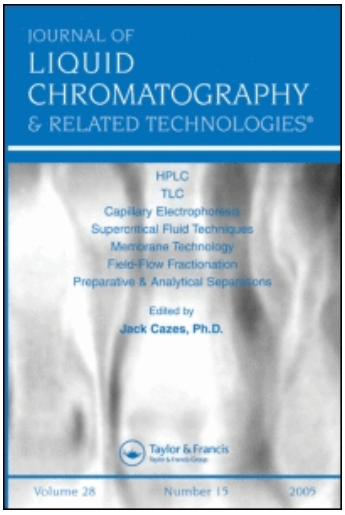

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ABSTRACT

A method of analysis using high performance liquid chromatography (HPLC) was developed for the separation and quantitation of the metabolites of stevioside: steviol-16,17α-epoxide, 15α-hydroxysteviol, steviolbioside, isosteviol, and steviol. The separation was carried out on a reversed-phase C18 Nova-Pack column with gradient elution of acetonitrile/water mixture. The applicability of the method was demonstrated in the detection and separation of stevioside and its metabolites found in blood, feces, and urine of hamsters after ingestion of stevioside.

INTRODUCTION

Stevioside, a major sweet diterpene glycoside of *Stevia rebaudiana*, has attracted much attention as a sweetener alternative.[1] It is now commonly used as a natural sweetener in beverages and foods,[2,3] being preferred over other non-caloric sucrose substitutes as it is heat-stable, somewhat resistant to acid hydrolysis, and non-fermentable.[4]

Several procedures for the assay of stevioside and other diterpene glycosides of Stevia have been reported including gas-liquid chromatography,[5] thin-layer chromatography,[6] and "thinchrography,"[7] a combination of rod-type thin-layer chromatography with flame ionization detection. A number of high performance liquid chromatography (HPLC) methods have been applied to plant material[8,9] and to food and beverage products[10,11] containing these sweet glycosides. A capillary electrophoresis (CE) method[12] has also been reported.

There are three possible metabolites of stevioside, namely, steviolbioside, 19-O-β-D-glucopyranosyl steviol, and steviol. Compadre et al.[13] reported that there are 22 possible derivatives of steviol and of these, only 15 have been studied by mass spectral analysis. We have developed a method to separate stevioside and its metabolites, namely, steviol-16,17α-epoxide, 15α-hydroxy-steviol, steviolbioside, isosteviol, and steviol.

The applicability of this method was demonstrated in the analysis for the presence of these metabolites in blood, urine, and feces of hamsters following force-feeding (gavage) with stevioside.

EXPERIMENTAL

Standard Compounds

Stevioside was extracted and purified from dried *S. rebaudiana* leaves as previously described.[14] Steviol, isosteviol, and steviolbioside were obtained from stevioside by oxidation,[15] acid hydrolysis,[16] and alkaline hydrolysis,[17] respectively. The 15α-hydroxysteviol and steviol-16,17α-epoxide were obtained from steviol by oxidation[18] and epoxidation.[16]

The molecular structures of all these standard compounds were confirmed by infrared spectral study, melting point determination, and elemental analysis. Their purities were tested by analytical HPLC prior to use in the present study.

Solvents

Acetonitrile used in this study was of HPLC grade and was purchased from Mallinckrodt Specialty Chemicals Co., Paris, KY, USA. Water was also of HPLC grade (18 MΩ-cm) filtered with a Millipore water purification unit. The solvents were degassed by sonication (Sonicor Instrument Co., Copiague, NY, USA) under reduced pressure.

Apparatus

The separations were carried out on a HPLC system from Waters Corporation (MA, USA). It consisted of two model 510 pumps, model 712 Waters autosampler, and a tunable absorbance detector. A Maxima chromatography software, together with a System Interface Module, were used for data acquisition and for controlling the pumps and autosampler.

Chromatographic Conditions

The column was a Waters reversed-phase C18 Nova-Pak (3.9 x 300 mm) column maintained at room temperature (ca. 25°C). A linear gradient was employed with conditions as follows: initial 70% water, 30% acetonitrile, 20 min, 35% water, 65% acetonitrile; 20.1 min 70% water, 30% acetonitrile and held for 9.9 min. The run time (injection to injection) was 30 min. The flow rate was 1.0 mL/min and absorbance monitored at 210 nm.

Standard Curve of Standard Compounds

Stock solutions of the standard compounds were prepared in 50% acetonitrile/water. A series of solutions containing 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 μg/100 μL of each standard was injected into the HPLC system. The injection for each concentration was done in triplicate. The areas of the peaks of each standard were used to make the respective standard curves. The detection limit of each standard was obtained from triplicate injections of the following amounts: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 μg per injection. The injection of each concentration was also done in triplicate.

Preparation of Samples

After 18 hours of fasting, hamsters were force-fed (gavage) with stevioside at a dose of 1 g/kg body weight. Blood was collected 3 hours later from the

abdominal artery and plasma was obtained by centrifugation at 2000 g for 20 min. To a 1.0 mL aliquot of plasma was added 3.0 mL of acetate buffer (pH 5.0),[19] which was then incubated with 6000 units of type H-5 β-glucuronidase together with 500 units of sulfatase (Sigma, St. Louis, USA) in a shaking water bath at 55°C for 3 hours.[20] It is known that the metabolites of xenobiotics are conjugated with glucuronic acid, sulfuric acid, acetic acid, or amino acid to form highly polar conjugates, hence facilitating their rapid excretion. Glucuronidation and sulfation comprise 95% of the total excreted metabolites.[21] As the HPLC separation in this work was developed for the non-conjugated forms of the metabolites of stevioside, it was necessary to add the enzymes to remove glucuronic acid and sulfuric acid groups prior to analysis. At the end of the incubation period, 10 mL of acetonitrile were added, and the solution was sonicated for 10 min, vortexed for 30 seconds and centrifuged at 2000 g for 20 min. The volume of the supernatant was reduced under N_2 flow and filtered through 0.45 μm nylon filter.[16] An aliquot of 100 μL of the filtrate was injected into HPLC system.

Urine and feces were collected at the end of 24-hour period and kept frozen at -80°C until analysis. Prior to freezing, the feces were dried in an oven at 40°C. Urine was centrifuged at 2000 g for 15 min. The pellet was re-suspended in a volume of acetate buffer (pH 5.0) equal to the original volume, and the solution was sonicated for 10 min, centrifuged and the supernatants pooled.[22] The feces were triturated in a marble mortar. A 0.1 g portion of the feces powder was dissolved in 4 mL 1:1 (v/v) methanol:acetate buffer (pH 5.0), which was then homogenized and sonicated for 20 min. Methanol was evaporated under N_2 flow. Both suspensions of urine and feces were incubated with 6000 units of type H-5 β-glucuronidase together with 500 units of sulfatase in a shaking water-bath at 55°C for 3 hours.[19] At the end of the incubation period 10 mL of acetonitrile were added, and the solution was vortexed for 30 sec and centrifuged at 2000 g for 10 min. The same procedure was conducted on the pellet. The supernatants were pooled and their volume reduced under N_2 flow. The sample was passed through 0.45 μm nylon filter[16] and an aliquot of 100 μL was injected into HPLC system.

RESULTS AND DISCUSSION

The molecular structures of the metabolites of stevioside and steviol are shown in Figure 1. There are three metabolites of stevioside (steviolbioside, 19-O-β-D-glucopyranosyl steviol and steviol), whereas the metabolites of steviol are steviol-16,17α-epoxide, 15α-hydroxysteviol and isosteviol. It can readily be noticed that the nucleus of each compound is a steviol.

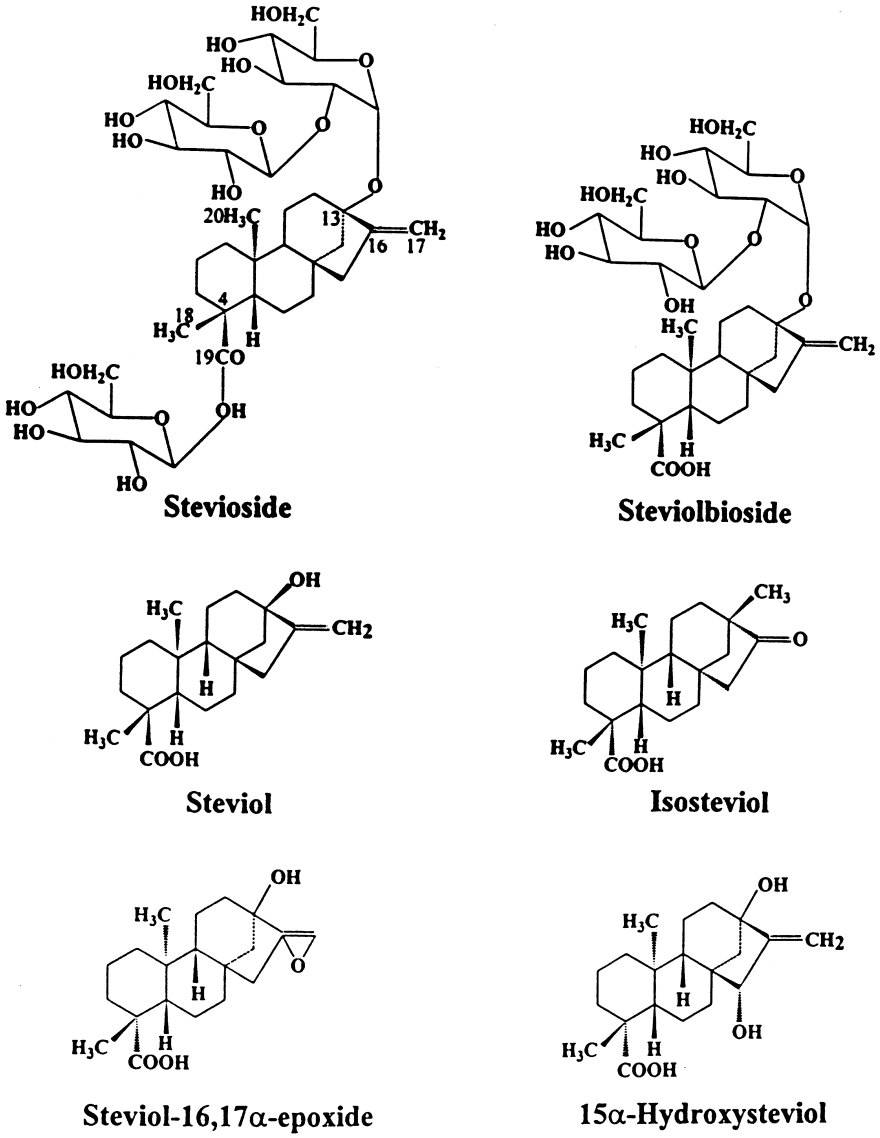

Figure 1. Structures of stevioside and its metabolites.

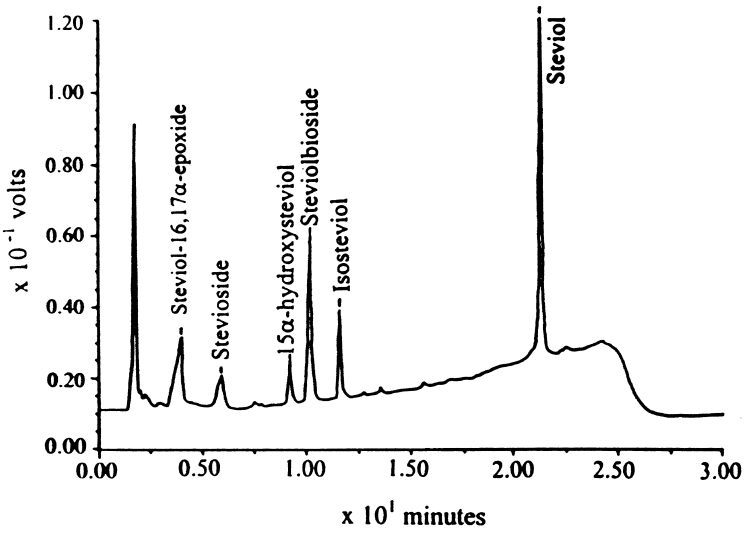

Figure 2. Chromatogram of the standard compounds.

Table 1

HPLC Characteristics of Some of the Metabolites of Stevioside

Compound	Retention Time (min.)	Slope (mVsec/μg)	y-Axis Intercept (mVsec)	Corr. Coeff.	Limit of Detection (μg)
Steviol-16, 17α-epoxide	3.60	188.89	-12.98	.9993	0.4
Stevioside	5.87	235.83	+45.39	.9996	0.4
15α-hydroxy-steviol	9.23	67.40	+0.47	.9990	0.6
Steviolbioside	10.18	284.38	-21.14	.9989	0.4
Isosteviol	11.63	33.60	-2.33	.9927	0.6
Steviol	21.29	623.05	+47.36	.9988	0.4

Figure 2 shows the HPLC separation of the standards of stevioside and its metabolites by the method of gradient elution. All of the compounds were readily resolved. The detection limit of the standards ranged from 0.4 to 0.6 μg as shown in Table 1. This separation and detection technique was highly reproducible.

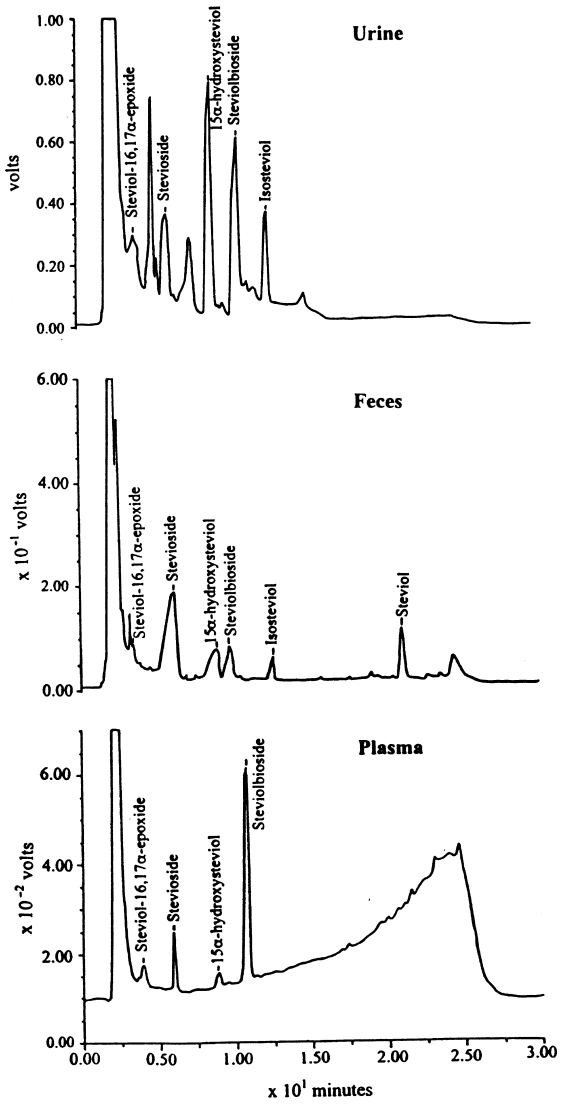

Figure 3. HPLC chromatograms of stevioside and its metabolites in feces, urine, and plasma.

The retention time determined for each solute is listed in Table 1, which also lists the slope, y-axis (peak area) intercept, and correlation coefficient of linear regression calculated for each standard. The table also shows the detection limit of each compound. The detector response was linear over the weight range mentioned in the experimental section.

The detection limit of stevioside and steviolbioside was the same as that of the HPLC method developed by Makapugay[23] which was 0.4 μg, whereas the detection limits of other metabolites cannot be compared because they have not been reported previously. The order of elution in this system was steviol-16,17α-epoxide, then stevioside, 15α-hydroxysteviol, steviolbioside, isosteviol, and steviol.

Figure 3 shows the chromatograms of metabolites of stevioside in urine, feces, and plasma which were collected 24 hours, 24 hours and 3 hours, respectively, after feeding of the hamsters with stevioside. The metabolites present in plasma, urine, and feces were the results of the digestion, metabolism, and excretion process of the ingested stevioside.

The chromatogram of urine shows the presence of steviol-16,17α-epoxide, stevioside, 15α-hydroxysteviol, steviolbioside, and isosteviol. All of these compounds were also detected in feces, including steviol. The chromatogram of plasma shows the presence of steviol-16,17α-epoxide, stevioside, 15α-hydroxysteviol, and steviolbioside.

Each of the peaks of the metabolites found in the above chromatograms corresponded to their respective peak in the chromatogram of the standards shown in Figure 2. Each chromatogram was also compared to the chromatogram obtained from control plasma, urine, and feces (not shown).

To our knowledge, the separation of stevioside metabolites described herein has never been reported before. This method of simultaneous separation thus offers a means for simple and accurate determination of the metabolites produced by digestion, biodisposition, and metabolism of stevioside and steviol.

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REFERENCES

1. B. Crammer, R. Ikan, "Stevia Sweeteners," in **Developments in Sweeteners-3**, T. H. Grenby, ed., Elsevier Applied Science, London, 1987, pp. 46-67.

2. L. E. Ishii, J. A Schwab, A. Bracht, Biochem. Pharmacol., **36 (9)**, 1417 (1987).

3. H. Fujita, T. Edahiro, Shokuhin Kogyo, **22**, 66 (1979).

4. A. D. Kinghorn, D. D. S. Soejarto,. "Stevia Sweeteners," in **Alternative Sweeteners**, L. O'Brien Nabers, R. C. Belardi, eds., Vol. 2., New York, Marcel Dekker, Inc.,1991, pp. 157-171.

5. H. Sakamoto, H. Kohda, K. Murakam, O. Tanaka, Yakugaku Zasshi, **95**, 1507 (1975).

6. J. Iwamura, R. Kinoshita, N. Hirao, Nippon Nogeikagaku Kaishi, **54**, 195 (1980).

7. H. Mitsuhasi, J. Ueno, T. Sumita, Shoyakugaku Zasshi, **32**, 209 (1978).

8. Y. Hashimoto, M. Moriyasu, S. Nakamura, S. Ishiguro, M. Komuro, J. Chromatogr., **161**, 403 (1978).

9. M. S. Ahmed, R. H. Dobberstein, J. Chromatogr., **236**, 523 (1982).

10. M. Hirakido, I. Nakajima, K, Nakajima, S Mizoiri, F. Endo, Shokuhin Esei Zasshi, **21**, 451 (1982).

11. M. S. Ahmed, R. H. Dobberstein, J. Chromatogr., **245**, 373 (1982).

12. J. Liu, S. F. Y. Li, J. Liquid Chromatogr., **18 (9)**, 1703 (1995).

13. M. Compadre, R. A. Hussain, N. P. D. Nanayakkara, J. M. Pezzuto, A. D. Kinghorn, Bio. Env. Mass Spectrometry, **15**, 21 (1988).

14. J. Adduci, D. Buddhasukh, B. Ternai, J. Sci. Soc. Thailand, **12**, 179 (1987).

15. T. Ogawa, M. Nozaki, M. Mitsui, Tetrahedron, **36**, 264 (1980).

16. J. M. Pezzuto, C. M. Compadre, S. M. Swanson, N. P. D. Nanayakkara, A. D. Kinghorn, Proc. Nat. Acad. Sci. USA, **82**, 2478 (1985).

17. H. B. Wood, R. Allerton, H. W. Diehl, H. G. Fletcher, J. Org. Chem., **20**, 875 (1955).

18. J. M. Pezzuto, **New Trends in Natural Products Chemistry**, Elsevier Science Pub., Amsterdam, 1986, p. 467.

19. T. Wikberg, A. Vuorela, P. Ottoila, J. Taskinen, Drug Metab. and Dispos., **211**, 81 (1993).

20. W. Schanzer, M. Konike, Anal. Chim. Acta, **275**, 23 (1993).

21. B. G. Katzung, **Basic and Clinical Pharmacology**, Prentice Hall, London, 1992, p. 54.

22. K. W. Turteltaub, M. G. Knize, S. K. Healy, J. D. Tucker, J. S. Felton, Fd. Chem. Toxic., **27**, 667 (1989).

23. H. C. Makapugay, N. P. D. Nanayakkara, A. D. Kinghorn, J. Chromatogr., **283**, 390 (1984).

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