OPINION OF THE SCIENTIFIC COMMITTEE ON FOOD ON THE TOLERABLE UPPER INTAKE LEVEL OF VITAMIN B2

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FOREWORD

This opinion is one in the series of opinions of the SCF on the upper levels of vitamins and minerals. The terms of reference given by the European Commission for this task, the related background and the guidelines used by the Committee to develop tolerable upper intake levels for vitamins and minerals used in this opinion, which were expressed by the SCF on 19 October 2000, are available on the Internet at the pages of the SCF, at the address: http://www.europa.eu.int/comm/food/fs/sc/scf/index_en.html.

1. INTRODUCTION

Riboflavin (vitamin B2) is chemically specified as a 7,8-dimethyl-10-(1'-D-ribityl) isoalloxazine. The free vitamin is a weak base normally isolated or synthesised as a yellowish-orange amorphous solid. Riboflavin is widely distributed in foodstuffs and all plant and animal cells contain it, but there are very few rich sources. Only yeast and liver contain more than 2 mg/100g. Other good sources are milk, white of egg, fish roe, kidney and leafy vegetables (Elmadfa and Leitzmann, 1998).

Riboflavin is a precursor of certain essential coenzymes such as flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD). In these coenzyme forms riboflavin functions as a catalyst for redox reactions including flavoprotein-catalyzed dehydrogenations that are either pyridine nucleotide dependent or independent reactions with sulphur-containing compounds, hydroxylations, oxidative carboxylations, dioxygenations and the reduction of oxygen to hydrogen peroxide. Flavo-coenzymes are also involved in the biosynthesis of niacin-containing coenzymes from tryptophan via FAD-dependent kynurenine hydroxylase, the FMN dependent conversion of the 5´-phosphates of vitamin B6 to pyridoxal 5´-phosphate and the FAD-dependent dehydrogenation of 5,10-methylene-tetrahydrofolate to the 5´-methyl product, with the vitamin B_{12}-dependent formation of methionine and sulphur amino metabolism.

2. NUTRITIONAL BACKGROUND

In foodstuffs riboflavin occurs free or combined either as FAD and FMN as a complex with protein. Protein bound riboflavin is hydrolysed in the gastrointestinal tract to free riboflavin, the form absorbed. At physiological concentrations the uptake of riboflavin occurs by an active, saturable transport system. At high levels of intake riboflavin is absorbed by diffusion (McCormick, 1989). The amount absorbed depends on the intake, it is increased by bile salts and when riboflavin is given orally, with food. Absorption rate of free riboflavin is 50-60% for a dose range of 2-25 mg (Elmadfa and Leitzmann, 1998). In plasma, riboflavin is bound to proteins, predominantly albumin, but also to immunoglobulins, and mainly found as FAD. Although the significance of this protein binding is not fully understood, the main function is the transport of riboflavin from plasma into the central nervous system (Steier *et al*, 1976; Natraj *et al*, 1988). Phosphorylation and dephosphorylation are features of intracellular metabolism.

In the cellular cytoplasm of most tissues, the small intestine, heart, liver and kidney, riboflavin is converted into the coenzymes FMN with flavokinase and FAD by the apoenzyme FAD-synthetase. In the body tissue riboflavin is predominantly present as the coenzyme FAD, which can be evaluated by determination of the blood FAD level. In healthy adults riboflavin accounts for 60-70% of the excreted urinary flavins (McCormick, 1989). The urinary excretion of riboflavin varies with intake, metabolism, and age. This is an alternative approach for determination of riboflavin status, because it reflects an excess of current intake beyond tissue requirements. A recent study of the pharmacokinetics of riboflavin uptake in human subjects indicated an upper limit of absorption from a single dose of 27 mg. The half-life of absorption was 1.1 h. Stool analysis to estimate riboflavin excretion was not done in this study. It also demonstrated relatively modest changes in plasma riboflavin or flavoenzymes following oral administration (Zempleni *et al*, 1996).

One of the methods commonly used for assessing riboflavin status involves the determination of the erythrocyte glutathione reductase (EGR) activity. The method generally preferred for the estimation of riboflavin status is the stimulation of FAD-dependent EGR *in vitro*, which relies on an associated oxidation of NADPH which can be readily monitored spectrophotometrically (Bates *et al*, 1986).

The second biochemical method used is the detection of riboflavin in the urinary excretion – a normal adult excretes 120 µg or more per 24 h. Less than 40 µg per 24 h (Horwitt, 1950) or 27 µg/g of creatinine (Sauberlich, 1999) is an indicator for riboflavin deficiency.

Red blood cell FAD and FMN (after modest hydrolysis from FAD) have been used as indicators of the cellular concentration of riboflavin in its form of coenzyme, since these forms comprise over 90% of riboflavin.

The highest mean intake of riboflavin from diet and supplements was reported for males aged 31-50 years: 6.9 mg/day. The highest reported intake at the ninety-fifth percentile was 11 mg/day in females over 70 years (Food and Nutrition Board, 1998).

The RDA (Recommended Daily Allowance) for riboflavin varies from 0.5-0.9 mg/day for children, 1.3 mg/day for male adults and 1.0-1.1 mg/day for female adults (Food and Nutrition Board, 1998); 0.8-1.6 mg/day children/male, 0.8-1.3 mg/day children/female, 1.3 mg/day male adults and 1.1 mg/day female adults (SCF, 1993), 0.7-1.6 mg/day children/male, 0.7-1.3 mg/day children/female, 1.2-1.5 mg/day male adults and 1.2 mg/day female adults (D-A-CH Referenzwerte, 2000).

3. HAZARD IDENTIFICATION

3.1. Studies on genotoxicity

Riboflavin and FMN were found not to be mutagenic in the Ames test with *Salmonella typhimurium* (strains TA97A, TA102, TA98, TA100). Both suspensions and plate overlay tests were conducted and assays were done with and without mammalian activation systems (Fujita and Sasaki, 1986; Kale *et al*, 1992).

DNA-damage was found in human cell cultures after multivitamin administration together with light. It was suggested that riboflavin was involved in this photodynamic damage, but only in synergism with other multivitamin components, because riboflavin solely was not able to damage DNA even in a 30 fold higher concentration (Ennever *et al*, 1983).

3.2. Special studies on reproduction

Weaned male and female rats were fed daily doses of 10 mg of riboflavin for 140 days. The animals were mated and normal litters were obtained from the riboflavin and control groups. At three weeks of age the offspring of the first generation were fed with 10 mg/kg bw/day of riboflavin. Daily feeding over periods of 140 days was continued for three generations. No differences in development, growth, maturation and reproduction of treated and control animals were observed. Autopsies at the end of the test period did not show any gross changes (Unna & Greslin, 1942).

Thirteen female rats were fed diets containing 100 ppm of riboflavin/day for two weeks, prior to mating and subsequently during gestation and lactation. Control rats received 4 ppm riboflavin in the diet. There was no difference between groups except an apparent decrease in the viability of the offspring in the high riboflavin group as a result of the loss of one litter (Schumacher *et al*, 1965).

No differences in the number per litter, mortality or weight gain of offspring in young female Wistar rats were found between diets containing 4 or 40 ppm of riboflavin during pregnancy and lactation (Le Clerc, 1974).

3.3. Toxicity studies

Early reports of some toxic effects with riboflavin in laboratory animals were due to the effects of the solvent and not caused by the vitamin. Unna and Greslin (1942) reported a lack of toxicity in rats receiving 10 g/kg orally, or 5 g/kg subcutaneously, and in dogs receiving 2 g/kg orally. The first reliable report of toxicity in animals for riboflavin was an investigation in rats receiving 0.6 g/kg intraperitoneally where the animals became anuric and riboflavin crystals were found in the renal tubes (Unna and Greslin, 1942). The monodiethanolamine salt of FMN was fed to groups of 10 weaned female rats five days per week for 29 weeks at doses of 1, 4, 10 and 40 mg/day (= 5, 20, 50 and 200 mg/Kg bw). No effects were observed at 5 and 20 mg/Kg bw levels. A slight decrease in haemoglobin concentration was observed at 50 mg/Kg bw. At 200 mg/Kg bw two rats died and the surviving eight animals showed slight anaemia and decreased weight gain (Randall, 1950). Groups of four rabbits each received 10 or 100 mg (5 or 50 mg/Kg bw) monodiethanolamine riboflavin by intravenous or intramuscular injection five days per week

for three weeks. One of the rabbits died with evidence of renal damage following seven intravenous injections at 50 mg/kg bw. No toxic effects were noticed after intramuscular injection (Randall, 1951). The administration of 25 mg/kg bw of riboflavin for five months did not cause any toxic effects in dogs (Unna and Greslin, 1942).

Riboflavin (chemically synthesised or produced by fermentation, food grades purity 98%) was examined in a sub-chronic 13-week oral feeding study in three groups of 16 male and 16 female Wistar rats at doses of 20, 50 or 200 mg/kg bw (SCF, 1998). There were no dose related differences regarding feed consumption, feed conversion efficiency and water intake. A 6% (<10%) growth retardation was found in female rats given 200 mg/kg bw/day riboflavin ex fermentation and males and females treated with 50 mg/kg bw/day riboflavin ex synthesis. No dose related changes in haematological parameters, urine analysis or clinical chemistry were noted, except for borderline variations in the haemoglobin concentration, red blood cell and reticulocyte counts in females with 200 mg/kg bw/day riboflavin ex synthesis. Gross and histopathological findings showed no significant treatment related lesions in any test group.

The LD_{50} after an intraperitoneal riboflavin injection was 340 mg/kg for the mouse and 560 mg/kg for the rat (Yoneda, 1984). Death, which occurs after 2-5 days, was from formation of riboflavin crystals in the kidney, leading to anuria and azotemia. Vitamin crystallisation in the kidney occurs when the riboflavin blood level exceeds 20 µg/ml in rats. Urinary levels of 150 µg/ml may be a sign of toxicity (Machlin, 1991).

The low toxicity following oral administration can probably be explained by the limited capacity of the intestinal absorption mechanism (Machlin, 1991).

There are no published data from studies using animal models or in humans that connect riboflavin with genotoxic, carcinogenic, teratogenic or reproductive toxic effects or with toxic effects in humans.

3.4. Mechanistic studies

Evidence of adverse effects associated with the group of flavins is based on *in vitro* studies showing involvement in the formation of active oxygen species and in the axonal degeneration on intense exposure to ultraviolet and visible light (Spector *et al*, 1995, Lucius *et al*, 1998).

3.5. Human studies

The few studies performed involving large doses of riboflavin were not designed to evaluate adverse effects, but identification of hazards as a first step is possible based on the studies done with high dose supplementation and large intakes of riboflavin.

The highest doses orally administered over a longer time period were in two studies by Schoenen *et al* In the first study, Schoenen *et al* (1994) reported no side effects in 49 patients treated for migraine with 400 mg/day of riboflavin taken with meals for at least 3 months. One patient, receiving riboflavin together with aspirin, withdrew from the study due to a gastric upset possibly due to aspirin. No side effects were reported by the other study participants.

In the second study (Schoenen *et al*, 1998), 55 patients with migraine were treated with 400 mg/day of riboflavin (or a placebo) in a random trial of 3 months duration. Minor adverse effects were observed in two cases in the riboflavin group (diarrhoea and polyuria). In the placebo group one case of abdominal cramps was reported.

For the treatment of methaemoglobinaemia due to NADH methaemoglobin reductase deficiency, 120 mg riboflavin/day were administered to the members of a family for a period of 10 months. The intake was reduced to 10-30 mg/day for longer periods; no side effects were observed (Hirano *et al*, 1981).

One 24 year old woman, suffering from chronic fatigue, received 100 mg/day riboflavin for two years without any side effects, and a 14 year old girl with the same disorder received 200 mg/day riboflavin for one year and 100 mg/day for the next 2 years without any side effects (Peluchetti *et al*, 1991).

A 57 year old epileptic woman, treated with barbiturates, was given daily 600 mg of riboflavin as a chronic treatment. There was a small electro-encephalographic abnormality, which was not associated with clinical symptoms and which disappeared 47 days after the treatment was completed (Santanelli *et al*, 1988).

The lack of harmful results from high doses of riboflavin can also be due to its physico-chemical properties – the solubility is limited and, especially, the capacity to absorb riboflavin from the gastrointestinal tract by humans is limited (Stripp, 1965; Zempleni *et al,* 1996).

Stripp (1965) found the single oral administration of 50-500 mg of the sodium salt of FMN without any adverse effect.

One case is described where a woman with multiple myeloma showed an impaired turnover and excretion of dietary riboflavin, causing yellow pigmentation of the skin and hair (Farhangi and Osserman, 1976).

4. DOSE RESPONSE ASSESSMENT AND DERIVATION OF A TOLERABLE UPPER INTAKE LEVEL (UL)

The absorption of riboflavin is limited when administered in high doses. Data on adverse effects from high oral riboflavin intake are not sufficient for a risk assessment. Given the lack of any demonstrated functional disorders or adverse structural effects in humans following excessive oral riboflavin intake and considering the limitation of intestinal absorption, the relevance of the mild effects shown in *in vitro* studies to human health *in vivo* is questionable.

Available subchronic data from human studies and on pharmacokinetics studies do not show reported effects on oral toxicity of riboflavin. Apart from a few minor gastrointestinal disorders, which are not clearly related to the riboflavin intake, it is free from serious adverse effects.

Although the studies of Schoenen *et al* (1994, 1998) involved an adequate number of subjects with a daily dose of 400 mg riboflavin, they included only self-reporting of adverse effects and did not include adequate assessment of parameters relevant to the detection of adverse effects (for example biochemical indices of hepatic or renal function). In consequence, these studies were not of sufficient quality and extent to be used for the determination of a Tolerable Upper Intake Level (UL).

The results of a 13 week feeding study in Wistar rats (SCF, 1998) show that a dose of 50 mg riboflavin per kg body weight can be considered as the NOAEL and does not contradict the current JECFA ADI value for riboflavin and riboflavin 5-phosphate of 0-0.5 mg/kg bw (JECFA, 1969). The SCF has not adopted an ADI for riboflavin, but regards its use as food colorant to be acceptable (SCF, 1977).

5. CHARACTERISATION OF RISK

The dietary intake of riboflavin from food was evaluated in the 1990s in different European countries. Mean riboflavin intake in the population from The Netherlands, based on data from the Dutch National Food Consumption Survey (n = 5958, 2-day estimated dietary record) is 1.54 mg/day (97.5 percentile: 2.87 mg/day) (Hulshof *et al*, 1997-1998). Italian data are based on the Italian survey (n = 2734, 7-day weighed record), with a mean intake of riboflavin of 1.6 mg/day (97.5 percentile: 2.7 mg/day) (Turrini, 1994-1996). In the Austrian Study on Nutritional Status (n = 2488, 24-h-recalls), the mean riboflavin intake is 1.49 mg/day (97.5 percentile: 3.29 mg/day) (Elmadfa *et al*, 1998). It should be noted that these studies were not designed to assess specifically the intake of riboflavin from supplements; however, the number of consumers of supplements is probably not sufficient to influence significantly the mean consumption of the total population.

In the UK (EVM, 2000), the mean riboflavin intake from all sources for men and women was 2.3 mg/d and 1.8 mg/d, respectively; riboflavin intake for adults (16-64 years old) from food supplements among supplement consumers only (4% of males, 8% of females) amounted on average to 5.2 mg/day in males and to 3.1 mg/day in females, based on data collected in 1986/87. In Ireland (IUNA, 2000), the mean riboflavin intake from all sources for adults (18-64 years old) was 2.1 mg/day (97.5th percentile: 4.6); mean riboflavin intake from food supplements among supplement consumers only (7% of males, 13% of females) was 2.4 mg/day in males and 3.9 mg/day in females.

No study has reported significant adverse effects in humans of excess riboflavin consumption from food or supplements. This does not mean that there is no potential for adverse effects from high intakes. Although it is not possible, based on the present database, to derive an UL for riboflavin, the limited evidence available from clinical studies indicates that current levels of intake of riboflavin from all sources do not represent a risk to human health.

6. REFERENCES

Bailey, A.L., Maisey, S., Southon, S., Wright, A.J., Finglas, P.M. and Fulcher, R.A. (1997). Relationships between micronutrient intake and biochemical indicators of nutrient adequacy in a "free-living" elderly UK population. Br. J. Nutr. 77: 225-242.

Bates, C.J. and Fuller, N.J. (1986). The effect of riboflavin deficiency on methylenetetrahydrofolate reductase (NADPH) (EC 1.5.1.20) and folate metabolism in the rat. Br. J. Nutr. 55: 455-464.

Cooperman, J.M. and Lopez, R. (1991). Riboflavin. In: Handbook of Vitamins. Ed: Machlin, L.J. Second Edition. Marcel Dekker, Inc. New York and Basel.

D-A-CH Referenzwerte (2000). Deutsche Gesellschaft für Ernährung, Österreichische Gesellschaft für Ernährung, Schweizerische Gesellschaft für Ernährungsforschung, Schweizerische Vereinigung für Ernährung: Referenzwerte für die Nährstoffzufuhr, Umschau/Braus Verlag.

Elmadfa, I. and Leitzmann C. (1998). Ernährung des Menschen, 3. Auflage. Verlag Eugen Ulmer, Stuttgart.

Elmadfa, I., *et al* (1998). Austrian Study on Nutritional Status, Österreichischer Ernährungsbericht.

Ennever, J.F., Carr, H.S. and Speck, W.T. (1983). Potential for genetic damage from multivitamin solutions exposed to phototherapy illumination. Pediatr. Res. 17: 192-194.

EVM, Expert Group on Vitamins and Minerals (2000). Review of riboflavin. VM/00/15/P. Food Standards Agency (UK).

In http://www.foodstandards.gov.uk/committees/evm/papers/evm15.pdf.

Farhangi, M. and Osserman, E.F. (1976). Myeloma with xanthoderma due to an IgG lambdamonoclonal anti-flavin antibody. N. Engl. J. Med. 294: 177-183.

Food and Nutrition Board (1998). Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. Institute of Medicine, National Academic Press, Washington, D.C.

Fujita, H., Sasaki, M. (1986). Mutagenicity test of food additives with *Salmonella typhimurium* TA97 and TA102 (I). Kenkyu Nenpo Tokyo Toritsu Eisei Kenkyusho 37; 447-452.

Hirano, M., Matsuki, T., Tanishima, K., Takeshita, M., Shimizu, S., Nagamura, Y. and Yoneyama, Y. (1981). Congenital methaemoglobinaemia due to NADH methaemoglobin reductase deficiency: successful treatment with oral riboflavin. Br. J. Haematol. 47: 353-359.

Horwitt, M.K., Harvey, C.C., Hills, O.W. and Liebert, E. (1950). Correlation of urinary excretion of riboflavin with dietary intake and symptoms of ariboflavinosis. J. Nutr. 42: 247-264.

Hulshof, K.F.A.M., *et al* (1997-1998). 3rd Dutch National Food Consumption Survey.

IUNA (Irish Universities Nutrition Alliance) (2000). The North/South Ireland Food Consumption Survey. Food Safety Promotion Board, Dublin.

JECFA (1969). Thirteen report. Joint FAO/WHO Expert Committee on Food Additives WHO FOOD report series no. 46A. ADD/7036. In (JECFA) 1954-1997. http://jecfa.ilsi.org.

Kale, H., Harikumar, P., Nair, P.M. and Netrawali, M.S. (1992) Assessment of the genotoxic potential or riboflavin and muminflavin A. Effect of metabolic enzymes, Mutat. Res. 298: 9-16.

Le Clerc (1974). Influence de la teneur du regime alimentaire en thiamine, en riboflvine et en vitamine B_6 sur la teneur des tissues de la ratte en lactation et des jeunes en ces memes vitamines. Ann. Nutr. Aliment. 23: 111-120.

Leske, M.C., Wu, S.Y., Hyman, L., Sperduto, R., Underwood, B., Chylack, L.T., Milton, R.C., Srivastava, S. and Ansari, N. (1995). Biochemical factors in the lens opacities. Case-control study. The Lens Opacities Case-Control Study Group. Arch. Ophthalmol. 113: 1113-1119.

Lucius, R., Mentlein, R. and Sievers, J. (1998). Riboflavin-mediated axonal degeneration of postnatal retinal ganglion cells in vitro is related to the formation of free radicals. Free. Radic. Biol. Med. 24: 798-808.

Madigan, S.M., Tracey, F., McNulty, H., Eaton-Evans, J., Coulter, J., McCartney, H. and Strain, J.J. (1998). Riboflavin and vitamin B-6 intakes and status and biochemical response to riboflavin supplementation in free-living elderly people. Am. J. Clin. Nutr. 68: 389-395.

McCormick, D.B. (1989). Two interconnected B vitamins: riboflavin and pyridoxine. Physiol. Rev. 69: 1170-1198.

Natraj, U., George, S. and Kadam, P. (1988). Isolation and partial characterisation of human riboflavin carrier protein and the estimation of its levels during human pregnancy. J. Reprod. Immunol. 13: 1-16.

Peluchetti, D., Antozzi, C., Roi, S., DiDonato, S. and Cornelio, F. (1991). Riboflavin responsive multiple acyl-CoA dehydrogenase deficiency: functional evaluation of recovery after high dose vitamin supplementation. J. Neurol. Sci. 105: 93-98.

Powers, H.J. (1999). Current knowledge concerning optimum nutritional status of riboflavin, niacin and pyridoxine. Proc. Nutr. Soc. 58: 435-440.

Randall, L.O. (1950). Toxicity of mono-diethanolamine salt of riboflavin monophosphoric acid ester dihydrate. Unpublished report of Hoffman-LaRoche, Inc. Submitted in pursuance of Fed. Register 38: 28581 (October 15, 1973) in connection with the review of over-the-counter vitamin, mineral and hematinic drug products.