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Identification of Steviol Glucuronide in Human Urine

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Stevioside (250 mg capsules) was given three times daily to 10 healthy subjects. Steviol glucuronide (steviol 19-O-β-D-glucopyranosiduronic acid; MM, 494.58; melting point, 198−199 °C) was characterized in the 24 h urine as the only excretion product of oral stevioside by MS, NMR, IR, and UV spectroscopy. This is the first report on the unambiguous identification of steviol glucuronide in human urine.

KEYWORDS: ***Stevia rebaudiana*** **(Bertoni) Bertoni (Asteraceae); stevioside degradation; steviol; steviol glucuronide**

INTRODUCTION

The natural sweetener stevioside is a diterpene glycoside extracted from the plant *Stevia rebaudiana* (Bertoni) Bertoni, which belongs to the Asteraceae family and is native to Brazil and Paraguay. Stevioside tastes about 300 times sweeter than 0.4 M sucrose and is noncaloric. In some parts of the world, including Japan, South Korea, Israel, Mexico, Paraguay, Brazil, Argentina, and Switzerland, stevioside is used to sweeten food products and beverages. In the United States, powdered *Stevia* leaves and refined extracts from the leaves have been used as a dietary supplement since 1995 (*1*). Recently, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) accepted a temporary allowable daily intake (ADI) of 0−2 mg steviol equivalents/kg body weight (BW) (*2*). However, the U.S. Food and Drug Administration (FDA), European Food Safety Agency (EFSA) (European Union), and Food Standards Australia and New Zealand do not accept this temporary ADI.

Bacteria isolated from the human colon are able to effect in vitro transformation of stevioside into steviol (*3*−*5*). In vivo degradation of stevioside into steviol occurs by bacterial action in the colon of pigs (*6*) and humans (*7*). Among the selected intestinal groups, bacteroidaceae were the most effective in hydrolyzing *Stevia* sweeteners to steviol (*5*). In the colon of pigs and humans, oral stevioside was completely degraded into steviol, which was the only metabolite found in the faeces (*6*−*8*). We report the identification of steviol glucuronide (steviol 19-O-β-D-glucopyranosiduronic acid) as an urinary excretion product after oral intake of stevioside by human volunteers.

MATERIALS AND METHODS

Chemicals. A commercial mixture of steviol glycosides was crystallized repeatedly from MeOH affording stevioside [19-O-β-D-glucopyranosyl-13-O(β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-steviol] in over 97% purity; impurities were 2.8% steviolbioside and a trace of rebaudioside A (0.2%). Steviol was made according to ref *9* and repeatedly crystallized from MeOH to more than 99% purity. All high-performance liquid chromatography (HPLC) grade solvents (H_2O, acetonitrile, $CHCl_3$, MeOH, EtOH, and *N*,*N*-dimethylformamide) were obtained from Acros Organics (Geel, Belgium), and acetone was obtained from Biosolve (Valkenswaard, The Netherlands). Triethylamine was from Acros, and 4-(bromomethyl)-7-methocoumarin [IUPAC name: 4-(bromomethyl)-7-methoxy-2*H*-chromen-2-one] was from Fluka (VWR, Haasrode, Belgium). β-Glucuronidase/sulfatase type H-2 from *Helix pomatia* digestive juice was from Sigma (Bornem, Belgium).

Subjects. To be included in this study, subjects had to be between 21 and 29 years old, be healthy as assessed by a medical questionnaire, and have visible veins facilitating blood take, and the women could not be pregnant, as determined from a pregnancy test at the beginning of the investigation. Persons using medications known to affect the blood pressure and those with diabetes were excluded (this was assessed by determining glucose in a urine sample and by a medical questionnaire). Before the start of the study, the weights and heights of the subjects were measured. The Medical Ethical Committee of the University Hospital Gasthuisberg Leuven approved the study protocol. After the purpose of this study was explained to the volunteers, all gave written informed consent to the protocol.

Ten healthy volunteers, female (five) and male (five), participated in the study. The volunteers were all between 21 and 29 years old. On average, the women were 23 ± 1 years old, 172 ± 5 cm in height, and weighed 65 ± 7 kg. On average, the men were 26 ± 2 years old, 175 ± 5 cm in height, and weighed 74 ± 11 kg. The body mass index

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(BMI) averaged 23.1 ± 0.9 kg/m^2. No power calculation was performed before the start of the study.

Study Design. After the selection procedure, the volunteers collected a 24 h control urine. Then, capsules with 250 mg of stevioside were given to the subjects three times a day with 8 h intervals for a period of 3 days. The volunteers were instructed to take the capsules with a glass of water. On the third day, the volunteers were asked to collect a 24 h urine sample (stevioside urine). The calorie intake between subjects was not considered, nor was their physical activity, as this was beyond the aim of this metabolism study.

From the 24 h control and stevioside urine, 20 mL was taken for the detection of different markers. Creatinine was determined using the Jaffé method (*10*). Sodium and potassium were detected by indirect potentiometric determination by means of ion selective electrodes (Roche/Hitachi Modular analyzers; Roche Diagnostics Belgium, Vilvoorde, Belgium). For the calcium determination, a complexometric method was used, based on the reaction of calcium with *o*-cresolphthalein complex in alkaline solution (*11*). For the analysis of urea, a kinetic UV assay was applied based on the coupled urease/glutamate dehydrogenase enzyme system (*12*).

Urine Fractionation. The total 24 h urine fraction (between 1124 and 2494 mL) was run over an Amberlite XAD-2 column at about 30 mL/min. The column bed volume was 200 mL, which is sufficient for the adsorption of all amphipathic molecules in a 24 h urine (Geuns et al., unpublished). The columns were then rinsed with 1 L of distilled water and then eluted with 400 mL of MeOH:acetone (50:50, v/v). The eluate was divided into four equal fractions, and the solvent was evaporated at reduced pressure at 50 °C.

Derivatization of Steviol-Containing Fractions. Fractions containing steviol and/or dihydroisosteviol used as an internal standard (IS) were completely dried; the residues were taken up in dry acetone and derivatized to form the 7-methoxy-coumarinyl esters as described (*13*). After derivatization, the samples were purified by thin-layer chromatography (TLC) using $CHCl_3$:MeOH (98:2) as the eluent. The blue fluorescent TLC band (UV 366 nm) corresponding with the ester derivative of steviol was scraped off and eluted with $CHCl_3$:MeOH (80:20). After evaporation of the solvent, the residue was dissolved in a known amount of MeOH and HPLC was done using a fluorescence detector (λ_{exc}, 321 nm; λ_{em}, 391 nm) (*13*). The identity of the steviol 7-methoxy coumarinyl ester was checked by MS.

Analysis of Bound Steviol. In preliminary experiments, steviol possibly bound as glucuronide and sulfate conjugates was obtained after hydrolysis by β-glucuronidase/sulfatase from *H. pomatia* digestive juice. Urine fractions were dissolved in 10 mL of MeOH:acetone (50:50) and dihydroisosteviol (200 μg) (IS) was added to 250 μL of the solution. The samples were then evaporated, and the residues were dissolved into 500 μL of acetate buffer, pH 5. Then, 50 μL of β-glucuronidase/sulfatase (5000 U/375 U) was added. The mixture was incubated for 6 h at 37 °C. Under these conditions, a complete conversion to steviol occurred. After hydrolysis, the samples were purified on C18 Extract Clean Columns (500 mg, Alltech, Belgium) that were conditioned before use with 3 mL of MeOH followed by 3 mL of H_2O. After application of the enzyme mixtures, the columns were rinsed with 3 mL of water and 3 mL of 50% MeOH. The steviol and IS were eluted with 5 mL of MeOH. A 250 μL amount of the latter fraction was evaporated under a stream of nitrogen at 50 °C. The completely dried residue was then derivatized as described above.

Isolation of Larger Amounts of Steviol Glucuronide. A column (35 mL bed volume) was prepared with 50 g of silica gel for column chromatography (Machery-Nagel Silica gel 60, 0.063−0.2 mm) suspended in ethyl acetate. About 600 mg of urine residue isolated from the Amberlite XAD-2 purification step (see above) was dissolved in 1 mL of MeOH, and this solution was adsorbed to 1 g of silica gel by evaporating the solvent under a gentle flow of N_2 and under continuous mixing. The silica gel-bound sample was applied onto the top of the column, which was eluted with a solvent mixture of ethyl acetate:ethanol:water (80:30:20). Fractions of 5 mL were collected. Samples of each fraction were analyzed by TLC using ethyl acetate:ethanol:water (80:30:20) as the solvent and compared with steviol glucuronide as a reference compound. In addition, each fraction was tested for the occurrence of steviol glucuronide and/or sulfate conjugates. To this end, 20 μL samples of each fraction were evaporated in an Eppendorf tube. Then, 150 μL of acetate buffer (pH 5) was added, followed by 20 μL of β-glucuronidase/sulfatase (2000 U/150 U). After enzymatic reaction for 15 h at 37 °C, the reaction mixture was freeze-dried and the residue was derivatized by reaction with 4-(bromomethyl)-7-methocoumarin (see above). All fractions containing enzyme-sensitive steviol conjugates eluted as a single large peak from the silica gel column, indicating the presence of only one steviol conjugate in the urine samples. Therefore, all of the fractions containing this steviol conjugate were pooled and the solvent was evaporated. The residue was subjected to preparative TLC, and about 10 mg of white crystalline material was isolated following elution of the TLC band with MeOH.

Table 1. Determinants of Tissue Damage before and after Stevioside Administration[a]

		stevioside		
	before	after 250 mg of stevioside with water		
plasma (U/L)	0 h	1 h	3 h	7 h
alkaline phosphatase	79 ± 9	80 ± 7.3	81 ± 7	87 ± 7
ALT/GPT[b]	14 ± 2.6	11 ± 3	10 ± 3.66	12 ± 3.33
creatine kinase	101 ± 31	74 ± 14.6	74 ± 13.6	76 ± 15.3
lactate dehydrogenase	184 ± 41.6	131 ± 27.3	132 ± 32.6	81 ± 23.6

[a] Blood was taken on the third day of the experiment. Values are means ± SEM (n = 9). [b] ALT/GPT, alanine aminotransferase or glutamic pyruvate transaminase.

Melting Point. The melting point (uncorrected) was measured on a Thermovar 9200 (type 300429) apparatus of Reichert-Jung (VWR).

Spectroscopic Techniques. *IR.* The IR spectrum was recorded in a KBr pellet (FTIR 1600 series of Perkin-Elmer, Zaventem, Belgium).

MS. Mass spectral analysis of steviol glucuronide was carried out using the LCQ Advantage ion trap mass spectrometer of Thermo Finnigan (San Jose, CA) in both positive ESI (+) and negative ESI (−) electrospray ionization modes. MS/MS spectra were generated by CID (collision-induced decomposition) of the $[M + 2Na - H]^+$ adduct ion formed by ESI (+) and of the $[M - H]^-$ molecular ion obtained by ESI (−).

NMR. ^{1}H and ^{13}C NMR spectra of steviol glucuronide were recorded on a Bruker AMX 400 spectrometer equipped with an inverse ^{1}H multinuclei probe and operating at 400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C measurements (Bruker, Rheinstetten, Germany). The spectra were run in CD_3OD as a solvent. The chemical shifts are reported in ppm vs TMS (tetramethylsilane) as an internal reference.

In the ^{13}C spectra, the septet of the CD_3OD signal was used as an internal reference and placed at δ 49 vs TMS. Besides the fully decoupled ^{13}C spectrum, the DEPT 135 pulse sequence was used to differentiate between C, CH, CH_2, and CH_3 signals.

Statistical Analysis. Data were analyzed using the general linear models procedure of SAS (SAS software; version 8.1; SAS Institute, Inc., Cary, NC) to test differences between stevioside and control groups with repeated measurements for the different time points. Results are expressed as means ± standard errors of the mean (SEM). Differences were considered statistically significant when the p value was less than or equal to 0.05.

RESULTS

The volume of 24 h urine samples averaged 36% higher after the stevioside intake (1561 ± 489 mL) as compared to the control condition (1150 ± 488 mL). However, this difference did not reach statistical significance (p = 0.06) because of the large interindividual variations. No significant differences were detected in electrolytes excreted in the 24 h urine (**Table 1**). Markers of tissue damage did not significantly differ between the control and the stevioside treatment (**Table 1**).

Following metabolic conversion of ingested stevioside, steviol glucuronide was isolated as the only metabolite detected in the

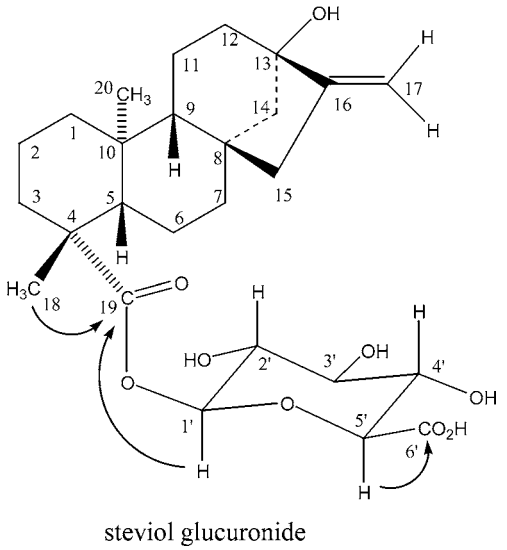

Figure 1. Structure of steviol glucuronide and important HMBC interactions.

collected urine. The purified, crystalline material had a melting point of 198−199 °C (uncorrected). The UV spectrum showed a maximum absorbance at 208 nm (methylene). The IR spectrum displays bands corresponding to the hydroxyl functions at 3416 (broad) and 1057 (broad) cm^{-1}, to the ester carbonyl group at 1725 cm^{-1}, and to the carboxylic acid at 1616 cm^{-1}.

ESI spectra were run in both positive ESI (+) and negative ESI (−) modes. In the ESI (+) spectrum, the disodium adduct ion $[M + 2Na - H]^+$ was the main peak observed at *m*/*z* 539. Further MS/MS analysis of this adduct ion revealed cleavage of the glycosidic ester linkage to form both the $[C(19)O_2Na_2]^+$ disodium adduct ion of steviol (*m*/*z* 363) and the complementary disodium adduct of the glucuronic acid moiety (*m*/*z* 221).

The ESI (−) spectrum displayed the molecular ion $[M - H]^-$ at *m*/*z* 493 as the main ion species, corresponding to deprotonation of steviol glucuronide. Other interesting ions were due to ion−molecule associations providing the dimer and trimer ion species $[2M - H]^-$, $[2M + Na - 2H]^-$, and $[3M + 2Na - 3H]^-$, observed at *m*/*z* 987, 1009, and 1526, respectively. Separate MS/MS analysis of the $[M - H]^-$ ion at *m*/*z* 493 led to cleavage of the glycosidic bond of the ester conjugate to produce two complementary ions at *m*/*z* 317 and *m*/*z* 175, representing the $[C(19)O_2]^-$ carboxylate anion of steviol and that of the glucuronic acid part, respectively.

The 19-*O*-acyl ester glycoside structure (**Figure 1**) proposed for steviol glucuronide is fully confirmed by the ^1H and ^{13}C NMR spectra, in which relevant δ and *J* values can be discerned for the terpene and glucuronide moieties. The ^1H NMR spectrum reveals the two exocyclic vinylidene protons on C17 at δ 4.93 and 4.77 (obscured by the OH of methanol at 25 °C). The methylene and methine protons absorb as overlapping multiplets between δ 2.3 and 0.7. The C18 and C20 methyl groups display two sharp singlets at δ 1.24 and 0.97.

The anomeric proton of the glucuronide appears as a downfield doublet at δ 5.46 ($^3J_{H1'-H2'} = 7.8$ Hz); this chemical shift value is consistent with an 19-*O*-acyl glycosidic ester linkage while the vicinal coupling indicates diaxial coupling of H1′ and H2′. Proton H5′ is observed as a doublet at δ 3.68 ($^3J_{H5'-H4'} = 9$ Hz); the signals for H2′, H3′, and H4′ appear as a multiplet (3 H) between δ 3.5 and 3.35.

The ^{13}C NMR spectrum of steviol glucuronide displays the following absorptions for the terpene moiety: two methyls, nine methylenes, two methines, four quaternary carbon atoms, two vinylic carbons for the exocyclic C16−C17 double bond, and an ester carbonyl carbon for C19. Two-dimensional (2D) ^1H, ^{13}C correlation spectroscopy via one bond $^1J_{CH}$ coupling (heteronuclear multiple quantum coherence, HMQC) or via two and three bonds coupling (heteronuclear multiple-bond correlation, HMBC) allowed unequivocal assignment of all C, CH, CH_3, and two CH_2 (C1′ and C3) carbon atoms. The other CH_2 signals were assigned by comparison with the values for free steviol reported (*14*). All ^{13}C absorptions found for the terpene moiety are fully comparable with those reported for steviol (**Table 2**).

Table 2. Assignment of Signals in the ^{13}C NMR Spectra of Steviol (*18*) and Steviol Glucuronide (This Work); δ Values All Relative to Internal $SiMe_4$

		steviol in $CDCl_3$ (*18*)	steviol glucuronide in CD_3OD (this work)	
		^{13}C	^{13}C	^1H correlation
CH_3	C18	28.8	29.0	1.24 (s)
	C20	15.4	16.4	0.97 (s)
CH_2	C1	40.5	41.9	1.89 and 0.85
	C2	19.0	20.2	1.42
	C3	37.8	39.1	2.21 and 1.04
	C6	21.8	22.9	1.87
	C7	41.2	42.8	1.54 and 1.43
	C11	20.5	21.4	1.76
	C12	39.5	40.6	1.46
	C14	47.4	47.3	2.13 and 1.28
	C15	47.0	48.9	2.18 and 2.07
CH	C5	56.9	58.7	1.12
	C9	53.8	55.4	0.99
C	C4	43.6	45.1	
	C8	41.8	42.8	
	C10	39.5	40.7	
	C13	80.4	80.8	
sp^2 C	C16	155.7	157.1	
	C17	103.0	103.3	4.97 and 4.77 (br. s)
	C19	183.5	178.0	
glucuronide part of steviol glucuronide	CH1′		95.4	5.46 (d)
	CH5′		77.4	3.68 (d)
	CH2′, CH3′, CH4′		78.6, 73.9, 73.5	δ 3.5 to 3.35 (m)
	CO_2D		176.6 (br)	

For the glucuronide part, five absorptions in the ^{13}C spectrum are identified as CHO carbon atoms by their correlation with the corresponding protons via the 2D HMQC method. Finally, 2D HMBC correlated spectroscopy was applied to reveal diagnostic ^2J and $^3J_{CH}$ couplings. Thus, the ester glycoside linkage CO(19)→O(1′) clearly appears from two $^3J_{CH}$ correlations observed between C19 (δ 178) and (i) the C18 methyl group (δ 1.24) and (ii) the anomeric proton (δ 5.46). The free carboxyl group (COOD) of the glucuronide moiety at δ 176.6 is identified by its $^2J_{CH}$ correlation with the H5′ doublet at δ 3.68.

DISCUSSION

Because of its molecular size, the uptake of stevioside by the intestinal tract is expected to be extremely low, as suggested by experiments with everted gastrointestinal sacs of rats (*15*) and Caco-2 cell layers in which the uptake of stevioside was less than 0.16% (*6*). Moreover, stevioside is not degraded by the enzymes of the intestinal tract (*3*, *4*). However, stevioside is degraded by bacteria commonly found in the colon resulting in free steviol that is easily absorbed (*5*−*7*, *15*).

No free steviol was detected in urine. After enzymatic hydrolysis of urine extracts by β-glucuronidase/sulfatase, steviol was found as the only aglycone present. There was no indication for the occurrence of, e.g., steviol sulfates. From in vitro

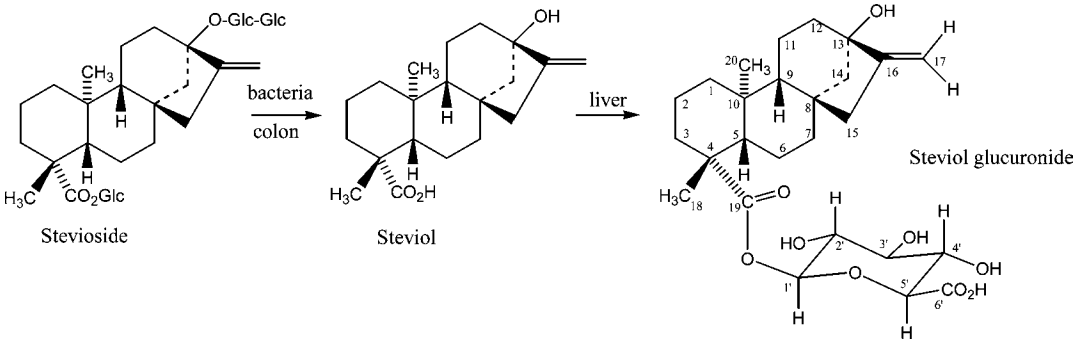

Figure 2. Hypothetical route from dietary stevioside to steviol glucuronide in human urine.

incubations of steviol with human liver microsomes, it was concluded that the transformation of steviol by human microsomes was very low and about four times lower than that by rat microsomes (*4*). As no other metabolites were found, the following excretion route is suggested (**Figure 2**). After degradation of stevioside to steviol by bacteria of the colon, part of the steviol is absorbed by the colon and transported to the liver by portal blood. In the liver, steviol glucuronide is formed, which is released into the blood and filtered out by the kidneys into the urine. Of the daily dose of 750 mg of stevioside, 300 mg of free steviol is formed in the colon (complete degradation). About 23 ± 2.7 mg of free steviol is directly excreted in the feces. About 277 mg of free steviol is taken up by the colon, of which about 101.8 ± 16.4 mg are recovered in the blood as steviol glucuronide and about 101.8 ± 21.3 mg are are excreted into the urine, also as steviol glucuronide. The total recovery of steviol is 226.6 ± 23.5 mg (75.5%). This is a recovery similar to the methodological recovery. The steviol glucuronide present in the blood is expected to be excreted in the urine during the next 24 h. The results suggest that there is no accumulation of steviol derivatives in the human body. In a metabolism study with volunteers using liquid chromatography−mass spectrometry, similar results were found, i.e., degradation of stevioside to steviol in the colon and excretion of a compound with the same molecular weight as steviol glucuronide (*7*). No other metabolites of steviol were detected in urine. However, no positive identification of the steviol glucuronide was done. It is known that glucuronide formation easily happens in the liver as is also the case with soy isoflavones that, after uptake, were metabolized to compounds, which were hydrolyzable with a combined β-glucuronidase and sulfatase enzyme preparation (*16*).

ABBREVIATIONS USED

ADI, allowable daily intake; BMI, body mass index; BW, body weight; EFSA, European Food Safety Agency (Europe); FDA, Food and Drug Administration (United States); HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple-bond correlation.

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Supporting Information Available: Figures of the IR spectrum, the ESI-MS, and the ^{1}H NMR spectrum. This material is available free of charge via the Internet at http://pubs.acs.org.

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