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Study of the Solubility and Membrane Permeability of Mechanochemically Obtained Solid Dispersions of Plant Flavonoids

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Abstract

Solid dispersions of plant flavonoids (dihydroquercetin, puerarin, rutin and genistein) with the arabinogalactan polysaccharide and the glycyrrhizic acid disodium salt are obtained by mechanochemical treatment. Ways of increasing their solubility and transmembrane permeability on artificial membranes have been studied. The formation of supramolecular systems – intermolecular complexes and micelles – was established by NMR relaxation upon dissolution of solid dispersions in water, their stability constants and thermodynamic parameters were estimated. The possibility of increasing the solubility of the studied flavonoids by a factor of 1.9–30 was shown. It was revealed that the measured rate of transmembrane transfer of flavonoid molecules through an artificial hydrophobic membrane did not correlate with an increase in their solubility. It is assumed that this is due to the structural features of the complexes, as well as the specificity of the flavonoid molecules themselves. The results obtained are qualitatively close to the available data on bioavailability and transmembrane transfer *in vivo*.

Key words: flavonoids, mechanochemical treatment, solubility, membrane permeability, supramolecular complexes, micelles

INTRODUCTION

Flavonoids form the most numerous class of natural phenolic compounds characterized by structural diversity, high and versatile biological activity, and low toxicity [1]. It is known that dihydroquercetin (DHQ), rutin, genistein and puerarin, belonging to the lasses of flavons and isoflavons, demonstrate a broad range of biological action: they exhibit antioxidant, cytoprotective, vasoprotective, anticancer, and cardioprotective activity [2], decrease the risk of cardiovascular diseases [3–7] and are used in parapharmaceutical and pharmaceutical preparations. However, the potential of the practical application in the composition of solid pharmaceutical dosage forms is limited by their low solubility and biological availability [8]. A significant direction in the investigation of flavonoids is the development of complex preparations on their basis.

It is known [9] that the biological availability of solid dosage forms for oral intake is determined by the water solubility of the active pharmaceutical ingredient and the rate of its absorption in the gastrointestinal tract (GIT). Parapharmaceutical preparations are close in their destination to pharmaceutical means, so traditional pharmaceutical technologies are used for their production.

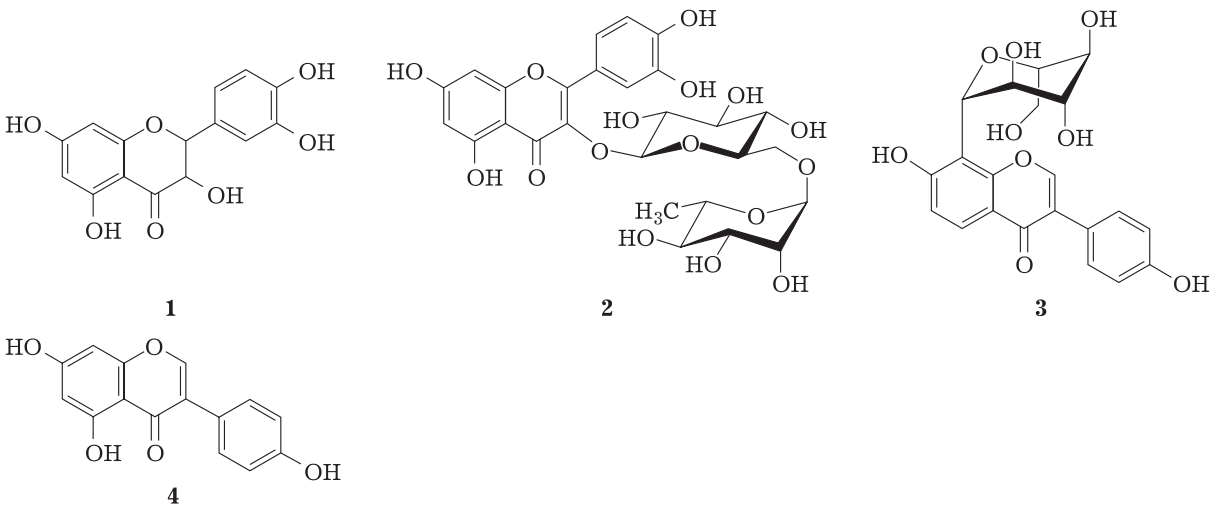

Fig. 1. Structural formulas of flavonoids: **1** – dihydroquercetin; **2** – rutin; **3** – puerarin; **4** – genistein.

A number of approaches exist to solve the problem of the low solubility of pharmaceutical substances (PS): a decrease in the size of particles (micronization), modification of the crystal structure (polymorphous transformations), the formation of co-crystals, the formation of water-soluble salts, ionization of the molecules of PS (pH change), preparation of the dispersions of PS with fillers (eutectic mixtures, solid dispersions (SD), solid solutions) *etc.* [10–12].

Previously we demonstrated in our works that the solubility characteristics of PS, including some of the flavonoids studied in the present work, may be improved due to obtaining their SD with specially selected water-soluble auxiliary substances through the joint mechanochemical treatment in ball mills [13–16].

The rate and degree of absorption of the biologically active substances (BAS) through the walls of the gastrointestinal tract into the blood flow are not less important factors affecting the efficiency of the action of solid oral forms. This absorption is measured as the value of BAS bioavailability calculated from the pharmacokinetic parameters. In the case of flavonoids under investigation, according to the available data [8], this value is estimated as 2–20 % of the theoretical value. Correspondingly, only a small fraction of these substances enters the systemic bloodstream and causes the expected biological/pharmacological effect. Therefore, the studies of factors promoting an increase in the biological availability of BAS are urgent.

In the present work, we studied the routes to the increased solubility and membrane permeability of flavonoid molecules (Fig. 1) through their inclusion into supramolecular systems – guest-host type compounds and micelles. The complex-forming agents were polysaccharide arabinogalactan (AG) and the derivative of glycyrrhizic acid (GA) – its disodium salt (Na_2GA) [17].

Arabinogalactan is a highly branched polysaccharide isolated from larch wood. Its structure may promote the inclusion of small molecules of GAS into the space between the side chains of the macromolecule. Glycyrrhizic acid is a water-soluble saponin isolated from the roots of licorice. Its molecules are amphiphilic and able to self-association in solution – they form micelles [17, 18]. Flavonoid molecules may be located either in the inner part of the formed micelle or at the outer surface of the self-associate.

EXPERIMENTAL

Materials

Solid dispersions were obtained on the basis of the substances of flavonoids: DHQ (LC INPF "Khimiya Drevesiny", Russia); rutin (Qingdao Samin Chemical Co., Ltd, China); puerarin (Shaanxi Pioneer Biotech Co., Ltd, China); genistein (Sigma-Aldrich). AG from daurian larch wood (Ametis JSC, Russia, TU 9325-008-70692152–08) and Na_2GA (Shaanxi Pioneer Biotech Co., Ltd, China) were used as the complex-forming agents (auxiliary substances).

Synthesis of solid dispersions

The synthesis of SD was carried out with the help of solid-phase mechanochemical technology.

The mechanochemical treatment of a mixture of the flavonoid substance and the auxiliary substance was carried out in a ball rotary mill according to the procedure described in [15]. The mass ratio of flavonoid to t the auxiliary substance was 1 : 10.

Methods of investigation

The concentrations of rutin and genistein in the solutions were determined by means of high performance liquid chromatography (HPLC) using Agilent 1200 chromatograph according to the procedure described in [15]. Acetate buffer (pH 3.4)/acetonitrile in the volume ratio of 55 : 45 was used as the eluent for genistein and in the ratio of 80 : 20 for rutin.

The concentrations of DHQ and puerarin in solutions were determined with the help of HPLC using a microcolumn chromatograph Milikhrom A-02 equipped with a UV detector, using a reverse-phase sorbent RP-18. The column temperature was +35 °C. For the analysis of DHQ solutions, the mobile phase was composed of 4 M $LiClO_4$ with 0.1 M $HClO_4$/acetonitrile in the volume ratio of 70 : 30. To analyze the solutions of puerarin, 0.1 % sodium citrate /acetonitrile in the volume ratio of 80 : 20 was used as the eluent.

Determination of the solubility of flavonoid substances and their complexes was carried out according to the procedure described in [14]. A weighted portion of the sample corresponded to the mass necessary for the achievement of the required concentration: genistein – 0.5 g/L, rutin – 2 g/L, dihydroquercetin – 5 g/L, puerarin – 10 g/L.

The formation of intermolecular complexes in the aqueous D_2O solutions was confirmed by means of ^1H dynamic NMR spectroscopy using a Bruker AVANCE III 500 spectrometer (Germany) at a frequency of 500 MHz at 30 and 50 °C. Measurement of the times of phase relaxation T_{ph} was carried out using a standard Car–Purcell–Meoboom–Gill (CPMG) sequence of the kind of

$$P_1(90°) - (\tau - P2(180°) - \tau)n$$

where P_1, P_2 are radio-frequency pulses at the rotation angle of 90° and 180°, respectively; τ – a fixed time delay equal to 0.5 ms; n – the number of cycles varying within the range from 0 to 2000. Using data obtained, we carried out an estimation of the constants of complex stability and the major thermodynamic parameters of complex formation.

Membrane permeability for flavonoids *in vitro* was determined with the help of the PAMPA model (parallel artificial membrane permeability assay). The investigation was carried out using artificial membranes formed on the basis of hexadecane according to the procedure described in [15].

RESULTS AND DISCUSSION

Investigation of solubility

The solubility parameters for flavonoids and their SD are listed in Table 1. An increase in solubility is observed in all cases; its value (1.9 to 30 times) depends on the nature of the complex-

TABLE 1

Parameters of the solubility of flavonoid substances and their complexes with the auxiliary substances, prepared by physical mixing and mechanical treatment

Flavonoid-AS (mass ratio)	C_{phys}, g/L (n)	$C_{m/t}$, g/L (n)
DHQ substance	2.1	
DHQ-AG (1 : 10)	2.1 (1)	3.5 (1.7)
DHQ-Na_2GA (1 : 10)	4.4 (2.1)	4.5 (2.1)
Rutin substance	0.052	
Rytin-AG (1 : 10)	0.052 (1)	0.099 (1.9)
Rutin-Na_2AG (1 : 10)	0.904 (17)	1.1 (21)
Puerarin substance	4.3	
Puerarin-AG (1 : 10)	5.0 (1.2)	5.3 (1.2)
Puerarin-Na_2GA (1 : 10)	9.1 (2.1)	9.0 (2.1)
Genistein substance	0.005	
Genistein-AG (1 : 10)	0.005 (1)	0.014 (2.8)
Genistein-Na_2GA (1 : 10)	0.010 (2)	0.150 (30)

Note. C_{phys}, $C_{m/t}$ – solubility of the complexes prepared by means of physical mixing and mechanical treatment, respectively; n – an increase in solubility, times.

forming substance and on the method used to prepare the compositions. One can see in the data presented in Table 1 that higher solubility values are achieved for complexes with Na_2GA than in the complexes with AG. Depending on the method of obtaining the complexes (mechanochemical or physical mixing), the solubility values are comparable. An exception is the complex of genistein with Na_2GA: the solubility of the physical mixture C_{fis} increased by a factor of two, while for the complex obtained by mechanochemical treatment the solubility $C_{m/o}$ increased by a factor of 30. The observed effect may be connected with the extremely low solubility of genistein substance and is characteristic of a number of other substances studied previously [17].

Investigation of the formation of complexes in aqueous solutions

Flavonoid substances possessing the highest solubility (DHQ and puerarin) demonstrate insignificant changes in the solubility in the presence of complex-forming agents. To confirm the formation of intermolecular complexes of these flavonoids with AG and Na_2GA in aqueous solutions, we used NMR relaxation, a method that proved itself to be effective [19–21]. It is known that the times of the spin-spin relaxation of magnetic nuclei of the molecules in solution are very sensitive to the intermolecular interaction and to the diffusion mobility of molecules [22]. This is due to the change of the time τ_c of rotational reorientation of molecules in the complex, which is estimated using Stokes–Einstein–Debye equation: $\tau_c = 4\pi a^3\eta/(3kT)$, where a is the size of molecules, η is viscosity, T is temperature, k is Boltzmann constant. When a molecule gets inside the complex, the time of proton relaxation decreases substantially as a result of deceleration of diffusion and rotational mobility.

Relaxation times of the aromatic protons of DHQ and puerarin were measured (see Fig. 1) in the aqueous solutions of SD with AG and Na_2GA, as well as in the solutions of initial substances. In all SD, a decrease in T_{ph} in comparison with the free molecule was observed. For example, for the complex DHQ-Na_2GA at a ratio of 1 : 10 a decrease in T_{ph} from 1.4 s for the free molecule to 0.58 s for DHQ in the complex was observed at a temperature of 30 °C (Fig. 2).

Serial dilution of the SD solution leads to an increase in the observed relaxation time, which is a consequence of the shift of equilibrium towards

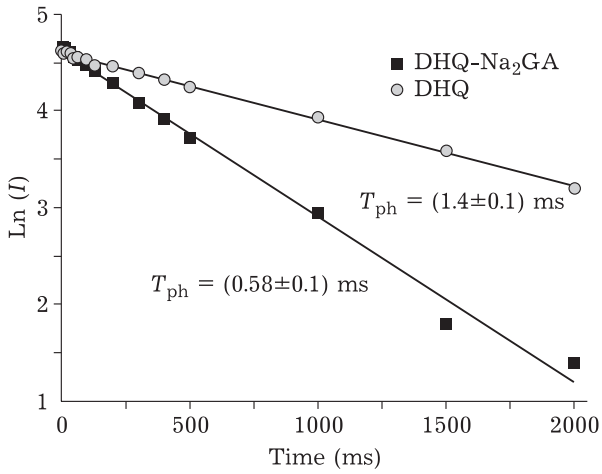

Fig. 2. Dependences of the intensity of echo signal I in the CPMG experiment (in the logarithmic scale) from the aromatic protons of DHQ on time in the aqueous solution of DHQ (0.4 mM) and in the solution of DHQ-Na_2GA complex (1 : 10, GA concentration 3 mM) at a temperature of 30 °C, pH 6.2. Points – experiment, continuous lines – calculation.

the free DHQ molecules in the reaction of complex formation:

$$DHQ + Na_2GA \leftrightarrow DHQ\text{-}Na_2GA$$

The stoichiometric coefficient n and the constant of complex stability K were estimated from the dependence of the time of proton relaxation in DHQ on GA concentration plotted in Benesi-Hildebrand coordinates:

$$\Delta_{max}/\Delta - 1 = 1/(K[Na_2GA]^n) \quad (1)$$

Here Δ and Δ_{max} are the observed and maximal changes of the rate of DHQ proton relaxation with respect to the free molecule: $\Delta = 1/T_{ph}(\text{obs.}) - 1/T_{ph}(\text{free})$. The concentration dependence plotted in the coordinates Δ_{max}/Δ *versus* $1/[Na_2GA]^n$ allows calculating n and K from the slope of the straight line. Experimental points are well described by the straight line with $n = 1$, which points to the stoichiometry of the complex 1 : 1. The constants of stability for the DHQ-Na_2GA complex may be estimated from the slope of the straight line at different temperatures: $K(30) = (125\pm20)$ M^{-1} and $K(50) = (340\pm50)$ M^{-1}. Relatively low values of stability constants obtained for DHQ, in comparison with the values for strongly hydrophobic molecules [23, 24], are most probably due to the hydrophilic properties of DHQ. An interesting feature of the DHQ-Na_2GA complex is an increase in its stability constant with an increase in temperature (125 → 340 M^{-1}), which points to the prevailing contribution from the entropy factor into the change of the free energy during the formation of the complex. Knowing the constant of stability for two temperature points, one may

TABLE 2

Time (T_{ph}, s) of spin-spin relaxation for aromatic protons of puerarin (PR) at T equal to 30 and 50 °C, during the dissolution of the initial substance and solid dispersions in water (initial concentration of Na_2GA – 2.3 mM) followed by dilution by a factor of 3 and 9. Error of fitting: less than 5 %

T, °C	PR	PR-Na_2GA	PR-Na_2GA/3	PR-Na_2GA/9
30	1.26	0.74	1.01	1.50
50	1.43	1.18	1.36	2.06

estimate the major thermodynamic parameters of complex formation: the change of Gibbs energy $\Delta_r G^o_T$, enthalpy $\Delta_r H^o_T$ and entropy $\Delta_r S^o_T$ according to equations

$$\Delta_r G^o_T = - RT \ln K \quad (2)$$

$$\ln (K_1/K_2) = \Delta_r H^o_T /R\ (1/T_2 - 1/T_1) \quad (3)$$

$$\Delta_r S^o_T = (\Delta_r H^o_T - \Delta_r G^o_T)/T \quad (4)$$

where K_1 and K_2 are the values of thee constant of complex stability measured at temperatures T_1 and T_2. As a result, the following values of thermodynamic parameters were obtained: the change of Gibbs energy $\Delta_r G^o$(30 °C) = −12 kJ/mol, enthalpy $\Delta_r H^o$ = +42 kJ/mol, and entropy $\Delta_r S^o$(30 °C) = +180 J/(mol · K). The error of the estimation is about 20 %.

The solid dispersion of puerarin with Na_2GA at a ratio of 1 : 10, prepared with the help of mechanochemical technology, exhibited a similar behaviour during the dissolution of the dry mixture in water. The data of the measurements for the aromatic protons of puerarin (the signals of all aromatic protons overlap, so the total intensity was measured) are presented in Table 2. One can see that the time of relaxation of the aromatic protons of puerarin in water is smaller than in the diluted solution of the complex, which points to the existence of puerarin in aqueous solutions in the form of self-associates. It should be stressed that variations of puerarin concentration from 3 to 0.3 mM do not lead to substantial changes in the proton relaxation time (±10 %). At the same time, a 9-fold dilution of the complex causes an increase in the observed relaxation time from 0.74 to 1.5 s at 30 °C. It may be assumed that puerarin molecules exist in aqueous solutions in the form of stable associates (maybe dimers), which are not decomposed after dilution. However, these associates are not formed after the dissolution of mechanochemically obtained SD of puerarin with Na_2GA. The dependence of the time of relaxation of protons in puerarin on the concentration of Na_2GA plotted in Benesi–Hildebrand coordinates (1) allowed estimating the stoichiometry and stability of the complex: n = 1, K(30 °C) = (480±50) M^{-1} and K(50 °C) = (1640±150) M^{-1}. Similarly to the complex of DHQ, the complex of puerarin demonstrates an increase in the constant of stability with an increase in temperature, which points to the prevailing contribution from entropy into the change of the free energy during the formation of the complex. According to the estimations of thermodynamic parameters of complex formation, the change of Gibbs energy $\Delta_r G^o_T$ = −16 kJ/mol, enthalpy $\Delta_r H^o_T$ = +50 kJ/mol, and entropy $\Delta_r S^o_T$ = +220 J/(mol · K) at 30 °C.

Similar studies of the SD with polysaccharide arabinogalactan point to the higher stability of these dispersions. A characteristic value of the constant of stability both for DHQ and for puerarin with AG is K(30 °C) = 10^4 M^{-1} and decreases with an increase in temperature. The accompanying changes of enthalpy and entropy of the formation of the complex with DHQ are: $\Delta_r H^o_T$ = −9.55 kJ/mol and $\Delta_r S^o_T$ =+39 J/(mol · K) at 30 °C, which points to the substantial role of Van der Waals interactions and desolvation of DHQ molecules.

Investigation of membrane permeability

At the early stages of drug development, it is important to possess the data on the transmembrane permeability of the potential medicinal preparations in order to predict oral bioavailability and pharmacokinetic properties. A rapid evaluation may be carried out using the PAMPA procedure. The basis of the method is the model of the penetration of molecules through artificial membranes. The application of this procedure allows predicting the rate of the passive diffusion of BAS through the epithelial cells of the walls of the gastrointestinal tract, hematoencephalic barrier and skin [25].

A reliable increase in the transmembrane transport of flavonoids is observed only for solid dispersions: DHQ-Na_2GA and genistein-AG (Fig. 3). However, the rates of transfer do not correlate quantitatively with the observed increase in the solubility of the indicated flavonoids. In the case

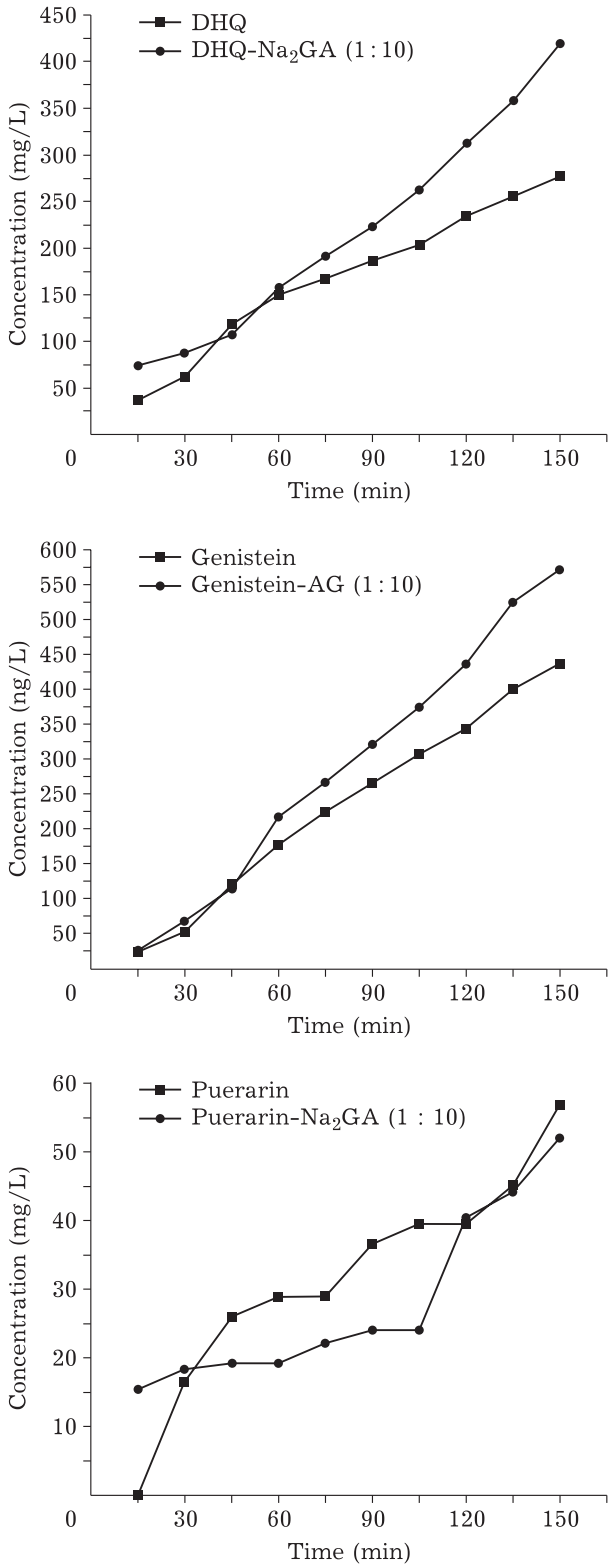

Fig. 3. Dynamics of the passive transport (diffusion) of flavonoid molecules from solutions through an artificial membrane.

of puerarin, the rates of transmembrane transfer both for the initial substance and for its complex with Na_2GA did not differ from each other within the experimental error. For the complexes of DGQ and puerarin with AG and for the complex of genistein with Na_2GA, the absence of transport was revealed. It was also established that rutin in all the cases, either as the initial substance or as the complex with AG and Na_2GA, does not diffuse through the artificial membrane.

For detailed interpretation of the observed differences between the rates of transport, further studies are necessary. Nevertheless, the obtained data coincide qualitatively with the results of the studies of membrane permeability for the culture of Caco-2 cells, demonstrating that the transport of flavonoids through a monolayer of these cells increases in the sequence: puerarin ~ rutin < dihydroquercetin < genistein [26].

It is necessary to stress that pharmacokinetics of polyphenol compounds, which is the basis determining bioavailability, in the human organism is due to a number of complicated processes including the formation of conjugates, fermentation in the large intestine, transformation in the liver. In addition, flavonoid molecules exhibit membrane-acting properties [27] and may affect the transport activity of carriers. So, it may be concluded that the results of the model experiment should not be interpreted unambiguously because the PAMPA procedure does not take into account metabolic transformations, active transport and other routes of transmembrane transfer.

CONCLUSION

SD of flavonoids (DHQ, rutin, genistein and puerarin) with complex-forming agents (AG and Na_2GA) at the mass ratio of 1 : 10 were prepared by means of mechanochemical treatment. The possibility to enhance their solubility by a factor of 1.9–30 was demonstrated. The formation of intermolecular complexes during the dissolution of the resulting SD in water was confirmed by means of NMR relaxation. The constants of stability and thermodynamic parameters of complexes were estimated from the concentration dependences of the relaxation time at different temperatures. The measured rate of transmembrane transport of flavonoid molecules through a model hydrophobic membrane does not correlate with an increase in their solubility. An increase in the membrane permeability is insignificant. This may be connected with the features of the structure of flavonoid molecules and supramolecular systems based on these molecules. The results generally

agree with the literature data on the low absolute bioavailability of the studied compounds, which may be due to the hindered transmembrane transport and metabolic processes.

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