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Development of a simple, rapid and reproducible HPLC assay for the simultaneous determination of hypericins and stabilized hyperforin in commercial St. John's Wort preparations

Gerlie C. de los Reyes, Robert T. Koda *

Department of Pharmaceutical Sciences, University of Southern California, 1985 Zonal Ave., Los Angeles, CA 90089-9121, USA

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Abstract

A reversed-phase HPLC method was developed and validated for the simultaneous determination of hypericins and stabilized hyperforin in St. John's Wort extract. The sample solution was prepared by extraction of the finely powdered extract with methanol–water (80:20, v/v) containing 5% HP-β-cyclodextrin, and adjusted to pH 2.5 with *ortho* phosphoric acid. Diluted extract solutions, maintained at 0 °C, were injected into a C_{18} column. The samples were eluted isocratically using a mobile phase consisting of acetonitrile and 0.3% v/v phosphoric acid (90:10, v/v) at a 1.5 ml/min flow rate with simultaneous fluorescence (315/590 nm, excitation/emission) and UV (273 nm) detection. Quantification of the marker compounds (hypericin, pseudohypericin, hyperforin) was achieved by use of standard curves generated by plotting peak heights versus concentrations. Validation studies demonstrated that this HPLC method is simple, rapid, reliable, and reproducible. The standard curves were linear over the concentration ranges, 0.5–2.5 μg/ml (hypericin), 0.35–1.6 μg/ml (pseudohypericin) and 5–50 μg/ml (hyperforin). The intra-day coefficients of variation obtained for hypericin, pseudohypericin and hyperforin were $\leqslant 4.4\%$, $\leqslant 5.4\%$, and $\leqslant 2.8\%$, respectively; inter-day CVs were $\leqslant 5.8\%$, $\leqslant 4.9\%$, and $\leqslant 2.5\%$, respectively. This method may be applied for the routine standardization of St. John's Wort products against hyperforin and the hypericins, the putative antidepressant principles in the herbal.

Keywords: Hypericin; Pseudohypericin; Stabilized hyperforin; Reversed-phase HPLC; Validation; Simultaneous assay

1. Introduction

Hyperforin (Fig. 1) is the focus of recent pharmacological and clinical studies on the antidepressant properties of St. John's Wort (*Hypericum perforatum*) [1,2]. The compound is a major component occurring in concentrations of 2–4% of

* Corresponding author. Tel.: +1-323-442-1473; fax: +1-323-442-3835.
E-mail address: rtkoda@hsc.usc.edu (R.T. Koda).

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the total extract [3]. Although this compound is now regarded as the true 'active' ingredient of St. John's Wort, it is relatively unstable in the presence of oxygen and light [4,5]. As such, the hypericins (Fig. 2) remain the popular marker substances for the standardization of the herbal product.

Several HPLC methods have been reported for the determination of hypericins [6–9] and hyperforin [10] in St. John's Wort extract. To our knowledge, only two HPLC methods for the simultaneous assay of hypericins and hyperforins in St. John's Wort crude extract [6] and plant sample [11] have been published. The previous methods required either mass spectroscopic detection or extraction of the plant sample with methanol for 2 h followed by a solid-phase cleanup. The apparent instability of hyperforin in extract solutions possibly precluded the comprehensive validation of these methods.

Recently, the stability of isolated hyperforin in solution and solid form was investigated in several antioxidant systems [12]. Hyperforin showed greater stability in polar solvents compared to nonpolar solvents. In addition, hyperforin was more stable in pH 2.0 methanolic solution than in alkaline pH 12 solution.

In this study, a simple, rapid, and reproducible HPLC assay method was developed and validated under conditions that were able to maintain the stability of hyperforin without compromising the recovery of the hypericins. This method is proposed for the routine simultaneous assay of hypericins and hyperforin in St. John's Wort products.

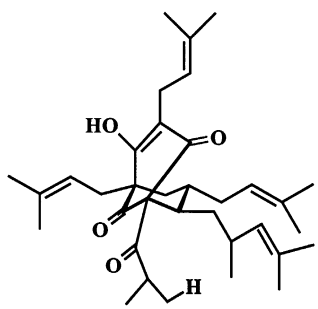

Fig. 1. The structure of hyperforin ($C_{35}H_{52}O_4$).

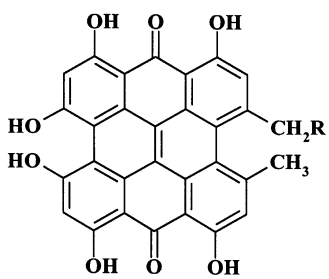

Fig. 2. The structure of the hypericins. $R = H$ (hypericin, $C_{30}H_{16}O_8$); $R = OH$ (pseudohypericin, $C_{30}H_{16}O_9$).

2. Experimental

2.1. Materials

Chemicals were purchased from suppliers as indicated: hyperforin, > 90% HPLC purity and pseudohypericin, 85.62% HPLC purity (Addipharma, Hamburg, Germany), hypericin, > 85% HPLC purity (Sigma-Aldrich, St. Louis, MO), anhydrous methyl alcohol, A.C.S., and acetonitrile, A.C.S. (Mallinckrodt AR, Paris, Kentucky), and *ortho* phosphoric acid, 85%, A.C.S. (Fisher Scientific, Fair Lawn, New Jersey). St. John's Wort extract, R3760Q, Lot No. 834322AA (Finzeberg, IN) was a gift from Leiner Health Products, Inc. (Carson, CA). A commercial St. John's Wort product containing 300 mg extract per capsule was purchased from a local pharmacy.

The chromatographic separation was performed using a HPLC system consisting of the following: Perkin–Elmer LC-410 pump, ISS-100 auto-injector, LC 90 UV spectrophotometric detector (273 nm), LS-4 fluorescence spectrometer, and LCI-100 laboratory computing integrator. During the HPLC assay, the samples were maintained at 0 °C using a MGW Lauda RM6 thermostat. Hyperforin and the hypericins were separated on a Discovery C_{18} (5μ particle size, 150 mm × 4.6 mm i.d.) reversed phase column obtained from Supelco (Sigma-Aldrich, Bellefonte, PA). The mobile phase consisted of acetonitrile and 0.3% v/v *ortho* phosphoric acid (90:10, v/v). The pH of the mobile phase was adjusted to pH

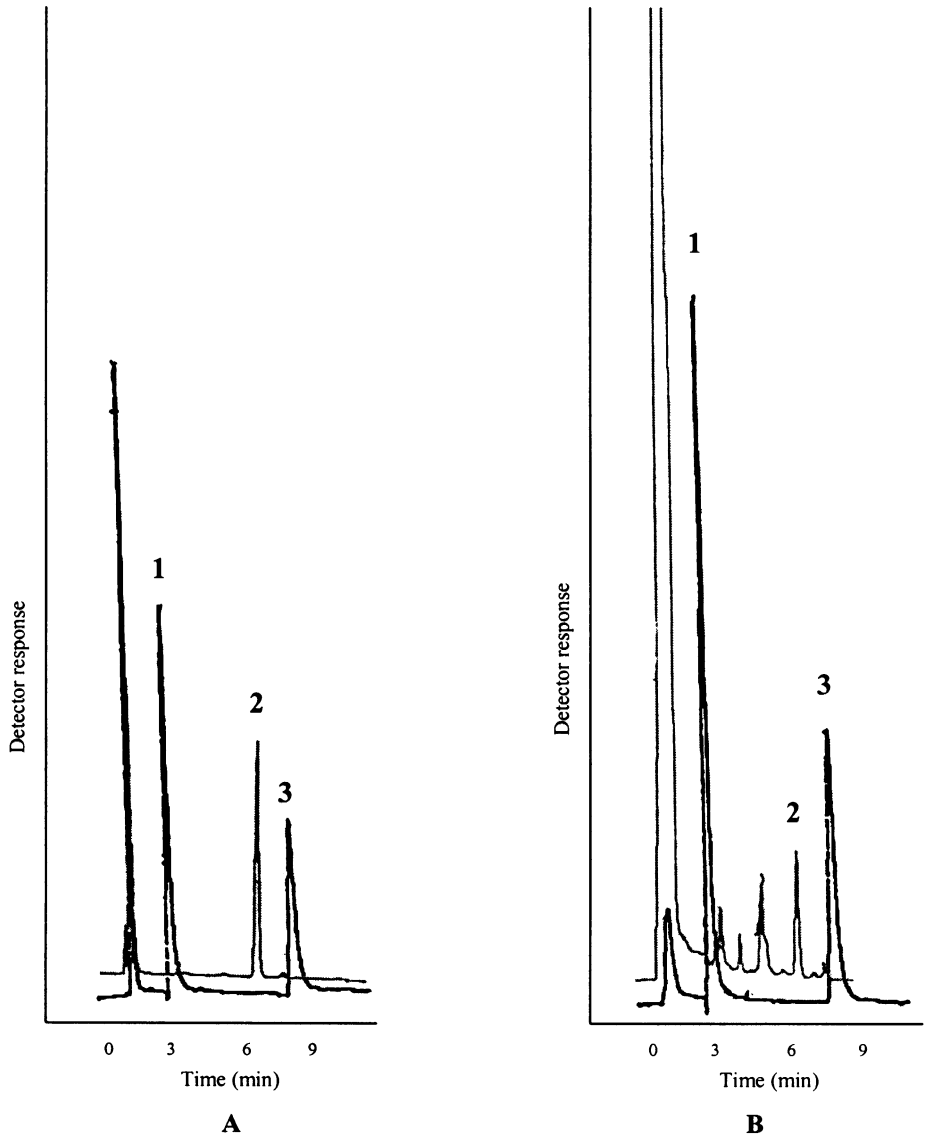

Fig. 3. Typical chromatograms of: A — standard solution containing (1) pseudohypericin 35 ng, (2) hyperforin 500 ng; (3) hypericin 50 ng; B — St. John's Wort extract solution (1:4 dilution). HPLC tracings were obtained by simultaneous UV (hyperforin) and fluorescence (hypericins) detection.

2.5 with 1 N phosphoric acid, and the solution was degassed by suction-filtration through a Nylon 100 membrane (Alltech, Deerfield, IL). The extraction solution consisted of 80% methanol in water, with 5% hydroxypropyl-β-cyclodextrin (HP-β-CD), adjusted to pH 2.5 with 85% *ortho* phosphoric acid.

2.2. Selection of extraction solution: efficiency versus hyperforin stability

To compare extraction efficiency and the potential to maintain hyperforin stability, several solvent systems were evaluated: solvent 'A' (methanol), 'B' (methanol, adjusted to pH 2.5

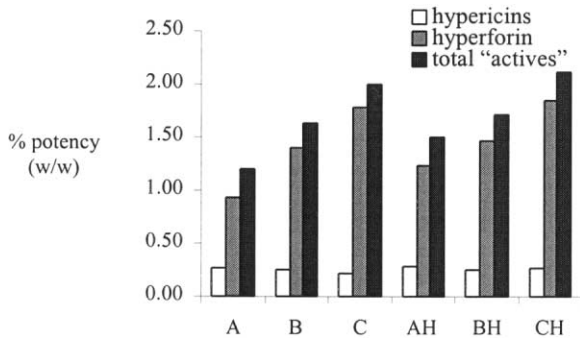

Fig. 4. Measured levels of hypericins (□) and hyperforin ■) in extract following sonication with various solutions: A — methanol, B — methanol, adjusted to pH 2.5 with *ortho*phosphoric acid, C — methanol–water (80:20), adjusted to pH 2.5; AH, BH, CH — A, B, and C with 5% hydroxypropyl-β-cyclodextrin. Total 'active' (■) is the sum of hypericins and hyperforin.

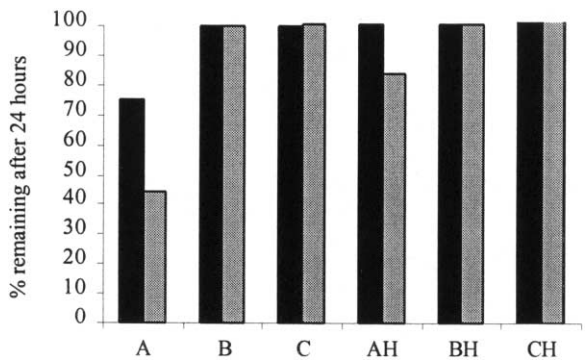

Fig. 5. Stability of hyperforin in extract solutions 24 h following sonication with various solutions: A — methanol, B — methanol, adjusted to pH 2.5 with orthophosphoric acid, C — Methanol-water (80:20), adjusted to pH 2.5; AH, BH, CH — A, B, and C with 5% hydroxypropyl-β-cyclodextrin. Storage at 4 °C (■) and 25 °C (▯).

with *ortho*phosphoric acid), 'C' (methanol–water, 80:20, v/v, pH 2.5), and their counterparts containing 5% HP-β-CD, 'AH', 'BH', and 'CH', respectively. Test extract solutions were prepared in each of the liquids as described above. These solutions were stored at 25 or 4 °C. Samples were collected at 0 and 24 h and stored at −70 °C until HPLC analysis.

2.3. Preparation of standard and sample solutions

Standard solutions containing hyperforin (5, 10, 25, 50 μg/ml), hypericin (0.5, 1.0, 2.5 μg/ml) and pseudohypericin (0.35, 0.7, 1.6 μg/ml) were prepared by serially diluting stock solutions with the extraction solution. Fifty microliters of each solution was injected into the HPLC column.

About 0.3 g of the crude extract powder, accurately weighed, was transferred into a 100-ml volumetric flask containing about 70 ml of the extraction solution. Following 15 min sonication, the preparation was filtered through cellulose paper into a second volumetric flask. The first flask was rinsed with extra solution and the wash was passed through the same filter to bring the filtrate to final volume. Fifty microliters of a 1:4 dilution of the preparation was injected into the HPLC column.

2.4. HPLC assay

A reversed-phase HPLC assay, suitable for the simultaneous determination of hypericins and stabilized hyperforin in extract solutions, was developed and validated. A simple extraction technique with aqueous methanol (pH 2.5) containing 5% HP-β-CD was used to prepare the test solutions. Hyperforin and the hypericins were eluted isocrat-

Table 1
Linear regression analysis parameters for quantification of hyperforin, hypericin and pseudohypericin

Marker compound	Concentration range	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
	(μg/ml)	slope	y-intercept	r^2	slope	y-intercept	r^2
Hyperforin	5.0–50.0	20.2	19.1	0.9993	19.9	21.9	0.9991
Hypericin	0.50–2.50	1062.0	12.7	0.9996	1126.0	58.1	0.9999
Pseudohypericin	0.35–1.60	4042.0	−230.0	1.0000	4051.0	−281.0	0.9997

Table 2
Intra-day and inter-day accuracy and precision of the assay for the quantification of hyperforin

Hyperforin	Intra-day ($n = 5$)		Inter-day ($n = 5$)	
Theoretical concentration (μg/ml)	Measured concentration (μg/ml)	CV (%)	Measured concentration (μg/ml)	CV (%)
5	4.9	2.8	5.1	1.0
10	10.4	2.7	10.2	1.2
25	26.3	2.5	24.2	2.5
50	49.0	1.5	49.7	1.0
Extract A (1:4)	5.8	5.6	5.8	6.8
Extract B (1:4)	7.2	2.6	7.0	4.5

Table 3
Intra-day accuracy and precision (% CV) of assay for quantification of hypericins; $n = 5$

Hypericin			Pseudohypericin		
Theoretical concentration (μg/ml)	Measured concentration (μg/ml)	CV (%)	Theoretical concentration (μg/ml)	Measured concentration (μg/ml)	CV (%)
0.50	0.48	4.4	0.35	0.34	5.4
1.00	1.02	4.2	0.70	0.69	2.0
2.50	2.49	3.0	1.60	1.60	3.8
Extract A (1:4)	1.76	0.4	Extract A (1:4)	1.38	2.5
Extract B (1:4)	1.23	1.3	Extract B (1:4)	0.97	1.8

Table 4
Inter-day accuracy and precision (% CV) of assay for quantification of hypericins; $n = 5$

Hypericin			Pseudohypericin		
Theoretical concentration (μg/ml)	Measured concentration (μg/ml)	CV (%)	Theoretical concentration (μg/ml)	Measured concentration (μg/ml)	CV (%)
0.50	0.49	5.8	0.35	0.35	3.3
1.00	1.01	1.0	0.70	0.69	1.7
2.50	2.50	3.4	1.60	1.60	4.9
Extract A (1:4)	1.71	0.9	Extract A (1:4)	1.45	0.9
Extract B (1:4)	1.20	1.7	Extract B (1:4)	1.47	1.9

ically from a C_{18} reversed-phase column using a mobile phase consisting of acetonitrile and 0.3% phosphoric acid (90:10, v/v) at a 1.5 ml/min flow rate with simultaneous fluorescence (315/590 nm, excitation/emission) and UV (273 nm) detection of hypericins and hyperforin, respectively. The total chromatographic analysis time per sample was 10 min with pseudohypericin, hyperforin, and hypericin eluting at retention times of about 3.0, 6.5, and 8.5 min, respectively (Fig. 3).

2.5. Assay validation

The standard solutions and the extract samples were processed in five replicates on a daily basis. All validation runs were performed on five consecutive days. Standard curves of peak height versus concentration were plotted and the equation of the regression line was determined. The actual concentrations, percent of observed versus theoretical concentration, within-day and be-

tween-day coefficients of variation (% CV) were determined.

3. Results and discussion

3.1. Selection of extraction solution: effect on extraction efficiency

Fig. 4 shows the levels of hyperforin and hypericins found in the samples following extraction with various solutions. The following orders of extraction efficiency was found for hyperforin: 'C' > 'B' > 'A', and for hypericins: 'A' > 'B' = 'C'.

Compared to 'C' (pH 2.5 methanol–water, 80:20, v/v) and 'B' (pH 2.5 MeOH), A (methanol) showed poor extraction efficiency for hyperforin. It is apparent from Fig. 4 that the acidic pH and the addition of water improved hyperforin extraction. The extraction of hypericins by 'B' and 'C' was lower but comparable (95%) to that afforded by 'A' (pure methanol).

Hydroxypropyl-β-cyclodextrin (HP-β-CD) was added to the three solutions A, B, and C to determine its solubilizing effect on the hypericins and hyperforin. Fig. 4 shows that the addition of 5% HP-β-CD to 'C' resulted in a further improvement in the extraction of hypericins by this solution compared to pure methanol ('CH' vs. 'A'). On the other hand, HP-ß-CD increased the measured level of hyperforin in methanol ('A' vs. 'AH') but not to the same extent as that resulting from extraction with low pH aqueous methanol ('AH' vs. 'C').

3.2. Selection of extraction solution: effect on hyperforin stability

The hypericins remain the preferred potency markers for the standardization of St. John's Wort products because of the unstable nature of hyperforins in extract solutions. Compared to the hypericins, hyperforin is highly susceptible to degradation, especially in the presence of light and oxygen [4,5]. The degradation of hyperforin from extract solutions was found to be greater from solutions of the isolated or pure form. Thus, the effect of various extraction solutions on the stability of hyperforin was evaluated. Fig. 5 compares the hyperforin concentrations after 24 h of storage at room and refrigerated temperatures. The stability of hyperforin was dependent on temperature and extraction solution used. For example, hyperforin in methanol solutions showed greater than 50% and 20% degradation at 25 and 4 °C, respectively after 24 h. These observations suggest that methanol extraction at room temperature is not suitable for the assay of hyperforin in extract samples. Hyperforin could be stabilized in pH 2.5 methanolic solution, with or without the addition of water. The addition of 5% HP-β-CD also improved its stability in methanol ('A' vs. 'AH') but not as effectively as low pH. On the other hand, low pH, addition of HP-β-CD, and water did not affect the stability of the hypericins (data not shown).

'C' (80% MeOH in water, pH 2.5) efficiently extracted hyperforin and maintained its stability in extract solutions. The addition of 5% HP-β-CD to this solution (as 'CH') improved hyperforin stability as well as ensured the complete extraction of the hypericins.

3.3. Assay validation

3.3.1. Linearity

Calibration curves were constructed by plotting peak heights against concentrations. Linear regression analysis was performed for each reference standard. Table 1 summarizes the slopes, y-intercepts, and correlation coefficients (r^2) obtained for the regression lines based on a minimum of three data points. Calibration curves constructed on five different days showed low inter-day variability over the concentration ranges studied. The correlation coefficients were all greater than 0.999, indicating a high degree of linearity. The inter-day CVs for the slopes were 2.6, 4.8, and 6.0% for hyperforin, hypericin and pseudohypericin, respectively, indicating satisfactory between-run reproducibility.

3.3.2. Precision and accuracy

The reproducibility of the proposed method was evaluated by replicate analyses of standard samples containing at least three different concen-

trations (Tables 2–4). The intra-day coefficients of variation ranged 1.5–2.8% (hyperforin) and 2.0–5.4% (hypericins). The inter-day CVs ranged 1.0–2.5% (hyperforin) and 1.0–5.8% (hypericins). The low values of the CVs ($< 10\%$) obtained for the standards and the extract solution reflect the high precision of the method. The assay is also accurate as indicated by the close agreement of the theoretical with the measured concentration values.

4. Conclusion

The proposed HPLC method demonstrated excellent accuracy and reproducibility. The sample preparation and assay procedure involved is simple, rapid, sufficient to maintain the stability of hyperforin in extract solutions, and offers a simultaneous assay for hyperforin and the hypericins. This method is recommended for the routine standardization of St. John's Wort products against these marker substances.

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