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SPECTROFLUORIMETRIC DETERMINATION OF HYPERICIN IN DRUGS AND VEGETABLE RAW MATERIALS

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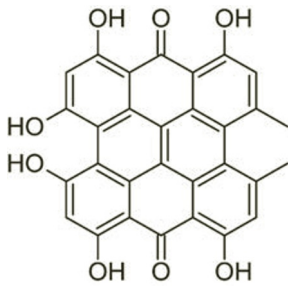
Hypericin was isolated from Diahyperon tincture for use as a standard sample. The chromatographic purity of the hypericin was confirmed by high-performance liquid chromatography/mass spectrometry. Hypericin has the only chromatographic peak with a retention time of 23.10 min and gave a characteristic signal for the molecular ion $[M - H^+]^-$ *with m/z 504.05 in the mass spectrum. Electronic absorption spectra of Diahyperon tincture and the MeOH extract of Hyperici herba (Hypericum perforatum) showed characteristic hypericin bands at 550 and 590 nm and a band at 665 nm that was due to the presence of chlorophyll. It was shown that excitation of hypericin in Diahyperon tincture at* λ_{ex} *= 470 nm gave an emission spectrum with characteristic hypericin bands at 593 and 640 nm while the emission spectrum of the MeOH extract of Hyperici herba showed the hypericin emission bands and a weak chlorophyll emission band at 670 nm. It was found that spectrophotometric and spectrofluorimetric determinations of hypericin in Diahyperon tincture and in Hyperici herba gave similar results that were consistent with the standardized values of the hypericin content in the studied samples.*

Keywords: *hypericin, Hyperici herba, electron spectroscopy, fluorescence spectroscopy.*

Introduction. Hypericin (4,5,7,4′,5′,7′-hexahydroxy-2,2′-dimethylnaphthodianthrone)



is a biologically active compound of the herb St. John's wort. Preparations based on hypericin are widely used in contemporary medicine as antidepressant, anti-inflammatory, antibacterial, antiviral, and photo-sensitizing drugs [1].

Chromatographic, chromato–mass-spectrometric, and spectrophotometric analytical methods are used for quantitative determination of hypericin in drugs and vegetable raw material. Most publications on the isolation and identification of hypericin and its derivatives have focused on thin-layer (TLC) and high-performance liquid chromatographic (HPLC) methods followed by mass spectrometric or spectrophotometric detection [2–6]. However, comparisons of the experimental results and their reproducibility are inadequate because columns with the same bonded phase but from different manufacturers were used for the extraction and identification so that the hypericin had different activities. HPLC with fluorimetric detection followed by confirmation of the results by mass spectrometry was used for extraction and quantitative determination of hypericin and its derivatives [7, 8]. The above methods are highly sensitive but require costly equipment and qualified personnel to perform the complicated sample preparation and analysis.

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A few studies focused on electroanalytical determination methods. For example, capillary electrophoresis was used to isolate total hypericins with subsequent amperometric detection after oxidation of the analyte on a glassy-carbon electrode at a potential of +1.1 V [9]. Spectrophotometric analytical methods for hypericin and its derivatives based on visible absorption spectra were the simplest and least expensive [10, 11]. A method for quantitative determination of total hypericins recalculated as hypericin in Hyperici herba included double extraction of hypericin from ground vegetable raw material by aqueous THF, evaporation, and dissolution of the dry residue in MeOH followed by measurement of the extinction at $\lambda = 590$ nm [12]. The main drawbacks of spectrophotometric determination of hypericins are the poor sensitivity and selectivity due to contributions of other constituents of the vegetable raw material to the analytical signal in the used wavelength range.

Hypericin is known to possess rather strong intrinsic fluorescence [13]. Therefore, spectrofluorimetric determination of it could be an alternative to existing analytical methods. Fluorescent methods are more sensitive and selective, have a broader range of determined concentrations, and are more reproducible than spectrophotometric methods. The simplicity of the apparatus and low equipment costs for performing spectrofluorimetric methods are also important.

The goal of the present work was to develop science-based methodology for quantitative determination of hypericin in drugs and vegetable raw material using fluorescence spectroscopy. For this, hypericin reference standard had to be obtained from Diahyperon tincture, the optimal conditions for spectrofluorimetric determination of hypericin in drugs and vegetable raw material had to be selected considering the presence in the *H. perforatum* extract of a significant amount of chlorophyll, and the results of spectrofluorimetric determination of hypericin had to be compared with those obtained from spectrophotometric analytical methods [12, 14].

Experimental. Diahyperon tincture (NPUP Dialek), which was the extract of Hyperici herba in 70% EtOH, was used to obtain hypericin reference standard. Hypericin was isolated using TLC on Silica gel 60 TLC chromatography plates (20 × 20 cm, Merck, Millipore). A continuous band of tincture (280 μL) was placed at the origin. The eluent was EtOAc:HOAc in a 50:5 ratio. After the chromatography was finished, the plate was dried in air in the dark. The band with $R_f = 0.61$ (hypericin) was scraped from the plate. Extraction of the obtained silica gel with EtOAc (2×) produced a solution of hypericin reference standard. The extract was evaporated to dryness in an RV 8 rotary evaporator (IKA) at 35°C.

LC-MS analysis of the isolated hypericin used a Waters Micromass ZQ 2000 LC-MS equipped with a Waters Symmetry C18 column (250 × 4.6 mm, 5 μm). The mobile phase was a gradient of MeCN (solution A) and aqueous NH_4OAc (0.01 M, solution B) (A:B, %: 0 min, 15:85; 0–5 min, 30:70; 5–10 min, 45:55; 10–15 min, 60:40; 15–20 min, 75:25; 20–40 min, 90:10). The flow rate was 1 mL/min.

Electronic absorption spectra in the range $\lambda = 450$–800 nm and extinction at 590 nm were recorded on a Specord 200 Plus spectrophotometer (Analytik Jena) using a 1-cm cuvette. Spectrofluorimetric measurements at $\lambda_{ex} = 470$ nm and emission at $\lambda_{em} = 592$ nm with slit width 2.5 nm were made on an FP-8500 spectrofluorometer (Jasco).

Hypericin solutions for constructing a calibration curve in the concentration range $(0.20–2.06)\cdot10^{-6}$ M were prepared by sequential dilution of the reference standard solution (in MeOH) of concentration $4.12\cdot10^{-6}$ M. Extract of Hyperici herba in MeOH was obtained by the literature method [12].

Results and Discussion. Isolated hypericin was dissolved in MeOH. The electronic absorption spectrum of the resulting solution was recorded (Fig. 1). Characteristic hypericin absorption bands with maxima at 550 and 590 nm were observed and agreed with the literature [11, 15]. The chromatographic purity of the reference standard was confirmed by the chromatogram and mass spectrum of the isolated hypericin (Fig. 2). Hypericin was the only chromatographic peak with retention time 23.10 min and gave a characteristic peak for the molecular ion $[M - H^+]^-$ with m/z 504.05 in the mass spectrum.

Electronic absorption spectra of Diahyperon tincture and the MeOH extract of Hyperici herba (Fig. 1) showed characteristic hypericin bands and an absorption band with $\lambda_{max} = 665$ nm due to the presence of chlorophyll [15–17]. The strong absorption band of chlorophyll could partially overlap the hypericin analytical band and distort the results of spectrophotometric hypericin determination in the extracts of the vegetable raw material.

The effect of chlorophyll on hypericin fluorescence was assessed by recording emission spectra of the MeOH extract of Hyperici herba at various excitation wavelengths (Fig. 3a). The emission spectra showed maxima at 592 and 640 nm that were characteristic of hypericin [15, 18, 19] and a maximum at 670 nm due to the presence of chlorophyll [20, 21]. An analysis of the obtained emission spectra allowed the hypericin excitation wavelength to be chosen as 470 nm, which was optimal for quantitative determination of hypericin in drugs and vegetable raw material because chlorophyll fluorescence was held to a minimum at this λ_{ex}. Hypericin emission spectra have also been recorded before at $\lambda_{ex} = 470$–476 nm [22–25]. Figure 3b shows that chlorophyll fluorescence was not observed in the emission spectrum of Diahyperon tincture.

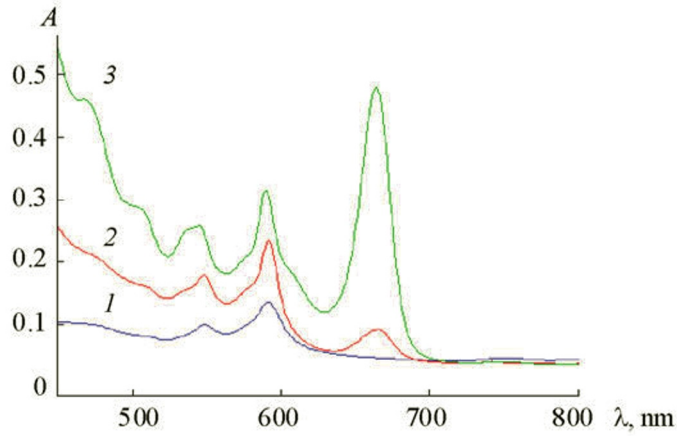

Fig. 1. Electronic absorption spectra of a MeOH solution of isolated hypericin (1), Diahyperon tincture (2), MeOH extract of Hyperici herba (3).

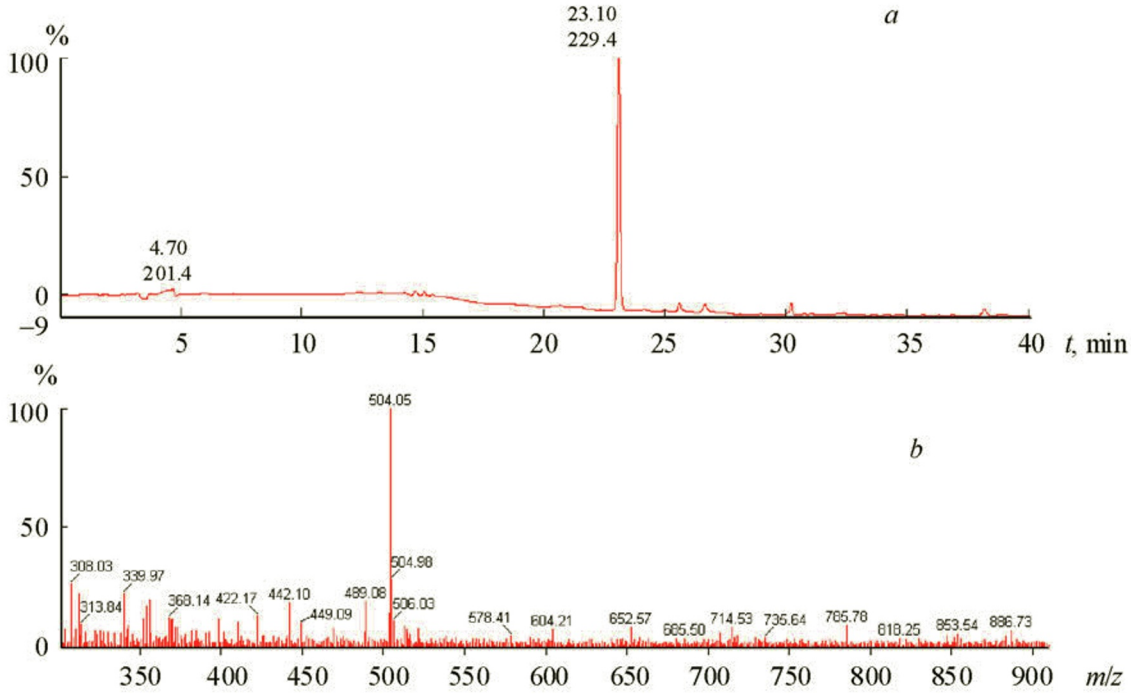

Fig. 2. Chromatogram (a) and mass spectrum (b) of isolated hypericin.

A calibration curve for the dependence of the fluorescence intensity of MeOH solutions of hypericin on its concentration was constructed for quantitative determination of hypericin in Diahyperon tincture and Hyperici herba (Fig. 4). The hypericin contents in the samples were determined from the obtained intensities of the analytical signal at λ_{em} = 592 nm taking into account the dilutions. The results for quantitative determination of hypericin obtained by fluorescence spectroscopy were compared with those for spectrophotometric analytical methods for Diahyperon tincture [14] and Hyperici herba [12] (Table 1). The specific absorption coefficient of hypericin at 590 nm in EtOH was 718; in MeOH, 870, according to the aforementioned regulatory documentation. The published molar absorption coefficients of hypericin were $(4.0–4.6)\cdot10^4$ $M^{-1}\cdot cm^{-1}$ in EtOH [22, 26, 27] and $(3.4–5.2)\cdot10^4$ $M^{-1}\cdot cm^{-1}$ in MeOH [28]. Spectrophotometric and spectrofluorimetric determination of hypericin in Diahyperon tincture and Hyperici herba gave similar results that agreed with the standard hypericin content in the studied samples [12, 14].

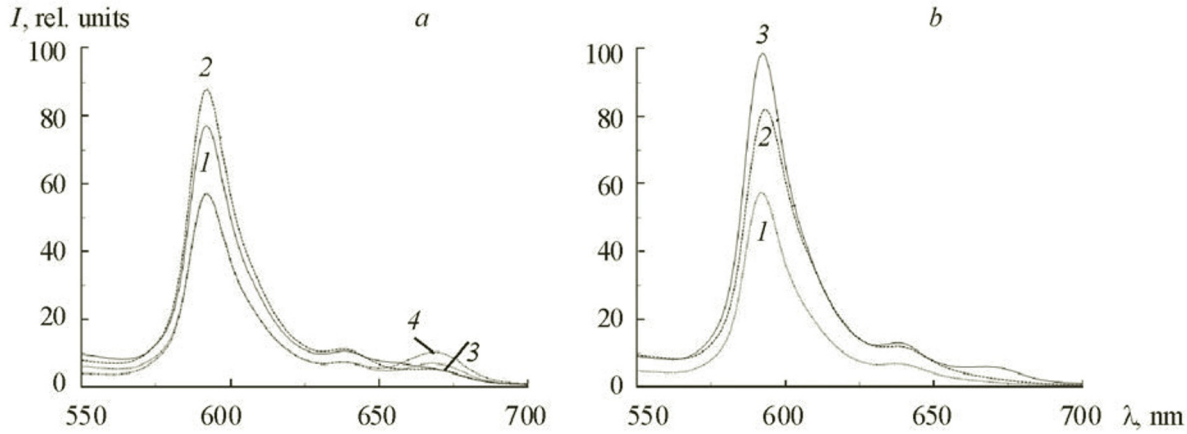

Fig. 3. Emission spectra of MeOH extract of Hyperici herba for λ_{ex} = 450 (1), 470 (2), 490 (3), and 510 nm (4) (a) and a solution of hypericin reference standard (1), Diahyperon tincture (2), and MeOH extract of Hyperici herba (3) for λ_{ex} = 470 nm (b).

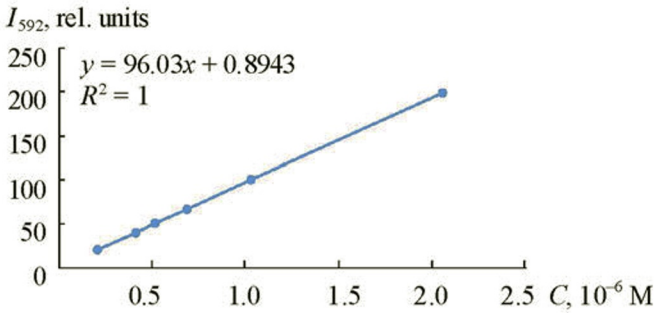

Fig. 4. Dependence of fluorescence intensity of hypericin in MeOH solutions on its concentration.

TABLE 1. Quantitative Determination of Hypericin in Diahyperon Tincture and Hyperici Herba

Hypericin-containing sample	Spectrofluorimetry		Spectrophotometry
	C, M	*X*, %	*X*, %
Diahyperon tincture	$3.25 \cdot 10^{-4}$	0.018 ± 0.0006	0.016 ± 0.0015
Hyperici herba	$5.33 \cdot 10^{-5}$	0.084 ± 0.0009	0.086 ± 0.0012

Note: *C*, molar concentration of hypericin in tincture/extract; *X*, hypericin content in tincture/extract; results are given as means of three independent measurements ± standard deviation.

Conclusions. Conditions for spectrofluorimetric determination of hypericin in hypericin-containing samples that avoided the effect of chlorophyll were selected using chromatographically pure hypericin obtained from Diahyperon tincture by TLC. Spectrophotometric determination of hypericin performed according to compendial methods showed that the results for the hypericin content in Diahyperon tincture and Hyperici herba agreed with the standard values. The similarity of the results obtained by the two spectral methods indicated that chlorophyll did not affect the quantitative determination of hypericin. Thus, fluorescence spectroscopy could be used as an alternative method for quantitative determination of hypericin in drug and vegetable raw material.

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