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Short communication

Simultaneous determination of quercetin, kaempferol and isorhamnetin accumulated human beast cancer cells, by high-performance liquid chromatography

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Abstract

Quercetin, kaempferol and isorhamnetin are the most important constituents in ginkgo flavonoids. A simple, rapid and sensitive high-performance liquid chromatography method was developed to simultaneously determine quercetin, kaempferol and isorhamnetin absorped by human breast cancer cells. Cells were treated with ginkgo flavonols and then lysed with Triton-X 100. The flavonols in the samples were measured by RP-HPLC with a C18 column after a simple extraction with a mixture of ether and acetone. The mobile phase contained phosphate buffer (pH 2.0; 10 mM) tetrahydrofuran, methanol and isopropanol (65:15:10:20, v/v/v/v). The ultraviolet detector was operated at 380 nm. The calibration curve was linear from 0.1 to 1.0 μM ($r > 0.999$) for each flavonol. The mean extraction efficiency was about 70%. The recovery of the assay was between 98.9 and 100.6%. The limit of detection was 0.01 μM for quercetin and kaempferol and 0.05 μM for isorhamnetin. The limit of quantitation was 0.1 μM (R.S.D. < 10%) for each flavonol. The intra- and inter-day coefficients of variation were less than 10% (R.S.D.). The validated method was applied to quantify quercetin, kaempferol and isorhamnetin in human breast cancer Bcap37 and Bcap37/MDR1 cells.

Keywords: Quercetin; Kaempferol; Isorhamnetin; High-performance liquid chromatography; Cells; Determination

1. Introduction

Ginkgo biloba is a dioecious tree with a history of use in herbal medicine. Although the seeds are most commonly employed in traditional Chinese medicine, in recent years the extracts of the leaves have been widely used as a phytomedicine in Europe and China and as a dietary supplement in the United States. The primary active constituents of the leaves include flavonol glycosides and unique diterpenes. Clinical studies have shown that ginkgo extracts exhibit therapeutic activity in a variety of disorders [1–8]. Recent studies conducted with various molecular, cellular and animal models have revealed that leaf extracts of *G. biloba* may have anticancer/chemopreventive activities and effects of apoptosis induction, antimutagenesis, modulation of cell cycle and overcoming breast cancer resistance protein mediated drug resistance in tumor cells [9–12], which are related to their antioxidant, anti-angiogenic and gene-regulatory actions. The results from cDNA microarray analyses have shown that the exposure of human breast cancer cells to a Ginkgo extract altered the expression of genes that are involved in the regulation of cell proliferation, cell differentiation or apoptosis [13]. The ginkgo flavonol aglycones mainly consist of quercetin, kaempferol and isorhamnetin (Fig. 1), and these are present in only trace amounts in the extract. However, after oral administration of *G. biloba* extract, aglycones could be formed by gastrointestinal microorganisms that metabolize flavonoid glycosides [14]. The biological function of these ginkgo flavonols has to depend on the extent to which

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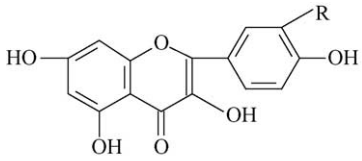

	R
Quercetin	OH
Kaempferol	H
Isorhamnetin	OCH_3

Fig. 1. Chemical structures of Ginkgo flavonol aglycones: quercetin, kaempferol and isorhamnetin.

they associate with the cells. Cytotoxicity of the flavonols on tumor cells depends on cellular accumulation of the drugs. In order to study the anticancer mechanism of flavonols, a method that can quantify the flavonols in cancer cells will be helpful.

In the past few years it has become apparent that many transport proteins such as P-glycoprotein (P-gp) play a major role in controlling the distribution, elimination and potentially the metabolism of some drugs [15,16]. For example, P-gp is believed to recognize a wide variety of structurally diverse lipophilic compounds and pump them out of cells [17]. Therefore, it is very important to identify substrates of transporter proteins in order to avoid drug–drug interaction arising from interactions with drug transport proteins. One of the assays is the cell based drug accumulation assay [18]. In this assay, the accumulation of P-gp substrate in cells that over-express P-gp was restricted by P-gp mediated efflux of the compound back into the extracellular fluid. Drug accumulation in the P-gp expressing cells was then compared with the accumulation in cells from the low P-gp-expression parental cell line. Since P-gp transported substrates out of the cell, P-gp substrates showed lower accumulation in P-gp expressing cells than in the P-gp-deficient cells.

Therefore, it is necessary to develop a method for quantify quercetin, kaempferol and isorhamnetin simultaneously in cells. Up to now, there were no reports about this but there were a few for determining quercetin only [19,20]. This paper proposes a specific, precise and sensitive HPLC method to measure the three ginkgo flavonols in breast cancer cells. We also report a preliminary investigation of the flavonols accumulated in Bcap37 and Bcap37/MDR1 cells.

2. Experimental

2.1. Chemicals and reagents

Quercetin, kaempferol, isorhamnetin and morin (purity >99.6%) were purchased from the China National Institute for the Quality Control of Pharmaceutical and Biological Products. Bcap37 human breast cancer cells were obtained from Cancer Institute, Zhejiang University. Bcap37/MDR1 cells (Bcap37 cells were infected with a recombinant plasmid containing MDR1 cDNA and high expressing P-gp) were developed by our lab [21]. RPMI-1640 medium, newborn calf serum, penicillin, streptomycin and trypsin were purchased from Invitrogen Life Technologies. Tetrahydrofuran, methanol and isopropanol were of HPLC grade. Triton-X 100 was purchased from Sigma Chem Co. (St. Louis, MO, USA). Bio-Rad DC Protein Assay kit was purchased from Bio-Rad Laboratories, Inc. All other chemicals and solvents were of analytical or chromatographic grade and obtained from commercial sources.

2.2. Cell culture

Bcap37 and Bcap37/MDR1 cells were grown in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated newborn calf serum. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 in air, and were subcultured every 3 days with 0.25% trypsin. The cells were harvested in their exponential growth phase. Bcap37 cells were washed with PBS twice and lysed by 10 ml of 0.1% Triton-X 100, which was used as blank cell lysis solution.

2.3. Instrument and chromatographic conditions

Chromatographic determinations of quercetin, kaempferol and isorhamnetin were performed using on Agilent 1100 System equipped with G1310A pump coupled to a manual injector with a 20 μl fixed loop, a Platinum EPS C_{18} 100A (250 × 4.6 mm i.d., 5 μm, purchased from Alltech Associates, Inc.) column with a guard column (10 mm × 4.6 mm i.d., 5 μm, packed with YWG-$C_{18}H_{37}$, purchased from Jiangsu Hanbon Science and Technology Co. Ltd.), and a G1314A VWD UV detector (Set at 380 nm). The chromatographic data were collected and processed on Agilent Chemstation version (No. G2170AA).

The mobile phase consisted of phosphate buffer (pH 2.0; 10 mM) -tetrahydrofuran-methanol-isopropanol (65:15:10:20, v/v/v/v) at a flow rate of 0.5 ml/min. The phosphate buffer used for the mobile phase was prepared from 10 mM potassium dihydrogen phosphate and adjusted to pH 2.0 with concentrated phosphoric acid. Morin was used as internal standard. The quercetin, kaempferol and isorhamnetin concentrations were calculated from the calibration curves.

2.4. Stock and working standard solutions

Analyses were accurately weighted, transferred to volumetric flasks and dissolved in methanol to make individual stock solutions of 1 mM of quercetin, kaempferol and isorhamnetin and 1 mg/ml of the internal standard morin. The solutions were stored at −20 °C, with protection from

light, and used for 3 months. Stock solutions of quercetin, kaempferol and isorhamnetin were diluted to 1, 2.5, 5, 7.5, 10 μM solutions with methanol, which were used for spiking cell lysis solution. These solutions were kept at 4 °C with protection from light when not in use.

2.5. Sample preparation

Fifty-microlitre of 0.01 mg/ml morin were added to 2.0 ml aliquots of cell lysis solution as an internal standard. One millilitre of 6 M hydrochloric acid was added into samples for precipitating proteins. The mixture was extracted with 3.0 ml of ether and acetone (14:1, v/v), vortexed for 2 min then centrifuged for 15 min at 2500 rpm. The upper organic layer was transferred into another test tube and evaporated to dryness with N_2. The residue was reconstituted in 80 μl mobile phase before analysis. Twenty microlitre of the sample was injected into the HPLC system.

2.6. Calibration curve

To prepare calibration curves, standard samples of quercetin, kaempferol and isorhamnetin were added to blank cell lysis solution to give final concentrations of 0.1, 0.25, 0.5, 0.75, 1 μM for each flavonol. Calibration curves were constructed by performing a linear regression analysis of the peak area ratios of the flavonols to the internal standard versus the flavonols concentrations.

2.7. Precision and accuracy

The intra-day precision of the assays performed in replicate ($n = 5$) were tested by using three concentrations of the individual flavonol, namely, 0.25, 0.5 and 0.75 μM. The inter-day precision of the assays was estimated from the results of three replicate assays on 5 different days within 1 week. The precision was evaluated by the relative standard deviation (R.S.D.).

2.8. Extraction efficiency

Extraction efficiency of quercetin, kaempferol and isorhamnetin from cell lysis solution was evaluated ($n = 5$) at 0.25, 0.5 and 0.75 μM by comparing the peak areas of an extracted sample containing a known amount of flavonols with the peak areas obtained from direct injections of the solution containing the same concentration of flavonols in pure solvent.

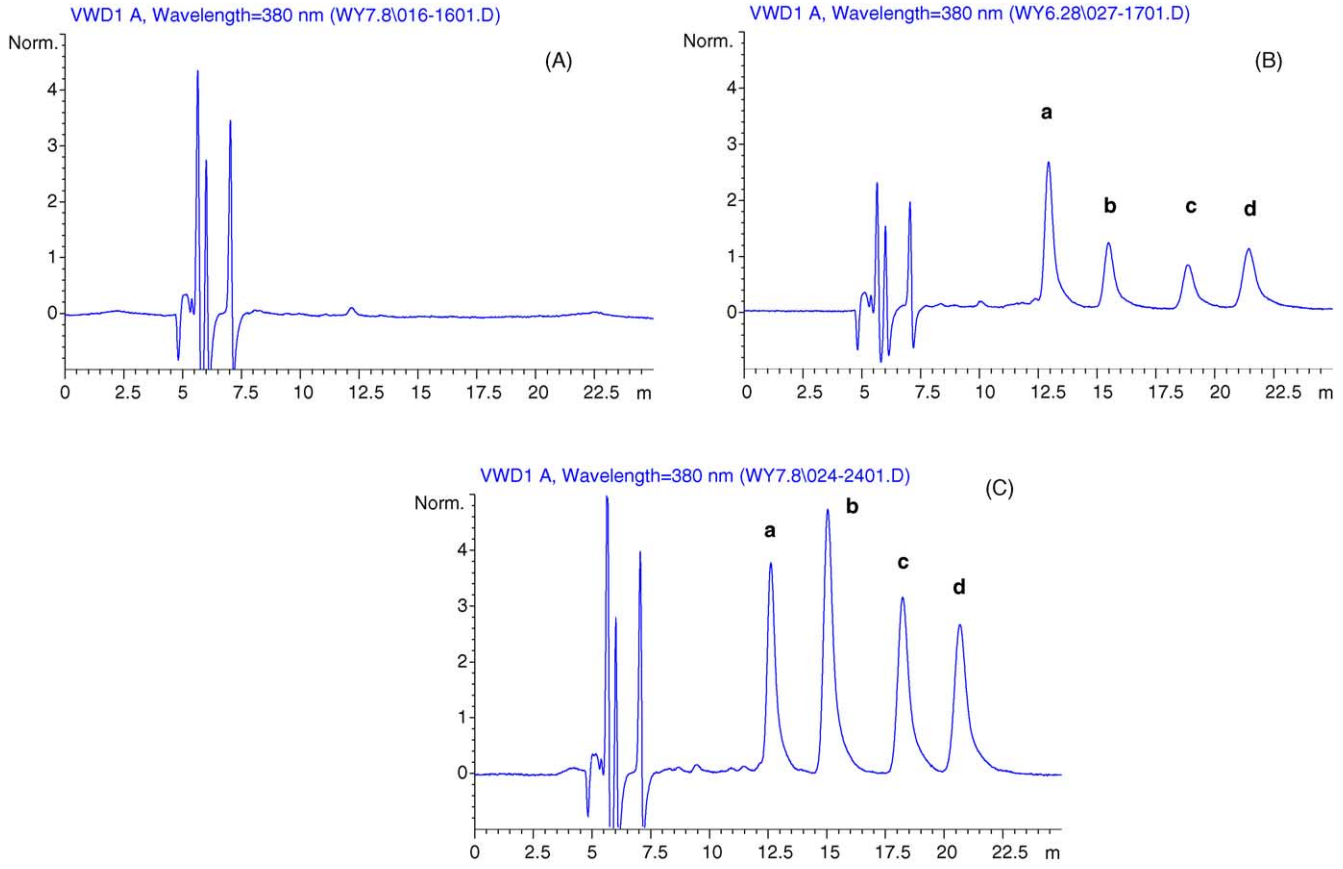

Fig. 2. Typical chromatograms of extracts of (A) blank cell lysis solution; (B) cell lysis solution spiked with 0.01 mg/ml morin, 0.25 μM quercetin, 0.25 μM isorhamnetin and 0.25 μM kaempferol; (C) three flavonols accumulated in Bcap37 cells at 45 min: (a) morin, (b) quercetin, (c) isorhamnetin, (d) kaempferol.

2.9. Stability of flavonol samples

The stability of quercetin, kaempferol and isorhamnetin was evaluated ($n = 3$) at concentrations of 25 μM in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated newborn calf serum at 37 °C for 1 h.

The stability of flavonols in blank cell lysis solution was investigated at three concentrations (0.1, 0.5 and 1 μM) at room temperature for 6 h.

2.10. Assay procedure

The Bcap37 and Bcap37/MDR1 cells were seeded at the density of 7.5×10^5/well to 35 mm i.d. tissue culture wells. After 24 h, the culture medium was aspirated, and replaced by RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% heat-inactivated newborn calf serum and 25 μM quercetin, 25 μM kaempferol and 25 μM isorhamnetin. After 15, 30 and 45 min, the medium was aspirated and the cells were washed three times with ice-cold PBS (pH7.4) to stop further uptake. One millilitre of 0.1% Triton X-100 was added to each well to lysis the cells and then the procedure was carried out according to the Section 2.5. The drug concentration in lysis solution was determined by reversed-phase HPLC. Values from cellular accumulation of the three flavonols were normalized to cellular protein content.

Protein content was determinated using a Bio-Rad DC Protein Assay kit, with bovine serum albumin as the standard.

3. Results

3.1. Chromatographic specificity

Separation of the three ginkgo flavonols from the internal standard in cell lysis solution was achieved using the C18 column. There were no interfering peaks co-eluting with the compounds of interest (Fig. 2A and B).

Table 2
Extraction efficiency of assaying flavonols from cell lysis solution (mean ± S.D., $n = 5$)

Target concentration (μM)	Extraction recovery (%)		
	Quercetin	Kaempferol	Isorhamnetin
0.25	68.3 ± 2.7	64.5 ± 3.6	69.0 ± 2.1
0.5	70.5 ± 6.2	72.6 ± 7.0	72.4 ± 6.6
0.75	74.3 ± 6.0	70.0 ± 4.2	71.5 ± 5.7

3.2. Calibration curves

Calibration curves for quercetin, kaempferol and isorhamnetin were constructed by analyzing a series of blank cell lysis solution, spiked with three flavonols at the concentration range from 0.1 to 1 μM. The procedure was carried out according to Section 2.5. Peak area ratios (y) of the quercetin, kaempferol or isorhamnetin versus the internal standard were measured and plotted against the concentration (x) of each flavonol. The calibration curves for quercetin, kaempferol and isorhamnetin were linear over the range of 0.1 to 1 μM. The regression equations of the calibration curves were $y = 3.3294\ x - 0.0102$ ($r = 0.9997$) for quercetin, $y = 5.6153\ x + 0.079$ ($r = 0.9997$) for kaempferol and $y = 1.5456\ x - 0.0248$ ($r = 0.9996$) for isorhamnetin.

3.3. Precision and accuracy

The precision and accuracy of the method were assessed by intra- and inter-assay validations ($n = 5$) at concentrations of 0.25, 0.5, 0.75 μM, according to Section 2. The results showed that the intra- and inter-day coefficients of variation were less than 10% (R.S.D.) (Table 1). The recovery of the assay was between 98.9 and 100.6%.

Table 1
Intra- and inter-assay precision and accuracy of flavonols from cell lysis solution (mean ± S.D.)

	Target concentration (μM)	Measured concentration (μM)		
		Quercetin	Kaempferol	Isorhamnetin
Intra-assay variability ($n = 5$)	0.25	0.24 ± 0.01	0.25 ± 0.01	0.25 ± 0.01
	R.S.D. (%)	3.7	4.4	1.4
	0.5	0.50 ± 0.03	0.49 ± 0.02	0.51 ± 0.02
	R.S.D. (%)	4.7	3.4	4.1
	0.75	0.74 ± 0.02	0.75 ± 0.03	0.74 ± 0.04
	R.S.D. (%)	2.3	4.1	5.8
Inter-assay variability ($n = 5$)	0.25	0.24 ± 0.03	0.25 ± 0.02	0.24 ± 0.02
	R.S.D. (%)	9.8	6.2	7.8
	0.5	0.52 ± 0.02	0.50 ± 0.03	0.49 ± 0.04
	R.S.D. (%)	4.6	4.9	7.6
	0.75	0.74 ± 0.06	0.75 ± 0.03	0.75 ± 0.03
	R.S.D. (%)	8.0	4.1	7.1

Table 3
Accumulation of quercetin, kaempferol and isorhamnetin in Bcap37 and Bcap37/MDR1 cells (mean ± S.D.)

Minutes	Quercetin (nmol/mg)		Isorhamnetin (nmol/mg)		Kaempferol (nmol/mg)	
	Bcap37	Bcap37/MDR1	Bcap37	Bcap37/MDR1	Bcap37	Bcap37/MDR1
15	0.63 ± 0.06	0.23 ± 0.04	0.62 ± 0.05	0.26 ± 0.03	0.44 ± 0.05	0.17 ± 0.02
30	0.86 ± 0.13	0.58 ± 0.05	0.67 ± 0.11	0.47 ± 0.04	0.48 ± 0.08	0.33 ± 0.03
45	0.83 ± 0.16	0.55 ± 0.01	0.59 ± 0.11	0.39 ± 0.01	0.44 ± 0.09	0.27 ± 0.01

3.4. Sensitivity

The limit of detection, defined as the lowest sample concentration which can be detected (signal-to-noise ratio = 3) was 0.01 μM for quercetin and kaempferol and 0.05 μM for isorhamnetin, and the limit of quantification, defined as the lowest sample concentration which can be quantitatively determined with suitable precision and accuracy (signal-to-noise ratio > 10) was 0.1 μM (R.S.D. < 10%) for each flavonol.

3.5. Extraction efficiency

A series of blank cell lysis solutions, spiked with various amounts of quercetin, kaempferol and isorhamnetin, were processed according to determined extraction efficiency. The mean extraction efficiency for quercetin, kaempferol and isorhamnetin from cell lysis solution varied from 64 to 74% (Table 2).

3.6. Stability of flavonol samples

No significant degradation was detected after the samples of the three flavonols in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated newborn calf serum were incubated at 37 °C for 1 h. There was no significant decomposition observed in cell lysis solution spiked with various amounts of quercetin, kaempferol and isorhamnetin at room temperature for 6 h.

3.7. Determination of three flavonols in the Bcap37 and Bcap37/MDR1 cells

The amount of quercetin, kaempferol and isorhamnetin accumulated in Bcap37 and Bcap37/MDR1 cells was determined by reversed-phase HPLC, and the values were normalized to cellular protein content (Table 3). A typical chromatogram from the cellular uptake experiment was shown in Fig 2C.

4. Discussion

Three organic solvents including methanol, tetrahydrofuran and isopropanol were used in the mobile phase to separate the three ginkgo flavonols extracted from cell lysates. The peak diffusion was reduced when the volumn ratio of tetrahydrofuran and isopropanol was 3:4 and the best peak performance was achieved by using pH 2.0 phosphate buffer in the mobile phase. Triton X-100 was used to lyse cells and liberate the analytes. Hydrochloric acid was added to samples to precipitate proteins and stabilize the analytes, which can improve the recovery. It is essential that acetone was added to the ether as a part of the extraction solvent in order to enhance the extraction efficiency and improve precision of the analytical method.

The HPLC method was used to determine the accumulation of the three ginkgo flavonols in human breast cancer cell, Bcap37 and Bcap37/MDR1 cells. Quercetin, kaempferol and isorhamnetin remain dissolved and stable under the cell culturing conditions. PBS was used to wash cells in order to remove flavonols adhering to the cell membranes after incubation with flavonol containing medium. No flavonols were detected by HPLC in the final wash solution. The values measured by the assay thus directly reflected the amount of flavonols that were taken up by the cells. Bcap37/MDR1 cells were the Bcap37cells that transfected with recombinant plasmid containing MDR1 cDNA and stable high expressing P-gp. The results indicated that the cellular flavonol concentration was saturated within 30 min, and the accumulated concentration of quercetin was higher than other flavonols. No metabolism or other structural changes of three flavonols was observed in Bcap37 and Bcap37/MDR cells. The accumulation concentrations of ginkgo flavonols in Bcap37 cells were more pronounced than those in Bcap37/MDR1 cells at 15, 30 and 45 min. These data suggest that the accumulation of ginkgo flavonols were affected by P-gp in Bcap37/MDR1 cells.

A validated simple HPLC method for the simultaneous determination of quercetin, kaempferol and isorhamnetin in breast cancer cells has been developed. The method was specific, precise and sensitive.

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