Investigation of quercetin stability in cell culture medium: Role in \textit{in vitro} experiment

Jun Hu\textsuperscript{1}, Long Chen\textsuperscript{1}, Fan Lei\textsuperscript{1}, Yu Tian\textsuperscript{1,2}, Dong-Ming Xing\textsuperscript{1}, Yu-Shuang Chai\textsuperscript{1}, Shuang Zhao\textsuperscript{1}, Yi Ding\textsuperscript{1,2} and Li-Jun Du\textsuperscript{1\ast}

\textsuperscript{1}Protein Science Laboratory of the Ministry of Education, Laboratory of Pharmaceutical Sciences, School of Life Sciences and School of Medicine, Tsinghua University, Beijing 100084, China.
\textsuperscript{2}Drug Discovery Facility, Tsinghua University, Beijing 100084, China.

Accepted 23 March, 2012

The aims of this work are to investigate the \textit{in vitro} stability of quercetin, a focused compound in food components and a candidate drug, and to supply the essential information for the experimental researches of quercetin. The stability of quercetin in Dulbecco's modified Eagle's medium (DMEM) and H\textsubscript{2}O at the cell culture pH range of 6 of 8 at continued time points, as well as 6 frequently used supplements in DMEM was investigated. The assay of high-performance liquid chromatography (HPLC) was used for quercetin determination. Considering the stable conditions of quercetin, the transport behavior of quercetin in Neuro-2a (N2a) cells was investigated at the dosages of 58.8 and 7.83 \mu g/ml at 37°C for different time points. It is shown that quercetin remained stable in DMEM at pH of 6 with or without 10 mM/L HEPES, 5% D (+)-glucose, 110 mg/L sodium pyruvate, antibiotics (50 IU/ml penicillin and 0.05 mg/ml streptomycin), or 10% fetal bovine serum (FBS), but was decreasing at pH of 7 or 8 with or without 3.7 g/L NaHCO\textsubscript{3}. Quercetin transported through N2a cells in 10 min in both dosages of 58.8 and 7.83 \mu g/ml. Our data demonstrate that the culture conditions of quercetin \textit{in vitro} should be considered to acquire the convictive results in the experiments.

\textbf{Key words}: Quercetin, stability, high performance liquid chromatography (HPLC), \textit{in vitro}, transportation.

\textbf{INTRODUCTION}

Flavonoids, the constituents of the regular diet, have a long evolutionary history in plant physiology, and the isolation and biological identification of it were first described by Gyorgyi (Armentano et al., 1936). Quercetin (structure is shown in Figure 1) is one of natural flavonoids of which number is more than 4000 (Hollman and Katan, 1999). It stays in many foods and has a wide range of medicinal effects. In recent years, quercetin has been receiving great attention on its biological activities, such as anti-tumor (Chien et al., 2009; Wong and Chiu, 2010), anti-oxidant (Chen et al., 2006; Arash, 2010), anti-inflammatory (Rogerio et al., 2010; El-Sayed and Rizk, 2009), antiviral (Walker et al., 2009), antibacterial (Hirai et al., 2010; Ramadan and Asker, 2009), analgesia (Kumar and Goyal, 2008; Lee et al., 2005), hypoglycemic (Torres-Piedra et al., 2010), protective effect from alloxan-induced DNA-damage in diabetic mellitus (Orsolic et al., 2011), positive effect at lower concentrations and negative at higher concentrations to myocardial and coronary function (Angelone et al., 2011), as well as activation of GABA(A) receptors to alter sleep-wake cycle. (Kambe et al., 2010; Kawabata et al., 2010). Especially, quercetin can scavenge the free radicals to reduce oxidant pressure in cerebral tissue and neurons. It possesses anti-apoptotic effect on the neurons, which are shown in the treatment of Alzheimer's disease, Parkinson's disease and stroke (Ansari et al., 2009; Hollman et al., 2010). From the literature described previously, quercetin is considered as a potential drug...
and food supplement for the development.

Quercetin is also reported for cancer treatment. The multidrug resistance (MDR) is thought to represent one of the main causes in cancer treatment failure, which involves an increased activity of ATP-binding cassette family transporters (ABC) (Ambudkar et al., 2003). P-glycoprotein (P-gp), encoded by the \textit{ABCB1} gene, is the most frequently over expressed ABC membrane transporter (Sauna et al., 2007). The phenomenon of MDR reversal caused by quercetin has been reported in many studies: The quercetin can enhance the bioavailability of antineoplastic either \textit{in vivo} or \textit{in vitro} (Jing et al., 2008; Kim et al., 1998; Shin et al., 2006). One of the explanations has been confirmed lately is that quercetin can inhibit P-gp function and \textit{ABCB1} gene expression in many cell lines (Borska et al., 2010; Limtrakul et al., 2005). However, chronic ingestion of 10 mg/kg quercetin decreased the bioavailability of simvastatin to a significant extent (Cermak et al., 2009) by increasing the expression of CYP3A4, which means there may be a drug interaction with quercetin.

Several methods of determination of quercetin have been published (Careri et al., 2003; Zu et al., 2006), including in biological samples (Jin et al., 2004; Jones et al., 1998). We studied the transport behavior of quercetin in our previous work (Liu et al., 2006). When we performed the experiment, we found quercetin was not stable in the culture. There might be the instability of quercetin in the cell culture in which would influence the \textit{in vitro} studies. The factors that cause quercetin unstable need to be revealed. Therefore, we conducted the experiment for quercetin stability. In this study, we investigated the stability of quercetin in common used cell culture medium and within several supplements, and revised our past procedure. Then we observed the transport behavior of quercetin in Neuro-2a (N2a) cells, as an exemplification to validate quercetin stable conditions. N2a cells are commonly used in Alzheimer's disease model (Zhang et al., 2006), and quercetin was reported could be accumulated in brain by oral administration (Ishisaka et al., 2011) and restored impaired phosphorylation of cyclic AMP response element-binding protein (CREB) in Aβ- expressing N2a cells (Xu et al., 2007).

**EXPERIMENTAL**

**Chemicals**

Quercetin was purchased from the National Institutes for Food and Drug Control (Beijing, China), with purity of 98%. Methanol and acetonitrile (high performance liquid chromatography, HPLC grade) were purchased from Baker (J.T. Baker, USA). Penicillin, streptomycin, sodium bicarbonate, sodium pyruvate, D (+) glucose, and HEPES were all purchased from Sigma Chemical Co. (St Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco. Deionized water was used throughout the experiment. Other organic solvents and reagents were of analytical-reagent grade.

**Instrumentation and chromatographic conditions**

The high performance liquid chromatography (HPLC) system consisted of two 515 HPLC pump (Waters, USA), a 996 photodiode array detector (Waters), a Rhodyne 7725i manual injector (Waters) and an Empower² Chromatography Work Station. Separation was carried out by using a Hypersil C₁₈ column (4.6 × 150 mm; 5 μm; Rainbow, China). The mobile phase was 0.1% phosphoric acid–acetonitrile (60:30, v/v), filtered through a 0.45 μm Millipore filter and degassed prior to use. The flow rate was 1.0 ml/min. Detection was performed at a wavelength of 371 nm with the column maintained at a constant temperature (25 ± 1°C).

**Standard solution and stability**

Quercetin was dissolved in methanol with 3 min ultrasonic dispersion in order to obtain a 60 μg/ml standard solution. To investigate the stability of quercetin at different pH, the solution was diluted 20 times by DMEM and H₂O at pH of 6, 7 and 8. DMEM was dissolved without Na₂CO₃. After DMEM was added with different supplements, the solutions and H₂O were adjusted to pH 6, 7 and 8 by 1 mol/L HCL and NaOH solutions. For viewing the stability of quercetin in commonly used cell culture supplements, the solution was diluted 20 times by DMEM added separately with 10 mM/L HEPES, 5% D (+)-glucose, 3.7 g/L sodium bicarbonate, 110 mg/L sodium pyruvate, antibiotics (50 IU/ml penicillin and 0.05 mg/ml streptomycin) and 10% FBS, adjusting the pH to 6. All the samples were injected 20 μl at 0, 10, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h after preparation. All the solutions were kept in the cellular incubator (Sanyo, Japan) with the atmosphere of 95% air and 5% CO₂ at 37°C all the time.

**Cell culture and sample preparation**

Mouse neuroblastoma Neuro-2a (N2a) cells were obtained from the American Type Culture Collection (USA). The cells were cultured in DMEM supplemented with 10% FBS, 50 U/ml penicillin, 100 mg/ml streptomycin and 1 mM/L sodium pyruvate in an atmosphere of 95% air and 5% CO₂ at 37°C (Chen et al., 2007). Grown cells were seeded in at a density of 1 × 10⁵ cells/ml in 24-well tissue culture
plates (Costar) at 37°C in 5% CO₂ and 95% air 24 h prior to experiments. Quercetin was dissolved in DMSO with the end concentration less than 0.4%. There was no cytotoxic effect of quercetin on cells during experimental period with 58.8 or 7.83 μg/ml. To investigate the quercetin transport behavior, the incubation medium was replaced by 1 ml/well DMEM with 10% FBS, containing 58.8 or 7.83 μg/ml quercetin (through a 0.22 μm Millipore filter), for the specific time periods (10, 30, 60, 120, 240, 360 and 480 min). At each time-point, the incubation medium was drawn out from each well, the neurons were washed three times with 0.2 ml of ice-cold PBS, and 200 μl of 0.25% trypsinase was added to lift the cells. Cells from 6 wells were collected together as one sample and the cell numbers were counted. Then, the cells were frozen at -80°C and thawed at room temperature to rupture cell membranes. The residual solution was freeze-thawed then the freeze-dried residue was dissolved in 100 μl of methanol, centrifuged at 12,000 g for 10 min at 4°C. A 20 μl aliquot was injected to the HPLC system for determining the concentration of quercetin in the cells. The quercetin concentration within the cells was calculated as follows (Meng et al., 2007): Quercetin content (μg/10⁶ cells) = (quercetin concentration in the sample × 0.1/ total cell number from 6 wells) × 10⁶.

Validation of method

The limit of detection (LOD) was determined as the lowest concentration that could be detected with acceptable accuracy and precision, which was achieved from the plot of three times the noise level. The limit of quantification (LOQ) was defined as the lowest concentration on the calibration curve for which assay precision (coefficient of variation, CV) was lower than 10% and was 10 times the noise level.

The reference substance was accurately weighed and dissolved in methanol to obtain matrix standard. For the transport behavior study, seven different concentrations of standard solutions were prepared at 0.58, 1.45, 2.9, 5.8, 14, 29 and 58 μg/ml. The calibration curves, was established by determining peak areas in blank cell frozen-thawed fluid processed as described previously. The content of quercetin in the samples was calculated using the regression parameters obtained from the standard curves. Calibration standards were included in every analytical batch of samples.

The precision of a quantitative method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings. It was measured by repeatedly injecting a readymade sample pool and expressed as relative standard deviation of the results. Analyses with three different concentrations (58, 5.8 and 0.58 μg/ml) of quercetin were performed. To determine the intra-day variance, the assays were carried out on the same samples at different times during one day. Inter-day variance was determined by assaying the spikes samples over three consecutive days. CVs were calculated from these values.

The recovery of quercetin from the samples was evaluated by using two different concentrations (58.8 and 7.83 μg/ml) covering the linear range of the calibration curve. Samples processed according to the method mentioned previously were spiked with different amounts of quercetin, and the resulting peak areas were compared with the standard of quercetin carried in mobile phase to provide the recovery values.

Statistical analyses

All values were expressed as mean ± SD. Data were statistically analyzed by ANOVA. The p-values less than 0.05 were considered statistic significance.

RESULTS AND DISCUSSION

Chromatograms and selectivity

Under the conditions described previously, the HPLC chromatograms of quercetin reference (5.8 μg/ml), blank cell frozen and thawed fluid, blank cell frozen and thawed fluid spiked with quercetin reference (3 μg/ml) and the quercetin sample with the cell culture are shown in Figure 2. The retention times of quercetin reference, quercetin spiked in cell frozen and thawed fluid and quercetin in the cell culture were all about 10 min, and UV spectrums of them were all the same. No interfering peaks were observed within the time in which quercetin was detected, indicating that the specificity and selectivity of the elaborated procedure were satisfactory.

Limit of quantification and calibration curve

The limits of detection (LOD, signal-to-noise ratio = 3:1) was 0.10 μg/ml, and the limits of quantification (LOQ) was 0.29 μg/ml for quercetin.

The calibration curve prepared in frozen-thawed cell fluid was linear over the concentration range 0.58 to 58 μg/ml. A typical equation of the calibration curve was as follows: \( y = 41857x + 10074 \) \( (x = \text{concentration of quercetin spiked with blank frozen-thawed cell fluid, } y = \text{the peak area of quercetin}) \) with a mean correlation coefficient \( r^2 \) of 0.9993, pointing to a good linear relationship between peak areas and concentrations of quercetin.

Precision, accuracy and recovery

The intra-day, inter-day precision and the accuracy by using three different concentrations (58, 5.8 and 0.58 μg/ml) were evaluated and the results are summarized in Table 1. The maximum CV value was 3.55% for intra-day precision and 3.84% for inter-day, indicating the good precision of the method. Moreover, the small difference (≤10%) noted between added levels and the estimated concentrations showed the appropriate accuracy of the method.

The loss of quercetin due to the extraction process was determined by comparing the data obtained by the direct injection of quercetin reference dissolved in mobile phase to those obtained after the whole extraction procedure. The recoveries were 102.6 ± 4.7% at 58.89 μg/ml and 93.8 ± 6.4% at 7.83 μg/ml. The freeze-dried samples were much more stable than the samples kept in normal saline (Liu et al., 2006), which lead to imperfect detection of quercetin in cells.

Stability of quercetin in different conditions

The effects of pH in DMEM and H₂O were studied by
Figure 2. Chromatograms and UV spectrums for the determination of quercetin in the medium and in the cells. (A) Chromatogram of the blank cell frozen and thawed fluid; (B) Chromatogram of quercetin reference (5.8 μg/ml); (C) Chromatogram of the blank cell frozen and thawed fluid spiked with quercetin reference (3 μg/ml); (D) Chromatogram of the quercetin reference (3 μg/ml) in DMEM medium at pH 6 for 24 h; (E) Chromatogram of the quercetin reference (3 μg/ml) in DMEM medium at pH 8 for 24 h; (F) Chromatogram of the quercetin sample within the cells 2 h after quercetin administration (58.8 μg/ml). The mobile phase was 0.2% phosphoric acid–acetonitrile (60:30, v/v) and the flow rate was 1.0 ml/min.

Table 1. Validation of the intra-day and inter-day assay.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Spiked concentration (μg/ml)</th>
<th>Measured concentration (μg/ml)</th>
<th>Accuracy (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day</td>
<td>0.58</td>
<td>0.59 ± 0.02</td>
<td>98.12</td>
<td>3.55</td>
</tr>
<tr>
<td></td>
<td>5.8</td>
<td>5.93 ± 0.17</td>
<td>98.53</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>58.47 ± 1.13</td>
<td>99.37</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td>0.58</td>
<td>0.57 ± 0.03</td>
<td>97.29</td>
<td>3.84</td>
</tr>
<tr>
<td>Inter-day</td>
<td>5.8</td>
<td>5.72 ± 0.21</td>
<td>98.35</td>
<td>3.38</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>58.68 ± 1.24</td>
<td>99.26</td>
<td>2.19</td>
</tr>
</tbody>
</table>

* Each value represents the mean ± SD (n = 3).
Figure 3. The effects of pH on the stability of quercetin. (A) Quercetin in DMEM; (B) Quercetin in H$_2$O at pH of 6, 7 and 8. The stability was evaluated by the time-residue curves. All the solutions were injected 20 μl at 0 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h after preparation. Each point and bar represents the mean ± SD (n=3).

diluting 60 μg/ml quercetin methanol solution with DMEM and H$_2$O at different pH. The relations between time and the residual percentage are presented in Figure 3. Quercetin in methanol was proved to be stable by our previous work (Liu et al., 2006). However, in DMEM, the stability was only emerged at pH of 6 (Figure 3A). When pH values were 7 and 8, the residual quercetin was greatly decreased in 1 h and gently decreased in next 5 h. Until more than 3 h later, the reduction became very slowly. After 24 h at last, the residue at pH of 7 remained only 49.75% of the original quercetin, but still 9.36% higher than that at pH of 8. While in H$_2$O (Figure 3B), quercetin was made the considerable reduction only at pH of 8, and its final residue was 50.83%, still more than
The effects of cell culture medium supplements on the stability of quercetin. The dosages of supplements in DMEM were: 10 mM/L HEPES, 3.7 g/L NaHCO$_3$, 5% D(+)glucose, 110 mg/L sodium pyruvate, antibiotics (50 IU/ml penicillin and 0.05 mg/ml streptomycin) and 10% FBS. The pH was adjusted to 6. The stability was evaluated by the time-residue curves. All the solutions were injected 20 μl at 0 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h after preparation. Each point and represents the mean ± SD (n = 3).

The residue in DMEM at the same pH. When the H$_2$O pH values were 6 and 7, the quercetin was stable. All the results indicate that pH is the crucial agent affecting stability of quercetin. Quercetin is more stable in weak acid than in weak base. Moreover, the constituents in DMEM affected the stability, because quercetin showed more stability in H$_2$O than in DMEM.

The effects of the commonly used cell culture supplements such as HEPES, sodium bicarbonate, glucose, sodium pyruvate, antibiotics (penicillin and streptomycin) and FBS were investigated in their regular used dosages. Since quercetin can be kept stable in DMEM of pH = 6, the supplements was added in DMEM and the final pH was adjusted to 6 (Figure 4). Most supplements, such as HEPES, glucose, sodium pyruvate, antibiotics and FBS scarcely affect the stability of quercetin. Their quercetin residual percentages almost did not change as the time went by in a whole day.

However, the time-residue curve of NaHCO$_3$ was similar like those of DMEM at pH of 7 and 8 (Figure 3A). The reduction occurred severely in 1 h and kept on decreasing gradually in the following time, though the final residue at 24 h was 33.19% higher than that of pH=8 in DMEM. Therefore, NaHCO$_3$ did significantly affect the stability of quercetin in DMEM.

Quercetin structure (Figure 1) possesses five phenol hydroxyl groups, which make it a weak acid. We found this structure is more stable in weak acid (Figure 3). However, it is less stable in DMEM than in H$_2$O at the pH of 7, and some studies administrating quercetin in DMEM at pH around 7 (Lu et al., 2006). It is known that quercetin is a potent scavenger of reactive oxygen species (ROS) that effectively prevent cell oxidation (Chen et al., 2006). Their antioxidant activities are governed by their structural characteristics and their ability to interact with ROS. The 3-OH quercetin radical possesses a large spin density on the C-2 atom, which causes the C-ring opening process and producing the deposit (Trouillas et al., 2006). We assume that the numerous constituents in DMEM may create a redox system, which caused the loss of quercetin content. Additionally, quercetin can generate a lot of complex formations with Ca$^{2+}$, Fe$^{3+}$, Zn$^{2+}$, etc by chelating (Abou-El-Sherbini and Hassanien, 2004; Leopoldini et al., 2006). So the metal ions in DMEM may affect quercetin content as well. In Figure 4, we obtained the direct evidence that NaHCO$_3$ dramatically affected the stability of quercetin in DMEM. It might involve with the buffer action of NaHCO$_3$. The result shows that the actual dosage in cell culture medium is less than the administration and in weak acidic DMEM without NaHCO$_3$ could enhance the stability of quercetin.

**Figure 4.** The effects of cell culture medium supplements on the stability of quercetin. Two time-concentration curves presents the quercetin in the cells fed with quercetin of the high dosage (58.8 μg/ml) and the low dosage (7.83 μg/ml) at 37°C for different time points (10, 30, 60, 120, 240, 360 and 480 min). Each point and bar represents the mean ± SD (n = 3).

**Figure 5.** Transport behavior of quercetin in N2a cells. Two time-concentration curves presents the quercetin in the cells fed with quercetin of the high dosage (58.8 μg/ml) and the low dosage (7.83 μg/ml) at 37°C for different time points (10, 30, 60, 120, 240, 360 and 480 min). Each point and bar represents the mean ± SD (n = 3).

**Transport behavior of quercetin in N2a cells**

Based on the aforementioned cell culture condition, the transport behavior of quercetin in N2a cells was observed in two administration concentrations: The high dosage was 58.8 μg/ml and the lower was 7.83 μg/ml. Figure 5 displays that quercetin could be detected at all the selective periods (10, 30, 60, 120, 240, 360 and 480 min) in both dosages. It also showed that quercetin could be...
transported into N2a cells rapidly either in the high or low dosage. However, the peak rates were different. The quercetin concentration was increased quickly and peaked at 1 h in the high dosage. Then the quercetin in the cells was fluctuated over the range of 44.78 to 54.81 μg/10⁶ cells in the following time. In comparison, the quercetin concentration of the low dosage was increased gently and peaked until 6 h, then decreased violently from 3.62 to 1.44 μg/10⁶ cells.

Quercetin is transported via GLUT1 and GLUT4, and its uptake is accelerated by exchanging with intracellular glucose (Cunningham et al., 2006; Vlachodimitropoulou et al., 2011). It has been proven that quercetin decreases both the expression and function of P-gp (p-glycoprotein) in a concentration dependent manner in a resistant and a sensitive to daunorubicin pancreatic cancer cell models (Borska et al., 2010). In N2a cells, the peak concentration of the high dosage was close to 16 times of the low dosage. However, the high dosage was only 7.5 times of the low dosage in the medium (Figure 5). Therefore, we demonstrated that the high dosage of quercetin revealed the faster peak, the weaker elimination and the higher absorbance of the N2a cells than the low dosage of quercetin. This dose-dependent phenomenon clues that the transport behavior was involved with P-gp inhibition of quercetin (Scambia et al., 1994), which attenuated the efflux of quercetin itself in the higher dosage (Chen et al., 2010).

Conclusion

In this study, we specified to investigate the stability of quercetin in DMEM with the supplements. We found quercetin was stable in weak acidic DMEM without NaHCO₃. We also investigated the transport behavior of quercetin in N2a cells, by which conformed the reliable culture conditions for quercetin in vitro. These results should be considered in the in vitro studies of quercetin in the future.

ACKNOWLEDGEMENTS

The present study was supported by the National Natural Science Foundation of China (30973896, 30801523 and 81073092), the National S&T Major Special Project for New Drug R&D of China (2009ZX09103-301, 2009ZX09502 and 2011ZX09101- 002-11) and the Foundation for Laboratories of Tsinghua University (LF 20103579).

REFERENCES


