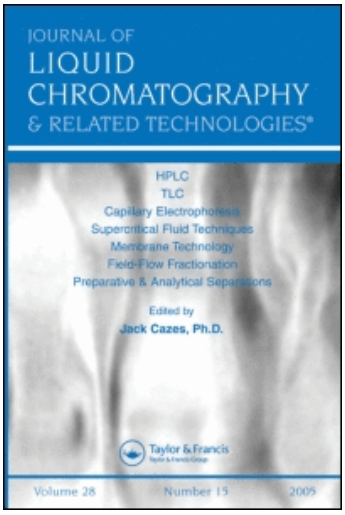

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF QUERCETIN IN PLASMA

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Online publication date: 18 January 2000

To cite this Article Khaled, Khaled A. and El-Sayed, Yousry M.(2000) 'HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF QUERCETIN IN PLASMA', Journal of Liquid Chromatography & Related Technologies, 23: 3, 455 — 465
To link to this Article: DOI: 10.1081/JLC-100101464
URL: http://dx.doi.org/10.1081/JLC-100101464

J. LIQ. CHROM. & REL. TECHNOL., 23(3), 455–465 (2000)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF QUERCETIN IN PLASMA

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ABSTRACT

A sensitive, reproducible, simple, and accurate high performance liquid chromatographic (HPLC) method for the quantitative determination of quercetin in plasma has been developed and validated. Sample preparation involves simultaneous precipitation of plasma proteins and extraction of quercetin and kaempferol (the internal standard) from 0.1 mL plasma. The separation was performed in a stainless steel Ultrasphere-ODS column with a mobile phase consisted of a mixture of 30% acetonitrile and 70% of 5% acetic acid in HPLC water. The mobile phase was pumped at a flow rate of 1.5 mL/min and the effluent was monitored at 375 nm. The retention times for the drug and the internal standard were found to be 3.5 and 6.2 minutes, respectively. Peak-area ratios of the drug to the internal standard were used for quantitation of quercetin in plasma samples. The limit of detection of drug in plasma was found to be 0.06 μg/mL. The intra-day coefficient of variation (CV) ranged from 4.35% to 9.47%, and the inter-day CV ranged from 1.77% to 6.38% at three different concentrations.

Mean absolute recoveries ranged from 96.52% to 101.19% and the relative recoveries ranged from 92.64% to 111.03% at three different concentrations. Preliminary stability tests showed that quercetin is stable for at least 8 weeks in plasma after freezing at -20°C. The method was applied for the determination of the pharmacokinetic parameters of quercetin after intravenous administration to three rats.

INTRODUCTION

Quercetin, 3,5,7,3′,4′ -pentahydroxyflavone, the major representative of the flavonol subclass of flavonoids, is a common dietary component. Quercetin has been frequently used as a model compound showing the protective properties of flavonoids.[1]

Quercetin has a wide range of biological activities, which include a strong and prolonged anti-inflammatory effect,[2] inhibition of histamine release from mast cells,[3] and prevention of the oxidation of low-density proteins resulting in the prevention of atherosclerosis plaque formation.[4] Quercetin has antiproliferative activity in vitro against ovarian, breast, and stomach cancer cell lines.[5-6] In addition, quercetin has been shown to promote relaxation of cardiovascular smooth muscles producing antihypertensive and antiarrhythmic effects.[7] All these activities suggest that quercetin could be a compound with potential clinical application.

Several methods were cited in the literature to determine quercetin and its metabolites in biological fluids. These include spectroscopic and fluorimetric methods,[8-9] and HPLC methods.[10-13] However, spectroscopic and fluorimetric methods suffer from lack of specificity and low sensitivity (limit of detection ≈ 0.2 μg/mL). On the other hand, the reported HPLC methods are highly sensitive but are time consuming and require tedious extraction and purification procedure.

The aim of the present work was to develop a simple, rapid, and sensitive method of analysis of quercetin in plasma. The method involves simultaneous acidification, deproteinization, and extraction of plasma and requires small plasma samples (0.1 mL). Kaempferol was used as an internal standard. The chemical structures of both quercetin and kaempferol are shown in Figure 1.

To avoid interference by plasma constituents, the analysis of the drug was performed at its second λ_{max} at 375 nm. The method was applied to determine quercetin plasma concentrations after intravenous administration of the drug to rats.

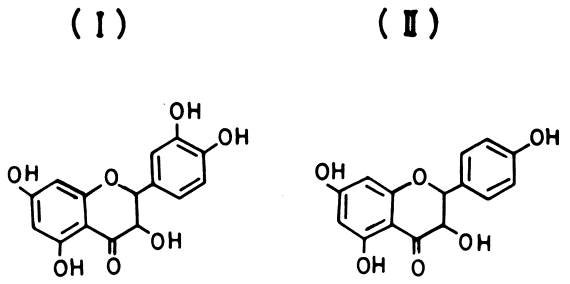

Figure 1. Chemical structures of quercetin (I) and kaempferol (II).

EXPERIMENTAL

Instruments

The high performance liquid chromatographic system consisted of a model LC-10 AD solvent delivery pump, a model CTO-10A column oven (both from Shimadzu Corp., Koyato, Japan); a model 486 variable wavelength detector, a model M730 recorder integrator data module (both from Waters Associates, Milford, MA, USA); and model 2E Rheodyne injector (Rheodyne Inc., Cotati, CA, USA). Chromatographic separation was performed using Ultrasphere-ODS steel column (150 mm length x 4.6 mm i.d., 5 μm particles).

Materials

Quercetin dihydrate (Winlab, Middlesex, UK), kaempferol HPLC grade (Fluka BioChemika, Switzerland), glacial acetic acid, perchloric acid, HPLC grade methanol, HPLC grade acetonitrile, polyethylene glycol 200 (PEG 200), and dimethyl sulfoxide (BDH Chemicals Ltd., Poole, UK). All materials were used as received without further purification. A volume of 0.1 mL of 35% perchloric acid was added to 10 mL of methanol to prepare acidified methanol used for deproteinization of plasma.

Standard Solutions

Quercetin dihydrate (equivalent to 10 mg of quercetin) was dissolved in 10 mL methanol. This stock solution was diluted 10, and 100-fold in methanol to

give the working standard solutions 100 and 10 μg/mL, respectively. The working internal standard (kaempferol) solution (130 μg/mL) was prepared by diluting 1 mL of the stock solution (1300 μg/mL) to 10 mL with methanol.

Chromatographic Conditions

The mobile phase was a mixture of acetonitrile : 5% acetic acid (30 :70%, v/v). It was degassed daily by passing it through a 0.45 μm membrane filter (Millipore, Bedford, MA,USA). The mobile phase was pumped isocratically at a flow rate of 1.5 mL/min at 25°C. The chart speed was 0.3 cm/min and the effluent was monitored at 375 nm and attenuation at 0.001 AUFS.

Analytical Procedure

One hundred microliter of plasma was transferred into 1.5 mL disposable polypropylene centrifuge tube, 20 μL of the internal standard solution (130 μg/mL) was added to the tube followed by the addition of 200 μL of the acidified methanol. The mixture was shaken on a vortex mixer for 5 min, and centrifuged at 12000 rpm in a microcentrifuge (MicroCentaur, MSE, UK) for 5 minutes. An appropriate aliquot from the supernatant (50 μL) was then injected into the loop injector. For plasma samples having, or expected to have, drug concentration ≤ 250 ng/mL, 1 ml of the supernatant was collected, from six centrifuge tubes, frozen in liquid nitrogen, and lyophilized (Freeze Dryer 4.5, Labconco corp., Kansas City, USA). The lyophilized material was reconstituted in 0.3 mL of methanol, vortexed for 5 min, and centrifuged at 12000 rpm for 5 min to ensure that no particulate matter would be injected into the column. A volume of 50 μL of the supernatant was injected.

Application

Male Sprague-Dawley rats weighing 250 g were used in the study. Quercetin (15 mg/kg) was injected intravenously. The drug was dissolved in a solvent mixture consisting of PEG 200 : DMSO (66 : 34 %, v/v). The rats were fasted overnight but allowed free access to water. Blood samples were collected from ether anaethesized rats, using cardiac puncture technique, into a heparinized glass tube before drug administration and at 5, 10, 20, 30, 60, 90, 120, 150, 180, 240, and 300 minutes after the drug was administered. Three rats were used for each sampling time. The plasma was separated after centrifugation for 10 min at 3000 rpm (Mistral 1000, MSE, UK). The plasma was, then, stored at –20°C pending analysis.

RESULTS AND DISCUSSION

The mobile phase used for the current assay provided good separation of the drug and its internal standard with no interference from other components in plasma. Figure 2 shows a typical chromatogram of: blank plasma (2-a1), blank plasma containing internal standard only (2-a2), and plasma containing both quercetin and the internal standard (2-b). The retention times were found to be 3.5 min and 6.2 min for quercetin and kaempferol, respectively. Increasing the percentage of acetonitrile in the mobile phase resulted in decreasing in retention times of both the analyte and the internal standard and interference with the endogenous plasma constituents may occur. The presence of 5% acetic acid in the mobile phase yielded sharp peaks. Keeping the temperature constant during the separation resulted in reproducible peaks. The role of the lyophilization process, in increasing the concentration of the injected drug to the column, is demonstrated in Figure 2-c, which shows the chromatogram obtained from the plasma sample before lyophilization (2-c1) compared to the chromatogram obtained from the same sample after it has been lyophilized (2-c2). Figure 2, also, shows a typical HPLC trace generated by analysis of kaempferol-spiked plasma obtained from a rat after two hours of intravenous administration of 15 mg/kg dose of quercetin (2-e) compared to control plasma sample with internal standard harvested after the injection of a blank bolus containing solvent system only (2-d).

Quantitation

The quantitation of the chromatograms was performed using peak-area ratios of the drug to the internal standard. For each assay an eight-point calibration curve was obtained by spiking 0.2 mL drug-free plasma samples (plasma standard curves) and 0.2 mL mobile phase samples (aqueous standard curves) with known quantities of the drug to obtain quercetin concentrations of 0.12, 0.24, 0.5, 1.0, 2.5, 5.0, 10.0, and 15.0 μg/mL. Calibration samples were processed identically as described. The concentrations and peak-area ratios were linearly related over this range. Each point on the standard curve was based on six determinations. Least squares regression analysis of the mean standard calibration lines for the mobile phase and plasma samples resulted in the following equations:

For mobile phase:

$$Y = 8.22488\,X - 0.03473 \quad \text{and} \quad r = 0.9999$$

and for plasma:

$$Y = 7.80236\,X + 0.18215 \quad \text{and} \quad r = 0.9998$$

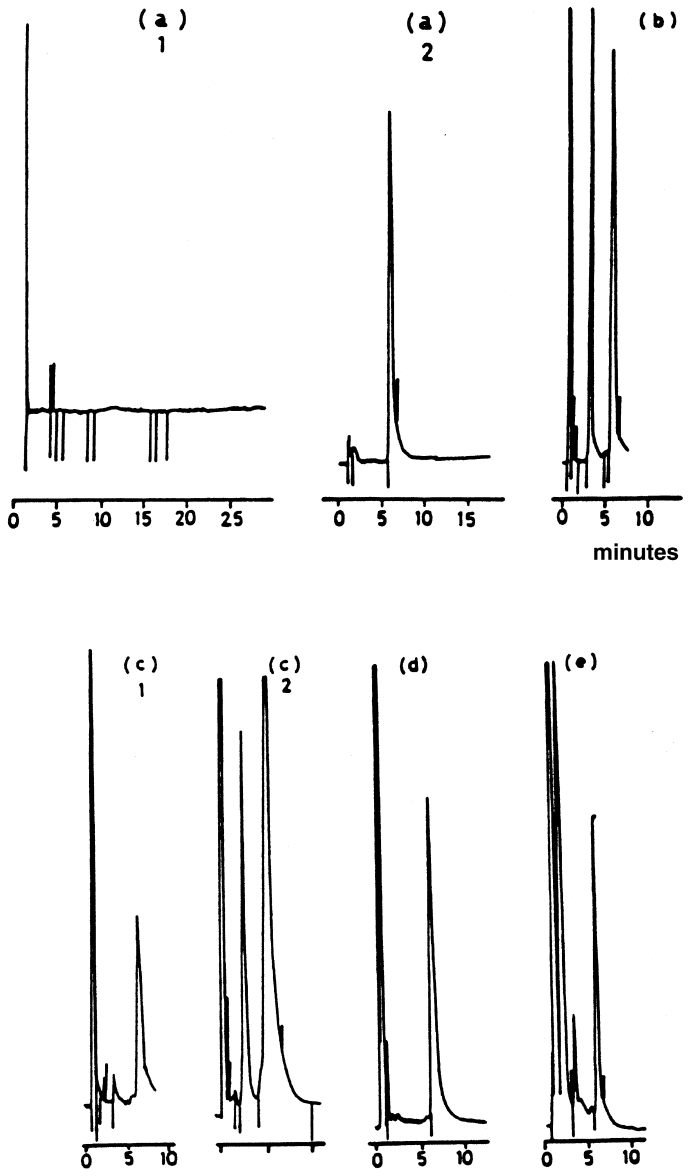

Figure 2. Chromatograms of drug-free rat blank plamsa (a); rat plasma spiked with quercetin and kaempferol (b); rat plasma containing low concentration of quercetin before and after lyophilization process (c); control plasma obtained after intravenous injection of drug free solvent mixture to the rats (d); and plasma obtained after intravenous injection of 15 mg/kg of quercetin to the rats (e).

Table 1

Intraday and Interday Precision of Quercetin in Plasma

Added Conc. (μg/mL)	Intra-Day[a] Measured conc. (μg/mL)	Bias[c] (%)	Inter-Day[b] Measured Conc. (μg/mL)	Bias[c] (%)
0.3				
Mean	0.296	-1.322	0.302	0.667
SD	0.028		0.012	
CV, %	9.472		3.974	
6.0				
Mean	6.020	0.361	6.037	0.617
SD	0.262		0.107	
CV, %	4.351		1.772	
12.0				
Mean	11.981	-0.158	11.137	-7.192
SD	0.556		0.711	
CV, %	4.641		6.380	

[a] Mean values represent 6 different plasma samples for each concentration.
[b] Interday precision was determined from 6 different runs over a 4 week period at each of the three concentrations. The concentration of each run was determined from a single calibration curve run on the first day of the study. [c] Bias = 100 X (measured conc. - added conc.)/added conc.

Standard curves of quercetin in plasma were constructed on six different days to determine the variability of the slopes and intercepts. The results showed little day-to-day variability of slopes and intercepts as well as good linearity over the plasma concentration range studied (r = 0.9998). The coefficient of variation of the slope was 1.423%.

Precision

The intraday precision (random analytical variation) was evaluated by replicate analysis of plasma samples containing quercetin at three different concentrations (0.3, 6.0, and 12.0 μg/mL). The intraday precision showed a coefficient of variation (CV) of 4.351% to 9.472% (Table 1). The interday precision (total analytical variation) was similarly evaluated over 4 weeks. The interday CVs varied from 1.772% to 6.380% (Table 1).

Table 2

Absolute and Relative Recoveries of Quercetin from Plasma*

Conc. (μg/mL)	Mean Peak Area Ratios Aqueous	Plasma	Absolute Recovery % Mean ±SD	Relative Recovery % Mean ±SD	Range of Relative Recovery
0.3	2.45	2.48	101.19±2.18	102.22±3.2	93.79-111.03
6.0	48.88	47.18	96.52±3.93	100.07±1.72	93.85-104.88
12.0	94.12	93.72	99.57±4.64	99.85±5.56	92.64-106.79

* Six replicate analyses of each concentration.

Table 3

Effect of Frozen Storage on the Quercetin Stability in Rat Plasma

Weeks	Measured Concentration (μg/mL)		
0	0.303	6.010	11.950
1	0.285	5.784	11.590
2	0.316	6.029	12.580
4	0.309	6.249	12.690
6	0.305	5.877	12.000
8	0.314	6.236	12.260
Mean	0.305	6.031	12.178
(±SD)	0.011	0.187	0.415
(CV, %)	3.651	3.101	3.408

Recovery

The accuracy of the assay was tested by determining the absolute and relative recoveries of quercetin from plasma. The absolute recovery of quercetin and the internal standard were determined by comparing the peak-area ratio of the drug obtained from spiked plasma with those obtained by the direct injection of pure standards of quercetin and kaempferol in the mobile phase at the same concentration levels. Three different concentrations were used (0.3, 6.0, and 12.0 μg/mL). Table 2 shows mean absolute recoveries of the drug ranged from 96.52% to 101.19 %.

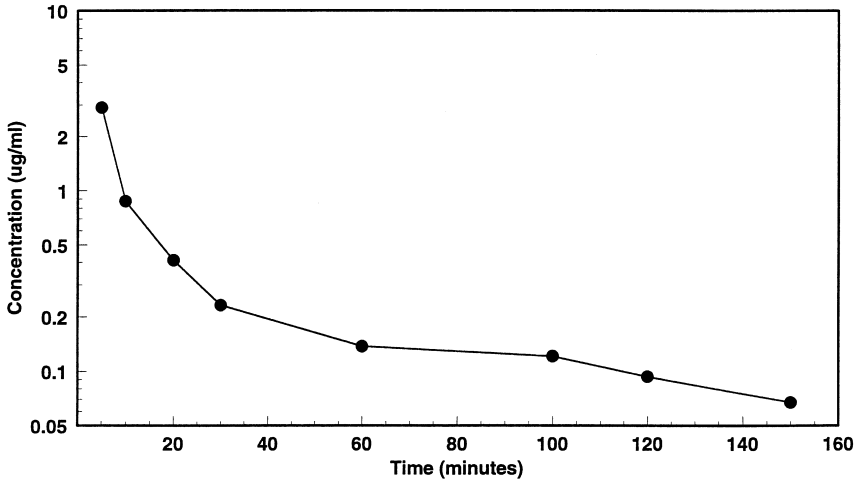

Figure 3. Mean plasma concentration-time profile of quercetin after intravenous injection of 15 mg/kg dose to three rats.

The absolute recovery was also determined from the mean slope of the mean standard curve in plasma over the mean slope of the standard curve in the mobile phase and found to be 94.86%. The relative recovery of quercetin was calculated by comparing the concentrations obtained from drug-supplemented plasma to the actual added concentrations. The relative recoveries ranged from 92.64 % to 111.03% (Table 2).

Limit of Detection

The limit for the determination of quercetin in plasma using the aforementioned method (defined as a signal-to-noise ratio of 3) was 0.06 μg/mL.

Stability

Stability study of plasma spiked with quercetin (0.3, 6.0, and 12.0 μg/mL) were performed over an eight-week period (Table 3).

Pooled plasma were divided in 0.2 ml lots and stored in a freezer at –20°C until the time of analysis. The results demonstrated that quercetin can be stored frozen in plasma for 8 weeks without significant degradation.

Table 4

Mean Pharmacokinetic Parameters of Quercetin After I. V. Injection of 15 mg/kg Dose to Rats

Parameter	Value
$AUC_{0\rightarrow\infty}$ [a] (μg.min/mL)	58.69
Cl[b] (mL/min/kg)	255.6
$AUMC_{0\rightarrow\infty}$ [c] (μg.min^2/mL)	3377
β[d] (min^{-1})	0.0094
$t_{1/2\beta}$ [e] (min)	73.72
MRT[f] (min)	57.54
V_{dc} [g] (liter/kg)	2.89
V_{dss} [h] (liter/kg)	14.7

[a] $AUC_{0\rightarrow\infty}$ = area under the plasma concentration-time curve from time zero to infinity. [b] Cl = clearance. [c] $AUMC_{0\rightarrow\infty}$ = area under the first moment curve from zero infinity. [d] β = terminal elimination rate constant. [e] $t_{1/2\beta}$ = terminal elimination half-life. [f] MRT = mean residence time. [g] V_{dc} = volume of the central compartment. [h] V_{dss} = volume of distribution at steady rate.

Application

Figure 3 shows the mean plasma concentration-time profile of quercetin after intravenous administration of 15 mg/kg to rats. Three rats were used for each sampling time. It is clear that the disposition of the drug is characterized by a rapid distribution phase followed by a slower terminal (elimination) phase. The calculated pharmacokinetic parameters using model-dependent (two body compartment) and model-independent approaches are tabulated in Table 4.

CONCLUSION

The HPLC method described in this study has the sensitivity, selectivity, reproducibility, and rapidity which makes it valuable in many applications, specifically in drug level monitoring and pharmacokinetic studies of quercetin.

ACKNOWLEDGMENT

The authors would like to express their appreciation for the valuable technical assistance of Mr. G. Mahrous.

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Received January 5, 1999
Accepted April 26, 1999
Manuscript 4982