Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 109–113

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Simultaneous determination of the flavonoids robinin and kaempferol in human breast cancer cells by liquid chromatography-tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 26 June 2010
Received in revised form
15 December 2010
Accepted 15 December 2010
Available online 23 December 2010

Keywords:
LC/MS/MS
Robinin
Kaempferol
Analysis

ABSTRACT

An accurate, precise and sensitive method was developed and validated for the simultaneous quantification of the flavonoid glycoside robinin, and its algycone kaempferol in human breast cancer MCF-7 cells. The application of liquid chromatography-tandem mass spectrometry (LC/MS/MS) with a TurboIonspray interface in negative mode under multiple reactions monitoring was investigated. Chromatographic separation was achieved on a C_{18} column using a mobile phase consisting of (A) water with 0.025% formic acid and 1 mM ammonium formate and (B) acetonitrile with 0.025% formic acid. Rutin was used as the internal standard for robinin and fisetin as the internal standard for kaempferol. The assay had a limit of detection of 0.1 ng/ml for both compounds when present in cell lysate. The calibration curves were linear from 1 to 250 ng/ml ($r > 0.999$) for each compound. The intra- and inter-day coefficients of variation were less than 10% and intra- and inter-day accuracies were within 11%. This assay was successfully applied in a robinin cellular uptake study to determine the intracellular concentrations of robinin in MCF-7 cells.

1. Introduction

Astragalus falcatus Lem, one of the species from *Astragalus* genus, is widely distributed in Turkey, Caucasus and the European part of the Russian Federation. The detailed chemical investigation on *A. falcatus L.* growing in Georgia has been performed in Iovel Kutateladze Pharmacochemistry Institute. Isolated from its leaves and flowers, the original preparation "Flaronin" has been formulated as tablets [1,2]. Flaronin has received drug approval by the Russian and Georgian Ministries of Health. This preparation exhibits hypotensive, anti-inflammatory and diuretic effects, lowers nitrogen and creatinine levels in blood and is recommended for the treatment of kidney diseases complicated by azotemia [1].

The major active constituent of Flaronin is a flavonoid glycoside—kaempferol 3-O-robinoside-7-O-rhamnoside, which is known as robinin (Fig. 1). Robinin has also been detected in several plants such as *Pueraria hirsuta L.* (kudzu vine), *Vinca erecta*, and *Astragalus shikokianus* [3,4]. Bokkenheuser et al. [5] reported that robinin is hydrolyzed to kaempferol (Fig. 1) by intestinal microflora. Although Flaronin is widely used due to its well-documented beneficial effects, very little is known regarding the bioavailability and disposition of its active constituents such as robinin. To evaluate the bioavailabilty of robinin and investigate the role of transporter proteins in its absorption profile, a specific, precise and sensitive method is required for the simultaneous quantification of robinin and kaempferol in biological fluids. To the best of our knowledge, although there are several published assays for the quantification of kaempferol, no assay has been established for robinin [6–8].

In the current study, an accurate, rapid and reproducible LC/MS/MS method was developed and validated to quantitate the concentrations of robinin and its aglycone—kaempferol in human breast cancer MCF-7 cells. To the best of our knowledge, this is the first validated comprehensive method for simultaneous determination of robinin and kaempferol in biological fluids with the necessary sensitivity to evaluate concentrations from both in vitro and in vivo studies.

2. Experimental

2.1. Materials and methods

Robinin was provided by the laboratory of Polyphenolic Compounds of the Pharmacochemistry Institute (Tbilisi, Georgia).

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometric; QC, quality control; I.S., internal standard.

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doi:10.1016/j.jpba.2010.12.021

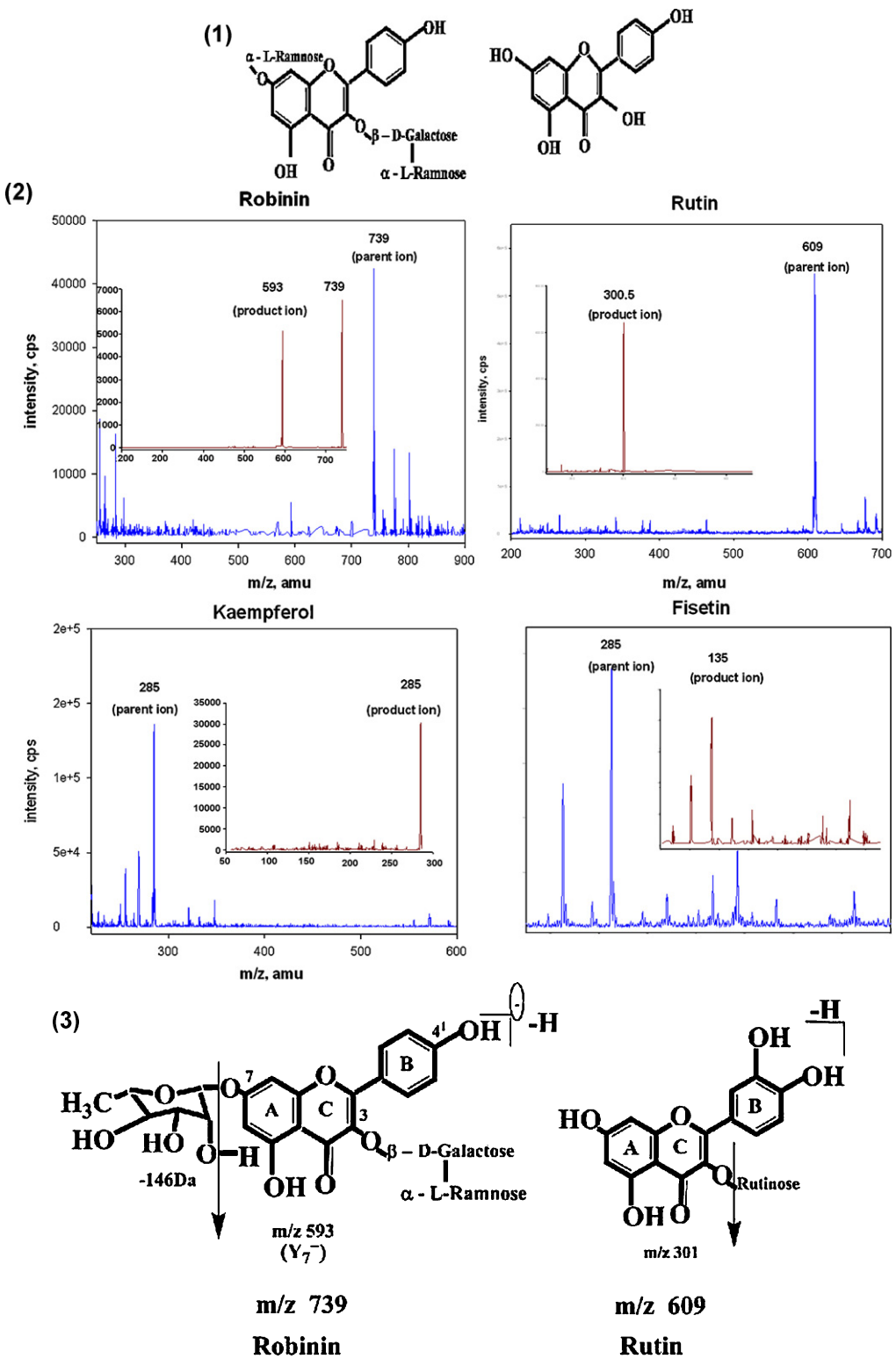

Fig. 1. (1) Chemical structures of robinin (left) and kaempferol (right); (2) Q1 full scan and product ion scan mass spectra of robinin, rutin, kaempferol and fisetin. Kaempferol was not fragmented in the analysis. The mass spectra were obtained by direct infusion of 1 μg/ml robinin and kaempferol in methanol/aqueous solution (50:50, v/v); (3) proposed fragmentation of robinin (left) and rutin (right).

Kaempferol, rutin and fisetin were purchased from Indofine (Hillsborough, NJ, USA). HPLC-grade acetonitrile and methanol were obtained from J.T Baker (Phillipsburg, NJ, USA). RPMI 1640 medium, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA, USA). All the other reagents or solvents used were commercially available and of reagent grade.

2.2. Cell culture

MCF-7 human breast cancer cells were obtained from the National Cancer Institute. MCF-7 cells were grown in a 5% CO_2 atmosphere at 37 °C and cultured in 75-cm^2 plastic culture flasks with RPMI 1640 medium supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin and 10% FBS. The MCF-7 cells were washed

with PBS three times and lysed by 10 ml of 0.5% X-100, which was used as blank cell lysis solution.

2.3. LC/MS/MS

LC/MS/MS was carried out on an Applied Biosystem API 3000 triple-quadruple tandem mass spectrometer (Applied Biosystem, Foster City, CA, USA) linked to a Turbo Ionspray interface and a Shimadzu Prominence liquid chromatograph. Analyst 1.4.2 software was used for data acquisition and processing. Chromatographic separation was achieved using XTerra MS C18 column (2.1× 100 mm i.d., 3.5 μm; Waters Corporation, Milford, MA, USA). The mobile phase consisted of 0.025% formic acid and 1 mM ammonium formate in water (A) and 0.025% formic acid in acetonitrile (B). The gradient elution profile had the following components: initial condition 16% B; gradient 16–75% B over 4 min; increased to 95% B and held for 1.4 min and finally returned to initial conditions for a total run time of 10 min. The injection volume was 35 μl and the flow rate was 0.2 ml/min. Rutin was used as the internal standard (IS) for robinin and fisetin was used as the IS for kaempferol.

Conditions for MS analysis of tested compounds included an ion spray voltage of −4200 V and a temperature of 350 °C. Nebulizer and curtain gas flow were set at 10 ml/min and 8 ml/min, respectively. The optimized declustering potential, focusing potential and entrance potential were −70, −300 and −10 V respectively. The collision cell exit potential was −15 V for robinin, fisetin and rutin, and −8 V for kaempferol. MS was performed in a negative ion mode using the multiple reactions monitoring (MRM). Selected parent-ion/product-ion pairs were monitored for each compound: robinin 739/593, rutin 609/300.5, fisetin 285/135 and kaempferol 285/285. Kaempferol did not produce an abundant product ion so it was not fragmented in the analysis.

2.4. Sample preparation

Five microliter of 2.5 μg/ml I.S. (rutin and fisetin) was added to 100 μl of robinin or kaempferol standards or quality control standards (QCs) and briefly vortex-mixed. 100 μl of methanol was then added to precipitate protein. To prevent oxidation of the flavonoids, 5 μl of ascorbic acid was added to each sample. After vigorous vortexing, the samples were centrifuged for 10 min with 14,000 rpm at 4 °C. One hundred μl of the supernatant was transferred into a 200 μl vial insert for LC/MS/MS analysis.

2.5. Calibration and validation

A stock solution of robinin or kaempferol was prepared at a concentration of 1 mg/ml in DMSO and was stored at −80 °C until use. Calibration standards were prepared in MCF-7 cell lysates at concentrations of 1, 2.5, 5, 10, 25, 50, 100, and 250 ng/ml, along with quality control samples at concentrations of 2, 20 and 200 ng/ml.

Recovery experiments were performed by comparing the peak area of robinin or kaempferol added to MCF-7 cell lysates with the same amount of robinin or kaempferol added to a mixture of methanol/water: 50/50 ($n = 3$). Three concentrations (2, 20 and 200 ng/ml) were evaluated in recovery experiments.

To determine the intra-day precision and accuracy of the method, quality control samples in cell lysates were analyzed three times on the same day. To determine the inter-day precision and accuracy, quality control samples were analyzed on three different days. Calibration curves for the cell lysates were run each day along with the quality controls. The precision was assessed by determining the coefficient of variation (C.V. %). The accuracy was calculated by the percent of measured concentration to the known concentration.

The calibration curves were constructed in the range of 1–250 ng/ml in cell lysates to cover the range of expected robinin or kaempferol concentrations in the samples. The calibration curves were obtained by analyzing the peak area ratios of robinin/kaempferol to I.S. and plotting these values against the robinin/kaempferol concentrations in cell lysates. To assess linearity, the line of best fit was obtained by weighted linear regression (weighting factor: $1/x^2$). The suitability of the calibration equation was confirmed by back-calculating the concentration of the calibration standards. The limits of detection and quantification were determined based on a signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively.

2.6. Stability of robinin

The stability of robinin was evaluated in RPMI 1640 medium and PBS ($n = 3$) at 37 °C for 1 h at a concentration of 10 μM and in blank cell lysis solution at 2, 20 and 200 ng/ml at room temperature for 6 h.

2.7. Application in a robinin uptake study

To evaluate the usefulness of our assay for the evaluation of the disposition of robinin in biological fluids, we analyzed cell samples obtained from the robinin uptake studies. MCF-7 cells were seeded in 6-well plates at a density of 1.6×10^6 cells/well. When the cells reached 85–90% confluence after 48 h, the cells were washed with PBS three times and then the medium was replaced by 1 ml of plain medium supplemented with 25, 50 and 100 μM robinin. After a 1-h incubation the medium was aspirated and the cells were washed three times with ice-cold PBS (pH 7.4). One ml of 0.5% Triton X-100 was added to each well to lyse the cells and then the procedure was carried out as described in Section 2.4. The drug concentration in the cell lysate was determined by LC/MS/MS and normalized by the cellular protein content. Protein concentration in cell lysate was determined using the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL).

3. Results and discussion

3.1. Evaluation of matrix effect

The matrix effect was evaluated during the assay validation. The peak area of investigated flavonoids obtained when they were spiked into blank sample extracts (i.e. final matrix after protein precipitation) was compared with the peak area of investigated flavonoids obtained when they were spiked into mobile phase. The ratio of these two peak areas was close to 1. Therefore the matrix effect for the assayed flavonoids was minimal in our study.

In addition, we used a diverter valve to prevent the early eluting salts from entering the LC/MS interface, with the aim of avoiding the ion suppression that may occur. However, we found minimal interference, even without a diverter valve, indicating that ionization suppression is minimal for our preparations.

3.2. Optimization of LC/MS/MS conditions

The MS parameters for each compound were optimized by direct infusion of standard solutions of robinin, kaempferol, rutin and fisetin (1 μg/ml) in methanol/aqueous solution (50:50). The collision energy (CE) was optimized to achieve the highest sensitivity and was chosen to obtain product ion mass spectra without total loss of the deprotonated parent molecular ion. The proposed fragmentation pathway of the glycosides robinin and rutin is provided in Fig. 1(3). The Q1 (first quadruple) full scan and product ion scan

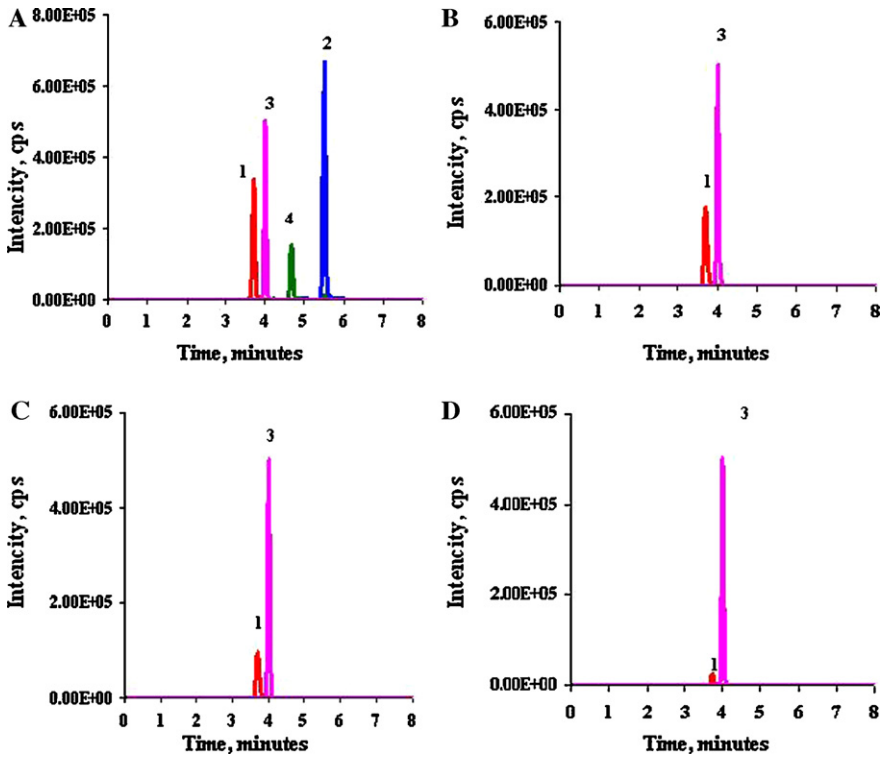

Fig. 2. Chromatograms of (A) cell lysis solution spiked with 100 ng/ml robinin (1) and kaempferol (2) and their internal standards rutin (3) and fisetin (4) at a concentration of 125 ng/ml; robinin present in MCF 7 cells at 60 min following the addition of robinin 100 μM (B), 50 μM (C) and 25 μM (D).

mass spectra of target compounds and their internal standards are presented in Fig. 1(2). The retention times of robinin, rutin, fisetin and kaempferol were 3.66 min, 4.06 min, 4.46 min and 5.51 min, respectively (Fig. 2(A)). The internal standards used were chosen considering the characteristics of the target compounds. Rutin was chosen as an internal standard for robinin since it is a glycoside (quercetin-3-rutinoside) and fisetin, an aglycone, was chosen as an internal standard for kaempferol.

3.3. Validation of the assay

Calibration data for each compound were obtained using the optimized MS/MS conditions described above. The curves for robinin (IS rutin) and kaempferol (IS fisetin) were linear over the concentration range of 1–250 ng/ml and showed good correlation with the concentrations of robinin: $y = 0.0093x - 0.0069$; $R^2 = 0.9993$ and kaempferol: $y = 0.0811x + 0.0905$; $R^2 = 0.9996$. For each calibration standard, the concentration was back calculated from the linear regression curve equation. In our study, we determined the limit of quantification based on two criteria: (1) at least 10 times response (peak value) compared to the baseline and (2) intra-day and inter-day precision and accuracy of this concentration within 20% and 80–120%, respectively. The LOQ of both investigated flavonoids in our study is 1 ng/ml. The limit of detection is 0.1 ng/ml for both robinin and kaempferol since this is the lowest concentration that can be reliably differentiated from background noise (signal to noise > 3).

Quality control samples representing low, medium, and high (2, 20, 200 ng/ml) concentrations were used to evaluate the accuracy and precision for robinin and kaempferol measurement on three consecutive days. Ten replicates of each quality control sample were analyzed together with a set of calibration standards. The intraday precision ranged from 1.91% to 7.39%, and the accuracy from 99.88% to 110.12%. The inter-day precision was 5.17–9.83% with an accuracy of 96.06–110.72. These data indicated that the developed LC–MS/MS method exhibited good precision (Table 1).

The series of blank cell lysis solutions, spiked with various amounts of robinin and kaempferol were prepared as described in Section 2. The mean recovery of robinin and kaempferol were 81.26 ± 2.25 and 65.80 ± 3.95 at a concentration of 2 ng/ml, 73.68 ± 1.75 and 59.56 ± 0.63 at a concentration of 20 ng/ml, and 70.19 ± 2.92 and 63.32 ± 1.36 at a concentration of 200 ng/ml, respectively.

3.4. Stability of robinin

No significant degradation was detected after the sample of robinin was incubated in RPMI 1640 medium at 37 °C for 1 h. There

Table 1
Intra-day and inter-day reproducibility of the LC–MS–MS analysis of robinin and kaempferol in MCF-7 cell lysates.

	Robinin						Kaempferol					
	Intra-day			Inter-day			Intra-day			Inter-day		
Nominal concentration (ng/ml)	2	20	200	2	20	200	2	20	200	2	20	200
Mean assayed concentration (ng/ml)	2.07	22.0	212	2.21	20.7	211	2.16	20.0	200	2.09	19.2	198
SD	0.15	0.42	7.29	0.21	1.07	15.6	0.08	0.72	9.03	0.11	1.89	12.0
Precision (CV%)	7.39	1.91	3.45	9.65	5.17	7.39	3.55	3.62	4.51	5.38	9.83	6.08
Accuracy (%)	103	110	106	111	103	105	108	99.9	100	105	96.1	98.8

Each individual value was the mean of triplicate determinations. Parameters were calculated as described in Section 2.

was no significant decomposition observed in cell lysis solution spiked with various amounts of robinin at room temperature when stored for 6 h.

3.5. Determination of robinin in MCF 7 cells lysates

The LC/MS/MS method presented here was successfully used to quantify robinin in cell samples from a robinin uptake study using human breast adenocarcinoma MCF-7 cells. PBS was used to wash cells in order to remove any flavonoid glycoside adhering to the cell membranes after incubation with medium and the test compound. Typical chromatograms from a cellular uptake experiment are shown in Fig. 2(B–D). When the cells were treated with 25, 50 and 100 μM robinin, the determined intracellular concentrations of robinin were 30 ng/mg protein, 60 ng/mg protein and 100 ng/mg protein respectively.

4. Conclusion

This is the first report of an LC/MS/MS assay for the simultaneous determination of robinin and kaempferol in human breast cancer cells. The method is specific, rapid, and highly sensitive. The assay has a limit of detection of 0.1 ng/ml for both compounds in cell lysates. The intra- and inter-day coefficients of variation of robinin and kaempferol were less than 10%. The assay was successfully applied in a robinin uptake study to determine the intracellular concentrations of robinin when MCF-7 cells were treated with 25, 50 and 100 μM robinin.

Acknowledgements

This project was supported in part by a Fulbright Scholarship awarded to Lia Tsiklauri under the international exchange program sponsored by the United States Government. Supported in part by a grant from the Susan G. Komen Foundation to MEM.

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