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Determination of hypericin and pseudohypericin in pharmaceutical preparations by liquid chromatography with fluorescence detection

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Abstract

The quantification of the naphthodianthrones, hypericin and pseudohypericin, to which most St. John's wort pharmaceutical preparations are standardized, can be accomplished with a high degree of specificity and sensitivity with LC–fluorescence. Extraction into ethanol is rapid and recovers more than 95% of both pseudohypericin and hypericin following 1 h of sonication. This LC–fluorescence method can separate and detect both pseudohypericin and hypericin in the presence of other constituents in *Hypericum perforatum* with a greater degree of specificity than LC–UV at either 236 nm or 592 nm and may even be more specific than LC–MS if only the molecular ion is monitored. LC–fluorescence is also more sensitive than LC–UV at either 236 nm or 592 nm. The LC–fluorescence method can quantify on-column amounts of hypericin and pseudohypericin as low as 0.18 ng with less than 5% deviation from known and method replication error (reproducibility) less than 5.5%. Hypericin and pseudohypericin can be accurately and reproducibly quantified from capsules, tablets and tinctures of St. John's wort.

Keywords: Hypericin; Pseudohypericin

1. Introduction

The use of St. John's wort (*Hypericum perforatum*) preparations, like many herbal medications is becoming widespread worldwide. Currently, St. John's wort encompasses more than 20% of the antidepressant market in Germany. St. John's wort has been used for decades, perhaps centuries, for the treatment of minor depression [1,2]. The active ingredients of this herb have not been clearly defined, but St. John's wort preparations are generally standardized to defined hypericin concentrations with the accepted standard of 0.3% [3]. A number of methods have been developed for the measurement of these compounds in a variety of media [4–12]. Many of the producers of St. John's wort preparations use the United States Pharmacopeia (USP) method [12], which is a simple UV spectrophotometric method or a liquid chromatographic method combined with UV detection. However, an interesting property of the naphthodianthrones like hypericin and its closely related derivatives such as pseudohypericin, is that they are capable of fluorescing [5,6,13]. Very limited research has been performed in the area of fluorescence spectrometry as a means of quantifying hypericin and pseudohypericin in phar-

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maceutical preparations. One published method used high-performance thin-layer chromatography (HPTLC)–densitometry (fluorescence) for determination of hypericin in Valena N extracts with St. John's wort [6]. Another method reported that fluorescence was no better than UV absorbance detection and that fluorescence was even less sensitive for the measurement of hypericin in plasma [5]. We report the use of gradient elution liquid chromatography (LC) and fluorometric detection which offers a higher degree of specificity and sensitivity when compared to standard UV detection procedures for the determination of the concentration of naphthodianthrones present in pharmaceutical preparations. In many instances fluorescence may even offer greater specificity then LC–mass spectrometry (MS).

2. Experimental

2.1. Materials

The hypericin used was obtained from Sigma (St. Louis, MO, USA) and was 97.3% pure. Triethylamine was obtained from Sigma. HPLC-grade methanol and acetonitrile were purchased from EM Science (Gibson, NJ, USA). Glacial acetic acid was purchased from BDH (Toronto, Canada) and was reagent grade. The anhydrous ethanol used was commercial grade and was purchased from Commercial Alcohols (Brampton, Canada). 0.07 *M* triethylamonium acetate was prepared by mixing 14.420 ml of triethylamine and 7.700 ml of glacial acetic acid with distilled deionized water to a final volume of 2 l. The pH was adjusted with acetic acid to 5.2–5.5.

2.2. Chromatographic analysis

The HPLC system consisted of a pump (SpectraSystem P4000; Thermo Separation Products, Freemount, CA, USA) and an autoinjector (WISP 712; Waters, Milford, MA, USA). The column used was a reversed-phase C_{18}, 250×4.6 mm, 5 μm column (Ultrasphere ODS; Beckman Instruments, San Ramon, CA, USA). The column effluent was monitored with UV and fluorescence detectors simultaneously. Ultraviolet detection at 236 nm was performed with a Hewlett-Packard Series 1050 variable-wavelength detector (Waldbronn, Germany) connected to a Chromjet Integrator (Spectra-Physics Analytical, San Jose, CA, USA). Ultraviolet detection at 236 nm and 592 nm was performed using a photodiode array detector (UV6000LP; Thermo Separation Products). Fluorescence detection was performed by a SpectraSystem FL 3000 (Thermo Separation Products). All detectors were ultimately connected to a computer and the data was analyzed by PC-1000 software (Thermo Separation Products). The chromatographic mobile phase used was derived from that used by Piperopoulos et al. [4] with the following changes. The initial conditions were A (methanol–acetonitrile, 5:4)–B (triethylammonium acetate buffer) (70:30). The initial conditions were held constant for 2 min before A was increased linearly to 90% over 8 min and then held constant for 4 min. Then A was further increased to 100% over 2 min and then held for 5 min. The system was returned to the initial conditions (A–B, 70:30) over 1 min and the conditions held constant for at least 4 min before injecting the next sample, giving a total run-time of 26 min. The flow-rate was 1 ml/min. The specificity of the chromatographic separation with each method of detection was determined by visual inspection and comparison of chromatograms as well as by mass spectrum ion scans for co-eluting compounds with a m/z ratio between 350 and 700.

2.3. Methods of detection

2.3.1. Fluorescence

The excitation and emission maxima were determined from scans performed on a SpectraSystem FL3000 fluorometer. Emission was evaluated over a wavelength range from 400 to 700 nm at 2-nm intervals and the maximum was determined to be 592 nm. The excitation wavelength was evaluated the wavelength range from 200 to 350 nm at 2-nm intervals. A maximum was observed at 236 nm.

2.3.2. UV

A photodiode array detector (UV6000LP) was used as the UV detector. This detector was fitted with a deterium lamp (wavelength range 190–350 nm) and a tungsten lamp (range 350–800 nm). The

maximum wavelength for UV absorbance, determined from a scan of the absorbance spectra from 200 to 600 nm identified two peak maxima at 236 and 592 nm.

2.3.3. Mass spectrometry

MS detection was only completed to evaluate assay specificity. Assay validation (accuracy and reproducibility and quantitative analysis was not completed with MS detection. A Perkin-Elmer Sciex API III+ triple quadrupole mass spectrometer (Toronto, Canada) was used in the evaluation of specificity of the method for hypericin and pseudohypericin. The MS conditions were identical to those described by Piperopoulos et al. [4] except that the collision gas densities were $2.82 \cdot 10^{14}$ atoms/cm^2 for hypericin and $4.20 \cdot 10^{14}$ atoms/cm^2 for pseudohypericin. As described by Piperopoulos et al. [4] the orifice plate was kept at 50°C, the operating voltage of the electrospray was −5.0 kV and the interface voltage was set at −650 V. Ion scans for co-eluting ions were performed over a range m/z 350–700 under both hypericin and pseudohypericin peaks and ion scans were performed throughout the chromatograph run time for protohypericin, pseudohypericin, hypericin and protopseudohypericin. Data were acquired and processed using the Sciex Mac Spec data system.

2.4. Evaluation of extraction efficiency

Naphthodianthrones were extracted from capsules and tablets. Since spiking a sample with hypericin and evaluating the extraction was not deemed appropriate because the spiked hypericin would not be within or bound to *Hypericum perforatum* material, the extraction procedure used was validated in three ways. First, the rate of extraction into ethanol was evaluated over time by drawing samples over 120 h. The contents of one capsule or one crushed tablet was placed in a 100-ml volumetric flask and the flask was filled to 100 ml with ethanol. In all studies, immediately following the 0 h sample, the mixture was sonicated in a Ney 300 Ultrasonik for 3 h and then was allowed to stand for 17 h followed by an additional 4 h of sonication. Eight to 10, 1-ml samples were drawn immediately following mixing and at various times the sampling period, which varied between 3 and 120 h. Each 1-ml sample was centrifuged (IEC HN-SII Model 2355; Damon IEC Division, Needham Heights, MA, USA) for 14 min at 2500 rpm (1800 *g*) following removal and 20 μl of the liquid layer was chromatographed using the gradient previously described.

Next, three capsules from the same brand were extracted using three different methods. The first sample was prepared by sonicating the mixture for 3 h and then allowing it to stand for 17 h followed by an additional 4 h of sonication. The second sample was left at room temperature for an equivalent period of time without sonication and the third sample was placed in a Soxhlet extractor for 3 h and then left to stand at room temperature for the remainder of the 24 h time period. After 24 h, a 1-ml sample was removed from each mixture, centrifuged (1800 *g*) and then 20 μl was chromatographed.

The final analysis was done with a sample that was extracted by sonication for 3 h, then allowed to stand for 17 h followed by an additional 4 h of sonication. The entire 100-ml volume was then filtered through a non-aqueous 0.5-μm type 0.5 filter (MicronSep Membrane Filters, Honeoye Falls, NY, USA). The retained residue was then placed in a 100-ml volumetric flask and extracted once again by sonication for 3 h, standing for 17 h followed by an additional 4 h of sonication. Following extraction a sample was removed, centrifuged for 14 min (1800 *g*) and then 20 μl chromatographed.

2.5. Standard curve preparation

Standard solutions of hypericin were prepared by weighing 0.60 mg of a 97.3% pure commercially available standard of hypericin. Quality control (QC) samples were prepared by weighing 0.40 mg of 97.3% pure hypericin. These samples were dissolved in absolute ethanol and standards were prepared by serial dilution. Standard curves were prepared in two different concentration ranges. For most samples, a standard curve of 10 standards was prepared with final concentrations of 14.595, 11.676, 8.757, 5.838, 2.919, 1.168, 0.876, 0.584, 0.292, 0.117 μg/ml and a blank. To determine the limit of quantitation (LOQ) of the LC–fluorescence method and measure the naphthodianthrone concentration in samples with a

concentration below 0.117 μg/ml, a second standard curve was constructed. The concentrations of these standards were 0.117, 0.088, 0.058, 0.029, 0.012, 0.009 μg/ml. Each standard was chromatographed in duplicate.

The column effluent was monitored simultaneously with UV and fluorescence detection. Chromatograms were recorded on computer. The hypericin peak area was determined and standard curves were constructed for both UV at 236 nm and 592 nm and fluorescence by linear regression of peak area and concentration. Acceptable standard curve performance was based on the guidelines set forth by Shah et al. [14]. Specifically, a linear correlation coefficient of 0.993 or greater, deviation of less than 15% {accuracy; [100×(observed−expected)/expected]} and sample replication or method reproducibility error, determined by the relative standard deviation (RSD), of less than 15%. The LOQ was defined as the lowest standard concentration or the concentration that can be determined with deviations from known and method reproducibility error of less than 20%.

2.6. Sample preparation

A total of two brands of capsules, tablets and tinctures were assayed in duplicate on 3 consecutive days. The mean content (μg) for capsules and tablets or concentration (μg/ml) for tinctures was calculated.

2.6.1. Capsules and tablets

The contents of the capsule were emptied into a 100-ml volumetric flask and the flask was filled with anhydrous ethanol to a final volume of 100 ml. Tablets were weighed and then ground with a mortar and pestle into a fine powder. The powder was transferred to a 100-ml volumetric flask with several washings of anhydrous ethanol. The volumetric flask was filled with anhydrous ethanol to a final volume of 100 ml.

Once 100-ml mixtures of the tablets or the contents of a capsule in ethanol were prepared they were sonicated for 3 h then left to stand sealed overnight for 17 h. The samples were then sonicated again for an additional 4 h. The resulting solutions were then allowed to return to room temperature before additional anhydrous ethanol was added to replenish losses due to evaporation for a final volume of 100 ml. The sample was then shaken several times and a portion was removed and centrifuged at 1800 *g* for 14 min. A sample of the liquid layer was removed and 20 μl chromatographed.

2.6.2. Tinctures

Alcoholic tinctures were centrifuged at 1800 *g* for 14 min and run directly unless the concentration was above the concentration of the highest standard in the standard curve. In these situations, the tincture was diluted 1:9 with anhydrous ethanol, centrifuged for 14 min and then a portion of the supernatant was withdrawn and 20 μl chromatographed.

3. Results

3.1. Specificity

The specificity of the assay was determined by visual inspection of chromatograms produced with fluorescence and UV detection as well as through analysis of LC–MS and LC–MS–MS ion scan data. The pseudohypericin and hypericin peaks observed in extracted samples following fluorescence and UV detection were visually inspected for co-eluting and near eluting peaks. Visual examination revealed no evidence of co-eluting peaks with fluorescence detection (Fig. 1a). However, co-eluting peaks were observed in chromatograms observed with UV detection at 236 nm. This is most readily apparent with hypericin and as the hypericin concentration increased, the broadening of the peak began to envelope other closely eluting compounds (Fig. 1b). Similarly, co-eluting peaks were observed in chromatograms observed with UV detection at 592 nm. Again, this was most readily apparent with hypericin and as the hypericin concentration increased, the broadening of the peak began to envelope other closely eluting compounds (Fig. 1c).

3.2. MS analysis

MS analysis of St. John's wort preparations revealed through ion scans that pseudohypericin (m/z 519) and hypericin (m/z 503) were the only

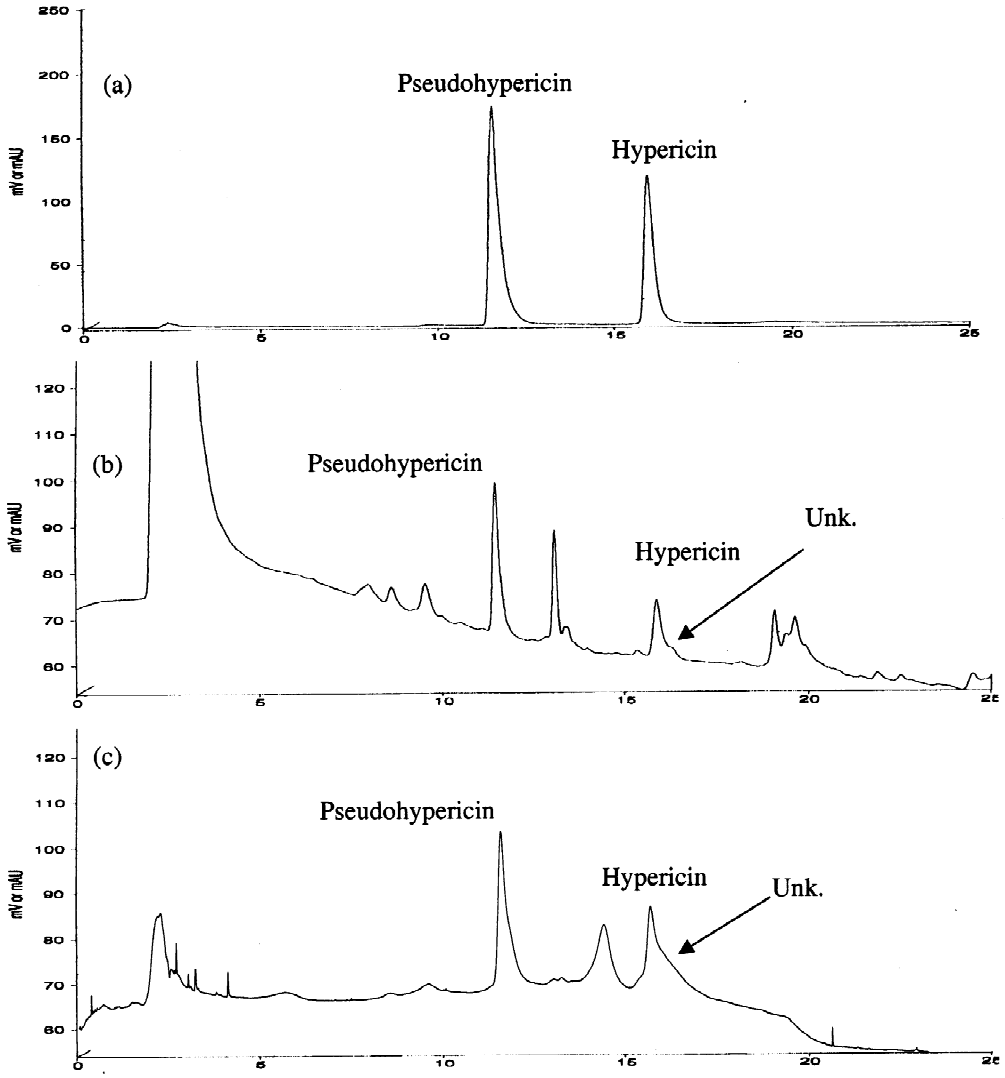

Fig. 1. Typical chromatogram for fluorescence (a, upper chromatogram), UV at 236 nm (b, middle chromatogram), and UV at 592 nm (c, lower chromatogram) detection. The UV chromatograms (b) and (c) show near-eluting peaks, identified as unknowns (Unk), in the hypericin peak. This near-eluting peak is not seen with fluorescence detection (a).

naphthodianthrones present in these samples. Protohypericin (m/z 505) [4] and protopseudohypericin (m/z 521) [4] were not observed. MS–MS analysis of the two peaks believed to be hypericin and pseudohypericin gave daughter ion spectra identical to those reported by Piperopoulos et al. [4], positively identifying both compounds in the sample.

Scanning for ions with m/z of 519 (pseudohypericin) and 503 (hypericin) revealed that there were other ions present in the samples with identical masses (m/z) as those expected for hypericin and pseudohypericin. MS analysis of the expected ion for pseudohypericin revealed several peaks with identical mass that eluted close to pseudohypericin (Fig. 2a). At higher concentrations one of these peaks became enveloped in the pseudohypericin peak (Fig. 2b). An additional co-eluting ion with m/z of 579 was also observed under the pseudohypericin peak. The presence of co-eluting peaks was further illus-

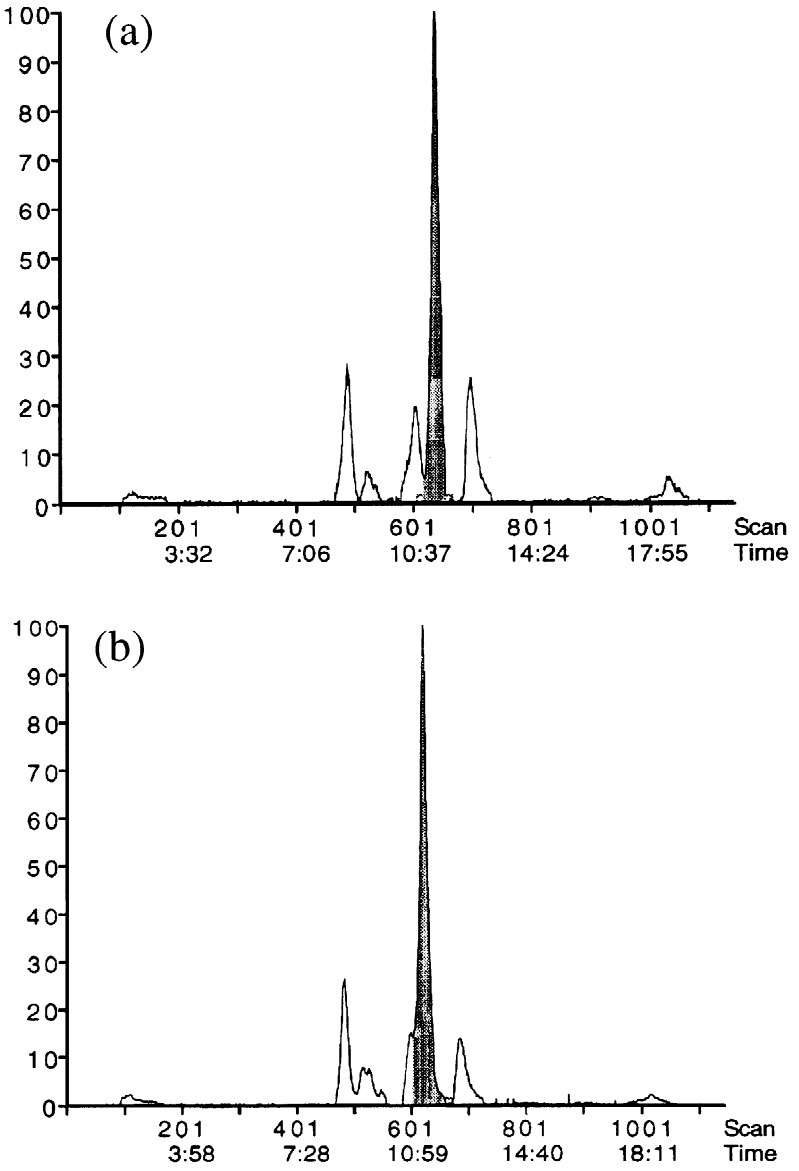

Fig. 2. Chromatogram with MS detection for ions with m/z of 519. Upper chromatogram shows a sample with a low concentration of pseudohypericin where pseudohypericin and a closely eluting peak, also with a m/z of 519 are nearly resolved. The lower chromatogram shows a sample with a higher concentration of pseudohypericin that envelops the closely eluting peak.

trated with MS analysis by two distinctive co-eluting ions of m/z 519 and m/z 555 under the hypericin peak (m/z 503).

3.3. Extraction validation

Results of comparisons between Soxhlet extraction, the method involving sonication and letting a sample stand in ethanol at room temperature revealed that method involving sonication was equivalent to Soxhlet extraction and more efficiently extracted hypericin and pseudohypericin than a sample allowed to stand for 24 h. Sequential sampling of a sonicated sample (Table 1) revealed that more than 90% of both hypericin and pseudohypericin were extracted with 30 min of sonication. However,

Table 1
Extraction of pseudohypericin and hypericin into ethanol from crushed tablets and capsules[a]

Time (h)	Hypericin	Pseudohypericin
0.00	7.34	7.11
0.08	45.24	44.91
0.33	89.33	95.15
0.50	93.09	109.05
3.00	98.13	101.97
24.00	100.00	100.00
120.00	101.54	102.75

[a] Expressed as the percent of the amount observed at 24 h. Individual values represent the mean value observed in one to four different studies. Data for times of 0.18, 0.75, 1.0, 1.33, 1.85, 2.22, 4, 17 and 20 h are not shown.

extraction for 24 h followed by filtration and re-extraction resulted in detection of an additional 2.4% of naphthodianthrones extracted. Therefore, although increases in the amount extracted after 3 h of sonication appeared minimal, since an additional 2.4% could be extracted even after 24 h, all samples were analyzed using the 24-h extraction procedure with sonication.

3.4. Fluorescence – accuracy, precision and LOQ

Analysis of standards indicated that standard curves were linear ($r>0.993$) in the concentration range from 0.117 to 14.595 μg/ml and from 0.009 to 0.117 μg/ml, both with insignificant *y*-intercepts (Table 2). Blanks indicated that the matrix (ethanol) had no peaks at the retention time of pseudohypericin or hypericin. Analysis of standards and QC samples demonstrated acceptable precision and accuracy (Table 2). All concentrations between 14.595 and 0.292 μg/ml had replicate error for samples analyzed in duplicate averaging less than 5.3%

Table 2
Reproducibility[a] and accuracy[b] for hypericin standards

Concentration (μg/ml)	UV at 236 nm LOQ: 584 μg/unit or 5.84 μg/ml	UV at 592 nm LOQ: 8.8 μg/unit or 0.088 μg/ml	Fluorescence LOQ: 0.88 μg/unit or 0.009 μg/ml
Mean within-day/between-day reproducibility of duplicate standards			
14.60	0.55/3.21	1.27/2.59	0.25/0.59
5.84	1.92/2.34	3.42/4.12	2.83/3.26
1.17	2.65/42.70	10.65/7.90	2.95/2.64
0.58	7.33/56.58	1.59/5.53	3.27/3.45
0.29	5.17/14.49	7.43/11.45	2.18/10.39
LOQ (μg/ml)	5.84	0.088	0.009
Within-/between-day reproducibility at the LOQ	1.92/2.34	6.92	4.35/5.47
Mean percent deviations for standards (sign ignored)			
14.60	3.09	1.11	0.94
5.84	3.26	4.86	2.75
1.17	1.97	3.89	2.82
0.58	1.44	6.43	3.59
0.29	9.12	13.78	7.18
LOQ (μg/ml)	5.84	0.088	0.009
Mean percent deviation at the LOQ	3.26	8.69	3.88
Typical standard curve endpoints			
Slope	42 561	270 714	443 118
Intercept	6841	−4953	16 066
(low concentrations)	(not run)	(390.3)	(−109.9)
r-Value	0.9985	0.9995	0.9998

[a] Error observed in duplicate determinations expressed as the relative standard deviation.
[b] Error expressed as percent deviation between observed result and known concentration.

within-day and less than 10.4% between-days. Similarly, accuracy (deviation from known) was less than 10% for all concentrations. At 0.117 μg/ml, between-day replication error and the percent deviation averaged 21.3% and 26.99%, respectively. Analysis of concentrations in a lower concentration range (0.009 to 0.117 μg/ml) indicated replicate errors of less than 6% for all samples within- and between-days and deviation from known of less than 4% (Table 2). Since the lowest concentration tested of 0.009 μg/ml had acceptable accuracy and reproducibility, this was taken as the LOQ. However, reproducible and accurate detection of lower concentrations with errors less than 15–20% [14], would appear to be possible.

3.5. UV – accuracy, precision and LOQ

Analysis of standards indicated that standard curves at both 236 nm and 592 nm were linear ($r>0.993$) in the concentration range from 0.117 to 14.595 μg/ml and from a concentration of 0.029 to 0.117 μg/ml at 592 nm. All standard curves had insignificant *y*-intercepts. (Table 2). Blanks indicated that the matrix (ethanol) had no peaks at the retention time of pseudohypericin or hypericin at either wavelength. For concentrations between 14.595 and 0.292 μg/ml, within-day replicate error for samples analyzed in duplicate, was less than 15%, but at 236 nm, poor estimates on 2 of 7 days resulted in unacceptably large between-day error (Table 2). There was less than 10% deviation from known (accuracy) for concentrations of 0.292 μg/ml or greater, but at 0.117 μg/ml the deviation from the known concentration approached 35% with the higher concentration range standard curve. Analysis of concentrations in the lower concentration range (0.009 and 0.117 μg/ml) indicated that UV absorbance at 236 nm was unable to detect the presence of either hypericin or pseudohypericin, while at 592 nm detection was possible, although it was not reproducible below a concentration of 0.088 μg/ml. Since the LOQ is the lowest concentration that can be determined with acceptable accuracy and precision [14], the LOQ of the method using UV detection at 236 nm was determined to be 5.84 μg/ml where the RSD was less than 3% between and within days and there was less than 4% deviation from the known concentration. If the two days of poor estimates are removed, the LOQ would be 0.292 μg/ml. With detection at 592 nm, with appropriate adjustment of the concentration range, the LOQ of the method was 0.088 μg/ml.

The specificity of the separation with UV detection at both 236 nm and 592 nm was less than satisfactory due to the presence of visibly near-eluting peaks, most obviously near the hypericin peak. These co-eluting peaks were more obvious at higher concentrations (Fig. 1b and c). This lack of specificity is further supported by the presence of near-eluting species seen under both the pseudohypericin and hypericin peaks when scanned using MS (Fig. 2). Poor specificity translates into an overestimate of the hypericin and pseudohypericin concentrations observed in the samples (Tables 3 and 4). For the single estimate where both UV at 236 nm and LC–fluorescence are above the LOQ, UV detection found 44.5% more hypericin (Table 3) and a similar amount of pseudohypericin (Table 4). For estimates where both UV at 592 nm and LC–fluorescence are above the LOQ, UV detection found at least 13.2% more hypericin (Table 3) and between 24 and 67% more pseudohypericin (Table 4).

3.6. Sample analysis

Fluorescence analysis of the St. John's wort preparations gave two distinct peaks (Fig. 1a), one for hypericin and the second for pseudohypericin verified by MS and by retention time for hypericin. Chromatography with UV detection at either 236 nm or 592 nm gave numerous peaks with several compounds eluting with similar or identical retention times to hypericin and pseudohypericin (Fig. 1b and c). The observed hypericin and pseudohypericin content of two different brands of St. John's wort capsules, tablets and tinctures are provided in Tables 3 and 4. UV analysis of the samples at both wavelengths generally resulted in higher concentrations of hypericin and pseudohypericin than those measured by fluorescence.

4. Discussion

With many countries contemplating the regulation and standardization of herbal products, reliable and accurate methods for analysis and quantification are

Table 3
Mean amount or concentration of hypericin in commercial formulations of St. Johns wort

Dosage form	Brand	Detection method		
		UV at 236 nm, LOQ: 584 μg/unit or 5.84 μg/ml	UV at 592 nm, LOQ: 8.8 μg/unit or 0.088 μg/ml	Fluorescence, LOQ: 0.88 μg/unit or 0.0088 μg/ml
Capsule (μg/capsule)	Twin Labs	530.13 μg[a]	529.51 μg	467.78 μg
	Herbtech	105.47 μg[a]	73.42 μg	1.10 μg[b]
Tablet (μg/capsule)	Your Life	292.29 μg[a]	292.83 μg	233.45 μg
	Holista	90.19 μg[a]	79.36 μg	25.74 μg
Tincture (μg/ml)	Sisu	40.76 μg/ml	54.24 μg/ml	28.20 μg/ml
	Naturally Nova Scotia	<5.84 μg/ml[a]	<0.088 μg/ml[b]	<0.0088 μg/ml[b]

[a] Below the limit of quantification on all measurements.
[b] Below the limit of quantification on one of the three measurements.

required. The current USP method of analysis used by many herbal manufacturers relies on spectrophotometric analysis. Inspection of chromatograms produced with UV detection especially at 236 nm but also at 592 nm indicates that pseudohypericin and hypericin are but two of the detected compounds. Even using LC with UV detection at 236 or 592 nm, while it improves specificity, does not result in specific detection of either pseudohypericin or hypericin. This results in over estimates of the hypericin and pseudohypericin concentrations in St. John's wort preparations.

A comparison of the assay validation for co-determined UV and fluorescence spectrometry of St. John's wort preparations indicates that fluorescence methods offer many advantages over other methods like UV at 236 nm and 592 nm. The higher degree of specificity eliminates many of the interfering compounds seen under UV analysis at both wavelengths and even under MS analysis. Fluorescence chromatograms gave only two distinct peaks during the chromatographic run while UV analysis at 236 nm and 592 nm gave numerous peaks. The two peaks observed in fluorescence chromatography corresponded to pseudohypericin and hypericin. The overestimates of hypericin and pseudohypericin in the samples analyzed by UV analysis at 236 nm and 592 nm can be avoided through the use of fluorescence due to its specificity for the naphthodianthrones. While UV analysis at 592 nm provides improved sensitivity over UV at 236 nm it is not as sensitive as fluorescence detection. UV analysis was only able to

Table 4
Mean amount or concentration of pseudohypericin in commercial formulations of St. Johns wort

Dosage form	Brand	Method of detection		
		UV at 236 nm, LOQ: 584 μg/unit or 5.84 μg/ml	UV at 592 nm, LOQ: 8.8 μg/unit or 0.088 μg/ml	Fluorescence, LOQ: 0.88 μg/unit or 0.0088 μg/ml
Capsule	Twin Labs	941.46 μg	1023.33 μg	823.35 μg
	Herbtech	8.12 μg[a]	32.93 μg	<0.88 μg[a]
Tablet	Your Life	444.14 μg[a]	520.63 μg	405.10 μg
	Holista	39.13 μg[a]	79.76 μg	47.73 μg
Tincture	Sisu	50.18 μg/ml	67.43 μg/ml	48.67 μg/ml
	Naturally Nova Scotia	<5.84 μg/ml[a]	<0.088 μg/ml[a]	<0.0088 μg/ml[a]

[a] Below the limit of quantification on all measurements.

accurately detect on column amounts of 117 ng or greater at 236 nm and 1.8 ng or more at 592 nm while fluorescence analysis was able to accurately detect on column amounts as low as 0.18 ng.

LC–fluorescence was also more specific than LC–UV at either wavelength and may actually be as specific as direct probe MS–MS.

Measures of accuracy and reproducibility (Table 2) are based on spiking ethanol standards and QCs with a known amount of hypericin. Due to the correlation between the concentration of pseudohypericin/hypericin and interfering substances, an inability to prepare a St. John's wort sample which was naphthodianthrone free but contained interfering substances, and the lack of a pure pseudohypericin standard, accuracy reported in Table 2 does not reflect the interference observed in chromatograms of samples or that seen with LC–MS analysis. The observation of interference and inaccuracy of the UV methods is based on analysis of LC–MS data and a comparison of the amount found in commercial formulations using UV and fluorescence detection.

5. Conclusion

After running several samples with this method it became evident that fluorescence is specific enough for simple quantification of total naphthodianthrones like hypericin and pseudohypericin that it could potentially be accomplished by simple fluorescence detection without chromatography. With only two peaks, identified as pseudohypericin and hypericin, eluting from the column when detected by fluorescence, the specificity of this method makes it an ideal choice for analysis of naphthodianthrones in pharmaceutical preparations. Chromatography is still required for specific quantification of the individual naphthodianthrones, unless direct probe MS–MS detection is used.

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