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In vitro metabolism of rebaudioside E under anaerobic conditions: Comparison with rebaudioside A

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ABSTRACT

The hydrolysis of the steviol glycosides rebaudioside (Reb) A and E, as well as steviolbioside (a metabolic intermediate) to steviol was evaluated *in vitro* using human fecal homogenates from healthy Caucasian and Asian donors. Incubation of each of the Rebs in both groups resulted in a rapid hydrolysis to steviol. Metabolism of 0.2 mg/mL sample was complete within 24 h, with the majority occurring within the first 16 h. There were no clear differences in the rate or extent of metabolism of Reb E relative to the comparative control Reb A. The hydrolysis of samples containing 2.0 mg/mL of steviol glycosides Reb A and Reb E tended to take slightly longer than 0.2 mg/mL samples. Herein, we report for the first time that there were no apparent gender or ethnicity differences in the rate of metabolism of any of the Rebs, regardless of the concentrations tested. Steviolbioside, an intermediate in the hydrolysis of Reb E to steviol was also found to be rapidly degraded to steviol. These results demonstrate Reb E is metabolized to steviol in the same manner as Reb A. These data support the use of toxicology data available on steviol, and on steviol glycosides metabolized to steviol (*i.e.*, Reb A) to underpin the safety of Reb E.

1. Introduction

Stevia rebaudiana (Bertoni) is a shrub indigenous to southern South America and is cultivated in many parts of the world (Carakostas et al., 2008; Ceunen and Geuns, 2013). Extracts of *S. rebaudiana* have been used commercially to sweeten foods in Japan and other Southeast Asian countries for a number of years (Ceunen and Geuns, 2013). Steviol glycosides have been identified as the compounds associated with the sweetness of stevia extracts. The stevia leaf contains several steviol glycosides with Reb A and stevioside being the most abundant. Additional steviol glycosides include Rebs B, C, D, M, F, steviolbioside, and dulcoside A. A novel steviol glycoside, Reb E, was recently identified in the leaf of the *S. rebaudiana* plant. Steviol glycosides are typically 200–350 times sweeter than sucrose. As a result, stevia sweeteners can be used to produce low and no-calorie products.

Steviol glycoside preparations containing not less than 95% total steviol glycosides and meeting specifications and purity criteria (also known by the common name stevia leaf extract), have been approved for use as sweeteners in food in Europe and elsewhere (EFSA, 2010; JECFA, 2010). Based on their review of the available data, the European Food Safety Authority (EFSA, 2010) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2010) established an "acceptable daily intake" (ADI) of 0–4 mg/kg body weight/day for steviol glycosides, expressed as steviol equivalents. This ADI is applicable to all steviol glycosides since they are all considered to be metabolized to the same common metabolite steviol. Recently, in the United States, stevia leaf extracts consisting of predominantly Reb A, Reb D and Reb M meeting the JECFA purity specification have been determined to be "generally recognized as safe" (GRAS) for use in various food applications (Food and Administration, 2008a,b).

Rebaudioside E (Fig. 1) is one of the glycosides of the diterpene derivative, steviol (ent-13-hydroxykaur-16-en-18-oic acid) which occurs naturally in the plant, *S. rebaudiana* (Bertoni) Bertoni (Compositae). As the structure of Reb E and Reb A are similar, it

Abbreviation: LLOQ, lower limit of quantitation.

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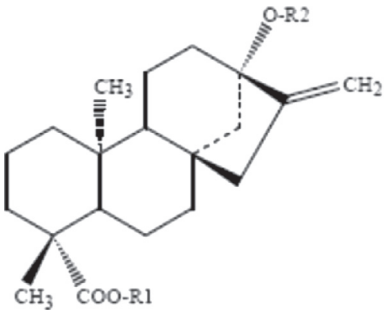

Test Article or Compound Name	R1	R2
Steviol (final metabolite)	H	H
Steviolbioside (metabolite intermediate)	H	β-Glc- β-Glc (2→1)
Rebaudioside A (positive control, CAS 58543-16-1)	ß-Glc	ß-Glc-ß-Glc (2→1) | ß-Glc (3→1)
Rebaudioside E (test article, CAS 63279-14-1)	β-Glc- β-Glc (2→1)	β-Glc- β-Glc (2→1)

Glc = β-D-glucopyranosyl

Fig. 1. Structures of steviol, rebaudiosides A, E, and steviolbioside.

was hypothesized that both of these compounds would be metabolized by gut microflora to the aglycone steviol (Renwick and Tarka, 2008). An *in vitro* model using human fecal homogenates has been shown previously to be representative of the *in vivo* metabolism of Reb A and other steviol glycosides (Gardana et al., 2003; Koyama et al., 2003; Purkayastha et al., 2014). Thus, the current studies presented here were conducted in order to demonstrate that the *in vitro* metabolism of Reb E is similar to that of Reb A. This would validate the use of safety data on steviol and Reb A/stevioside to support the safety of Reb E as was shown previously for Reb B, D, and M (Purkayastha et al., 2014). We also explored whether gender or ethnicity play a role in the rate of steviol glycoside metabolism. In order make this assessment, the rate and extent of steviol glycoside metabolism was compared between genders (male, female volunteers) and between healthy Caucasian and Asian volunteers. The results of these studies are reported herein.

2. Materials and methods

2.1. Study design

Experimental parameters including anaerobic culture bacterial density and anaerobic buffers have been optimized prior to this study and as previously described (Purkayastha et al., 2014). Temporal changes in the human microbiota and inter-individual variations in the composition of microbial content between genders and age groups have been described and reviewed in the literature (Hopkins and Macfarlane, 2002; Lozupone et al., 2012; Marteau et al., 2001). In this study, to provide a representative human fecal microbiota in the metabolism of rebaudioside E, the use of pooled human fecal homogenates from 6 Caucasians and 6 Asians consisting of equal male and female subjects were used to provide a human microbiota composite for evaluating the metabolic fate of rebaudioside E. An optimal human fecal anaerobic culture for investigating the metabolism of Reb E involved the use of pooled fecal homogenate ($n = 3$ donors) diluted 12.5-fold in an anaerobic phosphate buffer, followed by 3-fold dilution with Brain–Heart Infusion Broth (BHI) and the addition of Oxyrase™ for maintaining anaerobic conditions during incubation to achieve an overall 50-fold diluted pooled fecal homogenate.

Table 1
Evaluation of assay LLOQ and dilution integrity of steviol and steviolbioside in support of human fecal homogenate sample analysis.

LLOQ samples	Expected conc. (ng/mL)	Reported conc. (ng/mL)	% Bias
Steviol			
LLOQ 50 ng/mL-1	50.0	55.3	10.6
LLOQ 50 ng/mL-2	50.0	54.2	8.4
LLOQ 50 ng/mL-3	50.0	58.0	16.0
LLOQ 50 ng/mL-4	50.0	63.5	27.0
LLOQ 50 ng/mL-5	50.0	56.8	13.6
LLOQ 50 ng/mL-6	50.0	54.2	8.4
Mean		57.0	14.0
% CV		6.2	
Dilution integrity samples	Expected conc. (ng/mL)	Reported conc. (ng/mL)	% Bias
Dilution 10×-1	200	175	−12.5
Dilution 10×-2	200	185	−7.5
Dilution 10×-3	200	186	−7.0
Dilution 10×-4	200	188	−6.0
Dilution 10×-5	200	186	−7.0
Dilution 10×-6	200	195	−2.5
Mean		186	−7.1
% CV		3.5	
Dilution 100×-1	100	95.2	−4.8
Dilution 100×-2	100	99.9	−0.1
Dilution 100×-3	100	96.2	−3.8
Dilution 100×-4	100	99.2	−0.8
Dilution 100×-5	100	97.8	−2.2
Dilution 100×-6	100	104	4.0
Mean		98.7	−1.3
% CV		3.2	
Dilution 400×-1	100	105	5.0
Dilution 400×-2	100	99.4	−0.6
Dilution 400×-3	100	97.1	−2.9
Dilution 400×-4	100	105	5.0
Dilution 400×-5	100	107	7.0
Dilution 400×-6	100	113	13.0
Mean		104	4.4
% CV		5.4	

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