Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contacts with Food (AFC) on a request from the Commission related to

Coumarin

Question number EFSA-Q-2003-118

Adopted on 6 October 2004

SUMMARY

Coumarin is a naturally occurring flavouring substance. The Scientific Committee for Food (SCF) delivered an opinion on coumarin in 1994 (SCF, 1997) 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 (EEC, 1988) needed to be amended. The SCF concluded that coumarin was a carcinogen in rats via the oral route and possibly in mice, noting that adenomas and carcinomas of the liver and bile ducts and adenomas of the kidney have been observed in rats and adenomas and carcinomas of the lung and liver adenomas in mice. The Committee noted that a key issue in assessing the risk of coumarin to humans was deciding whether or not coumarin was genotoxic and that particularly strong reassurance was needed that coumarin was not genotoxic *in vivo* since, in addition to positive results from *in vitro* genotoxicity studies, an epoxide had been postulated as a metabolic intermediate. The available *in vivo* mutagenicity studies, while negative, were not of a high enough standard to provide sufficient reassurance that coumarin was not active *in vivo*. A further key consideration was whether the carcinogenicity seen in rats and mice, if due to an epoxide, was relevant to humans. The SCF concluded that the epoxide route could not be ruled out in humans and need only be a minor pathway for genotoxic/carcinogenic effects to be of concern.
The SCF in 1994 considered that further research, especially on the genotoxicity of coumarin, would be desirable, particularly if any proposals to raise the general limits from that then recommended (the lowest achievable limit of detection) were to be considered.

The SCF considered coumarin again in 1999 (SCF, 1999). Consideration of new data on liver metabolism did not reassure the Committee in 1999 that the epoxide-forming pathway was so minor in humans that no further concern with respect to genotoxicity was warranted. On the contrary, the new data on liver metabolism further supported the conclusions drawn in the opinion of the Committee in 1994 (SCF, 1997). Data from therapeutic use of coumarin also suggested that hepatotoxicity may occur in humans following coumarin treatment. No new data on genotoxicity as requested by the Committee in 1994 had become available, but data on the influence of human genetic polymorphism in the metabolism of coumarin reaffirmed concerns that a toxic epoxide intermediate may be produced in a significant proportion of the human population. Thus further information on genotoxicity was requested and the SCF

considered that a study of *in vivo* DNA-adduct formation in rats in the relevant target organs, liver and kidney would be appropriate.

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food has now been asked to review the toxicity in the light of the latest studies relevant to the safety assessment of coumarin in food products, particularly the specifically requested studies on possible DNA-adduct formation in kidney and liver, and to consider whether the previous opinions of the SCF need to be updated.

The results of the requested study on DNA-adduct formation in kidney and liver of rats demonstrate that coumarin does not bind covalently to DNA, supporting a non-genotoxic mode of action for tumour induction. The data now available allow the derivation of a Tolerable Daily Intake (TDI).

In addition to the requested studies, a number of recent publications relevant to the metabolism of coumarin in different species including humans were submitted. Studies on lung metabolism and morphology indicate that humans are unlikely to be susceptible to coumarin lung toxicity. Recent *in vitro* and *in vivo* studies show that the hepatotoxicity seen in rats is related to the 3,4- coumarin epoxide pathway, which is not a major metabolic pathway in humans. Originally it was thought that coumarin 3,4-epoxide was responsible for the liver toxicity, but in the mouse comparable levels of the epoxide are found but hepatotoxicity is negligible. From these comparative studies the Panel concluded that liver toxicity is not directly correlated to 3,4 coumarin epoxide/ortho-hydroxy phenyl acetic acid, but rather the balance between bioactivation and detoxification likely dictates the susceptibility of the animal species to coumarin-mediated liver toxicity.

Comparative studies in humans from South Europe and Asia show that a considerable number of individuals exhibit polymorphism, in that they have a considerable reduction in the 7-hydroxy coumarin pathway, which is normally the major metabolic route in humans that does not lead to the formation of 3,4 coumarin epoxide. However, it is not known what this reduction means with respect to the involvement of other metabolic pathways for coumarin. The Panel therefore concluded that hepatotoxic responses should be taken into account in setting a TDI and that in applying safety factors to the no-observed- adverse-effect level (NOAEL) for hepatotoxicity, it would be prudent to use a factor of 10 for potential interspecies variation, together with a factor of 10 for potential individual differences between humans. The overall NOAEL for liver toxicity in the most sensitive animal species, based on hepatotoxicity in a two year dog study, was 10 mg coumarin/kg bw/day. Applying a safety factor of 100, a TDI of 0 - 0.1 mg coumarin/kg bw can be established.

Conservative estimates of intake based on current maximum permitted concentrations in foodstuff suggest that present dietary intakes do not exceed the TDI.

KEYWORDS

Coumarin, hepatotoxicity, DNA adducts

BACKGROUND

The Scientific Committee on Food (SCF) issued an opinion on coumarin in 1994 (SCF, 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 (EEC Council Directive, 1988) needed to be amended. The opinion of 1994 of the Committee included a number of recommendations and indications on the need for 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.
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> 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 sister chromatid exchanges (SCEs) in CHO cells *in vitro* (NTP, 1993) (but the positive response without metabolic activation 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 in 1994 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, 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 *o*-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.
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> 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."

In 1999 the SCF considered new data on liver metabolism, but the Committee was not reassured that the epoxidation pathway is so minor in humans that no further concern with respect to genotoxicity was warranted (SCF, 1999). On the contrary, the new data on liver metabolism further supported the conclusions drawn in the opinion of the Committee in 1994. Data from therapeutic use of coumarin also suggested 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 reaffirmed concerns that a toxic epoxide intermediate may be produced in a significant proportion of the human population. Thus further information on genotoxicity was considered 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*. Since the 3,4-epoxide can be prepared synthetically, the Committee in 1999 considered that a study using [^{14}C]-ring-labelled coumarin of *in vivo* DNA-binding and DNA-adduct formation in the relevant target organs in rats, liver and kidney, would be more appropriate to resolve the issue of the genotoxic potential of coumarin. The Committee therefore required such studies to be submitted as soon as possible.

In this opinion the requested studies on DNA adduct formation in liver and kidney are evaluated and summarised as well as publications of additional studies relevant to the safety assessment of coumarin in food products and published after July 1999.

Terms of reference

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food is asked to re-evaluate the toxicity of coumarin in the light of the latest studies relevant to the safety assessment of coumarin in food products, particularly the specifically requested studies on possible DNA-adduct formation in the kidney and liver, and to consider whether the opinions on coumarin expressed by the SCF on 16 December 1994 and 22 September 1999 need to be updated.

ASSESSMENT

Chemistry

Coumarin is a naturally occurring flavouring substance. Coumarin is synonymous with 2H-1-benzopyran-2-one, 1,2-benzopyrone, cis-o-coumarin acid lactone, coumarinic anhydride, o-hydroxycinnamoic acid-δ-lactone, 2-oxo-2H-1-benzopyran, and is defined by the CAS number; 91-64-5. See figure 1 for the structural formula of coumarin and its major metabolites.

Occurrence and exposure

Coumarin is a naturally occurring compound present in a wide variety of micro-organisms and higher plants (Lake, 1999). Coumarin was first isolated from Tonka beans, and is found at high levels in some essential oils, particularly cassia leaf oil (up to 83,300 mg /kg) cinnamon leaf oil (40,600 mg/kg), cinnamon bark oil (7000 mg/kg) and in lavender oil and peppermint oil (20 mg/kg). Coumarin is also found in fruits (bilberry; 0.0005 mg/kg), green tea (1.2 – 1.7 mg/kg) and other foods, such as chicory (TNO, 1996). Many coumarin derivatives are also present in plants. Coumarin is listed as an "active principle" by the Council of Europe and the maximum permitted concentrations in foodstuffs are given in Annex II of European Directive 88/388/EEC (EEC, 1988). The general limit for coumarin in food and non-alcoholic beverages is 2 mg/kg, however, for alcoholic beverages and certain caramel confectionery, the permitted limit is 10 mg/kg and for chewing gum 50 mg/kg. The theoretical maximum daily intake (TAMDI) of coumarin was calculated to be about 4.1 mg/day or 0.07 mg/kg/day for a 60 kg person by Lake (1999).

Another estimate of intake has also been provided by Lake. The main source of coumarin in the diet is cinnamon. It would be more reasonable to assume that over a long period of time less than 5% of solid food would be flavoured with cinnamon or other ingredients capable of imparting the 2 mg/kg maximum concentration of coumarin. Considering an overall intake of 1.5 kg of solid food, this would reduce the

calculated maximum daily intake to about 1.3 mg/day or 0.02 mg/kg/day for a 60 kg person (Lake, 1999).

The Panel calculated a TAMDI of 1.5 mg coumarin/day which at a body weight of 60 kg would be equivalent to 0.025 mg coumarin/kg bw/day. The calculation was performed considering the concomitant consumption of 324 g of beverage in general, 133.4 g of food in general, 27 g of confectionery, 2 g of chewing gum and 20 g of alcoholic beverages, all containing coumarin at the current Maximum Permitted Concentration.

The Panel noted that coumarin is also found in fragrances and other cosmetic products, and since it is readily absorbed by the dermal route, exposure to coumarin from cosmetic products may be relatively high (twice as high as via food; Lake, 1999). This should not be ignored in an integrated risk assessment of coumarin.

BIOLOGICAL AND TOXICOLOGICAL DATA

Evaluation of the studies requested in the opinion of 22 September 1999 and of other relevant information since July 1999

Studies on genotoxic potential

The requested studies on DNA-adduct formation in the kidney and liver were submitted to EFSA. The ability of coumarin to covalently bind to the DNA in the target organs for tumour induction following long-term oral exposure was studied and evaluated in the liver and kidney of Sprague-Dawley (SD) and Fischer 344 (F344) rats. Four groups of 7 male rats were dosed with [^{14}C]-coumarin or non-radioactive coumarin. The [^{14}C]-radiolabel was located on the benzene ring. SD male rats were dosed with 60, 120, or 240 mg/kg [^{14}C]-coumarin (250 μ Ci/kg bw) or 240 mg non-radioactive coumarin/kg bw. F344 male rats were dosed with 25, 50, or 100 mg/kg [^{14}C]-coumarin (250 μCi/kg bw) or 100 mg non-radioactive coumarin/kg bw. The liver and kidneys were sampled 8 hours after exposure and DNA was extracted from both organs. Aliquots of the tissue homogenates and nuclear pellets were taken for liquid scintillation counting. Although large amounts of radioactivity were present in liver homogenates, there was no significant radioactivity in the DNA-fraction and thus it was concluded that there was no covalent binding to DNA. Indeed, the Covalent Binding Index (CBI) was 0.01 – 0.004 in all dosed groups, where CBI values $\leq$0.1 are usually considered as evidence for lack of direct interaction with DNA. Large amounts of radioactivity were present in kidney homogenates; however, there was no significant covalent binding to renal DNA from [^{14}C]-coumarin-treated rats of either strain. These data demonstrate that coumarin does not bind covalently to DNA (Swenberg, 2003).

The ability of coumarin to induce unscheduled DNA synthesis (UDS) in hepatocytes after *in vivo* administration to male SD rats was also studied. Results demonstrate that after oral administration at doses (32, 107 and 320 mg/kg bw) up to the maximum tolerated dose (MTD) of 320 mg/kg bw, coumarin does not induce UDS in male SD rat hepatocytes (Edwards *et al.*, 2000). In addition, coumarin was tested for its potential to cause genotoxic effects in mouse bone marrow cells using an *in vivo*

micronucleus assay (Api, 2001). Coumarin did not cause any increase in the incidence of micronucleated polychromatic erythrocytes in male or female mice at any of the dose levels tested 50, 100 and 200 mg/kg bw, whereas the positive control mitomycin C produced a significant increase. There was no evidence of coumarin or mitomycin C treatment related cytotoxicity to bone marrow cells. The results of this study indicate that coumarin is not able to induce micronuclei *in vivo* in mouse bone marrow (Api, 2001).

These new data indicate that coumarin is not genotoxic *in vivo*, supporting a non-genotoxic mode of action for tumour induction.

Studies on metabolism

A number of publications relevant to the metabolism of coumarin in different species, including man, have become available since 1999; they are reported below, after a brief summary of the information on coumarin metabolism considered in the previous SCF opinions.

Species differences in the hepatotoxic effects of coumarin are thought to be due to differences in its metabolism. The ranking of susceptibility to coumarin-induced hepatotoxicity is rat>mouse>human (Born *et al.*, 2002; Lake, 2002); in addition mouse is also prone to acute and chronic injury in the lung. Coumarin may be biotransformed by many different routes; however, the more relevant pathways are those shown in Fig. 1, leading to 7-hydroxycoumarin (7-HC) and to the coumarin 3,4-epoxide intermediate (CE), which spontaneously rearranges in an aqueous medium to *o*-hydroxyphenyl-acetaldehyde (*o*-HPA), by opening of the lactone ring to yield carbon dioxide. The aldehyde can be either oxidised to *o*-hydroxyphenylacetic acid (*o*-HPAA) or reduced to *o*-hydroxyphenylethanol (*o*-HPE).

Human metabolism of coumarin occurs predominantly to 7-HC, which together with its glucuronide and sulphate conjugates generally accounts for >60% of the urinary coumarin metabolites after oral administration. The reaction is catalysed by cytochrome P450 (CYP) 2A6, a polymorphic enzyme. The predominant reaction in rats is 3,4-epoxidation, *o*-HPA being the major product formed in liver microsomes and *o*-HPAA the major urinary metabolite. CE and *o*-HPA formation has been linked to acute lung toxicity and to mouse lung tumour induction. Marked strain differences have been reported in mouse 7-HC formation; however, in mice this pathway represents a limited fraction of total coumarin metabolism even in those strains showing relatively high levels of 7-HC formation.

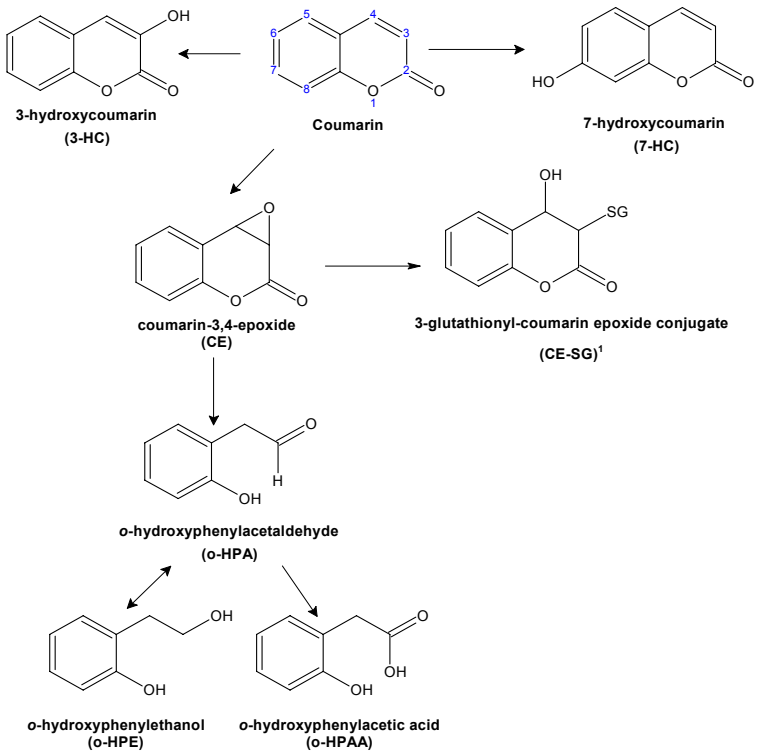

Figure 1. The major metabolic pathways in coumarin biotransformation

[1] The structure of the glutathione conjugate, that was actually described by Vassallo *et al* (2003), had a double bond at C3-C4 and did not contain the hydroxyl group at C4. According to Vassallo (personal communication, 2004) the double bond was re-introduced with a loss of water during sample clean-up.

Concerning metabolism in the lung, CE formation seems to be predictive of pulmonary damage. Indeed, *o*-HPA formation in mouse lung microsomes *in vitro* is comparable to that observed in mouse liver microsomes, being 20-fold higher than in rats. As the lung concentration achieved *in vivo* after an oral administration of coumarin is of similar magnitude in both species, the differential response of the rat and mouse lung to coumarin-induced toxic effects likely results from differences in the relative abundance of Clara cells and their metabolic activity (Born *et al.*, 2003). The lack of Clara cell toxicity in rat lung *in vivo* may be related to the absence of significant coumarin epoxidation in the tissue. The central role in catalyzing coumarin epoxidation in the mouse lung microsomes is played by CYP2F2, which is almost exclusively localized in mouse Clara cells (Born *et al.*, 2002). Furthermore, *o*-HPA formation was not detected in 10 individual lung microsomal samples of humans, indicating that humans are unlikely to be susceptible to coumarin-mediated pneumotoxicity (Caudill *et al.*, 2000).

After repeated oral coumarin administration (200 mg/kg), the mouse lung became tolerant to the toxic effects of coumarin; the results suggest that tolerance was not exclusively due to alterations in CYP-mediated metabolism (Born *et al.,* 1999).

A comparative study on metabolism and kinetics of coumarin in CD-1 and B6C3F1 mice and F344 rats clearly indicated that the differential tumour response in the mouse oral gavage and dietary assay is due to kinetic differences between the two routes of exposure, the lung concentration being 7-37-fold higher after the bolus

gavage doses of 50 or 200 mg/kg bw (Born *et al*., 2003) compared to a dietary intake of 1000 mg/kg feed.

The C_{max} for coumarin (at 200 mg/kg bw) in rat plasma was 4-fold lower than that in mouse; however, the AUC was similar in the two species, due to a longer half-life in the rat (20 h, *cf*. 4 h in the mouse), which may be due to enterohepatic circulation of coumarin and/or its metabolites, as a determinant for the higher susceptibility of rats to coumarin hepatotoxicity. Of the CE metabolites, only *o*-HPAA was detected in mouse and rat plasma and urine, indicating that CE is rapidly detoxified. Neither CE, nor *o*-HPA could be detected in the plasma of either species. (Born *et al.*, 2003). *In vitro* kinetics of coumarin 3,4-epoxidation in mouse and rat liver microsomes, as monitored by the spontaneous rearrangement to *o*-HPA, indicated that *o*-HPA formation is biphasic in rat and mouse liver microsomes, with a similar apparent high affinity K_m (about 40-50 μM) in the two species. However, the intrinsic clearance ($Cl_{int} = V_{max}/K_m$) of coumarin via epoxidation to *o*-HPA was 4-fold greater in mice than rats. These data indicate that hepatic toxicity does not directly correlate with the rate of CE plus *o*-HPA formation as observed in *in vitro* studies. Rather, the balance between bioactivation (epoxide formation and rearrangement to *o*-HPA) and detoxification (glutathione conjugation of the epoxide and oxidation of *o*-HPA to *o*-HPAA) likely dictates the *in vivo* susceptibility of a species to coumarin-mediated liver toxicity (Born *et al.*, 2000b).

This conclusion is supported by studies demonstrating that a known amount of CE is extensively detoxified by mouse (64% of the total) and rat (48%) liver cytosolic glutathione transferases (GSTs); on the contrary CE conjugate with GSH (CE-SG) was not as readily formed by human cytosol (5%), which produces almost exclusively *o*-HPAA (95-100%) as the major CE detoxification product. In addition to CE-SG, mouse liver cytosol produces only *o*-HPAA (35%), whereas *o*-HPAA (11%), *o*-HPE (25%) and a residual levels of *o*-HPA (2.5%) and *o*-HPE (25%) were measured in rat hepatic cytosol. (Vassallo *et al.*, 2003). The relatively low conversion of CE to CE-SG in human liver cytosol as compared to rat or mouse liver cytosol was also shown in a follow-up study (Vassallo *et al.*, 2004). In addition, it was also shown that the intrinsic clearance of *o*-HPA through oxidation to *o*-HPAA in mouse or human liver was 20 to 50 times higher than that in rat liver. In contrast, reduction of *o*-HPA to *o*-HPE appeared to be only of importance in rat liver, but not in those of mice and humans. As mouse liver microsomes produced CE at a higher rate than rat liver microsomes while the mouse is less sensitive than the rat for coumarin hepatotoxicity, the authors concluded that differences in detoxication of *o*-HPA are the determining factor for species differences in sensitivity to coumarin hepatotoxicity (Vassallo *et al.*, 2004).

The *in vitro* kinetics of CE formation was studied in human liver microsomes (HLM) from 12 individual donors, none of them with variant alleles for CYP2A6: *o*-HPA formation was described by a single enzyme model, with K_m values (range 1.3 to 7.4 mM) higher than in rodents, and lower Cl_{int} values (1/9 that of rat). The high coumarin concentration required for *o*-HPA production in HLM strongly suggest that humans are unlikely to produce toxicologically relevant concentrations of this metabolite following low level exposures (Born *et al.*, 2000a), when CYP2A6-mediated 7-hydroxylation greatly exceeds CE formation with K_m values ranging from 0.2 to 3.6 μM. Studies with human and rat recombinant enzymes indicated that the hepatic

CYPs catalysing CE formation are CYP2E1, and to a lower extent 1A1, 1A2 in both species. Coumarin 3-hydroxylation was mainly catalysed by rat CYP3A2 and by the human orthologue CYP3A4, confirming that CE and 3-hydroxycoumarin are the products of two different metabolic pathways (Born *et al.*, 2002).

In addition to the previously reported polymorphism discussed in the opinion of 1999, other studies in humans dealing with polymorphism and metabolism have since been published, such as the study of Cok *et al.* (2001). The biotransformation of coumarin to 7-HC has been studied in 50 Turkish volunteers, administered 2 mg coumarin, and the presence of total 7-HC (total + conjugated) was measured in urine samples. The presence of any other metabolites was not checked. Considerable interindividual variability in the metabolism of coumarin to 7-HC was shown. About 70 % of the subjects excreted less than 60 % of the administered dose, suggesting that within the Turkish population, a higher proportion of individuals show a reduced capacity to 7-hydroxylate coumarin than Northern European populations (i.e. 6% in the UK). Despite the limited number of analysed genotypes, results seem to indicate that individuals carrying the mutant CYP 2A6*2 and *3 alleles have a decreased capacity to form and excrete 7-HC. Although the frequencies in distribution of homozygous genotypes of CYP2A6*2 in Caucasians (1-3%) are lower than those initially reported, it has been shown that heterozygous individuals (*CYP2A6*1/*2*), have different kinetics in 7-HC formation, with CL_{int} values significantly lower than the wild type allele homozygous individuals (Inoue *et al.*, 2000). At present it is still unknown what pathway will take over the metabolism of coumarin in those individuals with decreased capacity to form 7-HC. It cannot be ruled out it could be 3,4-epoxidation, also considering that CYP other than 2A6, such as 2A13, expressed in human lung, nasal mucosa and trachea, has been reported to catalyse the formation of 7-HC and *o*-HPA (43 and 30% of total metabolites, respectively) to a comparable extent (Von Weymarn and Murphy, 2003).

Epidemiological data

The safety of the coumarin-containing drug SB-LOT (90 mg coumarin + 540 mg troxerutin per day for 16 weeks) was investigated in a randomized double-blind placebo-controlled clinical trial in 231 patients with chronic venous insufficiency. The placebo group consisted of 117 persons while the exposed group comprised 114 patients (Schmeck-Lindenau *et al*., 2003).

A sub-study (Burian *et al*., 2003) focussed on detection of a possible association between coumarin-induced hepatotoxicity and genotype of the CYP2A6 alleles. Monitoring of the hepatic status involved regular clinical measurements (ALAT, ASAT, AP and γ-GT; all in serum) of liver function parameters (LFT). Genotyping of CYP2A6 was carried out in 112 placebo- and 104 SB-LOT-treated participants by means of PCR and confirmed by DNA sequencing analysis. Variant CYP2A6*2 and CYP2A6*3 alleles, with reduced metabolic activity, were found in ten and six individuals, respectively (allelic frequencies: 0.023 and 0.014, respectively). In the placebo group, six individuals with a CYP2A6 allele and four with a CYP2A6*3 allele were present. The treatment group included four bearers of the 2A6*2 allele and two of the 2A6*3 allele). Homozygous patients with two defective alleles were not present (due to their low allelic frequency, experimental groups would need to be much larger (e.g. 10000) to find one homozygous patient with defective alleles).

Increased serum activities of liver enzymes were observed in patients in both treatment and placebo group, resulting in a risk for elevated LFT of 4.9% in the SB-LOT group as compared to 2.1% in the placebo group. The nine patients showing elevated LFT in the treatment group comprised eight persons who were wild-type homozygous and one who was heterozygous for CYP2A6. This latter person had the CYP2A6*2 allele and showed only elevated γ-GT, which was considered a only marginal indication of liver toxicity by the study authors. The other 5 heterozygous individuals in the treatment group did not show any sign of hepatotoxicity, similar to the rest of the wild-type homozygous persons treated with SB-LOT. As there was no significant difference in the incidence of liver dysfunction between heterozygotes with CYP2A6*2, CYP2A6*3 and wild-type homozygotes, the study authors concluded that heterozygosity in CYP2A6 is not a determinant of coumarin-associated liver dysfunction.

CONCLUSION

As reported in the previous opinions by the SCF, coumarin is a well known rat hepatotoxicant whereas mice are susceptible to lung injury; long term studies showed induction of liver and lung tumours at high doses, usually associated with hepatic and pulmonary toxicity. The available data on coumarin genotoxic potential, at the date of the previous opinions did not allow the exclusion with certainty of a genotoxic mechanism for coumarin-mediated carcinogenicity. For that reason the SCF had required studies to examine the potential of coumarin to induce DNA-adduct formation in liver and kidney after administration of [^{14}C]-ring-labelled coumarin to rats, to resolve the issue of the genotoxic potential of coumarin. These and some additional *in vivo* studies relevant to the toxicity of coumarin have now become available to the AFC Panel.

From the studies on adduct formation it can be concluded that coumarin *in vivo* does not bind covalently to DNA in target organs. This result is further supported by another study in which coumarin did not cause UDS in hepatocytes of male SD rats *in vivo* after administration of coumarin at a dose levels up to the MTD, as well by a negative micronucleus assay in mice. Therefore it is concluded that coumarin induces tumours by a mechanism, which is preceded by toxicity in the same target organ, and therefore allows a threshold-based approach and the establishment of a NOAEL.

Comparative studies on kinetics and metabolism of coumarin in humans and rodents have confirmed species differences, as reported in the previous SCF opinion (SCF, 1999): 7-HC formation, mediated by CYP2A6, is the major detoxifying pathway in human, whereas in rats and mice bioactivation to CE is the prevalent biotransformation reaction. From recent additional submitted data it is clear that CE is rapidly converted to *o*-HPA in rodents. In rats the detoxification process of *o*-HPA is slow compared to other animal species, explaining why in long-term studies liver toxicity and hepatic tumours were observed in rat only.

On the other hand, CE formation in mouse Clara cells and the relatively higher abundance of these cells in the terminal bronchiolar region have been reported as the major determinant for mouse susceptibility to lung toxicity and carcinogenicity. These

effects are clearly dependent on the mode of administration (gavage or dietary) and are species-specific with limited relevance to human health.

Since the latest studies show that coumarin is not an *in vivo* genotoxic agent, a threshold-based approach is justified and the no-observed adverse effect level (NOAEL) from long-term studies can be used to derive a tolerable daily intake (TDI) for coumarin.

To establish a NOAEL, it is necessary to consider the previously reported and evaluated long-term oral toxicity studies in animals. Therefore this part of the 1994 SCF opinion is included in this opinion as an annex and also the review of Lake (1999) is reconsidered. Several subchronic and chronic studies with mice and rats have been performed. The rat strains studied were Fischer 344, Osborne-Mendel and Sprague-Dawley. In most cases coumarin was administered via the diet. In the dietary studies, the no-effect levels for hepatotoxic effects ranged from doses equivalent to 50 to 130 mg coumarin/kg bw/day, whereas in mice the no-effect levels were higher (doses equivalent to 280 mg coumarin/kg bw/day or higher) (Lake, 1999; NTP, 1993). Other species, including gerbil and dogs, revealed hepatotoxic effects after dietary administration of 50 mg coumarin/kg bw/day or more. In the dog oral doses of 25 mg/kg bw/day for over 100 days caused histological liver damage, 10 mg/kg bw/day being a no effect level (Hagan *et al*, 1967). As in the rat, urinary excretion of 7-hydroxycoumarin is low in dogs, hamsters and some strains of mice (Cohen, 1979). Baboons only showed increased liver weight but no morphological or ultra-morphological changes after a dietary dose of 67.5 mg/kg bw/day. When coumarin was given by gavage to mice an increase of hepatocellular adenomas or combined adenomas and carcinomas was seen at dose levels of 50 and 100 mg/kg bw/day, but not at a dose level of 200 mg coumarin/kg bw/day in females. No such effects were found in males. Based on these tumour incidences, the NTP classified coumarin as producing some evidence of carcinogenic activity in the liver (NTP, 1993). From these data it can be concluded that the overall NOAEL is 10 mg coumarin/kg bw/day, based on the hepatotoxicity in dogs seen at the next higher dose level (25 mg coumarin/kg/bw/day).

In contrast to rodents and dogs, in humans the detoxifying pathway leading to 7-HC formation is prevalent. However a CYP2A6 polymorphism exists, particularly in Asia and Southern Europe, and people affected by this polymorphism show a decreased capacity to metabolise coumarin to 7-HC. In such individuals it is not known which pathway becomes more important and whether coumarin may be metabolised to a larger extent to CE and *o*-HPA. For this part of the human population the outcome of the rat and dog studies may be relevant.

The study by Burian *et al*. (2003) in humans appears to suggest that the polymorphism in coumarin epoxidation / 7-HC might be of very limited relevance for determination of the population spread in sensitivity. However, as no homozygous individuals with both alleles defective were represented in the group, this conclusion is of limited relevance, because it has been described that homozygosity for the defective allele has much greater impact on coumarin metabolism than heterozygosity (Hadidi 1997). In addition, in this study by Burian *et al*. (2003) coumarin metabolism data were not collected.

The Panel therefore concluded that hepatotoxic responses as observed in rodents and dogs should be taken into account in setting a TDI. In applying safety factors to the no-observed-adverse-effect level (NOAEL) for hepatotoxicity, it would be prudent to use a factor of 10 for potential interspecies variation, together with a factor of 10 for potential individual differences between humans. The overall NOAEL for liver toxicity in the most sensitive animal species, based on hepatotoxicity in a two year dog study, was 10 mg coumarin/kg bw/day. Applying a total safety factor of 100 to this overall NOAEL, the Panel concluded that a Tolerable Daily Intake (TDI) of 0 - 0.1 mg coumarin/kg bw can be established. Studies aimed at establishing which metabolic pathway would increase in the case of reduced 7-HC formation in humans would be helpful. Indeed, if the active pathway did not lead to hepatotoxic metabolites as observed for the 3,4-epoxidation of coumarin, a lower interspecies safety factor could be justified and applied.

Risk characterisation.

The TDI of 0.1 mg/kg bw would be equivalent to 6 mg for a person of 60 kg. The estimated theoretical maximum daily intake (TAMDI) of coumarin via food is 4.085 mg/day (0.07 mg/kg bw/day). Taking a more realistic intake scenario (Lake, 1999), or the TAMDI calculated by the Panel, the total daily intake would be 1.3 - 1.5 mg/day (0.02 mg/kg bw/day). Both intake scenarios are below the TDI.

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SCIENTIFIC PANEL MEMBERS:

Robert Anton, Sue Barlow, Dimitrios Boskou, Laurence Castle, Riccardo Crebelli, Wolfgang Dekant, Karl-Heinz Engel, Stephen Forsythe, Werner Grunow, Maria Heinonen, John Chr. Larsen, Catherine Leclercq, Wim Mennes, Maria Rosaria Milana, Iona Pratt, Ivonne Rietjens, Kettil Svensson, Paul Tobback, Fidel Toldrá,

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ANNEX 1. SCF opinion on coumarin of 16 December 1994.

The text below is from the Secretariat archives and the published version of the opinion can be found at
http://europa.eu.int/comm/food/fs/sc/scf/reports/scf_reports_36.pdf

CS/FLAV/55 Final

OPINION ON COUMARIN

(A CONSTITUENT OF NATURAL FLAVOURING SOURCE MATERIALS LIMITED BY ANNEX II OF FLAVOURINGS DIRECTIVE 88/388/EEC)

(expressed on 16 December 1994)

OPINION ON COUMARIN (A CONSTITUENT OF NATURAL FLAVOURING SOURCE MATERIALS LIMITED BY ANNEX II OF FLAVOURINGS DIRECTIVE 88/388/EEC)

(expressed on 16 December 1994)

Terms of reference

To review the toxicity of coumarin in the light of recently completed studies and to consider whether the limits for coumarin in food set out in Annex II of the flavourings Directive 88/388/EEC (EEC, 1988) need to be amended..

Background

Coumarin is a naturally occurring benzopyrone. It occurs in various plants including tonka beans and sweet clover and in several natural flavouring source materials. Coumarin itself was originally used as a flavouring substance until the direct use of coumarin in food was prohibited in the USA in 1954 following reports of hepatotoxic effects in rats and dogs. It is still used in fragrances and tobacco. More recently, it has been used in the medical treatment of high protein lymphoedema and chronic infections such as brucellosis and tuberculosis. Coumarin has also been investigated in the treatment of renal cell carcinoma, malignant melanoma and prostate cancer.

Coumarin is listed in the 3rd Edition of the Council of Europe 'Blue Book' (Council of Europe, 1981) as an 'active principle'. The Council of Europe defines an active principle as a constituent of a natural flavouring source material for which, due to existing toxicological data, it appears necessary to set a maximum concentration limit in foodstuffs as consumed. No substance included in the list of active principles is approved by the Council of Europe for use as a chemically-defined flavouring substance in its own right. For coumarin, the general limits are < 2mg/kg in food and beverages with specific exceptions of 10 mg/kg in "special" caramels and 10 mg/kg in alcoholic beverages. It should be noted that the Council of Europe considered that the general limit in food and beverages should be less than 2mg/kg, but that methods of analysis generally available at the time did not permit a lower level of detection.

The SCF examined the Council of Europe list of active principles in 1979 and considered that allowing the continued use of natural flavouring source materials with limitation of the active principles represented a practical approach (CEC, 1979). The Council of Europe limits were endorsed with the addition of another exception of 50 mg/kg in chewing gum. The SCF limits for coumarin are included in Annex II of the EC Flavourings Framework Directive 88/388/EEC. That annex sets 'maximum limits for certain substances obtained from flavourings and other food ingredients with flavouring properties present in foodstuffs as consumed in which flavourings have been used'. In other words, the limit only applies if flavourings have been added to a foodstuff but refers to the total amount of coumarin present in that foodstuff from either food ingredients or the flavouring. Annex II of the directive also contains a footnote indicating that the substances listed may not be added as such to foodstuffs or to flavourings.

Recently, a draft report of a US National Toxicology Program carcinogenesis bioassay on coumarin has been published (NTP, 1992a) and so the SCF has been asked to review coumarin. In the current review, the Committee has looked again at the earlier published toxicological studies on coumarin, as well as taking account of the recent NTP work and other studies published since our last review. The Committee was asked to consider whether the limits for coumarin in food, which apply when natural flavouring source materials are added to food, as set out in Annex II of the flavourings Directive, need to be amended. The SCF was not asked to consider coumarin as a chemically defined flavouring substance since it is currently prohibited for that purpose, nor was it asked to consider the context of its occurence solely as a natural toxicant in foodstuffs.

Discussion

Coumarin is hepatotoxic to rats and dogs, causing liver enlargement, focal necrosis, fibrosis and bile duct proliferation. Other species however appear to be more resistant to the hepatotoxic effects of coumarin.

In rats, clear evidence of hepatotoxicity has been seen after dietary administration of doses in the range 87-125 mg/kg bw/day and above (HRC, 1984; Brune, 1984; Cohen, 1979) and after oral gavage doses of 25 mg/kg bw/day and above (NTP, 1992a). In addition to hepatotoxicity, studies have also shown coumarin caused cholangiocarcinomas and hepatocellular carcinomas. These were seen in both sexes at dietary doses of 230-340 mg/kg bw/day in two chronic studies on Sprague-Dawley rats in which doses ranging from 13-283 mg/kg bw/day (HRC, 1984) or 10-340 mg/kg bw/day (Brune, 1984) were given. Cholangiocarcinomas were not observed at doses of 87-110 mg/kg bw/day and below in these two studies. No cholangiocarcinomas or hepatocellular carcinomas were seen in the one chronic study in F344 rats in which coumarin was given by gavage at doses of 25, 50 or 100 mg/kg bw/day (NTP, 1992a). Several of the earlier 90 day and 2 year rat dietary studies have shown 1000 ppm (approximately 50 mg/kg bw/day) to be a no effect level for histological liver damage (Cohen, 1979). Changes in liver weight and serum liver enzyme levels however are seen from lower doses of 10-25 mg/kg bw/day when given by gavage (NTP, 1992a; Cohen, 1979). One of the more recent 2-year dietary studies has shown minor, atypical changes in the bile ducts of Sprague-Dawley rats at doses down to 10 mg/kg bw/day, the lowest dose tested (Brune, 1984).

In the dog oral doses of 25 mg/kg bw/day for over 100 days caused histological liver damage, 10 mg/kg bw/day being a no effect level (Hagan *et al*, 1967). Studies in limited numbers of baboons have found no evidence of histological liver damage and only slight evidence of reversible biochemical, histochemical or ultrastructural changes after oral dosing at 50 and 100 mg/kg bw/day for 3 weeks or dietary administration of doses up to 67.5 mg/kg bw/day for 2 years (Cohen, 1979). It is not known whether doses higher than these would produce histopathological changes in the baboon.

Only slight liver enlargement and no hepatotoxicity was found in CD1 mice given up to 280 mg/kg bw/day via the diet for 2 years (HRC, 1983). In a 2-year gavage

study in B6C3F1 mice given 50, 100 or 200 mg/kg bw/day, syncytial alteration and centrilobular hypertrophy were seen in the liver at 200 mg/kg bw/day in females and in males at 100 mg/kg bw/day and above; syncytial alteration alone was seen at 50 mg/kg bw/day in male mice (NTP, 1992a). Significant increases in eosinophilic foci and hepatocellular adenomas were seen at 50 and 100 mg/kg bw/day but not at 200 mg/kg bw/day in female mice. In males, eosinophilic foci were increased at all doses but no hepatocellular adenomas were observed. In contrast to rats, no centrilobular necrosis was seen in mice of either sex. Similar increases in hepatocellular adenomas were seen in females in a 2-year gavage study in B6C3F1 mice using 3,4-dihydrocoumarin (which does not form an epoxide and causes no histopathological changes in rat liver) (NTP, 1992b). Also in contrast to the rat, coumarin did not cause liver necrosis in gerbils given a single i.p. dose of 125 mg/kg bw (Fentem *et al*, 1992), nor was it found to be hepatotoxic or hepatocarcinogenic in hamsters given up to 0.5% in the diet (approximately 600 mg/kg bw) for 2 years (Ueno and Hirono, 1981).

There are major interspecies differences in coumarin metabolism and these have been intensively studied to see if they might provide the explanation for the observed species differences in coumarin hepatotoxicity. In the rat, the major urinary metabolite is o-hydroxyphenylacetic acid from 3-hydroxycoumarin, whereas in baboons and man the major urinary metabolite is 7-hydroxycoumarin (Cohen, 1979). As in the rat, urinary excretion of 7-hydroxycoumarin is low in dogs, hamsters and some strains of mice (Cohen, 1979). One early theory of the mechanism of coumarin toxicity was that it would occur in species which did not form 7-hydroxycoumarin. However, the lack of hepatic effects in the hamster (Ueno and Hirono, 1981) suggests this is not the key difference. In the rat, recent studies indicate the involvement of a cytochrome-P450 generated metabolite in the hepatotoxicity of coumarin, possibly the formation of a hypothetical 3,4-epoxide intermediate in the oxidation of coumarin to 3-hydroxycoumarin (Lake *et al*, 1989; Fentem *et al*, 1992a). Other recent evidence has suggested that the conjugation of a coumarin metabolite with glutathione provides a detoxification pathway in the rat (Huwer *et al*, 1991). Whilst the epoxide hypothesis is plausible and there are several strands of evidence to support it, once again the lack of hepatotoxicity in the hamster is puzzling since, like the rat, hamster microsomes produce o-hydroxyphenyl-acetaldehyde and 3-hydroxycoumarin, both of which can be derived from the 3,4-epoxide (Lake *et al*, 1992a). o-Hydroxyphenyl-acetaldehyde is the major microsomal metabolite at high coumarin concentrations in many species including man (Lake *et al*, 1992a; Fentem *et al*, 1992b). 7-Hydroxycoumarin excretion in man is variable and can be as low as 10% (Cholerton *et al*, 1992). Alternative metabolic pathways used when 7-hydroxylation is low are unknown, but *in vitro* studies on human liver microsomes suggest that at high coumarin levels, when the 7-hydroxylation pathway is saturated then the o-hydroxyphenyl-acetaldehyde pathway may be favoured (Lake *et al*, 1992a; Fentem *et al*, 1992b). Clinical trials in man suggest that hepatotoxicity from coumarin is rare (Cox *et al*, 1989; Marshall, 1992), but it does occur. Thus the possibility that man may be susceptible to coumarin cannot be ruled out.

Tumours have been observed at sites other than the liver. In a 2-year oral gavage study on F344 rats given 25, 50 or 100 mg/kg bw/day (NTP, 1992a), coumarin

caused a dose-related increase in chronic nephropathy and a low incidence of renal adenomas (but not carcinomas) at 25 mg/kg bw/day (males) and 50 mg/kg bw/day (females). There was no clear dose response although, in males, this could have been due to the poor survival because of the nephropathy at the top two doses. Similar minor effects on the kidney were seen in a 2-year gavage study with 3,4-dihydrocoumarin in F344 rats (NTP, 1992b). No such effects were seen in the rat studies in which coumarin was given in the diet (HRC, 1984). In B6C3F1 mice given 50, 100 or 200 mg/kg bw/day coumarin by gavage for 2 years (NTP, 1992a), lung adenomas in both sexes and carcinomas in females were increased at the top dose. No such effects were seen in 2 year dietary studies in CD-1 mice given up to 280 mg/kg bw/day (HRC, 1983).

In mutagenicity studies *in vitro*, coumarin has tested negative in three Ames studies (Cohen, 1979), but positive or weakly positive in three others in one strain of Salmonella typhimurium, TA100, in the presence of metabolic activation (NTP, 1992a; Norman and Wood, 1981; Stoltz *et al.*, 1982). A further study using the Ames spot test was inadequate for evaluation (Florin *et al.*, 1980). Coumarin selectively inhibited excision repair in *E.coli* WP2 cells (Grigg, 1972). It increased the frequency of sister chromatid exchanges in Chinese hamster ovary (CHO) cells in the absence but not in the presence of metabolic activation and increased chromosome aberrations in CHO cells in the presence but not in the absence of metabolic activation (NTP, 1992a). Mutagenicity studies *in vivo*, comprising a sex linked recessive lethal assay in *Drosophila* (NTP, 1992a) and micronucleus tests in blood (NTP, 1992a) and bone marrow (Morris and Ward, 1992; Sterner and Korn, 1981) in mice, while negative, are of poor quality.

Conclusions

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 duct and adenomas of the kidney have been observed. In mice, adenomas and carcinomas of the lung and liver adenomas have been observed. In reaching our recommendations (see below) 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 the 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 occurences 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 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, 1992a) 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.

Recommendations

Taking into account the natural occurrence of coumarin in natural flavouring source materials, the carcinogenic activity of coumarin and the fact that a genotoxic mechanism cannot be excluded at this point in time, 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.

Further research

The Committee considered that further research on coumarin would be desirable, particularly if any proposals to raise the general limit from that now recommended (the lowest achievable limit of detection) were to be considered. In this regard, further mutagenicity information could be particularly helpful. The Committee understands that new *in vitro* mutagenicity studies have been carried out recently, under the auspices of the Research Institute for Fragrance Materials, 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 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 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 materials and foodstuffs to allow at least a rough estimate of coumarin intake in man.

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ANNEX 2. SCF opinion on coumarin of 22 September 1999.

***The text below is from the Secretariat archives and the published version of the opinion can be found at* http://europa.eu.int/comm/food/fs/sc/scf/out40_en.pdf**

EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate B - Scientific Health Opinions
Unit B3 - Management of scientific committees II

Scientific Committee on Food

SCF/CS/ADD/FLAV/61 final 29/9/99

Opinion on coumarin

(expressed on 22/9/99)

Rue de la Loi 200, B-1049 Bruxelles/Wetstraat 200, B-1049 Brussel - Belgium - Office: BE232 - 6/37.
Telephone: direct line (+32-2) 295.81.10 / 296.48.70, exchange 299.11.11. Fax: (+32-2) 299.48.91
Telex: COMEU B 21877. Telegraphic address: COMEUR Brussels.
http://www.europa.eu.int/comm/dg24/health/sc/scf/index_en.html

SCIENTIFIC COMMITTEE ON FOOD

SCF/CS/ADD/FLAV/61final

Opinion on coumarin

(expressed 22/9/99)

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-hydroxycinnamoic 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, CYP 2A6 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; Beamand *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 defiency) 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 ^{14}C 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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