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Development and validation of a fluorometric method for the determination of hesperidin in human plasma and pharmaceutical forms

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Abstract: A fluorometric method, based on the fluorescence properties of the aluminium(III)–hesperidin complex, for the determination of hesperidin in human plasma and pharmaceutical forms has been developed and validated. The complex shows a strong emission in the presence of the surfactant betain sulphonate SB 12 at 476 nm with excitation at 390 nm. The linearity range for pharmaceutical forms of hesperidin was 0.06–24.4 μg mL^{-1} with a limit of detection, *LOD*, of 0.016 μg mL^{-1} and a limit of quantification, *LOQ*, of 0.049 μg mL^{-1}. Recovery values in the range 99.3–99.7 % indicate good accuracy of the method. A linear dependence of the intensity of fluorescence of the complex on the concentration of hesperidin in plasma was obtained in concentration range from 0.1–12.2 μg mL^{-1}. The *LOD* was 0.032 μg mL^{-1} while *LOQ* was 0.096 μg mL^{-1}. Recovery values were in the range 98.4–99.8 %. The reliability of the method was checked by an LC–MS/MS method for plasma samples and an HPLC/UV method for tablets with direct determination of hesperidin after separation. Linearity range in determination of hesperidin in pharmaceutical forms was obtained in the range from 0.05 to 10.00 μg mL^{-1}. The *LOD* was 0.01 μg mL^{-1} and the *LOQ* was 0.03 μg mL^{-1}. The linearity range for the determination of hesperidin in plasma was 0.02–10.00 μg mL^{-1} with an *LOD* 0.005 μg mL^{-1} and an *LOQ* of 0.015 μg mL^{-1}. The good agreement between the two methods indicates the usability of the proposed fluorometric method for the simple, precise and accurate determination of hesperidin in clinical and quality control laboratories.

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Keywords: hesperidin; human plasma; tablets; fluorometry; LC–MS/MS.

INTRODUCTION

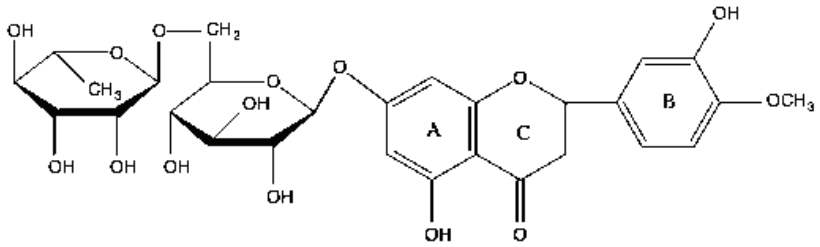
Flavonoids constitute a large group of naturally occurring phenolic compounds distributed in the plant kingdom. They are regularly consumed as food (*e.g.*, vegetables and fruits) and beverages such as tea and red wine. According to their chemical structure, they are usually subdivided into flavanols, anthocyanidins, flavones, flavanones and chalcones. These different flavonoids are reported to possess a wide range of biological activities, including anti-inflammatory, anti-oxidant, anti-allergic, hepatoprotective, anti-thrombotic, anti-viral, cardiovascular and anti-carcinogenic effects.[1]

Flavonoids are also effective metal ion chelators and form stable products that in several cases are highly fluorescent, a property which has been used in analytical methods for metal and ligand identification.[2,3] It is well known that they can chelate metallic ions such as beryllium (II),[4] aluminium(III),[5] iron(III),[6] and zinc(II) ion.[7] These complexes shown intense fluorescence signal which increases with metal concentration.

Hesperidin:

(2*S*)-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5--trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxy-2,3-dihydrochromen-4-one (Scheme 1),

is a flavanone-type flavonoid that is abundant in citrus fruit[8] and has been reported to exert a wide range of pharmacological effects.[9]



Scheme 1. The molecular structure of hesperidin.

Hesperidin has also been reported to possess anti-inflammatory, anti-allergenic, antihypertensive, antimicrobial, and vasodilatory properties and to decrease bone density loss.[10–12] Taking into account the biological effects of hesperidin, it is of interest to develop simple, accurate and precise methods for its determination in human plasma and pharmaceutical forms.

Various methods have been developed for the determination of hesperidin, such as high-performance liquid chromatography with UV/Vis and electrochemical detection modes,[13,14] liquid chromatography/mass spectrometry (LC–MS),[15] radio-immunoassey,[16] adsorptive-stripping voltammetry,[17] cathodic-stripping vol-

tammetry,[18] spectrophotometry,[19,20] spectrofluorometry with manual and flow injection methods[21] and pulse perturbation of an oscillatory reaction system.[22]

Perez-Ruiz *et al*.[21] developed and validated a spectrofluorimetrc method for the determination of hesperidin in orange peel and orange juice based on complexation between hesperidin and the aluminium(III)-ion in a micellar (sodium dodecylsulphate) medium. Manual and flow injection procedures were used for samples introduction using an excitation wavelength of 391 nm and an emission wavelength of 496 nm. The linear concentration range for hesperidin was between 5×10^{-7} to 2×10^{-5} mol L^{-1}, with a detection limit of 79 μg L^{-1}.

In this work, the same basic chemistry of the fluorescence reaction between aluminium (III)-ion and hesperidin for the determination of hesperidin in human plasma and pharmaceutical forms was employed. The zwiterionic surfactant sulphobetaine SB 12 (3-(*N*-hexadecyl-*N*,*N*-dimethylammonio)propane sulphonate) was used for fluorescence enhancement with an excitation wavelength of 390 nm and an emission wavelength at 476 nm. As comparative methods, an LC-MS/MS determination of hesperidin in human plasma and an HPLC/UV method for its determination in some pharmaceutical formulations were used.

EXPERIMENTAL

Materials and solutions

Aluminium nitrate, hesperidin ($C_{28}H_{34}O_{15}$; M_r = 610.56 g mol^{-1}; CAS number 520-26-3) (Fluka AG), methanol, NaOH, CH_3COOH (Merck), all *p.a*. grade, were used. SB 12 or (*n*-dodecyl sulphobetaine) (Serva, Germany) and sodium dodecylsuphate (SDS, Sigma Aldrich) were used. All reagents were employed without further purification. A stock solution of aluminium nitrate was prepared by dissolving $Al(NO_3)_3$ in doubly distilled water with the addition of an appropriate amount of nitric acid to prevent the initial hydrolysis of the aluminium(III) ion. The content of Al(III) ions was determined gravimetrically by precipitation with ammonia. A solution of hesperidin was prepared by dissolving a precisely measured mass of hesperidin in 70 vol. % methanol. A solution of SB 12 (0.5 mol L^{-1}) was prepared by dissolving a precisely measured mass of SB 12 in deionised water. A solution of SDS (0.5 mol L^{-1}) was prepared by dissolving a precisely measured mass of SDS in deionised water. These solutions were stored in a refrigerator.

Human pool plasma was obtained from the Department of Transfusion of the Clinical Hospital "Dr Dragiša Mišović", Belgrade, Serbia. Helopyrin tablets (nominal composition vitamin C 120 mg, bioflavonoids 20 mg, rutin 15 mg, excipients: microcrystalline cellulose, methylhydroxypropyl cellulose, Mg-stearate, starch hydrolysate) were from Rosch & Handel, (Vienna, Austria) and Vitamin C 1500 tablets with hesperidin were from American Nutrition Products.

Working solutions were prepared by dilution of stock solution and aluminium nitrate (1.00×10^{-3} mol L^{-1} $Al(NO_3)_3$) and hesperidin (1.00×10^{-4} mol L^{-1}).

Instruments

The fluorescence spectra were collected using a Fluorolog-3 spectrofluorimeter (Jobin Yvon Horiba, Paris, France) equipped with a 450 W xenon lamp and a photomultiplier tube. The samples were placed in a 1 cm optical path length quartz cuvette for spectral recording.

The slits on the excitation and emission beams were both set at 5 nm. The spectra were corrected for the dark counts. In each measurement, three scans with a one-second-integration time were averaged. The emission spectrum of the solvent (70 vol. % methanol) was subtracted. All measurements were performed at 25 °C controlled by a Peltier element. The pH measurements of were realised using a Metler Toledo mp 120 pH-meter (precision: ±0.01 pH unit) equipped with a combined glass electrode. All spectrofluorometric measurements were made in acetate buffers at pH 4.58 (in 70 vol. % methanol) which was prepared according to Perrin.[23]

Chromatographic measurements were carried out using the HPLC system Perkin Elmer PE200 (Norwalk, CT, USA), composed of a binary pump, an autosampler (injection volume 20 µL) and equipped with a Gemini C_{18} column (150×4.6 mm, 3 µm, Phenomenex, CA, USA), a column thermostat and variable UV–Vis detector operating at 280 nm. The experimental conditions were as follows: mobile phase A: 2% acetic acid; mobile phase B: acetonitrile, mixed in a linear gradient, 0–5 min: 85 % A, 15 % B; 25–30 min: 10 % A, 90 % B; 35–40 min: 85 % A, 15 % B; flow rate: 0.7 mL min^{-1}; injection volume: 20 µL.

Mass spectrometric conditions

A 3200 QTRAP MS/MS mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) with ESI ionisation (ESI and turbo-ion spray, TIS) was employed. The data were processed using Analyst TM software (PE Sciex). The negative ionization mode of the LC–MS/MS was used for monitoring selected multiple reaction (SRM) analyses with a dwell time 50 ms. The mass spectrometric parameters were optimized to maximize the SRM sensitivity by injecting a 100 µg mL^{-1} standard solution of hesperidin in methanol using a syringe. The optimized instrument conditions were as follows: interface temperature, 500 °C; ion spray voltage, 4500 V; curtain gas He at 10 psi; nebulizing gas N_2 at 40 psi; TIS gas He at 60 psi; declustering potential (DP), 57 V; entrance potential (EP), –5 V; collision energy (CE) –34 eV and collision cell exit potential (CXP), 9 V. The signal used to detect and quantify hesperidin was SRM transition at *m*/*z* 609 → 325. The mass spectrometer was operated at unit mass resolution for both the Q1 and Q3 quadrupoles.

RESULTS AND DISCUSSION

Complex formation between hesperidin and aluminium(III)-ion

Hesperidin and aluminium(III) ions upon reaction in methanolic solution form a complex in the pH range 3.0–7.0. The stoichiometry of the complex was estimated by the Job method[24] and by the mole ratio method.[25]

The reaction of hesperidin and aluminium(III) ions in methanolic solution in the pH range 3.0–7.0 leads to the formation of a complex, as evidenced by the large increase of fluorescence intensity (order of magnitude 10^5) and bathochromic shift (*ca*. 50 nm) of the emission band of hesperidin in the presence of aluminium(III) ions. The fluorescence spectra were recorded using 70 % v/v methanol as a blank. The excitation spectra were followed at λ_{em} = 490 nm and the excitation wavelength used for recording the emission spectra was λ_{ex} = 390 nm. The excitation (1′) and emission (1) spectra of the aluminium(III)–hesperidin solution and the excitation (2′) and emission (2) spectra of hesperidin are shown in Fig. 1.

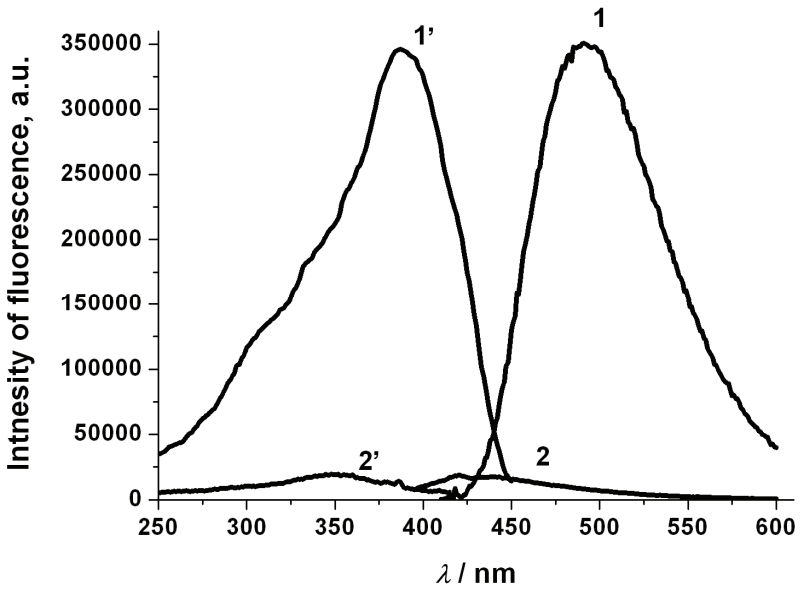


Fig. 1. Exitation (1′) and emission (1) spectra of the aluminium (III)–hesperidin complex and exitation (2′) and emission (2) spectra of hesperidin.

As indicated by the Job[24] method, the most probable stoichiometry of the complex formed at pH 4.58 is aluminium:hesperidin = 1:1. The mole ratio method[25] for the determination of the composition of the aluminium(III)–hesperidin complex formed at pH 4.58 confirmed the 1:1 ratio for the complex (Fig. 2).

Thus, the complexation equilibrium is not stepwise and the complex is formed in a single step.

The stability constant of the complex at pH 4.58 was estimated from the Job plot and the (conditional) stability constant was found to be log K = 8.26±0.02.

The formation of a stable aluminium (III)–hesperidin complex in methanolic solution in presence of SB 12 with enhanced fluorescence could be utilized for the quantitative determination of hesperidin in various matrices in trace amounts. It was decided to develop and validate a method for the determination of hesperidin in human serum and pharmaceutical dosages forms.

Method development

The critical parameters for the development of a fluorometric method involved the composition of the solvent, the concentration of aluminium, the concentration of SB 12, pH and the reaction time between aluminium(III) ion and hesperidin. The optimal values for these parameters were determined in order to attain the maximum fluorescence intensity.

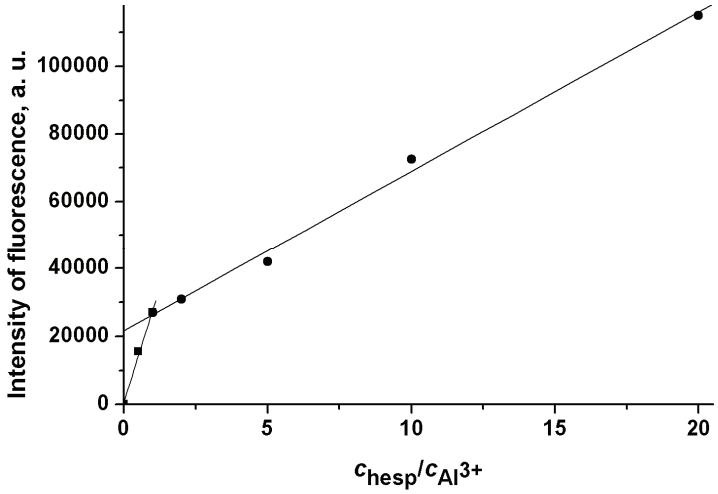


Fig. 2. Method of mole ratios. Dependence of intensity of fluorescence on ratio $c_{hesp}/c_{Al(III)}$.

The composition of the solvent influences the fluorescence intensity and the solubility of the complex. Solvent compositions examined were 30, 50, 70 and 90 vol. % of methanol. The optimal composition of the solvent was methanol:water 70:30 v/v because in this solvent, the maximum intensity of the fluorescence and solubility of the complex was observed.

Concentration of aluminium(III) ions also influences the intensity of fluorescence and for this optimisation, different concentrations of aluminium(III) ions in the range 1×10^{-6}–5×10^{-5} mol L^{-1} (c_{Al}:c_L = 1:1 to 50:1, pH 4.0) were examined. The fluorescence intensity increased with increasing concentration of aluminium(III) ion up to 5×10^{-5} mol L^{-1}.

Influence of pH on the fluorescence intensity of the aluminium(III)–hesperidin complex was examined in the range 3.0–7.0, as shown in Fig. 3. The pH dependence of fluorescence intensity exhibited a complex shape. At low pH values, the intensity decreased because protons tended to displace aluminium(III) ion. At pH values higher than 4.58, the intensity again decreased because more of the aluminium(III) ions were in the form of hydroxide complexes. The optimal pH value was around 4.5, which was used for all further experiments.

According to the work Peres-Ruiz *et al.*,[21] the type of surfactant has a great influence on the fluorescence intensity. They studied the influence of cationic, anionic and non-ionic surfactants on fluorescence intensity, but did not examine the influence of zwiterionic micellar media For this reason, the surfactant SB 12 was used in the present study. The concentration of surfactant was about three times that of the critical micellar concentration and it was shown that this surfactant increase the fluorescence intensity by` about 5 times compared to metha-

nolic solution with no addition of SB 12. With purpose of investigating the influence of surfactants on the fluorescence intensity of the aluminium(III)–hesperidin complex, the emission spectra of aluminium(III)–hesperidin complex without surfactants (Fig. 4, curve 1), and in presence of SDS (Fig. 4, curve 2), and SB 12 (Fig. 4, curve 3) were recorded. Based on the obtained results (Fig. 4), the significant influence of SB 12 on the fluorescence intensity was confirmed.

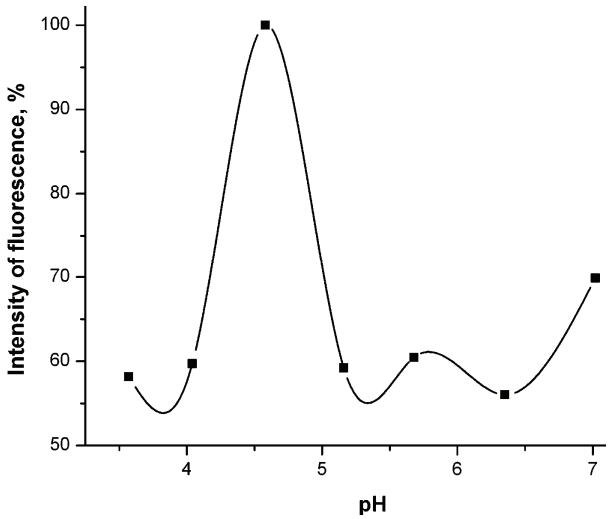


Fig. 3. Effect of pH on the intensity of the fluorescence of the aluminium(III)–hesperidin complex.

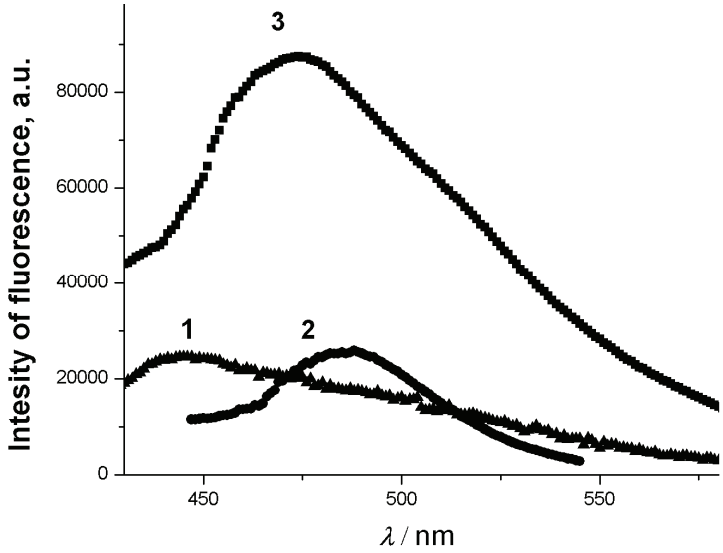


Fig. 4. Influence of surfactants on the intensity of the fluorescence of the aluminium(III)–hesperidin complex: emission spectra of the aluminium(III)–hesperidin complex without surfactants (1), emission spectra of complex in presence of SDS (2) and SB 12 (3).

For the study of the effect of the micelar medium (SB 12) on the excitation and emission spectra of the aluminium(III)–hesperidin complex in 70 % methanol was studied. The zwitterionic surfactant, SB 12 was tested at a concentration of about three times that of the critical micellar concentration. The fluorescence emission spectra shown in Fig. 5 were recorded in 70 vol. % methanol using an excitation wavelength of λ_{ex}= 390 nm. The emission spectra of the aluminium(III)–hesperidin complex (1) and of hesperidin (1′) are illustrated in Fig. 5a, while the corresponding spectra (2 and 2′, respectively) in the presence of SB 12 are presented.

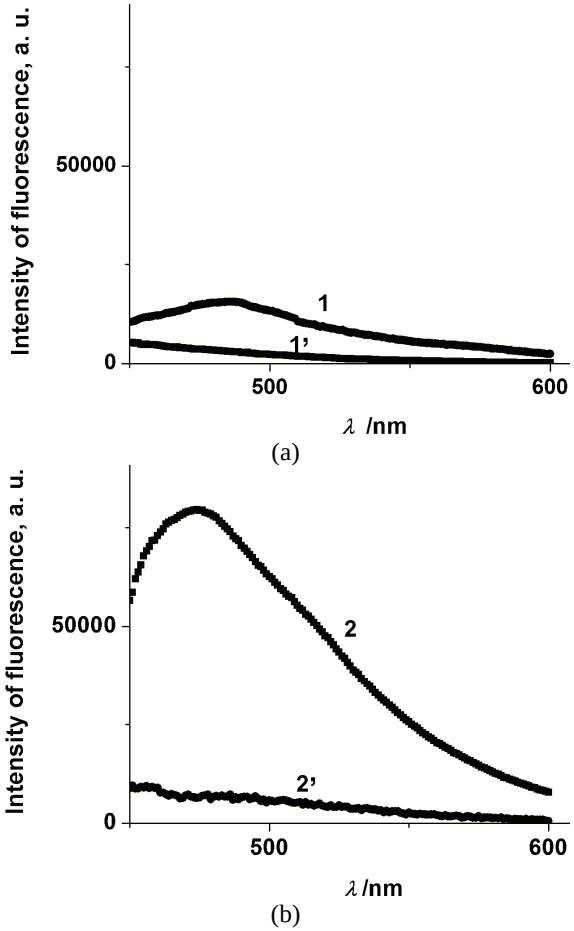


Fig. 5. a) Emission spectra of the aluminium(III)–hesperidin complex (1) and hesperidin(1′); b) emission spectra of the aluminium(III)–hesperidin complex (2) and hesperidin (2′), both in the presence of SB 12.

From Fig. 5, it could be seen that the wavelength of the emission maxima were hypsochromically shifted to λ_{em}= 476 nm and the intensity of the complex fluorescence increased by about four times in presence of SB 12. Due to this, all measurements were performed in the presence of SB 12 at this concentration.

Fluoromeric analysis of hesperidin in pharmaceutical preparations

The calibration curve method was used, requiring solutions containing constant concentrations of SB 12 and $Al(NO_3)_3$ and different concentrations of hesperidin in acetate buffer (using 70 vol. % methanol as the solvent) at pH 4.58. The blank was acetate buffer of pH 4.58.

The regression equation:

$$I_F = (4.06 \pm 0.01)c + (1.27 \pm 0.02) \quad (1)$$

(N = 9) was calculated with the aid of Origin, version 7, software, where I_F is fluorescence intensity in % (λ_{em} = 476 nm) and c is concentration of hesperidin in μg mL^{-1}. The good linearity of the calibration curve and the negligible scatter of experimental points are confirmed by the high correlation coefficient, r = = 0.99999. Linear dependence of the intensity of fluorescence of the complex was obtained in concentration range from 0.06–22.4 μg mL^{-1}.

LOD (limit of detection) and LOQ (limit of quantification). The limit of detection (LOD)[26,27] was calculated by establishing the minimum level at which hesperidin can be detected, according to the formula:

$$LOD = \frac{3.3 s_b}{a} \quad (2)$$

where s_b is the standard deviation of the intercept and a is the slope of the calibration line. It was found that the *LOD* is 0.016 μg mL^{-1}.

The limit of quantification (*LOQ*)[26,27] was determined by using the formula:

$$LOQ = \frac{10 s_b}{a} \quad (3)$$

Thus, hesperidin can be quantified at a concentration of 0.049 μg mL^{-1}.

Precision. The accuracy of the method was determined for four different hesperidin concentrations (Table I). The accuracy and repeatability of the method is fairly high as indicated by good recovery and low values of the *SD*.

TABLE I. The fluorimetric determination of hesperidin in aqueous–methanolic solutions (N = 5)

Added, μg mL^{-1}	Found, μg mL^{-1}	Recovery, %	$SD \times 10^3$	CV / %
0.305	0.303	99.3	2.45	0.81
0.611	0.609	99.7	1.71	0.28
1.222	1.218	99.7	2.92	0.24
6.110	6.085	99.6	7.8	0.13

The results of the obtained analysis are given in Table II.

Procedure for analysis of hesperidin in pharmaceutical preparations

Hesperidin in pharmaceutical forms was analysed by the proposed method. For the analyses of hesperidin in helopyrin tablets, ten tablets were weighed and

powdered using a pestle and mortar. A portion of the powder, equivalent to weight of one tablet, was dissolved in 100 ml 70 vol. % v/v of methanol and the solution was filtered through a Millipore membrane filter with pore size 0.45 μm. 0.25 mL of this solution, 0.35 mL of 0.5 mol L^{-1} SB 12 and 0.5 mL of 1×10^{-3} mol L^{-1} $Al(NO_3)_3$ were mixed in a 10-mL volumetric flask and diluted to the mark with acetate buffer of pH 4.58 (in 70 % v/v methanol). The fluorescence of the prepared solution was taken at λ_{ex} = 390 nm and λ_{em} = 476 nm. The blank was acetate buffer of pH 4.58.

TABLE II. The fluorimetric determination of hesperidin in pharmaceutical preparations

Tablets	Found, mg	Recovery, %	*SD* / %	*CV* / %
Helopyrin, declared 20 mg bioflavonoids	18.06	90.3	0.12	0.66
Vitamin C, content of hesperidin not declared	117.38	–	0.81	0.69

The method suitability was confirmed by taking the fluorescence spectra of the excipient mix (microcrystalline cellulose, methylhydroxypropyl cellulose, Mg-stearate and starch hydrolysate) with the addition of 0.5 mL 1.0×10^{-3} mol L^{-1} of $Al(NO_3)_3$ solution. In Fig. 6 the spectra of aluminium(III) ions + tablet solution and aluminium(III) ion + excipient mix are shown, from which it may be seen that excipients did not interfere with hesperidin determination. Under the chosen experimental conditions, the aluminium complex with rutin (and other citrus flavonoids in the tablets) did not produce measurable fluorescence, and thus, rutin does not interfere with the hesperidin determination.

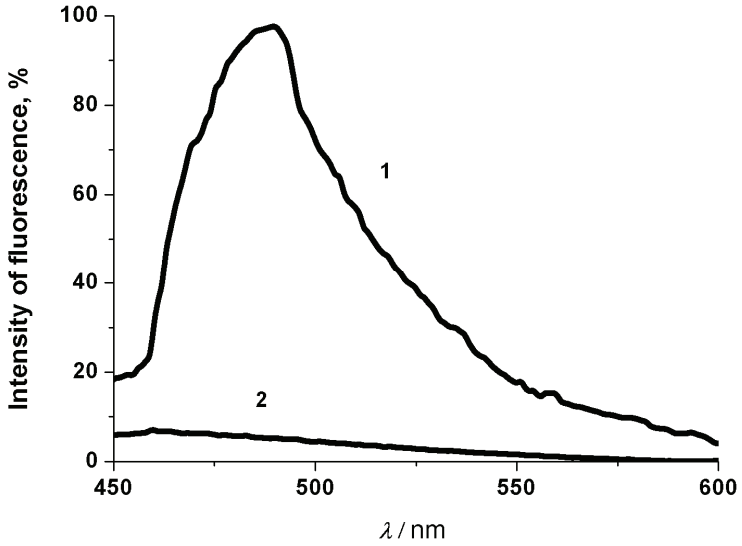


Fig. 6. Emission spectra of the aluminium–hesperidin complex in helopyrin tablets (1) and excipient mix (2). The blank was acetate buffer at pH 4.58.

The content of hesperidin in helopyrin tablets is in good agreement with content that Obendorf *et al*. obtained using the cathodic stripping voltammetry method.[18]

Determination of hesperidin in plasma

0.2 mL human pool plasma, 0.35 mL of 0.5 mol L^{-1} SB 12 and different volumes of hesperidin stock solution to give concentrations of 0.1–12.2 μg mL^{-1} were mixed in a 10-mL volumetric flask and diluted to the mark with pH 4.58 acetate buffer (in 70 vol. % methanol). After incubation (30 min), 0.5 mL of 1×10^{-3} mol L^{-1} $Al(NO_3)_3$ was added. The fluorescence of the prepared solutions were measured at λ_{ex} = 390 nm and λ_{em} = 476 nm. Plasma with SB 12 in acetate buffer (pH 4.58) served as the blank.

Linear dependence of the intensity of fluorescence of the complex on the concentration of hesperidin in diluted plasma samples was obtained in the interval 0.1–12.2 μg mL^{-1}. The regression equation was calculated (I_F is fluorescence intensity in %, and c is the concentration of hesperidin in μg mL^{-1}):

$$I_F = (4.12 \pm 0.02)c + (2.13 \pm 0.04) \quad (4)$$
$$(N = 7, r = 0.99998).$$

The *LOD* of hesperidin in plasma was calculated to be 0.032 μg mL^{-1}. The *LOQ* of hesperidin in plasma showed that it could be quantified at a concentration of 0.096 μg mL^{-1}.

Three different concentrations of hesperidin were added to human plasma in order to obtain concentrations of hesperidin in plasma in the range 0.122–6.110 μg mL^{-1}. These plasma samples were treated in the same way as for the calibration graph. The analytical recovery was 98.4–99.8 %. The low values of relative error (*RE*) and the relative standard deviation of determination (*RSD*) indicate very good reproducibility of the measurements. The results are given in Table III.

TABLE III. The fluorimetric determination of hesperidin in serum samples (N = 5)

Added, μg mL^{-1}	Found, μg mL^{-1}	Recovery, %	$SD\times10^3$	CV / %
0.122	0.120	98.4	1.08	0.86
0.611	0.610	99. 8	1.22	0.20
6.110	6.091	99.7	1.30	0.21

HPLC and mass spectrometric determination of hesperidin

Preparation of solutions for HPLC and mass spectrometric determination of hesperidin. A stock solution of hesperidin (1.0226 mg mL^{-1}) was prepared in methanol. A series of working standard solutions of hesperidin in the concentration range: 0.02–12.2 μg mL^{-1} was prepared by diluting the stock solution with methanol.

Solid phase extraction of plasma samples

Hesperidin was extracted from the plasma samples using LC-18 (500 mg) Supelco cartridges. The cartridges were preconditioned with 5 mL of methanol followed by 5 mL of Milli-Q water. Then 5 mL of sample was forced through the cartridge at a flow rate of about 0.5 mL min^{-1}. After loading, the SPE cartridge was washed with 5 mL of Milli-Q water and then dried under vacuum for 10 min. Finally, the sample was eluted with 2.5 mL of methanol. Human plasma samples were thermostated at 25 °C and after fortification with hesperidin were subjected to protein precipitation and liquid–liquid extraction. To a tube containing 1.0 mL of plasma was added 500 μL methanol or a standard solution of hesperidin. 2-Propanol (1.0 mL) was added to precipitate the proteins. The mixture was then vortex-mixed for 1 min and centrifuged at 10000×*g* for 10 min. The upper clear solution layer was collected and 500 μL water and 500 μL saturated solution of potassium chloride were added. After mixing for 15 s, 5 mL ethyl acetate was added and the sample was vortex-mixed for 1 min and shaken for 10 min. After centrifugation at 3500×*g* for 5 min, the upper organic layer was transferred to another tube and evaporated under nitrogen at 40 °C. The residue was reconstituted in 1 mL methanol and 20 μL was injected into the HPLC and MS system.[28] The extract was first injected onto a Gemini column in the HPLC/UV system with gradient elution and UV detection. The obtained results confirmed the presence of only hesperidin without interfering substances in the extract. The quantification was subsequently performed by injection of 20 μL of the extract into the MS system using the autosampler of the HPLC system but bypassing the chromatographic column.

To check the reliability of fluorescence method the direct HPLC determination of hesperidin in plasma samples LC–MS/MS and HPLC/UV methods in tablets, were developed as modifications of reported methods.[29] Two calibration curves were established for the determination of hesperidin: in methanol medium and in plasma spiked with known quantities of hesperidin.

Analysis of tablets

The helopyrin tablets were analysed for their hesperidin content. The tablets were prepared for analysis according to the procedure given for the fluorometric determination. No extraction procedure was used. Different aliquots of the tablet solutions were subjected to HPLC analysis using a Gemini column and gradient elution with UV detection. The chromatogram of Helopyrin tablets is shown in Fig. 7. The hesperidin peak appeared at a retention time of 13.74 min, as confirmed by comparison with the chromatogram of the standard substance and by checking the UV and mass spectra of the corresponding peak.

The calibration graph was constructed using working standard solutions of hesperidin. The regression equation of the calibration line was:

$$A = (4.83\pm0.05)\times10^4 c + (2.1\pm0.2)\times10^3 \quad (N = 10,\ r^2 = 0.9998) \quad (5)$$

where A is the area of the SRM transition and c is the sample concentration in μg mL^{-1}. The linear range was 0.05–10.0 μg hesperidin mL^{-1}. The *LOD*, calculated from the calibration line, was 0.01 μg mL^{-1} and the *LOQ* was 0.03 μg mL^{-1}. Three aliquots of tablet solution were subjected to chromatographic analysis. The results of the analysis are given in Tables IV and V.

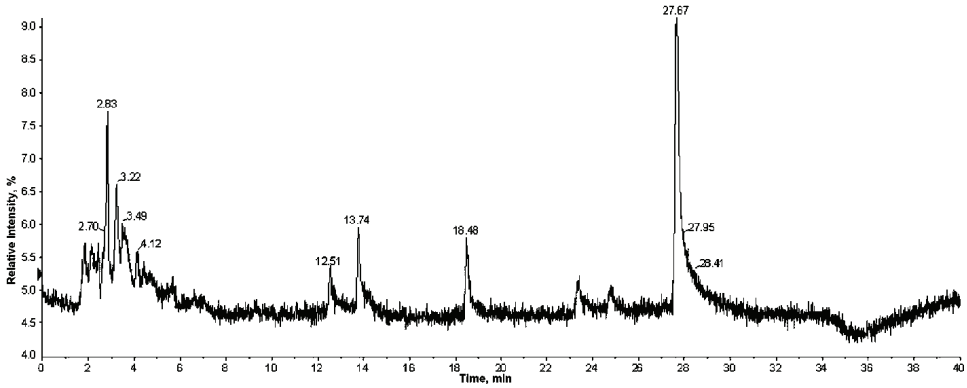


Fig. 7. Chromatogram of the helopyrin tablets.

TABLE IV. HPLC/UV Analysis of helopyrin tablets

Taken, μg mL^{-1}	Found, μg mL^{-1}	Recovery, %	*SD*	*CV* / %
1.5	1.26	84.00	0.25	0.17
3.0	2.84	94.66	0.36	0.12
6.0	5.75	95.83	0.39	0.07

TABLE V. HPLC/UV Analysis of combined Vitamin C tablets for their hesperidin content

Taken, μg mL^{-1}	Found, μg mL^{-1}	Recovery, %	*SD*	*CV* / %
0.1	0.08	75.00	0.3	8.3
0.2	0.2	88.00	0.7	3.65
0.3	0.3	95.33	0.2	0.76

Low recovery of determination of hesperidin in Vitamin C tablets could be explained by absorption of other substances present in tablets that absorb at the same wavelength as hesperidin and could not be removed by the SPE extraction.

Analysis of plasma samples

The mass spectrum consists of six signals of various intensities (Table VI).

Intensity (peak area) of *m/z* 609 → 325 transition was followed. The calculated equation of the calibration line was:

$$A = (4.83\pm0.02)\times10^4 c + (1.75\pm0.09)\times10^3 \quad (6)$$

TABLE VI. Mass spectrum of hesperidin

m/*z*	Abundance, %
609/343	31
609/325	72
609/301	100
609/265	16
609/253	12
609/174	24
609/151	32

where *A* is the area of the SRM transition and *c* is the sample concentration in μg mL^{-1}) for 10 samples. The linearity range was 0.02–10.0 μg mL^{-1}. The *LOD* was estimated from $n = 7$ replicate measurements of the samples in the concentration range 0.002–0.05 μg mL^{-1} and $n = 7$ replicate measurements of the reagent blank (methanol). This procedure provided an *LOD* estimate of 5.0×10^{-3} μg mL^{-1}, *i.e.*, 5.0 ng mL^{-1}. The *LOQ* may then be taken as 15.0 ng mL^{-1}.

The results of analysis of spiked plasma samples are presented in Table VII, from which the excellent recovery can be seen; thus, indicating the good accuracy of the method.

TABLE VII. Accuracy and precision of the HPLC/MS analysis of serum samples spiked with hesperidin ($N = 5$)

Taken, μg mL^{-1}	Found, μg mL^{-1}	Recovery, %	*SD*	*CV* / %
0.5	0.497	99.4	0.006	1.21
1.0	1.02	102.0	0.06	5.88
2.5	2.48	99.2	0.05	2.02
5.0	5.19	102.0	0.03	0.58

The good recovery for the determination hesperidin with the serum sample could be explain by the facts that the LC–MS/MS detection method is much more sensitive and specific compared with the other method and the concentration level was 10 to 100 times higher than in the serum examined by HPLC. This concentration range was used because it is the optimal concentration in human serum after oral dosage of tablets containing hesperidin.[30]

CONCLUSIONS

In this work, a simple, precise and accurate method for the determination of hesperidin in human plasma and tablets based on the fluorescence properties of the aluminium–complex in micellar media was developed. The reliability of the method was confirmed by parallel determination of hesperidin in human plasma using mass spectrometry while the determinations of hesperidin in tablets were compared with an HPLC/UV determination using gradient elution. The fluorometric determination of hesperidin in human plasma, after its complexation with

aluminium(III)-ion, provides good accuracy and precision and may be used for routine clinical analysis, since it is considerably simpler and less time consuming than mass spectrometric determinations. The fluorometric determination in dosage forms may be successful only when no other flavonoids which bind aluminium to adjacent 3-hydroxy and 4-keto groups (stoichiometry 1:1) are present.

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ИЗВОД

РАЗВОЈ И ВАЛИДАЦИЈА ФЛУОРИМЕТРИЈСКЕ МЕТОДЕ ЗА ОДРЕЂИВАЊЕ ХЕСПЕРИДИНА У ХУМАНОМ СЕРУМУ И ФАРМАЦЕУТСКИМ ПРЕПАРАТИМА

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Развијена је флуориметријска метода за одређивање хесперидина у хуманом серуму и фармацеутским препаратима која се заснива на флуоресценцији комплекса алуминијум(III)–хесперидин. Комплекс показује интензивну флуоресценцију у присуству сурфактанта SB 12 на 476 nm приликом ексцитације на 390 nm. Линеарна зависност интензитета флуоресценције од концентрације при одређивању хесперидина у фармацеутским препаратима добијена је у концентрационом опсегу 0,06–24,4 μg mL^{-1} са границом детекције од 0,016 μg mL^{-1} и границом квантификације од 0,049 μg mL^{-1}. Добијене „*recovery*“ вредности у интервалу 99,3–99,7 % показују велику прецизност методе. Линеарна зависност интензитета флуоресценције компекса од концентације хесперидина добијена је у концентрационом опсегу 0,1–12,2 μg mL^{-1} са границом детекције од 0,032 μg mL^{-1} и границом квантификације од 0,096 μg mL^{-1}. „*Recovery*“ вредности су добијене у опсегу 98,4 до 99,8 %. Поузданост методе проверена је LC–MS/MS методом за одређивање хесперидина у серуму, а HPLC/UV методом проверена је поузданост приликом одређивања хесперидина у фармацеутским препаратима. Линеарна зависност при одређивању хесперидина у фармацеутским препаратима добијена је у интервалу 0,05–10,00 μg mL^{-1}. Граница детекције је износила 0,01, а граница квантификације је 0,03 μg mL^{-1}. Линеарна зависност при одређивању хесперидина у хуманом серуму је добијена у интервалу 0,02–10,00 μg mL^{-1} са границом детекције од 0,005 и границом квантификације од 0,015 μg mL^{-1}. Добро слагање између ове две методе показује применљивост флуориметријске методе у клиничким лабораторијама и лабораторијама за контролу квалитета. Предложена флуориметријска метода је једноставна, поуздана и прецизна за одређивање хесперидина у хуманом серуму и фармацеутским препаратима.

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