ELSEVIER

Journal of Chromatography B, 765 (2001) 29–35

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of hyperforin, hypericin, and pseudohypericin in human plasma using high-performance liquid chromatography analysis with fluorescence and ultraviolet detection

Steffen Bauer[a,*], Elke Störmer[a], Hans-Joachim Graubaum[b], Ivar Roots[a]

[a]*Institute of Clinical Pharmacology, University Medical Center Charité Humboldt University Berlin, Schumannstrasse 20/21, 10098 Berlin, Germany*

[b]*PhytoPharm Consulting, Institut für Phytopharmaka GmbH, 13467 Berlin, Germany*

Received 24 April 2001; received in revised form 22 August 2001; accepted 23 August 2001

Abstract

Hyperforin, hypericin and pseudohypericin are the main ingredients of St. John's wort extract, which is available over the counter for treatment of mild to moderate depression. To facilitate clinical studies we developed two sensitive HPLC methods for determination of hypericin/pseudohypericin and hyperforin, respectively, in human plasma samples. The achieved limits of quantitation of 0.25 ng/ml for hypericin and pseudohypericin and 10 ng/ml for hyperforin were low enough to allow determination of pharmacokinetic parameters of the substances. Following liquid–liquid extraction of human plasma the samples were separated by isocratic reversed-phase HLPC and analyzed using fluorimetric detection for hypericin/pseudohypericin and UV detection for hyperforin.

Keywords: Hyperforin; Hypericin; Pseudohypericin

1. Introduction

Extracts of St. John's wort are becoming increasingly popular for the treatment of mild to moderate depression. Although the pharmacologic mechanism of this herbal remedy remains to be elucidated, therapeutic effects of extracts from St. John's wort have been confirmed in several studies compared to placebo or standard antidepressants [1–4]. St. John's wort extracts contain a variety of different compounds [5], with hypericin, pseudohypericin, and hyperforin (Fig. 1) presumed responsible for the drug's antidepressive activity [6,7]. In Germany hypericum extracts account for more than 25% of prescriptions of antidepressive drugs and are available as over-the-counter drugs [8]. Over the past years, an increasing number of reports on drug interactions of hypericum extract with various drugs emerged [9–12] generating a demand for robust, fast, simple, and sensitive methods for the quantitation of St. John's wort's ingredients in human plasma under the conditions of drug treatment.

Previously described methods by Chi and Fanklin [13,14] were validated only for concentration ranges of 5–100 ng/ml for hypericin and 150–300 ng/ml hyperforin, which are sufficient for determination of

*Corresponding author. Tel.: +49-30-4505-25127; fax: +49-30-4505-25910.

E-mail address: steffen.bauer@charite.de (S. Bauer).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00390-5

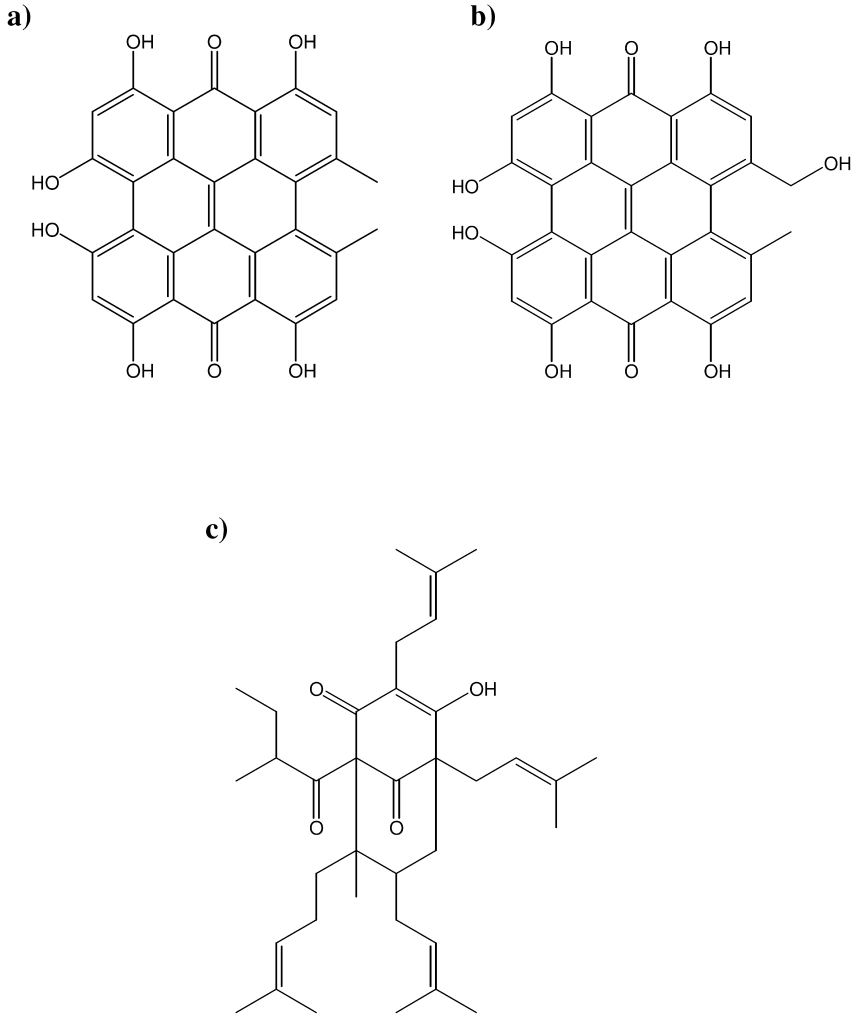

Fig. 1. Chemical structures of hypericin (a), pseudohypericin (b), and hyperforin (c).

peak plasma concentrations of the analytes, but do not satisfy the sensitivity requirements associated with pharmacokinetic measurements. Liebes et al. [15] have described a method with slow extraction steps and chromatography by gradient elution that is unfavorable for the processing of large sample sets. The high sensitivity of the method by Biber et al. [16] for the determination of hyperforin was obtained using tandem mass spectrometry (MS–MS) detection, a method not routinely available for pharmacokinetic measurements. The hypericin/pseudohypericin method presented here is based on the high-performance liquid chromatography (HPLC) procedure described by Kerb et al. [17] coupled with a new extraction procedure that markedly improved its reproducibility.

We present two methods developed to suit the requirements of clinical studies involving low-dose hypericum extract that allow a rapid, sensitive and reproducible determination of low concentrations of hypericin, pseudohypericin and hyperforin in human plasma.

2. Experimental

2.1. Chemicals and reagents

Hypericin (Rotichrom) was obtained from Roth (Karlsruhe, Germany), pseudohypericin was kindly provided by AnalytiCon (Berlin, Germany), and hyperforin was provided by TC BIOMED (Berlin, Germany). Sodium hydroxide pellets, sodium hydrogenphosphate, orthophosphoric acid (85%, extra pure), tetrahydrofuran, ethanol, methanol, acetonitrile, *n*-hexane, ethyl acetate, and pyridine were of HPLC or analytical grade and were purchased from Merck (Darmstadt, Germany). Nortestosteronepropionate was purchased from Steraloids (Newport, RI, USA).

2.2. Chromatography

2.2.1. Hypericin and pseudohypericin

Hypericin and pseudohypericin were quantified using a Shimadzu HPLC system (Duisburg, Germany) consisting of a pump LC 9A, an automatic sampler SIL 6B, a column heater CTO 6A and a fluorescence detector RF 551. Class LC10 software Version 1.6 (Shimadzu) was used for data analysis and processing. The compounds were separated at 60°C on a LiChrospher RP Select B column (5 μm, 250×4.6 mm I.D.) (VDS Optilab, Montabaur, Germany) with a guard column and quantified by fluorescence detection at 315/590 nm (ex/em). For preparation of the mobile phase 6.7 ml concentrated phosphoric acid was diluted with 950 ml distilled water, adjusted to a pH of 4.0 with sodium hydroxide (30% in water) and filled up to 1000 ml with distilled water. A 300-ml volume of this aqueous solution was combined with 450 ml methanol and 250 ml tetrahydrofuran to constitute the mobile phase. The mobile phase was prepared weekly and was delivered at a flow-rate of 0.75 ml/min. The substances were quantified using peak height.

2.2.2. Hyperforin

Hyperforin was quantified using a Shimadzu HPLC system consisting of a pump LC 10A, an automatic sampler SIL 10A, a column heater CTO 10A and a spectrophotometric detector SPD 10AV. Class LC10 software Version 1.6 (Shimadzu) was used for data analysis and processing. Samples were analyzed at 50°C on a Phenomenex Luna C18(2) column (5 μm, 250×4.6 mm I.D.) (Phenomenex, Aschaffenburg, Germany) with a guard column and quantified by UV detection at 273 nm. The mobile phase consisted of acetonitrile–0.01 *M* sodium hydrogenphosphate buffer (pH 2.4) (90:10). The mobile phase was prepared weekly and was delivered at a flow-rate of 1.5 ml/min. Hyperforin was quantified using its peak height ratio to an internal standard.

2.3. Sample preparation

2.3.1. Hypericin and pseudohypericin

In a 2-ml plastic tube 250 μl plasma was mixed with 500 μl mobile phase and 750 μl acetonitrile and extracted for 20 min at 40°C in an Eppendorf thermomixer 5437 (Hamburg, Germany). Samples were spun for 10 min at 3000 *g*, supernatants were transferred to a new tube and evaporated to dryness at 57°C under a stream of nitrogen. Samples were reconstituted in 75 μl mobile phase and 75 μl of a mixture of 10% pyridine in methanol and a volume of 75 μl was subjected to HPLC analysis.

2.3.2. Hyperforin

In a 10-ml glass tube 0.5 ml plasma was mixed with 30 μl internal standard solution (1 mg nortestosteronepropionate/100 ml methanol) and 6 ml of a mixture containing *n*-hexane–ethyl acetate (90:10) and extracted under agitation for 20 min at room temperature. Samples were spun for 5 min at 15 000 *g*, supernatants were transferred into a new tube and evaporated to dryness at 40°C under a stream of nitrogen. The dried extract was vortex-mixed with 1 ml extraction mixture and again evaporated to dryness. Samples were reconstituted in 100 μl mobile phase and a volume of 25 μl was subjected to HPLC analysis.

2.4. Preparation of stock solutions, calibration standards and quality control samples

2.4.1. Hypericin and pseudohypericin

A stock solution was prepared by dissolving 1.00 mg hypericin and 1.00 mg pseudohypericin in etha-

nol in a 20-ml volumetric flask. The solution was stored at −80°C in aliquots of 100 μl. For preparation of calibration standards 16 μl stock solution was evaporated to dryness and reconstituted in blank human plasma yielding the highest calibration standard with a concentration of 40 ng/ml hypericin and pseudohypericin, which was then used to generate standard samples with final hypericin and pseudohypericin concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0 ng/ml by serial dilution with blank plasma. For preparation of quality control samples appropriate aliquots of the hypericin/pseudo-

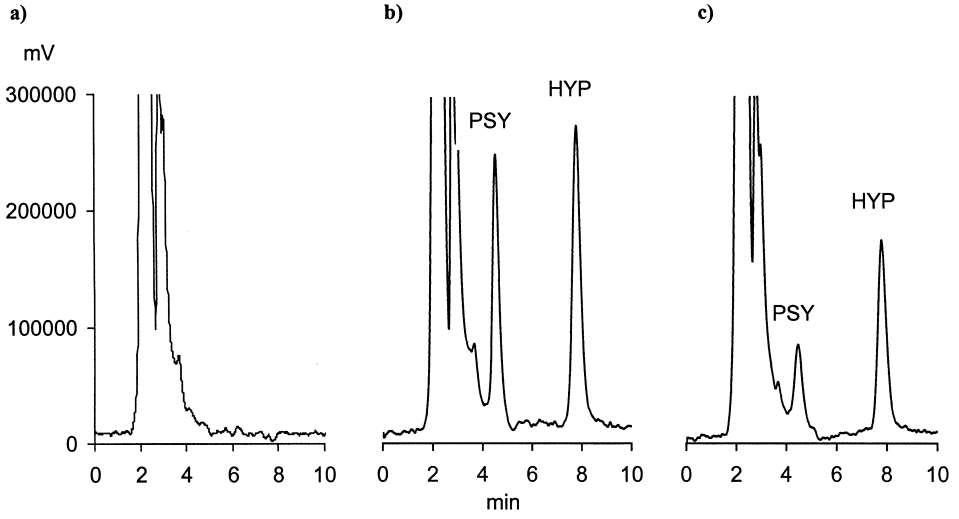

Fig. 2. HPLC trace of hypericin (HYP) and pseudohypericin (PSY) using fluorescence detection at 315/590 nm (ex/em). (a) Blank plasma sample; (b) calibration standard (5 ng/ml); (c) plasma sample 24 h post administration of hypericum extract (3.4 ng/ml hypericin, 1.5 ng/ml pseudohypericin).

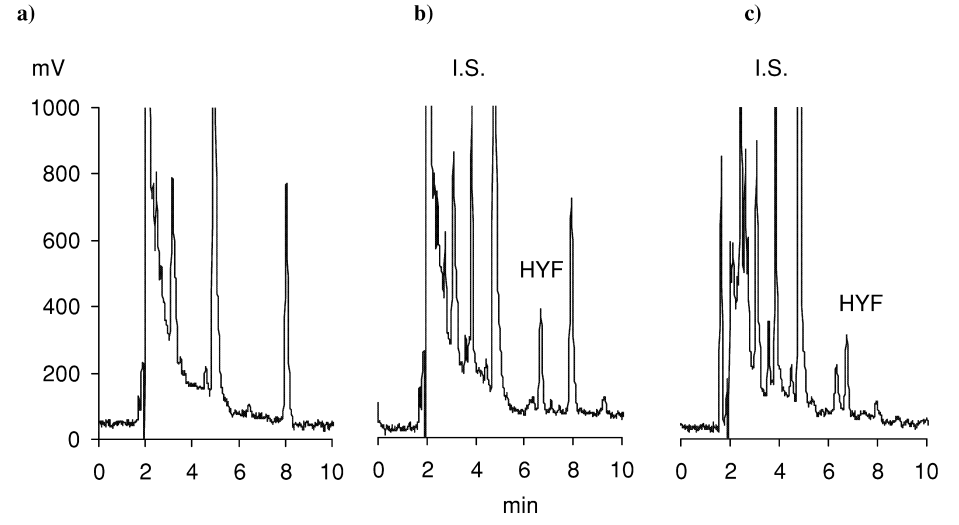

Fig. 3. HPLC trace of hyperforin (HYF) and nortestosteronepropionate (internal standard, I.S.) using UV detection at 273 nm. (a) Blank plasma sample; (b) quality control sample (150 ng/ml); (c) plasma sample 24 h post administration of hypericum extract (76.9 ng/ml hyperforin).

Table 1
Limits of detection and limits of quantitation for hypericin, pseudohypericin, and hyperforin

	Limit of detection (pg)	Limit of quantitation (pg/ml)
Hypericin	15	250
Pseudohypericin	10	250
Hyperforin	625	1000

Table 2
Assay linearity for the quantitation of hypericin, pseudohypericin, and hyperforin

		Mean	SD	RSD (%)	*n*
Hypericin	Slope	32 208	5389	16.7	14
	r^2	0.9976	0.0019	0.2	14
Pseudohypericin	Slope	26 580	3437	12.9	14
	r^2	0.9956	0.0133	1.3	14
Hyperforin	Slope	0.042	0.003	6.9	16
	r^2	0.9965	0.0050	0.5	16

hypericin stock solution were evaporated to dryness and reconstituted in blank human plasma. The final hypericin and pseudohypericin quality control concentrations were 2.5, 10.0, and 25.0 ng/ml. Calibration standards, blank plasma samples and quality control samples were stored in aliquots of 250 μl at −80°C until analysis.

2.4.2. Hyperforin

A hyperforin stock solution of 0.1 mg/ml was kindly provided by TC BIOMED and was stored protected from light at −20°C. For preparation of calibration standards and quality control samples appropriate aliquots of the hyperforin stock solution were evaporated to dryness and reconstituted in blank human plasma. The final hyperforin standard sample concentrations were 0, 25, 50, 100, 200, and 300 ng/ml, the quality control concentrations were 10, 75, 150, and 250 ng/ml. Calibration standards, blank plasma samples and quality control samples were stored in aliquots of 550 μl at −20°C until analysis.

3. Results

3.1. Separation and specificity

Hypericin and pseudohypericin were well separated under the HPLC conditions applied. Retention times were 4.5 min for pseudohypericin and 7.8 min for hypericin. Hyperforin and the internal standard nortestosteronepropionate were well separated with retention times of 3.8 min for the internal standard

Table 3
Intra-assay and inter-assay variability and accuracy of the quantitation of hypericin, pseudohypericin, and hyperforin

	ng/ml	Intra-assay variability			Inter-assay variability		
		n	RSD (%)	Accuracy (%)	*n*	RSD (%)	Accuracy (%)
Hypericin	0.5				20	17.8	102.2
	2.5	10	2.4	92.7	20	7.8	101.8
	10	10	1.1	106.7	20	7.6	113.9
	25	10	1.5	90.7	20	6.5	99.9
Pseudohypericin	0.5				20	16.0	91.0
	2.5	10	1.9	80.9	20	6.8	94.0
	10	10	2.2	96.1	20	5.5	104.3
	25	10	1.5	82.6	20	5.6	94.8
Hyperforin	10	10	10.7	99.5	12	16.1	104.7
	75	10	5.3	110.2	12	5.4	98.8
	150	10	7.1	113.9	12	10.1	95.4
	250	10	5.9	111.6	12	7.4	93.9

and 6.8 min for hyperforin. No interferences were observed in blank plasma samples. Figs. 2 and 3 show the chromatograms of hypericin/pseudohypericin and hyperforin, respectively, with a blank plasma sample (a), a calibration standard (b) and a plasma sample after administration of hypericum extract (c).

3.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD was determined as the amount of drug corresponding to a signal-to-noise ratio of 3:1. The LOQ was determined as the lowest concentration of the analyte in plasma that could be quantified with an inter-assay relative standard deviation (RSD) of $<$20% and an accuracy between 80 and 120%. The respective values for the three analytes are reported in Table 1.

3.3. Linearity

Assay linearity was evaluated up to hypericin and pseudohypericin concentrations of 40 ng/ml and hyperforin concentrations of 300 ng/ml. The mean slopes and r^2 values with SD and RSD are reported in Table 2.

3.4. Intra-assay and inter-assay variation

The intra-assay RSD for the three analytes ranged from 1.1 to 10.7% and the inter-assay RSD from 5.4 to 17.8%. The values are reported in Table 3

3.5. Accuracy

The accuracy of the measurements was determined using three quality control samples for each compound in every run and the results are reported in Table 3.

4. Discussion

We introduced two methods for the determination of hypericin/pseudohypericin and hyperforin in human plasma combining simple liquid–liquid extraction procedures with sensitive isocratic reversed-phase HPLC analysis with fluorescence and UV detection. LOQs of 0.25 ng/ml for hypericin and pseudohypericin, and 10 ng/ml for hyperforin were substantially lower than previously published [13,14]. Plasma concentration curves obtained over 12 h post administration of hypericum extract to healthy volunteers show that a reliable determination of pharmacokinetic parameters (i.e., area under the curve, $t_{1/2}$, clearance) does require quantitation of hypericin and pseudohypericin at concentrations below 1 ng/ml and of hyperforin at concentrations

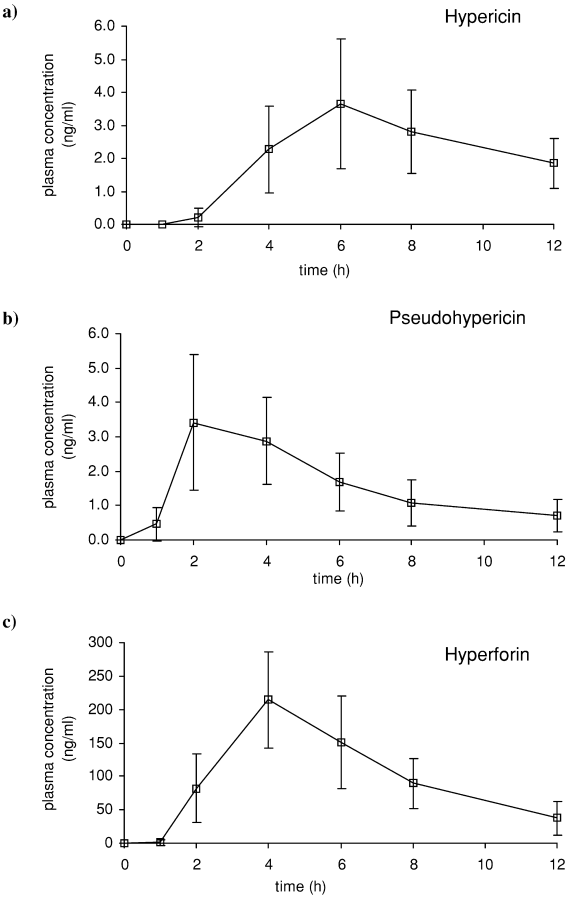

Fig. 4. Mean plasma concentration–time curves of (a) hypericin (n=9), (b) pseudohypericin (n=9), (c) hyperforin (n=10), 0–12 h after administration of hypericum extract to healthy volunteers. Data represent the mean±SD of 9 or 10 individuals, respectively.

below 50 ng/ml (Fig. 4). The improved extraction for the determination of hypericin and pseudohypericin requires reduced volumes of organic solvent compared to Kerb et al. [17]. Both methods have been applied to several clinical studies in our department [12,18,19] and have been found to be reliable and reproducible.

References

[1] E. Schrader, Int. Clin. Psychopharmacol. 15 (2000) 61.
[2] H. Sommer, G. Harrer, J. Geriatr. Psychiatry Neurol. 7 (1994) S9.
[3] D. Wheatley, Pharmacopsychiatry 30 (1997) 77.
[4] H. Woelk, Br. Med. J. 321 (2000) 536.
[5] A. Nahrstedt, V. Butterweck, Pharmacopsychiatry 30 (Suppl. 2) (1997) 129.
[6] S.S. Chatterjee, S.K. Bhattacharya, M. Wonnemann, A. Singer, W.E. Muller, Life Sci. 63 (1998) 499.
[7] V. Butterweck, F. Petereit, H. Winterhoff, A. Nahrstedt, Planta Med. 64 (1998) 291.
[8] M. Lohse, B. Müller-Oerlinghausen, in: U. Schwabe, D. Paffrath (Eds.), Arzneimittelreport '99, Springer Verlag, Berlin, Heidelberg, New York, 1999, p. 488.
[9] S.C. Piscitelli, A.H. Burstein, D. Chaitt, R.M. Alfaro, J. Falloon, Lancet 355 (2000) 547.
[10] F. Ruschitzka, P.J. Meier, M. Turina, T.F. Luscher, G. Noll, Lancet 355 (2000) 548.
[11] D. Durr, B. Stieger, G.A. Kullak-Ublick, K.M. Rentsch, H.C. Steinert, P.J. Meier, K. Fattinger, Clin. Pharmacol. Ther. 68 (2000) 598.
[12] A. Johne, J. Brockmöller, S. Bauer, A. Maurer, M. Langheinrich, I. Roots, Clin. Pharmacol. Ther. 66 (1999) 338.
[13] J.D. Chi, M. Franklin, J. Chromatogr. B 735 (1999) 285.
[14] J.D. Chi, M. Franklin, J. Chromatogr. B 724 (1999) 195.
[15] L. Liebes, Y. Mazur, D. Freeman, D. Lavie, G. Lavie, N. Kudler, S. Mendoza, B. Levin, H. Hochster, D. Meruelo, Anal. Biochem. 195 (1991) 77.
[16] A. Biber, H. Fischer, A. Romer, S.S. Chatterjee, Pharmacopsychiatry 31 (Suppl. 1) (1998) 36.
[17] R. Kerb, J. Brockmöller, B. Staffeldt, M. Ploch, I. Roots, Antimicrob. Agents Chemother. 40 (1996) 2087.
[18] A. Johne, J. Schmider, J. Brockmöller, A.M. Stadelmann, E. Störmer, S. Bauer, G. Scholler, M. Langheinrich, I. Roots, J. Clin. Psychopharmacol. (2001) in press.
[19] A. Maurer, A. Johne, S. Bauer, J. Brockmöller, F. Donath, I. Roots, M. Langheinrich, W.D. Hübner, Eur. J. Clin. Pharmacol. 55 (1999) A22.