A Simple and Reliable Semipreparative High-Performance Liquid Chromatography Technique for the Isolation of Marker-Grade Hyperforin from *Hypericum perforatum L.* Extract

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Abstract

The present work describes isolation of bioactive lipophilic constituent [namely, hyperforin from St. John's wort (*Hypericum perforatum L.*)], of approximately 98% purity by semipreparative high-performance liquid chromatography (LC). The extraction, isolation, and analysis of the collected compound is performed without the use of antioxidants and inert gas atmospheres at all the stages. Hyperforin, separated isocratically on a 12-µm semiprep column, is obtained in high purity, lyophilized after the removal of the organic phase, and preserved at a low temperature. The purity of the collected marker compound is estimated by the use of LC–mass spectrometry and spectroscopic techniques.

Introduction

Hyperforin (Figure 1) is one of the bioactive molecules (1–3) present in the lipophilic extract of St. John's Wort *(Hypericum perforatum)* and has been claimed to be the main active constituent of natural antidepressant preparations contained in this plant (4). An extraction and isolation procedure has been previously reported (5) using antioxidants to prevent peroxidative degradation of the final product. In this paper, we present an alternative method to obtain an equal and sufficiently high yield of hyperforin (1% in dry plant), which is simple, rapid, reproducible, and does not include the use of antioxidants.

Shade-dried herb (*Hypericum perforatum L.)* was refluxed with hexane without an antioxidant for 2 h. The hexane extract was concentrated and analyzed via analytical high-performance liquid chromatography (HPLC) (6). Separation of the molecule of interest was performed isocratically on a semipreparative HPLC system with a mixture of aqueous and organic solvents. The organic phase was removed on a rotary evaporator under reduced pressure. The aqueous part of the solvent system was lyophilized. The isolated molecule was dissolved in methanol and its purity ascertained via liquid chromatography coupled with negative ion electrospray-ionization (ESI)–tandem mass spectrometry (7). The purity of the compound was found to be approximately 98%, which was finally confirmed by ^{1}H and ^{13}C NMR. The hyperforin marker thus obtained was stored at –70°C.

Experimental

Materials

HPLC-grade acetonitrile, methanol, and water were from Ranbaxy India (Rankem, Mohali, India). Analytical-grade

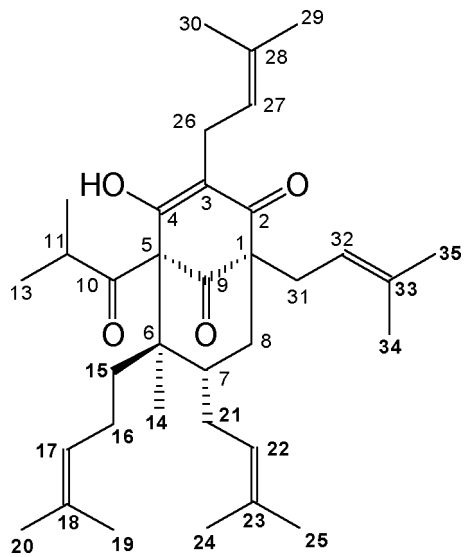

Figure 1. Structure of hyperforin ($C_{35}H_{52}O_4$).

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orthophosphoric acid and ammonium orthophosphate was from SDS Chemicals (Biosar, India).

Analytical HPLC instrumentation

A Gilson (Villiers Le Bel, France) HPLC with 305 pump and 10SC pump head, 306 manometric module, 115 UV detector set at 272 nm, Rheodyne (Cotati, CA) injector 77251 with 50-µL sample loop was used with a Merck (Darmstadt, Germany) Lichrospher RP-18 column (5-µm particle size, 4- × 100-mm size).

Semipreparative HPLC instrumentation

A Gilson semipreparative HPLC system consisting of a 305 pump with 25SC pump head, 306 manometric module, 7725i Rheodyne injector with a 300-µL sample loop, 116 UV detector set at 272 nm, and Rainin Dynamax semiprep column (5- × 100-mm, 12-µm particle size C_{18}) was used along with a Gilson 201 fraction collector.

Liquid chromatography–mass spectrometry

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

Extraction of lipophilic compounds

Soft parts of the plant material including blossoms, leaves, and shoots were shade dried and crushed. One hundred grams of this material was loaded for refluxing with *n*-hexane (100 mL) free of antioxidants. Two hours of refluxing produced hyperforin-rich lipophilic extract (4.7 g).

Analytical HPLC of the extract

An amount of 23.5 mg of hyperforin-rich extract was dissolved in methanol (25 mL). The profile of the extract was first checked on the analytical HPLC system. The mobile phase consisting of filtered, degassed, premixed acetonitrile–ammonium orthophosphate buffer (1.1 g/L, pH set at 2.5 by adding orthophosphoric acid) was pumped in the ratio of 90:10 at a flow rate of 0.8 mL/min. The chromatogram of the filtered extract was plotted on HP3395 integrator. The hyperforin peak was detected at a retention time (t_R) of 10.8 min. Traces of adhyperforin was also observed.

Isolation of hyperforin

After composition confirmation via analytical HPLC, the extract was concentrated to a volume of approximately 1 mL for use on the semipreparative HPLC. A premixed solvent system consisting of acetonitrile–water (90:10) was isocratically pumped at a flow rate of 3 mL/min. Three-hundred microliters of the extract was loaded on the Rheodyne injector. The peak of interest was observed at a t_R of 36.5 min (Figure 2). Hyperforin-rich solution (10 mL) was collected in clean preweighed flasks from 34 to 37.2 min at room temperature under normal atmosphere.

Processing of the collected fraction

The azeotrope phase was removed by rotary evaporation on Heidolf-make rotavapor under reduced pressure. With the hyperforin being thermolabile, the rotary evaporation was carried out at ambient temperature. Removal of the organic phase resulted in milky white liquid containing hyperforin and water.

To remove the aqueous part of the collected fraction lyophilization was carried out on Heto make bench top lyophilizer model DW3, without using nitrogen atmosphere.

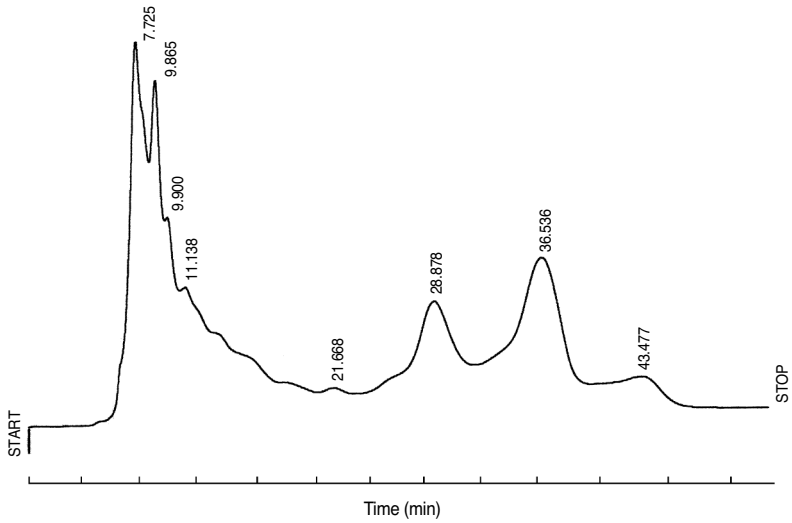

Figure 2. Chromatogram of *Hypericum perforatum* extract as analyzed by semipreparative HPLC. The peak had a t_R of 36.5 min and was collected and identified as hyperforin.

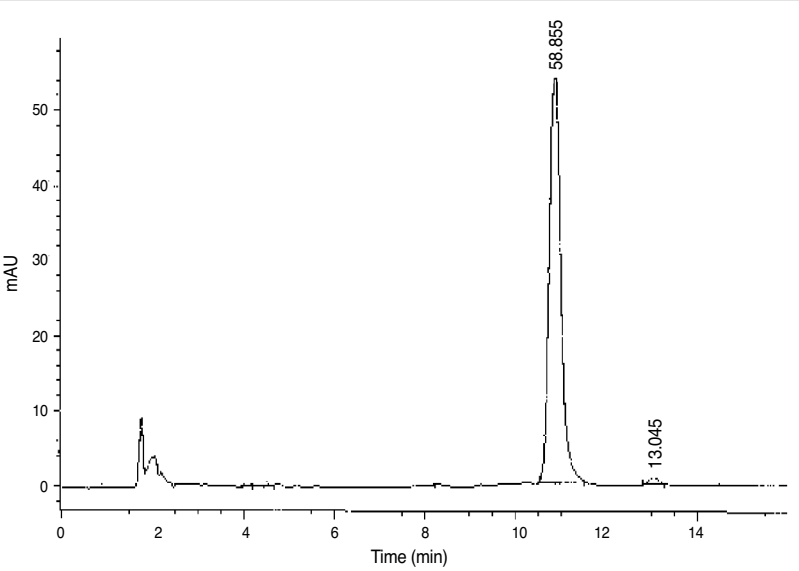

Figure 3. Chromatogram of collected hyperforin fraction. The peaks of hyperforin and adhyperforin have retention times of 10.8 and 13.0 min, respectively.

Approximately 5 h of lyophilization resulted in the complete removal of water and a clear compound sticking to the walls of the flask. The flask was again weighed, and the weight of the collected marker was determined (~ 2 mg). The flask was sealed properly and stored at –70°C.

Purity check of the collected fraction

To check the purity of the collected fraction, it was dissolved in HPLC-grade methanol by ultrasonication for 20 min. The volume was then diluted to 5 mL in a volumetric flask. The prepared solution was analyzed on an HP 1100 HPLC system (Agilent) linked to Bruker Daltonics Esquire 3000 mass spectrometer with an ESI source (LC–ESI–MS–MS). Ten microliters of the hyperforin solution was injected with an autoinjector on a Merck Chromolith C_{18} column (100- × 4-mm i.d.) and eluted using acetonitrile–ammonium orthophosphate buffer (1.1 g/L, pH set at 2.5 by adding orthophosphoric acid) at 90:10 with a flow rate of 0.8 mL/min. The hyperforin peak eluted at a t_R of 10.8 min, which exhibited a molecular ion peak at *m/z* 535 $[M–H]^-$ in the negative mode. The chromatogram is shown in Figure 3. Using a T-joint, part of the LC output was fed to the Bruker mass spectrometer, and mass spectra of all the peaks observed in the chromatogram were recorded.

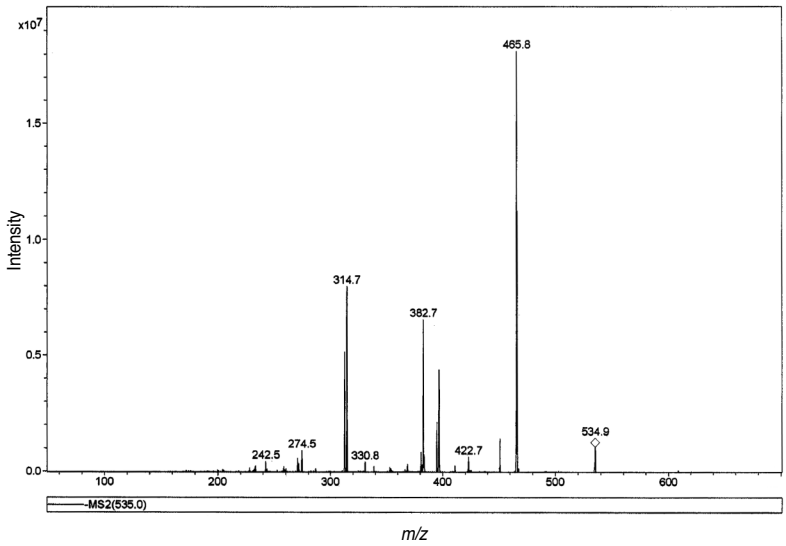

Figure 4. MS–MS of the peak at *m/z* 535. The peaks below *m/z* 535 are daughter-ion peaks of hyperforin. The fragment peaks at *m/z* 466 and 383 are attributable to the subsequent loss of the 2-methyl-but-2-enyl and 2-methyl-pent-2-enyl moieties, respectively. The peak at *m/z* 314 is attributable to the loss of the 2-methyl-but-2-enyl moiety.

Results and Discussion

The purity of the collected fraction was determined on the basis of LC. The solvent peaks were observed at a t_R of approximately 2 min. Hyperforin and adhyperforin eluted at a t_R of 10.8 and 13.0 min, respectively, which was confirmed simultaneously by comparison of total-ion chromatogram (TIC) with an LC–UV chromatogram. Complete overlap of the TIC and UV chromatograms pointed to the purity of the collected standard. The possibility of the presence of impurities with no UV-detectable chromophore was thus ruled out. MS–MS was also carried out (Figure 4) to further check the purity of the collected fraction. Peaks existing below a mass of 535 were thus identified as the daughter-ion peaks of hyperforin. Based on the area under the curve in the LC chromatogram, the percent purity of the hyperforin was determined to be approximately 99%. Also, cospiking of the lipophilic extract with the collected standard was performed, which indicated enhancement in the area of the peak of hyperforin.

The collected compound compares well physicochemically with the hyperforin obtained using antioxidants and inert gases, as reported earlier (5). A simple isocratic HPLC method has been described yielding a high-purity marker compound. Moreover, usage of antioxidants and inert gas (such as helium) in the process of isolation and purification has been determined to be of no extra advantage, as was emphasized in some earlier reports (5).

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