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Stability of daphnoretin *in vitro*

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The stability of daphnoretin in sodium phosphate buffers at different pH and temperature, and in different biological samples at 37 °C was investigated using HPLC with UV detector set at 345 nm. Daphnoretin degraded rapidly in alkaline environment and was stable in acidic environment. Daphnoretin was stable in simulated gastrointestinal liquid, stomach contents, gastric mucosa and colon contents; it was unstable in plasma, liver homogenates, small intestine contents, small intestinal mucosa and blind gut contents. The stability of daphnoretin in plasma and other biomaterials could have a significant impact on its absorption.

1. Introduction

Daphnoretin (7-hydroxyl-6-methoxy-3, 7′-dicoumaryl ether, Fig. 1), a daphnane type orthoester diterpene, is found exclusively in the species of Thymelaeceae, Rutaceae and Leguminosae, such as *Daphne mezereum* L., *Daphne gnidium* L., and *Wikstroemia indica* (Badawi et al. 1983). It shows potent inhibitory on the growth of tumor cells, such as Ehrlich′s ascites carcinoma cells (Hall et al. 1982), AGZY-84-a, Hep2 and HepG2 cell lines etc. (Yang et al. 2008; Lu et al. 2011). Further more, daphnoretin has demonstrated to inhibit Ehrlich ascites carcinoma growth significantly *via* inhibition of protein and DNA synthesis in Ehrlich ascites carcinoma cells (Liou et al. 1982). Daphnoretin could also suppress hepatitis B virus gene expression in human hepatoma cells (Chen et al. 1996), decrease myocardial consumption of oxygen (Zhang et al. 1993), and inhibit the expression of protein kinase C (Wang et al. 1995). In addition, the compound was reported to have antimicrobial (Cottiglia et al. 2001) and anxiolytic effects (Navarro-Garcia et al. 2007).

Research on daphnoretin, a prospecting new drug, is still limited. Li et al. (2009) determined daphnoretin in natural plant material and in Chinese medicinal preparations. Lie et al. (2005) described a HPLC method for the determination of daphnoretin in rat plasma. When daphnoretin (500 mg/kg) was given orally, the oral bioavailability was very low (about 0.15%).

In vitro stability is important for drug discovery (Di et al. 2004). Early screening of stability and other pharmaceutical properties provides important information for discovery teams to modify structures, diagnose *in vitro* and *in vivo* assay results and enhance the overall quality of development candidates (Kerns and Di 2003). However, there are no reports about the stability of daphnoretin in biological samples, which would be necessary for research and development of this anti-tumor drug. In this study, the stability of daphnoretin in sodium phosphate buffers (PBS) at different pH and temperature values, and in different biological samples at 37 °C was investigated in detail, using a HPLC method.

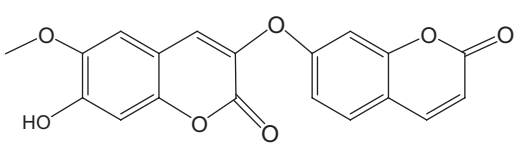

Fig. 1: Chemical structure of daphnoretin

2. Investigations, results and discussion

2.1. Method validation

Typical chromatograms of method selectivity are shown in Figs. 2 and Fig. 3 . No endogenous interference with daphnoretin and IS was observed in the tested biological materials.

The calibration curve was linear over the concentration range of 0.10–5.0 μg/mL for daphnoretin in PBS, simulated gastric liquid and simulated intestinal liquid; in plasma and other biomaterials, the calibration range was 0.20–5.0 μg/mL. The correlation coefficient (r) was higher than 0.99 for each calibration curve. The intra- and inter-day precision (RSD) were less than 13.7% and the accuracy (RE) was in the range of −12.9–13.1%. These results demonstrated the accuracy and reproducibility of the developed method. The average extract recoveries of daphnoretin at low, medium and high concentrations were 98.0%, 99.5%, 102.0% in PBS; 98.1%, 92.9%, 94.0% in simulated gastrointestinal liquid; and 77.6%–95.1% in plasma and other biomaterials.

The stability of daphnoretin in processed samples was evaluated. The RSD and RE were within 15%, suggesting that daphnoretin was stable after storage at 4 °C for 12 h.

2.2. Stability of daphnoretin in vitro

2.2.1. Stability in phosphate buffered saline

The stability of daphnoretin was investigated in PBS at 37 °C and different pH values from 1.20 to 10.97. The corresponding degradation profiles of daphnoretin are shown in Fig. 4. Under acidic conditions (pH 1.20–5.00), daphnoretin was found to be stable. With an increase in pH, the stability of daphnoretin decreased. When the pH value of the buffer solution was higher than 9.01, the decrease in the concentration level of daphnoretin

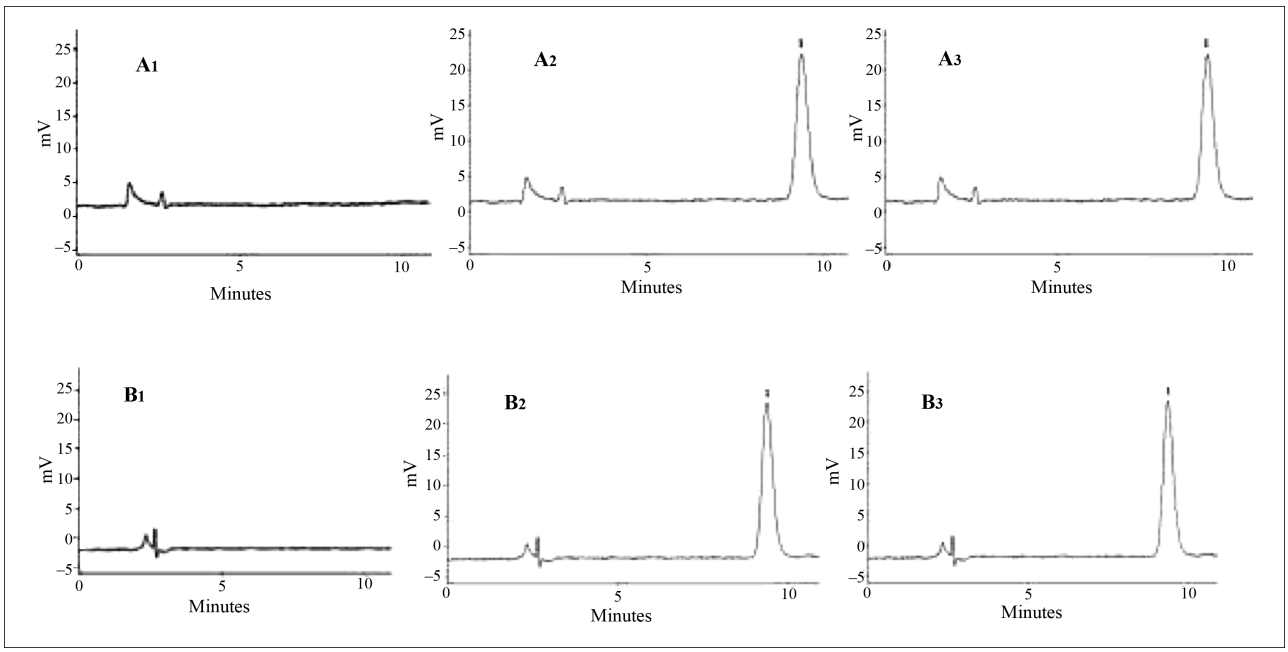

Fig. 2: Typical chromatograms of daphnoretin in simulated gastrointestinal liquid. A_1, B_1: blank samples of simulated gastric liquid and simulated intestinal liquid; A_2, B_2: blank samples of simulated gastric liquid and simulated intestinal liquid spiked with daphnoretin standard solution; A_3, B_3: samples obtained at 2 h after initiation of incubation in simulated gastric liquid and simulated intestinal liquid. Peak1: daphnoretin

was significant with an apparent first-order kinetic process after incubation at 37 °C. The half life ($t_{1/2}$) of degradation was calculated using the expression $t_{1/2} = 0.693/k$, where k is the slope found in the linear fit of the natural logarithm of the fraction remaining of the parent comound vs. incubation time. The results are presented in Table 1.

The effect of temperature on the stability of daphnoretin was also evaluated in PBS at pH 6.80, 8.00, 9.01, 10.02 and 10.97. The corresponding degradation profiles of daphnoretin at different temperatures are shown in Fig. 5. The hydrolytic kinetics parameters of daphnoretin are listed in Table 2. Hydrolysis rate of daphnoretin accelerated with an increase of temperature. When the temperature was increased from 45 °C to 75 °C, the hydrolysis rate constant of daphnoretin was increased from 5.6-fold to 21.0-fold. Within the experimental range, when the temperature was promoted by 10 °C, the hydrolysis rate constant increased by 1.4-fold to 5.0-fold, which was in line with Van 't Hoff rules. The apparent activation energy and Arrhenius equation of daphnoretin hydrolysis are listed in Table 3. The results showed a temperature-dependent degradation of daphnoretin. Temperature strongly affected the stability of daphnoretin and daphnoretin was stable at lower temperature.

2.2.2. Stability in simulated gastrointestinal liquid and other biomaterials

The stability of daphnoretin in simulated gastric liquid and simulated intestinal liquid was investigated. Daphnoretin was found stable in simulated gastric liquid and simulated intestinal liquid at 37 °C (Fig. 6). The results revealed that pepsase and trypase did not affect the hydrolysis of daphnoretin.

Daphnoretin was found stable in stomach contents, gastric mucosa and colon contents at 37 °C. On the other hand, daphnoretin was significantly and rapidly decomposed in 20% plasma, blind gut contents, small intestine contents, small intestinal mucosa, 20% liver homogenates at 37 °C. The concentration-time profiles of the degradation of daphnoretin in rat plasma and other biomaterials are given in Fig. 7.

The concentration-time profiles clearly demonstrated that daphnoretin degraded faster in small intestine contents (after 8 h of incubation, the degradation rate was $33.4 \pm 0.1\%$) than in other biological samples. After incubation in small intestinal mucosa, 20% plasma and 20% liver homogenates for 8 h, the degradation rate of daphnoretin was $20.5 \pm 0.2\%$, $22.5 \pm 0.1\%$ and $22.1 \pm 0.2\%$, respectively. And after incubation in blind gut contents for 12 h, the degradation rate of daphnoretin was $27.9 \pm 0.4\%$. The results indicated that in plasma and liver homogenates the hydrolysis reaction of daphnoretin was enzyme-catalyzed.

Pharmacokinetic studies have shown that daphnoretin has low oral bioavailability. Stability in the gastrointestinal tract, membrane permeability and liver first pass effect etc. can be main reasons of a low oral bioavailability of daphnoretin. Daphnoretin was relatively stable in simulated gastric liquid, stomach contents and gastric mucosa, suggesting that enzymes and microbes in the stomach were not involved in the metabolism of daphnoretin. The degradation rate of daphnoretin was below 3% in PBS at pH 6.8 and pH 8.0, thus, a variety of digestive enzymes and flora in small intestine contents, small intestinal mucosa and blind gut contents may be involved in the metabolism of daphnoretin. This study revealed the major degradation locations of daphnoretin were small intestine, small intestinal mucosa, blind gut, plasma and the liver.

Table 1: Hydrolytic kinetics parameters of daphnoretin in PBS at 37 °C

pH	Hydrolytic kinetics equation	k/h^{-1}	$t_{1/2}$/h	r
1.20	/	/	/	/
2.00	/	/	/	/
5.00	$\ln C = 1.375 - 0.0061\ t$	0.61×10^{-2}	113.6	0.9919
6.80	$\ln C = 1.330 - 0.0113\ t$	1.13×10^{-2}	61.3	0.9908
7.39	$\ln C = 1.332 - 0.0140\ t$	1.40×10^{-2}	49.5	0.9943
9.01	$\ln C = 1.324 - 0.0200\ t$	2.00×10^{-2}	34.6	0.9979
10.02	$\ln C = 1.359 - 0.0204\ t$	2.04×10^{-2}	34.0	0.9953
10.97	$\ln C = 1.281 - 0.0614\ t$	6.14×10^{-2}	11.3	0.9946

3. Experimental

3.1. Materials

Daphnoretin (98.5% pure) was obtained from the Laipudake Company (Guangzhou, China). Psoralen (internal standard, IS) was supplied by the

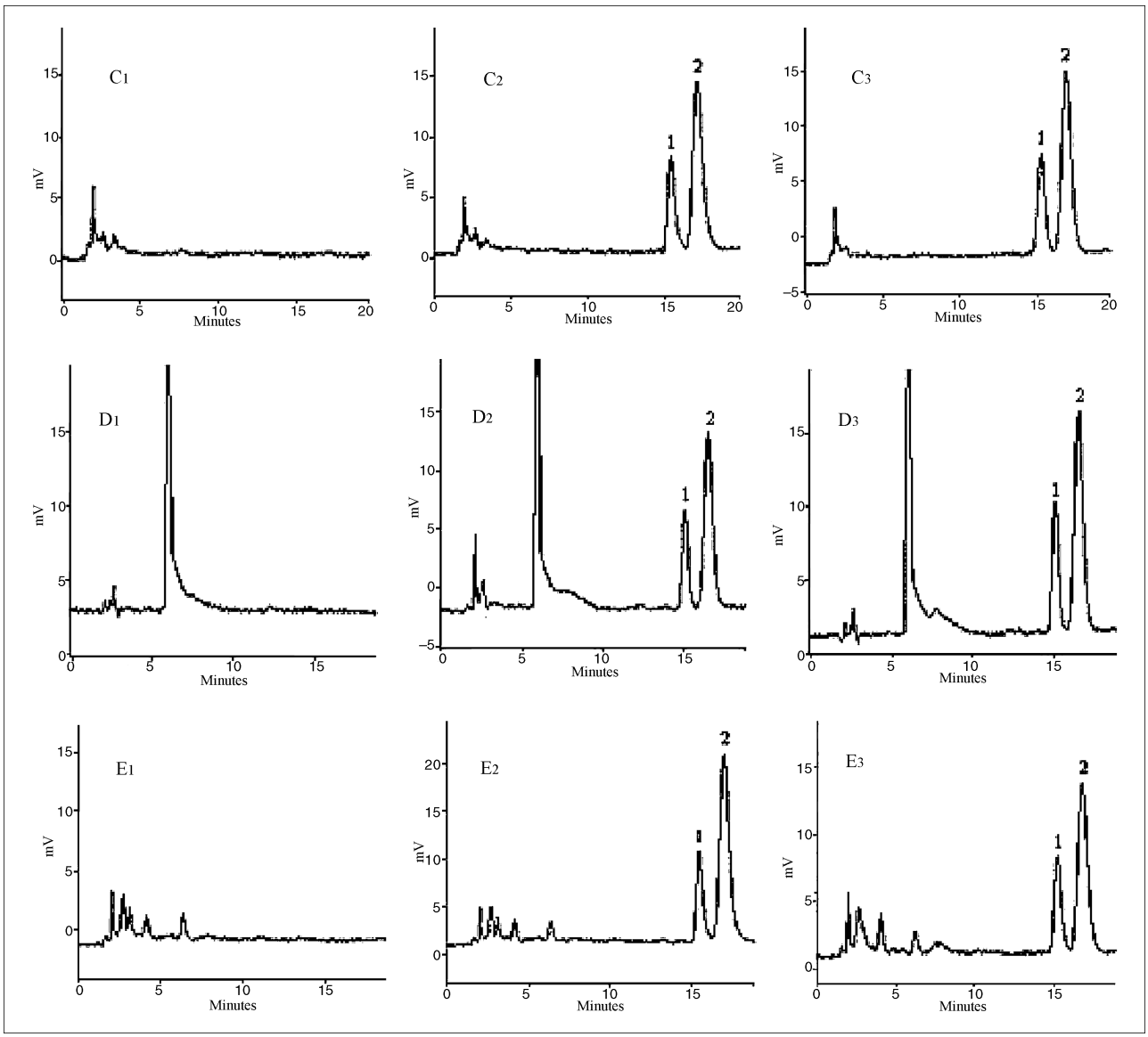

Fig. 3: Typical chromatograms of daphnoretin in blank samples of rat colon contents, 20% plasma and 20% liver homogenates blank samples of rat colon contents, 20% plasma and 20% liver homogenates spiked with daphnoretin (**C_2-E_2**); samples obtained at 3 h after initiation of incubation in rat colon contents, 20% plasma and 20% liver homogenates (**C_3-E_3**). Peak 1: psoralen(internal standard); Peak 2: daphnoretin

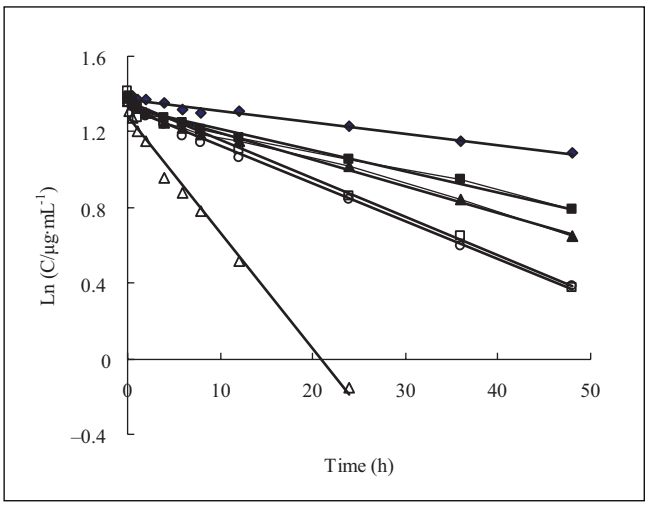

Fig. 4: Concentration-time profiles of the degradation of daphnoretin in different PBS pH at 37 °C *in vitro*. ♦: pH = 5.00; ■: pH = 6.80; ▲: pH = 7.39; □: pH = 9.01; ○: pH = 10.02; △: pH = 10.97

National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol was purchased from Shandong Yuwang Industrial Co., Ltd (Yucheng, China). Water was doubly distilled in the laboratory. All other reagents were analytical grade and from commercial sources.

3.2. Instrument and chromatographic conditions

The HPLC system was consisted of a Shimadzu LC-10A pump, a SPD-10AV detector and a column oven. Separation was performed on a Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm) protected with a guard column (10 mm × 4.6 mm) of the same packing material. The column oven was maintained at 40 °C; the flow rate was 1.0 mL/min; UV detector was set at 345 nm. Injection volume was 20 μL.

The mobile phase was methanol-0.05% H_3PO_4 (54:46, *v/v*) (for the analysis of daphnoretin in PBS, simulated gastric liquid and simulated intestinal liquid) or methanol-0.4% formic acid (48:52, *v/v*) (for the analysis of daphnoretin in plasma and other biomaterials).

3.3. Preparation of standard solutions and quality control samples

Stock solutions of daphnoretin (200.0 μg/mL) as well as IS (100.0 μg/mL) were prepared by dissolving an appropriate amount of substance in methanol. The stock solution of daphnoretin was further diluted with methanol to give a series of working standards at concentrations of 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 μg/mL. The IS working solution (40.0 μg/mL) was also prepared by diluting a 100.0 μg/mL stock solution of IS with methanol.

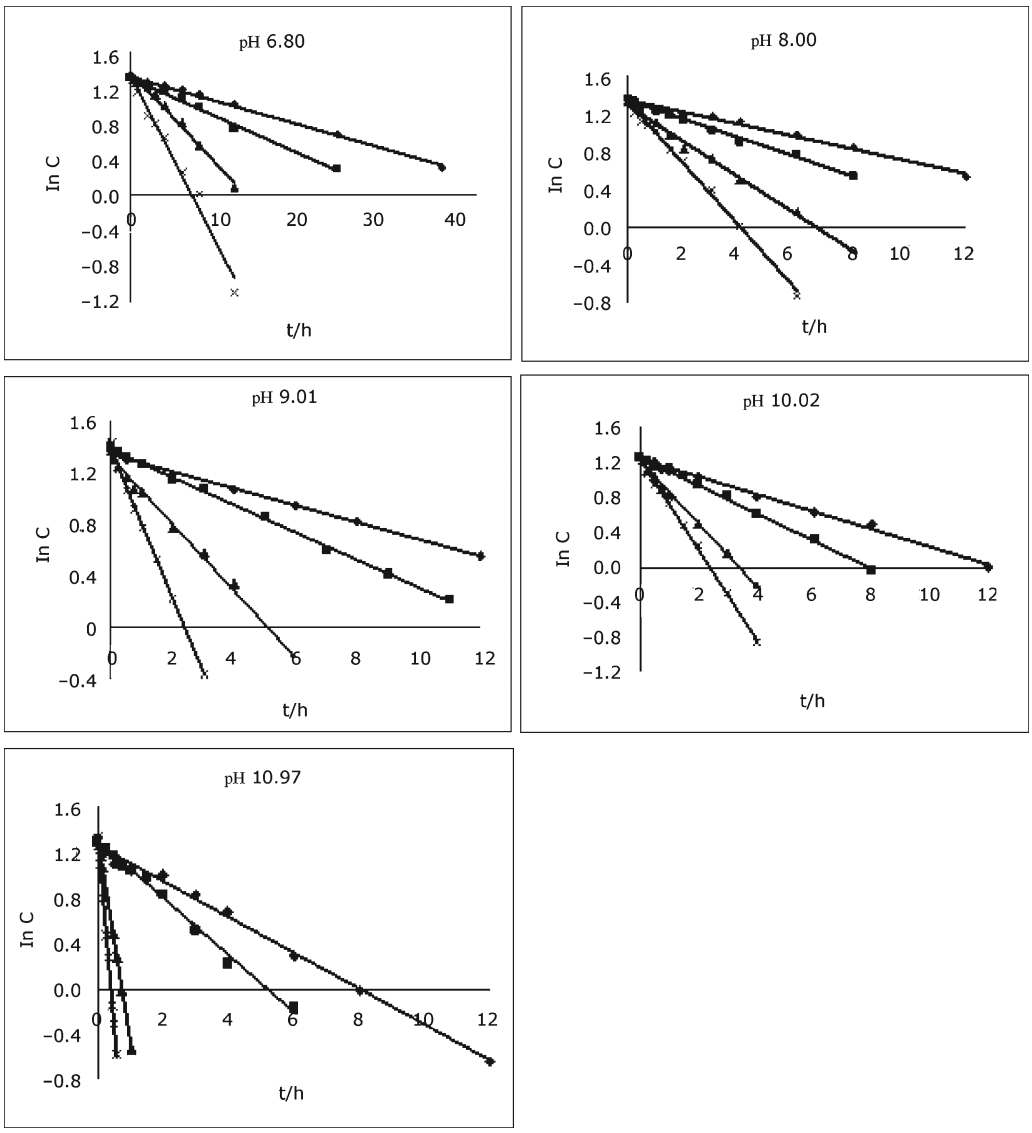

Fig. 5: Concentration-time profiles of the degradation of daphnoretin at different temperature and in the same PBS. ♦: 45 °C; ■: 55 °C; ▲: 65 °C; × : 75 °C

The quality control (QC) solutions were prepared at concentrations of 2.0, 10.0 and 40.0 μg/mL for PBS, simulated gastric liquid and simulated intestinal liquid; 5.0, 20.0 and 40.0 μg/mL for plasma and other biomaterials in the same way. All the solutions were kept at 4 °C and brought to room temperature before use.

Both calibration standard samples and QC samples were prepared by spiking 200 μL blank biological samples (firstly put them in 100 °C water bath for 1 min to inactivate enzymes) with 20 μL working solution during validation and the pharmacokinetic study.

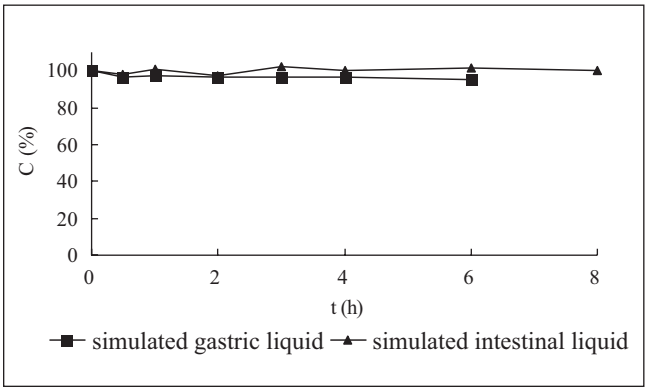

Fig. 6: Concentration-time profiles of the degradation of daphnoretin in simulated gastric liquid and simulated intestinal liquid at 37 °C *in vitro*

Table 3: Apparent activation energy and Arrhenius equation of daphnoretin hydrolysis

pH	Arrhenius equation	Ea / (kJ·mol^{-1})	*r*
6.80	lnk = 16.433–6392.7 / T	53.16	0.9922
8.00	lnk = 19.546–7161.1 / T	59.54	0.9998
9.01	lnk = 22.695–8106.8 / T	67.41	0.9975
10.02	lnk = 23.364–8246.7 / T	68.57	0.9993
10.97	lnk = 34.980–11779.0 / T	97.94	0.9910

3.4. Preparation of simulated gastric liquid and simulated intestinal liquid

Simulated gastric liquid was prepared by mixing pepsase 10 g with diluted hydrochloric acid 16.4 mL, and then diluted the mixture to 1000 mL by water.

Simulated intestinal liquid was prepared by mixing pancreatin 10 g with potassium dihydrogen phosphate buffer (dissolve potassium dihydrogen phosphate 6.8 g in 500 mL water, adjusted to pH 6.8 with 0.1 mol/L sodium hydroxide), and then diluted the mixture to 1000 mL by water.

3.5. Animals

Sprague-Dawley rats, 220 ± 20 g, were from the Experimental Animal Center of Shenyang Pharmaceutical University [SCXK (Liao) 2009–004]. They were housed in a room with controlled temperature and humidity and had

Table 2: Hydrolytic kinetics parameters of daphnoretin at different conditions

pH	t/°C	Hydrolytic kinetics equation	k/h^{-1}	$t_{1/2}$/h	*r*
6.80	45	lnC = 1.3297–0.0277 t	2.77×10^{-2}	25.02	0.9984
	55	lnC = 1.3064–0.0426 t	4.26×10^{-2}	16.27	0.9968
	65	lnC = 1.3788–0.1032 t	1.03×10^{-1}	6.72	0.9947
	75	lnC = 1.2848–0.1554 t	1.55×10^{-1}	4.46	0.9959
8.00	45	lnC = 1.3214–0.0516 t	5.16×10^{-2}	13.43	0.9963
	55	lnC = 1.3379–0.1000 t	1.00×10^{-1}	6.93	0.9970
	65	lnC = 1.2926–0.1921 t	1.92×10^{-1}	3.61	0.9973
	75	lnC = 1.346 6–0.3621 t	3.62×10^{-1}	1.91	0.9955
9.01	45	lnC = 1.347 0–0.0664 t	6.64×10^{-2}	10.44	0.9983
	55	lnC = 1.396 4–0.1246 t	1.25×10^{-1}	5.56	0.9992
	65	lnC = 1.318 7–0.2598 t	2.60×10^{-1}	2.67	0.9972
	75	lnC = 1.379 0–0.5824 t	5.82×10^{-1}	1.19	0.9988
10.02	45	lnC = 1.225 1–0.0787 t	7.87×10^{-2}	8.81	0.9965
	55	lnC = 1.259 2–0.1608 t	1.61×10^{-1}	4.31	0.9990
	65	lnC = 1.211 9–0.3590 t	3.59×10^{-1}	1.93	0.9973
	75	lnC = 1.254 1–0.7243 t	7.24×10^{-1}	0.96	0.9968
10.97	45	lnC = 1.254 1–0.1568 t	1.57×10^{-1}	4.42	0.9964
	55	lnC = 1.339 8–0.3020 t	3.02×10^{-1}	2.29	0.9949
	65	lnC = 1.322 8–1.8138 t	1.81	0.38	0.9983
	75	lnC = 1.335 1–3.3246 t	3.32	0.21	0.9983

free access to food and water until 12 h prior to the experiment, when their food was withdrawn. The protocol of the animal study was in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

3.6. Sample collection

Rat blood was collected from the abdominal aorta into heparinized tubes, and then was centrifuged at 15,000 rpm for 4 min to obtain plasma. The plasma was diluted to 20% with chilled 0.9% NaCl and was kept on ice before the incubation.

After death of the animal, the stomach, small intestine, colon, blind gut and liver were immediately removed and placed in chilled buffer solution. All subsequent preparations were carried out at 4 °C. The stomach, small intestine, colon and blind gut were carefully rinsed with hydrochloric acid solution (pH = 1.2), PBS (pH = 6.8), PBS (pH = 8.0), respectively, and collected the respective contents. The stomach and small intestine were longitudinally opened, and the mucosa scraped off with the back of a razor-blade. The liver was washed in PBS at pH 7.4, trimmed of adhering tissue, and cut into small pieces. The liver was added with 5 volumes (10 volumes for the mucosa) of chilled phosphate buffer at pH 7.4 [hydrochloric acid solution (pH = 1.2) for gastric mucosa and phosphate buffer at pH 6.8 for small intestine mucosa, w/v] and homogenized under ice-cooling with a FJ 200-S homogenizer (23,000 rpm, Shanghai, China). The contents and the mucosa were prepared to 0.1 g/mL. The homogenates and the prepared contents were centrifuged at 1,5000 rpm (4 min at 4 °C) to remove particulate material, the fatty layer floating on the top of the homogenates was discarded and the supernatants were used for the incubations. All the samples were placed on ice until use.

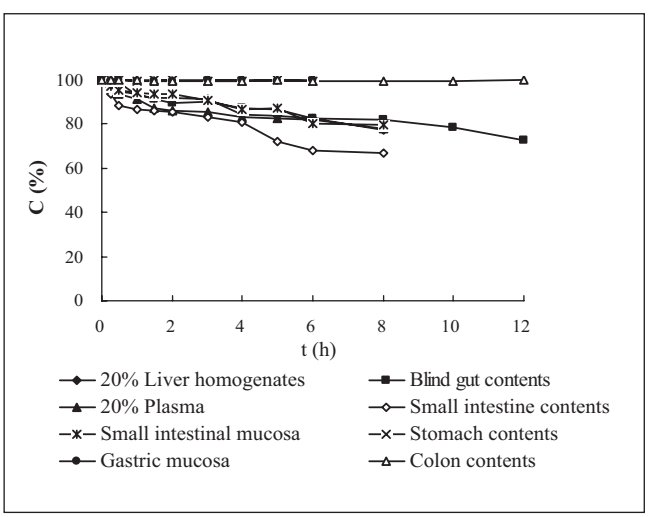

Fig. 7: Concentration-time profiles of the degradation of daphnoretin in plasma and other biomaterials at 37 °C *in vitro*. *The time points where the statistically significant differences between the results obtained at plasma, blind gut contents, small intestine contents, small intestinal mucosa and liver homogenates were proven ($p < 0.05$), and no statistical difference between the results obtained at stomach contents, gastric mucosa and colon contents ($p < 0.05$). Results are shown as a mean ± S.D.(n = 3)

3.7. Sample preparation

Physiological buffers (simulated gastric liquid and simulated intestinal liquid samples) of 200 μL were spiked with methanol (800 μL) and were vortexed for 3 min. After centrifugation at 15,000 rpm for 4 min, an aliquot of 20 μL supernatant was directly injected into the HPLC system.

Rat biological samples (200 μL) were vortex-mixed with 20 μL of IS solution (40.0 μg/mL) and 600 μL of methanol. After centrifugation at 15,000 rpm for 4 min, an aliquot (20 μL) of the supernatant was directly injected into the HPLC for analysis.

3.8. Method validation

The analytical method was tested with respect to following validation parameters: selectivity, linearity, accuracy, precision, recovery and stability. The selectivity was evaluated by processing and analyzing blank materials obtained from six different sources.

Duplicate calibration curves were analyzed each run. For each standard calibration curve, the peak area ratio of daphnoretin to IS was calculated and plotted against normal daphnoretin concentrations. The curves were fitted by least squares linear regression with $1/x^2$ weighing. Concentrations of daphnoretin in samples were calculated from the calibration curves. Precision and accuracy of the method were determined from QC samples. These samples were extracted in five replicates on three consecutive days. Assay precision was calculated by ANOVA. The intra- and inter-day precisions (RSD) were required to be below 15%, and the accuracy (RE) to be within ± 15%. The extraction recovery was determined by comparing analyte peak areas from extracted QC samples to those of drug-free extracts spiked with daphnoretin at equivalent concentrations. The stability of the samples was evaluated after short-term (4 °C for 12 h) storage. The concentration of daphnoretin was obtained by freshly prepared calibration curve.

3.9. In vitro stability study of daphnoretin

3.9.1. Stability in PBS

The drug-free PBS at different pH values of 1.20, 2.00, 5.00, 6.80, 7.39, 9.01, 10.02, 10.97 (preincubated at 37 °C for 10 min) were spiked with the stock solution to get a final concentration of 4.0 μg/mL of daphnoretin. Thereafter,

these samples were incubated at 37 °C in a water bath. During the whole stability experiment, the samples were constantly stirred (130 spin/min). In the different time periods, 200 μL aliquots of the incubation mixtures were taken and placed in 100 °C water bath for 1 min to stop the reaction. Samples corresponding to time zero were prepared manually separately. All the incubations were carried out in triplicate. The samples were stored at 4 °C until analysis.
On the other hand, stability of daphnoretin in PBS (at different pH values of 6.80, 8.00, 9.01, 10.02 and 10.97) were incubated at 45 °C, 55 °C, 65 °C, and 75 °C in water bath. Then samples were prepared as described above.

3.9.2. Stability in simulated gastrointestinal liquid and other biomaterials

Daphnoretin in simulated gastrointestinal liquid and other biological samples at 4.0 μg/mL was incubated at 37 °C. During whole stability experiment, the samples were constantly stirred (130 spin/min). At defined time intervals, 200 μL of each incubation mixtures was removed and placed in 100 °C water bath for 1 min to stop the reaction. The samples were processed as described previously. A triplicate experiment was performed for each biological sample. The samples were stored at 4 °C until analysis. The stability of daphnoretin was determined as the decrease in the initial concentration as a function of time.

3.10. Statistics

Statistical Product and Service Solutions (SPSS 16.0, SPSS Inc., Chicago, USA). was used in the statistical analysis. To evaluate the statistical differences between results obtained in different biological samples ANOVA test was used. The results were expressed as means ± S.D.

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