

# Development of a High-Performance Liquid Chromatographic Method for the Quantitative Determination of Hyperforin

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**Abstract:** An RP-HPLC method with photodiode array detection was established for the determination of major constituent, hyperforin in St. John's Wort extract samples. The hyperforin was separated by using gradient mode consisting of 0.2 % formic acid in water and 0.2% formic acid in methanol at a flow rate of 1.0 mL/min. Under these conditions, a plot of integrated peak area versus concentration of hyperforin was found to be linear over the range of 1.0 – 100.0 µg/mL, with a relative standard deviation of 0.16 – 0.88%. The limit of detection was 10 ng on column and the limit of quantitation was 20 ng on column. The determination of the hyperforin content in a commercially available St. John's Wort extracts exhibited a mean content of 2.0 – 27.0 % w / w. Recovery experiments led to a mean recovery rate of 98.12 ± 0.94 %. The proposed method is not time-consuming, sensitive and reproducible and is therefore suitable for routine analysis of hyperforin in herbal medicinal products.

**Keywords:** *Hypericum perforatum*; St. John's Wort; Pharmaceutical analysis; Hyperforin

## 1. Introduction

St. John's Wort (*Hypericum perforatum*) is a herbaceous perennial plant of the Hypericaceae family, which is distributed in Europe, Asia, and Northern Africa, and naturalized in the USA. St. John's Wort extract preparations have been used for a long time as an effective alternative to synthetic anti-depressants for treating mildly to moderately severe depressions. Although the mechanism of its anti-depressant activity has not yet been completely elucidated, the anti-depressant efficacy has been confirmed in most clinical studies. Numerous controlled clinical trials conducted

during the past decade have confirmed the therapeutic use of alcoholic extracts of St. John's Wort for the treatment of mild to moderate depressive disorders, and those pharmacological and therapeutic properties have been well documented and reviewed [1-4].

St. John's Wort extracts contain at least six major natural product chemical classes, including naphthodianthrones (hypericin and pseudohypericin), flavonoids (rutin, hyperoside, isoquercitrin, quercitrin and quercetin), phloroglucinols (hyperforin and

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adhyperforin), biflavonoids (I3, II8-biapigenin, I3', II8-biapigenin), proanthocyanidins and chlorogenic acid [5]. Hyperforin is the main source of pharmacological effects caused by the consumption of alcoholic extracts of *St. John's Wort* in the therapy of depression. It offers many effects comparable to that of synthetic antidepressants like the uptake inhibition of 5-hydroxy tryptamine, norepinephrine, and dopamine. But in addition the  $\gamma$ -aminobutyric acid and L-glutamate uptake is also inhibited. However, several studies indicate those flavones, e.g. rutin, and also the naphthodianthrones hypericin and pseudohypericin take part in the antidepressant efficacy [6-8]. Against this background it is reasonable—though not demanded by the approval authorities—to examine the pharmaceutical quality (e.g. batch-to-batch reproducibility, stability) of *St. John's Wort* extracts on the basis of their hyperforin content and hence, the availability of a robust method allowing the analysis of hyperforin content in extract is highly desirable.

Although several HPLC methods have been reported for the determination of the major constituents, e.g. rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudohypericin, hyperforin and hypericin in *St. John's Wort* [9-12], these methods either employed non-organic solvents, use of organic acids, buffers in higher percentage, which may deteriorate efficiency of column or HPLC instrument [9-10] or required more than 60 min for HPLC separation [9, 11-17] or less sensitivity of method. The published methods so far do not offer simple, rapid, precise, accurate and economical way for the routine analysis for quantification of Hyperforin in commercial samples of extract as well as formulation and hence, due to increase in the interest in *St. John's Wort* research, a high-performance liquid chromatographic method with photodiode array detection has been developed.

## 2. Experimental

### 2.1. Materials and methods

Hyperforin was isolated, purified and structural confirmation was carried out. The working standard purity was determined to be more than 97.0 % by HPLC and hence, was considered as reference standard for the analysis purpose.

Methanol and formic acid were HPLC grade from Merck Specialty Private Ltd (Mumbai, India). Deionized water was obtained with an in-house Milli-Q Nanopure (Millipore, Bedford, MA, USA). The commercial extracts of *Hypericum perforatum* L. were procured from two manufacturers of India, which were coded as IE-1 and RE-1.

### 2.2. Equipment

The HPLC system consisted of a Waters liquid chromatograph equipped with an auto sampler, a photodiode array (996) detector and column (Kromasil 100, Barcelona, Spain). The absorption was measured in a full spectrum (200–800 nm) or at 270 nm for hyperforin. The chromatographic data was recorded and processed with Waters Millennium 2001<sup>®</sup> software.

### 2.3. Chromatography

Analyses were carried out at 25°C on a Kromasil 100 C18 column (3.5  $\mu$ m, 4.6 $\times$ 150 mm, serial no. N45672; batch TR-013407) (Teknokroma, Barcelona, Spain). The mobile phases consisted of water (A, containing 0.2% Formic acid) and methanol (B, 0.2% Formic acid). The analyses followed a linear gradient program. Initial conditions were 10% A; 0–30 min, changed to 0% A; 30–35 min, maintained in to 0% A; 35–36 min, to 10% A; 36–45 min, equilibration with 10% A; The flow-rate was kept at 1.0 ml/min, temperature of column was set at 25°C  $\pm$  2°C and the injection volume was 20  $\mu$ l. The peak in the HPLC chromatogram of *St. John's Wort* extract was tentatively identified by comparing

the retention time and UV spectra of hyperforin in the samples with reference standard of hyperforin. The peak purity was checked by PDA software routines.

#### 2.4. Standard solutions

Hyperforin, reference standard, in the range of 10.0 mg was accurately weighed or transferred into a 10-ml volumetric flask and dissolved in methanol to obtain solution with 1mg / ml concentration of Hyperforin. This solution was then further diluted to obtain the concentration – 1 µg/ml to 100 µg/ml of Hyperforin stock solution and stored at –20°C and brought to room temperature before use. In the same way, three sets of control for hyperforin was prepared from a separate stock, so as to lie in the lowest, middle and highest regions of the calibration curves. Further standard solutions are prepared freshly each day by appropriate dilution of stock solution with methanol for intraday as well as interday analysis.

#### 2.5. Sample solutions (Evaluation of commercial extracts)

##### 2.5.1. Test sample preparation (IE-1)

St. John's Wort commercial extract (100 mg) was exactly weighed into a 10-ml borosilicate glass tube with a polypropylene screw cap (Fisher Scientific, cat. # 14-962-26F) and extracted with 10 ml of methanol (10 ml x 2) using a sonicator under strict exclusion of light in water bath (~23°C) for 2 min and allowed to stand for 5 mins. The mixture was then filtered through Whatmann no.42 filter paper and resulting filtrate (I) were pooled, concentrated to 7-8 ml volume and transferred to a 10-ml volumetric flask and made up to volume with methanol. Then 20 µl of the resulting solution was subjected to HPLC analysis and the concentration of the major constituent, Hyperforin, in the extract – IE-1 was calculated based on the equations for the

calibration curves. Hyperforin is known to be highly photosensitive. Thus, all steps were performed protected from light by using amber glass utensils.

##### 2.5.2. Test sample preparation (RE-1)

St. John's Wort commercial extract (6.0 mg) was exactly weighed into a 10-ml borosilicate glass tube with a polypropylene screw cap (Fisher Scientific, cat. # 14-962-26F) and extracted with 10 ml of methanol (10 ml x 2) using a sonicator under strict exclusion of light in water bath (~23°C) for 2 min and allowed to stand for 5 mins. The mixture was then filtered through Whatmann no.42 filter paper and resulting filtrate (I) were pooled, concentrated to 7-8 ml volume and transferred to a 10-ml volumetric flask and made up to volume with methanol. Then 20 µl of the resulting solution was subjected to HPLC analysis and the concentration of the major constituent, Hyperforin, in the extract – RE-1 was calculated based on the equations for the calibration curves. Hyperforin is known to be highly photosensitive. Thus, all steps were performed protected from light by using amber glass utensils.

#### 2.6. Linearity and limit of quantitation

For a long-term use of the analytical method a rigorous validation is indicated and requires the following procedures. For the preparation of calibration curve the stock solution was diluted freshly with methanol to obtain a set of 14 calibration standards. These standards were measured and the integrated peak areas were plotted against the corresponding concentrations of the injected standards. The complete procedure was repeated on three consecutive days. The so obtained three calibration curves were used to calculate a mean calibration graph. The limit of quantification was defined as that lowest concentration where accuracy better than 20.0% was achieved [18].

## 2.7. Intraday and interday analysis using Hyperforin

Three different concentrations using a different stock solution of hyperforin were prepared (4.0; 40.0 and 80.0  $\mu\text{g/mL}$ ). For the determination of the intraday precision and accuracy three replicates of the standard solution were analyzed at the same day. The precision and the accuracy of the interday analysis were determined by analyzing the standard solution on 3 different days.

## 2.8. Stability

St. John's Wort extract – IE-1 (100 mg) was extracted with methanol (10 ml  $\times$  2) as described. After filtration and concentration, the concentrated solution was transferred into a 10-ml volumetric flask and made up to volume with methanol. The sample solution was put in the dark at 4°C and analyzed on consecutive days (24, 48, 72 and 96 hr) to observe the stability of sample solutions.

## 2.9. Robustness and Ruggedness studies

Robustness and ruggedness parameters were applied by making small deliberate changes of the conditions (mobile phase composition, column temperature, different lot of stationary

phase, analyst and equipment) to validate the method.

## 3. Results and discussion

### 3.1. Chromatography

Under the current gradient conditions, Hyperforin along with other phytoconstituents of *H. perforatum* extract were eluted within 45 min. Figure 1, 2 and 3 shows the typical LC chromatograms of working standard of hyperforin and extracts of St. John's Wort sample such as RE-1 and IE-1 at 270 nm respectively. Figure 4 shows the UV spectrum of working standard hyperforin along with its UV maxima at 273.9 nm and peak purity at three different levels of peak.

### 3.2. Limit of detection and limit of quantitation

The limit of detection (LOD) was obtained by successively decreasing the concentration of hyperforin as long as a signal-to-noise ratio of 3:1 appeared. The LOD was found to be 10 ng on column (volume of injection is 20  $\mu\text{L}$ ; corresponding to a concentration of 500 ng / ml). The limit of quantitation (LOQ) was found to be 20 ng on column (volume of injection is 20  $\mu\text{L}$ ; corresponding to a concentration of 1  $\mu\text{g/mL}$  hyperforin

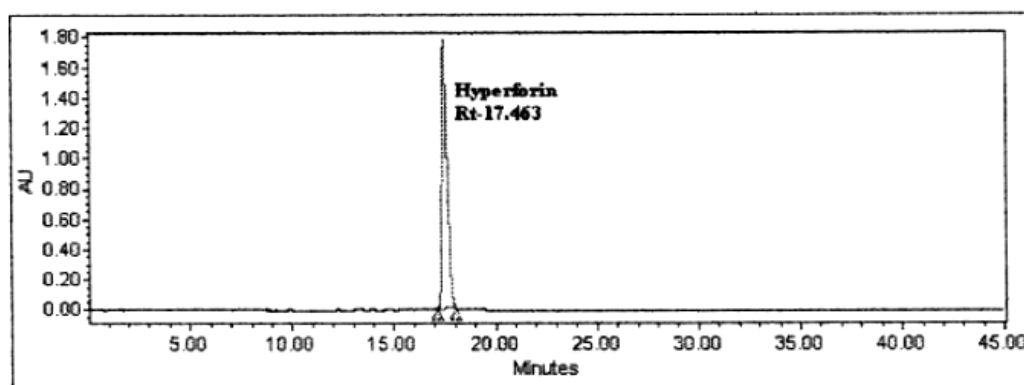


Figure 1. LC Chromatogram of working standard Hyperforin

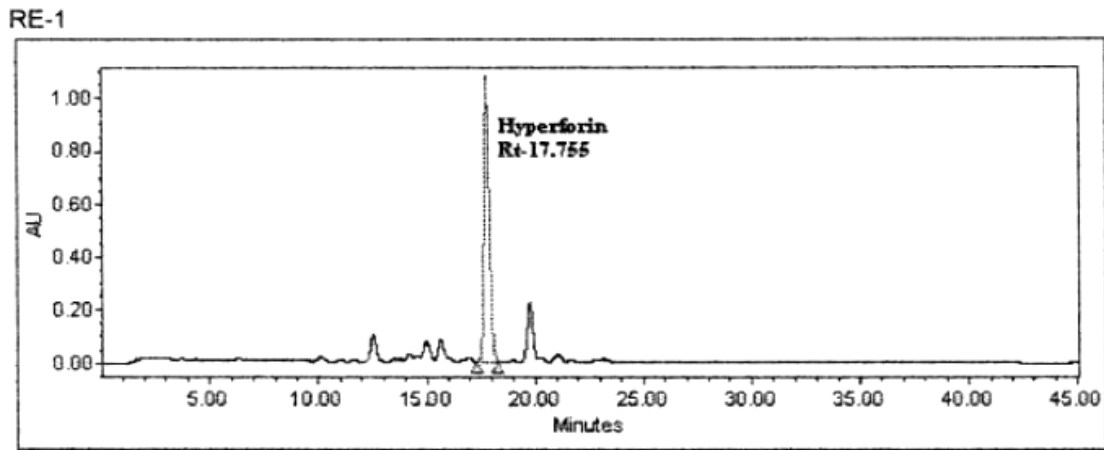


Figure 2. LC Chromatogram of RE-1, extract of St. John's Wort

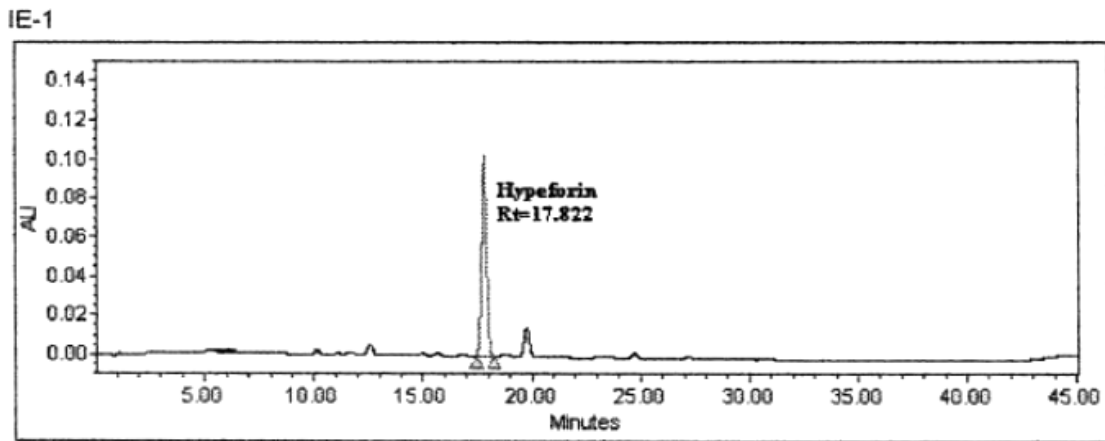


Figure 3. LC Chromatogram of IE-1, extract of St. John's Wort

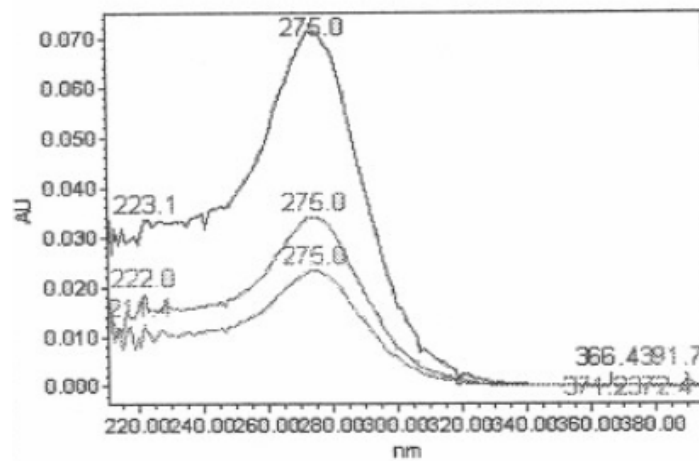


Figure 4. UV spectrum of working standard Hyperforin

### 3.4. Linearity and reproducibility

The calibration was based on the duplicate analysis of calibration working solutions at fourteen concentration levels on 3 consecutive days for hyperforin (1.0 - 100.0 µg/ml) with regression ( $r^2$ ) more than 0.9972. The repro-

ducibility of the method was evaluated by analyzing three sets of controls ( $n=3$ ) on 3 separate days ( $n=3$ ) and calculating the relative standard deviation (RSD). As shown in Table I, the RSD (%) were founded in the range of 0.16 – 0.88%.

Table 1. Precision and accuracy of the recalculated calibration samples

Given (µg/mL)	Found, mean ± S.D. (µg/mL)	Precision (R.S.D., %)	Accuracy (% Deviation)
5	4.96 ± 0.69	0.809	- 0.80
7.5	7.52 ± 0.94	0.887	+ 0.26
10	9.93 ± 0.64	0.626	- 0.70
12.5	12.59 ± 0.15	0.160	+ 0.72
15	14.90 ± 0.42	0.600	- 0.66

### 3.5. Intraday and interday analysis using Hyperforin

Furthermore the precision and accuracy of the intraday and interday analysis were inves-

tigated on the basis of a set of standard solution. The results given in Table II stands for a quite good trueness of the proposed method particularly considering interday and intraday analysis.

Table2. Intraday and interday precision and accuracy of hyperforin

Given (µg/ml)	Intraday		Interday	
	Precision (R.S.D., %)	Accuracy (Percent deviation)	Precision (R.S.D., %)	Accuracy (Percent deviation)
4	3.950	+ 2.79	0.898	- 0.64
40	0.151	- 0.11	0.523	+ 0.18
80	0.443	- 0.31	1.962	- 1.38

### 3.7. Stability

It has been well known for some time that some constituents, especially hyperforin, in St. John's Wort, are highly sensitive to oxidation [19-20]. Several oxidized forms of hyperforin have been isolated and characterized [21-23]. However, in contrast with the high sensitivity to oxidation in its pure form, hyperforin in dried herbs or preparations containing St. John's Wort extract was more stable [20]. In the current assay, analyses of stability sam-

ples in methanol on consecutive days (24, 48, 72 and 96 hr) revealed that the major constituents in the methanolic extract of St. John's Wort are stable in solution form with relative standard deviation (RSD (%))2.09 ( $n = 3$ ) for hyperforin at 4°C respectively.

### 3.8. Robustness and Ruggedness studies

The method was found to be re-producible from one analyst to another. The low values of R.S.D. (2.152 % - 2.874%) obtained after

small deliberate changes of the conditions (mobile phase composition, column temperature, different lot of stationary phase, analyst and equipment) used for the method indicated its robustness.

### 3.9. Sample analysis

Three sets of samples were analyzed for hyperforin in St. John's Wort extracts (IE-1 and RE-1), according to the method described above. The average content of hyperforin in extracts, IE-1 and RE-1 was found to be in the range of 2.45 - 2.61 % w/w and 26.88 - 27.47 % w/w respectively.

### 4. Conclusions

The need for quality assurance, including confirmation of the label strength and content uniformity has long been recognized even for herbal medicinal products. A high-performance liquid chromatography method has been developed for the detection and quantitation of major constituents of St. John's Wort using a photodiode array detector. Analysis of St. John's Wort samples with the proposed method does assure prolong life of column and system due to lower percentage of acid in mobile phase and hyperforin can be quantitated successfully, using standard calibration curve. The method was found to be specific and suitable for routine analysis because of its simplicity, and reproducibility. The relative standard deviation for the investigated St. John's Wort extracts indicates that the method is precise and reproducible.

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