Absorption, Distribution, Metabolism and Excretion of Stevioside in Rats

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Kunio NAKAYAMA, Daigo KASAHARA and Fumihiro YAMAMOTO

(Omiya Research Laboratory, Nikken Chemicals Co. Ltd.,
1-346, Kitabukuro-cho, Omiya, Saitama, Japan)

[³H] Stevioside was administered orally at a dose of 125 mg/kg to Wistar rats, and its disposition and metabolism were studied. The level of radioactivity in the blood increased slowly to a maximum of 4.83 μ g/ml at 8 hours, exhibiting a biological half-life of 24 hours. At 1 hour, the highest concentration was observed in the small intestine, followed by the stomach and cecum in that order. At 4 hours, the concentration in the cecum was markedly higher than those of other tissues. Radioactivity remaining in the body at 48 hours was 30.7% of the dose. At 120 hours, the percentages of radioactivity excreted into the feces and expired air were 68.4% and 23.9%, respectively, while radioactivity excreted into the urine was only 2.3%. Radioactivity excreted into the bile at 72 hours was 40.9% of the original dose. From the results of biliary and fecal excretion, it was concluded that entero-hepatic circulation occurs in the body.

TLC analysis of the intestinal contents, feces and bile showed that stevioside is decomposed by cecal flora to steviol and sugars, indicating that steviol and these sugars are absorbed from the cecum, distributed throughout the whole body, and excreted mainly into feces and expired air.

Key words: stevioside; absorption; distribution; metabolism; excretion; rat; cecal flora; steviol; feces; respiratory air

Introduction

Stevioside is a white crystalline powder, having a molecular weight of 804.90, and is approximately 300 times sweeter than sucrose. It has been used as a natural sweetener. Subacute toxicity tests in rats given large dosages of stevioside,¹⁾ and food mixed with stevioside,²⁾ indicated that this compound is safe. It is not mutagenic,³⁾ and has little effect on reproductive ability⁴⁾.

Stevioside is a glycoside, which is composed of glucose, sophorose and steviol (tetracyclic diterpene). When it is hydrolyzed with 0.4%

hydrochloric acid, steviol, 13-*O*- β -D-glycopyranosyl steviol, 19-*O*- β -D-glycopyranosyl-13-*O*- β -D-glycopyranosyl steviol and steviolbioside are produced.⁵⁾ It was suggested that stevioside may be degraded in the stomach.⁶⁾ Furthermore, it has been reported that stevioside is degraded by rat cecal bacteria to steviol, and that almost all steviol absorbed from the intestine is excreted into feces through the bile.⁷⁾

Since the disposition of stevioside in the body has not yet been researched, we studied the absorption, distribution, metabolism and excretion after oral administration of [³H] stevioside to rats.

Materials and Methods

Chemicals

Stevioside (Tama Seikagaku Co.) was labelled with tritium gas by New England Nuclear and the crude product had an initial specific radioactivity of $1.15 \mu\text{Ci/mg}$. After recrystallization from ethanol, [^3H] stevioside was separated by preparative thin-layer chromatography (Merck PLC plate silica gel 60, $20 \times 20 \text{ cm}$, thickness 2 mm) with a solvent system of chloroform-methanol-water (15:6:1, v/v). The silica gel at the position of stevioside was scraped off, and [^3H] stevioside was extracted with water. Silica gel column chromatography (column size $30 \times 170 \text{ mm}$) of the extract was carried out by eluting twice with the same solvent system as described above, and the fractions containing [^3H] stevioside were collected. [^3H] Stevioside was obtained by drying the fractions in a vacuum. The radiochemical purity (95%) was determined by radio thin-layer chromatography (TLC) on silica gel 60 plate (Merck) with the following solvent systems:

- (I) chloroform-methanol-water (15:6:1, v/v)
- (II) *n*-butanol-pyridine-water (6:4:3, v/v)
- (III) *n*-propanol-water (2/1)-ethyl acetate (35:65, v/v)

Specific radioactivity was 13.2 or $46.1 \mu\text{Ci/mg}$. Authentic samples of stevioside, steviolbioside, steviol, isosteviol were provided by Tama Seikagaku Co. and glucose was purchased from Wako Junyaku Co.

Animals and treatment

Male Slc: Wistar rats weighing about 300 g were used for the biliary excretion experiment and those weighing about 180 g for the other animal experiments. Animals were fasted for 16 hours before the start of the test, but water was given *ad libitum*. The animals were fed chow (CA-1, Japan Clea) 6 hours after administration of [^3H] stevioside. The temperature and humidity of the room were maintained at $22 \pm 2^\circ\text{C}$ and $50 \pm 5\%$, respectively.

[^3H] Stevioside diluted with cold stevioside was suspended in 2% gum arabic solution to make a 2.5% suspension. A solution of [^3H] stevioside ($125 \text{ ml}/5 \text{ ml/kg}$; $10.4\text{--}120.0 \mu\text{Ci/kg}$) was given to each rat by means of a stomach

tube.

Radioactivity in blood

Blood was collected from the jugular vein without anesthesia at 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 48, 72, 96 and 120 hours.

Urinary and fecal excretions

Animals were housed individually in metabolic cages during the experiment, and urine and feces were collected at 24-hour intervals for 120 hours.

Biliary excretion

In some animals, the bile duct was cannulated with polyethylene tubing under ether anesthesia, and [^3H] stevioside was administered orally after awakening. Each rat was fixed in a Bollman cage, and the bile was collected at 24-hour intervals for 72 hours.

Excretion in expired air

Animals were kept individually in glass metabolic cages to determine the amount of tritium-labelled water in the expired air. The exhaled tritium-labelled water was collected in a cold trap and then the air was passed through gas-scrubbing towers filled with ethanolamine.

Distribution of radioactivity

Animals were sacrificed by decapitation at 1, 4, 24 and 48 hours after dosing. Blood was collected in a heparinized tube. The tissues were removed and the digestive organs were washed with 20 ml of saline solution.

Metabolites in the gastro-intestinal contents

The gastro-intestinal contents were mixed with 20 ml of methanol, the supernatant was separated, and 10 ml of a mixture of methanol-water (1:1, v/v) was added to the residue. The residue was extracted 4 times with the same solvent. The extracts were collected and concentrated in a vacuum. Then the extracts were subjected to TLC and compared with the authentic substances described above. The radioactivity of the chromatogram was determined by a TLC radiochromato-scanner (Aloka TLC-2D) and each peak of the chromatogram was examined by comparison with spots of authentic substances that were detected by using iodine vapor.

Metabolites in feces

The feces were collected for 72 hours after dosing and extracted with 4 times their weight of methanol-water (1:1, v/v) mixture. Extrac-

Fig. 1. Blood levels of radioactivity after oral administration of [^3H] stevioside to rats

Radioactivity was converted $\mu\text{g/ml}$ equivalent of stevioside. Each plot with the vertical bar represents mean $\pm$ S.E. for 3-7 rats. Animals were given [^3H] stevioside orally at a dose of 125 mg/5 ml/kg (99.1 or 141.0 $\mu\text{Ci/kg}$). Blood was collected from the jugular vein of each rat without the use of anesthesia.

tion was repeated 5 times and the supernatant was collected, concentrated and subjected to TLC.

Metabolites in bile

The collected bile was incubated with and without β -glucuronidase (Sigma Type H-5) in 0.2 M acetate buffer (pH 5.0) for 24 hours at 37°C. The same volume of methanol was added to the hydrolysate and the supernatant was collected. Extraction was repeated 4 times with a mixture of methanol-water (1:1, v/v). The extracts was concentrated and subjected to TLC.

Radioassay

The tissues, urine, feces and bile were treated in a sample oxidizer (Aloka ASC-111 or Packard 306), and radioassay was performed with a liquid scintillation counter (Aloka LSC-703). The tritium water collected from the expired air was directly placed in a scintillator and the radioactivity was measured (expressed as stevioside equivalent, $\mu\text{g/g}$ or $\mu\text{g/ml}$).

Results

Blood level

The blood level in rats given [^3H] stevioside orally is shown in Fig. 1. The blood level was 0.73 $\mu\text{g/ml}$ at 0.5 hour, reached a peak of 4.83 $\mu\text{g/ml}$ at 8 hours and then decreased slowly with an elimination half-life of 24 hours.

Excretion

Excretions of radioactivity into urine, feces

Table 1. Cumulative Excretion of Radioactivity after Oral Administration of [^3H] stevioside to Rats

Time (hr)	Feces (n=3)	Urine (n=3)	Expired air (n=3~5)
24	49.4 $\pm$ 0.9 ^{a)}	2.0 $\pm$ 0.2 ^{a)}	2.3 $\pm$ 1.6 ^{a)}
48	60.3 $\pm$ 1.3	2.2 $\pm$ 0.2	6.8 $\pm$ 5.5
72	63.8 $\pm$ 1.6	2.2 $\pm$ 0.2	23.9 $\pm$ 0
96	67.0 $\pm$ 1.9	2.3 $\pm$ 0.2	—
120	68.4 $\pm$ 2.1	2.3 $\pm$ 0.2	—

^{a)} Expressed as % of dose. Values each represent the mean $\pm$ S.E. The number of examples in each experiment is shown in parenthesis. The animals were given [^3H] stevioside orally at a dose of 125 mg/5 ml/kg (27.0 or 45.5 $\mu\text{Ci/kg}$).

and expired air are shown in Table 1. Fecal excretion of radioactivity was 68.4% of the dose within 120 hours, and was far higher than urinary excretion which was 2.3%. Although excretion into expired air was low up to 48 hours, it reached 23.9% at 72 hours. Biliary excretion was low up to 24 hours, but it rapidly increased to 20.8% at 48 hours and reached 40.9% of the dose at 72 hours.

Tissue level

The concentrations of radioactivity in the tissues of rats (represented as stevioside equivalent, $\mu\text{g/g}$ or $\mu\text{g/ml}$) are shown in Table 2.

The level in the blood obtained by decapitation was low (0.21 $\mu\text{g/ml}$) at 1 hour, but increased to 3.78 $\mu\text{g/ml}$ at 4 hours, then remained

Table 2. Tissue Levels of Radioactivity after Oral Administration of [³H] stevioside to Rats

Tissue	Radioactivity Concentrations ^{a)}			
	1 hr	4 hr	24 hr	48 hr
Liver	1.92± 0.46	9.16± 0.85	5.74± 0.58	5.47±1.09
Spleen	0.49± 0.13	2.35± 0.11	2.15± 0.49	4.66±0.80
Kidney	0.78± 0.27	4.20± 0.46	3.17± 0.69	3.32±0.58
Heart	0.30± 0.10	2.85± 0.26	2.01± 0.58	2.33±0.15
Lung	0.43± 0.27	2.47± 0.15	1.91± 0.28	2.76±0.35
Brain	0.23± 0.08	2.02± 0.38	1.87± 0.54	2.52±0.41
Pancreas	1.31± 1.05	2.75± 0.35	2.31± 0.43	2.74±0.15
Testis	0.15± 0.07	2.17± 0.46	1.86± 0.27	2.60±0.29
Ad. G.	0.06± 0.05	13.79± 0.38	11.84± 1.29	21.23±4.17
Stom.	179.06± 9.47	20.83± 4.31	1.98± 0.30	2.67±0.28
S. In.	221.66±18.23	43.04±12.33	8.79± 1.76	5.68±0.54
Cecum	93.90±45.59	282.96±30.29	39.62±13.67	25.80±5.10
L. In.	10.91± 3.54	19.53± 4.32	11.86± 3.28	11.00±3.48
Muscle	1.30± 0.21	3.13± 0.46	1.93± 0.44	2.26±0.19
Fat	1.60± 0.67	17.69± 1.91	11.60± 1.56	15.31±1.37
Blood	0.21± 0.04	3.78± 0.59	2.56± 0.11	44.83±0.70

^{a)} Expressed as μg equivalent of stevioside per g (or ml). Values each represent the mean±S.E. for 3 rats given [³H] stevioside orally at a dose of 125 mg/5 ml/kg (27.0 or 120.0 $\mu\text{Ci/kg}$).

Ad.G.: adrenal glands; Stom.: stomach; S. In.: small intestine; L. In.: large intestine

the same at 24 hours and 48 hours. The levels of most tissues were slightly higher than that of the blood, but showed similar patterns to that in the blood. In the stomach and the small intestine, the highest levels were observed at 1 hour (179.06 and 221.66 $\mu\text{g/g}$, respectively), being about 1000 times higher than that in the blood. In the cecum and large intestine, the levels were 93.90 and 10.91 $\mu\text{g/g}$, respectively, at 1 hour, being about 1/2 and 1/20 of those of the stomach and small intestine. At 4 hours, the cecal and large intestinal levels had increased to 282.96 and 19.53 $\mu\text{g/g}$, being about 70 times and 5 times the blood level, respectively. In contrast, the stomach and small intestinal levels decreased to about the same level as in the blood after 24 and 48 hours. At 48 hours, the cecal level was 25.80 $\mu\text{g/g}$, about 5 times higher than the levels in other tissues. The large intestinal level decreased to 11.86 $\mu\text{g/g}$ at 24 hours and even after 48 hours, it remained unchanged about 2 to 5 times higher than the blood level.

Distribution in the digestive organs

Radioactivity levels in the gastro-intestinal tract and contents are shown in Table 3.

Most of the radioactivity was present in the

Table 3. Distribution of Radioactivity in the Gastrointestinal region and Contents after Oral Administration of [³H] stevioside to Rats

Specimen	Radioactivity recovered ^{a)}			
	1 hr	4 hr	24 hr	48 hr
Stom.	0.9±0.1	0.1±0.0	0.0±0.0	0.0±0.0
S. In.	3.5±0.6	0.8±0.2	0.2±0.0	0.1±0.0
Cecum	0.3±0.1	0.9±0.1	0.2±0.1	0.1±0.0
L. In.	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0
St. C.	9.8±1.2	0.5±0.1	0.4±0.1	0.6±0.0
S. I. C.	65.0±5.7	4.4±0.8	2.3±0.1	1.7±0.3
Ce. C.	3.2±2.8	33.1±1.8	2.4±0.8	1.4±0.3
L. I. C.	0.1±0.0	1.0±0.1	0.9±0.2	1.0±0.2

^{a)} Expressed as percentage of administered dose.

Each value represents the mean±S.E. for 3 rats that are identical to those in Table 2.

Stom.: stomach; S. In.: small intestine; L. I.: large intestine; St. C.: stomach contents; S. I. C.: small intestine contents; Ce. C.: cecal contents; L. I. C.: large intestinal contents

digestive contents and less than 1% of the dose was found in the tissues except for the small intestine at 1 hour. The distribution in the small intestinal contents was 65.0% of the dose at 1 hour, and this was the highest amount. At

Fig. 2. Thin-layer radiochromatograms of gastrointestinal contents in rats given [^3H] stevioside orally

Typical chromatograms after development with solvent system I: chloroform-methanol-water (15:6:1, v/v)

Authentic substances, A: Glucose; B: Stevioside; C: Steviolbioside; D: Steviol; E: Isosteviol

the same time the distributions in the contents of stomach, cecum and large intestine were 9.8, 3.2 and 0.1% of the dose, respectively. The distribution in the cecal contents reached 33.1% of the dose at 4 hours, while that in the small intestinal contents was only 4.4% of the dose. In contrast, the recoveries of radioactivity in the stomach contents and large intestinal contents were only 0.5 and 1.0% of the dose, respectively, at the same time. The radioactivity of other tissues remained low. The recoveries in the contents of the small intestine, cecum and large intestine decreased to 2.3, 2.4 and 0.9% of the dose, respectively, at 24 hours. The amount remaining in the other tissues was less than 1.0% of the dose. The recovery at 48 hours was almost the same as that at 24 hours. The total amount remaining in the main tissues and gastro-intestinal contents (shown in Table 2 and Table 3) was 83.0 and 42.0% at 1

and 4 hours after dosing, respectively, but fell to 7.3 and 5.9% after 24 and 48 hours, respectively.

Metabolites in the gastro-intestinal contents

Typical thin-layer radiochromatograms of metabolites in the gastro-intestinal contents are shown in Fig. 2. Stevioside was detected as a major component of the stomach contents at 1 hour and minor metabolites were also detected. In the small intestinal contents, stevioside and metabolites accounted for 33 and 67% of total radioactivity, respectively. The highest peak obtained in solvent system I was at the position of glucose, but the peak appeared at different positions in solvent systems II and III (data not shown). Stevioside in the cecal contents was not located clearly because of its low radioactivity. Radioactivity in the large intestinal contents was so low that the metabolites in the contents were not examined.

Stevioside, steviolbioside and steviol accounted for 7.6, 8.0 and 7.5%, respectively, of the radioactivity in the small intestinal contents at 4 hours, while they accounted for 39.4%, 16.7% and 5.1%, respectively, in the cecal contents at the same time. At 24 hours, stevioside was not detectable in the cecal contents, but steviol and unidentified metabolites accounted for 15.6 and 68.0% of the radioactivity, respectively. The peak at the position of unidentified metabolites was different from those in solvent systems II and III. Analysis of metabolites in contents other than the cecal contents was impossible because of the low radioactivity.

Metabolites in feces

A typical radiochromatogram of metabolites in the feces is shown in Fig. 3. Steviol was found to be a major metabolite, accounting for 39.2% of the radioactivity in the feces. In contrast, the radioactivity levels of stevioside and the unidentified substances were so low that their ratio could not be calculated.

Metabolites in bile

Typical radiochromatograms of metabolites in the bile are shown in Fig. 4. An unidentified metabolite accounted for most of the radioactivity in the untreated bile up to 24 hours, and the same unidentified metabolite was detected in the bile treated with β -glucuronidase. The un-

Fig. 3. Thin-layer radiochromatogram of the 0-72 hr feces of rats given [^3H] stevioside orally

Typical chromatograms after development with solvent system I. Authentic substances: the same substances as in Fig. 2.

identified metabolite, however, disappeared after treatment with 0.2 M acetate buffer (pH 5.0) and steviol appeared as a major metabolite, representing 51% of the radioactivity in the bile. On the other hand, the untreated bile obtained from 24 to 48 hours after administration contained an unidentified metabolite that accounted for approximately 88% of the radioactivity. At

Fig. 4. Thin-layer radiochromatograms of the 0 to 24 hr and 24 to 48 hr bile of rats given [^3H] stevioside orally

Typical chromatograms after development with solvent system I. Authentic substances: the same substances as in Fig. 2.

the same time, steviol accounted for 63% of the radioactivity after treatment with β -glucuronidase. When the bile was treated with 0.2 M acetate buffer (pH 5.0), steviol accounted for most of the radioactivity, as was the case in the bile collected from 48 to 72 hours (data not shown).

Discussion

The disposition and metabolism of [^3H] stevioside after oral administration to rats were studied. Radioactivity in the blood increased slowly and reached the maximum concentration at 8 hours after administration. Thereafter, the radioactivity decreased gradually, showing a half-life of 24 hours. The change of blood radioactivity with time suggests that stevioside is absorbed very slowly for a long time. From the results of organ distribution, it is supposed that most of the radioactivity is not absorbed but remains in the small intestinal contents at 1 hour after administration. At 4 hours, most of the radioactivity has reached the cecum, and correspondingly the radioactivity decreased to 4.4% in the small intestinal contents. In addition, the tissue level in the cecum was very high. The blood and organ distribution results indicate that the radioactive substances are mainly absorbed in the cecum, and the rate is higher than that of the small intestine.

At 24 hours, radioactivity levels in the gastro-intestinal contents and tissues were low. The other organs showed lower radioactivity than the gastro-intestinal contents and tissues. The total amount remaining unexcreted was 46.3% of the dose at 24 hours. It seems likely that low levels of radioactivity are widely distributed throughout the whole body at this time, since radioactivity was not very high in the main organs. The distribution of radioactivity in the gastro-intestinal contents and tissues at 48 hours was similar to that at 24 hours. The excretion of radioactivity increased to 69.3% of the dose, and that remaining in the body fell to 30.7% of the dose. Further, in view of the increase of excretion of radioactivity at 72 hours, it is suggested that radioactivity will eventually be eliminated entirely from the body. Fecal excretion reached half of the dose at 24 hours and 68% at 120 hours. This indicates that the fecal

route is the major route of radioactivity excretion after oral administration of [^3H] stevioside. Radioactivity in the expired air increased sharply to 24% of the dose at 72 hours. The increase of respiratory excretion thus occurred at a much later time, but the respiratory excretion of radioactivity (as tritium water) was next highest after the fecal excretion. The biliary excretion of radioactivity also started to increase at a later time.

Suzuki et al.⁽⁶⁾ have suggested that stevioside may be hydrolyzed in the stomach. Nabeta et al.⁽⁵⁾ reported that steviol and other decomposition products can be obtained by refluxing stevioside with 0.4% hydrochloric acid for 5 hours. It is evident from our data that most of the stevioside does not change within 1 hour, and also does not stay in the stomach for a long time. Therefore, this indicates that stevioside may not be hydrolyzed by acid in the stomach of the rat. The formation of the metabolite was observed in the small intestinal contents shortly after administration, but the metabolite quickly disappeared from the intestine. We were not able to determine whether the metabolite was absorbed or further metabolized in the intestine.

On the other hand, it was observed that stevioside changed to steviolbioside and steviol in the cecal contents at 4 hours. However, a small quantity of steviol and a large quantity of the unidentified metabolite were observed in the cecal contents at 24 hours. That metabolite was the same metabolite which was found in the bile. This suggests that the metabolite in the cecal contents at 24 hours originated from biliary excretion. Wingard et al.⁽⁷⁾ have reported that stevioside is decomposed by the cecal flora to steviol. Steviol is then absorbed from the lower part of the intestine and excreted into the feces.

From the results mentioned above, it appears that orally administered stevioside is not absorbed readily from the upper part of the small intestine, but the metabolites, formed by bacterial flora in the small intestine and primarily in the cecum, are absorbed from the lower part of the intestine. Therefore, the increase in blood radioactivity was somewhat delayed (it began at 4 hours), and the excretions into the bile and expired air began to rise at 8 hours. It was found that biliary metabolites were mostly steviol con-

jugates. The steviol conjugate was hydrolyzed by weak acid, but not by β -glucuronidase in the 0 to 24 hour bile. In contrast with this, the conjugate was hydrolyzed by both acetate buffer and β -glucuronidase in the 24 to 48 hour bile, and steviol was released. The kinds of conjugates involved could not be identified. However, it is clear that different steviol conjugates are present in the 0 to 24 hour bile and the 24 to 48 hour bile. The nature of the difference requires further study.

It is thought that tritium water is hardly contained in bile. If it were excreted into the bile, it might be reabsorbed from the intestine and then urinary excretion of radioactivity would gradually increase. Urinary excretion of radioactivity, however, was very low and was almost completed within 24 hours.

Steviol was found to be a major metabolite in the feces. Small amounts of stevioside, steviolbioside and the afore-mentioned unidentified metabolite were also found. Consequently, it is inferred that a part of stevioside administered orally is excreted into the feces, but most of it is decomposed by bacterial flora in the cecum to steviolbioside, steviol and glucose, and they are absorbed as they are or after further decomposition. Absorbed glucose is metabolized and excreted in expired air as carbon dioxide and water, while most of the steviol is changed to the conjugate in the liver and excreted into the

intestinal tract through the bile. The conjugates are decomposed by the intestinal flora and free steviol is formed. Some of the free steviol is excreted, but a part of it remains in the body because of reabsorption through the enterohepatic circulation, although the amount is small.

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