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Simultaneous determination of hypericin and hyperforin in human plasma with liquid chromatography–tandem mass spectrometry

Klaus-Dieter Riedel, Karin Rieger, Meret Martin-Facklam, Gerd Mikus, Walter E. Haefeli, Jürgen Burhenne*

Department of Internal Medicine VI, Clinical Pharmacology and Pharmacoepidemiology, University of Heidelberg, Im Neuenheimer Feld 410, D-69120 Heidelberg, Germany

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Abstract

A selective and sensitive method for the simultaneous determination of hypericin and hyperforin—the two main active ingredients of St. John's Wort (SJW) extract—in human plasma depending on liquid/liquid-extraction and LC/MS/MS detection has been developed, validated after specifying the stability of the photosensitive hypericin in plasma samples during light exposure and applied to samples of a patient. After extraction with ethyl acetate/*n*-hexane in the darkness, sample extracts were chromatographed isocratically within 6 min on a Kromasil RP-18 column. The analytes were detected with tandem mass spectrometry in the selected reaction monitoring mode using an electrospray ion source. The limit of quantification was 0.05 ng/mL for hypericin and 0.035 ng/mL for hyperforin. The accuracy of the method varied between 101.9 and 114.2% and the precision ranged from 4.7 to 15.4% (S.D., batch-to-batch) for both analytes. The method was linear at least between 0.05 and 10 ng/mL for hypericin and between 0.035 and 100 ng/mL for hyperforin. Using this method hypericin and hyperforin were determined successfully in a patient over seven days following discontinuation of exposure with therapeutic doses of St. John's Wort extract.

Keywords: Hypericin; Hyperforin; Stability to light

1. Introduction

St. John's Wort (SJW), a widely used medicinal plant, is an effective treatment of mild to moderate depression [1]. It may, however, cause substantial changes of dose requirements of co-administered drugs and thus prompt therapeutic failure of, e.g. oral contraceptives [2] or the immunosuppressant cyclosporine A [3] because two of its major constituents (hypericin, hyperforin [4]) may act as potent inducers of the efflux transporter P-glycoprotein as well as the drug metabolising cytochrome P450 isozymes CYP3A4, CYP2C9, and CYP1A2 [5–7].

Interindividual variability of the enzyme-inducing effect of SJW is large [8] as is the pharmacokinetic variability of hypericin and hyperforin [9,10]. However, thus far the concentration–effect relationship of the inducing compounds of SJW has not been characterised in vivo. It is therefore unknown what concentrations are actually required to prompt induction and whether pharmacokinetic variability may explain differences in the extent of induction. The effect of an enzyme inducer including SJW is concentration-dependent [6,7], may evolve at rather low plasma concentrations [11], and, as shown for antiepileptic drugs, may persist even when only subtherapeutic concentrations are present [11,12]. After discontinuation of SJW induction is maintained for several days [13] compatible with the interpretation that also in the case of hypericin and hyperforin subtherapeutic plasma concentrations are sufficient to maintain induction.

After repeated administration of 900 mg/d SJW extract corresponding to 750 μg/d hypericin, peak plasma concentrations (median (range)) of hypericin were 8.8 ng/mL (5.7–22.1 ng/mL) and the elimination half-life was 41.3 h

* Corresponding author. Tel.: +49 6221 56 36395; fax: +49 6221 56 5832.
E-mail address: juergen_burhenne@med.uni-heidelberg.de (J. Burhenne).

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(30.1–71.4 h) [9]. In contrast hyperforin peak plasma concentrations after repeated administration of 900 mg/d SJW extract containing 5% hyperforin were 246 ± 22.3 ng/mL (mean ± S.E.M.) and its elimination half-life was 11.2 ± 1 h [10].

Extrapolated from its pharmacokinetics, the expected plasma concentrations 5 days after discontinuation of SJW are in the range of 1 ng/mL for hypericin and 0.1 ng/mL for hyperforin. Taking further into account that the concentrations of these two components in commercially available dry extracts are highly variable [14], an even lower limit of quantification (LOQ) of 0.5 ng/mL for hypericin and 0.05 ng/mL for hyperforin may be required for their quantification.

It was thus the objective of this study to develop a most sensitive method suited to evaluate the concentration–effect relationship of the two inducing ingredients and to apply the assay to patient plasma samples drawn over several days after discontinuation of SJW.

Several analytical methods depending on high-pressure liquid chromatography (HPLC) coupled to ultraviolet or fluorescence detection have been described for hypericin and hyperforin in different biological matrices such as plasma, urine, and blister fluid [10,15–19]. The most sensitive methods described LOQ values of 0.2 ng/mL for hypericin [17] and 1.0 ng/mL for hyperforin [10] for the determination in serum or plasma. Two LC/MS/MS methods for the determination of hypericin and hyperforin in biological matrices have been published [20,21]. However, Pirker et al. [20] used the mass spectrometer only for identification purposes whereas the quantification was done with the fluorescence signal and the data of Piperopoulos et al. [21] represented MS/MS results of naphthodianthrones in plant extracts.

In commercial dried extracts and capsules of SJW the photostability of hypericin and hyperforin is limited [16,22]. This fact is also important for the handling of plasma samples. We therefore verified the stability of the drug in plasma samples exposed to sunlight.

This paper describes a new rapid method for the quantification of low hypericin and hyperforin concentrations in human plasma considering procedures for sample handling which avoid the degradation of the compounds by light. In contrast to all previously published methods this method is more than 20-fold more sensitive than the most sensitive method for hyperforin [10] and four-fold more sensitive than the most sensitive fluorescence method for hypericin [17].

2. Patient, materials, and methods

2.1. Patient

After approval of the study protocol by the Ethics Committee of the Medical Faculty of Heidelberg and obtaining written informed consent, we have collected plasma samples on days 1, 3, and 7 of hospitalisation of an obese 72-year old female patient. She was admitted to internal medicine because of an acute state of exhaustion, suffered from depression, arterial hypertension, mild heart failure, and osteoporosis, and indicated to have been exposed to SJW (two tablets per day of Jarsin® containing 300 mg SJW dry extract) up to the day of admission. Blood sampling tubes (Monovette™/NH_4^+-heparinate, Sarstedt, Nuembrecht, Germany) were protected from light using brown coloured transport sample containers (Ref. no. 78/898.300, Sarstedt, Nuembrecht, Germany) immediately after blood sampling. Collected tubes were centrifuged and the plasma was directly transferred into the freezer to store at −20 °C until analysis.

2.2. Materials

Reference compounds hypericin potassium salt (E-006/20396, purity >90%) and hyperforin 2-aminoisobutanol salt (HY–453, purity >98%) were generous gifts from Dr. Willmar Schwabe (Karlsruhe, Germany) (Fig. 1A and B). All reagents and solvents used for the chromatographic, spectroscopic, and sample procedures were of analytical or higher quality and were purchased from E. Merck (Darmstadt, Germany).

2.3. Standard solutions, calibration, and quality control samples (QC)

Hypericin and hyperforin were separately weighed into a volumetric flask (10 mL) considering the differences of the purities of hypericin and hyperforin. From each of these solutions aliquots were pipetted into a separate flask and diluted. From this analyte solution separate dilutions were prepared yielding final concentrations of 10, 6.75, 3.50, 0.95, 0.35, 0.050, and 0.0035 ng/mL for hypericin and 100, 67.5, 35.0, 9.50, 3.50, 0.50, and 0.035 ng/mL for hyperforin when 0.5 mL plasma were spiked with 25 μL of these solutions. The lowest calibration point for hypericin at 0.0035 ng/mL was not used to establish the calibration curve. The diluent for all calibration solutions was a mixture of acetonitrile/water (3/1, v/v).

Quality control samples of hypericin and hyperforin were prepared as calibration samples but with different weighing thus leading to final concentrations in plasma of 6.26, 3.25, and 0.14 ng/mL for hypericin and 66.5, 34.5, and 1.48 ng/mL for hyperforin.

2.4. Sample preparation

The complete sample preparation was done in a darkened room to avoid degradation of the analytes by light [22]. Five milliliters of ethyl acetate/*n*-hexane (7/3, v/v) was added to the plasma samples (0.5 mL) and shaken overhead for 10 min. After centrifugation at 2000 × *g* (10 min) the organic layer was separated and evaporated to dryness by a gentle stream of nitrogen at 40 °C. Reconstitution of the extract was done

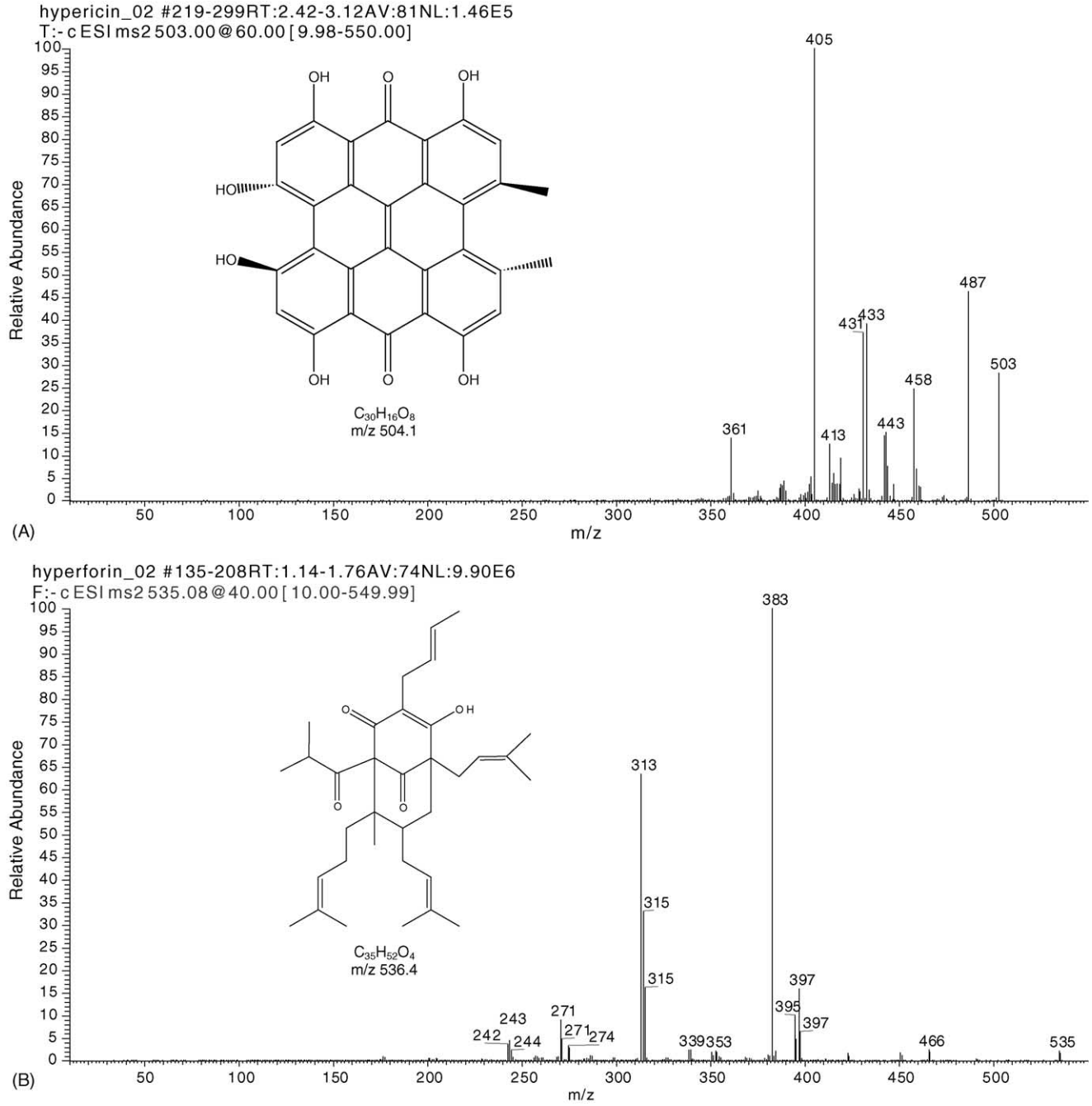

Fig. 1. (A) Tandem mass spectrum (daughter ion scan) of the hypericin parent ion *m/z* 503 performed with ESI (negative mode) and CID at 62 V using Ar at 4.8 mbar and chemical structures and codes of hypericin. (B) Tandem mass spectrum (daughter ion scan) of the hyperforin parent ion *m/z* 535 performed with ESI (negative mode) and CID at 40 V using Ar at 4.8 mbar and chemical structures and codes of hyperforin.

by addition of 200 μL acetonitrile/water (3/1, v/v) and instrumental analysis was performed within 8 h.

2.5. Stability of hypericin with and without sunlight exposure

Plasma samples were spiked to a hypericin concentration of 30 ng/mL and exposed to sunlight (at room temperature in glass vessels behind the laboratory window) for 0, 1, 2, 4, and 8 h. This procedure simulated a worst-case scenario when the sample preparation will be processed at a bench exposed to sunlight. In parallel, samples with the same concentration were standing at room temperature for 3 h but protected from sunlight by tightly enwrapping the sample vessels with aluminium foil. Subsequently all samples were extracted as described before. The chromatographic procedure and quantification of the drug was done with an HPLC/fluorescence method described by Bauer et al. [19]. This fluorescence

method was validated before starting of the development of the LC/MS/MS method, but was not sensitive and selective enough to determine hypericin and hyperforin concentrations when applied to samples of a clinical study [23].

2.6. Instrumental analysis parameters

The HPLC system consisted of a quaternary LC pump (TSP Model P4000, Thermo Electron, Dreieich, Germany) with degasser and a sample cooling (5 °C) autosampler (TSP Model AS3000) with integrated column heater. Injection volume was 20 μL. For isocratic chromatographic separation at 40 °C a Kromasil C_{18} column 100 A 3 μm, 70 mm × 2 mm i.d. with integrated guard column was used. The eluent (25% A/75% B) consisted of 0.1% (by volume) aqueous acetic acid including 5 mM ammonium acetate (A) and acetonitrile (B). The flow rate was 0.4 mL/min and was introduced without splitting into the electrospray ion source (ESI) of a triple-stage quadrupole mass spectrometer (Finnigan TSQ 7000 with API-2 ion source and performance kit, Thermo Electron, Dreieich, Germany). ESI interface parameters were as follows: middle position, spray voltage −4.5 kV, sheath gas (N_2) 60 psi, aux gas (N_2) 20 scales, capillary heater temperature 350 °C. The voltages responsible for the spray focus (heated capillary, skimmer lens, lens L11) were optimised. For this purpose aqueous solutions of pure hypericin (0.1 mg/mL) and hyperforin (0.1 mg/mL) were introduced continuously into the LC eluent via a syringe pump (at 5 μL/min). The intensity of the $[M-H]^-$ ion was monitored and adjusted to maximum. In the full scan MS mode 1.20 kV multiplier voltage was chosen. Selected reaction monitoring (SRM) measurements were performed at 1.63 kV multiplier voltage. MS/MS transitions monitored in the negative ion mode were *m/z* 503.0 → *m/z* 405.0 for hypericin and *m/z* 535.1 → *m/z* 382.9 for hyperforin. The parameters influencing these transitions were optimised: the Ar pressure in the collision quadrupole q2 was set to 4.8 mbar, the collision energy (CID-voltage) on q2 was adjusted to 62 and 40 V for hypericin and hyperforin, respectively. The resolution on the parent quadrupole q1 was slightly decreased in order to increase the sensitivity.

2.7. Validation of the analytical method

Analytical method validation was performed in accordance to the recommendations published by the U.S. Food and Drug Administration (FDA) [24,25]. Accuracy was calculated on the basis of the quotient of the averaged measurements and the nominal value and expressed in percent. Precision was defined as the ratio of the standard deviation and the mean calculated value in percent. These values are given within-batch and batch-to-batch. For this purpose analytical batches ($n = 3$) each containing seven calibration samples, 18 quality control samples at three different concentrations, six quality control samples for LOQ, and six samples of blank plasma from different individuals for specificity testing were analysed. From these values accuracy and precision of the method were calculated.

The robustness of the method was verified using the accuracy of all QC samples and the deviation of retention times for hypericin and hyperforin of all QC samples measured within the validation procedure ($n = 3$ batches) and within the determination of study samples from [23] ($n = 4$ batches). These seven batches were measured within 3 weeks.

3. Results and discussion

3.1. Sample preparation

Liquid/liquid extraction with ethyl acetate/*n*-hexane (7/3, v/v) was chosen for a fast and easy sample preparation. The dried residue from 0.5 mL samples was reconstituted with 200 μL acetonitrile/water (3/1, v/v) yielding recoveries from plasma for hypericin of 37% at 3.25 and 6.26 ng/mL, which was in accordance to Pirker et al. [20]. The corresponding recoveries for hyperforin were 63 and 68% at concentrations of 34.5 and 66.5 ng/mL.

3.2. Stability of hypericin in plasma samples

Hypericin and hyperforin are sensitive to light with hypericin being the more sensitive compound [22]. In order to evaluate the stability of hypericin which is important for sample handling in clinical studies spiked plasma samples were exposed to direct sunlight. Under these conditions the degradation of hypericin followed first order kinetics with a degradation constant k_1 of 0.387 h^{-1} corresponding to a half-life of 1.8 h. Within 8 h, more than 90% of hypericin is degraded. In contrast, the hypericin concentration in light-protected sample vials remained unchanged over a period of 3 h (Fig. 2), which was selected as a presumed maximum duration for pre-analytical sample handling.

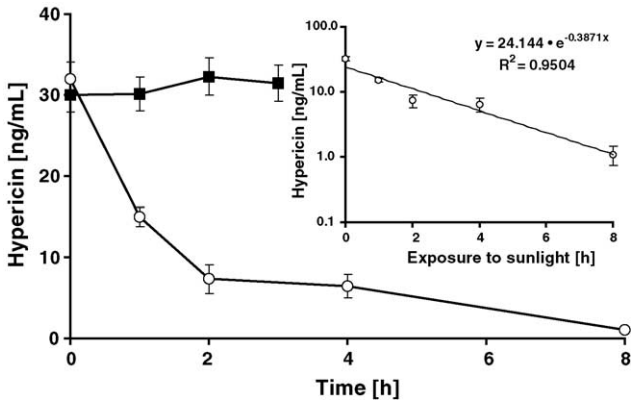

Fig. 2. Stability of hypericin in spiked plasma samples during exposure to sunlight (mean ± S.D., $n = 3$ samples). Open symbols: samples exposed to sunlight. Solid symbols: samples protected from sunlight.

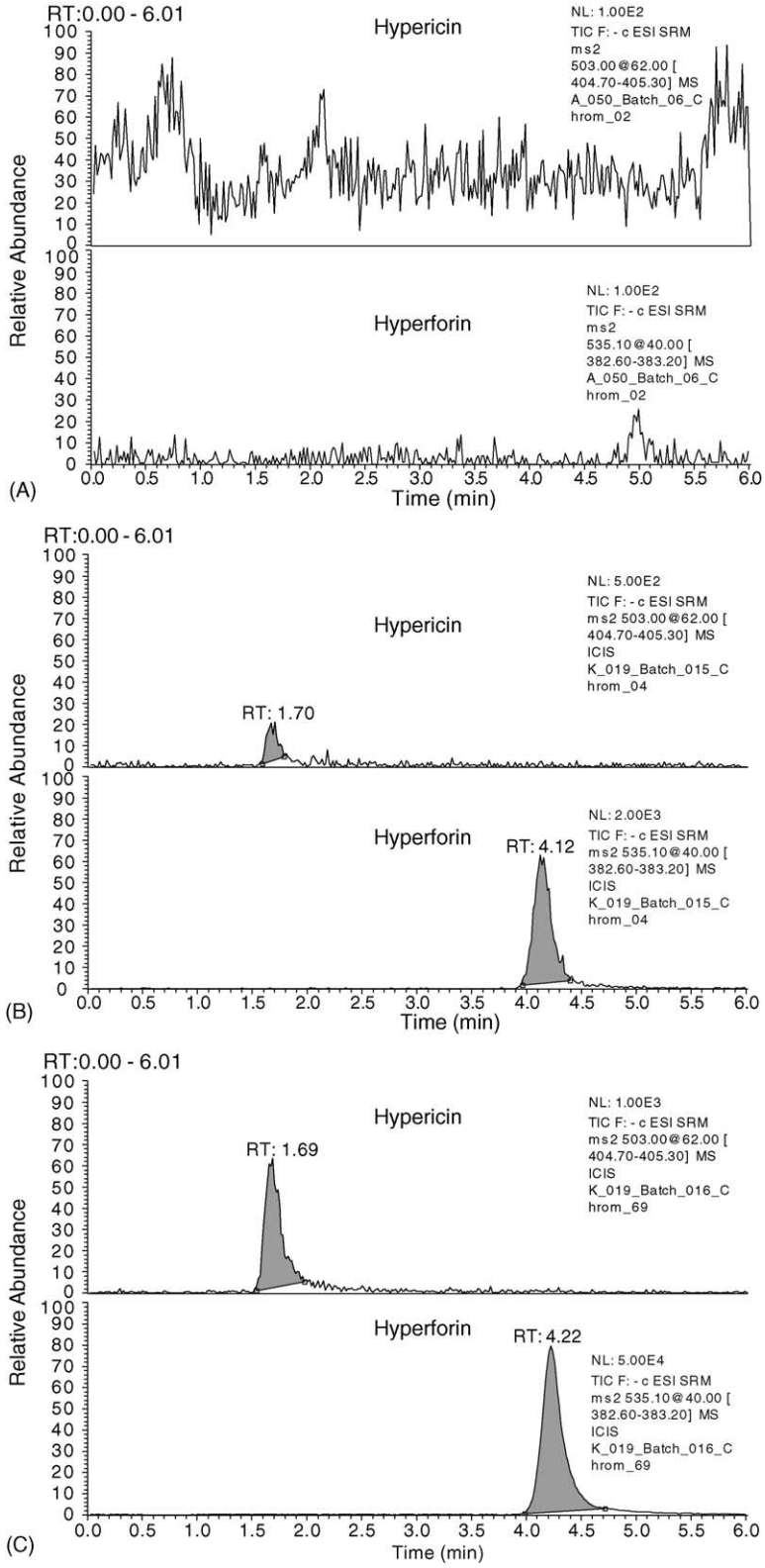

Fig. 3. LC/MS/MS-chromatograms of human plasma extracts. (A) Blank plasma. (B) Spiked plasma at 0.05 ng/mL hypericin and 0.5 ng/mL hyperforin. (C) Patient plasma containing 0.40 ng/mL hypericin and 16.6 ng/mL hyperforin. Upper traces: hypericin, 503.0 → 405.0, CID 62 V. Lower traces: hyperforin, 535.1 → 382.9, CID 40 V.

Table 1
Results of the QC samples of the analytical method validation ($n = 3$ batches; $n = 6$ replicates within each batch)

Batch number		Hypericin			Hyperforin		
		0.14[a]	3.25[a]	6.26[a]	1.48[a]	34.5[a]	66.5[a]
Within batch							
1	Mean (ng/mL)	0.12	3.15	5.58	1.64	36.5	64.7
	Precision (% CV)	2.7	7.9	11.3	4.9	4.3	4.3
2	Mean (ng/mL)	0.15	3.58	7.03	1.70	37.7	72.7
	Precision (% CV)	11.4	4.3	5.7	4.1	9.7	3.1
3	Mean (ng/mL)	0.16	3.79	6.96	1.72	38.8	73.7
	Precision (% CV)	14.3	8.6	8.3	4.2	6.1	2.6
Batch-to-batch							
	Mean (ng/mL)	0.14	3.51	6.52	1.69	37.7	70.4
	Precision (% CV)	15.4	10.3	13.1	4.7	7.1	6.7
	Accuracy (%)	101.9	108.0	104.1	114.2	109.3	105.9

[a] Adjusted concentration (ng/mL).

3.3. Performance and validation of the analytical method

The HPLC parameters were optimised for the fast detection of hypericin and hyperforin with mass spectrometry by choosing a short and narrow reversed phase HPLC column. The solvent system, which contained only volatile compounds, was reduced to the specific requirements of the ESI source. In Fig. 3A and B selected blank and spiked blank chromatograms as well as a real plasma sample (Fig. 3C) are shown: The high amount of acetonitrile in the eluent (75%) was adjusted for an elution of hypericin and hyperforin within 5 min and resulted in short chromatograms (6 min) with maximum peak half widths of 30 s. Additionally this led to a fast elution of interfering compounds preventing contamination of the analytical column. Using selective and sensitive tandem mass spectrometric detection no matrix interference was observed in the blank plasma of six different individuals. In general, co-eluting matrix is able to influence the ionisation process (ion suppression) and to contaminate the heated capillary, resulting in higher variation particularly at the lower limit of quantification (LOQ). Both were not the case and no increase of the ESI spray current was measured, which can be observed when suppressing ions or matrix compounds co-elute with the analytes. The LOQ for hypericin was 0.05 ng/mL and for hyperforin 0.035 ng/mL with an accuracy of 20%. The signal-to-noise ratio at LOQ was at least 5:1. Without cleaning of the heated capillary more than hundred measurements were possible.

The analytical method validation was performed with three analytical batches according to [24,25] with the following results: Within-batch precision for hypericin (data for hyperforin are in parentheses) ranged between 2.7 and 14.3% (2.6–9.7%) (S.D.). The overall precision ($n = 18$) was 15.4% (4.7%) in the low, 10.3% (7.1%) in the middle, and 13.1% (6.7%) in the high quality control samples. The overall accuracy of the QC-samples ranged between 101.9% (105.9%) and 108.0% (114.2%) in the three validation batches (Table 1). The correlation coefficients (r^2) from the calibration curves of the validation batches were at least 0.995 or higher for both compounds. The robustness expressed in the overall accuracy of the QC samples from seven analytical batches, which were measured within 3 weeks showed accuracies varying between 89.9 and 114.2%. The retention times of hypericin and hyperforin taken from calibration and QC samples of seven analytical batches were 1.63 min ± 3.3% (hypericin) and 4.20 min ± 2.6% (hyperforin).

This assay was first developed using an internal standard (Ro-31-8959/048, Roche Products Limited, Hertfordshire, England), which is a compound normally used for the HPLC/UV determination of saquinavir in biological matrices. This compound showed extraction recoveries similar to hyperforin and can easily used under these LC and ESI-negative parameters. However this analysis revealed reproducible extraction recoveries making the obligatory use of an internal standard dispensable.

3.4. Application of the method to samples of a single patient

The hyperforin concentration on days 1, 3, and 7 of hospitalisation of the patient was 90.2, 23.5, and 16.6 ng/mL, respectively. The corresponding hypericin concentrations were 2.55, 1.17, and 0.40 ng/mL (see Fig. 3C) resulting in an estimated half-life of slightly more than 2 days.

4. Conclusion

This LC/MS/MS assay combined with liquid/liquid extraction is highly sensitive, precise, and fast for the determination of hypericin and hyperforin in human plasma samples. Care must be taken during sample preparation, which must be done in the darkness because hypericin is sensitive

to sunlight. The method is calibrated and validated in the concentration range of therapeutic interest. Applying (1) the results of the pharmacokinetic studies of Kerb et al. [9] and Biber et al. [10], who both used a SJW dose effective for treatment of mild to moderate depression [1], and (2) the LOQ-values of our method, hypericin may be detected up to about 12 days and hyperforin about 5 days after discontinuation of SJW. To cover such a long time period is important because the induced state following SJW discontinuation has been shown to last 2 weeks or even longer in patients treated with cyclosporine A [3,13]. This suggests that even small and possibly subtherapeutic concentrations of SJW ingredients are capable of maintaining enzyme induction. It further emphasises the importance of having a method at hand that is more sensitive than the currently available fluorescence methods.

In conclusion, we have developed a sensitive method suited to evaluate the concentration–effect relationship of the two inducing ingredients of SJW in human plasma. This assay reached the demands of a clinical study [23] in which secret intake of SJW had to be proven after several days resulting in plasma concentrations around 0.1 ng/mL for hypericin and hyperforin.

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