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HPLC analysis of flavokavins and kavapyrones from *Piper methysticum* Forst.

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Abstract

A simultaneous HPLC separation of the six major kavapyrones and the flavokavins A–C in an ethanolic extract of *Piper methysticum* was carried out on a Symmetry C18 column. For quantitative determinations of the flavokavins, calibration curves with correlation coefficients between 0.9986 and 0.9998 were established. Detection limit for each flavokavin of 0.5 ng per injection was measured at 355 nm. The precision of the HPLC analysis was verified by six determinations of the content of flavokavins in the kava extract. Flavokavins A–C contents of 0.62 ± 0.01 mg/100 mg, 0.34 ± 0.01 mg/100 mg and 0.14 ± 0.003 mg/100 mg ethanolic kava extract was found, respectively. From the corresponding relative standard deviation of 1.53, 1.99 and 2.30% the confidential interval ($P = 95$) of the mean value was calculated for each flavokavin. The accuracy of the method was proven by recoveries between $99.2 \pm 0.3\%$ and $101.1 \pm 0.4\%$ for the flavokavins A–C.

Keywords: HPLC; Kava; Flavokavins; Kavapyrones

1. Introduction

Preparations of *Piper methysticum* Forst. are used for the treatment of mild and moderate states of anxiety [1–4]. The efficacy and the compatibility of the kava preparations could be verified in clinical studies [5–11], although serious side effects, e.g. hepatitis and acute liver failure were observed recently [12–16]. For this reason European drug control authorities took the decision to withdraw the registration of all kava preparations for herbal medicinal products. Nevertheless kava preparations are still available as food supplement. The kavapyrones are considered to be the active substances and are used for the standardization of this herbal medicines.

Several HPLC methods for separation and quantification of kavapyrones were published. For a good and rapid separation of crude drug extracts silica gel columns were used [17]. Furthermore, RP materials were taken for analysis [18]. Separation of dihydromethysticin and kavain was not achieved with a Prodigy® ODS column [19]. However, Shao et al. [20] obtained a complete separation of the six major kavapyrones within 34 min with a YMCbasic column, while Ganzera et al. [21] were successful with a Luna C8 column within 40 min [20,21]. A computer-assisted optimisation of a HPLC analysis of kavapyrones and Luna C18 column was published by Schmidt and Molnar [22]. Moreover, the separation of kavapyrone enantiomers was described with a ChiraSpher® NT column [23,24]. Capillary electrophoresis using the micellar electrokinetic chromatography technique (MEKC) revealed a baseline separation of the kavapyrones within 15 min [25].

Besides of the kavapyrones the chalkones flavokavins A–C (Fig. 1) are further constituents of *Piper methysticum* Forst. with pharmacological activities [26]. A single qualitative HPLC method for the separation of flavokavins A–C was published. In contrast a complete separation of the kavapyrones was not achieved [19]. So far only a semiquantitative determination of the flavokavins in a dichloromethane extract of *Piper methysticum* was described by Lazar [27] using DC with visual quantitative evaluation [27]. An amount of approximately 0.1% flavokavins A and B was found.

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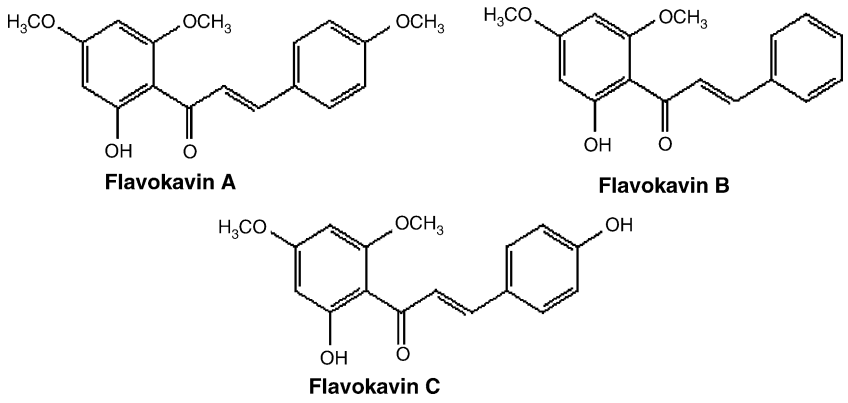

Fig. 1. Flavokavins of *Piper methysticum* Forst.

This work describes a HPLC method for the simultaneous separation of both the kavapyrones and flavokavins and the quantification of the flavokavins.

2. Experimental

2.1. Chemicals

Acetonitrile (LiChrosolv®), isopropanol (SupraSolv®), methanol (Prepsolv®), ethanol (LiChrosolv®) and acetic acid (p.a.) were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

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

2.3. Chromatography

2.3.1. Method 1

Running conditions: analytical column: Symmetrie® C18 (150 mm × 3.9 mm, 5 μm, Waters, Eschborn); eluent: A, 0.1% acetic acid; B, isopropanol/acetonitrile = 7/3 (w/w); mobile phase: 0–26 min 27% B (isocratic elution); 26–35 min to 100% B (linear gradient); 35–50 min B 100% (isocratic elution); flow-rate: 0.5 ml/min; temperature: 30 °C; absorbance: 240 and 355 nm.

2.3.2. Method 2

Running conditions: analytical column: LiChrospher® RP select B (125 mm × 4 mm, 5 μm, Merck, Darmstadt); eluent: 0.1% acetic acid/isopropanol/acetonitrile = 60/28/12 (w/w/w); flow-rate: 0.6 ml/min; temperature: RT; absorbance: 355 nm.

2.4. Plant extract

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. 100.00 mg of the remaining kava extract (drug/extract ratio: 14.5:1) was dissolved in 100.00 ml methanol and after filtration (0.45 μM), 20 μl of the solution were investigated by HPLC.

2.5. Reference substances

Kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin and desmethoxy-yangonin were obtained from Krewel-Meuselbach (Eitorf, Germany). The kavapyrones were identified by NMR and mass spectrometry [28]. The flavokavins A–C were synthesized in our lab according to Hänsel et al. [29] and were identified by NMR, IR and mass spectrometry [29–31]. Methanolic stock solution of flavokavin A (25 μg/ml), flavokavin B (10 μg/ml) and flavokavin C (5 μg/ml) were diluted stepwise and investigated by HPLC to generate the calibration curve.

2.6. Peak identification

Retention times and UV spectra of the reference substances were used to identify the peaks in the chromatograms (for k' values see Table 1).

3. Results and discussion

To achieve a complete separation of both the kavapyrones and the flavokavins a spheric 5 μm Symmetry® C18 material was used. For detection of the kavapyrones (Fig. 2a) a wavelength of 240 nm was chosen. The flavokavins were detected at 355 nm, because this wavelength showed the optimal ratio of the peak area of the three flavokavins A–C to each other (Fig. 2b).

A temperature dependent HPLC analysis in the range of 20 and 40 °C showed the best separation of the kavapyrones at 30 °C. Minor temperature variations had an unusually large influence on the chromatographic resolution which was also found for the separation of the kavapyrone enantiomers on ChiraSpher NT material [24]. Values for the relative retention times (α), capacity factors (k') and chromatographic resolutions (R), calculated from the HPLC analysis of the kava extract (Fig. 2), are summarized in Table 1.

For validation of the quantitative determination of the standards “flavokavin A”, “flavokavin B” and “flavokavin C” two

Table 1
Chromatographic parameters of kavapyrones and flavokavins

Compound	Capacity factor (k')	Relative retention time (α)	Chromatographic resolution (R)
Methysticin (1)	8.47	–	–
Dihydromethysticin (2)	9.07	$\alpha_{1/2} = 1.071$	$R_{1/2} = 1.48$
Kavain (3)	12.62	$\alpha_{2/3} = 1.391$	$R_{2/3} = 7.21$
Dihdyrokavain (4)	14.13	$\alpha_{3/4} = 1.120$	$R_{3/4} = 2.47$
Yangonin (5)	19.13	$\alpha_{4/5} = 1.353$	$R_{4/5} = 6.57$
Desmethoxyyangonin (6)	21.71	$\alpha_{5/6} = 1.135$	$R_{5/6} = 3.15$
Flavokavin C (7)	24.52	$\alpha_{6/7} = 1.129$	$R_{6/7} = 5.77$
Flavokavin A (8)	26.26	$\alpha_{7/8} = 1.070$	$R_{7/8} = 7.67$
Flavokavin B (9)	26.65	$\alpha_{8/9} = 1.015$	$R_{8/9} = 2.06$

different HPLC-methods were used with a Symmetry® C18 column (method 1) and a LiChrospher® 60 RP-select B column (method 2). As a criterion for the precision, standard deviations of six determinations and confidence intervals ($P = 95$) of the mean values for the content of flavokavins A–C were calculated for each method. An average content of $99.22 \pm 0.33\%$ for flavokavin A, $99.42 \pm 0.42\%$ for flavokavin B and $98.82 \pm 0.48\%$ for flavokavin C was found.

Calibration curves were established for flavokavins A–C with the method 1 and correlation coefficients between 0.9986 and 0.9998 were found. The scope of work of each flavokavin is summarized in Table 2. The detection limit was defined by the flavokavin concentration, which yielded a three-fold peak height in comparison to the random noise of the baseline. A value of 0.5 ng per injection for each flavokavin was found using a detection wavelength of 355 nm.

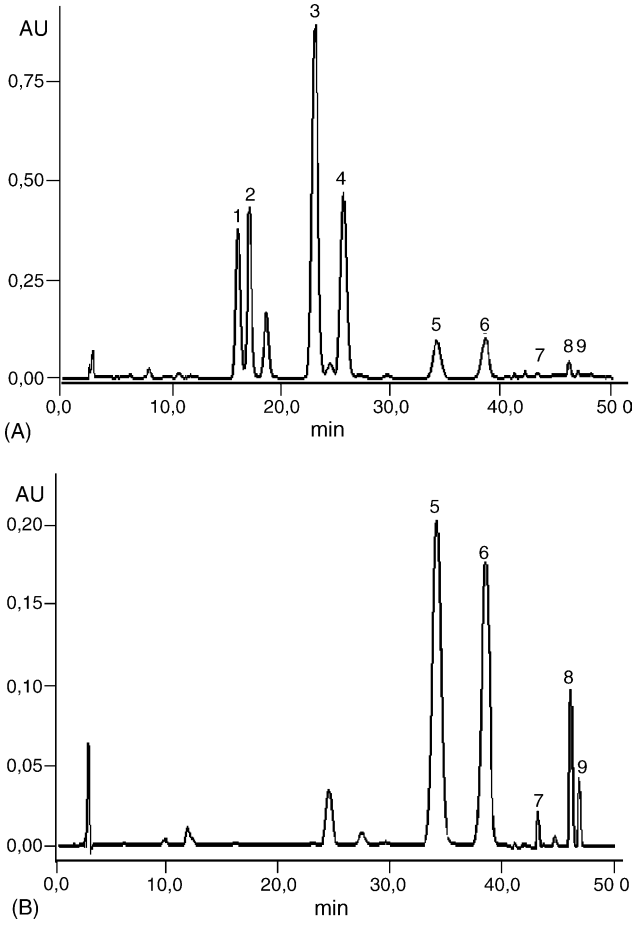

Fig. 2. HPLC-chromatogram of *Piper methysticum* Forst. extract: (A) absorbance at 240 nm and (B) absorbance at 355 nm. (1) Methysticin, (2) dihydromethysticin, (3) kavain, (4) dihydrokavain, (5) yangonin, (6) desmethoxyyangonin, (7) flavokavin C, (8) flavokavin A, and (9) flavokavin B.

For the determination of the total flavokavin amount in the kava extract method 1 was used. The precision was verified by establishing the standard deviation and the confidence interval ($P = 95$) from six determinations of the content of flavokavins in 100.0 mg kava extract under repeated conditions. A content of 0.62 ± 0.01 mg/100 mg kava extract for flavokavin A, 0.34 ± 0.01 mg/100 mg kava extract for flavokavin B and 0.14 ± 0.003 mg/100 mg kava extract for flavokavin C was found. From the corresponding relative standard deviations of 1.53, 1.99 and 2.30% the confidential intervals ($P = 95$) of the mean values were calculated (Table 3). A limit of quantification (signal-to-noise ratio of 10:1) of 1.5 ng per injection for each flavokavin was found at a wavelength of 355 nm.

The accuracy of the quantitative flavokavin determination was verified by analysis of the recovery. Defined amounts of the flavokavins were added to six samples of the kava extract and analysed by HPLC. The resulting flavokavin concentrations ranged between 80 and 120% of the nominal value. The mean of recovery yielded a value of $99.2 \pm 0.3\%$ for flavokavin A, $101.1 \pm 0.4\%$ for flavokavin B and $100.1 \pm 0.3\%$ for flavokavin C.

The total amount of flavokavins of 1.1% in the ethanolic kava extract was higher than in the dichloromethane extract with a content of 0.1% [27], although the flavokavins are more soluble in dichloromethane than in ethanol. The low flavokavin content might be due to different quality of plant material or to a insufficient semiquantitative visual evaluation of the DC chromatogram.

Table 2
Regression data and analytical parameters of flavokavins

Compound	Equitation of the calibration curve	Correlation coefficient	μg/ml
Flavokavin A	$f_{(X)} = 264.8x - 32.2$	0.9998	2.5–25.0
Flavokavin B	$f_{(X)} = 217.0x + 20.8$	0.9986	1.0–10.0
Flavokavin C	$f_{(X)} = 221.0x - 0.4$	0.9996	0.5–5.0

Table 3
Evaluation of the quantitative determination of flavokavins

Compound	Content (mg/100 mg kava extract)[a]	Confidence interval ($P = 95$) (mg/100 mg kava extract)[a]	Added (μg)	Detected (μg)	Recovery (%)	Mean of recovery (%)[a]
Flavokavin A	0.62 ± 0.01	0.61–0.63	797.04	785.57	98.6	99.2 ± 0.3
			805.98	800.63	99.3	
			638.30	635.61	99.6	
			627.17	622.48	99.3	
			493.20	488.80	99.1	
			499.45	495.51	99.2	
Flavokavin B	0.34 ± 0.01	0.33–0.35	444.50	450.94	101.5	101.1 ± 0.4
			424.32	426.54	100.5	
			354.24	358.47	101.2	
			348.16	351.70	101.0	
			272.72	275.92	101.2	
			276.13	279.90	101.4	
Flavokavin C	0.14 ± 0.003	0.14–0.15	185.47	186.29	100.4	100.1 ± 0.3
			176.98	176.67	99.8	
			147.96	147.73	99.8	
			145.40	145.94	100.4	
			114.12	114.33	100.2	
			115.56	115.32	99.8	

[a] All values are expressed as mean of at least six distinct analyses.

The HPLC method used for this work describes for the first time a simultaneous separation of the kavapyrones and flavokavins from the kava extract and enables the validation of a quantitative flavokavin determination from plant material, extracts and kava preparations.

References

[1] M. Blumenthal, W.R. Busse, A. Goldberg, J. Gruenwald, T. Hall, C.W. Riggins, R.S. Rister, The Complete German Commision E Monographs, American Botanical Council, Austin, 1998, pp. 685.
[2] M.H. Pittler, E. Ernst, Cochrane Database Syst Rev. 2 (2002) CD003383.
[3] Y.N. Singh, N.N. Singh, CNS Drugs 16 (2002) 731.
[4] M.H. Pittler, E. Ernst, J. Clin. Psychopharmacol. 20 (2000) 84.
[5] H. Woelk, O. Kapoula, S. Lehrl, K. Schröter, P. Weinholz, Z. Allgemeinmed. 69 (1993) 271.
[6] E. Lehmann, E. Kinzler, J. Friedemann, Phytomedicine 2 (1996) 113.
[7] H.-P. Volz, M. Kieser, Pharmacopsychiatry 30 (1997) 1.
[8] K.M. Connor, J.R. Davidson, Int. Clin. Psychopharmacol. 17 (2002) 185.
[9] A. Girmann, R. Lee, B. Kligler, Am. J. Obstet. Gynecol. 188 (2003) S56.
[10] R. Teschke, W. Gaus, D. Loew, Phytomedicine 10 (2003) 440.
[11] U. Malsch, M. Kieser, Psychopharmacology 157 (2001) 277.
[12] S. Russmann, B.H. Lauterburg, Ann. Intern. Med. 135 (2001) 68.
[13] F. Stickel, H.M. Baumuller, K. Seitz, D. Vasilakis, G. Seitz, H.K. Seitz, D. Schuppan, J. Hepatol. 39 (2003) 62.
[14] C.L. Humberston, J. Akhtar, E.P. Krenzelok, J. Toxicol. Clin. Toxicol. 31 (2003) 109.
[15] R.B. Brauer, M. Stangl, J.R. Stewart, R. Pfab, K. Becker, J. Clin. Psychiatry 64 (2003) 216.
[16] P.J. Gow, N.J. Connelly, R.L. Hill, P. Crowley, P.W. Angus, Med. J. Aust. 178 (2003) 442.
[17] L. Gracza, P. Ruff, J. Chromatogr. 193 (1980) 486.
[18] R.M. Smith, H. Thakrar, T.A. Arowolo, A.A. Shafi, J. Chromatogr. 283 (1984) 303.
[19] X.G. He, L.Z. Lin, L.Z. Lian, Planta Med. 63 (1997) 70.
[20] Y. Shao, K. He, B. Zheng, Q. Zheng, J. Chromatogr. A 825 (1998) 1.
[21] M. Ganzera, I.A. Khan, Chromatographia 50 (1999) 649.
[22] A.H. Schmidt, I. Molnar, J. Chromatgr. A 948 (2002) 51.
[23] G. Boonen, M.A. Beck, H. Häberlein, J. Chromatogr. B 702 (1997) 240.
[24] H. Häberlein, G. Boonen, M.A. Beck, Planta Med. 63 (1997) 63.
[25] M. Lechtenberg, B. Quandt, F.J. Kohlenberg, A. Nahrstedt, J. Chromatogr. A 848 (1999) 457.
[26] O. Meissner, Analytik und pharmakologische Bedeutung der Flavokavine aus *Piper methysticum* Forst., ISBN 3-8322-0661-2, Thesis, University of Marburg, 2002.
[27] J. Lazar, Beitrag zur Chemischen Zusammensetzung des Kawatranks (aus Piper metysticum Forst.), Thesis, Freie Universität Berlin, 1983.
[28] H. Achenbach, W. Regel, Chem. Ber. 106 (1973) 2648.
[29] R. Hänsel, G. Ranft, P. Bahr, Z. Naturforsch. 18(b) (1963) 370.
[30] J.F. Stevens, A.W. Taylor, G.B. Nickerson, M. Ivancic, J. Henning, A. Haunold, M.L. Deinzer, Phytochemistry 53 (2000) 759.
[31] W.D.H. Ranjith, N.P. Dhammika Nanayakkara, I.A. Khan, Phytochemistry 59 (2002) 429.