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Opinion on

STEVIOSIDE AS A SWEETENER

(adopted on 17/6/99)

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Terms of Reference

To re-evaluate the safety of stevioside (13-[(2-O-β-D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid-4α-β-D-glucopyranosyl ester), a glycoside of the diterpene derivative steviol, extracted and refined from *Stevia rebaudiana Bertoni* leaves, in use as a sweetener in light of additional information received.

Background

Stevioside is a high intensity sweetener, 250-300 times that of sucrose, intended to be used in a wide range of low or reduced calorie food products and beverages. *Stevia rebaudiana* is native in South America. Both the plant and extracts of the plant have been used for several years as a sweetener in South America, Asia, Japan and China.

Stevioside was considered by the Committee for the first time during its comprehensive review of sweeteners in 1985 (1). The review on the safety of stevioside was updated by the Committee in 1989 (2). In both these opinions, several questions of concern were raised by the Committee regarding the purity of the extracts that had been tested, the metabolism of stevioside, mutagenicity of metabolites, questionable chronic toxicity and carcinogenicity studies, and finally fertility and teratogenicity studies not performed according to Good Laboratory Practice. In conclusion, the Committee could not accept its use based on the submitted documentation and stevioside extracts from *Stevia rebaudiana* leaves were considered as toxicologically not acceptable.

Since that time the Commission has received a further application (3) and a subsequent addendum to the application (4) to use stevioside, extracted and refined from *Stevia rebaudiana Bertoni* leaves, as a sweetener.

In addition to the limited information submitted by the petitioner, other published studies were considered.

The FAO/WHO Joint Expert Committee on Food Additives and Contaminants (JECFA) considered stevioside in 1998 but concluded that it was not possible to allocate an Acceptable Daily Intake on the data available (5).

Nature of stevioside preparations

Stevioside is isolated and purified from *Stevia rebaudiana Bertoni* leaves. A range of stevioside preparations of varying purity have been proposed or are in use outside the EU. The leaves naturally contain a complex mixture of eight sweet diterpene glycosides, including stevioside, steviolbioside, rebaudiosides (A,B,C,D,E) and dulcoside A. However, for the majority of toxicological studies, a precise composition of the extract that has been tested has not been adequately defined. In particular, studies on preparations of stevioside of higher than 95% purity are limited in number.

Most studies have been conducted using crude extracts of *S. rebaudiana* or low purity mixtures of stevioside and other sweet glycosides of *S. rebaudiana*. The present application refers to stevioside isolated and purified from *S. rebaudiana* leaves after multiple and selective extractions, followed by recrystallisation, resulting in a stevioside purity higher than 95%, with rebaudioside A as the main impurity (≤2%).

Toxicological evaluation

Metabolism studies: After oral application of radio-labelled stevioside 1.5 % of the radioactivity is excreted in the urine of intact rats, whereas in rats with a ligated bile duct 96 % of the radio-activity is excreted in the urine (6). This indicates enterohepatic circulation of stevioside and/or its metabolites with an elimination half-life of 24 hrs.

After oral administration of stevioside to the rat, a major part seems to be degraded by the gut flora to steviol (6). In vitro studies using the rat intestinal microflora have shown that the degradation, within 2 days, of stevioside and rebaudioside A to steviol are approximately 100% and 65%, respectively (7). Stevioside is not absorbed, but steviol is readily absorbed, later excreted in the bile as conjugates which subsequently are excreted in the faeces. (6,7). Wingard et al (7) also concluded that degradation of stevioside may occur in man. Degradation of stevioside was recently shown to occur by various digestive enzymes from the gastrointestinal tract of different animal species (8). The microflora of the human faeces metabolised stevioside to both steviol and steviol-16,17α-epoxide (8).

Thus, steviol and its epoxide may be formed and subsequently absorbed. Since steviol is mutagenic in vitro (see later), its formation is of potential concern. Pharmaco-kinetics (ADME-studies) in humans, with particular reference to the intestinal uptake and metabolic pattern after oral administration would be essential for the safety evaluation of stevioside.

Mutagenicity studies: Several studies on stevioside crude crystals are available and indicate that the compound is not mutagenic. This has repeatedly been shown in several bacterial assays, i.e. the Ames test in *S. typhimurium*, forward mutation test in *B. subtilis*, spore rec assay and tests for chromosomal aberration in mammalian cells in CHL and human lymphocytes in vitro (5).

However, the stevioside metabolite steviol shows mutagenic activity in the forward mutation assay using *S. typhimurium* TM677 in the presence of a metabolic activation system, as well as in CHL cells in vitro (gene mutations and chromosomal aberrations) (9,10). Steviol was negative in the mouse micronucleus test (10). Thus, since metabolism studies using the human intestinal microflora show that bio-transformation to steviol may occur (see metabolism studies above), additional mutagenicity studies are needed. Steviol needs to be further studied in mammalian systems in vivo.

Acute toxicity: Stevioside and steviol have very low acute oral toxicity in the mouse, rat and hamster (11-13).

Subchronic toxicity: Three subchronic oral toxicity studies in the rat have been published, two in Japanese (12,14) and another in Korean (15) of which only English summaries are available. In one of the rat studies (14), a 13-week dose-finding study,

cell necrosis in the liver of all treated males was reported (lowest dose equivalent to 155 mg/kg bw). However, the Committee was not able to evaluate these studies.

Chronic toxicity and carcinogenicity: Yamada et al (16) have performed a chronic study on stevioside. The study was performed in F344 rats and the duration of the investigation was 22 months for the male rats and 24 months for the females. A purified hot water extract of *S. rebaudiana*, containing the equivalent of about 95% total sweet glycosides (74.5% stevioside and 16.3% rebaudioside), were given at doses of 0.1, 0.3 and 1%, each dose group consisting of 70 males and 70 females. The high dose was based on an estimated human intake of 4 mg/kg bw and an added safety factor of 100. The major findings, that were more common in rats given the *S. rebaudiana* extract, were reduced spermatogenesis, decreased seminal vesicle weight, interstitial cell proliferation in the testes, medullary cell proliferation in the adrenal glands, atrophy of the thymus, inflammatory lesions in the trachea and lungs, age-related changes of the kidneys (such as degeneration of tubular epithelium, hyaline casts and glomerular sclerosis) and pigmentation and increased haematogenesis of the spleen. The above information was drawn from an extensive summary provided by the petitioner. However, the summary information was insufficient to assess if this study adequately investigated carcinogenic aspects.

Xili et al have performed a combined chronic and carcinogenicity study, in Wistar rats, using a stevioside powder of 85% purity (17). Stevioside was given in the diet at 0, 0.2, 0.6 and 1.2%, equivalent to 100, 300 and 600 mg/kg bw/day, each group consisting of 45 males and 45 females. No treatment-related effects were observed, and the incidence of non-neoplastic and neoplastic changes were unrelated to the level of stevioside in the diet. Consequently, the NOEL was the highest dose, i.e. 600 mg/kg bw/day. However, in view of a lack of toxic effects and not adequately described chemical composition of the test compound, the relatively low purity and doses of the stevioside that was used in the study, it was not possible for the Committee to evaluate if this study adequately investigated carcinogenic aspects.

A new carcinogenicity study, performed in F344 rats, was recently published using a purified stevioside extract (95.6% purity) (18). The doses were equivalent to 155, 310, 625, 1250 and 2500 mg/kg bw/day, each group consisting of 50 males and 50 females. No identification and quantification of impurities in the extract were reported. It was concluded that stevioside was not carcinogenic in F344 rats under these experimental conditions. However, almost all male rats including controls developed interstitial cell tumours in the testis. One major concern in previous studies, as well as in the cited study, is the effects on the male reproductive system (see below). However, a possible treatment related effect on the testicular system can not be evaluated in a strain of rats that normally seems to develop testicular changes. It was previously suggested by the Committee that a chronic oral toxicity and carcinogenicity study should be performed in another rat species than F344. This still seems to be needed with the compound for which approval is sought.

Fertility and teratogenicity: Leaves of *S. rebaudiana* have been used by the Paraguayan Indians in tea as a male contraceptive. Furthermore, extracts of *S. rebaudiana* (10 ml of a 5 % extract) given to rats in the drinking water have been reported to induce infertility for periods of up to two months (19). A target organ toxicity directed to the

male reproductive system (see above for chronic toxicity studies) that subsequently also could affect fertility can not be excluded from the animal studies.

Several studies on *S. rebaudiana* extracts report effects on the male reproductive system, such as reduced spermatogenesis, decreased seminal vesicle weight and interstitial cell proliferation in the testes (16). Administration of aqueous *S. rebaudiana* extracts (corresponding to 0.667 g dried leaves/ml, 2ml/rat twice a day) for 60 days to the rat decreased seminal vesicle weight by about 60% (20). Mazei-Planas and Kuc (19) showed that a water decoction of *S. rebaudiana* extracts reduced fertility to 21% compared to 100% in control rats. Fertility remained reduced (47%) after a 50 to 60 days recovery period. However, in most old studies of reproduction performance the administered dose has been low and not comparable to those used in other toxicological studies. Furthermore, the administered stevioside extracts have chemically not been adequately described.

The petitioner states in the application that available results do not indicate that stevioside induces embryotoxic or teratogenic effects and this statement is scientifically supported by three studies, one in the hamster (21) and two in the rat (22, 23). Hamsters were fed with stevioside (500, 1000 and 2500 mg/kg bw/day, 90% purity) during which time they were mated and allowed to bear three litters (21). Fertility, mating performance, pregnancy, number of fetuses, as well as growth and fertility of the offspring were not affected by the treatment regimen. However, teratogenic effects were not studied. In one of the rat studies (22), Wistar rats were given stevioside in the diet (0.15, 0.75 and 3%, equivalent to 150, 750 and 3000 mg/kg bw/day, 96% purity). Males were treated for 60 days before and during the mating period and females for 14 days before the mating period and for 7 days during gestation. Results showed no treatment related effects on fertility or mating performance, and the foetuses did not develop any malformations. In the other rat study (23) stevioside (95.6% purity), given at doses of 250, 500 and 1000 mg/kg bw/day from day 6 through day 15 of pregnancy, induced no teratogenic effects. However, when steviol, the metabolite of stevioside, was given to hamsters (20 per group) on days 6-10 of pregnancy at doses of 500-1000 mg/kg bw/day it induced toxicity (24). The number of live foetuses per litter and mean foetal weight decreased. The maternal kidneys showed a dose-dependent increase in severity of convoluted tubules in the kidneys. The no-effect level for maternal and foetal toxicity was 250 mg/kg bw/day.

Thus, it can be concluded that steviol, but not stevioside, seems to induce developmental toxicity at high doses. However, the data suggest that there may be effects of stevioside on male reproductive performance. Since stevioside seems to affect the male reproductive organ system and also has been claimed to act as a contraceptive additional studies are needed and should be performed with the specific stevioside preparation for which approval is sought.

Special studies: In rats there seems to be a vasodilator effect resulting in decreased mean arterial pressure and lowering of renal vascular resistance (25). The authors concluded that it is possible that stevioside acts on arterial pressure and renal function as a calcium antagonist as is the case for verapamil. However, before a final conclusion of possible effects of stevioside on renal and cardiovascular function could be made definitive clinical studies are needed.

The effects of stevioside and steviol on the carbohydrate metabolism are not entirely clear. Some cited references indicate effects on blood glucose levels and liver glycogen content (5, 26), but results are questionable.

Conclusion

In the safety assessment of the specific stevioside preparation for which approval is sought, several questions of concern were raised by the Committee regarding the specifications of the extracts that had been tested, questionable chronic toxicity and carcinogenicity studies, and possible effects on the male reproductive system that could affect fertility. Furthermore, steviol, one metabolite of stevioside, that is produced by the human microflora is genotoxic and induces developmental toxicity. The Committee is not satisfied with the submitted documentation and has concern about possible toxicity. Areas that need further studies are stated in the above opinion. The Committee reiterates its earlier opinion that the substance is not acceptable as a sweetener on the presently available data.

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