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Use of an on-line, precolumn photochemical reactor in high-performance liquid chromatography of naphthodianthrones in *Hypericum perforatum* preparations

Alexander H. Schmidt*

Steiner and Co., Deutsche Arzneimittel Gesellschaft, Ostpreussendamm 72/74, 12207 Berlin, Germany

Abstract

A method has been developed for the determination of naphthodianthrones in *Hypericum perforatum* L. extracts and phytopharmaceutical preparations by high-performance liquid chromatography combined with on-line, precolumn photochemical conversion followed by photodiode-array detection. The chromatographic separation was performed on a C_{18} column under isocratic reversed-phase conditions. An on-line, precolumn photochemical reactor equipped with a knitted PTFE reaction coil around a visible light source was used in order to transform the light sensitive naphthodianthrones, protohypericin and protopseudohypericin, very easily into the non-protoforms, hypericin and pseudohypericin, respectively. Two UV chromatograms (photochemical reactor ‘‘on’’ and ‘‘off’’) were compared and were quite useful in characterizing the sample. Validation studies demonstrated that this HPLC method is simple, rapid, reliable and reproducible. The time-consumptive manual irradiation of the samples is omitted by this automated on-line irradiation step. The developed method was successfully applied to the quality control of *Hypericum perforatum* L. extracts and its phytopharmaceutical preparations.
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1. Introduction

Photochemical derivatization is one of the simplest derivatization methods in liquid chromatography, but used least often and generally unfamiliar to analytical chemists. This derivatization technique uses the impact of light on a molecule and results in structural changes (rearrangements, dimerizations, isomerization, and so on). An overview of chemical reactions, which are initiated, forced or catalyzed by light, was published by Krull and LaCourse [1] and Liu et al. [2].

In the early years, operating problems that limited the routine use of these instruments included reactor coil heating, leaking and large dead volumes. An extensive review of Brinkman et al. considered the design, construction, and application of post-column reactors [3] and Neue published research on using PTFE tubes in a 3-dimensional knitted form to minimize peak broadening in a photochemical reactor [4,5]. Nowadays, a few commercial photochemical reactors are available and nearly 100 publications on this subject show the potential advantage of the technique [6–8].

In nearly all publications a photochemical reactor

*Fax: +49-30-712-5012.
E-mail address: aschmidt@steinerarznei-berlin.de
(A.H. Schmidt).

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was used in an on-line, post-column approach for a wide variety of organic and inorganic compounds. That means analytes eluting from the LC column are photochemically converted [8].

In this study the photochemical reactor was used to convert the light sensitive biosynthetic precursor of naphthodianthrones after their injection into a HPLC system before they were separated on an analytical column (on-line, precolumn mode).

Hypericum perforatum L., popularly known as St. John's Wort, is a plant used in the production of extracts for phytopharmaceutical preparations and has enjoyed a tremendous surge in interest and sales for the treatment of depression.

The therapeutic use of alcoholic extracts of St. John's Wort for the treatment of mild to moderate depressive disorders, somatoformic disturbances, including symptoms such as restlessness, anxiety and irritability, have been confirmed on the basis of detailed pharmacological data and clinical studies [9]. The randomized controlled studies have provided evidence that *Hypericum perforatum* extracts are as effective as standard antidepressants in mild-to-moderate depression (e.g. Ref. [10]). A review of clinical studies of *Hypericum perforatum* extracts in depressed patients was recently published by Kasper [11].

A large number of compounds with biological activity have been isolated from the extract including a broad range of flavonoids (rutin, hyperoside, isoquercitrin, quercitrin and quercetin), bioflavonoids (biapigenin and amentoflavone), naphthodianthrones (hypericin, pseudohypericin) and phloroglucinols (hyperforin and adhyperforin) [12]. The characteristic constituents are the naphthodianthrones, mainly hypericin and pseudohypericin and their hydrated proto-forms, which occur as the biosynthetic precursors protohypericin and protopseudohypericin, and which are subsequently transformed into the cyclic compounds by exposure to light (Fig. 1).

The naphthodianthrones and phloroglucinols are expected to be the active ingredients of St. John's

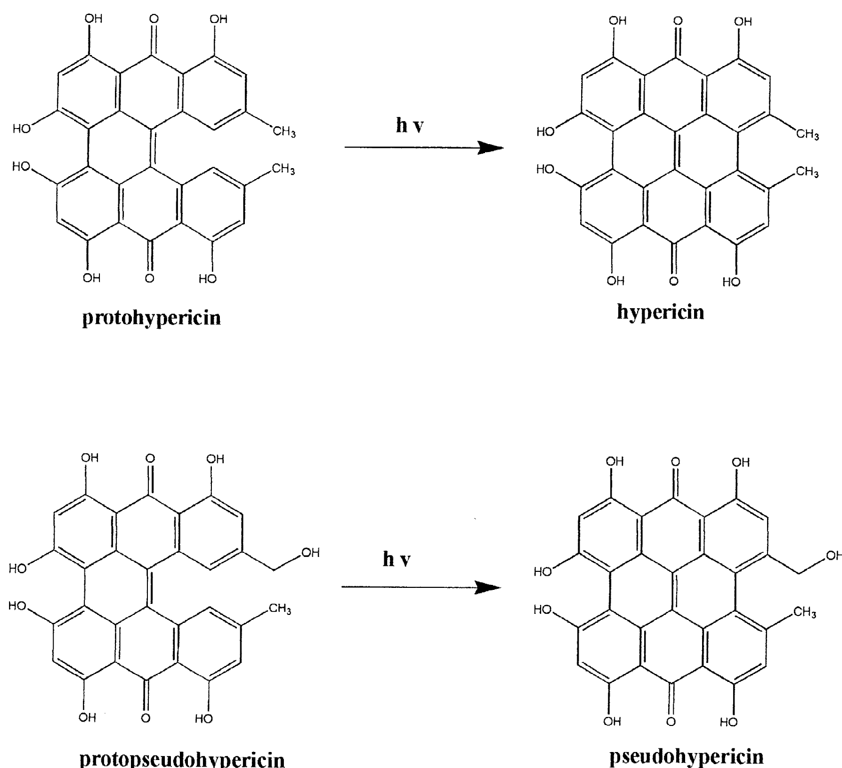

Fig. 1. Structures of naphthodianthrones in *Hypericum perforatum* and scheme of reactions.

Wort. The phloroglucinol hyperforin is relatively unstable in the presence of oxygen and light, and as such, the naphthodianthrones, commonly calculated as total hypericin, remain as popular marker substances and are used as a quality control measure in standardized extracts and phytopharmaceuticals.

Among various analytical techniques, a number of HPLC methods to determine the amount of naphthodianthrones have been published in recent years using either UV, fluorescence or mass spectrometric detection [13–26]. In most publications the sample preparation was performed in the absence of light, which is difficult to handle in a routine lab. To overcome this problem a few publications deal with the irradiation of the sample to daylight before analyzing for total hypericin [24–26]. Due to the research of Sirvent et al. the conversion of the protoforms to pseudohypericin and hypericin was completed within the first 2 h of illumination [15], but this time-consumptive manual irradiation of the samples extend the sample preparation time.

Our continuing interest in phytopharmaceutical analysis using high-performance liquid chromatography (HPLC) led us to develop an improvement for the analysis of total hypericin in *Hypericum perforatum*. On the basis of encouraging results of recent research in photochemical derivatization of naphthodianthrones it makes sense to determine the amount of total hypericin after exposure of the sample to light in order to allow the quantitative photo-conversion of the protoforms. Therefore, the irradiation of the sample was automated by means of a photochemical reactor to reduce sample preparation time and to improve the accuracy of the assay.

2. Experimental

2.1. Chemicals

Methanol and ethyl acetate were of HPLC grade and all other chemicals were reagent grade or better and purchased from Merck (Darmstadt, Germany). Water was purified by a Milli-Q water purification system (Millipore, Eschborn, Germany). A 0.1 *M* phosphate buffer (pH 2.1) was prepared by adding orthophosphoric acid to a solution of 0.1 *M* NaH_2PO_4 in water up to the desired pH.

2.2. Equipment

Chromatographic analysis of naphthodianthrones was performed using a HPLC system (Waters, Eschborn, Germany) equipped with a 600E multisolvent delivery system with column heater, 717plus autosampler and a 996 photodiode-array detector. Chromatograms were monitored at a wavelength of 590 nm. UV spectra were taken in the range of 210–600 nm.

The photochemical reactor Beam Boost 6808 (ICT, Vienna, Austria), equipped with knitted PTFE reaction coils (5, 10 and 15 m×0.3 mm I.D.) and a visible light lamp source Lumilux de Lux Daylight 36 W/12 (Osram, Munich, Germany), emission maximum 435+545 nm, was used for precolumn on-line irradiation and installed between autosampler and the analytical column. Unless otherwise stated, a 15-m knitted reactor coil was used, which results in an irradiation time of about 138 s at a flow-rate of 0.45 ml/min. By changing the length of the knitted reactor coil and/or the mobile phase flow-rate, the irradiation time in the photochemical reactor was varied.

For system control, data acquisition and data processing we used the Millennium[32] client/server software version 3.20 (Waters).

2.3. Chromatographic conditions

Chromatography was performed using a Nucleosil 100 C_{18} column packed with 5-μm particle size and dimensions of 250×3 mm (Macherey-Nagel, Düren, Germany). The mobile phase consisted of methanol–ethyl acetate–0.1 *M* phosphate buffer pH 2.1 (67:16:17, v/v). The flow-rate was 0.45 ml/min and the column temperature was 32 °C. The UV chromatograms of naphthodianthrones were recorded with the visible light lamp of the photochemical reactor switched ''off'' and ''on'' and compared.

2.4. Standards

A reference standard of hypericin (Addipharma, Hamburg, Germany) was accurately weighed into a 100-ml volumetric flask and dissolved in methanol to prepare a stock solution. The stock solution was stored at 4 °C and brought to room temperature

before use. The solution of the standard was found to be stable over the investigated period of 4 weeks at 4 °C.

Pseudohypericin, used in previous studies [19,21], gave the same calibration curve and UV–Vis spectrum as hypericin and therefore the hypericin calibration curve was used for hypericin and pseudohypericin.

2.5. Sample preparation

Two hundred mg *Hypericum perforatum* extract (STEI300, Steiner, Berlin, Germany) was exactly weighed into a 100-ml Erlenmeyer flask and extracted with 50 ml methanol by means of an Ultra-Turrax T25 (IKA, Staufen, Germany) for 10 min at room temperature. The mixture was transferred into a 100-ml volumetric flask and made up to volume with methanol. An aliquot of the preparation was filtered through a 0.45-μm PTFE membrane filtration cartridge (Gelman Sciences, Dreieich, Germany) into a vial and transferred into the HPLC autosampler.

Powdered samples of the phytopharmaceutical dosage form (Aristo 350 capsules, Steiner), equivalent to about 200 mg of *Hypericum perforatum* extract, were treated in the same way.

2.6. Peak identification

The hypericin peak in the chromatogram was identified by comparison of the retention time and the UV spectra of the peak with those from the hypericin reference standard.

Because of the lack of the other naphthodianthrones, pseudohypericin, protopseudohypericin, and protohypericin were identified by comparison of the UV–Vis spectrum with literature data [21,27].

2.7. Method validation

The analytical method was fully validated in compliance with the guidelines Q2A and Q2B [28] issued by the International Conference on Harmonization (ICH).

3. Results and discussion

3.1. Sample analysis

Routine analysis of naphthodianthrones in *Hypericum perforatum* extracts and its phytopharmaceutical preparations were carried out by on-line, precolumn irradiation in a photochemical reactor followed by photodiode-array detection. Baseline separation of all naphthodianthrones was achieved within 20 min. As shown in Table 1 several samples were analyzed according to the method described above. The average content of total hypericin in the *Hypericum perforatum* extracts and a commercial product was found to be 0.2% (w/w) for the extract and 0.7 mg for the capsule, respectively.

Table 1
Results for *Hypericum perforatum* extracts and preparations. Results in μg/100 mg for the extract and μg/capsule for the preparation

	Batch no.	Amount (μg/100 mg)			Amount (μg/capsule)		
		Pseudo-hypericin	Hypericin	Total hypericin	Pseudo-hypericin	Hypericin	Total hypericin
Hypericum perforatum extracts							
STEI300	WB10628	125.1	71.0	196.1			
STEI300	WB60210	143.4	52.2	195.6			
STEI300	WB92221	146.5	42.3	188.8			
Commercial products							
Aristo 350	7402748				461.8	236.9	698.7
Aristo 350	7405629				508.5	191.7	700.2
Aristo 350	7407939				542.7	196.9	739.6

Table 2
Experimental data collected with four different reactor coil lengths

Length of reactor coil (m)	Irradiation time (s)	Concentration (μg/ml)				
		Proto-pseudo-hypericin	Pseudo-hypericin	Proto-hypericin	Hypericin	Total hypericin
0	0	1.118	4.595	0.317	2.772	8.544
5	46	0.664	5.618	0.184	2.932	9.139
10	92	0.255	6.623	0.095	3.181	9.895
15	138	0.086	6.996	0.086	3.199	10.109
20 (15+5)	184	0.086	6.987	0.086	3.208	10.109

To achieve optimum conditions, the effect of the irradiation time on the protoforms of the naphthodianthrones was investigated. Experimental data collected with four different reactor coil lengths shows that both protoforms of the naphthodianthrones, protopseudohypericin and protohypericin, were completely converted into hypericin and pseudohypericin, respectively, by using the 15 m×0.3 mm I.D. knitted PTFE reactor coil. Under such conditions the irradiation time lasts 138 s for a mobile phase flow-rate of 0.45 ml/min. A longer irradiation time by using a longer reactor coil or reducing the flow-rate had no influence on the amount of total hypericin (see Table 2). Fig. 2 shows two chromatograms with the photochemical reactor lamp switched "off" and "on". The visual inspection of the chromatogram shows that photo-conversion of the proto-forms was complete.

3.2. Validation of the method

3.2.1. Specificity and selectivity

The studies show that the method was sufficiently specific to *Hypericum perforatum* extracts and its

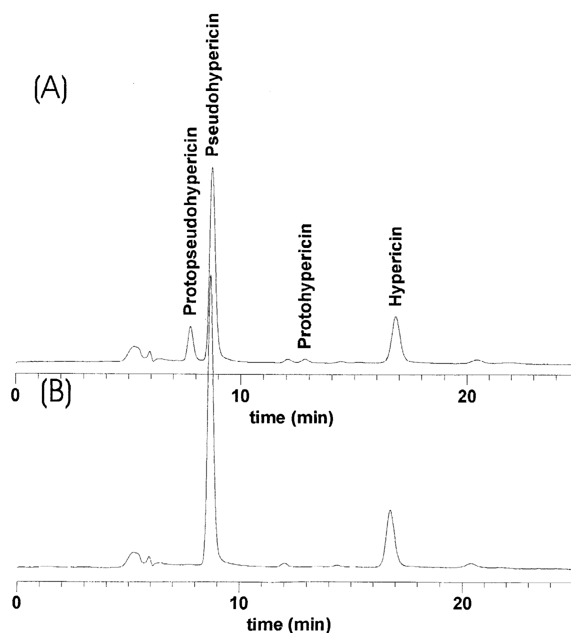

Fig. 2. Chromatograms of *Hypericum perforatum* recorded at 590 nm show the non-irradiated (A) and irradiated (B) experiment. For further details, see text.

preparations. The resolution factors for the hypericin peak and pseudohypericin peak were >3 from the nearest resolving peak. The method was also selective as all the peaks were pure, which was proved through photo-diode array studies.

3.2.2. Linearity

The calibration curve of hypericin was constructed at six concentration levels over the range 1–10 μg/ml by plotting peak areas against concentrations. Linear regression analysis was performed for the reference standard hypericin. The calibration curve followed the equation of $y = a + bx$ with y being the peak area, x the sample amount, the intercept $a = -9680.39$ and the slope $b = 112444.72$. The correlation coefficient was 0.99996 and indicated a high degree of linearity. The limits of detection and quantification, calculated from the residual standard deviation of the calibration curve, were 0.098 μg/ml and 0.297 μg/ml, respectively.

3.2.3. Accuracy and precision

The average recovery of hypericin was 100.26% with an RSD of 1.02% and reflected the high accuracy of the method. The intra-day RSDs were 0.56% and 0.94% for hypericin and pseudohypericin, respectively, indicating a high repeatability.

The inter-day coefficients of variation were 1.08% and 0.75% for hypericin and pseudohypericin, respectively. The statistical mean-*t*-test shows no significant differences of the variances or of the means, which confirm that the method is precise.

4. Conclusions

It has been demonstrated that the use of a photochemical reactor for the on-line, precolumn irradiation of the biosynthetic precursors protopseudohypericin and protohypericin shortens the sample preparation time. In addition, the on-line irradiation technique improves the accuracy of measuring the amount of total hypericin in *Hypericum perforatum* extracts and its phytopharmaceutical preparations. Because this method can be used with the photochemical reactor switched "on" and "off", the chromatograms can be compared.

The method was validated in compliance with the ICH guidelines Q2A and Q2B. The validation studies demonstrated that this HPLC method is accurate, precise, specific and selective. The developed method was successfully applied to the quality control of *Hypericum perforatum* extracts and its phytopharmaceutical preparations.

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