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STEVIA REBAUDIANA

I. USE OF *p*-BROMOPHENACYL BROMIDE TO ENHANCE ULTRAVIOLET DETECTION OF WATER-SOLUBLE ORGANIC ACIDS (STEVIOLBIOSIDE AND REBAUDIOSIDE B) IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

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SUMMARY

A method is described for the analysis of stevioside and rebaudioside A from *Stevia rebaudiana* Bert. These compounds were hydrolyzed to steviolbioside and rebaudioside B, respectively, and reacted with *p*-bromophenacyl bromide and crown reagent to yield the corresponding chromophoric esters, which were separated and quantitated by high-performance liquid chromatography. More generally, a method is described for maximizing ester yields from water-soluble organic acids by using methanol as the reaction solvent and adjusting reaction pH and time.

INTRODUCTION

The leaves of *Stevia rebaudiana* Bert. (Compositae) have been reported to contain the sweet-tasting diterpene glycosides steviolbioside, stevioside, rebaudiosides A–E and dulcoside A (Fig. 1), with stevioside and rebaudioside A being the major constituents[1–9]. Since removal of saccharin from the U.S. market by the Food and Drug Administration is apparently inevitable[10], the latter two compounds are attractive potential non-caloric sweetening agents.

When this study commenced, the published assay procedures for stevioside and rebaudioside A were restricted to the use of thin-layer chromatography (TLC) or gas–liquid chromatography[11–13]. The latter technique requires the enzymatic hydrolysis of diterpene glycosides to steviol (Fig. 1), or acid hydrolysis and methylation of diterpene glycosides to isosteviol (Fig. 1) methyl ester. A more satisfactory high-performance liquid chromatographic (HPLC) separation of stevioside and rebaudioside A was therefore sought.

In the present work, mild alkaline hydrolyses of stevioside and rebaudioside A

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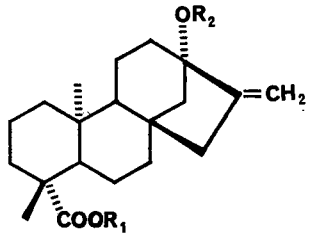

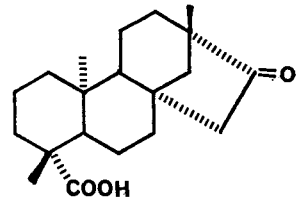

ISOSTEVIOL

COMPOUND	R_1	R_2	COMPOUND	R_1	R_2
Steviol	-H	-H	Rebaudioside C (Dulcoside B)	-G	$-G^2_3{}^1Rh$, ^1G
Steviolbioside	-H	$-G^2{}^1G$			
Stevioside	-G	$-G^2{}^1G$	Rebaudioside D	$G^2{}^1G$	$-G^2_3{}^1G$, ^1G
Rebaudioside A	-G	$-G^2_3{}^1G$, ^1G	Rebaudioside E	$G^2{}^1G$	$-G^2{}^1G$
Rebaudioside B	-H	$-G^2_3{}^1G$, ^1G	Dulcoside A	-G	$-G^2{}^1Rh$

G = glucose

Rh = rhamnose

Fig. 1. *Stevia rebaudiana* diterpene glycosides and their hydrolysis products.

yield the corresponding acids, steviolbioside and rebaudioside B (Fig. 1), respectively[1]. The phenacyl esters of these acids were then prepared in order to enhance their ultraviolet (UV) detection following HPLC. Phenacylation has previously been used successfully to esterify fatty acids[14], C-4 carboxylic acids[15], prostaglandins[16] and penicillins[17]. Purified *p*-bromophenacyl bromide (PBPB) and 18-crown-6-(1,4,7,10, 13,16-hexaoxacyclooctadecane) (crown reagent) are commercially available and reasonably inexpensive[18]. The recommended procedure for esterification of acids involves dissolution of the acid in methanol and titration to the phenolphthalein endpoint with methanolic potassium hydroxide. Following evaporation of methanol *in vacuo*, PBPB and crown reagent in acetonitrile are added. The solution is heated at 80° for 15 min and the resulting reaction mixture is chromatographed directly[18].

Although several workers have stated that small quantities of water or methanol do not adversely affect the reaction[14,15], the derivatization method was generally found to be unsuccessful for organic acids which are insoluble in acetonitrile. This communication describes the first successful esterification of water-soluble organic acids with PBPB and crown reagent using methanol as the reaction solvent. The HPLC separation of the *p*-bromophenacyl esters of steviolbioside and rebaudioside B from a crude extract of *S. rebaudiana* is also reported.

EXPERIMENTAL

Plant material

Stevia rebaudiana Bert. seeds were provided by Stevia, Inc. (Arlington Heights, Ill., U.S.A.) and grown at the University of Illinois Pharmacognosy and Horticulture Field Station, Lisle, Ill., U.S.A. Plants were authenticated by Mr. Floyd A. Swink of the Morton Arboretum, Lisle, Ill., 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, Ill., U.S.A.

Apparatus

UV and infrared (IR) spectra were recorded using a Beckman (Beckman Instruments, Irvine, Calif., U.S.A.) Model DB-G grating spectrophotometer and a Beckman IR-18A spectrophotometer with polystyrene calibration at 1601 cm^{-1} (KBr pellet), respectively.

Liquid chromatographic separations were conducted using a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A liquid chromatograph equipped with a Rheodyne (Berkeley, Calif., U.S.A.) Model 7120 syringe-loading sample injector and 20-μl sample loop, a Waters Assoc. Model 450 variable-wavelength UV spectrophotometer, and a Texas Instruments (Houston, Texas, U.S.A.) Servo/Riter II portable recorder. Separations were carried out using a Waters Assoc. 30 × 0.4 cm I.D. μBondapak C_{18} column.

For TLC analyses, glass-backed silica gel GF_{254} plates (20 × 20 cm, 0.25 mm thick) were obtained from E. Merck (Darmstadt, G.F.R.). A Chromaflex TLC streaker (Kontes of Illinois, Evanston, Ill., U.S.A.) was used to apply mixtures to TLC plates for preparative separations.

Chemicals

All chemicals and solvents used in this investigation were reagent grade. Solvents for HPLC were redistilled in glass.

PBPB and crown catalyst were obtained from Regis (Morton Grove, Ill., U.S.A.).

Stevioside and rebaudioside A were extracted from the defatted leaves of *Stevia rebaudiana* using methanol. The concentrated methanol extract was chromatographed over a column of silica gel 60 PF_{254} (E. Merck) using chloroform–methanol–water (45:9:1) as the initial solvent system, followed by chloroform–methanol–water (45:15:2) (ref. 7). Stevioside and rebaudioside A, following elution from the column and recrystallization twice from warm methanol, were found to be identical (mixture m.p., IR, TLC) to authentic standards.

Stevioside and rebaudioside A were hydrolyzed to steviolbioside and rebaudioside B, respectively, by refluxing with 10% aqueous potassium hydroxide at 100° for 1 h. Following neutralization with glacial acetic acid, the precipiated compounds were each recrystallized twice from methanol, and were found to exhibit the same mp and TLC behavior as that reported in the literature[1,7,13].

Esterification of steviolbioside and rebaudioside B

Method A. 4 μmoles of the appropriate acid in methanol were neutralized to

the phenolphthalein end-point with 0.01 % methanolic potassium hydroxide. Following evaporation to dryness, the residue was redissolved in 2 ml of solvent (see below), 5 μmoles of PBPB and 0.25 μmole of crown reagent (each dissolved in acetonitrile) were added, and the mixture was refluxed at 80° for 30 min. The reaction solvents used were: A_1, acetonitrile; A_2, dioxane; A_3, dimethylsulfoxide; A_4, pyridine; A_5, acetonitrile–water (3:2); A_6, acetonitrile–water (1:1); A_7, acetonitrile–methanol–water (5:2:3); A_8, acetonitrile containing 1.0 % Tween 80; and A_9, methanol.

Method B. The "pH" of a solution containing 4 μmoles of the appropriate acid in 2 ml of methanol was adjusted to the indicated value using 0.01 % methanolic potassium hydroxide or 0.01 % methanolic hydrochloric acid and Alkacid short-range pH paper (Fisher Scientific, Itasca, Ill., U.S.A.). PBPB (5 μmoles) and crown reagent (0.5 μmole) were added and the mixture was refluxed at 65° for 3 h. The "pH" values used were: B_1, 5.0; B_2, 6.0; B_3, 7.0; B_4, 8.0; B_5, 8.5; B_6, 9.0; B_7, 9.5; B_8, 10.0; and B_9, 11.0. Aliquots were analyzed by TLC to determine ester yields.

Thin-layer chromatography

Reaction mixtures were spotted on silica gel GF_{254} plates and developed with chloroform–methanol (17:3). After development, chromatograms were air-dried, examined under short-wave UV light, sprayed with anisaldehyde–sulfuric acid reagent[19] and heated at 100° for 5 min to visualize steviolbioside, rebaudioside B and their *p*-bromophenacyl esters.

Preparation of steviolbioside and rebaudioside B p-bromophenacyl esters for HPLC

Quantities of the esters were prepared using method B_5 (pH 8.5) to esterify steviolbioside and rebaudioside B (10 mg of each). After refluxing for 3 h, the reaction mixtures were concentrated *in vacuo* and each was applied to six silica gel GF_{254} TLC plates. Following development with chloroform–methanol (17:3) and air-drying, ester zones were visualized under short-wave UV light and scraped from the TLC plates. Esters were eluted from the adsorbent with methanol, and each solution was filtered and evaporated to dryness *in vacuo*.

High-performance liquid chromatography

The operating conditions for HPLC were: ambient temperature; flow-rate of eluting solvent, acetonitrile–water (3:2), 0.5 ml/min; wavelength of UV detector, 258 nm; recorder chart speed, 6 in./h at 1.0 a.u.f.s. Standard solutions of the *p*-bromophenacyl esters of steviolbioside and rebaudioside B were injected onto the column and their retention times determined.

Beer's law standard curves were obtained by injecting different quantities (3.25, 7.5, 15.0 and 30 μg per 20-μl injection) of each ester onto the column in triplicate and measuring the resulting peak heights.

Hydrolysis and esterification of Stevia rebaudiana extracts

Powdered leaves of *S. rebaudiana* (1.0 g) were defatted by maceration twice with petroleum ether (30 ml). The marc was boiled with distilled water (25 ml), filtered and re-extracted with distilled water (25 ml) twice. The combined filtrates were diluted to 100 ml with distilled water. An aliquot (10 ml) of this filtrate was extracted twice with chloroform (5 ml each) and the chloroform extract discarded. 10 % aqueous

potassium hydroxide (5 ml) was added to the aqueous extract, and the mixture was refluxed on a steam bath for 1.5 h. The reaction mixture was rendered acidic (pH 5) by the addition of glacial acetic acid, and extracted three times with chloroform–methanol (2:1) (6 ml each). The combined organic extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in methanol (10 ml) and 0.01% methanolic potassium hydroxide (to pH 8.5), PBPB (5 mg in 2 ml of methanol) and crown reagent (2.25 μmoles) were added. The reaction mixture was refluxed on a water bath at 65° for 1 h and evaporated to dryness *in vacuo* to yield a residue which was dissolved in methanol for HPLC analysis.

RESULTS AND DISCUSSION

No detectable quantities of either ester were obtained with methods A_1 through A_4. Methods A_5 through A_8 produced partial esterification (approximately 10–20% yields for each ester) as determined by the partial disappearance of starting material and appearance of new spots on thin-layer chromatograms. The R_F values for rebaudioside B (RB), steviolbioside (SL), rebaudioside B *p*-bromophenacyl ester (RBPE) and steviolbioside *p*-bromophenacyl ester (SLPE) were 0.11, 0.31, 0.53 and 0.66, respectively. When methanol (method A_9) was used as the reaction solvent, yields of approximately 40% were achieved for both esters after heating for 2 h. Increasing the reaction time to 4 h did not increase the yields for any of these methods. It appears that the low yields produced by methods A_1 through A_8 were caused by the limited solubility of RB and SL in the reaction solvents. Therefore, methanol was selected as the reaction solvent, and the pH of the reaction mixture was varied. Methods B_1, B_2 and B_9 produced no detectable esterification of either RB or SL after 3 h of heating. Method B_3 (pH 7.0), B_4 (pH 8.0), B_5 (pH 8.5), B_6 (pH 9.0), B_7 (pH 9.5) and B_8 (pH 10.0) all produced partial esterification, giving approximate yields of 60, 80, 90, 80, 60 and 25%, respectively, of RBPE and SLPE as determined by TLC. The reduced yields of esters at more alkaline pH probably result from partial hydrolysis of the esters after they are formed.

Under the conditions used for HPLC in this study, RBPE and SLPE were well separated, giving retention times of 8.6 and 10.6 min, respectively. Quantitation was also readily achieved by HPLC since the detector response was linear for both RBPE and SLPE in concentrations of 4–30 μg. For the RBPE standard curve, the slope was 1.32, the *y* axis (peak height) intercept was +4.34, and the reliability was 0.994. The standard curve for SLPE gave a slope of 3.31, a *y* axis intercept of +3.41, and a reliability of 0.998. Minimum and maximum amounts amenable to accurate quantitation were 1 and 155 μg for RBPE and 1 and 62 μg for SLPE, respectively. It should be noted that stevioside, SL, RB and rebaudioside A cannot be detected under these conditions.

After a suitable HPLC separation of RBPE and SLPE from other components of the reaction mixture was developed (Fig. 2A), the effects of reaction pH and reaction time were more accurately determined using HPLC. The pH and reaction time profiles for SLPE are shown in Figs. 3 and 4. These data show that the maximum yield is obtained at pH 8.5 using a reaction time of 1 h (92.7 $\pm$1.7% yield). The paucity of rebaudioside A isolated from the plant precluded additional work with RBPE. However, the preliminary TLC results showed that a maximum yield was also obtained for RBPE under these same conditions.

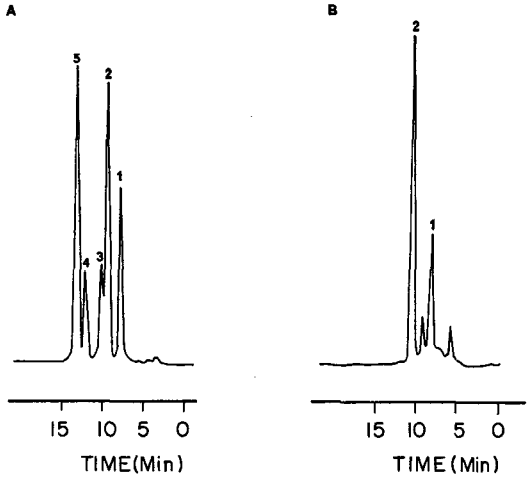

Fig. 2. Separation of rebaudioside B and steviolbioside *p*-bromophenacyl esters. A = reaction mixture containing a ten-fold excess of PBPB; B = hydrolyzed, esterified *Stevia rebaudiana* extract. Operating conditions: column, μBondapak C_{18}; mobile phase, acetonitrile–water (3:2); flow-rate, 0.5 ml/min; detector, UV spectrophotometer (258 nm). Peaks: 1 = rebaudioside B *p*-bromophenacyl ester (RBPE); 2 = steviolbioside *p*-bromophenacyl ester (SLPE); 3 = *p*-bromophenacyl alcohol; 4 = PBPB impurity; 5 = PBPB.

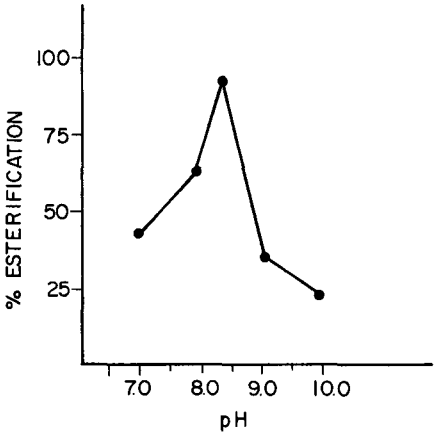

Fig. 3. The effects of pH on yields of steviolbioside *p*-bromophenacyl ester (SLPE) as determined by HPLC assay.

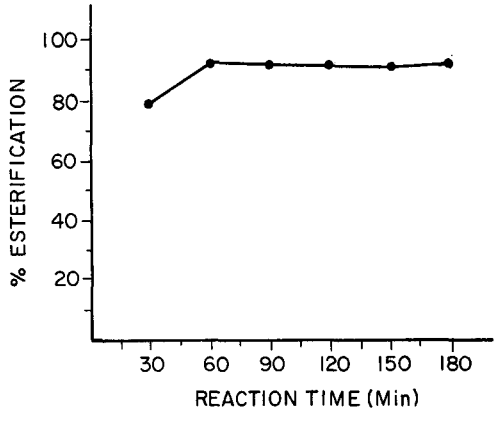

Fig. 4. The effects of reaction time on yields of steviolbioside *p*-bromophenacyl ester (SLPE) as determined by HPLC assay.

The reported HPLC conditions also produced reasonably good resolution of RBPE and SLPE from other compounds present in the hydrolyzed and esterified aqueous extract of *S. rebaudiana*, as shown in Fig. 2B. A major disadvantage of the method is that rebaudiosides D and E would be converted to RBPE and SLPE, respectively, during hydrolysis and esterification, and would therefore be quantitated as the latter compounds. In view of the reported separation of stevioside and rebaudio-

side A on a Shodex OHpak M-414 column[20], a simpler procedure to obviate this problem was developed, and will be reported at a later date. Nevertheless, the procedures described herein have potentially more general utility. The results for RBPE and SLPE suggest that preparation of phenacyl esters of water-soluble organic acids using the phenolphthalein end-point as the reaction pH and acetonitrile or acetonitrile–methanol as the reaction solvent may produce extremely poor yields. However, the use of methanol as the reaction solvent (when the potassium salt of the acid is soluble) and appropriate adjustment of reaction pH and time may maximize ester yield.

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