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RESEARCH NOTE

Rapid Quantification of Cellular Flavonoid Levels using Quercetin and a Fluorescent Diphenylboric Acid 2-amino Ethyl Ester Probe

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Abstract Flavonoids are abundant in fruits and vegetables and have various biological activities. A rapid, sensitive method for assessing cellular quercetin levels is necessary because cellular levels are often low. A new method for measuring cellular flavonoid levels is proposed using diphenylboric acid 2-aminoethyl ester (DPBA), a fluorescent probe, and quercetin, a representative flavonoid. This spectrofluorometry-based assay is simple and allows rapid quantification of the cellular quercetin content with high sensitivity. CHO-K1 cells were incubated with quercetin for 5 min, and then the cellular quercetin concentration was assayed using DPBA spectrofluorometry. Results, compared with high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), and conventional spectrophotometry measurements, were highly correlated with HPLC data (R>0.99). The method is more sensitive than TLC and conventional spectrophotometry. Spectrofluorometric measurement using DPBA is a simple, rapid, and sensitive method for assessing cellular quercetin levels.

Keywords: flavonoids, spectrofluorometry, fluorescence activated cell sorter (FACS), diphenylboric acid 2-aminoethyl ether (DPBA), quercetin

Introduction

Flavonoids are a class of secondary plant metabolites that contain 2-phenyl-chrome flavonone, which confers a characteristic yellow color on the compounds (1). They are present in large quantities in various plant materials, including fruits and vegetables. Flavonoids are ingested in significant quantities, and mean daily consumption by humans is 10-100 mg. The most abundant dietary flavonoid found in plants is quercetin, which is a flavonol. Onions, black tea, apples, and black currants contain large amounts of quercetin, ranging from 20-400 mg/kg (2). The average American consumes 20 mg of quercetin daily (3). Many review studies have investigated quercetin. Various biological functions of quercetin (both *in vitro* and *in vivo*) have been reported, including hypolipidemic, hypoglycemic, anti-inflammatory, anti-allergy, and anti-viral effects (4-7).

The bioavailability of quercetin in food is limited and the cellular concentration is usually low. Quercetin is taken up by cells predominantly as an aglycone, and only 20%-30% of dietary quercetin aglycone is absorbed after consumption, resulting in micromolar intracellular and plasma concentrations. Flavonoid levels in plant materials are routinely determined using spectrophotometry (8,9); however, this method has limited sensitivity. Thus, a more sensitive method for quantification of cellular and plasma flavonoid levels is needed (10). High-performance liquid chromatography (HPLC) is a conventional quantification method that identifies individual flavonoids in various samples; however, it requires instrumentation and significant time and effort for measurement optimization (11-14).

A new rapid approach for quantifying cellular quercetin levels (as a representative flavonoid) using the fluorescent probe diphenylboric acid 2-aminoethyl ester (DPBA) is reported. DPBA forms a spontaneous complex with common flavonoid compounds, and then emits fluorescence. Thus, it has been used widely to examine the accumulation, localization, and movement of flavonoids in living plants (15). Here, DPBA staining was used to quantify cellular quercetin levels in cultured mammalian cells with a spectrofluorometric assay.

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Materials and Methods

Cell culture CHO-K1 cells were grown in Dulbecco's modified Eagle's medium (Thermo Scientific, Bremen, Germany). First, they were incubated in an F-12 nutrient mixture (Thermo Scientific) containing 10% heat-inactivated fetal bovine serum (Thermo Scientific) and 1% penicillin-streptomycin (Thermo Scientific) at 37°C in a 5% CO_2 incubator to 60%-80% confluence. Then, they were treated with various concentrations of quercetin.

Sample preparation CHO-K1 cells were seeded in 6 well plates at 1×10^6 cells/well the day before treatment. Cells were stimulated with quercetin (Sigma, St. Louis, MO, USA) at various concentrations (5, 10, 20, 50, and 100 μM) for 5 min, washed twice with PBS, and covered with 1 mL of methanol (J.T. Baker, Phillipsburg, NJ, USA). Next, the cells were collected and incubated at 65°C for 1 h. After centrifugation (1730MR; Gyrozen, Seoul, Korea) at 27,000×*g* for 2 min, the supernatants were collected and concentrated using a SpeedVac concentrator (N-Biotek, Bucheon, Korea). Following solvent evaporation, 100 μL of methanol was added to dissolve the cellular extract. A total of 10 μL of each sample was used for HPLC and thin layer chromatography (TLC). An amount of 1 μL of each sample was used for spectrophotometry. Samples for spectrofluorometry were diluted in 100 μL of DMSO and 95 μL of this solution was used in the assay.

HPLC A Waters HPLC system (Milford, MA, USA) with a 2996 photodiode array detector and an 717 plus autosampler was used for analysis. Quercetin was analyzed using an OptimaPak RP-C18 column (250×4.6 mm i.d., 5 μm; RStech, Daejeon, Korea). Formic acid (Daejung, Siheung, Korea), acetonitrile (J.T. Baker) 2-propanol (Merck, Darmstadt, Germany), and water (triple-distilled) reagents were used for preparation of the mobile phase. Elution of quercetin was monitored at 370 nm. The run time was 25 min between injections. The samples were not subjected to any clean-up steps. The injection volume was 10 μL. The mobile phase used was 1% formic acid (aqueous solution):acetonitrile:2-propanol (70:22:8, v/v/v). The mobile phase flow rate was 0.25 mL/min. Separation was performed at room temperature (24±1°C). Quercetin was eluted within 20 min.

Spectrophotometry and spectrofluorometry A total of 1 μL of the cellular extract was added to test tubes containing 0.2 μL of 10% aluminum nitrate (Sigma), 0.2 μL of 1 M aqueous potassium acetate (Junsei Chemical, Tokyo, Japan), and 8.6 μL of 80% ethanol. After 40 min of incubation at room temperature, the absorbance of the sample was measured at 415 nm to determine the total flavonoid concentration using a NanoDrop spectrophotometer (ND-1000; Thermo Scientific). For DPBA-stained spectrofluorometry, 100 μM DPBA was added to cellular extracts at 1:1 (v/v), and then the optical density was measured using an excitation filter at 485 nm and an emission filter at 535 nm.

TLC In total, 10 μL of cellular extract prepared as described for HPLC was applied to a TLC silica gel plate ($60F_{254}$; Merck) using capillary tubes. Quercetin standard solutions and samples extracted from CHO-K1 cells incubated with various concentrations of quercetin were spotted on the plate and developed using a mobile system of methanol:water:formic acid (40:57:3, v/v/v). Before developing the TLC plate, the chamber was saturated with solvent for 30 min at room temperature. To visualize flavonoid spots, the plate was stained with 0.5% DPBA (Sigma) in methanol by spraying, and quercetin spots were visualized under UV light at 365 nm.

Assessment of cellular flavonoid distribution using fluorescence activated cell sorter (FACS) analysis CHO-K1 cells were plated in 6-well plates at a density of 1×10^6 cells/well and grown for 24 h. Next, the cells were treated with 0, 5, 10, 20, 50, or 100 μM quercetin for 5 min and stained with DPBA. The cells were then washed twice with PBS and detached from the plate using trypsin-EDTA (Thermo Scientific). The collected cells were centrifuged (1730MR; Gyrozen) at 350×*g* for 3 min to form a pellet, washed twice, and resuspended in 1 mL of FACS buffer (0.1% BSA and 0.02% sodium azide in PBS). Each sample was then analyzed using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) to determine the FL-1 fluorescence. Data analysis was performed using Cell Quest Pro software (BD Biosciences). A total of 10,000 cells were counted in each group. Data are displayed in histogram form (Fig. 2B). Results were calculated using the geometric mean of each peak and displayed as a bar graph (Fig. 1E) after excluding the baseline value (the 0 μM group). Fluorescence images indicated formation of cellular quercetin-DPBA complexes after 5 min of treatment. Cells were fixed using 4% formaldehyde (Duksan, Seoul, Korea), washed twice, and observed under fluorescence microscopy (Eclipse Ti; Nikon, Tokyo, Japan) using UV wavelengths.

Statistical analysis All experiments were done at least in triplicate. All data are expressed as the mean±standard error of the mean (SEM). Comparisons between groups were performed using a one-way analysis of variance (ANOVA) with log values of the data. Differences at $p<0.01$ were considered to be statistically significant.

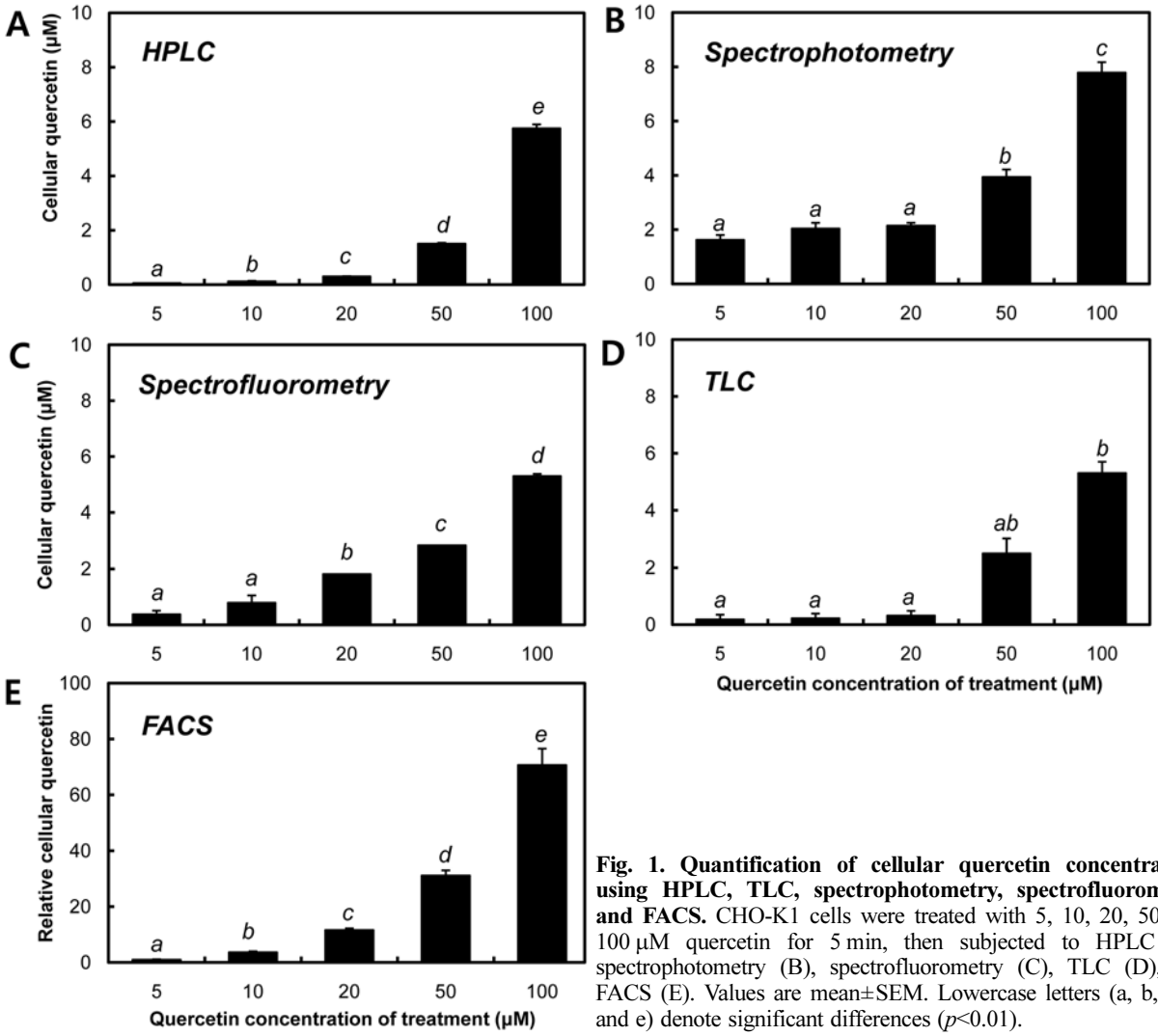

Fig. 1. Quantification of cellular quercetin concentrations using HPLC, TLC, spectrophotometry, spectrofluorometry, and FACS. CHO-K1 cells were treated with 5, 10, 20, 50, and 100 μM quercetin for 5 min, then subjected to HPLC (A), spectrophotometry (B), spectrofluorometry (C), TLC (D), and FACS (E). Values are mean±SEM. Lowercase letters (a, b, c, d, and e) denote significant differences (p<0.01).

Results and Discussion

A rapid and sensitive method for quantifying the amount of cellular quercetin would be useful for studying cellular biological functions, toxicity, and bioavailability (16). Therefore, a new spectrofluorometry-based method for quercetin quantification using a DPBA fluorescent probe is proposed.

First, conventional HPLC and spectrophotometric assays of extracts from CHO-K1 cells incubated with various concentrations of quercetin (5, 10, 20, 50, and 100 μM) (Fig. 1A, 1B) were performed, and the sensitivity of each technique was judged using analysis of variance (ANOVA). Cellular quercetin concentrations should depend on the initial treatment concentrations. The entire HPLC analysis result showed a significantly different cellular quercetin level, within 5 to 100 μM range of quercetin treatment (p<0.01, Fig. 1A). Spectrophotometric analytical data, which is frequently used for flavonoid quantification, was not useful for detection of the correct concentration of quercetin in cells exposed to a concentration less than 20 μM quercetin for 5 min (Fig. 1B). The cellular concentrations of quercetin after 5, 10, and 20 μM treatments were not significantly different, even after a 20× sample concentration (p>0.01).

Use of DPBA increases the sensitivity of flavonoid detection by formation of complexes with 3-hydroxyflavones via a condensation reaction (15). The spectrofluorometric method was able to measure low cellular quercetin concentrations and was as sensitive as HPLC. The correlation coefficient of HPLC versus spectrofluorometry results was highest (Table 1). The correlation coefficients of HPLC versus spectrophotometry results and HPLC versus spectrofluorometry results were 0.993 and 0.997, respectively. Spectrofluorometry takes advantage of DPBA-quercetin complexes to improve the sensitivity. Furthermore, this technique requires only 5 min for formation of the DPBA-quercetin complex, while conventional spectrophotometry requires 40 min of incubation to form aluminum-quercetin complexes. Thus, DPBA-based spectrofluorometry

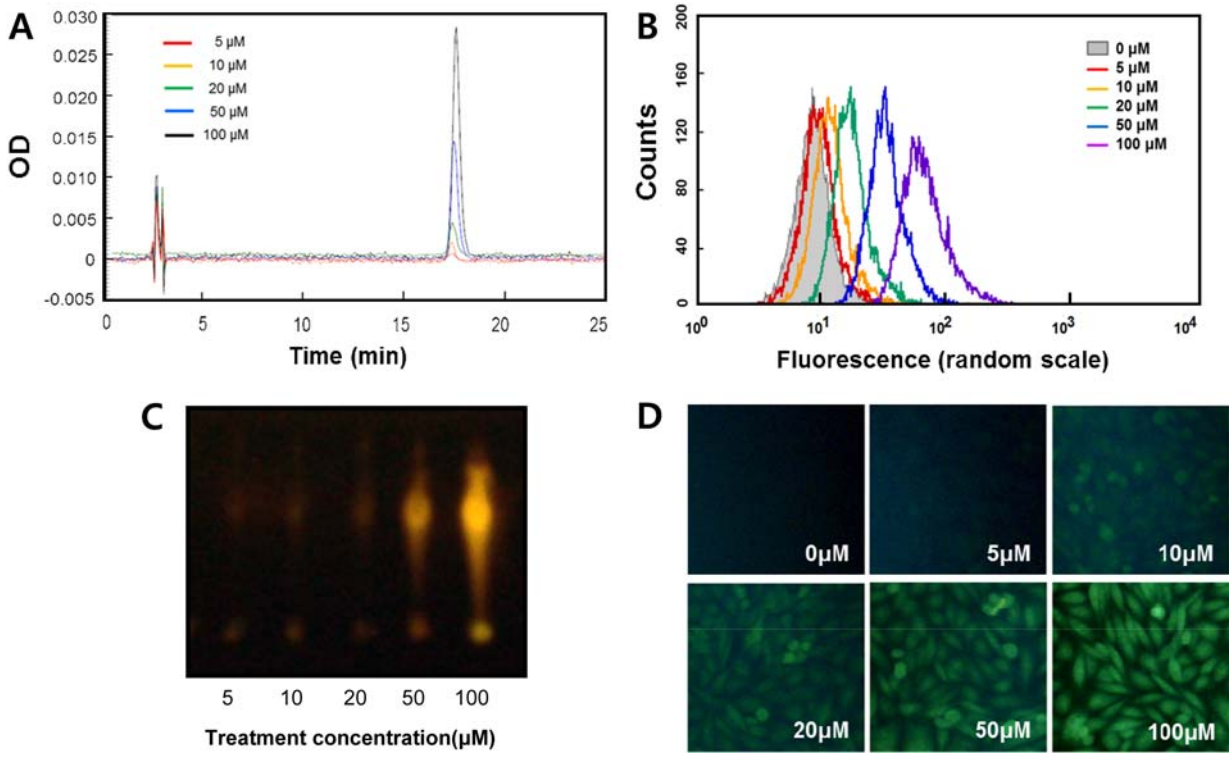

Fig. 2. Chromatographs and FACS results. (A) HPLC; (B) FACS; (C) Separation of cellular extracts on a TLC plate; (D) DPBA staining of cellular quercetin

saves time.

The DPBA-based TLC and FACS assay results were also compared to the spectrofluorometry results. Measurement of the cellular distribution of quercetin using FACS analysis showed the highest sensitivity among the three DPBA methods. This suggests that FACS analysis is an accurate method for assessing the quercetin content of cells incubated with quercetin relative to untreated controls. The cellular quercetin distribution measured using FACS analysis showed a strong positive correlation (>0.98) with the quercetin concentrations quantified using HPLC (Fig. 1D and Table 1). Although FACS analysis was used to determine cellular quercetin concentrations relative to controls, the sensitivity was high.

The TLC method using DPBA was faster, but far less sensitive than HPLC. Quercetin levels in cells incubated with quercetin concentrations less than 20 μM for 5 min were not correctly measured, and the quercetin levels in cells treated with 5, 10, and 20 μM quercetin were not statistically different (p>0.01).

The cellular quercetin concentrations were 0.06, 0.12, 0.30, 1.5, and 5.75 μM using HPLC, and 0.13, 0.25, 0.64, 1.87, and 5.35 μM using spectrofluorometry in CHO-K1 cells incubated with 5, 10, 20, 50, and 100 μM quercetin for 5 min, respectively (Fig. 1A, 1C). Spectrofluorometry tended to overestimate cellular flavonoid levels when quercetin concentrations were lower than 1 μM. TLC chromatographs and FACS results are shown in Fig. 2.

In conclusion, we propose a rapid method of quantifying cellular quercetin levels using a fluorescent DPBA probe. Spectrofluorometry has several advantages over conventional techniques. It is a rapid, and the resulting data are highly correlated (R>0.99) with HPLC data. Also, it is a simple procedure, compared to HPLC and TLC (HPLC and TLC require optimization) (17). In addition, DPBA has broad specificity for the major flavonoids rutin, luteolin, morin, kaempferol, and myricetin. Thus, it can be used to detect multiple flavonoids (18). FACS analysis does not require

Table 1. Correlations of flavonoid concentrations measured using HPLC and spectrophotometry, spectrofluorometry, TLC, and FACS[1)]

	Spectrophotometry	Spectrofluorometry	TLC	FACS[2)]
HPLC	0.993±0.006	0.997±0.008	0.978±0.012	0.983±0.003

[1)]Values are the mean±SEM of the correlation value.
[2)]Correlation coefficients were calculated using the flavonoid concentration quantified using HPLC and the relative cellular quercetin levels measured using FACS.

the extraction step that is essential for HPLC and TLC. Hence, spectrophotometry is the fastest technique, although FACS analysis only shows cellular quercetin concentrations relative to controls. DPBA-based spectrofluorometric analysis of cellular quercetin can be applied in many areas of quercetin research, including studies of health functionality, toxicity, and bioavailability.

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