/w).

Using similar conditions, six samples were prepared and injected into the HPLC system in triplicate. Both the single compounds and the sum of the kavapyrones were statistically evaluated (Table 2). The R.S.D. values of single kavapyrones were between 1.06% and 2.39%, with 1.97% for the total determination. A nominal value of 40 mg kavapyrones per tablet was given for the phytopharmacon. The analyses revealed a mean value of 39.62 mg per tablet, calculated by the sum of the single kavapyrone amounts. Taking the correlation factors into consideration, a similar value of 39.69 mg per tablet was found, calculated as kavain. For a certainty of 95%, a confidence interval ($P=95$) of $\mu = 39.6 \pm 0.82$ mg/tablet was found.

The accuracy of the quantitative kavapyrone determination was verified by the analyses of six samples ranging from 80% to 120% of the analysed value of 39.6 mg/tablet. The mean of recovery yielded a value of 99.7% with a confidence interval ($P=95$) of $\mu = 99.7 \pm 1.78\%$. The R.S.D. value was 1.71%.

Table 2
Evaluation of the quantitative determination of kavapyrones

Kavapyrone	Content of single kavapyrones in the phytopharmacon (mg/tablet)[a]	R.S.D. of the kavapyrone determination (%)[a]	Confidence interval ($P=95$)	Content of kavapyrones calc. as kavain (mg/tablet)[a]	Recovery (%)[a]	R.S.D. of recovery (%)[a]
Desmethoxyyangonin	4.10	1.06	$\mu = 4.10 \pm 0.05$ mg/tablet	4.23	99.6	1.45
(+)-Dihydrokavain	7.78	2.26	$\mu = 7.78 \pm 0.18$ mg/tablet	7.92	99.7	2.09
Yangonin	8.92	1.75	$\mu = 8.92 \pm 0.16$ mg/tablet	8.87	99.3	2.10
(+)-Kavain	6.15	1.98	$\mu = 6.15 \pm 0.13$ mg/tablet	6.15	99.8	1.28
(+)-Dihydromethysticin	6.75	2.25	$\mu = 6.75 \pm 0.16$ mg/tablet	6.75	99.7	1.05
(+)-Methysticin	5.92	2.39	$\mu = 5.92 \pm 0.15$ mg/tablet	5.77	99.3	1.99
Sum of kavapyrones[b]	39.62	1.97	$\mu = 39.62 \pm 0.82$ mg/tablet	39.69	99.7	1.71

[a] All values are expressed as mean of at least six distinct analyses.
[b] Nominal value: 40 mg kavapyrones/tablet.

Acknowledgments

This research was financially supported by Krewel-Meuselbach, Eitorf, Germany.

References

[1] Bundesanzeiger Nr. 101 vom 1.6.1990, monograph of the commission E.
[2] B. Geßner, Z. Cnota, Phytother. 15 (1994) 30.
[3] D. Johnson, A. Frauendorf, K. Stecker, U. Stein, TW Neurol. Psychiatr. 5 (1996) 349.
[4] E. Kinzler, J. Krömer, E. Lehmann, Arzneim.-Forsch./Drug Res. 41 (1991) 584.
[5] E. Holm, U. Staedt, J. Heep, C. Kortsik, F. Behne, A. Kaske, I. Mennicke, Arzneim.-Forsch./Drug Res. 41 (1991) 673.
[6] H. Häberlein, G. Boonen, M.-A. Beck, Planta Med. 63 (1997) 63.
[7] H. Achenbach, W. Regel, Chem. Ber. 106 (1973) 2648.
[8] R. Hänsel, H. Woelk, Spektrum Kava-Kava, Aesopus, Basel, 1996.