Determination and Quantitation of Five Cucurbitane Triterpenoids in *Momordica charantia* by Reversed-Phase High-Performance Liquid Chromatography with Evaporative Light Scattering Detection

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Abstract

A simple and specific analytical method for the quantitative determination of five cucurbitane-type triterpenoids isolated from the fruit of *Momordica charantia* is developed. The triterpenoids present in the fruits of *Momordica charantia* are separated with an acetonitrile (0.1% acetic acid)–water (0.1% acetic acid)–methanol (0.1% acetic acid) gradient at a flow rate of 0.5 mL/min. The high-performance liquid chromatography separation was performed on a Phenomenex C18 reversed-phase column. By using an evaporative light scattering detector, the main triterpenoids of *Momordica charantia* could be detected at levels as low as 10 µg/mL. The method was validated for precision, repeatability, and accuracy. The relative standard deviation was between 0.6–4.4%. The method was sensitive, quick, and accurate for the determination of main triterpenes and saponins in *Momordica charantia,* and can be used for quality control of *Momordica charantia* and its related dietary supplements.

Introduction

Bitter melon, *Momordica charantia* L. (Cucurbitaceae), is a widely cultivated plant and grows in tropical areas, including Asia, East Africa, the Caribbean, and South America. The fruits of this plant have been used not only as a vegetable, but also as traditional medicine for the treatment of bitter stomachic, as a laxative, an anti-diabetic, an anthelmintic in children, treatment of feverish conditions, and an antiviral for both measles and hepatitis (1). Various studies have shown that the saponin fraction of *Momordica charantia* inhibits the increase of blood glucose and serum neutral fat (2–6). One recently published investigation indicated that the major cucurbitane triterpenoids from its dried fruits, 3β, 7β-25-trihydroxycucurbita-5-23 (*E*)-dien-19-al and 5β, 19-epoxy-3β, 25-dihydroxycurubita-6, 23 (*E*)-dienen had significant antidiabetic activity *in vivo* (3) and lipid abnormalities in HepG2 cells (7). Cucurbitane-type triterpenoids and related glycosides are the major chemical constituents in this plant and exist in the fruits, seeds, leaves, and vines of *M. charantia* (8–11). The antidiabetic and antitumor activity of *M. charantia* has aroused interests in its chemical analysis and in the development of dietary supplement products. Therefore, it is necessary to develop analytical methods for the identification of *M. charantia* plant material, for the quality assurance of dietary products, as well as the chemical fingerprinting of *M. charantia*. To date, there is no such method reported for the analysis of cucurbitane triterpenoids in this plant by LC–ELSD, except for the analysis of a single compound, momordicoside A by Shui Wang (12). It is important to note that momordicoside A occurs widely in plants of Cucurbitaceae family. In our current investigation, we have developed and present a reliable and sensitive method for the identification and quantification of five major cucurbitane-type compounds (Momordicoside A, **1**; Momordicoside L, **2**; Momordicoside F_2, **3**; Momordicoside, K., **4**; and 3β,7β,25-trihydroxy cucurbita-5, (25E)-dien-19al, **5**) (Figure 1) from the fruits of *M. charantia* utilizing liquid chromatography–evaporative light scattering detector (LC–ELSD).

Experimental

Plant material and chemicals

The standard compounds **1–5** were isolated at the National Center for Natural Products Research (NCNPR), the identity and purity was confirmed by chromatographic (TLC, HPLC) methods, by the analysis of the spectral data (IR, 1D- and 2D-NMR, HR-ESI-MS) and comparison with published spectral data (8,9). The percent purity of compounds **1–5** are 98.2%, 92.5%,

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87.2%, 96.4%, and 99.1%, respectively. Acetonitrile, methanol, water, and glacial acetic acid were HPLC grade purchased from Fisher Scientific (Fair Lawn, NJ). Plant materials studied were from 3 different populations of *M. charantia* (MC-1 to MC-3) (Voucher numbers: 2774, 2945, 3190) procured from India. Voucher specimens of all samples are deposited at the National Center for Natural Products Research (NCNPR), The University of Mississippi, Mississippi.

Chromatographic instrument and conditions

HPLC experiments were performed on a Waters 2695 Alliance Separations Module (Waters, Milford, MA) connected with a Sedex 55 ELS detector (Sedex, Alfortville, France) using a Gemini column (150 × 4.6 mm; 5 µm particle size) from Phenomenex (Torrance, CA), and maintained at 25°C. The mobile phase consists of methanol (A), acetonitrile (B), and water (C), all containing 0.1% acetic acid, which were applied in the following gradient elution: 0–5 min, 10% A, 25% B, 65% C; 5–36 min, 10% A, 25% B, 65% C to 4% A, 70% B, 26% C; 36–38 min, 4% A, 70% B, 26% C to 100% B, then held for 5 min. Each run was followed by a 5 min washing procedure with 100% acetonitrile. The flow rate was adjusted to 0.5 mL/min, and the injection volume was 10 µL. Re-equilibration was with 10% A, 25% B, 65% C for 15 min. Total run time was 43 min. The ELS detector was adjusted to 40°C, at a gain of 11 and with a nitrogen pressure of 2.1 bar.

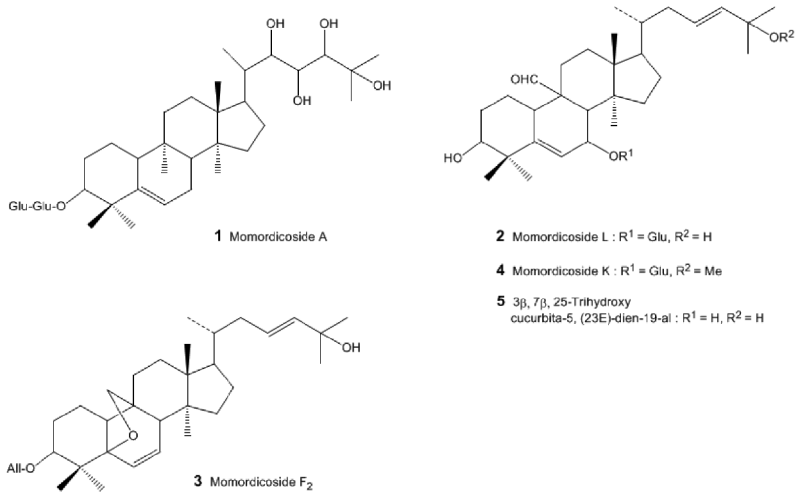

Figure 1. Structure of standard compounds (**1**–**5**).

Standard Preparation

One milligram of each standard compound was dissolved in 1.0 mL methanol (stock solution). Five additional calibration levels were prepared by diluting the stock solution with methanol. Within the range of concentrations injected (800.0–50.0 µg/mL for compounds **1**, **3**, and **4**, 1000–125 µg/mL for compound **2** and 500.0–30.0 µg/mL for compound **5**, respectively) the detector response is a function of the mass and follows an exponential relationship (the log of response versus log of concentration is linear).

Sample preparation

Finely powdered dried plant materials (1.0 g) of *M. charantia* samples were sonicated in 5.0 mL of methanol–water (90:10, v/v) at 35°C for 25 min, followed by centrifugation for 15 min at 9000 rpm. The supernatant was transferred into a 50-mL flask. The procedure was repeated four times. The combined supernatants were evaporated under nitrogen gas utill the volume was less than 6 mL. The concentrated extracts were moved into a 10.0-mL volumetric flask, and the previous flask was rinsed with 3 mL methanol–water (90:10, v/v). The rinsing solvents were combined with the concentrated extracts. The final volume was adjusted to 10.0 mL with methanol–water (90:10, v/v) and mixed thoroughly.

Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.45-µm nylon membrane filter. The first 1.0 mL was discarded, and the remaining volume was collected in an HPLC sample vial. Each sample solution was injected in triplicate.

Precision

Precision (intra- and inter-day assay) of the method was determined by analyzing five individual samples of *M. charantia* on three consecutive days. The samples were extracted and assayed under optimized conditions (Table I).

Table I. Calibration Data, Range, LOD, LOQ for Compounds 1–5 and Content of Triterpenoids by Intra- and Inter-Day Precision of One Sample (MC-1, *M. charantia*) Assayed Under Optimized Conditions*

Analyte	Regression Equation	Concentration Range(µg/mL)	r^2	LOD (µg/mL)	LOQ (µg/mL)	Intra-Day (n = 5) Day 1	Intra-Day (n = 5) Day 2	Intra-Day (n = 5) Day 3	Inter-Day (n = 15)
1	Y = 1.75 e + 000X – 5.02 e-001	50–800	0.999	30	50	0.234 (2.31)	0.235 (0.63)	0.234 (2.19)	0.234 (0.93)
2	Y = 1.61 e + 000X – 5.11 e-001	125–1000	0.998	60	110	0.152 (3.73)	0.154 (3.74)	0.152 (4.40)	0.152 (0.38)
3	Y = 1.73 e + 000X – 3.14 e-001	50–800	0.999	30	50	0.085 (2.42)	0.088 (3.64)	0.086 (3.31)	0.086 (0.63)
4	Y = 1.61 e + 000X – 1.57 e-001	50–800	0.996	30	50	0.110 (2.98)	0.110 (3.96)	0.111 (4.28)	0.110 (0.68)
5	Y = 1.84 e + 000X + 1.84 e-001	30–500	0.998	10	30	0.036 (0.76)	0.033 (2.08)	0.036 (2.09)	0.035 (0.77)

* Calibration data = regression equation and correlation coefficient (r^2); Content = Values in mg/100 mg of dry plant material weight; % relative standard deviations are given in parentheses.

Recovery

The accuracy of the method was determined by analyzing the percentage recovery of the main constituents in extract of *M. charantia*. The sample (MC-1) was spiked with 0.1 mL of standard stock solution (1.0 mg/mL) containing compounds **1–5**. The spiked sample was extracted and assayed under optimized conditions.

Results

To demonstrate the feasibility of our method, we analyzed three populations of *M. charantia*. The fruit extracts (MC-1 to MC-3) were analyzed using LC–ELSD, and the relative concentrations of triterpenoids are shown in Table II. Of the three populations of plant materials, only the MC-1 sample provided a clear assignment of all five triterpenoids. Compound **1** was not detected in MC-2 and MC-3 fruit extracts. Furthermore, Compound **5** was not detected in the MC-3 plant sample.

Discussion

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed phase C18 column. The different columns tried were: Gemini C18, Lichrosphere 5 RP 18, Luna C18, Luna Phenyl-Hexyl, and Synergi POLAR-RP. The best results were observed with the Gemini C18 column using water, acetonitrile, and methanol, all containing 0.1% acetic acid as mobile phase. The optimal separation temperature for this method was determined to be 25°C with a flow rate of 0.5 mL/min. Increasing the column temperature to 30°C or more reduced the sensitivity of peaks remarkably. By LC–ELSD, the gradient was changed to a slightly concave one that enhanced the resolution.

The separation of a standard mixture containing five cucurbitane triterpenoids is shown in Figure 2. The compounds were isolated from *M. charantia* (for peak assignments see Figure 2, for corresponding structures see Figure 1) and represent the major triterpenoids of this species. By using a methanol– acetonitrile–water gradient as eluent and reversed phase C-18 material as stationary phase, a runtime of less than 43 min was required to separate all five triterpenoids because momordicoside F2, momordicoside K, and 3β, 7β, 25-trihydroxycucurbita-5,(23*E*)-dien-19-al held the similar structures and retention times.

For a successful separation of triterpenoids isolated from *M. charantia*, not only did the separation conditions need to be carefully investigated, but also the method of detection. As these compounds are not sensitive to UV absorption, an ELS detector is considered a better alternative because this method of detection is based on mass and not UV absorbance, making these compounds easily detectable.

M. charantia preparations are available throughout the United States as dietary supplements. According to USP, test procedures for the assessment of the quality of pharmaceutical products require the determination of certain analytical parameters like accuracy, precision, peak purity, linearity, and limit of detection. The accuracy of our method was confirmed by determining the recovery. One sample (MC-1) was spiked with known amounts of the standard compounds **1–5**. Compared to the theoretical amounts, the recovery rates were found to be 98.2% for **1**, 103.2% for **2**, 98.3% for **3**, 97.3% for **4**, and 102.9% for **5**. An indicator for precision is the relative standard deviation (RSD). All samples were injected in triplicate, and the standard deviation of compounds **1–5** was below 1.5% for all samples. Intra- and inter-day variation of the assay was determined and showed to be lower than 4.5%, with a maximum RSD of 4.39% (Table I). Peak purity and identity were verified by studying PDA and ELS data, as well by spiking samples with reference compound and by comparing their retention times. No indications of impurities were found. Calibration data indicated the linearity of the detector response for standard compounds **1**, **3**, **4** from 800.0 to 50.0 µg/mL, for compound **2** from 1000–125 µg/mL, and for compound **5** from 500.0 to 30.0 µg/mL. Table I shows the calibration data, calcu-

Table II. Content of Triterpenoids Found in Three Populations of *M. charantia*

Analyte	Three Populations of *M. charantia**		
	MC-1	MC-2	MC-3
1	0.234	ND†	ND
2	0.152	0.437	DUL
3	0.086	0.194	DUL
4	0.110	DUL	DUL
5	0.035	DUL	ND

* Values in mg/100 mg of dry plant material weight.
† ND = not detected; DUL = detected under limits of quantitation.

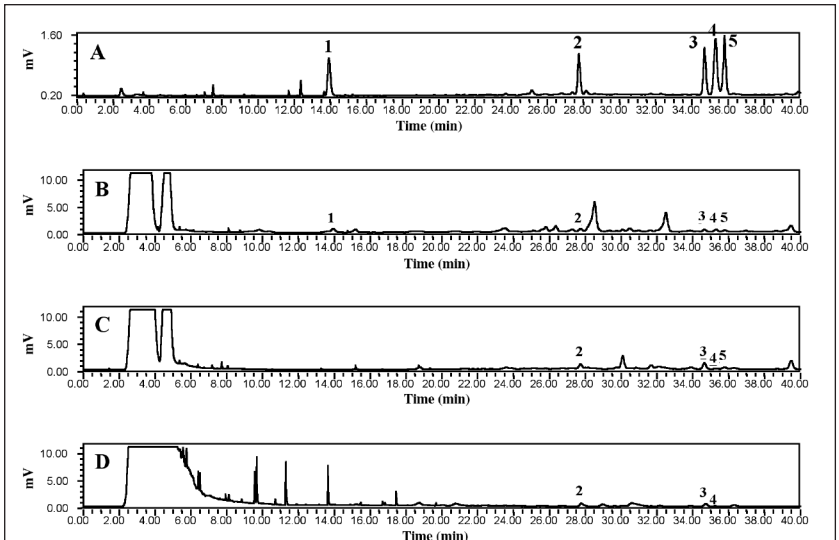

Figure 2. A typical HPLC–ELS chromatogram of standard compounds (**1–5**) (A), three different populations of *Momordica charantia* (B–D).

lated limit of detection and limit of quantitation (the limit of detection and the limit of quantitation were determined by serial dilution based on a signal to noise ratio of 5:1 and of 10:1, respectively).

Conclusions

The HPLC–ELSD method described in this paper is the first detailed report of an analytical method capable of determining five main triterpene and triterpenoid glycosides of *M. charantia*. The developed method allows a reliable and accurate qualitative and quantitative analysis of different populations of *M. charantia*. It fulfills the requirements of a validated method. The method described in this paper can be applied as an analytical tool for determining the authenticity of *M.* charantia plant material and will assist in the quality and safety assessment of commercial botanical products.

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