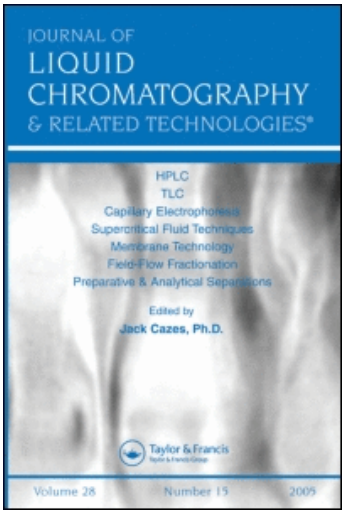

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RAPID ISOCRATIC HPLC ANALYSIS OF HYPERICINS

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ABSTRACT

We describe a new method for the analysis of hypericins from crude extracts of *Hypericum perforatum* (St. John's Wort) using HPLC. Hypericin and pseudohypericin and related compounds were separated in 15 minutes using a simple isocratic elution method on a modified phenyl column. This isocratic method permits the quantitative and rapid analysis of hypericins in crude acetone samples.

In addition, we report on the photoconversion of protohypericins to hypericins, as well as the stability of hypericin and pseudohypericin as a function of light, time, and storage conditions.

INTRODUCTION

Hypericum perforatum L., also known as St. John's Wort, is a herbaceous perennial belonging to the Hypericaceae family.[1] Its use has widely increased on the general market for the herbal "treatment" of depression, more than doubling its market in the US between 1995 and 1997.[2] Analysis of *Hypericum* samples has become increasingly important since hypericins have been used as the measure of extract potency. Most analytical determinations of hypericin have been carried out using thin layer chromatography (TLC) and visible spectrophotometric methods which employ extinction coefficients for sample quantitation. Both of these methods are insufficient in determining an accurate measurement of the hypericins mainly due to interference from other constituents in the extracts; in addition, separation of pseudohypericin and hypericin has been nearly impossible to achieve using these methods.

There are a few reports describing the use of HPLC analysis of hypericins. Earlier methods required lengthy run times and complicated gradient elution systems using solvent mixtures.[3,4] Another method described for phytotherapeutic beverages is not repeatable in our lab using field samples,[5] and the most recently published method does not show significant resolution of the protohypericins.[6]

This study was conducted to establish a robust, rapid method for the quantitative determination of hypericins using HPLC. Additionally, we report on the stability of hypericins as a function of light, time, and storage conditions.

EXPERIMENTAL

Hypericin Standards

An authentic hypericin standard was obtained from Sigma Chemical Company (St. Louis, MO) and pseudohypericin was obtained from Calbiochem (La Jolla, CA). The standards were run separately to determine retention times and then mixed together in HPLC grade methanol to an initial concentration of 1 mg/mL. The test mixture was then serially diluted and injected into the column to evaluate performance under various separation conditions and to generate a concentration curve for sample calibrations.

All samples were filtered through a 0.45 μm PFTE filter (Gelman Sciences) prior to injection onto HPLC. Both nylon and PVDF filters retained a significant amount of hypericins on filter membranes whereas no retention was found when samples were filtered through 0.2 or 0.45 μm PFTE filters.

Hypericum perforatum Extract Preparation

All operations were carried out in the fume hood under low light conditions. For the initial extraction, 100 g (dry weight) samples of crushed *H. perforatum* leaves, flowers, and stem material (kindly provided by Bighorn Botanicals, Noxon, Montana) was combined with approximately 1 L of $CHCl_3$. The material was allowed to steep overnight with constant stirring. The chlorophyll-saturated $CHCl_3$ fraction was filtered off and an additional 1 L of $CHCl_3$ was combined with the sample. This was repeated for a total of 3 times until little green coloration was present in the $CHCl_3$ extract.

The sample was allowed to air dry to remove residual $CHCl_3$, then it was mixed with approximately 1 L of acetone, and allowed to stir overnight. The acetone was decanted and replaced with fresh solvent at 24 h, for a total of 4 days, followed by sonication for 20 min before allowing it to stir overnight. The acetone extracts were combined, evaporated to dryness on a rotoevaporator, removed to a tared vial and dried to constant weight, then reconstituted in MeOH to 10 mg/mL. The sample was then filtered through a 0.45 μm filter prior to HPLC analysis.

Fresh plant materials were also prepared using field-collected samples of *H. perforatum*. Randomized 5 g fresh weight samples were dried at either 45°C and 60°C until constant weight was achieved, then extracted and analyzed as above.

HPLC Columns

Several analytical columns, including C-18 (Supelco, 5 μm, 250 x 4.6 mm) and Diazem-phenyl™ (Metachem, 5 μm, 250 x 4.6 mm), were tested for adequate hypericin separation using an isocratic reverse phase analysis. Initial analyses were performed without guard columns, but the final separation method employed a Diazem-phenyl™ guard column cartridge placed in tandem with the analytical column. Mobile phase consisted of acetonitrile:methanol:water:phosphoric acid (48:40:10:2) at a flow rate of 1 mL/min with a column temperature of 30°C, and a total run time of 15 min per sample. Instrumentation consisted of a Beckman Model 126 binary pump, Beckman model 168 diode array detector, and Beckman Model 507 autosampler. with instrument control via a Gateway Pentium computer running Beckman System Gold v. 6.07 software. Primary detection of hypericins was set at 590 nm, with secondary detection at 254 nm. Retention times and absorption spectra were compared to authentic standards to confirm presence of hypericins. An automated fraction collector (Foxy, ISCO, Inc.) was also employed to collect hypericins based on retention times and to confirm conversion of protoforms to their corresponding hypericins.

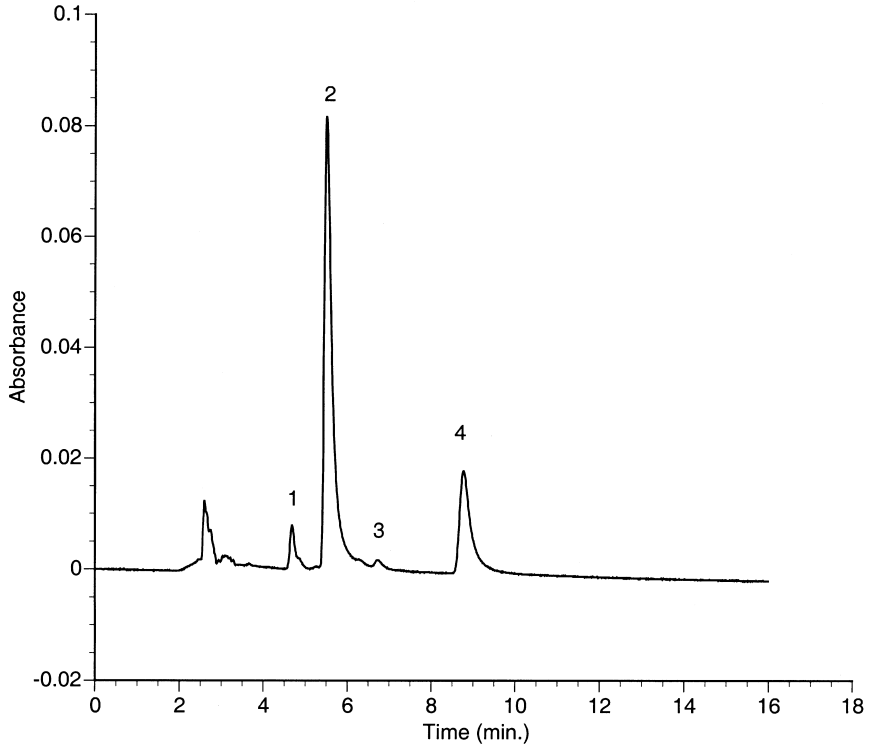

Figure 1. Chromatogram of a *Hypericum* crude extract. Detection at 590 nm where [1] is protopseudohypericin, [2] is pseudohypericin, [3] is protohypericin, and [4] is hypericin.

Stability Assays

For the light stability assays, a solution of hypericin was reconstituted from the dried, crude extract containing hypericins at a concentration of 10 mg/mL in acetone. The solution was placed in clear glass vials and subjected to high light intensity (234.4 $\mu E/m^2/s$) for a period of 2 weeks. Aliquots were taken from the sample at specified times, filtered, and analyzed by HPLC.

The storage assays were conducted similarly. A 10 mg/mL extract solution was placed in an amber vial so that light would not significantly affect decomposition of the sample. Samples were placed at 25°C, 4°C, and –18°C. Aliquots were taken from each sample at predetermined times and subsequently filtered and analyzed via HPLC.

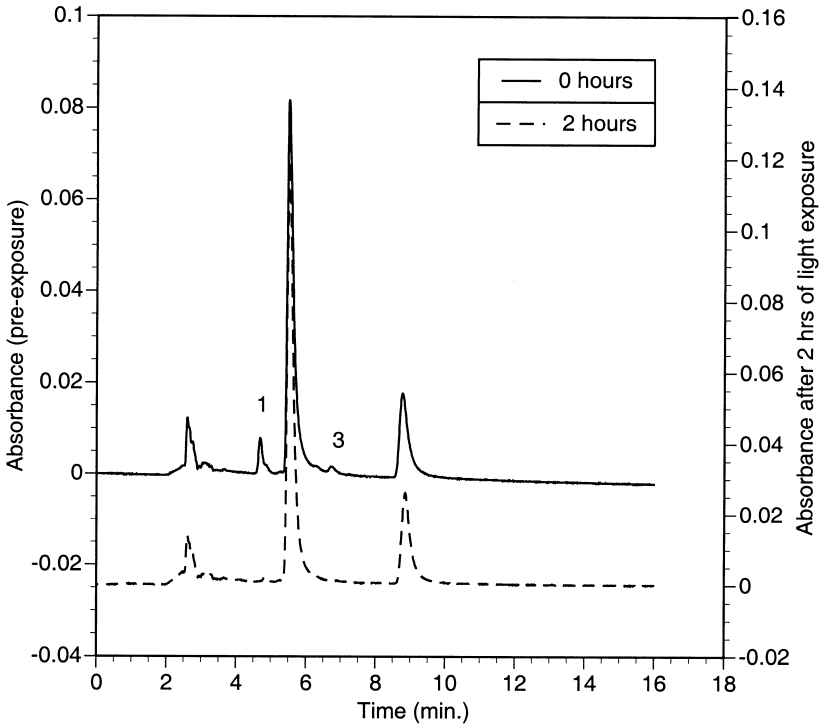

Figure 2. Comparison of secondary compounds before (solid line) and after (dashed line) illumination. Detection at 590 nm. Notice conversion of protoforms ([1] protopseudohypericin and [3] protohypericin) to hypericins.

RESULTS

HPLC Methodology for Hypericin Determination

Baseline resolution of all hypericins was achieved within 15 min using the Diazem-phenyl column under isocratic conditions eluted with a mobile phase of acetonitrile:methanol:water:phosphoric acid (48:40:10:2) (Figure 1), whereas a C-18 column did not achieve adequate resolution of the hypericins under the same conditions. Using the Diazem-phenyl system, pseudohypericin eluted at 5.3 minutes with hypericin eluting at 8.7 minutes, as verified by retention time and scangraphics of external standards. We also observed peaks at 4.6 min and 6.2 min which we tentatively assigned to be protopseudohypericin and protohypericin, respectively, by comparing scangraphics to published spectra.[6,7]

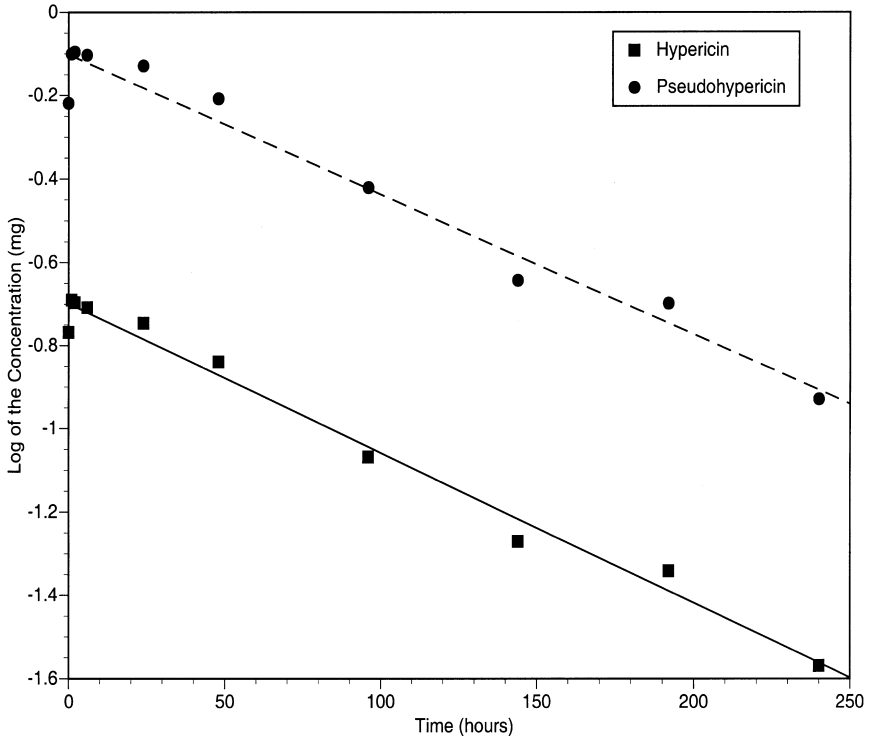

Figure 3. Graph depicting linear degradation relationship of hypericin (solid line) and pseudohypericin (dashed line) as a response to light. Each point is an average of 3 samples.

When the hypericin mixture was placed in the presence of light, peaks corresponding to protohypericin (6.2 min) and protopseudohypericin (4.6 min) were barely detectable within 1 hour, while corresponding increases in the concentration of hypericin and psuedohypericin were noted (Figure 2).

Conversion of the proto forms to pseudohypericin and hypericin was complete within the first 2 hours of illumination.

Samples eluting at the retention times of protopseudohypericin and protohypericin were also collected from repetitive injections of the initial hypericin mixture, then exposed to light and rechromatographed to confirm conversion of the protoforms to hypericin and pseudohypericin, respectively.

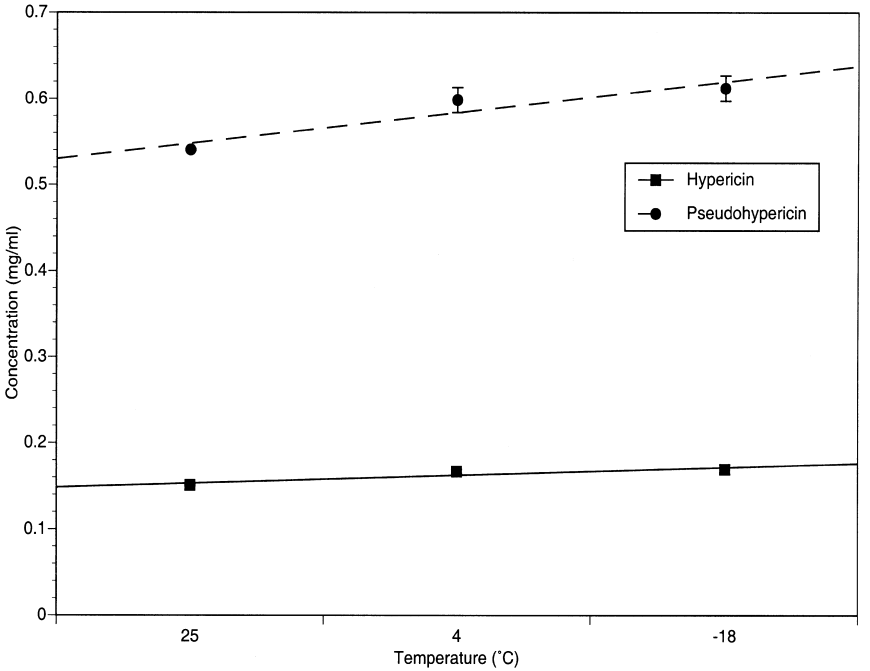

Figure 4. Stability of hypericin (solid line) and pseudohypericin (dashed line) as a function of temperature. Results are shown after storage for 15 days in a crude methanol solution (10 mg/mL). Each point represents 2 replicates.

Stability of Hypericin and Pseudohypericin

No interconversion of hypericin and pseudohypericin was seen in a combined sample of 10mg/mL hypericin and pseudohypericin which was dissolved in methanol, then exposed to light for up to 2 weeks. The small increase within 1 hour of the hypericin concentration is due to light dependent conversion of the protoforms as described above (Figure 3). Both compounds, however, appeared to convert to more polar degradation products that eluted as a large peak at approximately 2 minutes following the solvent front. The degradation of both hypericin and pseudohypericin followed first order kinetics with a rate constant of -0.00359 μg per day (Figure 3).

In solution, concentrations of hypericin and pseudohypericin remained relatively stable for a period of 2 weeks at temperatures of –18°C, 4°C, and 25°C. Over a longer period of time, however, hypericin solutions stored at –18°C showed less degradation in comparison to those stored at higher temperatures (Figure 4). These results may indicate that hypericin and pseudohypericin may be heat labile in solution. However, freshly prepared plant materials dried at either

45°C or 60°C showed no difference in hypericin and pseudohypericin concentrations, suggesting that the dried plant materials may preserve more active constituents. Further studies are needed to determine whether solutions remain stable over longer storage periods.

DISCUSSION

Although several HPLC methods have been published detailing the quantitation of hypericin and other constituents in *Hypericum perforatum* extracts,[6,8,10] most of these methods rely on extensive sample preparation, gradient elution systems, or long chromatographic runs per sample. With the use of an isocratic eluant and a phenyl-based column, we have developed a rapid HPLC method which has baseline resolution of all the major hypericin peaks, including the protoforms of the hypericins. This method should be useful for the rapid screening of large numbers of relatively crude samples, especially since determination of hypericins content has generally been used for standardization of extracts as well as phytotherapeutic products of St. John's wort. We were also able to demonstrate the conversion of protopseudohypericin and protohypericin to pseudohypericin and hypericin, respectively, upon light exposure, and thus verify the tentative assignment of these peaks based on scangraphics and photoconversion to the active forms.

Hypericin solutions appeared to be stable up to 2 weeks regardless of storage temperature, although some degradation was observed with longer storage times, especially at 25°C. Exposure to light also caused losses in both pseudohypericin and hypericin with time, and both compounds displayed similar decay kinetics. Although the identity of the breakdown products was not part of this study, the material appears to be more polar in nature based on its elution profile. However, it still retains absorption at 590 nm, thus the presence of these breakdown products in extracts would lead to overestimation of hypericins if only spectrophotometric detection methods were employed for extract quantitation.

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REFERENCES

1. R. Upton, A. Graff, E. Williamson, D. Bunting, D. M. Gatherum, E. B. Walker, V. Butterweck, U. Liefländer, A. Nahrstedt, H. Winterhoff, J. Cott, "St. John's Wort, *Hypericum perforatum*: Quality Control, Analytical and Therapeutic Monograph," in **HerbalGram**, R. Upton, ed., 1997, pp. 1-32.

2. M. O'Hara, D. Kiefer, K. Farrell, K. Kemper, Archives of Family Medicine, **7**, 523-536 (1998).

3. V. J. Hölzl, E. Ostrowski, Deutsche Apotheker Zeitung, **23**, 1227-1230 (1987).

4. H. Häberlein, K. P. Tschiersch, S. Stock, J. Hölzl, Pharmazeutische Zeit., **137**, 169-174 (1992).

5. L. Liebes, Y. Mazur, D. Freeman, D. Lavie, G. Lavie, N. Kudler, S. Mendoza, B. Levin, H. Hochster, D. Meruelo, Anal. Biochem., **195**, 77-85 (1991).

6. H. Kurth, R. Spreemann, Advances in Therapy, **15**, 117-1128 (1998).

7. H. Schuett, **Morphologische, Phytochemische und Botanische Untersuchungen zur Selektion Hypericin-, Pseudohypericin- und Flavonoidreicher Hypericum Perforatum L. Staemme**, 1996.

8. M. Brolis, B. Gabetta, N. Fuzzati, R. Pace, F. Panzeri, F. Peterlongo, Journal of Chromatography A, **825**, 9-16 (1998).

9. G. Piperopoulos, R. Lotz, A. Wixforth, T. Schmierer, K. P. Zeller, Journal of Chromatography B., **695**, 309-316 (1997).

10. V. A. Zevakova, V. I. Glyzin, T. B. Shemeryankina, A. V. Patudin, K. Prir, Soedin, 138 (1991).

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