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In vitro metabolism of rebaudioside B, D, and M under anaerobic conditions: Comparison with rebaudioside A

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ABSTRACT

The hydrolysis of the steviol glycosides rebaudioside A, B, D, and M, as well as of steviolbioside (a metabolic intermediate) to steviol was evaluated *in vitro* using human fecal homogenates from healthy donors under anaerobic conditions. Incubation of each of the rebaudiosides resulted in rapid hydrolysis to steviol. Metabolism was complete within 24 h, with the majority occurring within the first 8 h. There were no clear differences in the rate or extent of metabolism of rebaudioside B, D, or M, relative to the comparative control rebaudioside A. The hydrolysis of samples containing 2.0 mg/mL of each rebaudioside tended to take slightly longer than solutions containing 0.2 mg/mL. There was no apparent gender differences in the amount of metabolism of any of the rebaudiosides, regardless of the concentrations tested. An intermediate in the hydrolysis of rebaudioside M to steviol, steviolbioside, was also found to be rapidly degraded to steviol. The results demonstrate that rebaudiosides B, D, and M are metabolized to steviol in the same manner as rebaudioside A. These data support the use of toxicology data available on steviol, and on steviol glycosides metabolized to steviol (*i.e.*, rebaudioside A) to substantiate the safety of rebaudiosides B, D, and M.

1. Introduction

Stevia rebaudiana (Bertoni) is a shrub indigenous to southern South America and which is cultivated in many parts of the world (Gardana et al., 2003; Koyama et al., 2003; Carakostas et al., 2008). Extracts of *S. rebaudiana* have been used commercially to sweeten foods in Japan and other Southeast Asian countries for a number of years (Koyama et al., 2003). Steviol glycosides have been identified as the compounds associated with the sweetness of Stevia extracts. These steviol glycosides include a series of rebaudiosides (*e.g.*, A, B, C, D, E, and F), stevioside, steviolbioside, and dulcoside A. These substances are about 200–300 times the sweetness of sucrose (Soejarto et al., 1982; Scheline, 1991; Hanson and De Oliviera, 1993). Stevioside and rebaudioside A have until recently been the glycosides of most commercial interest with respect to their sweetening properties (Carakostas et al., 2008; EFSA, 2010; JECFA, 2010).

Steviol glycoside preparations containing not less than 95% steviol glycosides, including components such as stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, dulcoside A, rubusoside and steviolbioside, and meeting specifications and purity criteria, 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 4 mg/kg body weight/day for steviol glycosides, expressed as steviol equivalents. This ADI is applicable to all steviol glycosides since they are considered to all be metabolized to the same common metabolite steviol. In the United States, rebaudioside A and several mixtures of steviol glycosides meeting the JECFA specification have been established to be "generally recognized as safe" (GRAS) for use in various food applications (U.S, 2008a,b; U.S, 2009a,b,c,d; U.S, 2010a,b; U.S, 2011a,b,c; U.S, 2012a,b,c).

Abbreviations: %CV, percent coefficient of variation; ADI, acceptable daily intake; BHI, brain heart infusion broth; DMSO, dimethyl sulfoxide; F1, F2, female fecal homogenate samples #1 and #2; FDA, United States Food and Drug Administration; GRAS, generally recognized as safe; HPLC, high performance liquid chromatography; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LC/MS, liquid chromatography/mass spectrometry; LLOQ, lower limit of quantitation; M1, M2, male fecal homogenate samples #1 and #2; QC, quality control; v/v, volume-to-volume.

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Critical to the establishment of the safety of rebaudioside A/stevioside was the consideration that both of these compounds were metabolized by gut microflora to the aglycone steviol (Renwick and Tarka, 2008). The ADI for all steviol glycosides was based on steviol. Thus, the toxicology database for stevioside and/or rebaudioside A can extend to other steviol glycosides shown to be metabolized to steviol (Carakostas et al., 2008; Renwick and Tarka, 2008).

Since the publication of the EFSA (2010) and JECFA (2010) opinions on steviol glycosides for use in foods, there has been research into the commercial use of other rebaudiosides, including rebaudioside B, D, and M as sweeteners for use in food based upon improvements in their sensory characteristics (Prakash et al., 2012; Nikiforov et al., 2013). Notifications of GRAS status of both rebaudioside D and M (also known as rebaudioside X) have been submitted to the United States Food and Drug Administration (FDA) for review (GRAS Associates, 2013; PureCircle Ltd., 2013). Rebaudiosides B, D, and M differ from rebaudioside A only in the number of glucose moieties, and/or the type of glycosidic bond, present in the esters of steviol. The structures of each of steviol, steviolbioside, and the rebaudiosides A, B, D, and M are presented in Fig. 1.

The determination of the safety of rebaudiosides B, D, and M is largely dependent upon the toxicology and clinical database available on steviol and on other steviol glycosides, such as rebaudioside A and stevioside, which are known to be completely metabolized to steviol (Koyama et al., 2003; Carakostas et al., 2008; 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 rebaudioside A and other steviol glycosides (Gardana et al., 2003; Koyama et al., 2003; Renwick and Tarka, 2008). Given this, the demonstration that rebaudiosides B, D, and M are also anaerobically metabolized completely to steviol by gut microflora would validate the use of safety data on steviol and rebaudioside A/stevioside to support the safety of rebaudiosides B, D, and M. As a result, an *in vitro* study using pooled human fecal homogenates was conducted to show that rebaudiosides B, D, and M, and an intermediate metabolite, steviolbioside, are metabolized to steviol in the same manner as rebaudioside A. The results of this study are reported herein.

2. Materials and methods

2.1. Materials

2.1.1. Test articles

Rebaudioside B (purity of 96.5%, Batch no. L200412P), rebaudioside A (purity 99.3%, Batch no. DR0311329), rebaudioside D (purity 96.46%, Batch no. PT301211), rebaudioside M (purity 97.33%, Batch no. PT140312), steviolbioside (purity 96.81%, Batch no. PT08111), and steviol (purity 97.37%, Batch no. PT270312) were provided by PureCircle Ltd. (Negeri Sembilan, Malayasia).

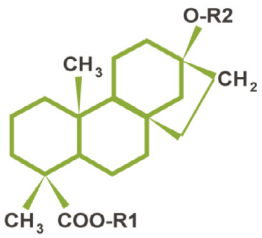
2.1.2. Solvents, reagents and liquid chromatography/mass spectrometry (LC/MS) solutions

Anaerobic phosphate buffer was prepared in house by BRI Biopharmaceutical Research. Brain heart infusion broth (BHI) (ID Nos. LCI-3108 and LCI-2989) and Oxyrase™ for the broth were obtained from Oxyrase, Inc. (Mansfield, OH USA). Dimethyl sulfoxide (DMSO) (purity > 99.9%) (Sigma Aldrich, St. Louis, MO, USA) was used as the solvent for steviol and for rebaudioside B and D. Deionized water (prepared in house by BRI Biopharmaceutical Research) was the solvent for rebaudioside A. The solvent for rebaudioside M was formamide (purity > 99.5%, Batch No. 6996TMV) (Sigma Aldrich, St. Louis, MO, USA). A 1:1 mixture of formamide and DMSO was used as the solvent for steviolbioside.

Abietic acid (internal standard for LC/MS) assay was obtained from Sigma Aldrich (St. Louis, MO USA). All reagents, including ammonium hydroxide, methanol, ammonium acetate in deionized water, and acetonitrile were of high performance liquid chromatography (HPLC) grade.

2.1.3. Human fecal samples

For the testing of rebaudiosides A, B, D, and steviol, human fecal materials were collected from six healthy male and six healthy female volunteer donors without known gastrointestinal disease and without previous ingestion of stevia-based natural sweeteners. The number of volunteers included was largely based on increasing the *n* values used in previous published research (Gardana et al., 2003; Koyama et al., 2003). The volunteers had not been exposed to laxatives or antimicrobial drugs for at least 7 days prior to the



Compound	R1	R2
Steviol	H	H
Rebaudioside A	β-Glc-	β-Glc-β-Glc (2→1) | β-Glc (3→1)
Rebaudioside B	H	β-Glc-β-Glc (2→1) | β-Glc (3→1)
Rebaudioside D	β-Glc-β-Glc (2→1)	β-Glc-β-Glc (2→1) | β-Glc (3→1)
Rebaudioside M	β-Glc-β-Glc (2→1) | β-Glc (3→1)	β-Glc-β-Glc (2→1) | β-Glc (3→1)
Steviolbioside	H	β-Glc-β-Glc (2→1)

Fig. 1. Structures of steviol, rebaudiosides A, B, D, and M, and steviolbioside.