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Relationship between vasorelaxation of flavonoids and their retention index in RP-HPLC

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In the present study the relationship between the vasodilation of flavonoids and their retention index (RI) was investigated. Retention index (RI) values of 18 flavonoids, including flavones, flavonols, flavanones, flavanols and isoflavones were measured by an RP-HPLC method based on alkan-2-ones. Vasorelaxation of the flavonoids was measured by measuring the reduction in tension by flavonoids in rat thoracic aorta precontracted by phenylephrine (PE, 10^{-6} mol/L). The results demonstrated that the potency of the vasorelaxation of flavonoids was positive by correlated with RI. The regression equation was $Rex = -11.68 + 0.11\ RI$ ($n = 18$, $r = 0.915$, $P < 0.01$).

1. Introduction

Flavonoids are polyphenolic compounds that occur ubiquitously in plants, especially those used in traditional medicine, such as Folium Ginkgo, Flos Chrysanthemi and Radix Scutellariae. They have been isolated from various traditional medicines and their remarkable biological activities have attracted extensive attention from researchers. It have been reported that they exhibit a wide range of biological effects, including antiviral (Li et al. 2002), anti-inflammatory, antinociceptive (Toker et al. 2004), anti-allergic and antioxidant activity and vasodilatory actions (Formica and Regelson 1995; Quettier et al. 2003). There has been a great deal of interest in vasorelaxation by flavonoids. Studies have shown that the vasorelaxation of flavonoids depends very much on their structure (Ajay et al. 2003; Herrera et al. 1996). However, since flavonoids induce vasorelaxation via a series of mechanisms (Jiang et al. 2005), and flavonoids differ from one another in the orientation of the substituents, the degree of unsaturation, the type of sugar moiety attached and the position of the benzenoid substitution, it is difficult to summarize the factors contributing to vasorelaxation by flavonoids. Up to the present, structure-activity research has been limited to qualitative studies of these relationships (Ajay et al. 2003).

Octanol-water partition coefficients (logP) of compounds as related to their chemical structure can always be applied to the study of quantitative structure-activity relationships (QSAR); however, it is difficult to measure logP of flavonoids by the classical shake-flask method (Hansch et al. 1967) because of their poor capacity to dissolve in both octanol and water. We measured logP by an RP-HPLC method based on compounds with known logP (Chen et al. 2004), but some researchers think this method is not acceptable. Baker and Ma (1979) developed a retention index system based on the alkan-2-ones as a method of standardization of retention data which would enable the interlaboratory comparison of results and subsequent use of reference databases for identification. Previous reports have demonstrated that the retention behavior of chemical substances in HPLC can be used in QSAR studies to replace the hydrophobicity parameter of drugs (Baker et al. 1980).

In the present study, retention index values of 18 flavonoids were determined by an RP-HPLC method, while vasorelaxation were evaluated by measuring the tension reduction induced by flavonoids in rat thoracic aorta precontracted by phenylephrine (PE, 10^{-6} mol/L), and subsequently, the relationship between the vasorelaxant activity of flavonoids and their retention behavior was studied.

2. Investigations, results and discussion

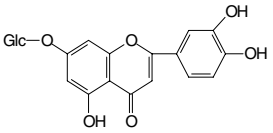
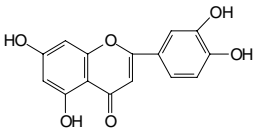
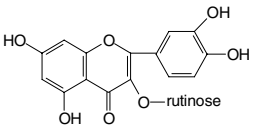
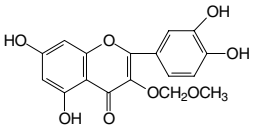
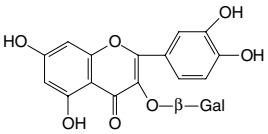
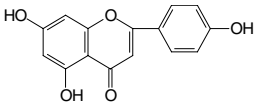
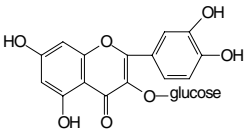
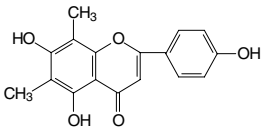
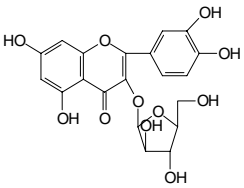
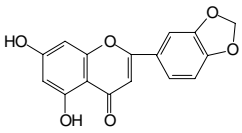
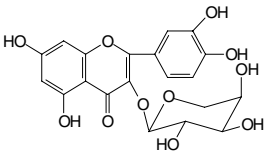
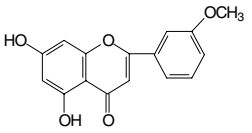
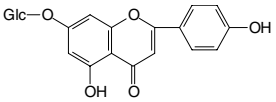
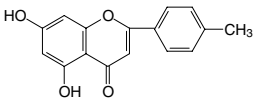
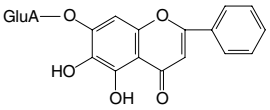
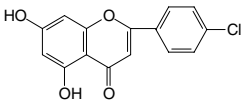
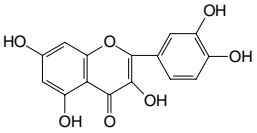
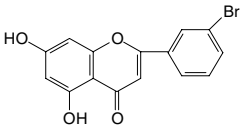
2.1. RI values of the flavonoids

RI values of samples were calculated by an equation based on the retention times of the alkan-2-ones used and the test compounds. The structures of the 18 flavonoids and the results are listed in the Table. We found that glycoside flavonoids had lower RI values than aglycone flavonoids because of the poly-hydroxyl groups of their glycosyl groups. In addition, the introduction of groups such as $-Br$, $-Cl$, $-CH_3$, $-OCH_3$ increased the RI values.

2.2. Relationship between vasorelaxant activity of flavonoids and their RI value

Vasorelaxation by the flavonoids in rat thoracic aorta precontracted by PE is listed in the Table. The data show that the flavonoids differed in their vasorelaxant activity. Aglycone flavonoids showed stronger vasorelaxant activity than the corresponding glycoside, e.g. the maximal relaxation (Rex%) of luteolin was 70.2%, and that of luteolin-7-*O*-α-D-glucoside was only 20.5%. Glycosylation of quercetin reduced its vasodilator activity. The Rex% of flavonoids

Table: Retention index (RI) of flavonoids determinated by RP-HPLC and their vasorelaxation effects (Rex) against PE induced contractions in rat aortic rings

Compd.	Structure	RI	Rex(%)	Compd.	Structure	RI	Rex(%)
1		356	20.5 ± 3.5	**10**		616	70.2 ± 2.3
2		365	24.7 ± 2.7	**11**		641	71.6 ± 7.0
3		385	34.0 ± 4.7	**12**		692	78.0 ± 2.9
4		388	48.6 ± 2.9	**13**		760	64.4 ± 3.5
5		414	35.1 ± 3.2	**14**		836	91.3 ± 3.4
6		433	24.7 ± 4.3	**15**		871	80.8 ± 5.6
7		439	36.2 ± 3.0	**16**		933	77.2 ± 5.9
8		494	20.1 ± 2.3	**17**		953	92.7 ± 4.1
9		573	45.9 ± 2.5	**18**		982	95.0 ± 2.0

Rex data were expressed as mean ± SE, n = 8. Comparisons were made by Student's t test, P values less than 0.05 were considered significant. The concentrations of the flavonoids were 36 μmol/L.

with lipophilic groups, such as compounds **14**–**18**, were more than 70%. In general, compounds had stronger activity with increasing RI values. Compound **18**, which has the maximum RI value, was identified as having the most potent vasodilator activity of the flavonoids studied. The relaxation ratio was plotted against the RI value (Fig.). The regression equation was $Rex = -11.68 + 0.11$ RI ($n = 18$, $r = 0.915$, $P < 0.01$). It is obvious that there is a positive correlation between the relaxation ratio and the RI value.

Since the general structure-activity relationship and the mechanism of action of these compounds were not evident, the positive correlation between the relaxation ratio and the RI value cannot be explained precisely. But the results in this study suggest that the introduction of lipophilic groups into the basic ring skeleton of flavonoids improves their vasorelaxant activity. The results may provide a basis for finding more effective flavonoids from traditional medicine for antihypertensive effects and for the study of the mechanism of vasorelaxation of flavonoids.

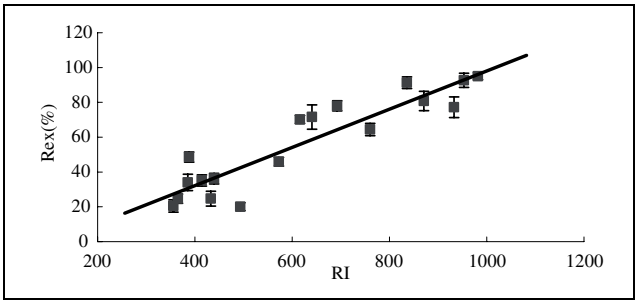


Fig.: Positive correlation between vasorelaxant activity of flavonoids and their RI values

3. Experimental

3.1. Materials

The alkan-2-ones used and test compounds **1**, **2**, **8**, **9**, **10** and **13** were purchased from commercial sources. Compounds **1** and **7** were separated from *Chrysanthemum morifolium* Ramat. Compounds **3**–**6**, **11**, **14**–**18** were synthesized by the Institute of Medicine, Zhejiang University, China. Phenylephrine (PE) and acetylcholine (Ach) were purchased from Sigma Chemical Co. Methanol, HPLC grade, as purchased from Merck Co. Ltd. The other reagents were of analytical purity.

Male Sprague-Dawley rats (220–250 g) were obtained from the Experimental Animal Center of Zhejiang Academy of Medical Sciences. All procedures were approved by the Animal Care Committee.

3.2. Equipment

Agilent 1100 HPLC system (Agilent Co.Ltd., USA), equipped with an auto-sampler, an on-line vacuum degasser, G1311A quaternary pump, a thermostatted column compartment, a photodiode array detector (DAD) and Agilent Chemstation software.

3.3. Measurement of the RI of the samples

3.3.1. Chromatographic conditions

The analysis was performed on an Agilent Zorbax SB-C_{18} column (4.6 mm 250 mm, 5 μm). The optimum separation in HPLC was carried out with a mobile phase composed of methanol: water (60 : 40, v/v) at a flow-rate of 1.0 ml/min. The volume of sample injected was 20 μl, the column temperature was set at 30 °C, and the isocratic elution condition was monitored at 280 or 350 nm, according to the UV absorption of the sample.

3.3.2. RI evaluation

Each of the flavonoids and alkan-2-ones was dissolved in methanol to a concentration of 0.1 mg/ml and then injected into the HPLC apparatus. The retention times of the flavonoids and alkan-2-ones were recorded. The capacity factors (K'_x) and RI values of the samples were calculated using the equations as follows:

$$K'_x = (t_x - t_0)/t_0$$

$$RI = 100\,N + 100(\log K'_x - \log K'_n)/(\log K'_{n+1} - \log K'_n)$$

where x = flavonoid measured, n = alkan-2-one eluting immediately before x, n + 1 = alkan-2-one eluting immediately after x and N = carbon number of alkan-2-one n. The K′ values were calculated using the retention time of $NaNO_2$ as a dead time.

3.4. Characterization of the vasorelaxant responses to flavonoids (Kubota et al. 2001)

3.4.1. Preparation of rat thoracic aorta rings

Male Sprague Dawley rats were killed by stunning and cervical dislocation and the thoracic aorta was rapidly removed and placed in a 4 °C Krebs-Henseleit solution (pH 7.4) of the following composition (mmol/L): NaCl 118.0, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0. After the adhering fat and connective tissue were removed, the aorta was cut into ring segments approximately 3 mm wide. Care was taken to avoid abrading the intimal surface in order to maintain the integrity of the endothelial layer. Each ring preparation was mounted vertically under a resting tension of 2 g in a 10 ml water jacketed organ bath filled with Krebs-Henseleit solution and attached to a LH-663 force-displacement transducer connected to a data acquisition system (PowerLab, AD Instruments). The bath solution was maintained at 37 °C and continually bubbled with a 95%O_2–5%CO_2 gas mixture. Each preparation was allowed to equilibrate for 60 min prior to the initiation of experimental procedures, and during this period the incubation media were changed every 15 min. After the equilibration period, the aortic rings were allowed to achieve maximal tension by exposure to an isotonic potassium solution, high K^+ (6×10^{-2} mol/L). After three successive responses to KCl for periods of 10 min each, the rings were constricted with phenylephrine (PE, 10^{-6} mol/L) to test the endothelial integrity with a single addition of acetylcholine (Ach, 10^{-5} mol/L). Only endothelium-intact rings, exhibiting more than 70% relaxation to Ach, were used for the experiments.

3.4.2. Determination of the vasorelaxant response to flavonoids

The aortic rings were precontracted with 10^{-6} mol/L phenylephrine, and the relaxant responses to the different flavonoids at a terminal concentration of 36 μmol/L were recorded, and the maximal relaxation (Rex%,with standard error, SE) was calculated. Before the final determination was taken, concentration-response curves had been obtained to check the terminal concentration in which the flavonoids inhibited the PE-induced contraction in rat thoracic aorta in a concentration-dependent manner.

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