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Opinion on coumarin

(expressed on 22/9/99)

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SCIENTIFIC COMMITTEE ON FOOD

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Terms of Reference

To review the toxicity of coumarin in the light of the latest studies and to consider whether the opinion on coumarin expressed by the SCF on 16 December 1994 has to be amended accordingly.

Background

Coumarin considered in this opinion is also known under different chemical names such as 2H-1-benzopyran-2-one, 1,2-benzopyrone, cis-o-coumarin acid lactone, coumarinic anhydride, o-hydroxycinnamic acid- δ -lactone, 2-oxo-2H-1-benzopyran, and is defined by the CAS number; 91-64-5.

The SCF delivered an opinion on coumarin in 1994 (Scientific Committee for Food, 1997). At that time the SCF reviewed the toxicity of coumarin , in order that the Commission could consider whether the limits for coumarin in food set out in Annex II of the flavourings Directive 88/388/EEC (Council Directive, 1988) needed to be amended. The opinion of 1994 of the Committee includes also a number of recommendations and indications on further research. The Committee concluded as follows.

“It may be concluded that coumarin is a carcinogen in rats via the oral route and possibly in mice. In rats, adenomas and carcinomas of the liver and bile ducts and adenomas of the kidney have been observed. In mice, adenomas and carcinomas of the lung and liver adenomas have been observed. In reaching the recommendations, the Committee gave particular weight to the occurrence of liver toxicity, including cholangiocarcinomas and hepatocellular carcinomas, confirmed in two chronic rat studies in which coumarin was administered by dietary route. The results of the NTP gavage studies are more difficult to interpret: in rats only general liver toxicity, not tumours were seen, together with kidney adenomas, as well as nephropathy which is common in ageing rats; in the mouse significant dose-related increases were seen in lung tumours in both sexes, but significant increases in liver tumours were only seen in the low and mid-dose females. Both liver and lung tumours are common spontaneous occurrences in the strain of mouse used. Whilst not consistent with the dietary studies, the results from the gavage studies did not lessen our concern about the toxicity of coumarin.

A key issue in assessing the risk of coumarin to man is deciding whether or not coumarin is genotoxic. Particularly strong reassurance is needed that coumarin is not genotoxic *in vivo* when, in addition to positive *in vitro* studies, an epoxide has been postulated as a metabolic intermediate. The requirement for metabolic activation for a positive response in *Salmonella typhimurium* TA100 and in a study of chromosomal aberrations in Chinese hamster ovary (CHO) cells is consistent with the idea that

activation to an epoxide may be required. However, metabolic activation did not appear to be required for the induction of SCEs in CHO cells *in vitro* (NTP, 1992) but the positive response without S9 was weak and was not dose-related. The available *in vivo* mutagenicity studies, while negative, are not of a high enough standard to provide sufficient reassurance that coumarin is not active *in vivo*.

A further key consideration is whether the carcinogenicity seen in rats and mice, if due to an epoxide, is relevant to man. The Committee concluded that the epoxide route cannot be ruled out in man and need only be a minor pathway for genotoxic/carcinogenic effects to be of concern”.

The SCF formulated the following recommendations: “Taking into account the natural flavouring source materials, the carcinogenic activity of coumarin and the fact that a genotoxic mechanism cannot be excluded at this point of time (1994), the Committee recommends that:

- i) the general limit in food and beverages for coumarin, which applies when it is present because natural flavouring source materials containing coumarin have been added, should be reduced to the currently achievable limit of detection for coumarin of 0.5 mg/kg.
- ii) action should be taken to reduce the higher levels which are currently permitted in certain traditional products”.

The Committee also expressed the wish to see further research carried out and commented as follows.

“The Committee considered that further research on coumarin would be desirable, particularly if any proposals to raise the general limits from that now recommended (the lowest achievable limit of detection) were to be considered. In this regard, further information on the mutagenicity of coumarin could be helpful. The Committee understands that new *in vitro* mutagenicity studies have been carried out recently, under the auspices of the Research Institute for Fragrance Material, USA, on 7-hydroxycoumarin and ortho-hydroxyphenylacetic acid, which are the major metabolites in man and rat respectively. Final reports on these studies are pending. The Committee wishes to see these reports, but it should be stressed that these studies are on the end-stage metabolites only and thus they do not address the concern about the possible formation of an active epoxide intermediate. To address this concern, in the first instance, an *in vivo* bone marrow micronucleus test in mice and an *in vivo* liver UDS (unscheduled DNA synthesis) study in rats would be helpful.

Further research to address the more difficult issues could also be helpful but the Committee recognises that the resolution of these questions is less certain and could involve extensive work. These issues include questions whether the 3,4-epoxide is indeed responsible for the toxicity of coumarin, the extent to which it is produced in other species including man, whether a good animal model for man can be found from the metabolic viewpoint, what proportion of the human population has low 7-hydroxylase activity and how coumarin is metabolised in these people.

Finally, no clear assessment of the likely risk to man will be possible without quantitative information on the levels of coumarin in various natural flavouring source material and foodstuffs to allow at least a rough estimate of coumarin intake in man.”

Evaluation of additional relevant information on coumarin since 1994

In order to evaluate the newly available toxicity data since 1994 (Scientific Committee, 1997) these data are summarised. The relevance of these data is discussed in the light of information lacking as indicated by the Committee in 1994.

There were a number of new studies on absorption, distribution, metabolism and elimination of radioactively labelled coumarin, both after dermal and oral intake (Born et al., 1997c; Beckley-Kartey et al., 1997; Hawkins et al., 1996a, b, c). The majority of the studies were *in vitro* or *in vitro ex in vivo* experiments with liver microsomes, cytochromes or precision-cut organ slices of mammals and human (Ratanasavanh et al., 1996; Born and Lehman-McKeeman, 1998 ; Price et al., 1995; Lake et al., 1995, 1996; Steensma et al., 1995; Koenigs et al., 1997). There are two major metabolic pathways for coumarin and there are interspecies differences in which route predominates. The 7-hydroxylation pathway produces the non-toxic, urinary metabolite 7-hydroxycoumarin (Lake, 1999) . The 3-hydroxylation pathway produces 3-hydroxycoumarin and this is thought to occur via a toxic, 3,4-epoxide intermediate. Studies with precision-cut liver showed that the metabolism of coumarin in calf, Cynomolgus monkey and human is quite similar in that the major metabolic pathway is the coumarin 7-hydroxylation, whereas the major metabolic pathway in rat is the coumarin 3-hydroxylation (Steensma et al., 1995; Koenigs et al., 1997). Nevertheless, these studies also showed that coumarin 3-hydroxylation takes place to a minor extent in humans.

Studies with microsomes gave similar results (Lovell et al., 1998; van Iersel et al., 1994). A study with microsome samples from 12 different humans demonstrated a great variation in the involvement of the coumarin 7-hydroxylation and coumarin 3-hydroxylation pathways, leading to a great variation in metabolites (Van Iersel et al., 1994). From the studies (Drager et al., 1997; Bogan et al., 1997; Born et al., 1997a) dedicated to the involvement of cytochrome types, it can be concluded that P450 CYP2A6 is involved in the 7-hydroxylation of coumarin, whereas at present the cytochrome type(s) responsible for the 3-hydroxylation of coumarin is not yet identified.

Some investigators demonstrated that there are humans who are homozygous or heterozygous for the genetic variants of CYP2A6 such as CYP2A6_{v1} and CYP2A6_{v2} (Salguero et al., 1995; Hadidi et al., 1997, 1998). Those allelic variants had a lower capacity for coumarin 7-hydroxylation and therefore they produced a higher level of 2-hydroxyphenylacetic acid indicating a higher involvement of the coumarin 3-hydroxylation. Worldwide the allelic frequency of CYP2A6_{v1} varied from 11 – 20 % and of CYP2A6_{v2} from 2.5 – 7 % with an exceptional high frequency of 28 % in Japan. On the basis of these frequencies it can be concluded that polymorphism contributes considerably either directly or indirectly to the variation of the involvement of coumarin 3-hydroxylation pathway and thus to the risk of producing toxic metabolites. In addition it was demonstrated that in people who suffered from hepatitis virus A

infection the 7-hydroxylation pathway of coumarin was inhibited (Pasanen et al., 1997).

A further key piece of new information is the demonstration that the 3,4-epoxide, which has not hitherto been isolated, has now been synthesised and that o-hydroxyphenylacetaldehyde (o-HPA), which is normally a prominent rat liver microsomal metabolite, is spontaneously (within 20 minutes) formed by opening of the ring of coumarin-3,4-epoxide in aqueous solution (Born et al., 1997b, Born and Lehman-McKeeman, 1998), supporting the suggested pathway in which coumarin-3,4-epoxide is the short lived reactive intermediate of 3-hydroxycoumarin pathway to o-HPA.

Some new studies on the hepatotoxicity both *in vitro* (Born et al, 1998b), and *in vivo* in (sub)chronic studies in rats, mice and hamsters (Cotrell et al., 1996; Lake and Grasso, 1996; Carlton et al 1996) and studies on lung toxicity (effect on Clara cells) in mice and rats (Born et al., 1998a; Fix et al., 1998) generally did not add any information to what was known before 1994. Increased tumour incidences (cholangiofibroma, cholangiocarcinoma, and parenchymal liver cell tumours) in rats were seen at high dose levels and on the basis of the dose-related decrease in food-intake and body weight gain it was claimed that these carcinogenic effects occurred above the maximum tolerated dose (Carlton et al., 1996).

There were no new genotoxicity studies on coumarin which specifically addressed the SCF's 1994 request. The genotoxicity data on the major end-stage metabolites in man and rats, 7-hydroxycoumarin and o-hydroxyphenylacetic acid respectively, showing a negative response, were considered not relevant in relation to the possible genotoxicity of coumarin and its intermediate metabolites including 3,4-coumarin epoxide (San and Wagner, 1994; San and Raabe, 1994).

A study on UDS in precision-cut liver slices of humans showed that coumarin in concentration ranging from 0.05 – 5.0 mM had no effect on the degree of the UDS in the human slices (Lake et al., 1996; Beaman et al., 1998). However, in the light of the variation in the 7-hydroxylation of coumarin in humans, such a single study with a limited number of humans is not considered adequate for the safety assessment of the human population as a whole.

In a number of cases hepatotoxicity has been reported in patients treated with coumarin as therapeutic (e.g. against lymphoedema or other protein oedemas) compounds (Cox et al., 1989; Casley-Smith and Casley-Smith, 1985, 1986; Koch et al., 1995; Beinssen, 1994; Morrison and Welsby, 1995). Recently investigators concluded on the basis of their survey that there was a strong signal for potential hepatotoxicity of coumarin (likely due to the production of reactive metabolites in some patients exhibiting a coumarin-7-hydroxylase deficiency) that caused the authority to withdraw coumarin from the market in France (Andrejak et al., 1998).

Conclusions

Consideration of the new data on liver metabolism did not reassure the Committee that the 3-hydroxylation pathway is so minor that no further concern with respect to genotoxicity is warranted. On the contrary, the new data on liver metabolism further support the conclusions drawn in the opinion of the Committee in 1994. Data from therapeutic use of coumarin also suggest that hepatotoxicity may occur in humans following coumarin treatment. No new data on genotoxicity as requested by the Committee in 1994 were available. The data on the influence of human genetic polymorphism in the metabolism of coumarin reaffirm concerns that a toxic epoxide intermediate may be produced in a significant proportion of the human population. Thus further information on genotoxicity is necessary.

In its 1994 opinion the Committee suggested that an *in vivo* bone marrow micronucleus test in mice and an *in vivo* UDS study in rats would be helpful. However, since the epoxide intermediate might be very short-lived, such studies might not resolve the issue of the potential for genotoxicity *in vivo*. Given that the 3,4-epoxide can be prepared synthetically, the Committee now considers that a study of *in vivo* DNA-binding and DNA-adduct formation in rats in the relevant target organs, liver and kidney, using ¹⁴C ring-labelled coumarin, would be more likely to resolve the issue of the genotoxic potential of coumarin. The Committee now requires such studies to be submitted as soon as possible.

The Committee also noted in its previous opinion in 1994 that the then currently achievable analytical limit of detection for coumarin was 0.5 mg/kg in food. The Committee understands that lower limits of detection are now achievable in certain food matrices, and recommends that this issue be reviewed.

The Committee is aware that if the present general limit for coumarin in food of 2 mg/kg were to be reduced, an increased number of traditional food products are likely to exceed the limit. In considering the need for exceptional limits for certain traditional food products, it should be noted that the issue of genotoxicity is not yet resolved. The Committee is therefore of the view that any exceptions to the general limit should be restricted to specific traditional products, bearing in mind their frequency and amounts of consumption, and not based on general food categories.

The Committee will review coumarin as soon as the additional requested data become available. In the meantime, the Committee would not wish to see current extent and levels of use increase.

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