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Determination of St. John's wort flavonoid-metabolites in rat brain through high performance liquid chromatography coupled with fluorescence detection

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Abstract

Flavonoids with the quercetin structure are widely distributed throughout the plant kingdom. Some effects such as their anti-oxidative and radical scavenging capacities are broadly discussed in literature. Furthermore, some *Hypericum* flavonoids show activity in depression-relevant animal model assays. So far, only one study concerning the pharmacokinetic profile of *Hypericum perforatum* (St. John's wort, SJW) flavonoids has been reported, but no data concerning their bioavailability in the CNS is on-hand. Thus, we developed a method for the quantification of quercetin, tamarixetin and isorhamnetin, both metabolites of quercetin, in very low concentrations in the rat brain in order to investigate the ability of flavonoids to cross the blood–brain barrier. The brain samples for analysis were taken 4 h after feeding an oral dose of an alcoholic SJW extract or pure isoquercitrin. We found the presence of quercetin and isorhamnetin/tamarixetin after feeding a SJW extract at 7 ng/g brain and 35 ng/g brain, respectively. In addition, we examined blood plasma taken from the same rats to correlate plasma and brain levels. The plasma levels were 350 ng/mL for quercetin and 1006 ng/mL for isorhamnetin/tamarixetin after intake of SJW extract.

Keywords: Quercetin; Flavonoids; *Hypericum perforatum*; St. John's wort; CNS; HPLC

1. Introduction

Extracts of the perennial plant *Hypericum perforatum* L., known as St. John's wort (SJW), are well known for treating mild to moderate depression [1–3]. The importance of alcoholic SJW extract is reflected by the amount of the drug sold. In 2003, the daily doses of SJW preparation sold in Germany was 89.9 millions [4], and in the USA the volume of sales of SJW preparations during 2001 was USD $24 million [5]. The pharmacologically active ingredients of SJW are the phloroglucinol derivatives hyperforin and adhyperforin, the naphthodianthrones hypericin and pseudohypericin, the flavonoids hyperoside, rutin, quercitrin, isoquercitrin and biapigenin and the tanning agent procyanidin. Published data strongly indicate that, beside hyperforin, hypericins and some flavonoids contribute to the antidepressant effect of the extract [1,6–13]. Flavonol glycosides (see Fig. 1), with quercetin as aglycone, represent up to 8% of the dry SJW extract and some of them show pharmacologically interesting effects in vitro and/or in vivo.

Singh et al. [14] found a potential correlation between quercetin content and a catecholamin-*O*-methyl-transferase (COMT)/monoaminoxidase (MAO)-inhibition. In addition, Butterweck et al. [15] reported that isolated flavonoids significantly reduce the immobility time in the forced swim test, a sensitive depression animal model. Noeldner et al. confirmed these findings by screening different constituents of SJW extract in this test [16]. Furthermore, he found a correlation between the rutin content and the antidepressant activity of SJW extract [17].

Pharmacokinetic investigations in humans as well as in animals showed that the intact flavonoids are not detectable in plasma after oral administration. Absorption mechanism and metabolism are still not completely understood, but seem to be similar for all SJW flavon-glycosides, i.e. deglycosilation in the small intestine and, after absorption, glucuronidation of the quercetin aglycone. Methylation in position 3′ or 4′ in the

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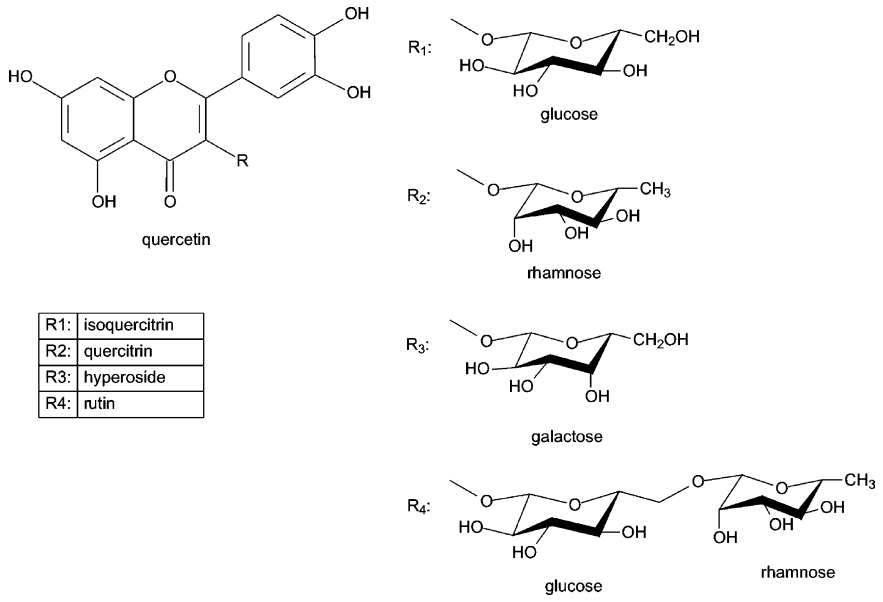

Fig. 1. Important quercetin flavonoids of *Hypericum perforatum*.

liver is possible and leads to isorhamnetin and tamarixetin. Furthermore, some sulphate conjugates can be found in human and animal plasma. Based on these findings, it seems unlikely that either intact flavonol glucosides or free aglycones appear in plasma or tissue, but their numerous metabolites should be present [18,19].

For the determination of flavonoids in plasma, many HPLC methods are described in literature. Among them, HPLC methods with electrochemical [18,20–24], mass spectrometric (MS) [25–27] and fluorescence detection [28–30] show adequate sensitivity. HPLC-UV–vis methods have also been reported [23,26,27,31–34]. Hollman et al. [29,30,35] published a robust and practicable methodology for quercetin analysis in plasma using fluorescence detection; the LOD of 5 ng/mL is, however, not sensitive enough for brain analysis. Based on the work of Hollman, we have developed a HPLC method to detect quercetin as well as isorhamnetin and tamarixetin in low concentrations in rat plasma and brains. Before analysis, the brain and plasma samples undergo an acidic hydrolysis in order to transform the metabolites into their agylca quercetin, isorhamnetin and tamarixetin. Fluorescence detection with a post-column complexation with aluminium nitrate resulted in a LOD of 0.4 ng/mL for quercetin and 0.05 ng/mL for isorhamnetin/tamarixetin. With this method it was for the first time possible to investigate the ability of SJW flavonoids to cross the blood–brain barrier in in vivo animal assays.

2. Experimental

2.1. Reagents, chemicals and animals

SJW extract (containing 1.35% isoquercitrin, 0.38% quercitrin, 3.26% rutin, 1.83% hyperoside all calculated as rutin), isoquercitrin and the flavon-free fodder were provided by Dr. Willmar Schwabe Arzneimittel, Karlsruhe. Methanol grade HPLC, acetic acid (100%) and hydrochloric acid 30% (Suprapure) were purchased from Merck, Darmstadt. Acetonitrile grade HPLC from Fisher Scientific, Loughborough, UK. Chloracetic acid (99%), sodium chloroacetate (98%), tris buffer (99.9%) and ethanol grade HPLC were purchased from Sigma-Aldrich, Taufkirchen; *tert*-butylhydroquinone (HPLC) and isoquercitrin (HPLC) from Fluka, Steinheim; aluminium nitrate nonahydrate (p.a.) from Acros, Geel, Belgium; isorhamnetin and tamarixetin (HPLC) from Roth, Karlsruhe. Water was purified by a Milli-Q system (Millipore, Bredford, MA).

The animal study was accredited by Regierungspräsidium Karlsruhe (accreditation number 35-9185.82/740/97). The animals were male NMRI-rats weighing 212–250 g from the breeder Janvier, Le Genest, France. They were maintained under standardised housing conditions (21 °C, 60% relative humidity, light from 6:00 to 18:00) for a period of at least 5 days prior to the start of the experiment. The animals received a standard flavonol-free diet and water ad libitum.

2.2. Sample collection

The animal assay was performed by Dr. Willmar Schwabe Arzneimittel, Karlsruhe. Extract and isoquercitrin suspensions were made in 0.2% agar gel and administered to the animals via pharyngeal tube. Dosage of the extract was 1600 mg/kg, the dosage of the isoquercitrin was 100 mg/kg. Blood and brain samples were taken 4 h after feeding. The brain, separated from the brain stem, was carefully washed with ice cold tris buffer (5 mM, pH 7.4), weighed and homogenized using a Potter-S, Braun (1 mL buffer/100 mg brain). Blood samples were collected directly into a Li-Heparin-Monovette (Fa. Sarstedt) containing 16 I.E. heparin/mL blood. The homogenates and the blood were stored for approximately 12 h at −20 °C before analysis.

2.3. Sample preparation

Brain and blood samples were defrosted slowly. Six hundred microlitres of the brain homogenate were mixed with 600 μL methanol, 400 μL *tert*-butyl hydroquinone (TBHQ) solution (5 mg/mL in methanol) and 400 μL concentrated HCl. The reaction mixture was vortexed for 20 s. Blood samples were centrifuged in order to get the plasma, and then 30 μL of plasma were mixed with 1200 μL of methanol. This mixture was vortexed for 20 s and 400 μL of TBHQ solution (5 mg/mL in methanol) and 400 μL HCl were added. The mixture was vortexed again for 20 s. The vials were sealed tightly with caps, inserted into a preheated aluminium block (Liebisch, Bielefeld) at 90 °C for 2 h, allowed to cool, centrifuged at 3700 × *g* for 10 min and then the supernatant was transferred to the HPLC system for the separation.

2.4. Quantitation and standards

A stock solution of quercetin and isorhamnetin was used as external standard and quality control. Standard curves were obtained at two levels (1–30 and 0.4–1 ng/mL) for quercetin and at three levels (1–50, 0.4–1 and 0.05–0.5 ng/mL) for isorhamnetin. Standard solutions were prepared directly before the preparation of brain and blood. Standard solutions contain 100 μg/mL of TBHQ and 20% HCl. Methanol 50% was used as a solvent for quercetin, and ethanol 50% for isorhamnetin. As quality control sample to monitor the reliability of the method we used pure methanol, blank brain samples, blank plasma samples, quercetin in methanol in the concentrations 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 10, 20, 40 and 50 ng/mL and isorhamnetin in methanol in the concentrations 1, 2, 3, 5 and 20 ng/mL. Quality controls with quercetin and isorhamnetin contain 100 μg/mL TBHQ and 20% HCl. Quality control vials were mixed randomly with the vials containing the brain and plasma samples.

2.5. Chromatographic instrumentation and conditions

HPLC analysis was carried out in a Varian ProStar instrument (Varian, Walnut Creek, USA). Seventy percent acetonitrile/methanol (100/380) (Phase A) and 30% chloracetic acid–sodium chloracetate buffer pH 2.5 (Phase B) at a flow rate of 1 mL/min were used as mobile phases. Solvents were degassed by an on-line degasser of the ProStar System. The column used was a Discovery® HS PEG 5 μm, 15 cm × 0.46 cm (Supelco, Taufkirchen) protected by a Discovery® guard column 5 μm, 2 cm × 0.4 cm. The column effluent was mixed with aluminium nitrate 1.5 M in methanol containing 7.5% glacial acetic acid at a flow rate of 0.4 mL/min using. A mixing T-adapter made of CTFE and a PTFE reaction coil (5 m × 0.5 mm i.d.) were used for mixing, so that quercetin, isorhamnetin and tamarixetin-aluminium complexes were formed. The aluminium nitrate solution was purged with a Knauer HPLC-Pumpe 64. The fluorescence of the resulting complexes was measured at 485 nm using a Varian ProStar 363 fluorescence detector with excitation wavelength set at 422 nm.

3. Results and discussion

Former studies showed that in vivo only traces of unconjugated quercetin [19,27,36] or intact quercetin glucosides [18,20,36,37] are present in animal or human plasma and tissue. Nearly all quercetin in plasma is either glucuronidated, sulphated and/or methylated [18,22,26,27] and numerous metabolites are detectable. In order to ease the analytical approach, the plasma and brain samples were treated for acid hydrolysis using hydrochloric acid, prior to the HPLC separation. This procedure reduced the number of metabolites to the three aglycons quercetin, isorhamnetin and tamarixetin. We separate quercetin from isorhamnetin and tamarixetin (these two analytes coelute) with a PEG column.

In order to detect the analytes at low concentrations, a post column complexation with aluminium nitrate was achieved using a reaction coil. These complexes provoke an intensive fluorescence signal. The advantage of this method is the use of an isocratic run over a short time with a satisfying peak resolution and width (even though we injected 200 μL in a primary flow of 1 mL/min).

3.1. Specificity and limit of quantitation

Specificity of the method was verified by comparing the chromatograms of six blank brain samples before and after spiking with quercetin and isorhamnetin. In brain and plasma blanks, no signal at the retention times of these flavonoids was detected.

The lowest concentration of the analyte measured with acceptable precision (relative standard deviation ≤10%) is defined as lower limit of quantification (LLOQ). The LLOQs were 0.4 and 0.05 ng/mL for quercetin and isorhamnetin, respectively.

3.2. Linearity, precision and accuracy

Linearity and precision were proven comparing the slope and the correlation coefficient of four calibration curves on four different days. Good linearity for the assay ($r^2 > 0.998$) was found over the investigated calibration range of 0.4–30 ng/mL for quercetin and 0.05–50 ng/mL for isorhamnetin. The relative standard deviations of the slopes of the four calibration curves were below 6%.

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

3.3. Recovery and stability

Recovery of quercetin and isorhamnetin from brain and plasma was determined using a spiked solution of isorhamnetin in ethanol and isoquercitrin in methanol ($n = 6$) at 6 ng/mL, compared with brain and plasma blanks. Recovery was measured

Table 1
Values of the inter- and intra-day precision and accuracy assay

	Nominal concentration (ng/mL)	Intra-day ($n = 4$)		Inter-day ($n = 8$)	
		R.S.D. (%)	Bias (%)	R.S.D. (%)	Bias (%)
Quercetin	3	10.12	−1.62	9.18	−4.62
	12.5	0.43	0.1	0.87	−0.42
	25.8	1.63	3.29	4.23	0.44
Isorhamnetin	1.8	2.44	3.0	5.43	−0.43
	16.5	13.2	5.88	5.29	1.55
	50	4.44	1.92	3.37	0.74

with isorhamnetin and isoquercitrin on two different days to demonstrate inter-day reproducibility. Isoquercitrin was used to simulate the in vivo metabolites of quercetin. Recovery of quercetin and isorhamnetin from brain and plasma on both days was obtained with a relative standard deviation lower than 15% (Table 2).

Stability of quercetin and isorhamnetin in the HPLC injection medium was tested at room temperature for 48 h, stability of isoquercitrin and isorhamnetin in brain was tested at −20 °C for 14 days at three standard level of concentration (low, medium, high). Stability of quercetin flavonoids in plasma is evidenced by literature [20,37]. Stability of quercetin in the HPLC injection medium was 31 h, whereas isorhamnetin was stable for 48 h. In the brain matrix, isoquercitrin as isorhamnetin did not degrade within 14 days when stored at −20 °C (Table 3).

Table 2
Values of the recovery of quercetin and isorhamnetin

	Original content (ng/mL)	R.S.D. (%)	Recovery (%)
Quercetin from plasma ($n = 6$)	7.5 (day 1)	0.73	82.39
	6.7 (day 2)	1.52	83.91
Mean			82.79
Quercetin from brain ($n = 6$)	5.858 (day 1)	1.16	87.56
	5.858 (day 2)	1.44	88.15
Mean			87.95
Isorhamnetin from plasma ($n = 6$)	8.94 (day 1)	2.12	98.06
	8.54 (day 2)	1.17	100.24
Mean			99.15
Isorhamnetin from brain ($n = 6$)	8.62 (day 1)	2.48	66.98
	11.05 (day 2)	2.42	68.79
Mean			67.88

3.4. Application in brain and blood samples

After validation was completed, the suitability of the approach for the determination of quercetin and isorhamnetin/tamarixetin in rat plasma and brain after an oral dose of SJW extract or pure isoquercitrin was tested. One rat received 1600 mg/kg SJW extract, containing about 100 mg flavonoids, another rat 100 mg/kg isoquercitrin. Samples were taken 4 h

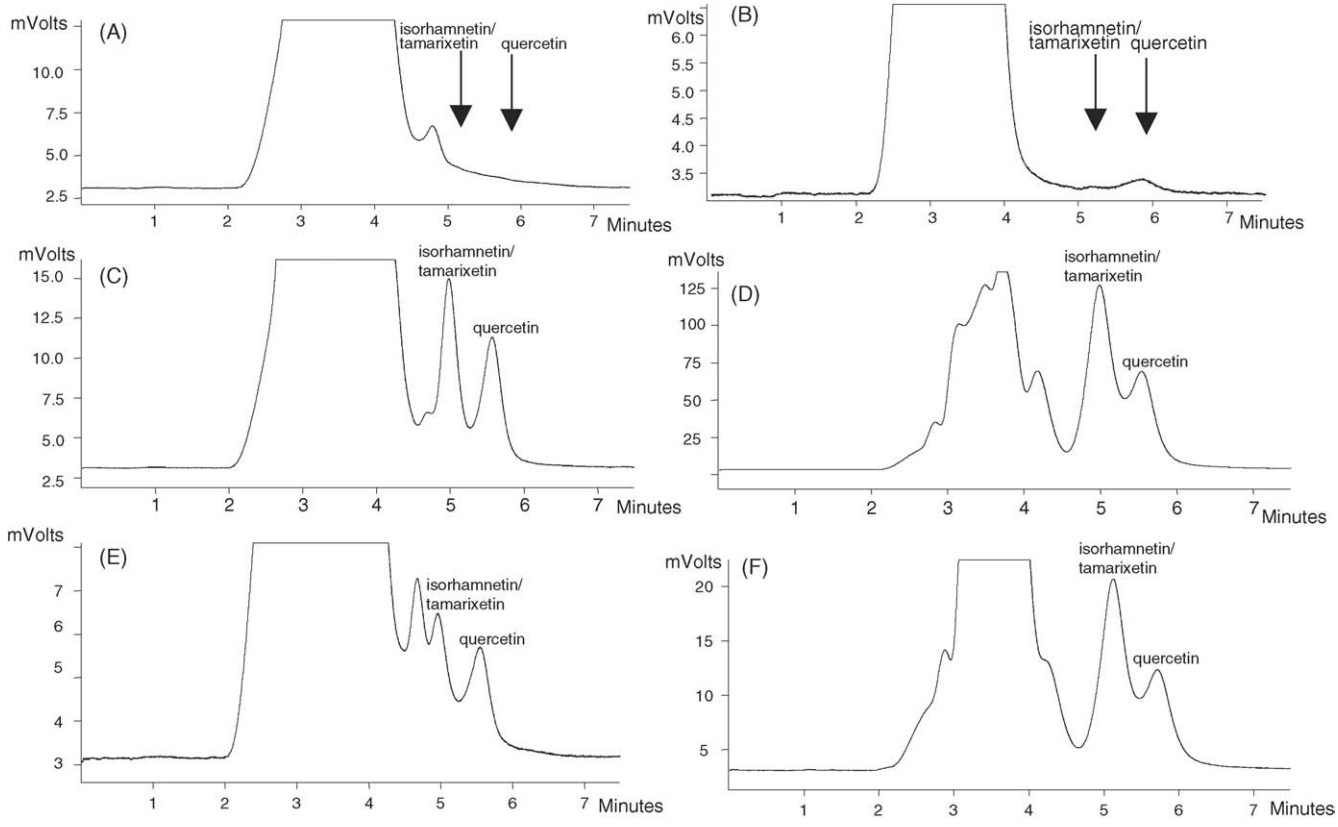

Fig. 2. HPLC chromatograms of brain and plasma samples 4 h after feeding of isoquercitrin (C and D, respectively) and SJW extract (E and F, respectively). A and B correspond to brain and plasma blanks.

Table 3
Values of the stability of the analytes under different conditions

	Content (ng/mL)	R.S.D. (%)	Bias (%)
Stability of quercetin in the HPLC injection medium for 31 h ($n = 2$)[a]	20	1.6	−3.48
Stability of isorhamnetin in the HPLC injection medium for 48 h ($n = 3$)	1	9.48	−23.6
	3	7.99	19.2
	10	2.6	−0.71
	30	2.38	0.93
Long term stability of isorhamnetin in brain at −20 °C for 14 days ($n = 3$)	1.8	9.08	−16.18
	16.5	5.66	−9.34
	50	3.56	4.59
Long term stability of isoquercitrin in brain at −20 °C for 14 days ($n = 3$)	3	8.62	−7.55
	12.5	4.46	−7.92
	25.8	4.16	−3.61

[a]The stability of quercetin under the conditions used is proved by literature [31], so we looked after the stability for only one concentration finding the properties approved.

after feeding and showed brain-levels of 35 ng/g (ng/g brain) quercetin and 7 ng/g isorhamnetin/tamarixetin for SJW extract and 119 ng/g quercetin and 72 ng/g isorhamnetin/tamarixetin for isoquercitrin. For plasma, the quercetin and isorhamnetin/tamarixetin levels were 6726 and 2279 ng/mL, respectively, after intake of isoquercitrin, and 1006 and 350 ng/mL after intake of SJW extract (see Fig. 2).

4. Conclusion

SJW extracts are complex mixtures of different constituents. Published data indicate that sufficient amounts of hyperforin, hypericins and flavonoids are necessary for the pharmacological activity and clinical efficacy of the extract. In order to achieve an antidepressive effect, it is important that these compounds are able to cross the blood–brain barrier. So far, the CNS bioavailability of hyperforin in vivo has been demonstrated [6,38]. We were, for the first time, able to demonstrate that metabolites of SJW flavonoids also reach the CNS of a rodent after an oral dose of SJW extract. However, the absorption mechanism as well as the molecular mode of action of the flavonoids in the antidepressant therapy still remain unclear and have to be investigated in further studies.

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