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Note

Stevia rebaudiana

II. High-performance liquid chromatographic separation and quantitation of stevioside, rebaudioside A and rebaudioside C*

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Stevioside, rebaudioside A, and rebaudioside C (dulcoside B) are the major and the sweetest diterpene glycosides isolated from the leaves of *Stevia rebaudiana* Bert[1]. The potential use of these glycosides in the United States as non-caloric sweetening agents stimulated the authors to devise a simple, efficient, and sensitive procedure for their quantitation in plant material. Several procedures have been published for the assay of *S. rebaudiana* total diterpene glycosides by gas–liquid chromatography of the aglycones[2,3] or by thin-layer chromatographic separation of stevioside, followed by colorimetric estimation[4]. None of these methods was deemed satisfactory. The first successful high-performance liquid chromatographic (HPLC) separation of stevioside and rebaudioside A was reported by Hashimoto and coworkers[5,6] while our study was in progress. A deficiency in one of their procedures will be related herein.

A previous publication by the authors described the HPLC assay of stevioside and rebaudioside A by hydrolysis of the esterified sugars, followed by formation of the chromophoric *p*-bromophenacyl esters[7]. However, it was noted that in our former procedure rebaudiosides D and E produced the same ester derivatives as rebaudioside A and stevioside, respectively. This communication reports the quantitative extraction of diterpene glycosides from *S. rebaudiana*, and a direct, efficient HPLC procedure for the quantitation of underivatized stevioside, rebaudioside A, and rebaudioside C.

EXPERIMENTAL

Plant material

Stevia rebaudiana Bert. (Compositae) was grown from seed; mature plants were authenticated by Mr. Floyd A. Swink of the Morton Arboretum, Lisle, IL, U.S.A. Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy and Pharmacology, University of Illinois at the Medical Center, Chicago, IL, U.S.A.

* Patent applied for.

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Apparatus

Liquid chromatographic separations were conducted with a Waters Model 6000A liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Rheodyne Model 7120 syringe-loading sample injector and 100-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) a Waters Model 450 variable wavelength UV spectrophotometer, and a Texas Instruments Servo/Riter II portable recorder (Texas Instruments, Houston, TX, U.S.A.). Separations were carried out on two Waters 30 × 0.78 cm I.D. Protein I-125 columns.

Chemicals

All chemicals and solvents used in this investigation were reagent or certified grade. Solvents for HPLC were redistilled in glass and degassed prior to use.

Stevioside and rebaudioside A were isolated from the leaves of *Stevia rebaudiana* as previously described[7]. Rebaudioside C was obtained from Professor M. Kobayashi, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan.

Stevia rebaudiana extraction for HPLC analysis

Dried, powdered (30 mesh) *S. rebaudiana* leaves (0.5 g) were continuously and sequentially extracted in a micro-soxhlet apparatus with chloroform (15 ml) for 3 h (extract A), and with methanol (15 ml) for 5 h (extract B); the plant marc was then refluxed with distilled water (15 ml) for 1 h (extract C). Each extract was evaporated to dryness *in vacuo* and the resulting residues were redissolved in chloroform (extract A, 10 ml) or in 1-propanol–water (49:1) (extract B, 50 ml; extract C, 25 ml) for chromatographic analysis.

For comparative purposes, *S. rebaudiana* was also extracted by the method of Hashimoto *et al.*[5]. Calcium carbonate (0.3 g) and distilled water (6.0 ml) were added to the dried, powdered leaves (1.0 g) and the slurry was mixed well prior to 15 h of maceration. The mixture was heated at 50°C for 4 h, cooled, diluted with acetonitrile (18 ml), filtered, and evaporated to dryness *in vacuo* to yield extract D. The residue was redissolved in 1-propanol–water (49:1) (100 ml) for HPLC analysis.

Thin-layer chromatography (TLC)

Extract A (20 μl), as well as HPLC column eluates of stevioside and rebaudiosides A and C were spotted on precoated silica gel GF_{254} plates (20 × 20 cm, 0.25 mm thick; E. Merck, Darmstadt, G.F.R.) and developed with chloroform–methanol–water (30:20:1). After development, chromatograms were air-dried, sprayed with anisaldehyde–sulfuric acid reagent[8], and heated at 100°C for 5 min to visualize *Stevia* diterpene glycosides.

High-performance liquid chromatography

The operating conditions for HPLC were: ambient temperature; flow-rate of eluting solvent, 1-propanol, 1 ml/min; wavelength of UV detector, 210 nm; recorder chart speed, 3 in./h at 0.04 a.u.f.s. Standard solutions of stevioside, rebaudioside A, and rebaudioside C were injected onto the column and their retention times determined.

Beer's law standard curves were obtained by injecting different quantities of

stevioside (5, 10, 15, 20, 30, 50, 60, 80, and 100 μg per 100 μl injection), rebaudioside A (5, 10, 20, 25, 50, and 100 μg per 100 μl injection), and rebaudioside C (4, 6.25, 12.5, 20, 25, 50, and 100 μg per 100 μl injection) onto the column in triplicate and measuring the resulting peak heights.

RESULTS AND DISCUSSION

Previous attempts by these authors to separate stevioside (SS), rebaudioside A (RA), and rebaudioside C (RC) or their benzoyl, 4-nitrobenzoyl, or 3,5-dinitrobenzoyl derivatives[9] on μPorasil, μBondaPak C_{18}, μBondaPak NH_2, μBondaPak CN, and μStyragel, 500-Å columns (Waters Assoc.) with numerous solvent systems having a UV cut-off below 210 nm were totally unsuccessful. A mixture of SS, RC, and RA was originally separated on a single Protein I-125 column, giving retention times of 19.7, 23.2, and 27.5 min, respectively. However, these compounds were poorly resolved when an aqueous extract of *S. rebaudiana* leaves was applied to the column. Therefore, attempts were made to purify the extract prior to HPLC. Extraction of the plant material with 1-butanol produced a simpler extract, but was found to be nonquantitative for the diterpene glycosides. Extraction of impurities from an aqueous extract with ethyl acetate or chloroform–methanol (9:1) prior to HPLC did not significantly improve resolution. Treatment of the aqueous extract with charcoal, calcium hydroxide, barium hydroxide, or lead acetate did remove impurities from the extract, but it resulted in the partial adsorption and/or precipitation of the diterpene glycosides as well.

Satisfactory resolution of SS, RC, and RA was finally achieved on two protein columns in series, after the plant material was extracted with chloroform (extract A) prior to methanol extraction (extract B). A typical HPLC chromatogram is shown in Fig. 1. TLC of extract A showed that no detectible quantitites of diterpene glycosides

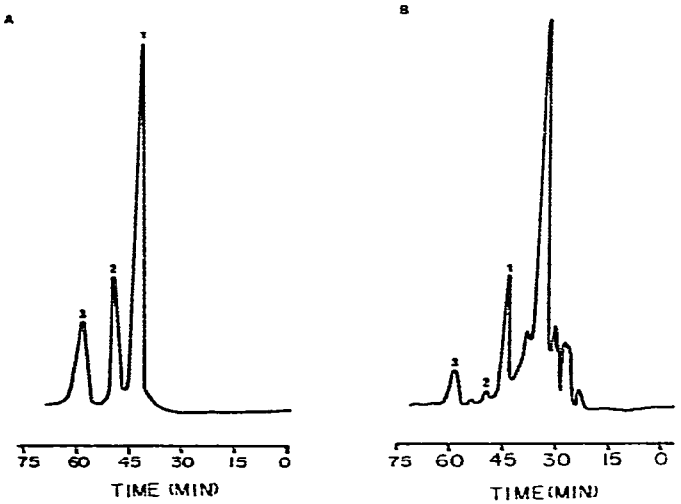

Fig. 1. Separation of stevioside, rebaudioside C, and rebaudioside A. A. Mixture of standards; B. *Stevia rebaudiana* aqueous extract (extract B). Operating conditions: two Protein I-125 columns in series; mobile phase, 1-propanol; flow-rate, 1 ml/min; detector, UV spectrophotometer (210 nm). Peaks: 1 = stevioside (SS); 2 = rebaudioside C (RC); 3 = rebaudioside A (RA).

were removed by this purification step. In addition, an aqueous extract (extract C) of the plant marc after methanol extraction was devoid of diterpene glycosides. Since these glycosides are more soluble in water than in organic solvents, these results indicate that SS, RA, and RC were quantitatively extracted by methanol in this procedure.

Under the HPLC conditions finally used in this study, SS, RC, and RA gave retention times of 43.2, 49.5, and 59.0 min, respectively, and linear detection responses when concentrations of 5–100 μg were employed. Beer's law standard curves gave slopes of 1.68 (SS), 1.22 (RC), and 0.91 (RA); *y* axis (peak height) intercepts of −1.16 (SS), +0.52 (RC), and +0.35 (RA); and correlation coefficients of 0.999 (SS, RC, and RA) by linear regression analysis. The minimum detectible quantities of SS, RC, and RA were 1.0, 2.0, and 2.0 μg, respectively. TLC analysis of SS, RC, and RA separated from extract B by this HPLC column showed a single spot for each compound (R_F = 0.30, 0.24, and 0.18, respectively) which co-chromatographed with the corresponding standard.

An analysis of extract D showed that the published procedure of Hashimoto *et al.*[5] removed fewer impurities from the plant. However, comparison with extract B showed that the diterpene glycosides were not quantitatively extracted by their procedure, yielding only 52.78 ± 0.13% of the extractible SS, 43.32 ± 0.08% RC, and 48.61 ± 0.04% RA. These low yields probably result from the small volume of water used for plant extraction.

The data demonstrate that SS, RC, and RA can be quantitatively extracted from *S. rebaudiana* and rapidly and accurately analyzed by HPLC with the procedures described in this communication. They also demonstrate the versatility of the Protein I-125 column in separating water-soluble compounds other than proteins.

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