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Determination of hypericin and pseudohypericin from *Hypericum perforatum* in rat brain after oral administration

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Abstract The popularity of St. *John*'s Wort (S*J*W) extracts for treating mild to moderate depression has increased over the last decades and great effort has been devoted to identify the active principle of S*J*W extract. Previous investigations suggest the contribution of at least three classes of compounds, the phloroglucinols, the quercetin flavonoids, and the phenanthroperylenequinones, to the clinical efficiency of S*J*W extracts. Up to now, a plausible molecular mechanism of action has been described only for the phloroglucinols. For the flavonoids and the phenanthroperylenequinones different targets were proposed on the basis of pharmacological studies. The vast majority of these targets are located in the CNS and therefore increasing interest in the question of the CNS availability of these substances arose. Recently, the ability of phloroglucinols and flavonoids to penetrate the blood brain barrier could be demonstrated. For the phenanthroperylenequinones an examination of CNS bioavailability is still missing.

The aim of this work is to close this gap by developing and validating a HPLC method with electrochemical detection for the quantification of the phenanthroperylenequinones in brain tissue of rodents after oral application of S*J*W extract or pure hypericin. In our study, the phenanthroperylenequinone content in the CNS tissue was below the lower limit of detection of the analytical method and was thus lower than 16 pmol/g brain for hypericin and lower than 52 pmol/g brain for pseudohypericin after oral administration of 1600 mg/kg S*J*W extract or pure hypericin (5 mg/kg).

Keywords St. *John*'s Wort; Hypericin; Natural products; Electrochemistry; High pressure liquid chromatography.

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Introduction

Depression is one of the most frequent psychiatric disorders with an estimated lifetime prevalence rate of about 17% [1, 2]. Reliably diagnosed it can be effectively treated with tricyclic antidepressants (*e.g.*, imipramine), *MAO*-inhibitors (*e.g.*, moclobemide) and selective serotonine reuptake inhibitors (*e.g.*, fluoxetine). Alternatively to these classical treatments extracts of *Hypericum perforatum* L. (St. *John*'s Wort, S*J*W) have attained widespread popularity in many countries for treatment of mild to moderate depression in the last years. The antidepressive efficacy of alcoholic S*J*W extracts has been documented in several clinical studies [3–5], showing equipotent effects to classical antidepressants implying only little side effects.

Due to these findings and by reason of the wide spectrum of different components existing in the extract the interest in identifying pharmacological active molecules has increased. Clinical studies, as well as *in vitro* and *in vivo* studies, suggest the involvement of the phloroglucinol derivative hyper-

forin in the effectiveness of the extract. Hyperforin is found to be CNS bioavailable [6] and mechanisms of action are discussed, like uptake inhibition of serotonin (*5-HT*), norepinephrine and dopamine (*D*), or modifying neurotransmitter storage in synaptic vesicles. These non-selective effects on neurotransmitter concentration in the synaptic cleft are caused by elevating the intracellular sodium concentration, which strongly affects the neurotransmitter re-uptake [7–10].

The second class of molecules strongly suggested to contribute to the antidepressive effect of S*J*W extract is represented by the quercetin flavones isoquercitrin, rutin, hyperoside, and quercitrin. As for hyperforin, CNS availabilty for quercetin metabolites was shown by *Paulke et al.* [11, 12], but the molecular mechanism of action remains unclear. There are speculations of a potential *COMT*/*MAO* inhibition caused by quercetin flavones [13, 14] as well as an involvement in endocrine effects [15, 16]. In addition, *in vivo* data show antidepressive effects of several quercetin flavones in *Porsolt*'s forced swim test (FST) [17, 18]. Furthermore, S*J*W extracts without rutin or with a very low rutin content are completely inactive in the FST [19].

For a long time the phenanthroperylenequinones (Fig. 1) were considered to be mainly responsible for the antidepressive effect of the S*J*W extract. Whereas early *in vitro* studies reported an inhibition of *MAO-A* and *MAO-B* caused by hypericin [20], later studies disproved these findings [21].

In a systematic *in vitro* receptor screening *Butterweck et al.* found inhibitory effects of hypericin and pseudohypericin on specific binding to D_3 and D_4 receptors of rats. Moreover, hypericin, but not pseudohypericin, inhibited binding to rat β_1 and β_2 receptors. On the other hand an affection of the serotonin transporter, the norepinephrine transporter or the *5-HT* receptor could not be shown for the phenanthroperylenequinones [22]. In another study *Butterweck et al.* found an increase of serotonin and a decrease of norepinephrine in the hypothalamus of rats after 8 weeks daily application of S*J*W extract as well as pure hypericin [23]. These effects may suggest a contribution of the phenanthroperylenequinones to the antidepressive effect of S*J*W extract, but imply a satisfying CNS bioavailability of the phenanthroperylenequinones. Until now, there is no evidence that phenanthroperylenequinones are able to penetrate the blood brain barrier (BBB). On the contrary, in a study of *Fox et al.* no hypericin was found in the cerebrospinal fluid after an *i.v.* administration of pure 5 mg/kg pure hypericin to primates [24].

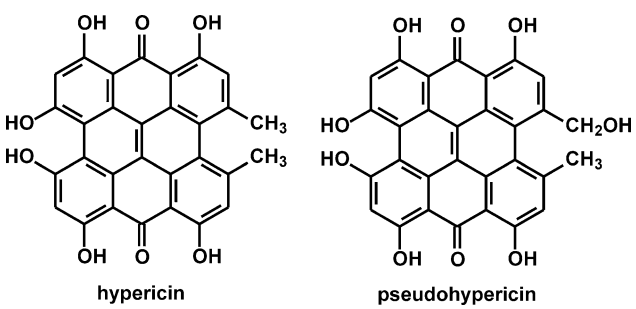

Fig. 1 Structure of S*J*W phenanthroperylenequinones

Beyond these findings, as for the quercetin flavones, some endocrine effects of S*J*W extract and pure hypericin are reported in addition to the affection of the hypothalamus pituary adrenal axis (*HPA* axis) [15]. But the coherency between *HPA* axis activity and depression is discussed very controversially. There is no evidence that a reduction of *HPA* axis activity improves depressive symptoms [25].

These results prompted us to explore the CNS availability of hypericin and pseudohypericin to elucidate the role phenanthroperylenequinones are playing in the clinical efficacy of S*J*W extract more precisely. Using HPLC we analyzed plasma and brain samples of rats fed with S*J*W extract and pure hypericin.

Many methods are described for the determination of phenanthroperylenequinones in plasma using high-performance liquid chromatography coupled with various detectors like UV [26], fluorescence [26–30], and ESI-MS [31, 32]. The chemical structure of the phenanthroperylenequinones encouraged us to develop a HPLC method with electrochemical detection (ECD), taking advantage of the high sensitivity of ECD, which is necessary for quantification of hypericins in low concentrations in tissue like those of the CNS.

The development of a HPLC-ECD method resulted in a lower limit of detection (LLOD) of 0.3 ng/cm^3 and 0.7 ng/cm^3 for hypericin and pseudohypericin and a lower limit of quantification (LLOQ) of 0.5 ng/cm^3 for hypericin and 1 ng/cm^3 for pseudohypericin.

Results and discussion

Validation

Validation was carried out in accordance with the international guidelines for validation ICH Q2A and ICH Q2B.

The specificity of the method was verified by comparing the chromatograms of three blank brain samples before and after spiking with hypericin and pseudohypericin. In brain blanks, no signals at the retention times of the phenanthroperylenequinones were detected.

The lowest concentration of the analytes measured with acceptable precision (relative standard deviation $\leq 7\%$) is defined as lower limit of quantification (LLOQ). The LLOQs were 0.5 and 3 ng/cm^3 for hypericin and pseudohypericin. The lowest concentration of the analytes detectable with a signal to noise ratio better than 1:3 is defined as limit of detection (LOD). The LODs were 0.3 and 0.7 ng/cm^3 for hypericin and pseudohypericin.

Linearity and precision were proven comparing the slope and the correlation coefficient of four calibration curves on four different days. Good linearity ($r^2 > 0.9994$) was found over the investigated calibration range of 0.5–50 ng/cm^3 for hypericin and 3–50 ng/cm^3 for pseudohypericin ($r^2 > 0.9959$). The relative standard deviations of the slopes of the four calibration curves were below 8.8%.

The intra-day precision and accuracy as well as the inter-day precision and accuracy were proven by comparing two differently spiked brain samples ($n = 4$) The values were situated within the range of the calibration curve. Intra-day and inter-day precision and accuracy were found acceptable, with relative standard deviations lower than 15%. Values are given in Table 1.

Table 1 Values of intra- and inter-day precision and accuracy in brain

	NC/ ng/cm^3	Intra-day ($n = 4$)		Inter-day ($n = 8$)	
		RSD/ %	Bias/ %	*RSD*/ %	Bias/ %
Hypericin	6.5	1.30	0.18	2.07	0.24
	30	6.59	0.63	4.56	−1.51
Pseudohypericin	6	3.54	2.56	4.01	0.06
	25	4.81	−1.88	7.60	10.23

NC Nominal concentration

Recovery of hypericin and pseudohypericin compared to blank samples were determined using a spiked solution of hypericin and pseudohypericin in methanol ($n = 6$) at a concentration of about 5 ng/cm^3 and 8 ng/cm^3. Recovery was measured on three different days to demonstrate inter-day reproducibility. On each day recovery of hypericin and pseudohypericin was determined with a relative standard deviation lower than 15%. The recovery values for hypericin were found to be 90.13%, whereas the recovery for pseudohypericin, was determined to be 61.75%. The values for the recovery assay are listed in Table 2.

Table 2 Values of the recovery of hypericin and pseudohypericin

	Original content/ ng/cm^3	*RSD*/ %	Recovery/ %
Hypericin from Plasma ($n = 6$)	80.15 (day 1)	7.78	58.84
	80.15 (day 2)	10.89	64.36
	70.84 (day 3)	7.54	61.69
Mean			61.63
Hypericin from Brain ($n = 6$)	4.94 (day 1)	6.16	91.21
	5.4 (day 2)	7.00	90.01
	5.15 (day 3)	7.50	89.18
Mean			90.13
Pseudohypericin from plasma ($n = 6$)	70.91 (day 1)	5.31	67.82
	70.91 (day 2)	4.68	68.20
	64.93 (day 3)	8.09	63.68
Mean			66.57
Pseudohypericin from brain ($n = 6$)	12.03 (day 1)	7.58	63.71
	12.54 (day 2)	7.53	59.79
Mean			61.75

Table 3 Stability of the analytes under different conditions

	Content/ ng/cm^3	*RSD*/ %	Bias/ %
Stability of hypericin in the HPLC injection system for 71.25 h ($n = 6$)	5.746	6.06	8.08
Stability of pseudohypericin in the HPLC injection system for 122.05 h ($n = 6$)	11.22	5.94	6.51
Long term stability of hypericin in brain at −20°C for 32 days ($n = 6$)	2.2	5.01	11.36
	14.88	7.69	−4.33
Long term stability of pseudohypericin in brain at −20°C for 13 days ($n = 6$)	4.9	7.88	4.49
	14.17	10.97	5.22

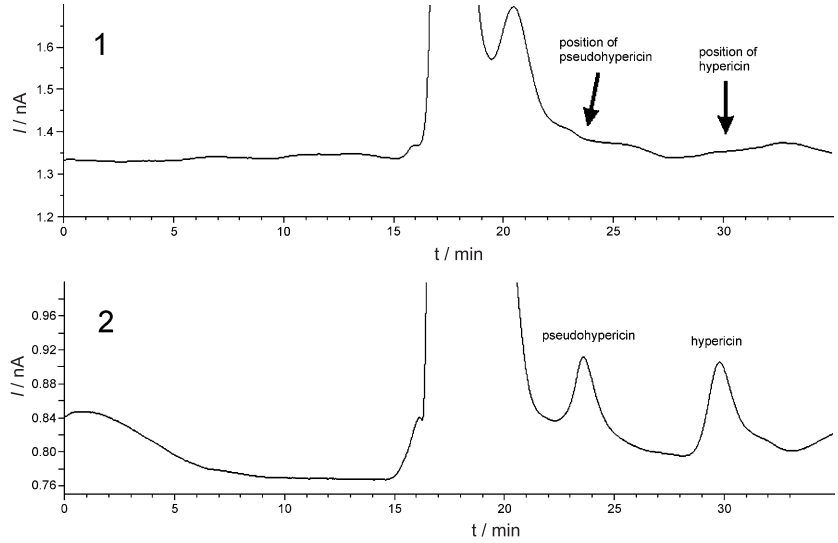

Fig. 2 Chromatogram of a brain sample after gavage of pure hypericin (5 mg/kg *b.w.*) (*1*) and after spiking with pseudohypericin ($NC = 12.75\,ng/cm^3$) and hypericin ($NC = 6.375\,ng/cm^3$) (*2*). *NC* Nominal concentration; *b.w.* body weight

Hypericin and pseudohypericin were found to be stable in the HPLC injection system at room temperature for 71 and 122 h. Long term stability of hypericin and pseudohypericin in brain at −20°C was verified at two concentration levels (low and high) for the period of 32 days for hypericin and 13 days for pseudohypericin. The chosen concentrations of 2.2 and 14.88 ng/cm^3 for hypericin and 4.9 and 14.17 ng/cm^3 for pseudohypericin were found to be stable over the observed times. The stability of hypericin and pseudohypericin standards in methanol stored at room temperature was observed over 3 days for hypericin and 2 days for pseudohypericin (see Table 3).

Animal study

In consideration of the poor solubility of hypericin in the stomach [33], plasma samples were taken together with the brain samples to prove satisfying concentrations of hypericin and pseudohypericin in the plasma. According to literature the plasma contents of hypericin and pseudohypericine were 80 ng/cm^3 and 28 ng/cm^3 after S*J*W extract application and 325 ng/cm^3 after administration of pure hypericin.

In order to examine the CNS bioavailability of hypericin an animal study was performed applying orally 1600 mg/kg S*J*W extract (1.34 mg/kg hypericin, 2.82 mg/kg pseudohypericin) and 5 mg/kg pure hypericin, respectively. As can be seen in Fig. 2, neither hypericin nor pseudohypericin could be detected in brain after application of S*J*W extract and pure hypericin, respectively. Taking into account the sensitivity of the analytical method, the hypericin and pseudohypericin content must be lower than 16 and 52 pmol/g brain.

Conclusion

Today, albeit the molecular mechanism of action is not clarified in detail yet, the clinical effectiveness of S*J*W extract in treating mild to moderate depression being proved by several clinical studies is beyond controversy [3–5, 34]. It could be shown that the phloroglucinoles [6–9] and quercetin flavones play the predominant role for the antidepressive activity in the extract [19]. For both classes of compounds the CNS bioavailability following oral administration of S*J*W extract was demonstrated by *Keller et al.* [6] and *Paulke et al.* [11]. However, the contribution of phenanthroperylenequinones to antidepressive efficacy is still under discussion.

About 15 years ago the phenanthroperylenequinones were thought to be the active principles of S*J*W extract, but until now no possible molecular mechanism of action for the phenanthroperylenequinones is scientifically proven and pharmacological data concerning the antidepressive effectiveness are rather weak [34]. However, all suggested mechanisms presuppose the ability of the phenanthroperylenequinones to penetrate the BBB and reach their target in the CNS tissue. Our results clearly demon-

strate that the phenanthroperylenequinones concentration in the CNS tissue is very low. In consequence probably a peripheral antidepressive effect like affecting the *HPA* axis may be taken into consideration, although the involvement of *HPA* axis in the aetiology of depression is controversially discussed [25]. Based on these results the role of the phenanthroperylenequinones for the clinical efficacy of SJW extract has to be critically discussed.

Experimental

Male NMRI-rats from the breeder *Janvier*, Le Genest, France, were provided by *Dr. Willmar Schwabe* Arzneimittel, Karlsruhe (accreditation by Regierungspräsidium Karlsruhe, accreditation number 35–9185.82/740/97). The rats were divided into two groups of 6 rats, each fed with 1600 mg/kg SJW extract or 5 mg/kg pure hypericin. SJW extract and hypericin were administered with 10 cm^3/kg of 0.2% agarose (vehicle) given *via* a pharyngeal tube. Brain samples were taken 4 h after feeding. The brain, separated from the brain stem, was carefully washed with ice cold tris buffer (5 m*M*, *pH* 7.4) and homogenized using a Potter-S, Braun (1 cm^3 buffer/100 mg brain). The brain homogenate was stored at −20°C subsequently.

For sample preparation the brain was defrosted slowly. 500 mm^3 of the brain homogenate were mixed with 500 mm^3 tetrahydrofuran and 30 mm^3 methanol. The reaction mixture was vortexed for 20 sec and centrifuged with $3030 \times g$ for 10 min in order to separate the precipiated out proteins. 850 mm^3 of the supernatant was taken on a 1 cm^3 Extrelut™ column and extracted with 6 cm^3 diethylether. The diethylether was blown off with nitrogen at 36°C and the remainder was mixed with 1 cm^3 methanol. The mixture was vortexed for 20 sec and treated with supersonic for 15 min. At last the mixture was centrifuged again with $3030 \times g$ for 10 min and the supernatant was injected into the HPLC system for analysis.

HPLC analysis was carried out on a Waters instrument (Waters, Milford, USA) containing a waters 600 controller, an In-Line Degasser AF, a waters 717plus autosampler and a waters *2465* electrochemical detector. A light reaction coil (own construction, light color 950, 36 W) was installed between the autosampler and the analytical column to convert potentially existing protohypericin and protopseudohypericin into hypericin and pseudohypericin [35]. Three mobile phases consisting of 45% methanol (phase A), 25% tetrahydrofuran (phase B) and 30% 75 m*M* phosphate buffer *pH* 2.8 (phase C) were used at a flow rate of 0.4 cm^3/min. The column used was a combination of a Discovery™ HS PEG 5 μm, 15×0.46 cm (Supelco, Taufkirchen) protected by a Discovery™ guard column 5 μm, 2×0.4 cm and a Chromolith SpeedROD™ RP18e, 5×0.46 cm. Separation temperature was set at 35°C. The amperometrical detection was performed with +0.93 V at 35°C.

For quantification a stock solution containing hypericin and pseudohypericin was used as external standard and spike solution. Standard curves were obtained at 0.5–50 and 10–200 ng/cm^3 for hypericin and 3–50 and 10–200 ng/cm^3 for pseudohypericin. The standard solutions were prepared directly before the preparation of brain and plasma.

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