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Absorbance data of hypericin and pseudohypericin used as reference compounds for medicinal plant analysis

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The evaluation of the absorbance data of hypericin and pseudohypericin revealed the molar/specific coefficients of absorbance in methanol-pyridine (99 : 1, v/v) at the maximum of the longest wavelength to be 51936/1030 and 43486/836, respectively. The absorbance data of hypericin were also determined in methanol. They were not significantly different from those in the presence of pyridine. The decrease of the coefficients by water addition was found to be the same for hypericin and pseudohypericin. It was concluded that hypericin and pseudohypericin reveal the same homoassociation behavior.

1. Introduction

The standardization of the commercial extracts of Hyperici herba is mostly based on the content of naphthodianthrones. Using hypericin as reference substance, its physicochemical properties are a matter of concern and its consistent quality is a precondition for reproducible standardization. As most quantification methods apply absorption measurement for detection, absorbance data of hypericin and pseudohypericin are of interest. Calibration graphs of hypericin are used for the calculation of both hypericin and pseudohypericin. Consequently, unknown differences in the absorption properties of the two compounds would lead to wrong results. Literature data of the molar coefficients of absorbance of hypericin are not consistent (Table 1A) and only few data are published for pseudohypericin (Table 1B). Various factors have been discussed to influence the absorption spectra of hypericin, as homoassociation, pH of the solvent and its tautomeric and conformational state.

Homoassociates of hypericin molecules reveal another molar coefficient of absorbance than monomolecularly dissolved hypericin, as has been shown by Falk and Meyer [1]. Hypericin dissolves monomolecularly in common polar solvents up to concentrations of 10^{-3} mol/l. Under certain conditions, however, it forms homoassociates, for example in the presence of water. Increasing percentages of water in dimethylsulfoxide-water mixtures lead to a decrease of the intensity of the long wavelength absorption band. Its extinction coefficient in water was reported to be about one fourth of that in pure dimethylsulfoxide [1]. Burel and Jardon [2] and Lavie et al. [3] did similar experiments with ethanol-water mixtures. They described as well water addition to decrease the intensity of the absorption spectra. Lavie et al. [3] determined the extinction coefficient of hypericin in water to be only one eighth of that in ethanol. Wynn and Cotton [4] mentioned homoassociation to occur likewise in nonpolar organic solvents such as toluene, chloroform and hexane. Homoassociation can be broken down by the alkali metal cations potassium and sodium, according to Burel et al. [5]. Xia et al. [6] observed that the addition of anthraquinones to hypericin solutions lowered the intensity of the absorption spectrum of hypericin, but did not change the shape of the spectrum. They thought aggregation to be responsible for the finding.

The form of the homoassociates depends on the tautomeric state of hypericin. The homoassociates of the 7,14-dioxo tautomer exhibit a stacking pattern due to the hydrophobic effect of the aromatic core. Hydrogen bonding is responsible for the formation of the homoassociates of the 1,6-dioxo tautomer (Fig. 1). In contrast to the homoassociates of the 7,14-dioxo tautomer, the homoassociates of the 1,6-dioxo tautomer show high extinction coefficients and narrow absorption bands [1, 7].

The aggregation behavior of pseudohypericin has not been described yet, although being of some importance with regard to its quantification as hypericin. The quantification of hypericin and pseudohypericin by VIS spectroscopy and HPLC/DAD in extracts of Hyperici herba does not give the same results. As the two methods do not use the same solvent systems, it was speculated that different association behavior of hypericin and pseudohypericin could be one of the factors responsible. If the homoassociation of pseudohypericin in water was more pronounced than that of hypericin, the molar coefficient of absorbance of pseudohypericin in watery solvents would be lower. Therefore, HPLC/DAD results would be lower than spectroscopic results, as VIS spectroscopy [8] does not make use of water, but most HPLC methods do [9–11].

Ionization was shown by Falk and Schmitzberger [12] to influence absorbance data of hypericin. They found that concentrated solutions of the monoanion of hypericin (1×10^{-2} mol/l) revealed a characteristic UV-VIS spectrum. In diluted solutions (1×10^{-5} mol/l), however, the

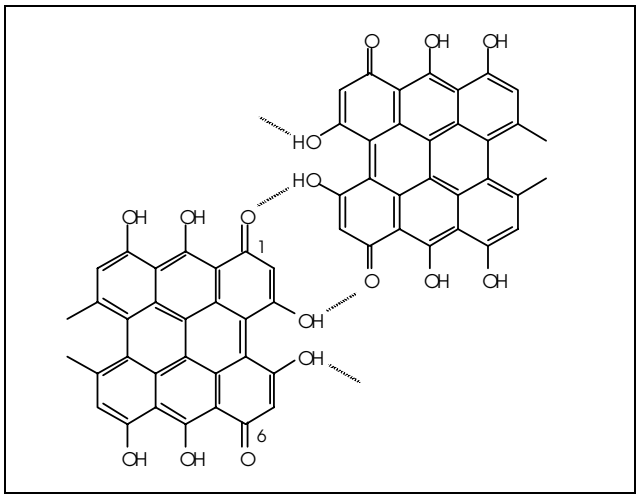

Fig. 1: Intermolecular hydrogen bonding of the 1,6-dioxo tautomer

Table 1A: Overview of absorbance data of hypericin described in the literature

Solvent	λ (nm) (ε)	c (mol/l)	Ref.	Solvent	λ (nm) (ε)	c (mol/l)	Ref.
Acetone	596/551/514/477		[4]	Methanol	588/545/509/471/384/328/282		[4]
Acetone	596(51190)/552(23550)/514(8190)/481(11260)	1×10^{-5}	[1]	Methanol	588		[25]
Acetone	597		[21]	Methanol	589(43450)/546(20860)/509(7390)/472(12170)	1×10^{-5}	[1]
Acetone	596(36790)/350(17640)		[22]	Methanol	630(24000)/588(22800)/547(13400)	1×10^{-2} B	[12]
Acetone	592		[23]	Methanol	589(43600)/546(24700)/509(10000)/472(13800)	2×10^{-5} B	[12]
Acetone	599/583/554/542/513/475		[24]	Methanol	590(36260)/548(17980)		[22]
Acetonitrile	594/550		[6]	Pyridine	602(49410)/557(22230)/518(7410)/484(10870)	1×10^{-5}	[1]
Acetonitrile	594/550/512/478/384/336/286		[4]	Pyridine	601/556		[37]
Acetonitrile	594(39560)/550(18990)/512(6730)/478(9890)	1×10^{-5}	[1]	Pyridine	601/557		[38]
Acetonitrile	594		[25]	Pyridine	600/557/518/484/455/388/335		[39]
Benzyl alcohol	596/551/515/480/390/335		[4]	Pyridine	603/559/520		[35]
Butanol	592/546/511/476/385/333/286		[4]	Pyridine	603/558/520/483	A	[24]
Carbon tetrachloride	609/568/479/395/330/286		[4]	Tetrahydrofuran	600/555/516/482/385/339/286		[4]
Chloroform	595/570/484/397/330/286		[4]	Tetrahydrofuran	601(55150)/556(23160)/516(8280)/481(13800)	1×10^{-5}	[1]
Cyclohexane	613/570/484/332/289		[4]	Toluene	609/570/481/331		[4]
Dimethylformamide	598/554	1.9×10^{-5}	[26]	Water	598(6000)/558(10000)	4.4×10^{-5}	[32]
Dimethylformamide	598/554/515/482/386		[4]	Water	598/560/324/285		[4]
Dimethylformamide	600(46060)/554(20730)/516(6910)/482(10870)	1×10^{-5}	[1]	Acetone-water (95 : 5)	593/551/512/480/450/387/350		[39]
Dimethylsulfoxide	600/550/516/481/454/388/335		[7]	Acetone-water (9 : 1)	596/551/514/477		[24]
Dimethylsulfoxide	598(39500)/554(17600)/514(5600)/480(9400)/450(7700)/388(10800)/343(25900)/334(26100)		[27]	Acetone-ether (33 : 67)	599/584/555/542/455		[24]
Dimethylsulfoxide	598(39500)		[28]	Acetone-ether (25 : 75)	599/583/569/542/529/454		[24]
Dimethylsulfoxide	600(48950)/555(22030)/516(7340)/482(11260)	1×10^{-5}	[1]	Ether + trace pyridine	589/573/546		[24]
Dimethylsulfoxide	598		[25]	Ether-pyridine (8 : 2)	599/555/517		[24]
Dimethylsulfoxide	600		[21]	Chloroform-hexane (8 : 2)	601/563/525/489/388/332		[29]
Dimethylsulfoxide	599(43000)/555/514/480/384/343/333/281		[29]	Ethanol-water (6 : 94)	586		[21]
Dioxane	590/547/510/471/330/283		[4]	Ethanol-water (8 : 2), pH 0.6	580(40000)/535(22000)/460(25000)/320(33000)		[40]
Ethanol	592/549	1.9×10^{-5}	[26]	Ethanol-water (8 : 2), pH 8.6	590(50000)/550(24000)/470(16000)/340(35000)	B	[40]
Ethanol	590(52000)/545		[30]	Ethanol-water (8 : 2), pH 13.5	470(18000)/365(41500)	C	[40]
Ethanol	591/547/510/475/382/332/285		[4]	Dimethylsulfoxide-acetic acid (9 : 1)	587/545/461/437/322	5×10^{-6}	[7]
Ethanol	591(37410)/549(22800)/510(7760)/476(12130)	1×10^{-5}	[1]	Dimethylsulfoxide-water (8 : 2), pH 0.45	586(2900)/544(1600)/507(700)/461(2000)/320(3000)		[41]
Ethanol	591		[21]	Dimethylsulfoxide-water (8 : 2), pH 8.8	599(3700)/555(1800)/545(1300)/516(800)/388(1200)/333(2500)		[41]
Ethanol	592(41000)/548(20400)/511(7600)/476(10700)/379(10700)/327(29300)		[31]	Acetic acid	579/567/538/503/453/430/320/279		[4]
Ethanol	591/578/547/510/477/383/337/326/284		[29]	Acetic anhydride	593/552/512/475		[39]
Ethanol	590(46000)/547(22000)/510/474/383/340/285/234	4.4×10^{-5}	[32]	Ethanol + H^+	581/569/539/504/454/430/321/280		[4]
Ethanol	590(40000)/510(9500)/308(21200)		[33]	Formic acid	578/537/501/454/432/321/279		[4]
Ethanol	586		[23]	Lactic acid	581/569/540/504/445/433/320		[4]
Ethanol	590(41600)/548(23500)/509(8700)/473(13000)/375(83000)/331(26200)/284(36800)	6.31×10^{-5}	[34]	Methanol-10 mM HCl	580		[25]
Ether	580/569/540/507/452/428/326/280/230		[4]	Methanol-0.1 N KOH (1 : 1)	625/460		[22]
Ether	582/569/541/530/506/455		[24]	Conc sulphuric acid	650/597/500/325/238/220	A	[4]
Ethyl acetate	592/549/511/477/384/334/285		[4]	Conc sulphuric acid	658		[25]
Ethyl Acetate	592(43450)/548(17580)/510(5990)/475(6700)	1×10^{-5}	[1]	Conc sulphuric acid	645/598/506		[35]
Ethyl Acetate	597/554/516	A	[35]	Water, pH 13	650		[25]
Hexane	613/570/486/333/292		[4]				
Methanol	590(45650)		[36]				

A: compound not defined properly; B: monoanion of hypericin; C: dianion of hypericin

Table 1B: Overview of absorbance data of pseudohypericin described in literature

Solvent	λ (nm) (ε)	c (mol/l)	Ref.
Dimethyl-sulfoxide	598(42100)		[28]
Methanol	590(43100)/546(24800)/509(10300)/470(13600)/446(12700)/384(13300)	2×10^{-5} B	[12]
Methanol	634(24200)/589(23200)/465(13700)	1×10^{-2} B	[12]

B: monoanion of pseudohypericin

spectrum approached that of non-ionized hypericin (Table 1A).

The conformational state of hypericin is a further factor affecting the absorption spectra. The propeller and butterfly conformers were reported to reveal small spectroscopic shift differences [13].

Finally, tautomerism of hypericin has to be considered as well, when absorbance data are looked at. The absorption spectra of the tautomer with the carbonyl groups in position 1 and 6 reveal fairly intensive bands between 400 and 500 nm and long wavelength absorption bands, which are hypsochromically shifted compared to the tautomer with the carbonyl groups in positions 7 and 14 [7, 14]. The following observations have been done by Kapinus et al. [7]. Both tautomeric forms (Fig. 2) can occur in the solid state. Transformation from one tautomer into another only occurs in solution. The 7,14-dioxo tautomer was shown to be the most stable hypericin isomer. It can be reverted to the 1,6-tautomer by acidification. Dissolution of the 1,6-dioxo tautomer in polar solvents as dimethylsulfoxide leads to its conversion to the 7,14-dioxo form. Saturated solutions of the 1,6-dioxo tautomer in tetrahydrofuran (or other low to moderate polar organic media) reveal some stability. Dilution, rise in temperature, addition of pyridine and dimethylsulfoxide revert the 1,6-dioxo to the 7,14-dioxo tautomer in tetrahydrofuran as well. The 1,6-dioxo tautomers form homoassociates, which are stabilized by efficient intermolecular hydrogen bonding (Fig. 1). Dilution, rise in temperature, addition of pyridine and dimethylsulfoxide disturb the hydrogen bonds by solvating the hypericin molecules, and therefore enhance the dissociation of the homoassociates. After dissociation, isomerization can take place. The stabilizing effect of the intermolecular hydrogen bonds on the 1,6-dioxo tautomeric form is most prominent in saturated solutions and in the solid state.

The aim of this study was to investigate the absorbance data of hypericin and pseudohypericin as literature data were not consistent. It was investigated if the addition of 1% pyridine to methanol influenced the molar coefficient of absorbance, as it was preferred to prepare extract solutions of Hyperici herba without pyridine. Effects on the absorption data would make it necessary to add the same concentration of pyridine to the extracts. As it was reported for hypericin that increasing percentages of water in ethanol-water mixtures went along with a decrease of its extinction coefficient (see above), it was investigated if it was the same for pseudohypericin. Differing data would implicit that the homoassociation behavior of the two substances are not alike.

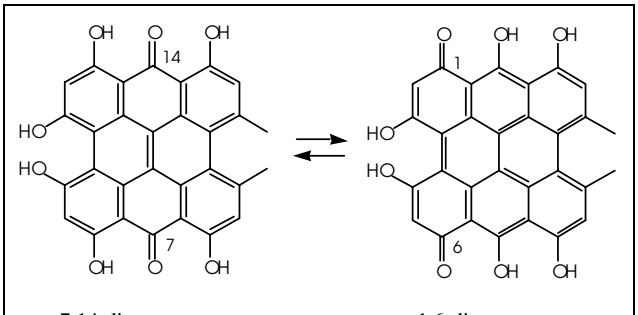

Fig. 2: Tautomeric forms of hypericin

2. Investigations, results and discussion

2.1. Absorbance data of hypericin and pseudohypericin

Absorbance data of hypericin were recorded in methanol and methanol-pyridine (99 : 1, v/v). The absorbance of pseudohypericin was measured in methanol-pyridine (99 : 1, v/v). As most HPLC/DAD methods and VIS spectroscopy use the maximum of the longest wavelength absorption band of hypericin for quantification, most attention was given to the absorption maximum and the molar coefficient of absorbance of this band.

The recorded position of the absorption maxima of hypericin (588 nm) and pseudohypericin (589 nm) were in accordance with literature data of methanolic solutions (Table 1A/B [4]). However, the molar coefficient of absorbance (ε) of the isolated hypericin in methanol was higher ($\varepsilon = 51712$ at 588 nm) than those described in literature (Tables 1 and 2: $\varepsilon = 45650$ or lower). The commercially available hypericin from Roth Company revealed a smaller ε-value as well ($\varepsilon = 33603$ at 588 nm, Table 2). Differing molar coefficients of absorbance could be caused by varying degrees of polymerization and degradation or other impurities. The isolated hypericin was assumed purer than the hypericins with lower ε-values, as the presence of impurities with higher ε-values than hypericin seemed to be less likely. Experiences of one's own showed hypericin only sparingly soluble in methanol, sonication and time (up to four days) being necessary for full dissolution [15]. Not completely dissolved hypericin could be another reason for low ε-values.

Table 2: Absorbance data of hypericin in methanol

λ (nm) (ε)	Reference
588(51712)/545(24393)/472(13544)	hypericin from isolation
589(43450)/546(20860)/472(12170)	[1]
588(33603)	hypericin from Roth Company

ε molar coefficient of absorbance

Table 3: Absorbance data of hypericin in methanol determined using six calibration curves with six concentration levels each

λ_{max} (nm)	$A^{1\%}$	ε (l mol^{-1} cm^{-1})	s_{rel} (%)
588	1025	51712	1.22
545	484	24393	1.22
472	269	13544	1.01
328	578	29173	1.31
285	755	38082	1.47

$A^{1\%}$ coefficient of absorbance for 1% (w/v) solution
ε molar coefficient of absorbance

Table 4: Absorbance of hypericin at 588 nm in methanol-pyridine (99 : 1, v/v) determined using six calibration curves with six concentration levels each

λ_{max} (nm)	$A^{1\%}$	ε (l mol^{-1} cm^{-1})	s_{rel} (%)
588	1030	51936	1.08

$A^{1\%}$ coefficient of absorbance for 1% (w/v) solution
ε molar coefficient of absorbance

Table 5: Absorbance data of pseudohypericin in methanol-pyridine (99 : 1, v/v) determined using six calibration curves with six concentration levels each

λ_{max} (nm)	$A^{1\%}$	ε (l mol^{-1} cm^{-1})	s_{rel} (%)
589	836	43486	0.47
546	412	21468	0.93
473	250	13025	6.30
327	518	26817	1.74

$A^{1\%}$ coefficient of absorbance for 1% (w/v) solution
ε molar coefficient of absorbance

The results presented in Tables 3 and 4 show that the addition of 1% pyridine to methanol does not significantly influence the molar coefficient of absorbance (ε) of hypericin at 588 nm. The solubility, however, can be enhanced considerably adding small amounts of pyridine [15]. Consequently, if VIS spectroscopy is used as a quantification method for hypericin in extracts of Hyperici herba, standard solutions of hypericin can be prepared with methanol-pyridine (99 : 1, v/v), also if the extracts are made with pure methanol.

Hypericin and pseudohypericin revealed tiny differences in the location of their absorption maxima (Tables 3 to 5). Furthermore, they showed different ε-values in the maximum of their longest wavelength, being 51936 and 43486 for hypericin and pseudohypericin, respectively, in methanol-pyridine (99 : 1, v/v). Most quantification methods use the calibration curve of hypericin to quantify pseudohypericin. Consequently, calibrated contents are too low, as far as detection is done by absorption measurement (for further discussion see 3).

2.2. Influence of water addition to methanol solutions on the molar coefficient of absorbance of hypericin and pseudohypericin

The molar coefficients of absorbance (ε) hypericin and pseudohypericin decreased with increasing amounts of water (Fig. 3). In 80%, 60% and 40% aqueous methanol the ε-values for the long wavelength absorption band were 90%, 65% and 31%, of the ε-value in pure methanol, respectively. The decrease of the intensity caused by water addition was more pronounced at the longest wavelength than at 546 nm. The ratios of the absorption at 588 nm to the absorption at 546 nm were 2.1, 2.0 and 1.6 (1.5 for pseudohypericin) for the solvents 100% methanol, 80% and 60% aqueous methanol, respectively. The absorption maxima of the hypericin/pseudohypericin spectra in 20% aqueous methanol were shifted to 594/595 nm and 555/555 nm. In 40% aqueous methanol, only the absorption band at 546 nm was shifted to 554 nm. As the results were the same for hypericin and pseudohypericin, it was concluded that the compounds did not differ in their tendency for homoassociation. Therefore, different association behavior of hypericin and pseudohypericin was not

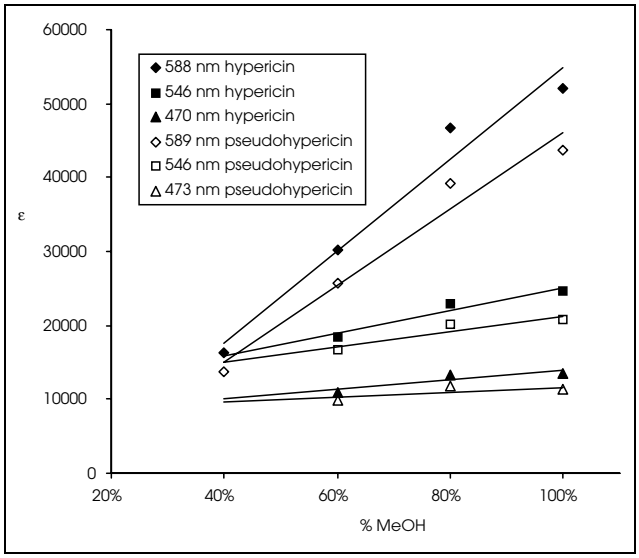

Fig. 3: Molar coefficient of absorbance (ε) of hypericin ($c = 8 \times 10^{-6}$ mol/l) and pseudohypericin ($c = 8 \times 10^{-6}$ mol/l) in aqueous methanol at different wavelengths

Table 6: Determination of the hypericin (H) and pseudohypericin (P) concentrations of the test solutions by HPLC and VIS spectroscopy

Test solution	HPLC		VIS spectroscopy	
	H (μg/ml)	P (μg/ml)	H (588 nm) (μg/ml)	P (589 nm) (μg/ml)
100% MeOH	3.98	3.79	3.99	3.83
80% MeOH	4.05	4.03		
60% MeOH	4.04	3.84		
40% MeOH	4.01	3.84		
20% MeOH	3.99	3.89		

Theoretical concentrations were 3.98 μg/ml hypericin and 3.96 μg/ml pseudohypericin

the explanation for the differences of VIS spectroscopic and HPLC results in the quantification of hypericin and pseudohypericin.

It was investigated if the water-methanol ratio of the test solutions influence the results determined by HPLC. The HPLC method of Krämer and Wiartalla [10] was applied to determine the concentration of hypericin and pseudohypericin. The test solution 100% methanol was examined by VIS spectroscopy as well. The calibration curves used were $y = 86.321x - 75.557$ (HPLC) and $y = 1017.2x + 0.0049261$ (VIS spectroscopy) for hypericin and $y = 58.236x - 6.5581$ (HPLC) and $y = 867.38x + 0.0011542$ (VIS spectroscopy) for pseudohypericin.

The results indicated that the water-methanol ratio of the test solutions did not influence the HPLC results (Table 6).

Identical results of HPLC and VIS spectroscopy indicated the reference compounds not to be responsible for differing results of the two methods, analyzing extracts of Hyperici herba.

3. Discussion

Based on the results of this investigation, hypericin reveals a higher molar coefficient of absorbance (51936) than pseudohypericin (43486). As the quantification of pseudohypericin is generally done with the calibration

graph of hypericin, evaluated contents are too low. Therefore, it should be worked with standard solutions of hypericin and pseudohypericin. However, as the availability of pure pseudohypericin is limited, the use of a conversion factor is reasonable as far as HPLC is applied for quantification. Referring to the calibration graphs of hypericin and pseudohypericin of section 2.2, the conversion factor (F) is 0.8 (F = area pseudohypericin per 10 ng/area hypericin per 10 ng). The factor is valid for the HPLC method of Krämer and Wiartalla [10].

The ratio of pseudohypericin to hypericin in extracts of Hyperici herba is not always the same, depending on the extracting solvent [16] and the origin of the drug material. Schütt [17] found the ratio to vary from 1.5 to 7.5, depending on the origin of the drug material. As separate determination of hypericin and pseudohypericin cannot be done with VIS spectroscopy, it is not possible to determine a conversion factor for VIS spectroscopy. Results will always contain some uncertainty and therefore, HPLC/DAD is the more precise method.

The specific coefficient of absorbance ($A^{1\%} = 870$) used for quantification by the German Drug Codex [8], the Ph. Helv. 8 [18] and the new monograph of the European Pharmacopoeia [19] is lower than that of hypericin in methanol-pyridine (99 : 1, v/v) determined in this study ($A^{1\%} = 1030$), but slightly higher than the value of pseudohypericin ($A^{1\%} = 836$). As the drug generally contains more pseudohypericin than hypericin, the applied value can be used as approximation.

It could be shown that the absorbance properties of hypericin are not influenced by the addition of 1% pyridine. Therefore, it is possible to prepare standard solutions of hypericin with methanol-pyridine (99 : 1, v/v) and extract solutions with pure methanol.

4. Experimental

4.1. Materials and solvents

Hypericin and pseudohypericin were isolated from an ethanol-water (1 : 1, m/m) dry extract (Ze117, batch EX-24-482-95) from Hyperici herba which was obtained from Zeller Company (CH-Romanshorn). For details of the isolation procedure as well as for MS and NMR data see [15].

UV-VIS (MeOH) of hypericin = λ nm (ε) 588 (51712), 545 (24393), 472 (13544). HPLC purity [20] was 100% at the wavelengths 254, 366, 450, 555 and 590 nm. At 290 nm occurred an additional signal at 48 min, revealing an UV spectrum with absorption maxima at about 220 and 310 nm. Referring to the area integration values, the HPLC purity at 290 nm was 93% for hypericin.

UV-VIS (MeOH-pyridine (99 : 1, v/v)) of pseudohypericin = λ nm (ε) 589 (43486), 546 (21468), 473 (13025). The HPLC purity of pseudohypericin was the same as for hypericin (see above).

The commercial sample of hypericin was from Roth Company (D-Karlsruhe); it was hypericin Rotichrom® and of TLC quality (lot 31628333).

Methanol, ethyl acetate and tetrahydrofuran were of HPLC quality (Romil Chemicals, GB-Shepshed). *Ortho*-phosphoric acid 85% (Ph. Helv. VI/Ph. Eur.) was purchased from Hänseler (CH-Herisau). Pyridine (p.a.) was from Fluka (CH-Buchs) and sodium dihydrogen phosphate (z.A.) from Merck (CH-Dietikon). De-ionized water was obtained using an NANO-pure cartridge system (Skan, CH-Basel).

4.2. Instrumentation and quantification

4.2.1. UV/VIS spectroscopy

UV/VIS spectra were measured on a Kontron-Uvikon 930 spectrophotometer (Kontron Instruments, CH-Zürich).

4.2.2. HPLC

HPLC analyses were performed using a Hewlett Packard instrument (Model 79994A analytical workstation, model 1090 liquid chromatograph, model 1040 diode-array detector). For chromatographic separations a Knauer (D-Berlin) prepacked cartridge column (250 × 4 mm I.D., 5 μm) filled with Spherisorb S ODS2 and a guard column (10 × 4 mm I.D.) of the same material were used.

4.2.3. Quantification by HPLC

The quantitative determination of hypericin and pseudohypericin was performed using the external standard method. The standard solutions of hypericin were used for the quantification of both hypericin and pseudohypericin. The calculation graphs were generated by a least squares regression method. All calculations were based on area counts. Over the selected concentration range of the standard solutions, the calibration curve showed a linear detector response. The correlation coefficient was 1.000. The external standard solutions were injected three times into the HPLC system for analysis.

4.3. Procedure

4.3.1. Absorbance data of hypericin and pseudohypericin

Standard solutions of hypericin: Six stock solutions were prepared for each solvent (methanol and methanol-pyridine (99 : 1, v/v)). 0.500 mg hypericin were dissolved in the corresponding solvent in a 100.0 ml volumetric flask, sonicated 3 times for 10 min and stored for 4 days at room temperature. Five different dilutions were prepared from each stock solution. The concentration range for hypericin in methanol and methanol-pyridine (99 : 1, v/v) was 1.27–5.56 μg/ml and 1.30–6.30 μg/ml, respectively. The molar and specific coefficient of absorbance of hypericin was determined in methanol-pyridine (99 : 1, v/v).

Standard solutions of pseudohypericin: Six stock solutions were prepared using methanol-pyridine (99 : 1, v/v) as solvent. 0.500 mg pseudohypericin were dissolved in 100.0 ml solvent in a 100.0 ml volumetric flask and sonicated 3 times for 5 min. As the solubility of pseudohypericin in methanol is better than that of hypericin, but its stability at room temperature is worse [15], the dilution of the stock solutions was done after 18 h. The concentration range was 1.20–5.90 μg/ml.

UV/VIS: UV/VIS spectra were recorded from 200 to 600 nm.

4.3.2. Influence of water addition to methanol solutions on the molar coefficient of absorbance of hypericin and pseudohypericin

Test solutions: 1.99 mg hypericin were dissolved in methanol-pyridine (99 : 1, v/v) in a 100.0 ml volumetric flask. The stock solution was stored three days at room temperature and sonicated three times for 10 min. Solutions of 20, 40, 60 and 80% aqueous methanol were prepared by diluting 10.0 ml of the stock solution to 50.0 ml with the corresponding amount of methanol and water (v/v). The 100% methanol solution was prepared by diluting 5 ml stock solution to 25.0 ml. The final hypericin concentration was 3.98 μg/ml.

0.99 mg pseudohypericin were dissolved in methanol-pyridine (99 : 1, v/v) in a 50.0 ml volumetric flask. The solution was sonicated for 15 min and stored over night at room temperature. The further procedure was according to the preparation of the hypericin solutions. The final pseudohypericin concentration was 3.96 μg/ml.

Standard solutions: To determine the hypericin and pseudohypericin content of the test solutions five standard solutions of each reference compound in methanol-pyridine (99 : 1, v/v) were prepared. The concentrations of the standard solutions were between 1.39 μg/ml and 5.54 μg/ml. They were used both for HPLC and UV/VIS spectroscopy.

UV/VIS: UV/VIS spectra were recorded from 200 nm to 600 nm.

HPLC: The applied HPLC method was the method developed by Krämer and Wiartalla [10]. The composition of the mobile phase was methanol-ethyl acetate-buffer (1893.4 : 526 : 618.4, m/m). The buffer consisted of 13.8 g sodium dihydrogen phosphate in 1 litre water adjusted to pH 2.1 with *ortho*-phosphoric acid 85%. The run time was 15 min, the flow rate 0.8 ml/min, the column temperature 25 °C and the injection volume 20 μl. The detection wavelength of the DAD was set at 590 nm.

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