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Short communication

High performance liquid chromatographic analysis of St. John's Wort with photodiode array detection

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Abstract

An RP-HPLC method with photodiode array detection was established for the determination of major constituents (rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudohypericin, hyperforin and hypericin) in St. John's Wort dietary supplements. The samples were extracted with methanol by means of sonication in low temperature. The extraction was rapid, with two steps of sonication (30 min each) recovering more than 99% of the major constituents in St. John's Wort samples. The major components were separated by RP-18 chromatography column using a 60-min water–acetonitrile–methanol–trifluoroacetic acid gradient. The quantification was performed by using external standards. Sample preparation and stability of methanolic extract of St. John's Wort were extensively explored. It is worth noting that the major constituents in the methanolic extract of St John's Wort, especially hypericin and pseudohypericin, might be retained by some filter cartridges during the filtration. The current method may serve as a valuable tool for the QA/QC of St. John's Wort dietary supplements.

Keywords: St. John's Wort; *Hypericum perforatum*; Flavonoids; Phloroglucinols; Naphthodianthrones

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. It has become very popular because of its reported beneficial effects as an antidepressant. 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]. Concerns have also been raised recently over the interactions between St. John's Wort and certain prescribed medicines [5].

Chemically, 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 adhyperforin), biflavonoids (I3, II8-biapigenin, I3′, II8-biapigenin), proanthocyanidins

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and chlorogenic acid [6]. The standardization of St. John's Wort is normally based on the content of hypericins (0.3–0.5%) and hyperforin (3.0%), which have been reported to mainly contribute to the pharmacological effects of St. John's Wort [7–11]. However, a recent publication showed that flavonoids might also make an important contribution to the antidepressant activity of St. John's Wort [12]. As the biological effects of St. John's Wort are being considered from the whole mixture of its major constituents rather than a single compound, the availability of a robust method allowing the analysis of the entire 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 [13–16], these methods either employed non-organic solvents [13,14] or required more than 60 min for HPLC separation [13,15,16]. Few publications are available to specify the sample preparation, reproducibility and stability. Because of the increasing interest in St. John's Wort, a high-performance liquid chromatographic method with photodiode array detection has been developed for the determination of the major constituents in St. John's Wort. Sample preparation, stability, and reproducibility were targeted in the current research.

2. Experimental

2.1. Chemicals

Rutin, quercetin and I3, II8-biapigenin were isolated and purified in the Program for Collaborative Research in the Pharmaceutical Sciences (PCRPS), College of Pharmacy, University of Illinois at Chicago, IL 60612, USA. Quercitrin, isoquercitrin, hyperoside, hypericin and pseudohypericin were purchased from Indofine (Somerville, NJ, USA). Hyperforin was obtained from Chromadex (Laguna Hills, CA, USA). The reference standard purity was determined to be more than 93% by HPLC.

Methanol, acetonitrile, and trifluoroacetic acid (TFA) were HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was obtained with an in-house Nanopure® water system (Barnstead, Newton, MA, USA).

2.2. Apparatus

The HPLC system consisted of a Waters Alliance 2690 liquid chromatograph equipped with an autosampler, a photodiode array (996) detector and a temperature controller for sampler and column (Waters, Milford, MA, USA). The absorption was measured either as a full spectrum (200–800 nm), at 270 nm for most constituents, or at 590 nm for naphthodianthrones (hypericin and pseudohypericin). The chromatographic data were recorded and processed with Waters Millennium 2000® software.

2.3. Chromatography

Analyses were carried out at 20°C on a Waters YMC ODS-AQ™ RP-18 column (5 μm, 120 Å, 4.6×250 mm, serial # 0425503853) (Waters, Milford, MA, USA), which was protected by a Waters Delta-Pak RP-18 guard column (Waters Technology Ireland, Wexford, Ireland). The mobile phases consisted of water (A, containing 20% methanol and 0.5% TFA) and acetonitrile (B, containing 10% methanol and 0.5% TFA). The analyses followed a linear gradient program. Initial conditions were 90% A; 0–20 min, changed to 30% A; 20–25 min, to 10% A; 25–30 min, to 0% A kept to 60 min; 60–65 min, went back to 90% A. The flow-rate was kept at 1.0 ml/min, and the injection volume was 10 μl. The peak in the HPLC chromatogram of St. John's Wort extract was tentatively identified by comparing the retention time and UV spectra of the peaks in the samples with those of reference standards. The purity of each peak was checked by PDA software routines.

2.4. Standard solutions

Reference standards (0.2–2.0 mg), rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudohypericin, hyperforin and hypericin, were accurately weighed or transferred (hyperforin, which was obtained in methanolic solution) into a 10-ml volumetric flask and dissolved in methanol to make a stock solution. The stock solution was stored at −20°C and brought to room temperature before use.

Calibration standard working solutions were freshly prepared by diluting the stock solution with methanol in appropriate quantities. In the same way, three sets of controls for isoquercitrin, quercitrin, quercetin, pseudohypericin, hyperforin and hypericin were prepared from a separate stock, so as to lie in the lowest, middle and highest regions of the calibration curves.

2.5. Evaluation of extraction efficiency

St. John's Wort powder (200 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 using a sonicator under strict exclusion of light in cool water bath (~11°C) for 30 min (the temperature of the sonicator bath increased to ~23°C after 30 min). The mixture was centrifuged at 7 *g* for 10 min, and resulting supernatant (I) was transferred to a 10-ml volumetric flask and made up to volume with methanol. The residue in the tube was extracted with a second 10 ml of methanol as described, and the resulting supernatant (II) was transferred to another 10-ml volumetric flask and made up to volume with methanol. The above procedure was repeated two more times. The concentrations of the major constituents in the resulting extracts (I, II, II and IV) were calculated based on the equations for the calibration curves, and the extraction efficiency was compared.

2.6. Comparison of the filtration loss of the major constituents in methanolic extracts of St. John's Wort after filtration through different cartridges

St. John's Wort powder (200 mg) was extracted with methanol (4×10 ml) as described. After centrifugation, the supernatants were combined into a 50-ml volumetric flask and made up to volume with methanol. Then 10 μl of the mixture was directly subjected to HPLC analysis ($n=3$). Meanwhile, a 1-ml aliquot of extract was filtered through five different membrane filtration cartridges: (i) nylon filter (0.2 μm); (ii) polypropylene filter (PP, 0.45 μm); (iii) Anotop inorganic membrane filter (0.2 μm) from Whatman, Clifton, NJ, USA; (iv) polytetrafluoroethylene filter (PTFE, 0.45 μm); and (v) polyvinylidene fluoride filter (PVDF, 0.22 μm) from Millipore, Molsheim, France. The filtrates were analyzed by HPLC. Five duplicate testing samples were prepared by using each type of cartridge. The contents of major constituents were calculated based on the calibration curves and the relative loss (%, w/w) for each constituent in the process of filtration was calculated by comparing the content (%, w/w) of these major constituents found in the different filtrates to that found in the sample solution obtained with centrifugation only.

2.7. Stability

St. John's Wort powder (200 mg) was extracted with methanol (4×10 ml) as described. After centrifugation, the supernatants were combined into a 50-ml volumetric flask and made up to volume with methanol. The sample solution was put in the dark at room temperature and analyzed on consecutive days (24, 48 and 72 h) to observe the stability of sample solutions.

2.8. Quantification of major constituents in St. John's Wort powder and tablets

Fine powder (200 mg) or a finely pulverized tablet (650 mg) of St. John's Wort was exactly weighed and extracted with methanol (4×10 ml) as described. After centrifugation, the supernatants were combined into a 50-ml volumetric flask, respectively, and made up to volume with methanol. Then 10 μl of the resulting supernatant was subjected to HPLC analysis and the content of major constituents was calculated based on the calibration curves.

3. Results and discussion

3.1. Chromatography

Under the current gradient conditions, all compounds were eluted within 60 min. Fig. 1 shows the typical chromatograms of methanolic extract of a St. John's Wort sample at 270 (Fig. 1a) and 590 nm (Fig. 1b). The components in the extracts correlated directly with retention order and UV results previously reported by Holzl et al. [13] and Gray et al. [17].

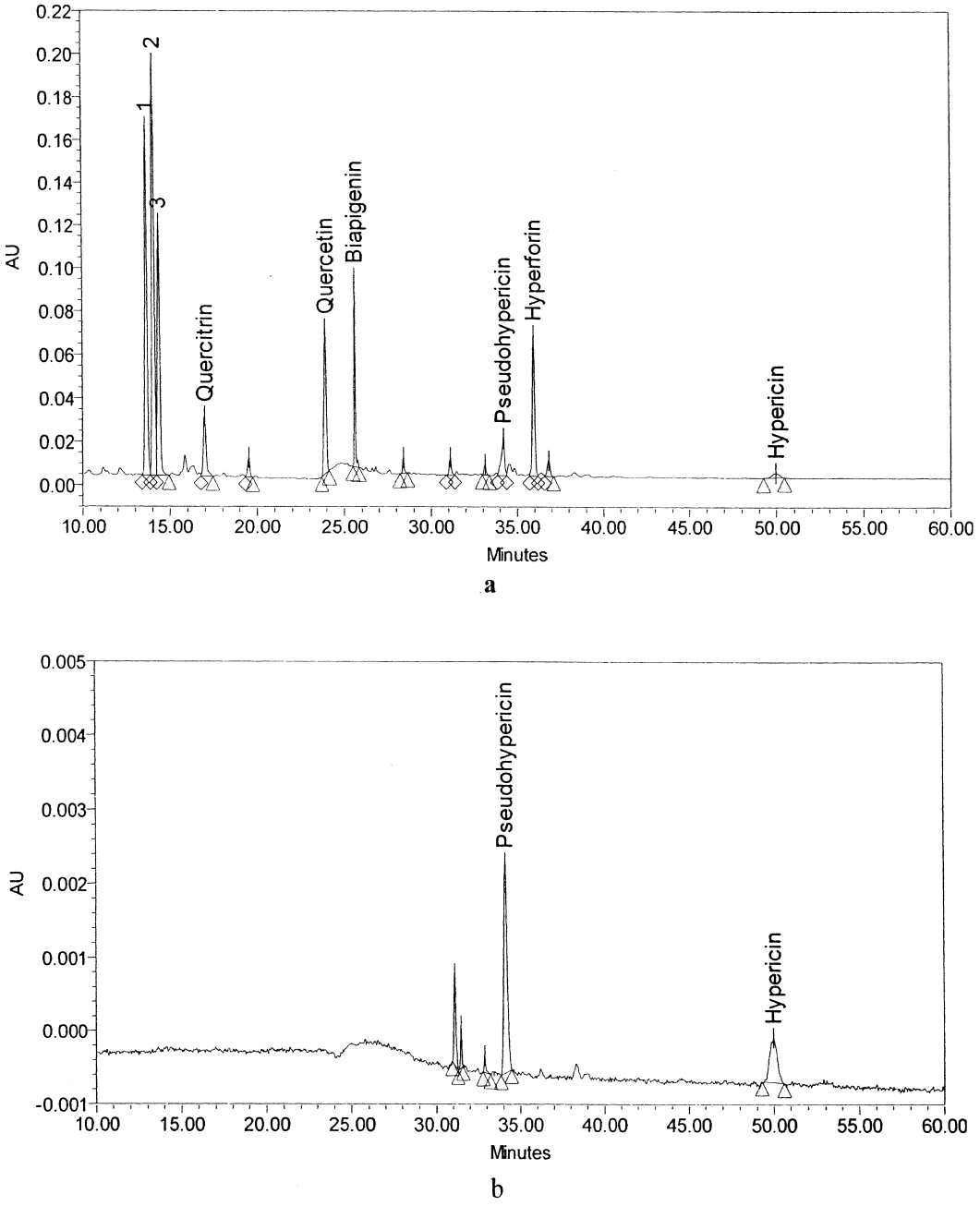

Fig. 1. Typical HPLC chromatograms of the methanolic extract of St. John's Wort with detection wavelength set at 270 nm (**1a**) and 590 nm (**1b**). The retention times of rutin (**1**), hyperoside (**2**), isoquercitrin (**3**), quercetrin, quercetin, biapigenin, pseudohypericin, hyperforin and hypericin were observed at 13.66, 14.08, 14.38, 16.66, 23.72, 25.59, 34.55, 35.95 and 49.97 min, respectively. The tentative identification of biapigenin was carried out by comparing the retention time of the peak with that of the reference standard, but the calibration curve was not established due to insufficiency of the standard.

Table 1
Reproducibility

Compound		Actual value (μg/ml)	Day 1			Day 2			Day 3		
			Observed value (μg/ml)[a]	RSD (%)	RE (%)	Observed value (μg/ml)[a]	RSD (%)	RE (%)	Observed value (μg/ml)[a]	RSD (%)	RE (%)
Isoquercitrin	QC-1	10.22	11.57±0.11	0.46	13.21	11.57±0.04	0.15	13.2	11.49±0.03	0.19	12.39
	QC-2	51.11	50.28±0.16	0.32	−1.63	49.95±0.63	0.35	−2.27	50.28±0.69	0.14	−1.63
	QC-3	92.0	96.78±0.29	0.30	5.20	95.92±0.30	0.31	4.26	96.53±0.23	0.24	4.93
Quercitrin	QC-1	4.09	4.40±0.17	0.50	7.55	4.53±0.01	0.21	10.76	4.40±0.01	0.16	7.57
	QC-2	20.43	19.36±0.07	0.43	−5.27	19.19±0.07	0.37	−6.07	19.49±0.01	0.59	−4.61
	QC-3	36.78	37.21±0.12	0.32	1.18	36.94±0.09	0.25	0.43	37.16±0.09	0.24	1.03
Quercetin	QC-1	20.18	21.86±0.06	0.48	8.30	22.23±0.13	0.08	9.13	21.80±0.18	0.26	8.03
	QC-2	100.90	94.99±0.40	0.31	−5.86	94.49±0.39	0.42	−6.35	95.09±0.58	0.35	−5.76
	QC-3	181.62	182.30±0.37	0.20	0.37	180.82±0.51	0.28	−0.43	181.91±0.49	0.27	0.16
Pseudohypericin	QC-1	1.99	2.07±0.05	0.78	3.94	2.07±0.07	2.92	3.96	1.90±0.04	2.70	−4.50
	QC-2	9.95	9.27±0.06	1.89	−6.82	9.16±0.10	1.01	−7.95	9.17±0.08	0.77	−7.88
	QC-3	17.91	17.31±0.37	2.11	−3.32	17.11±0.35	2.05	−4.43	17.04±0.24	1.39	−4.87
Hyperforin	QC-1	4.84	4.94±0.03	0.52	2.10	4.94±0.02	0.39	2.11	4.78±0.05	0.28	−1.33
	QC-2	24.21	21.85±0.10	0.47	−9.75	22.39±0.13	0.56	−7.52	23.01±0.06	0.34	−4.88
	QC-3	43.57	42.00±0.26	0.62	−3.61	42.33±0.16	0.39	−2.84	43.44±0.22	0.52	−0.30
Hypericin	QC-1	2.04	2.08±0.09	2.00	−2.74	2.12±0.04	3.24	−0.71	2.16±0.07	2.58	1.10
	QC-2	10.19	9.51±0.14	0.90	−6.0	9.41±0.05	0.75	−7.62	9.79±0.10	0.86	−3.90
	QC-3	18.34	17.10±0.29	1.68	−6.75	16.56±0.40	2.38	−9.69	17.21±0.12	0.70	−6.17

[a] The data represent the mean±SD of six observations.

3.2. Linearity and reproducibility

The calibration was based on the duplicate analysis of calibration working solutions at seven concentration levels on 3 consecutive days for rutin (5–100 μg/ml), hyperoside (5–100 μg/ml), isoquercitrin (5–100 μg/ml), quercitrin (2–40 μg/ml), quercetin (10–200 μg/ml), pseudohypericin (1–20 μg/ml), hyperforin (2.5–50 μg/ml) and hypericin (1–20 μg/ml) with regression (r^2) more than 0.996. The reproducibility 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 (%)) and relative errors (RE (%)). As shown in Table 1, the RSD (%) and the RE (%) were founded to be less than 3.24 and 13.21% (Table 1), respectively.

3.3. Evaluation of extraction efficiency

The extraction efficiency is summarized in Table 2. In four steps (30 min each) of sonication, more than 99% of the major constituents could be extracted in step one (I) and step two (II). The sample extraction time (4×30 min) was much less compared to water-bath shaking (3×5.6 h) or sonication (3×1.5 h) with ethanol-acetone as the solvent [15,16].

Table 2
The percentages (%±SD) of major constituents found in the consecutively extracted methanolic solutions of St. John's Wort ($n=3$)

Extraction	Rutin	Hyperoside	Isoquercitrin	Quercitrin	Quercetin	Pseudohypericin	Hyperforin	Hypericin
First	96.38±0.18	96.40±0.17	95.24±0.24	97.59±0.16	97.33±0.31	96.05±0.24	97.89±0.13	94.98±0.35
Second	3.29±0.17	3.30±0.16	4.21±0.22	2.35±0.13	2.6±0.29	3.79±0.20	2.08±0.13	4.83±0.22
Third	0.24±0.01	0.22±0.02	0.41±0.02	0.09±0.02	0.07±0.02	0.03±0.02	0.27±0.03	ND[a]
Fourth	0.08±0.01	0.07±0.0	0.14±0.01	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]

[a] Not detected.

3.4. Comparison of filtration loss of major constituents in methanolic extract of St. John's Wort after filtration through different cartridges

In the process of sample preparation of St. John's Wort, a filtration step has sometimes been employed in the literature, including filtering the alcoholic extracts through a filter paper [16], a mixed solid-phase cartridge (MSP, C-18 and Florisil) [17], a nylon syringe filter [15], a polytetrafluoroethylene (PTFE) membrane [14] and a non-aqueous type filter [18]. But there is concern that naphthodianthrones (hypericin and pseudohypericin), like resveratrol in grapes (unpublished data), might be retained by some kinds of filters in the course of filtration because there are two ketone and six phenoxy functional groups in these molecules. In the current study, filtrations with nylon, PP, Anotop, PTFE and PVDF cartridges were compared with centrifugation for filtration loss. As shown in Table 3, nearly 100% of pseudohypericin and hypericin were retained by the nylon filter in the process of filtration. Also, the flavonoids were found to be lost to different degrees in this process. But no significant filter absorption loss was observed using PTFE, PP, Anotop or PVDF.

3.5. 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 samples on consecutive days (24, 48 and 72 h) revealed that the major constituents in the methanolic extract of St. John's Wort are stable with relative standard deviation (RSD (%)) of 0.88, 0.89, 1.05, 1.50, 0.81, 1.30, 1.74 and 0.10% ($n=9$) for rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudohypericin, hyperforin and hypericin, respectively.

3.6. Sample analysis

Three sets of samples were analyzed for St. John's Wort powder and tablet, respectively, according to the method described above. The average content of rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudohypericin, hyperforin and hypericin in the powder and tablet were found to be 2.52, 2.19, 1.44, 0.31, 0.71, 0.11, 1.62, 0.09, and 0.79, 0.69, 0.43, 0.10, 0.21, 0.028, 0.61, 0.029%, respectively.

4. Conclusions

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. With this method, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudo-

Table 3
The filtration losses (%) of major constituents in the methanolic extract of St. John's Wort after filtration through different cartridges compared with that obtained with centrifugation ($n=5$)

Compound	Centrifugation, mean±SD (%)	Nylon		PP		Anotop		PVDF		PTFE	
		Found (mean ±SD) (%)	Loss (%)	Found (mean ±SD) (%)	Loss (%)	Found (mean ±SD) (%)	Loss (%)	Found (mean ±SD) (%)	Loss (%)	Found (mean ±SD) (%)	Loss (%)
Rutin	2.518±0.003	2.382±0.055	5.39	2.516±0.003	0.201	2.518±0.004	−0.001	2.514±0.001	0.16	2.539±0.010	−0.851
Hyperoside	2.185±0.006	2.04±0.047	6.62	2.180±0.007	0.21	2.187±0.004	−0.084	2.183±0.003	0.121	2.20±0.019	−0.685
Isoquercitrin	1.437±0.013	1.326±0.032	7.73	1.439±0.006	−0.081	1.429±0.008	0.574	1.433±0.002	0.27	1.448±0.010	−0.779
Quercitrin	0.306±0.001	0.284±0.007	7.17	0.306±0.003	−0.015	0.306±0.004	−0.093	0.303±0.002	0.782	0.307±0.0008	−0.456
Quercetin	0.708±0.006	0.621±0.011	12.25	0.698±0.004	1.316	0.70±0.002	1.766	0.696±0.0001	1.61	0.714±0.003	−0.813
Pseudohypericin	0.105±0.000	ND[a]	100	0.106±0.001	−0.583	0.107±0.003	−1.39	0.104±0.002	1.099	0.106±0.002	−0.616
Hyperforin	1.617±0.004	1.576±0.042	2.56	1.586±0.008	1.901	1.581±0.003	2.18	1.577±0.0002	2.47	1.624±0.003	−0.448
Hypericin	0.091±0.006	ND[a]	100	0.092±0.003	−1.48	0.086±0.003	5.0	0.094±0.001	−3.70	0.089±0.006	1.377

[a] Not detected.

hypericin, hyperforin and hypericin were successfully quantitated, using standard calibration curves. The method was found to be specific and suitable for routine analysis because of its simplicity, and reproducibility. Sonication is recommended for St. John's Wort sample preparation, by placing the sample (4× 30 min) in a cool water bath with methanol as the solvent in the dark and centrifuging the sample extract without filtration through a membrane filter.

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