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Comparative toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol in rats

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ABSTRACT

The toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol were examined in rats for comparative purposes to determine whether toxicological studies conducted previously with stevioside would be applicable to the structurally-related glycoside, rebaudioside A. Single, oral doses of the radiolabelled compounds were extensively and rapidly absorbed with plasma concentration–time profiles following similar patterns for stevioside and rebaudioside A. Elimination of radioactivity from plasma was essentially complete within 72 h. All plasma samples had similar metabolite profiles; the predominant radioactive component in all samples was steviol, with lower amounts of steviol glucuronide(s) and low levels of one or two other metabolites. Rebaudioside A, stevioside, and steviol were metabolized and excreted rapidly, with the majority of the radioactivity eliminated in the feces within 48 h. Urinary excretion accounted for less than 2% of the administered dose for all compounds in both intact and bile duct-cannulated rats, and the majority of the absorbed dose was excreted *via* the bile. After administration of the compounds to intact and bile duct-cannulated rats, radioactivity in the feces was present primarily as steviol. The predominant radioactive compound detected in the bile of all cannulated rats was steviol glucuronide(s), indicating de-conjugation in the lower intestine. Overall, the data on toxicokinetics and metabolism indicate that rebaudioside A and stevioside are handled in an almost identical manner. These studies support the use of toxicological safety studies conducted with stevioside for the safety assessment of rebaudioside A.

1. Introduction

Rebaudioside A (13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid β-D-glucopyranosyl ester), and the related compound, stevioside (13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] kaur-16-en-18-oic acid β-D-glucopyranosyl ester), are major steviol diterpene glycosidic constituents of the leaves of the plant *Stevia rebaudiana* Bertoni which have traditionally been used to sweeten food and beverages in South America (Soejarto et al., 1982; Gardana et al., 2003). Rebaudioside A has been reported to be metabolized to the aglycone, steviol (kaur-16-en-18-oic acid, 13-hydroxy-, (4α)-) *in vitro* by rat cecal bacteria and human intestinal microflora (Wingard et al., 1980; Gardana et al., 2003; Koyama et al., 2003a). Stevioside has also been reported to be metabolized to steviol *in vivo* in all species tested including rats, pigs, and humans (Nakayama et al., 1986; Cardoso et al., 1996; Geuns et al., 2003, 2007) and in *in vitro* studies involving human intestinal microflora (Gardana et al., 2003; Koyama et al., 2003a). Nakayama et al. (1986) reported that high percentages of radioactivity from an oral dose of radiolabelled stevioside administered to Wistar rats were found in the feces and bile, while a low percentage of the dose was eliminated in the urine. The authors concluded that enterohepatic circulation of steviol metabolites occurs following oral administration of stevioside. Similarly, a study conducted in Sprague–Dawley rats dosed with either stevioside or a stevia mixture demonstrated absorption, metabolism and excretion of steviol, the latter primarily in the feces (Sung, 2002; Koyama et al., 2003a). Wingard et al. (1980) reported that steviol was extensively absorbed, and excreted unchanged in the feces.

The present study was conducted to compare the absorption, plasma profiles, metabolism and excretion characteristics of ^{14}C-rebaudioside A, ^{14}C-stevioside and ^{14}C-steviol following single oral

Abbreviations: AUC, area under the curve; C_{max}, concentration maximum; cm, centimeter; g, gram; kg, kilogram; h, hours; HPLC, high pressure liquid chromatography; *k*, terminal rate constant; L, liter; LSC, liquid scintillation; M, molar; min, minute; μL, microliter; mL, milliliter; mm, millimeter; mg, milligram; mmHg, millimeters mercury; min, minute; *n*, number; nm, nanometer; rpm, revolutions per minute; T_{max}, time to maximum concentration; TLC, thin layer chromatography; UK, United Kingdom; UV, ultra violet; v, volume.

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doses administered to intact and bile duct-cannulated male and female rats. The objective was to determine whether previously conducted toxicology studies with stevioside would be applicable to the safety assessment of rebaudioside A.

2. Materials and methods

2.1. Materials

All radiolabelled compounds were synthesized by Huntingdon Life Sciences with ^{14}C in the $=CH_2$ group of the steviol moiety. Uniformly labelled rebaudioside A [sp. act. 56.7 mCi/mmol; radiochemical purity >97% by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC)], stevioside (sp. act. 53.2 mCi/mmol; radiochemical purity >97% by TLC and HPLC), and steviol (sp. act. 24.49 and 25.56 mCi/mmol, respectively; radiochemical purity >97% by TLC and HPLC). Non-radioactive rebaudioside A, stevioside, and steviol (purity 98.5%, 99.3%, and 100.0%, respectively) were sourced from Cambrex, Charles City, IA (USA). Radiolabelled and non-radiolabelled materials were co-dissolved to adjust the specific activity to the appropriate level.

2.2. Animals

Male and female Sprague–Dawley (Crl:CD(SD)) rats were obtained from Charles River UK Ltd. (Margate, Kent, UK). The studies were conducted by Huntingdon Life Sciences Ltd., Cambridgeshire, UK in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

For the pharmacokinetic portion of the study (111 animals per sex), rats were selected for dosing if they showed the expected weight gain during a 5-day acclimatization period and appeared healthy upon clinical observation. The rats were 6–9 weeks old at the time of dose-administration. Males weighed 196–228 g and females weighed 188–240 g. All rats were provided with VRF1 diet (manufactured by Special Diet Services, Witham Essex, UK) *ad libitum* except for a period of 12 h prior to dosing. During the pharmacokinetic portion of the experiment, and after dosing, rats of each sex were housed in groups of up to six in stainless steel battery cages with suspended mesh floors. All animals were weighed before dosing and prior to sacrifice.

The treatment of the animals in the metabolism and excretion portion of the study was similar to that of the pharmacokinetics portion, except that the males ($n = 35$) weighed from 202 to 257 g and females ($n = 35$) weighed from 180 to 236 g. The metabolism portions of the experiment were carried out in both intact and bile duct-cannulated male and female rats. Intact rats were housed in a manner identical to the rats in the pharmacokinetic portion of the study during the 5-day acclimatization period. They were subsequently housed individually in glass metabolism cages designed to allow the separate collection of urine and feces, after commencement of dosing.

Five animals per sex per test substance underwent bile duct cannulation using appropriate anaesthesia and surgical procedures. Prior to dosing, the cannula loop was opened and the flow of bile confirmed. From the time of opening and throughout the sample collection period, replacement bile salts (cholic acid and taurocholic acid: each 3.33 g/L in 5% sodium bicarbonate saline solution) were infused *via* the duodenal cannula at a rate of 0.65 mL/h. Bile duct-cannulated animals were housed individually in polypropylene cages designed to accept the swivel mechanism of the cannula after their surgery, and after dosing, these rats also were housed in glass metabolism cages.

2.3. Dosing

The same dose levels were used in the pharmacokinetic and metabolism and excretion portions of the study. The dose level of rebaudioside A (5 mg/kg body weight) was determined on the basis of the highest estimated daily human intake of rebaudioside A (Renwick, 2008). The dose levels of stevioside (4.2 mg/kg body weight) and steviol (1.6 mg/kg body weight) were equivalent on a molar basis to that of rebaudioside A. The formulations were administered by gavage at a rate of 5 mL formulation/kg body weight.

2.4. Pharmacokinetics

To determine the concentrations of radioactivity in the plasma, a single oral dose was administered to three rats per sex per substance (Experiment 1) and blood samples (approximately 0.3 mL) were taken from the tail vein of each animal at 0.5, 1, 4, 8, 12, and 24 h after dosing. An additional sample was taken 72 h (at the time of death) after dosing by cardiac puncture under terminal anaesthesia. Blood samples were added directly to tubes containing lithium heparin as an anticoagulant and plasma was separated by centrifugation. Duplicate weighed aliquots of approximately 50 μL of each plasma sample were taken for liquid scintillation counting (LSC).

To obtain plasma metabolite profiles, four animals per sex per test compound each received a single dose of the test compound (Experiment 2). Sample times were established following the analysis of the Experiment 1 samples. Blood samples (approximately 7–9 mL) were collected from two animals per sex at two different times after dosing by cardiac puncture under terminal anaesthesia. Blood was collected from the rats receiving ^{14}C-rebaudioside A at 8 and 24 h after dosing. Blood was collected from the rats receiving ^{14}C-stevioside at 4 and 8 h after dosing for males, and at 8 and 12 h after dosing for females. Blood was collected from the rats receiving ^{14}C-steviol at 0.5 and 8 h after dosing. Blood samples were added directly to glass tubes containing lithium heparin as an anticoagulant and the plasma samples were pooled by sex and sample time. Duplicate weighed aliquots (rebaudioside A and stevioside, approximately 250 μL; steviol, approximately 50 μL) of each pooled plasma sample were analyzed by LSC. Prior to chromatographic analysis by normal phase TLC, 2 mL of acetonitrile were added to 1 g of each pooled sample to precipitate plasma proteins. Following centrifugation, and resuspension of the precipitate in 200 or 250 μL of water, duplicate 25 μL aliquots of these solutions were taken for LSC to determine the recovery of radioactivity through the protein precipitation procedure. An average recovery rate of 65% was determined; since the major component detected in these extracts following each test compound was steviol (see later), it is likely that this sparingly-soluble compound also represented the majority of the unrecovered radioactivity, although no control extractions of plasma fortified with ^{14}C-steviol were conducted.

In the main study on plasma pharmacokinetics (Experiment 3), 27 rats per sex per test compound received a single oral dose of the test substance and blood samples (as much as possible) were collected from three animals per sex per compound by cardiac puncture under terminal anaesthesia at 0.25, 0.5, 1, 2, 4, 8, 24, 28, and 72 h after dosing. Blood samples were taken in the same manner from three undosed animals per sex, which were used as control samples for the unlabelled HPLC analyzes. Blood samples were added directly to tubes containing lithium heparin as an anticoagulant and plasma was separated from the blood cells by centrifugation. Duplicate weighed aliquots (rebaudioside A and stevioside, approximately 500 μL; steviol, approximately 250 μL) were taken for LSC and the remaining sample was divided into 0.5 mL portions which were stored at <−15 °C pending analysis for steviol and steviol glucuronide(s) by HPLC.

2.5. Metabolism and excretion

A single dose of each test substance was administered to five intact rats per sex and five bile duct-cannulated rats per sex. For each intact animal, urine was collected separately into solid CO_2-cooled containers from 0 to 6 and 6 to 24 h after dosing and at 24-h intervals thereafter, up to 96 h. The interiors of the metabolism cages were washed with distilled water every 24 h and these washings were retained. Feces were collected from 0 to 24, 24 to 48, 48 to 72, and 72 to 96 h after dosing. For each cannulated rat, bile was collected at intervals of 0–3, 3–6, 6–9, 9–12, 12–24, and 24–48 h. The samples were stored frozen at −15 °C until analyzed. Urine and feces were collected at intervals of 0–24 and 24–48 h post-dosing. Again, the cages were washed with water and the water washings collected. At 96 and 48 h after dosing, the intact and cannulated rats, respectively, were killed by cervical dislocation. The gastrointestinal tract (including contents) was removed from the carcasses of the intact rats and retained with the remaining carcasses. For the cannulated rats, the gastrointestinal tract (including contents) and the liver were removed from the carcasses and retained with the remaining carcasses.

The total weights of urine, bile, and cage wash samples were measured and duplicate weighed aliquots were analyzed by LSC. The total gastrointestinal tract and liver of each animal were weighed, homogenized and triplicate weighed aliquots were taken for combustion/LSC. Each carcass was weighed and solubilized in a mixture of distilled water, methanol, and Triton X-405 (6:3:1 by volume) containing sodium hydroxide (80 g/L) at 50 °C until digestion was complete. The total weight of the digest was measured and duplicate aliquots were analyzed by LSC.

Fecal samples were extracted with acetone and water by homogenization using an Ultra Turrax homogenizer. Following centrifugation, the supernatant was decanted, the total weight of the extract solution was measured, and duplicate weighed aliquots were analyzed by LSC. Following extraction, the total weight of fecal debris was measured and triplicate weighed aliquots were taken for combustion/LSC. Feces samples that were not extracted were homogenized with water; the total weight of the homogenate was measured, and triplicate weighed aliquots were taken for combustion/LSC.

For metabolism purposes, proportionate pools of feces extracts (0–48 h, males and females, separately) were prepared from intact animals dosed with ^{14}C-rebaudioside A, ^{14}C-stevioside, or ^{14}C-steviol, and from cannulated animals dosed with ^{14}C-rebaudioside A or ^{14}C-stevioside. Likewise, proportionate pools of bile samples collected during the 48-h period (males and females, separately) were prepared from selected cannulated animals dosed with ^{14}C-rebaudioside A, ^{14}C-stevioside, or ^{14}C-steviol. These samples were analyzed directly by TLC.

In order to confirm the identity of steviol glucuronide(s), portions of the pooled bile samples were incubated with a β-glucuronidase/sulfatase enzyme mixture (from *Helix pomatia*) in 0.2 M sodium acetate buffer (pH 5) at 37 °C for about 24 h. Either the hydroxyl-, carboxyl-glucuronide conjugates, or a mixture of both, could be detected, although the exact conjugate could not be identified once it had been cleaved by the action of β-glucuronidase. Control incubations were prepared similarly but also contained the β-glucuronidase-specific inhibitor (D-saccharic acid-1,4-lactone monohydrate). Further aliquots were taken to test for sulfatase activity by adding a few crystals of *p*-nitrocatechol sulphate and incubating at 37 °C for an additional hour.

2.6. Analytical methods

Radioactivity was measured by LSC using either LKB-Wallac model 1219, Rackbeta Spectral, Wallac model 1409, or model 1410 liquid scintillation counters with automatic quench correction. Radioactivity in amounts less than twice background levels was considered to be below the limit of accurate determination. Aliquots of liquid samples (dose solution, HPLC fractions, plasma, urine, cage washes, bile, fecal extracts, and carcass digests) were mixed with suitable scintillator solutions prior to LSC. Solid samples (fecal homogenates and residues, homogenized gastrointestinal tract and liver samples) were combusted in an automatic sample oxidiser prior to LSC. For the calculation of concentrations of radioactivity in plasma, aliquot weights and LSC data were recorded and processed using the DEBRA automated laboratory data capture and processing system (version 5.2a (200), LabLogic Systems Ltd., Sheffield, UK).

HPLC was performed in the pharmacokinetic portion of the study using either 3:1 (v/v) water:acetonitrile (Method 1) or 0.1% trifluoroacetic acid in water:acetonitrile (1: 1, v/v) (Method 2) as the mobile phase and a Jupiter C18, 15 cm × 4.6 mm column with ambient column temperature and a flow rate of 1 mL/min. In addition to these two HPLC methods, the metabolism and excretion portion of the study used a biphasic gradient from 90% 0.01 M ammonium acetate containing 0.1% trifluoroacetic acid (solvent A) and 10% acetonitrile containing 0.1% acetic acid (solvent B) to 40% solvent A and 60% solvent B over 15.5 min which was held for 6 min prior to re-establishing initial conditions. The column used was a Synergi 4μ Max-RP, 150 × 2 mm column, with ambient column temperatures and a flow rate of 0.2 mL/min (Method 3). In each case, the eluate passed through a UV absorbance detector, with a detection wavelength of 210 nm, and a radioactivity detector with solid scintillant cell. Laura software (version 1.4, LabLogic Systems Ltd.) was used to acquire output data from the detectors. Method 1 was used to measure the radiochemical purity of ^{14}C-rebaudioside A and ^{14}C-stevioside. Method 2 was used to measure the radiochemical purity of ^{14}C-steviol and for the analysis of selected samples from experimental Group 2. Method 3 was used for analysis of selected fecal extracts and bile samples. Fractions of the column eluate were collected and radioassayed by LSC for quantitative analysis. The proportion of the total net eluted radioactivity in each fraction and the recovery of radioactivity from the column were calculated.

Normal phase TLC was carried out on pre-layered, glass-backed silica gel plates of 0.25 mm thickness. The developing solvent systems were (ratios by volume): dichloromethane:methanol (20:1) (A), ethyl acetate:hexane (1:1) (B), chloroform:methanol:water (15:10:2) (C), and butan-1-ol:acetic acid:water (4:1:1) (D). All solvent systems were prepared fresh just prior to use. The measurement of the radiochemical purity of the test substances used solvent systems A, B, and C. Solvent system D was used for the analysis of plasma samples from Group 2 and for the analysis of fecal extracts and bile, including co-chromatography with test and reference substances. Two-dimensional chromatograms of the developed plates were obtained using an image analyzer. TINA software (version 2.09, Raytest Isotopenmessgerate GmbH) was used to generate linear scaled radiochromatograms. Non-radiolabelled test and reference substances were detected either by their quenching of the UV fluorescent indicator on the TLC plate or by exposing the plate to iodine vapour. Steviol glucuronide was used as a non-radiolabelled chromatographic reference.

The concentrations of unlabelled steviol and steviol glucuronide(s) were measured in each plasma sample from Group 3 by a validated LC–MS analytical method (Burton, 2007). The method was linear over the range 1–1000 ng/mL of steviol or steviol glucuronide(s) with coefficients of variation of <5% at concentrations of 10–750 ng/mL.

2.7. Calculations

Mean maximum observed plasma concentrations of radioactivity, steviol, and steviol glucuronide(s) (C_{max}) and their times of occurrence (T_{max}) were taken directly from the observed values. Areas under the mean concentration–time curves up to the time of the last quantifiable sample (AUC_t) and up to 72 h post-dose (AUC_{72}) were estimated by the linear trapezoidal rule. Areas under the mean concentration–time curves to infinite time (AUC_∞) were calculated by means of the equation: $AUC_\infty = AUC_t + C_{last}/k$ where C_{last} is the predicted concentration at the time of the last quantifiable sample. The terminal rate constants (k) were estimated by linear regression of log concentration against time.

3. Results

3.1. Pharmacokinetics

3.1.1. Experiment 1

Experiment 1 served as a pilot study to provide preliminary concentration–time data used to select appropriate sampling times for Experiments 2 and 3. Average concentrations of total radioactivity in plasma from Experiment 1 animals are reported in Table 1. The peak concentrations of radioactivity were at approximately 8, 4, and 0.5 h following doses of ^{14}C-rebaudioside ^{14}C-stevioside A, and ^{14}C-steviol, respectively.

3.1.2. Experiment 2

Experiment 2 was conducted to provide samples at two times after dosing for investigation of metabolic profiles. Plasma obtained from the rats in Experiment 2 was analyzed by normal phase TLC to determine the proportions of radioactive components present. The results of these analyses are presented in Tables 2–4 for rats dosed with rebaudioside A, stevioside, and steviol, respectively. Qualitatively, all plasma samples had similar metabolite profiles, with the predominant radioactive component in all samples identified as steviol. Lower amounts of steviol glucuronide(s), and even lower amounts of one or two (in most cases) unidentified metabolites, were detected following administration of each of the three test compounds. Based on their chromatographic properties, the unidentified metabolites were of slightly higher polarity than steviol.

3.1.3. Experiment 3

The mean concentrations of radioactivity in the plasma samples from animals in Experiment 3 dosed with ^{14}C-rebaudioside A, ^{14}C-stevioside, and ^{14}C-steviol are given in Fig. 1 for males and in Fig. 2 for females. For each substance, the concentration–time curves of radioactivity were largely similar. The limits of detection were lower in Experiment 3 compared to Experiments 1 and 2, and were 8.9 ng/mL for steviol and 10 ng/mL for steviol glucuronide(s).

Following the administration of ^{14}C-rebaudioside A and ^{14}C-stevioside, mean plasma concentrations of radioactivity for both compounds declined between 15 min and 1 h in both sexes. Concentrations increased from 1 h after dosing to between 2 and 8 h (depending on the substance and sex) up to the overall C_{max} values before declining once more. After a single oral dose of

Table 1
Mean plasma concentrations (standard deviation) of total radioactivity after a single 5 mg/kg oral dose of ^{14}C-rebaudioside A, 4.2 mg/kg oral dose of ^{14}C-stevioside, or 1.6 mg/kg oral dose of ^{14}C-steviol (Experiment 1)

Time (h post-dosing)	Male			Female		
	^{14}C-rebaudioside A[a,b]	^{14}C-stevioside	^{14}C-steviol	^{14}C-rebaudioside A	^{14}C-stevioside	^{14}C-steviol
0.5	35 (6)[c]	15 (2)	142 (139)	22 (8)	12 (11)	82 (38)
1	22 (8)	ND	79 (18)	28 (3)	ND	80 (18)
4	32 (9)	33 (16)	74 (46)	34 (19)	36 (26)	52 (18)
8	47 (9)	28 (11)	47 (15)	74 (23)	55 (13)	45 (25)
12	32 (11)	ND	17 (4)	61 (10)	37 (17)	30 (8)
24	ND	ND	NS	19 (5)	ND	NS
72	ND	ND	ND	ND	ND	ND

NS = no sample; ND = not detected.
[a] A mean was calculated when there were at least two measurable values (except for 0.5 h stevioside in females where one sample was ND and was regarded as 0).
[b] $n = 3$.
[c] Results are expressed as ng steviol equiv./g.

Table 2
Proportions of radioactive components in plasma after a single oral dose of ^{14}C-rebaudioside A (5 mg/kg) (Experiment 2)

Component	Males						Females					
	8 h after dosing			24 h after dosing			8 h after dosing			24 h after dosing		
	ppb[a]	%[b]	%[c]	ppb[a]	%[b]	%[c]	ppb[a]	%[b]	%[c]	ppb[a]	%[b]	%[c]
Total radioactivity	110			20			206			60		
Steviol glucuronide(s)	7	6	12	1	5	15	12	6	10	2	3	4
P1	–	–	–	–	–	–	1	1	1	–	–	–
P2	4	3	6	1	2	7	5	2	4	1	1	2
Steviol	47	42	82	5	25	78	110	54	86	34	56	93
Others[d]	–	–	–	–	–	–	0.4	0.1	0.3	–	–	–

[a] ng steviol equiv./g (taking into account the recovery through the protein precipitation step).
[b] % plasma radioactivity (taking into account the recovery through the protein precipitation step).
[c] % chromatographed radioactivity.
[d] Regions of the chromatogram that did not contain any discrete radioactive components.

Table 3
Proportions of radioactive components in plasma after a single oral dose of ^{14}C-stevioside (4.2 mg/kg) (Experiment 2)

Component	Males						Females					
	4 h after dosing			8 h after dosing			8 h after dosing			12 h after dosing		
	ppb[a]	%[b]	%[c]	ppb[a]	%[b]	%[c]	ppb[a]	%[b]	%[c]	ppb[a]	%[b]	%[c]
Total radioactivity	50			28			91			31		
Steviol glucuronide(s)	4	7	9	4	16	19	8	9	12	3	8	12
P1	–	–	–	–	–	–	1	1	1	–	–	–
P2	4	8	11	1	3	4	2	2	2	2	6	8
Steviol	30	60	79	17	62	71	51	55	78	16	50	73
Others[d]	1	1	2	2	6	7	4	5	7	2	5	7

[a] ng steviol equiv./g (taking into account the recovery through the protein precipitation step).
[b] % plasma radioactivity (taking into account the recovery through the protein precipitation step).
[c] % chromatographed radioactivity.
[d] Regions of the chromatogram that did not contain any discrete radioactive components.

Table 4
Proportions of radioactive components in plasma after a single oral dose of ^{14}C-steviol (1.6 mg/kg) (Experiment 2)

Component	Males						Females					
	0.5 h after dosing			8 h after dosing			0.5 h after dosing			8 h after dosing		
	ppb[a]	%[b]	%[c]	ppb[a]	%[b]	%[c]	ppb[a]	%[b]	%[c]	ppb[a]	%[b]	%[c]
Total radioactivity	41			18			118			57		
Steviol glucuronide(s)	7	17	27	3	16	24	21	18	25	6	10	14
P1	0.4	1	2	–	–	–	2	2	2	1	1	2
P2	3	8	12	1	6	10	2	2	2	1	3	5
Steviol	14	35	58	8	44	66	59	50	70	31	54	79
Others[d]	0.1	0.3	1	0.1	1	1	1	1	1	0.4	1	1

[a] ng steviol equiv./g (taking into account the recovery through the protein precipitation step).
[b] % plasma radioactivity (taking into account the recovery through the protein precipitation step).
[c] % chromatographed radioactivity.
[d] Regions of the chromatogram that did not contain any discrete radioactive components.

^{14}C-steviol, the C_{max} occurred in the first sample taken at 15 min after administration. Concentrations declined rapidly between 15 min and 1 h. A small increase was observed 2 h after administration followed by further decline. The increase in mean concentration observed in females 8 h after administration was attributed to one high value.

The concentrations of total steviol and steviol glucuronide(s) were also measured in plasma by HPLC in the Experiment 3 animals and these data were compared with the average total radioactivity.

Following administration of ^{14}C-rebaudioside A, all mean plasma concentrations of steviol in male rats were below the limit of detection of 8.9 ng/mL (although some individual values were greater), whereas the concentrations of steviol exceeded the limit of detection in all female rats at 4 and 8 h (means of 19.2 and 28.9 ng/mL, respectively) after dosing. All mean plasma concentrations of steviol glucuronide(s) were below the limit of detection (10 ng/mL) following administration of ^{14}C-rebaudioside A. Steviol and/or steviol glucuronide(s) was detected in 14 of the 54 plasma samples from males and females combined. Excluding the result for one outlier animal (where the measured steviol concentration was considerably higher than the total radioactivity), steviol concentrations accounted for between 32.5% and 74.3% (average 52.3%) of the total plasma radioactivity.

Following administration of ^{14}C-stevioside to male rats, plasma concentrations of steviol above the limit of detection were found in two of three animals at 4 h (mean concentration 20.3 ng/mL) and also at 8 h (mean concentration 18.3 ng/mL). In females, steviol concentrations above the limit of detection were observed in 2/3, 3/3, and 3/3 animals at 2, 4, and 8 h (mean concentrations 40.0, 45.7, and 79.9 ng/mL, respectively). A single mean concentration of steviol glucuronide(s) above the limit of detection was observed

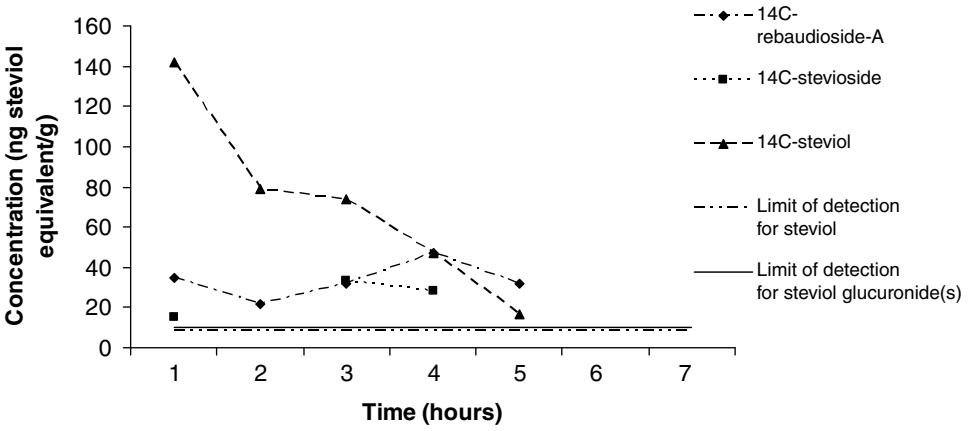

Fig. 1. Mean concentrations of radioactivity in the plasma of male rats following a single 5 mg/kg oral dose of ^{14}C-rebaudioside A, 4.2 mg/kg oral dose of ^{14}C-stevioside, and 1.6 mg/kg oral dose of ^{14}C-steviol (Group 3).

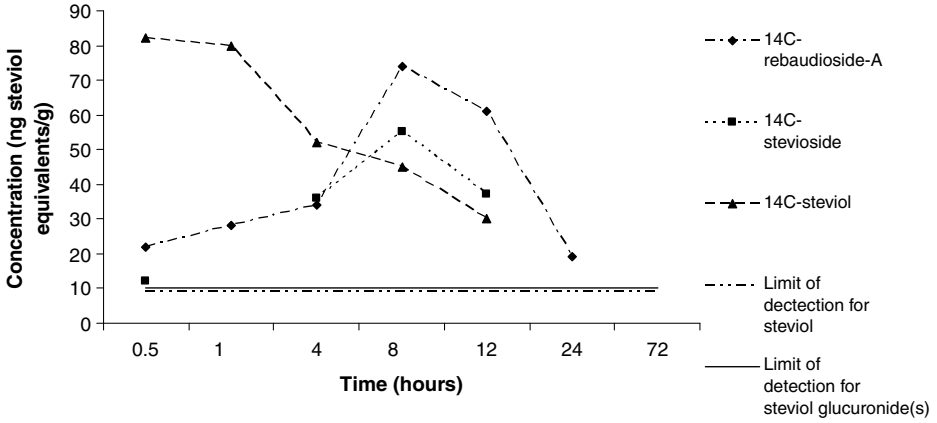

Fig. 2. Mean concentrations of radioactivity in the plasma of female rats following a single 5 mg/kg oral dose of ^{14}C-rebaudioside A, 4.2 mg/kg oral dose of ^{14}C-stevioside, and 1.6 mg/kg oral dose of ^{14}C-steviol (Experiment 3).

8 h after administration to both males and females (11.5 and 21.6 ng/mL, respectively), based on data for 2/3 and 3/3 animals, respectively. Steviol and/or steviol glucuronide(s) was detected in 13 of the 54 plasma samples from males and females combined, with concentrations representing between 69.0% and 108% (average 86.2%) of the total plasma radioactivity.

When ^{14}C-steviol was administered, measurable concentrations of steviol and steviol glucuronide(s) generally followed the concentrations of total radioactivity in plasma, with initial declines observed from 15 min and smaller secondary peaks at 1 or 2 h. Concentrations of steviol were above the limit of detection in at least 2/3 animals at each time point between 15 min and 24 h, but undetectable thereafter. The data were generally similar between the sexes, with the only appreciable difference, at 15 min, corresponding to a similar difference in total radioactivity concentration at this time. Steviol glucuronide concentrations were above the limit of detection in at least 2/3 animals at each time point between 15 min and 4 h (except for females at 1 and 2 h where it was detected in 1/3 animals), but undetectable thereafter. The concentrations were generally similar between the sexes. Of analyzable radioactivity (% chromatographed radioactivity), at 0.5 h post-dosing steviol and steviol glucuronide(s) accounted for 85.6% and 95.0% in males and females, respectively. At the 8 h post-dosing interval, steviol and its glucuronide conjugate(s) accounted for 89.3% and 92.4% of the analyzable radioactivity, respectively.

The pharmacokinetic parameters for total radioactivity measured in Experiment 3 are reported in Table 5. The observed peak plasma concentrations of total radioactivity in both males and females given stevioside or rebaudioside A occurred between 2 and 8 h after dosage and were slightly higher for stevioside than rebaudioside A. The peak plasma concentrations of steviol were detected in the first sample taken and therefore the data may underestimate the true C_{max} value; the C_{max} values were similar to those of the glycosides. For both sexes, the calculated AUC_{∞} and observed C_{max} values for rebaudioside A were lower than for stevioside. The AUC values for females given rebaudioside A or stevioside were higher than the corresponding values for males. The AUC values for steviol were similar for males and females. The AUC values for females, but not males, were lower for steviol than for stevioside and rebaudioside A, possibly because a significant contribution to the AUC for steviol was not measured prior to the first sample. The half-lives for total radioactivity were between 5 and 16 h for all three compounds in both sexes.

The pharmacokinetic parameters for steviol and steviol glucuronide(s) *per se* are not reported due to limited detection of these metabolites in the plasma of animals administered rebaudioside A or stevioside.

3.2. Metabolism and excretion

3.2.1. Excretion of radioactivity

The mean recoveries of radioactivity in the urine, feces, gastrointestinal tract, and carcass following a single oral dose of ^{14}C-rebaudioside A, ^{14}C-stevioside, and ^{14}C-steviol in male and female

Table 5
Pharmacokinetic parameters derived from mean total radioactivity concentrations in plasma following the administration of single oral doses of ^{14}C-rebaudioside A, ^{14}C-stevioside, and ^{14}C-steviol (Experiment 3)

Parameters for total radioactivity	Administered substance					
	Rebaudioside A		Stevioside		Steviol	
	Male	Female	Male	Female	Male	Female
C_{max} (ng equiv./g)	90	177	101	279	114	264
T_{max} (h)	2	8	4	8	0.25	0.25
AUC_{72} (ng equiv. h/g)	645	3329	1617	4287	1251	1604
AUC^{∞} (ng equiv. h/g)	630	3349	1607[a]	4359	1926[a]	1926[a]
k (h^1)	0.1462	0.0721	0.0795[a]	0.0460	0.0437[a]	0.0427[a]
$T_{1/2}$ (h)	5	10	9[a]	15	16[a]	16

C_{max} – maximum observed plasma concentration; T_{max} – time of maximum observed plasma concentration; AUC_{72} – AUC calculated from 0 to 72 h after administration; AUC_{∞} – AUC extrapolated to infinity using the terminal slope.
[a] Not all of the criteria for reliability were met (see the Section 2).

intact animals are presented in Table 6. Most of the radioactivity was detected in the feces, and accounted for about 97–98 % of the dose for both rebaudioside A and stevioside, and about 90% for steviol. For all compounds, the majority of the fecal radioactivity was excreted in the first 24 h (64–89%) with a further 10–22% excreted in feces between 24 and 48 h. Recovery of the radioactivity from the urine and cage wash fluid ranged from 1% to 3%. Radioactivity in the gastrointestinal tract at 96 h after administration ranged from 0.02% to 1.20%. No radioactivity was detected in the carcasses of rats of either sex at 96 h after administration of ^{14}C-rebaudioside A, while 0.01–0.10% was recovered from carcasses of rats dosed with ^{14}C-stevioside and ^{14}C-steviol. For the three test compounds, the mean total radioactivity recovered ranged from 90.1% to 100.2%.

In cannulated rats (Table 7) the majority of the radioactivity was recovered in the bile rather than in the feces. In both male and female rats, about 70–80% of the administered ^{14}C-rebaudioside A or ^{14}C-stevioside were recovered in bile within 24 h. Although there was limited excretion of radioactivity in the first 3 h of dosing, most of the biliary excretion occurred within the first 12 h after dosage. In contrast, the biliary excretion of radioactivity after ^{14}C-steviol was much more rapid, with about 50% and 70% of the dose eliminated in the bile of male and female rats within the first 3 h. The total recovery in bile over 48 h was 97–98% of the administered dose of steviol. For both rebaudioside A and stevioside, the remaining radioactivity was recovered in the feces (21–30%) and the urine and cage washings (1–2%). For steviol, only 1–2% of the administered dose was recovered in the feces, while the urine and cage washings accounted for a further 1% of the dose. Very low levels of radioactivity were detected at 96 h in the gastrointestinal tract and liver; no radioactivity was detected in the carcasses of any dose group. For the three test compounds, the mean total radioactivity recovered ranged from 98.8% to 104.4%.

The extent of absorption was estimated by summing the mean total amount of radioactivity in the bile, urine (including cage wash fluid), liver, and the remaining carcass. Using this method, approximately 70.7% and 82.0% of the dose of ^{14}C-rebaudioside A was absorbed by males and females, respectively, 77.9% and 80.5% of the ^{14}C-stevioside dose was absorbed by males and females, respectively, and 97.2% and 99.4% of the ^{14}C-steviol dose was absorbed by males and females, respectively.

3.3. Chromatographic analysis

Urine was not analyzed chromatographically, because it did not contain an appreciable proportion of the administered dose in any treatment group.

Fecal extract pools were prepared separately for each dose group and sex using the samples collected during the first 48 h after administration. The pooled samples were analyzed directly by TLC (system D; see Section 2). The results of the chromatographic analysis of fecal samples for the intact and bile duct-cannulated rats are presented in Tables 8 and 9, respectively.

For intact animals administered ^{14}C-rebaudioside A (Table 8), the two predominant radioactive components in the feces were rebaudioside A (29% and 19% of the dose for males and females,

Table 6
Average excretion of radioactivity in intact rats after a single oral dose of ^{14}C-rebaudioside A, ^{14} C-stevioside, or ^{14}C-steviol (5, 4.2, or 1.6 mg/kg, respectively)[a,b]

Sample	^{14}C-rebaudioside A		^{14}C-stevioside		^{14}C-steviol	
	Male	Female	Male	Female	Male	Female
Total urine (0–96 h)	1 (1)	1 (0.32)	1 (0.43)	1 (1)	3 (1)	3 (1)
Total cage wash (0–96 h)	0.11 (0.09)	0.29 (0.16)	0.32 (0.26)	0.09 (0.06)	0.32 (0.34)	0.09 (0.06)
Total urine and cagewash (0–96 h)	1 (1)	1 (0.35)	1 (1)	1 (1)	3 (1)	3 (1)
Feces (h)						
0–24	86 (10)	89 (5)	87 (8)	82 (6)	67 (5)	64 (3)
24–48	10 (6)	8 (4)	10 (6)	14 (3)	19 (3)	22 (2)
48–72	1 (2)	1 (1)	2 (1.26)	2. (1)	4 (1)	1 (0.33)
72–96	0.21 (0.40)	0.12 (0.10)	0.21 (0.22)	1 (1)	4 (2)	1 (0.39)
Total feces	97 (2)	98 (1)	98 (1)	98 (4)	93 (5)	87 (2.22)
Gastrointestinal tract and contents	0.10 (0.20)	0.03 (0.03)	0.02 (0.03)	1.2 (2)	0.13 (0.03)	0.23 (0.11)
Remaining carcass	ND	ND	0.04 (0.08)	0.07 (0.15)	0.10 (0.03)	0.01 (0.03)
Total recovery	98.4 (0.47)	98.5 (1)	99.0 (1)	100.2 (0.33)	96.5 (6)	90.1 (2)

ND = not detected.
[a] Results are expressed as % dose.
[b] Mean (standard deviation) based on five animals/sex/group.

Table 7
Excretion of radioactivity in bile duct-cannulated rats after a single oral dose of ^{14}C-rebaudioside A, ^{14}C-stevioside, or ^{14}C-steviol (5, 4.2, or 1.6 mg/kg, respectively)[a,b]

Sample	^{14}C-rebaudioside A		^{14}C-stevioside		^{14}C-steviol	
	Male	Female	Male	Female	Male	Female
Bile (h)						
0–3	1 (0.36)	9 (15)	2 (1)	3 (2)	69 (15)	51 (4)
3–6	28 (9)	29 (2)	42 (11)	42 (7)	22 (4)	35 (7)
6–9	23 (6)	27 (13)	25 (7)	21 (4)	5 (5)	5 (2)
9–12	13 (3)	12 (6)	7 (2)	10 (1)	0.45 (0.16)	4 (7)
12–24	4 (2)	4 (3)	2 (1)	5 (4)	0.15 (0.09)	2 (3)
24–48	0.10 (0.01)	0.08 (0.05)	0.04 (0.02)	0.09 (0.10)	0.05 (0.03)	0.11 (0.14)
Total bile	69 (2)	80 (3)	77 (4)	79 (6)	97 (9)	98 (5)
Total urine (0–48 h)	1 (0.48)	2 (0.32)	1 (0.23)	1 (1)	0.47 (0.24)	1 (1)
Cagewash (0–48 h)	0.21 (0.29)	0.05 (0.02)	0.05 (0.02)	0.03 (0.04)	0.01 (0.02)	0.01 (0.01)
Total urine and cagewash (0–48 h)	1 (1)	2 (0.33)	1 (0.22)	1 (1)	0.48 (0.26)	1 (1)
Feces (h)						
0–24	30 (3)	20 (2)	23 (4)	22 (6)	1 (0.23)	1 (0.35)
24–48	0.32 (0.14)	1 (1)	0.34 (0.12)	2. (2)	0.07 (0.04)	0.06 (0.04)
Total feces	30 (3)	21 (2)	23 (4)	24 (4)	2 (0.19)	1 (0.34)
Liver	0.01 (0.01)	0.01 (0.01)	<0.01 (0.01)	0.01 (0.01)	0.01 (0)	0.01 (0)
Gastrointestinal tract and contents	0.02 (0.01)	0.02 (0.02)	0.03 (0.02)	0.02 (0.01)	ND	0.01 (0.01)
Remaining carcass	ND	ND	ND	ND	ND	ND
Total recovery	101.0 (1)	103.0 (1)	101.2 (1)	104.4 (2)	98.8 (9)	100.8 (5)

ND = not detected.
[a] Results are expressed a % dose.
[b] Mean (standard deviation) based on three animals/sex/group selected randomly from the five animals/sex/group that completed the *in vivo* phase.

respectively) and steviol (44% and 57% of the dose for males and females, respectively). Stevioside and steviol glucuronide(s) were also detected, but at levels of <5%. Steviol and steviol glucuronide(s) were the predominant radioactive components in the feces of intact animals administered ^{14}C-stevioside, accounting for 56% and 14% of the dose, respectively, in males, and 72% and 10% of the dose, respectively, in females; stevioside accounted for 12% and 2% of the dose in males and females, respectively. Steviol was the predominant radioactive component of the feces of rats dosed with ^{14}C-steviol, accounting for 69% and 74% of the dose in males and females, respectively. Rebaudioside A, stevioside, and steviol glucuronide(s) were not detected.

In the bile duct-cannulated animals (Table 9) administered ^{14}C-rebaudioside A, the predominant radioactive component of the feces was steviol, accounting for 25% and 16% of the dose for males and females, respectively. Rebaudioside A, stevioside, and steviol glucuronide(s) were also detected but at levels of <2%. Steviol was also the predominant radioactive component in the feces of cannulated rats dosed with ^{14}C-stevioside, accounting for 18% and 16% of the dose for males and females, respectively. The feces from cannulated rats given ^{14}C-steviol were not analyzed due to the low levels of radioactivity present (Table 7).

Pooled bile samples were prepared for each dose group and sex using the samples collected during the 48-h period following administration and analyzed directly by TLC (system D; see Section 2) (Table 10). Steviol glucuronide conjugate(s) was/were the predominant radioactive component(s) in the bile of rats dosed with ^{14}C-rebaudioside A, ^{14}C-stevioside, or ^{14}C-steviol. The parent glycoside compounds and steviol were also detected in bile at levels <1% in all groups. Identities of rebaudioside A, stevioside, steviol, and steviol glucuronide(s) in feces and bile were established by chromatographic comparisons. The identity of steviol glucuronide(s) in bile was further confirmed by incubation of bile with an enzyme mixture containing β-glucuronidase (see Section 2). Unidentified compounds in bile represented about 9%, 3% and 5% for rebaudioside A, stevioside and steviol, respectively.

No sex differences were apparent in the routes or extents of elimination, or in the pattern of metabolites excreted.

Table 9
Radioactive components in the feces of bile duct-cannulated animals expressed as % dose

Administered substance	Rebaudioside A		Stevioside	
Sex	Male	Female	Male	Female
Rebaudioside A	0.3	ND	ND	ND
Steviol glucuronide(s)	0.4	1	0.4	1
Steviol	25	16	18	16
Others[a]	3	1	2	3

Data for steviol are not given as the feces were a negligible proportion of the administered dose. ND = not detected.
[a] Total radioactivity in regions of the chromatogram other than those specified.

Table 8
Radioactive components in the feces of intact animals expressed as % dose

Administered substance	Rebaudioside A		Stevioside		Steviol	
Sex	Male	Female	Male	Female	Male	Female
Rebaudioside A	29	19	ND	ND	ND	ND
Stevioside	3	4	12	2	ND	ND
Steviol glucuronide(s)	2	3	14	10	ND	ND
Steviol	44	57	56	72	69	74
Others[a]	14	10	11	9	15	9

ND = not detected.
[a] Total radioactivity in regions of the chromatogram other than those specified.

Table 10
Radioactive components in bile expressed as % dose

Administered substance	Rebaudioside A[b]		Stevioside[b]		Steviol[c]	
	Male	Female	Male	Female	Male	Female
Rebaudioside A	0.61	0.51	ND	ND	ND	ND
Stevioside	0.61	0.51	0.4	1.1	ND	ND
Steviol glucuronide(s)	59.5	71.7	68.9	68.7	90.5	91.1
Steviol	0.6	0.8	0.4	0.2	0.5	0.8
Others[a]	8.9	7.2	3.8	2.1	5.2	4.5

ND = not detected.
[a] Total radioactivity in regions of the chromatogram other than those specified.
[b] Bile collected between 0 and 24 h.
[c] Bile collected between 3 and 12 h.

4. Discussion

Comparisons of the pharmacokinetic parameters of total radioactivity following administration of ^{14}C-rebaudioside A, ^{14}C-stevioside, and ^{14}C-steviol (Table 1 and Figs. 1 and 2) indicate that the pharmacokinetics of ^{14}C-rebaudioside A and ^{14}C stevioside are similar, while the pharmacokinetics of ^{14}C-steviol differ, especially in the rate of absorption.

The differences in pharmacokinetic curves noted between the three substances support the previous reports (Wingard et al., 1980; Gardana et al., 2003; Koyama et al., 2003a) that the glycoside units are cleaved from the aglycone portion of the molecule by intestinal microflora prior to absorption. Because rebaudioside A contains one more additional glucose unit (a total of four) than stevioside, it was anticipated that peak plasma levels would be lower in comparison to stevioside based on the likely increased time for microbial hydrolysis. As expected, steviol was rapidly absorbed since no prior hydrolysis was necessary.

Qualitatively, all plasma samples had similar metabolite profiles, with the predominant radioactive component in all samples identified as steviol (Tables 2–4). Lower amounts of steviol glucuronide(s) and still lower amounts (in most cases) of one or two, unidentified metabolites were also recorded. The chromatographic properties of the unidentified metabolites indicated that they were of intermediate polarity compared with steviol and its glucuronide(s), and were probably oxidized steviol metabolites as previously proposed by Koyama et al. (2003b).

The observed and calculated PK parameters (Table 5) indicate that the peak plasma concentration (C_{max}) and AUC for stevioside were similar but slightly higher than those of rebaudioside A. This would indicate that there was slightly greater formation of steviol from stevioside than from rebaudioside A; this is consistent with the recovery of smaller amounts of the parent glycoside in the feces of intact rats given stevioside (Table 8).

The plasma kinetic data (Table 5) indicate a sex difference in the C_{max} and AUC for the total radioactivity, which was less apparent for steviol and was not reflected in the excretion data (Table 6) or the metabolism data (Tables 8–10). A possible explanation for the data is that more free steviol entered the circulation of female rats following oral administration of the glycosides, or possibly due to more extensive first-pass conjugation in the gut wall or liver of male rats. However the plasma data (Tables 2 and 3) did not show higher levels of glucuronide(s) in male rats. An alternative explanation would be that the systemic clearance of radioactivity, due to oxidation, conjugation and biliary excretion, might be higher in males than in females; however, this would be expected to result in a sex difference after steviol administration, which was not found.

The extent of absorption from the gastrointestinal tract was estimated from the different amounts of radioactivity in the urine and bile of bile duct-cannulated animals, and retained in the liver and carcass (Table 7). Approximately 71% and 82% of a 5 mg/kg body weight dose of ^{14}C-rebaudioside A was absorbed by males and females, respectively, compared with 78% or 81% of an equivalent dose of ^{14}C-stevioside absorbed by males and females, respectively. In contrast, 97% or 99% of an equivalent dose of ^{14}C-steviol was absorbed by males and females, respectively.

Steviol was quantitatively the most significant radioactive component in the feces of intact rats dosed with ^{14}C-rebaudioside A, ^{14}C-stevioside, or ^{14}C-steviol (Table 8). In intact animals administered either ^{14}C-rebaudioside A or ^{14}C-stevioside, the only other significant components identified were the parent glycoside and steviol glucuronide(s). Steviol was also the principal radioactive component in the feces of bile duct-cannulated rats treated with ^{14}C-rebaudioside A or ^{14}C-stevioside (16–25% of the dose) (Table 9). The absence of the parent glycoside in the feces of bile duct-cannulated rats given rebaudioside A may have arisen from *ex vivo* hydrolysis under the conditions of the experiment.

Steviol glucuronide(s) was/were the predominant radioactive component(s) in the bile (Table 10) of cannulated rats treated with ^{14}C-rebaudioside A, ^{14}C-stevioside, or ^{14}C-steviol. Because of the incomplete absorption of the glycosides, the percentage of steviol glucuronide(s) was much greater in the bile of rats dosed with ^{14}C-steviol (>90% of the dose) than those administered either ^{14}C-rebaudioside A or ^{14}C-stevioside (55–69% of the dose). The metabolite profile following administration of ^{14}C-rebaudioside A, ^{14}C-stevioside, and ^{14}C-steviol is indicative of rapid first pass Phase II metabolism (*i.e.*, glucuronidation). Steviol glucuronide(s) are subsequently eliminated in the bile and de-conjugated in the gastrointestinal tract prior to excretion in the feces. Some of the released steviol may undergo reabsorption and uptake, conjugation and elimination by the liver as part of enterohepatic circulation.

The results of the current study indicate that rebaudioside A, stevioside, and steviol are extensively absorbed after oral dosing. In agreement with the results reported by Gardana et al. (2003), Geuns et al. (2003, 2007) and Koyama et al. (2003a), both rebaudioside A and stevioside appear to be metabolized to steviol by gut microflora. Steviol appears to be metabolized to steviol glucuronide(s) within the body and excreted into the gastrointestinal tract via the bile. These observations support the results reported by Geuns et al. (2007), where administration of stevioside to volunteers resulted in the detection of steviol glucuronide(s) in the plasma but not of free stevioside, free steviol, or other free steviol metabolites. Due to the low levels of steviol glucuronide(s) and high levels of steviol in the feces of intact rats, it appears that the majority of steviol glucuronide(s) is/are hydrolyzed back to steviol by intestinal microflora. This supports the hypothesis of enterohepatic circulation reported in a previous study (Nakayama et al., 1986). However, the plasma concentration–time curves and especially the rate of biliary excretion indicate that enterohepatic recirculation is limited in extent.

Rebaudioside A, stevioside, and steviol all appear to be rapidly excreted as steviol, primarily in the feces. Previous administrations of stevioside to pigs and humans were reported to result in the rapid excretion of steviol *via* the urine and feces (Geuns

et al., 2003, 2007). Analyses of urine from human volunteers administered stevioside reported the detection of only steviol glucuronide (Geuns et al., 2006, 2007). The results from the toxicokinetic and metabolism studies of both rebaudioside A and stevioside indicate a close similarity in handling of all steviol glycosides in the Sprague–Dawley rat, which corroborate the metabolic results from previously reported studies administering stevioside in the Wistar rat (Nakayama et al., 1986). The similarity in the handling of steviol glycosides across rat strains would be predicted given the similarity in metabolic disposition across all animal species including humans, where it has been shown that administration of rebaudioside A and stevioside results in metabolism to steviol prior to absorption followed by extensive glucuronidation to steviol glucuronide.

In summary, pharmacokinetic and metabolic results from radiolabelled studies conducted in intact and bile duct-cannulated animals indicated that rebaudioside A was absorbed and metabolized in a similar manner to that of stevioside following oral administration. The data from the intact animal studies demonstrated that the majority of the dose was excreted rapidly in the feces for rebaudioside A, stevioside, and steviol with limited urinary elimination. Studies conducted in bile duct-cannulated animals provided additional data indicating that both of these glycosides are extensively absorbed (approximately 80%) and subsequently eliminated in the bile as steviol glucuronide(s). The remainder of the dose was excreted directly *via* the feces. In contrast, steviol was shown to be essentially completely absorbed and subsequently eliminated in the bile. The similarities in the kinetics and metabolism of rebaudioside A and stevioside support the use of previous toxicological studies conducted with stevioside for the safety evaluation of rebaudioside A.

Conflict of interest statement

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References

Burton, D.A., 2007. Validation of an analytical method to measure steviol and steviol glucuronide in rat plasma. Huntingdon Life Sciences Confidential Report No. CNX 0011/063636.

Cardoso, V.N., Barbosa, M.F., Muramoto, E., Mesquita, C.H., Almeida, M.A., 1996. Pharmacokinetic studies of ^{131}I-stevioside and its metabolites. Nucl. Med. Biol. 23, 97–100.

Gardana, C., Simonetti, P., Canzi, E., Zanchi, R., Pietta, P., 2003. Metabolism of stevioside and rebaudioside A from *Stevia rebaudiana* extracts by human microflora. J. Agric. Food Chem. 51, 6618–6622.

Geuns, J.M., Augustijns, P., Mols, R., Buyse, J.G., Driessen, B., 2003. Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. Food Chem. Toxicol. 41, 1599–1607.

Geuns, J.M., Buyse, J., Vankeirsbilck, A., Temme, E.H., 2007. Metabolism of stevioside by healthy subjects. Exp. Biol. Med. 232, 164–173.

Geuns, J.M., Buyse, J., Vankeirsbilck, A., Temme, E.H., Compernolle, F., Toppet, S., 2006. Identification of steviol glucuronide in human urine. J. Agric. Food Chem. 54, 2794–2798.

JECFA, 2006. Safety evaluation of certain food additives. Prepared by the Sixty-third Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) WHO Food Additive Series 54. World Health Organisation, Geneva.

Koyama, E., Kitazawa, K., Ohori, Y., Izawa, O., Kakegawa, K., Fujino, A., Ui, M., 2003a. In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. Food Chem. Toxicol. 41, 359–374.

Koyama, E., Sakai, N., Ohori, Y., Kitazawa, K., Izawa, O., Kakegawa, K., Fujino, A., Ui, M., 2003b. Absorption and metabolism of the glycosidic sweeteners stevia mixture and their aglycone, steviol, in rats and humans. Food Chem. Toxicol. 41, 875–883.

Nakayama, K., Kasahara, D., Yamamoto, F., 1986. Absorption distribution metabolism and excretion of stevioside in rats. Shokuhin Eiseigaku Zasshi 27, 1–8.

Renwick, A.G., 2008. The use of a sweetener substitution method to predict dietary exposures for the intense sweetener rebaudioside A. Food Chem. Toxicol. 46/7S, S61–S69.

Soejarto, D.D., Kinghorn, A.D., Farnsworth, N.R., 1982. Potential sweetening agents of plant origin. III: Organoleptic evaluation of Stevia leaf herbarium samples for sweetness. J. Nat. Prod. 45, 590–599.

Sung, L.H., 2002. Report on pharmacokinetic (PK) studies in rats. Unpublished report from Sunlabel Pte Ltd., Singapore. Submitted to WHO by the Ministry of Health and Welfare, Japan, 2002 (Cited in: JECFA, 2006).

Wingard Jr., R.E., Brown, J.P., Enderlin, F.E., Dale, J.A., Hale, R.L., Seitz, C.T., 1980. Intestinal degradation and absorption of the glycosidic sweeteners stevioside and rebaudioside A. Experientia 36, 519–520.