Preparative High-Performance Liquid Chromatographic Separation of Naphthodianthrones from St. John's Wort

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Abstract

St. John's Wort (*Hypericum perforatum*), a perennial flowering plant, has been used medicinally for thousands of years and has most recently been identified as an effective treatment for mild to moderate depression and neuralgic disorders. This work presents a procedure for the isolation of naphthodianthrones from St. John's Wort by an accelerated extraction and separation of marker compounds by preparative high-performance liquid chromatography (HPLC) with photodiode array detection. The accelerated extraction method minimizes the extraction time and increases the yield, and the marker compounds obtained by preparative HPLC are of 98% purity. The compounds are characterized by liquid chromatography–mass spectrometry (electrospray ionization) and NMR spectra.

Introduction

St. John's Wort extracts (*Hypericum perforatum*) are used mainly as oral antidepressants. The antidepressant effect is assumed to result from a synergism of several active constituents (i.e., naphthoanthroquinones flavonoids, phloroglucinols, xanthones, and essential oils) (1). In continuation of work on lipophilic contents of *H. perforatum* (2), a study was undertaken on naphthodianthrones and photosensitive constituents of the plant (e.g., pseudohypericin, hypericin, protohypericin, and protopseudohypericin) (Figure 1). The concentration of the reddish dianthrone pigment in the plant ranges from 0.02% to 2%, depending on harvesting period, drying process, storage, and analytical extraction procedure (3). In this study, plant material is extracted using an accelerated solvent extraction (ASE) method to shorten the extraction time and increase the recovery of the target compounds. The described ASE does not require the use of a chlorinated solvent (as in the conventional extraction method) and reduces solvent consumption. Moreover, the recovery of the photosensitive compounds was enhanced using methanol. There are several reports concerning the determination and analysis [i.e., high-performance liquid chromatography (HPLC)] of hypericin in St. John's Wort (4–6). In this study, a new method of extraction and separation using preparative HPLC is described.

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Experimental

Materials

HPLC-grade methanol, ethyl acetate, and water were purchased from Ranbaxy India (Rankem, Mohali, India). Reagent-grade chloroform and methanol and analytical-grade

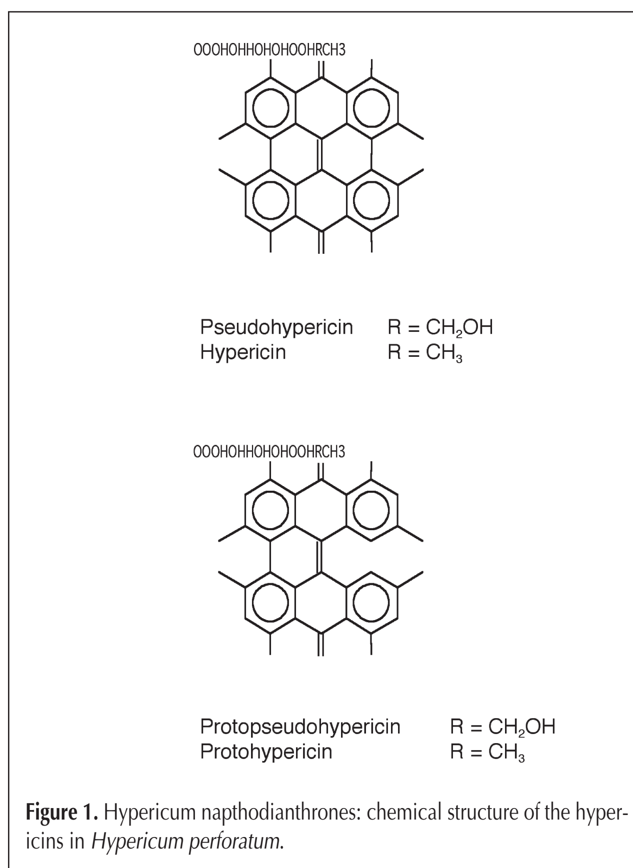

Figure 1. Hypericum napthodianthrones: chemical structure of the hypericins in *Hypericum perforatum.*

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orthophosphoric acid and amino orthophosphate were from SDS Chemicals (Biosar, India) and were used for extraction and column chromatography. The adsorbent for chromatography was silica gel 60 (Qualigens Chemicals, Mumbai, India). ASE was conducted with a Dionex 200 ASE (Dionex, Sunnyvale, CA). Mass spectra of the constituents were measured using a Bruker Daltronics Esquire 3000 system (Bruker, Bremen, Germany) with electrospray ionization (ESI) ion source.

Analytical HPLC instrumentation

A Gilson HPLC system (Gilson, Villers Le Bel, France) with a model 305 and 7010 SC PUMP head 306 manometric module, 115 UV detector set at 272 nm, Rheodyne (Cotali, CA) injector 7725i with 50-µL sample loop was in conjunction with a Merck (Darmstadt, Germany) Lichrosphere RP-18 column (4- × 100-mm, 5-µm particle size).

Preparative HPLC instrumentation

A preparative HPLC (Waters Prep LC 4000 system, Milford, MA) with a Bondapack preparative C_{18} column (25- × 100-mm size, 15–20-µm particle size, 125-Å pore size) combined with a Bondapack preparative C_{18} guard Pak insert (size 25- × 10-mm) with similar particle and pore size as that of the Bondapack cartridges.

The detection at 589 nm was achieved with a Waters 996 photodiode array detector, and the analysis was supported by Millennium chromatography manager software version 2.15 (Waters). The mobile phase used for the preparative HPLC consisted of MeOH–EtOAc–H_2O (67:16:17, v/v), and the pH was adjusted to 3 with acetic acid. A flow rate of 15 mL/min and column temperature of 30°C were used. The total analysis time was 7 min.

Liquid chromatography–mass spectrometry

Liquid chromatography (LC)–mass spectrometry (MS) experiments were performed on a Bruker Esquire 3000 ion trap MS with an ESI interface, connected to an Agilent HPLC system (Agilent Technologies, Palo Alto, CA) with 1100 series binary pump, photodiode array detector, automatic sample injection module, and thermostatic column oven (Agilent).

Extraction of the St. John's Wort blossoms/leaves

Each sample consisted of 100 g (dry weight) of crushed St. John's Wort leaves and flowers (collected from North Himalaya). They were extracted with ordinary Soxhlet extraction as well as ASE using methanol. The ASE method (Table I) resulted in a drastic reduction in time and increased recovery over Soxhlet. ASE at 100°C was also preferred over ASE at 32°C (see Table II).

Analytical HPLC of the extract

Naphthroquinones extract (25 mg) was dissolved in methanol (25 mL). The mobile phase, which consisted of methanol–ethyl acetate–water (67:16:17), was filtered and degassed. The pH of the water was adjusted to 2.5 with ammonium orthophosphate buffer (1.1 g/L) by adding orthophosphoric acid. The HPLC flow rate was 0.8 mL/min, the pseudohypericin and hypericin were detected at a retention time (t_R) of 3.9 min and 11.4 min, respectively, (Figure 2).

Sample preparation for preparative HPLC

The crude extract was further enriched in naphthodianthroquinone content by passing through a silica gel column

Table I. Methanol Extracts of St. John's Wort

Extraction method	Contents (g)*	Time
Soxhlet	7.8	38 h
ASE	8.0	15 min

* Aerial parts (100 g): twigs, leaves, and blossoms.

Table II. Results of Methanol Extraction by ASE at Different Temperature

Temperature (°C)	Dried wt. of plant	Detection of hypericin in extract (%)	Detection of hypericin in dried plant (%)
32	10 g	0.039	0.097
100	10 g	0.42	0.12

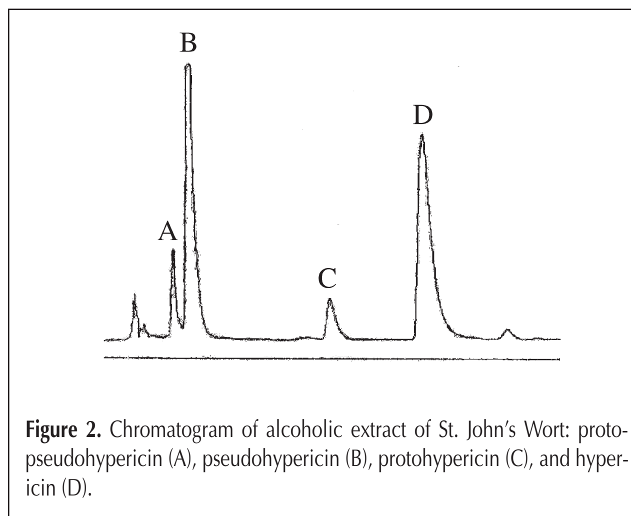

Figure 2. Chromatogram of alcoholic extract of St. John's Wort: protopseudohypericin (A), pseudohypericin (B), protohypericin (C), and hypericin (D).

Table III. Recovery of Naphthodianthrones by Preparative HPLC

Batch	Total no. of injections	Concentration of sample (mg/mL)	Total wt. of sample injected (mg)	Hypericin recovered (mg)	ψ-Hypericin recovered (mg)
1	13	13.2	686	22	13
2	13	16–18	842	11	14
3	11	19	836	9	15
4	12	20	960	15	15

and eluting with $CHCl_3$ and methanol, successively. The red colored methanol fractions were pooled and concentrated on a rotavapor. A 15–20-mg/mL solution was prepared in methanol in a 25-mL volumetric flask, and a 4-mL solution was injected into the HPLC (Figure 3).

Separation of the compounds

The first eluted peak was collected at 4 min as a deep red colored fraction. The second peak eluted was light pink in color and was collected at 6 min. The collected fractions were concentrated on a rotavapor (Table III). To check the purity of the collected fraction, samples were dissolved in HPLC-grade methanol. The solution was analyzed via LC–ESI-MS–MS. A 10-µL solution of the first peak and the second peak eluate were injected separately, using an autoinjector, on a Merck Chromolith C18 column (100- × 4-mm i.d.) and eluted using methanol–ethylacetate–water (67:16:17). The pH of the water was adjusted to 3 by acetic acid with a flow rate 0.8 mL/min. The first fraction was identified as pseudohypericin, eluted at 12.4 min, showing a molecular ion peak at *m/z* 518.8 [M^+–H] and a second fraction as hypericin eluted at 17.3 min, which exhibited a molecular ion peak at *m/z* 502.8 [M^+–H] in the negative mode.

Results and Discussions

The purity of the collected fraction was determined on the basis of analytical HPLC. Pseudohypericin and hypericin eluted at t_R of 12.4 and 17.4 min, respectively, which was confirmed simultaneously by comparison of the total ion chromatograms (TICs). The purity of the collected fractions at t_R 18.4 min was confirmed by running parallel LC–UV chromatograms and TICs with standard samples. The purity was also confirmed by direct ESI-MS under negative ESI conditions (Figure 4). These conditions were chosen. as hypericin and related compounds showed very little tendency to form $[M+H]^+$ quasimolecular ions by addition of protons but, on the other hand, were likely to deprotonate with production of $[M–H]^-$ ions (7). The negative ion mass spectrum showed two major ionic species at *m/z* 502.8 and 518.8, corresponding to $[M–H]^-$ molecular ions of hypericin and pseudohypericin, respectively. The ionic species at *m/z* 502.8 of hypericin appear to have been obtained by collision-induced dissociation.

Based on the area under the curve in the LC chromatograph, the purity of the pseudohypericin and hypericin was determined to be approximately 98% for both. Final confirmation and identification of pseudohypericin and hypericin was carried out using ^{1}H NMR, ^{13}C NMR, and mass spectral studies of the samples obtained by preparative HPLC.

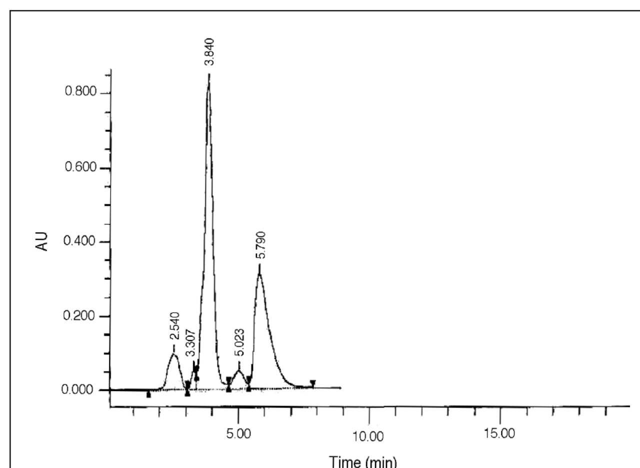

Figure 3. Chromatogram of alcoholic extract of St. John's Wort extract as analyzed by preparative HPLC on Millennium chromatography manager software version: 2.15 pseudohypericin (3.3–4.25 min) and hypericin (5.05–7.00 min).

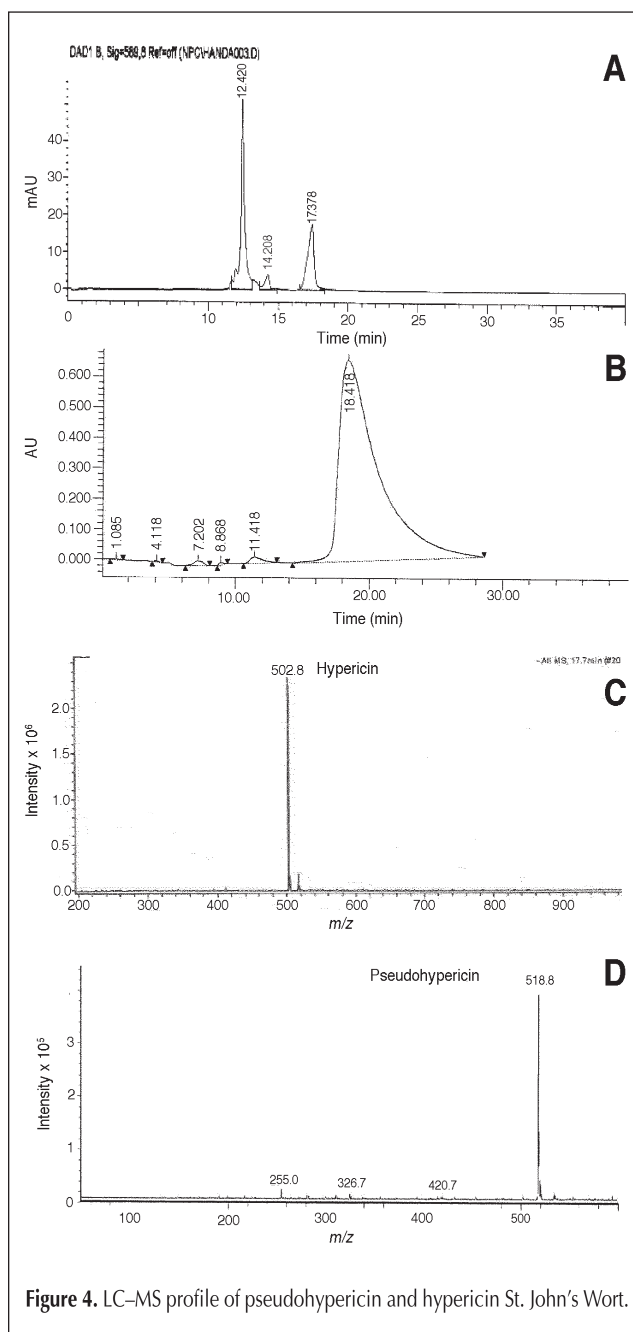

Figure 4. LC–MS profile of pseudohypericin and hypericin St. John's Wort.

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