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Solubility of hypericin in methanol and methanol-pyridine

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The solubility of hypericin in methanol and methanol-pyridine (99 : 1, v/v) was determined. The addition of pyridine turned out to enhance the solubility of hypericin. In pure methanol only 37.17 μg/ml could be dissolved. In comparison, 320.91 μg hypericin were soluble in one ml methanol-pyridine (99 : 1, v/v).

1. Introduction

As hypericin still plays an important role in standardization and quality control of *Hypericum perforatum* L., the preparation of standard solutions is a matter of concern. A major problem represents the bad solubility of hypericin in most common solvents. Moreover, the solubility properties of hypericin described in literature are not consistent. Reasons for the differences are discussed controversially. Sattler [1] determined the solubility of hypericin to be 40 μg/ml in water and 500 μg/ml in methanol; the lowest solubility (10 μg/ml) showed the investigated buffer systems (pH = 7.4). Butterweck [2] found similar results for the solubility of hypericin in water (60 μg/ml). She discussed the enhanced solubility of hypericin and pseudohypericin in extract solutions, explaining the finding by the presence of solubilizers as procyanidin B2 and C1, forming better soluble naphthodianthrone-procyanidin-complexes. Falk and Schmitzberger [3] presented another explanation. It was deduced from NMR data that hypericin was present in the plant as phenolate ion, potassium being the counter-ion. As the non-ionized hypericin from Roth Company was only sparingly soluble in common solvents, salt formation was made responsible for the easy extractability from the plant. The hypericin isolated from blossoms of *Hypericum perforatum* allowed them the preparation of solutions containing at least 5 mg hypericin/ml methanol. Stock [4] mentioned differing solubility properties of synthesized hypericin from Roth Company and hypericin isolated from Hyperici herba. She found the isolated hypericin to be better soluble than the synthetic one and assumed different amounts of water of crystallization to be responsible. The synthetic hypericin used by Liebes et al. [5] allowed the preparation of solutions of 5 mg hypericin in 1 ml ethanol absolute. It can be speculated that the hypericins with different solubility properties reveal different tautomeric forms. Strong hydrogen bonds are present in the 1,6-tautomer (Fig. 1); therefore, this is possibly the form with lower solubility.

Salts of hypericin are generally soluble in polar solvents [6]. Addition of 1% pyridine to methanol improves the solubility of hypericin as well [7], the cause of which might be the formation of hypericin-pyridine-complexes or ionization. In this context, pKa values of hypericin are of interest. The subject has been discussed controversially in literature (for references see [8]). Various methods have been applied to derive pKa values of hypericin such as ^{1}H NMR [3], UV/VIS spectroscopy [9, 10], MS [11], electrophoresis [8] and UV/VIS spectroscopy after derivatization of hypericin [8, 12]. From experiments, it was concluded that three main ionization steps, which are coupled in part to homoassociation equilibria, characterize the protonation and deprotonation behavior of hypericin. The protonation of C=O groups was assigned to the pKa value of –6 and the deprotonation of one of the hydroxyl groups in position 3 and 4 to the pKa value of 2 (Fig. 2). The pKa value

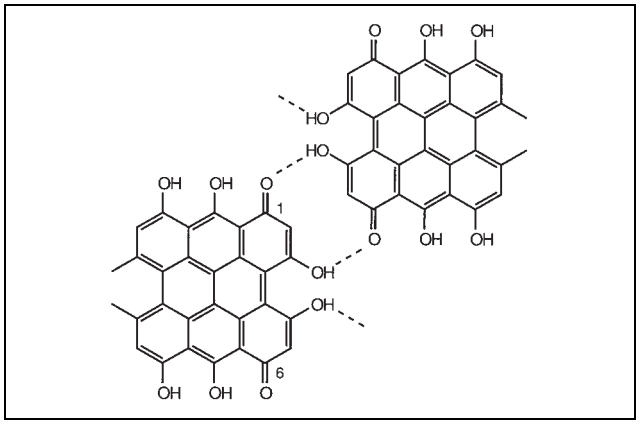

Fig. 1: Intermolecular hydrogen bonding of the 1,6-dioxo tautomer

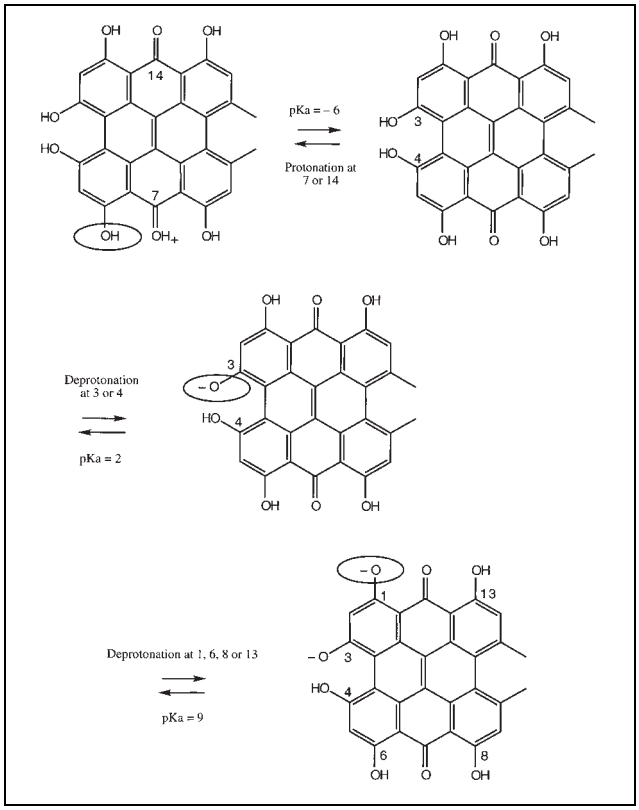

Fig. 2: Protonation and deprotonation behavior of hypericin

of 9 was found to characterize the second deprotonation step, which generates a diphenolate ion by ionization of one of the hydroxyl groups in position 1, 6, 8 and 13 [12]. These results indicate that hypericin is present as an anion under most circumstances, due to its rather low pKa value of 2. However, the finding that salts of hypericin are better soluble in common organic solvents, would be easier to explain by a higher pKa value, as found for example by Eloy et al. [13]. They depicted two deprotonation equilibria (pKa = 7 and 11). The protonation of the carbonyl groups was proposed to have a pKa of 1.

The aim of this study was the investigation of the solubility of hypericin in methanol. It was investigated as well, to which degree the addition of 1% pyridine improved the solubility.

2. Investigations and results

2.1. Determination of the range of linearity

The standard solutions were used to determine the range of linearity. In VIS spectroscopy at 589 nm and 590 nm, solutions obeyed Lambert-Beer's law in the concentration range of 0.9984 to 14.98 μg/ml; the detector itself was the limiting factor. In HPLC, linear detector response was given in the whole concentration interval examined from 0.9984 to 99.84 μg/ml. Correlation coefficients were 0.99999 and 1.00000 (Table 1).

2.2. Determination of the solubility of hypericin in methanol and methanol-pyridine

Methanol: HPLC could be used for the determination of the concentration of the undiluted supernatant (A) and the five times diluted sample (B). A was not in the linear range of VIS spectroscopy. The detection wavelength was 589 nm. The mean value of the solubility of hypericin in methanol was 37.17 μg/ml (Table 2). This finding is not consistent with the result of Sattler [1]. She determined the solubility of hypericin to be 500 μg/ml in pure methanol. About the causes of the differences can only be speculated. Polymerization can diminish solubility. The high molar coefficient of absorbance of the isolated hypericin in methanol at 588 nm (ε = 51712) [14] does not support this hypothesis, as association would decrease the coefficient. Salt formation and coisolated solubilizers from the extract have been discussed to enhance solubility. The ^{1}H NMR of Sattler [1] did not reveal any impurities, or a resonance at 18.4 ppm, which can be typical for ionized hypericin. However, as D_2O is usually present in deuterated solvents, the OH groups in position 3 and 4 were assumed to be involved in an isotope exchange with D_2O, resulting in the occasional absence of their resonances in ^{1}H NMR spectra [15, 16]. Stock [4] assumed varying amounts of water of crystallization to influence solubility.

Methanol-pyridine: HPLC could be used for the determination of the concentration of the undiluted supernatant (C) and the fifty times diluted sample (D). C was not in the linear range of VIS spectroscopy. The mean value of the solubility of hypericin in methanol-pyridine (99 : 1, v/v) was 320.91 μg/ml (Table 3). The results showed that the solubility of hypericin in methanol-pyridine is about nine times better than in pure methanol. The improved solubility of hypericin in the presence of pyridine may be explained by the formation of hypericin-pyridine-complexes. As absorption spectra of hypericin in methanol and methanol-pyridine were the same, the finding could not be explained by ionization of hypericin or the accelerated breakdown of homoassociates by pyridine due to enhanced solvatization [16].

Table 1: Determination of linearity in VIS spectroscopy and HPLC

Detection mode		Regression curve	Correlation coefficient (r)
VIS	589 nm	y = 1008x − 0.002326	0.99999
spectroscopy	590 nm	y = 971.5x − 0.002601	0.99999
HPLC	590 nm	y = 930326x − 10.38	1.00000

x being the concentration in [g/100 ml]

3. Discussion

Hypericin is only sparingly soluble in methanol and other common solvents. Addition of 1% pyridine, sonication and waiting time before dilution (about four days) are means to prevent incomplete dissolution leading to wrong calibration graphs. Stability tests proved that the addition of 1% pyridine and a waiting time of four days do not affect hypericin solutions, when excluded from light. The

Table 2: Determination of the solubility of hypericin in methanol

Detection method	Sample	Absorption at 589 nm	Peak area	Sample concentration (μg/ml)	Solubility of hypericin in methanol	
					(μg/ml)	(μmol/l)
VIS	A	3.1767		–	–	–
spectroscopy	B	0.7657		7.571	37.85	75.03
HPLC	A		3388.152	36.53	36.53	72.42
	B		680.416	7.425	37.13	73.60

Table 3: Determination of the solubility of hypericin in methanol–pyridine (99 : 1, v/v)

Detection method	Sample	Absorption at 589 nm	Peak area	Sample concentration	Solubility of hypericin in methanol–pyridine (99 : 1, v/v)	
				(μg/ml)	(μg/ml)	(μmol/l)
VIS	C	3.7849		–	–	–
spectroscopy	D	0.6514		7.571	321.89	638.10
HPLC	C		29465.796	36.53	321.67	637.66
	D		552.596	7.425	319.16	632.69

degradation of pseudohypericin, however, is slightly accelerated by pyridine and therefore not recommendable for standard solutions of pseudohypericin [14]. Solubility is less a problem with pseudohypericin, as the additional hydroxyl group makes it better soluble in common solvents. It could be shown that the absorbance properties of hypericin are not influenced by the addition of 1% pyridine [14]. 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 analytical data of the compounds see Wirz [14]. Methanol 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 was 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. Standard solutions

2.496 mg Hypericin were dissolved in methanol-pyridine (99 : 1, v/v) in a 25.0 ml volumetric flask. The solution was stored at room temperature for five days, shaken and sonicated 5 min each day. From this stock solution, eight standard solutions were prepared, their concentrations ranging from 0.9984 to 99.84 μg/ml.

4.3.2. Test sample in methanol

To prepare a saturated solution, 0.464 mg hypericin were distributed in methanol in a 5.0 ml volumetric flask. The sample was kept at room temperature for five days, shaken and sonicated 5 min each day. Then, the sample was centrifuged (5 min at 2000 rpm) and the supernatant (A) pipetted off. 1 ml of the supernatant was diluted with methanol to 5.0 ml (B).

4.3.3. Test sample in methanol-pyridine

To prepare a saturated solution, 3.494 mg hypericin were distributed in methanol-pyridine (99 : 1, v/v) in a 5.0 ml volumetric flask. The sample was kept at room temperature for five days, shaken and sonicated 5 min each day. The sample was centrifuged (5 min at 2000 rpm) afterwards and the supernatant (C) pipetted off. 1 ml of the supernatant was diluted with methanol-pyridine (99 : 1, v/v) to 50.0 ml (D).

4.3.4. VIS spectroscopy

Absorptions were measured at 589 and 590 nm.

4.3.5. HPLC

The modified method of Kerb et al. [17] was applied. Solvents used were solvent A (methanol) and solvent B (methanol-tetrahydrofuran-buffer (9 : 6:5, v/v)). The buffer consisted of 13.8 g sodium dihydrogen phosphate in 1 l water adjusted to pH 4.0 with ortho-phosphoric acid 85%. The elution profile was: 0–12 min 100% B, 12–17 min 100% A and 17–27 min 100% B. The flow rate was 0.8 ml/min for solvent B and 1 ml/min for solvent A. The column temperature was set at 25 °C and the injection volume at 25 μl. The detection wavelength of the DAD was set at 590 nm.

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