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Short communication

Determination of quercetin and kaempferol in human urine after orally administrated tablet of ginkgo biloba extract by HPLC

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Abstract

A sensitive, simple and accurate method was developed for determination of quercetin and kaempferol in human urine by reversed phase high performance liquid chromatography. The urine samples were analyzed on C_{18} column. Quercetin and kaempferol were analyzed simultaneously with good separation. UV detector was set at 380 nm. There was a linear relationship between chromatographic area of analytes and concentration of analytes over the concentration range 1.638–81.90 and 1.872–93.60 ng/ml for quercetin and kaempferol, respectively. The recovery of the assay was 99.7 ± 6.2 and 97.4 ± 7.2% for quercetin and kaempferol, respectively. The within-day and between-day coefficients of variation were less than 9.7 and 16.5% (RSD), respectively. The limit of detection was 1.0 ng/ml for quercetin and 1.1 ng/ml for kaempferol. The limit of quantitation was 1.61 ± 0.11 ng/ml ($n = 5$) for quercetin and 1.85 ± 0.11 ng/ml ($n = 5$) for kaempferol. The method developed has been applied to determine quercetin and kaempferol after orally administrated tablet of Ginkgo biloba extract in human urine.

Keywords: Flavonoids; High performance liquid chromatography; Disposition; Ginkgo biloba extract

1. Introduction

Flavonoids are phenolic substances isolated from a wide range of plants. Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. Quercetin and Kaempferol (Fig. 1), the polyphenol with potential health effects, are absorbed by humans. The chemical composition of Ginkgo biloba has been well studied. Documented pharmacological activities, including antidepressant, antiviral, and antibacterial effects, provide supporting evidence for several of the traditional uses stated for Ginkgo biloba [1,2]. Many pharmacological activities appear to be attributable to the flavonoid

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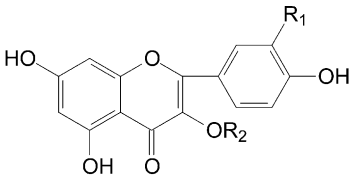

	R 1	R2		
Quercetin	H	H	$C_{15}H_{10}O_6$	286
Kaempferol	OH	H	$C_{15}H_{10}O_7$	302

Fig. 1. Chemical structure of Quercetin, Kaempferol.

constituents including quercetin and kaempferol. There were scarce reports about analytical methods for determination of quercetin and kaempferol simultaneously in human urine and blood [3] but there were some for determination of quercetin only [4–6]. We were used to following the method published and trying SPE method but failed. One reason is that it is difficult to extract the Ginkgo flavones. In this paper, a RP-HPLC with UV detector was developed to study disposition of quercetin and kaempferol from Ginkgo biloba extract (GBE) in human.

2. Materials and methods

2.1. Materials

Quercetin (Q) and Kaempferol (K) were purchased from the National Institute for Drugs and Bioproducts Quality Control (Beijing, China). Tablets of GBE were obtained from Zhejiang CONBA Pharmaceutical Manufacturing Company, Ltd. (Hangzhou, China). All other chemicals and solvents were of an analytical or chromatographic grade and obtained from commercial sources.

2.2. Chromatography

The modular HPLC equipment was composed of a LC-10AT *VP* with SPD-10A *VP* (Simadau, Japan) and N2000 data system(Zhejiang University, China). The analytical column was a Platinum EPS C_{18} 100A (250 × 4.6 mm i.d., 5 μm) with a guard column (10 × 4.6 mm, i.d., 5 μm; packed with YWG-$C_{18}H_{37}$). The mobile phase was a mixture of phosphate buffer(pH 2)-tetrahydofuran–methanol–isopropanol (70:15:10:20, v/v/v/v). The flow rate of mobile phase was set at 0.7 ml/min. The wavelength of UV detector was set at 380 nm.

2.3. Preparation of reference standards solution

The references of quercetin and kaempferol were weighed accurately then dissolved in methanol and diluted to 33.0 μg/ml for both of quercetin and kaempferol. All standard solutions were stable for at least 2 months when stored at −20 °C.

2.4. Assay procedure

1 ml of 25% HCl was added into 4.0 ml of human urine and mixed well. 5.0 ml ether was added after the urine sample was hydrolyzed for 30 min at 80 °C water bath. The sample hydrolyzed was extracted for 5 min rotationally and then centrifuged for 10 min. The 4.0 ml ether was transferred to a clean test tube and then evaporated to dryness under nitrogen stream. The residue was reconstituted with 100 μl mobile phase and an aliquot of 20 μl of the resulting solution was injected into the HPLC system.

3. Results

3.1. Chromatogrphic specificity

The chromatogram of quercetin and kaempferol showed a good separation with high column efficiency for those compounds (Fig. 2). The peaks of quercetin and kaempferol were confirmed with reference standards, No interference appeared at the peak positions of quercetin and kaempferol.

3.2. Calibration curves

Calibration curves were constructed by analyzing a serials of blank human urine, spiked with quercetin and kaempferol at the concentration 1.638, 2.73, 5.46, 10.92, 38.22, 54.6, 81.90 ng/ml and 1.872, 3.12, 6.24, 12.48, 43.68, 62.40, 93.60 ng/

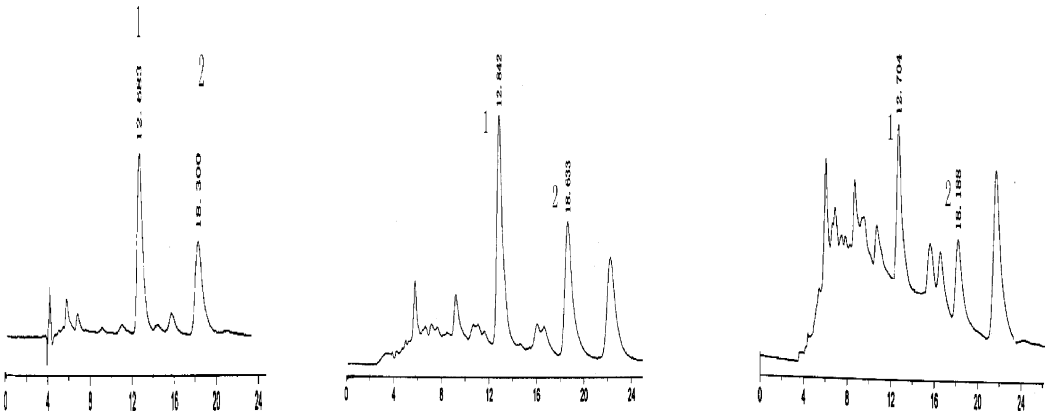

Fig. 2. Chromatogram of quercetin (1) and kaempferol (2). (A) Chromatogram of tablet of Ginkgo biloba extrat. (B) Chromatogram of blank urine spiked with quercetin (1) and kaempferol (2) standard. (C) Chromatogram of urine sample after a single oral administration of tablet of Ginkgo biloba extrat 4 h.

ml, respectively. Preparation and extraction of the urine samples were carried out as described under Assay procedure. Peak area (y) of the quercetin and kaempferol was measured and plotted against the concentration (x) of each flavoid. The linearity of the calibration curves was in the range from 1.638 to 81.90 and 1.872 to 93.60 ng/ml for quercetin and kaempferol, respectively. The regression equations of the calibration curves were $y = 3271.7x + 53\,675$ ($r = 0.9984$) for quercetin and $y = 3300.5x + 28\,191$ ($r = 0.9978$) for kaempferol.

3.3. Recovery studies

Human blank urine was spiked with 1.638, 10.92 and 54.6 ng/ml of quercetin, respectively, and 1.872, 12.48 and 62.45 ng/ml of kaempferol, respectively. Five samples for each concentration were processed as described under Assay procedure. The peak area of quercetin and kaempferol were compared with those obtained by calibration curves constructed with blank urine.

The recoveries of each compound are summarized in Table 1. The average recovery of this analytical method was 99.7 and 97.4% for quercetin and kaempferol, respectively.

3.4. Precision studies

The drug-free human urine, spiked with different concentrations of quercetin (1.638, 10.92 and 54.6 ng/ml) and kaempferol (1.872, 12.48 and 62.45 ng/ml), were used for precision studies. We determined intra-assay variability by analyzing samples in heptaplicate, and determined inter-assay variability by analyzing samples in heptaplicate on five separate days, according to the procedure described under Assay procedure. The relative standard deviations were calculated.

Table 1
Recovery and precision for assay of quercetin and kaempferol ($x \pm s$, $n = 5$)

Concentration spiked (ng/ml)	Recovery (%)	Precision	
		Intra-assay	Inter-assay
Quercetin			
1.638	104.1 ± 7.94	1.608 ± 0.11	1.803 ± 0.15
10.92	95.9 ± 7.31	10.65 ± 0.60	10.29 ± 0.10
54.60	99.1 ± 3.32	54.54 ± 0.85	53.67 ± 2.77
Kaempferol			
1.872	94.9 ± 10.4	1.85 ± 0.11	1.70 ± 0.28
12.48	97.7 ± 7.89	11.93 ± 0.56	12.45 ± 1.41
62.45	99.7 ± 3.39	63.18 ± 1.43	61.40 ± 2.81

The within- and between-day coefficients of variation were 6.9 and 9.7% for quercetin, respectively, and 5.9 and 16.5% for kaempferol. This showed that the precision of this method for analysis of quercetin and kaempferol in human urine was satisfied (Table 1).

3.5. Sensitivity

4 ml of blank human urine spiked with low concentration of quercetin and kaempferol used for sensitivity test. Five samples for each compound were processed as described under Assay procedure. The limit of detection (LOD) of the assay was measured as 1.0 ng/ml for quercetin and 1.1 ng/ml for kaempferol based on S/N = 3. The limit of quantitation (LOQ) calculated from calibration curves was 1.61 ± 0.11 ng/ml ($n = 5$) for quercetin and 1.85 ± 0.11 ng/ml ($n = 5$) for kaempferol.

3.6. Assay of quercetin and kaempferol in human urine

Urine samples were collected after seven healthy volunteer administrated orally six tablets (1.134 mg Q and 1.233 mg K per tablet) of GBE at 0, 1, 2, 4, 6, 8, 10, and 12 h. The urine volume was measured and stored in sealed containers at −20 °C. 4.0 ml of urine was transferred to a 15 ml test-tube and processed according to Assay procedure. The average concentrations in urine of quercetin and kaempferol after administration drug 4 h were 34.38 ± 22.62 and 44.45 ± 26.72 ng/ml ($n = 7$), respectively. The experimental results showed the accumulative excretion amounts of kaempferol are more than that of quercetin (Fig. 3).

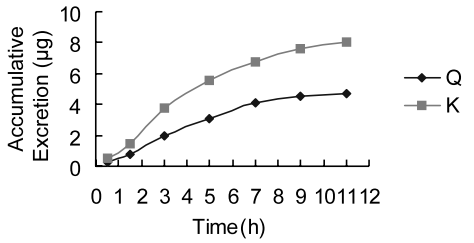

Fig. 3. The accumulative excretion amounts (μg) of quercetin and kaempferol in human urine.

4. Discussion

4.1. Mobile phase

To achieve good separation of the Ginkgo flavonoids from interfering compounds in the human urine, three organic solvents including methanol, tetrahydrofuran and isopropynol had to be used in mobile phase. The peak diffusion was reduced with tetrahydrofuran and isopropynol(3:4, v/v) and the best peak performance was got by using pH 2 phosphate buffer in the mobile phase. Decreasing acidity of mobile phase resulted in tailed peaks. The symmetrical peaks and good separation among quercetin, kaempferol and interfering compounds in the urine with reasonable retention times were achieved by using a mixture of phosphate buffer (pH 2)–tetrahydrofuran–methanol–isopropynol (70:15:10:20, v/v/v/v).

4.2. Hydrolyzing urine samples

The human urine contains the flavone glycosides and free flavone after GBE tables are administrated. The free flavones were analyzed only in this study so the flavone glycosides were hydrolyzed before analysis. The two samples were prepared for the glycosides hydrolysis test. One is the blank urine spiked rutin, the other is the human urine administrated with GBE tables. Then the two samples were hydrolyzed at different concentration HCl and different temperature for indicated time. After heating at 80 °C for 0.5 h with 25% HCl, the rutin was hydrolyzed completely and the maximum amount of quercetin was recovered. So acid hydrolysis was carried out by adding 1 ml 25% HCl to 4 ml urine and heating at 80 °C for 0.5 h.

In addition, we compared the urine samples hydrolyzed by acid, -glucuronidase and sulfase with untreated urine samples. No quercetin and kaempferol were detected in untreated samples. A few amounts of quercetin and kaempferol were detected in the urine samples hydrolyzed by sulfatase. The higher amounts of quercetin and

kaempferol were found in the urine samples hydrolyzed by acid and β-glucuronidase. It is supported the view that quercetin and kaempferol are excreted in the human urine mainly as glucuronides.

4.3. Dissolve solvent

The solvents used to dissolve the residues before HPLC analysis influenced the chromatogram quality of quercetin and kaempferol. The solvents including acetonitrile, methanol, acetone and mobile phase were tested and the results showed the good peaks shape and resolution for analytes were found by using mobile phase as a dissolving solvent. Kaempferol was stable only for 12 h in the mobile phase, therefore, the residues were dissolved prior to injection.

A RP-HPLC established in this paper was simple, accurate and precision, in addition, the method is economic. The method has been used to study excretion of quercetin and kaempferol from GBE in human. The results indicated that the excretion of kaempferol is more rapid than that of quercetin and the main metabolic pathway of quercetin and kaempferol is glucuronidation.

Acknowledgements

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