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4 Public statement on the use of herbal medicinal products 5 containing estragole

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1. Introduction (Problem statement)

In 2005 (EMEA/HMPC/138386, 2005), the HMPC prepared the 'Public statement on the use of herbal medicinal products containing estragole'. There are a large number of plants and their preparations which contain estragole, sometimes in very high amounts. From the European perspective, the most interesting plants are *Foeniculum vulgare* Mill. (both fruit and essential oil) and *Pimpinella anisum* L. (fruit).

HMPC concluded on the basis of the available toxicological data that estragole is a naturally occurring genotoxic carcinogen with a DNA potency similar to the one of safrole. There is a general consensus that the mechanism of action of genotoxicity and carcinogenicity is the dose dependent production reactive metabolite, the sulfate conjugate of the 1'-hydroxy estragole, and its subsequent binding to DNA and eventual genotoxic and carcinogenic sequelae. The metabolic activation and DNA binding occur also in human experimental systems. However, as the HMPC concluded, that the profiles of metabolism, metabolic activation, and covalent binding are dose dependent and that the relative importance diminishes markedly at low levels of exposure (i.e. these events are not linear with respect to dose). In particular, rodent studies show that these events are minimal probably in the dose range of 1-10 mg/kg body weight, which is approximately 100-1000 times the anticipated human exposure to this substance.

For the above reasons HMPC concluded that the present exposure to estragole resulting from consumption of herbal medicinal products (short time use in adults at recommended posology) does not pose a significant cancer risk. Nevertheless, HMPC noted the need of further studies to define both the nature and implications of the dose-response curve in rats at low levels of exposure to estragole. In the meantime exposure of estragole to sensitive groups such as young children, pregnant and breastfeeding women should be minimised. Also, toxicological assessment of preparations for topical and external use needs further investigation because data on absorption through the skin are missing.

1.1. Estragole in plants and plant preparations

Estragole (1-allyl-4-methoxybenzene, molecular formula: $C_{10}H_{12}O$, molecular mass: 148.20 g/mol, CAS.-No.: 140-67-0) is a volatile phenylpropanoid belonging to a group of alkenylbenzenes such as eugenol, isoeugenol, methyleugenol, safrole, isosafrole, anethole, elemicin, myristicin, apiole. A comprehensive perspective on structural and metabolic variations of alkenylbenzenes was recently published by Rietjens *et al*. (2014).

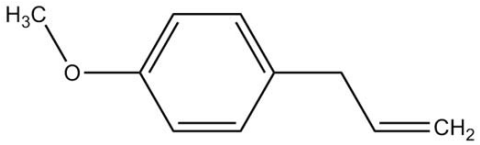

Fig. 1: Structural formula of estragole

Estragole is a major or minor component of a large number of plants or plant parts used for herbal medicinal products, botanicals and flavourings (Iten and Saller, 2004; EFSA, 2009). Table 1 provides some of the most important plants containing estragole. It is of importance to note that many of these plant sources contain a number of other alkenylbenzenes or other components which may affect the kinetics or dynamics of estragole. These potential matrix effects are being described in appropriate sections when research findings are available.

Table 1: Main occurrence of estragole in plants and/or essential oils (modified from EFSA, 2009, based principally on Council of Europe publications)

Botanical name	**Common name**	**Essential oil in plant (%)/estragole in essential oil (%)**	**Estragole in part of plant used (%)**
Agastache foeniculum (Pursh.) Ktze. (syn. *Lophantus anisatus A. anethiodora, A. anisata*) (Lamiaceae)	Anise hyssop, Giant hyssop, Liquorice mint	? / 74	
Anthriscus cerefolium (L.) Hoffm. ssp *cerefolium* (Apiaceae)	(Garden) chervil	0.9 in fruit/up to 85	max. 0.8
Artemisia dranunculus L. (Asteraceae)	Tarragon	0.25-1 in herb/60-75	0.7
Foeniculum vulgare Mill. subsp. vulgare var. *vulgare* (syn. *Foeniculum vulgare* Mill. var. dulce (Mill.) Batt. et Trab.) (Apiaceae)	Sweet fennel, Roman fennel	? / 1.5-5.0	
Foeniculum vulgare Mill. subsp. vulgare var. vulgare (syn. *Foeniculum vulgare* var. vulgare) (Apiaceae)	Bitter fennel, Common fennel	2-6 in fruit/3.5-12.0	0.3
Illicium verum Hook f. (Magnoliaceae)	Star-anise	5 in fruit/5-6l	max. 0.25
Melissa officinalis L. (Lamiaceae)	Lemon balm	no info/6.3	
Myrrhis odorata (L.) Scop (Apiaceae)	Sweet chervil	no info/up to 75	
Ocimum basilicum L. (Lamiaceae)	Sweet basil	0.8 in herb/20-89	approx. 0.4
Pimpinella anisum L. (Apiaceae)	Anise, Sweet cumin	1-4 in fruit/1-5	max. 0.04

In the earlier EMEA public statement (EMEA 2005) a large number of other plants, mainly essential oils, which contain estragole, were listed.

1.2. Exposure to estragole from herbal medicinal products and food

A major factor of relevance for the risk assessment and actions to take, is to evaluate the background exposure to alkenylbenzenes (and other related and relevant substances) from foodstuffs and food commodities of the consumer. Some official estimates of daily intake of estragole in foodstuffs indicate that baseline exposures are in the range of 0.5-5 mg estragole per day from the average food intake (Table 2). There probably exist large individual (and possibly regional) differences in estragole intake.

Table 2: Intake of estragole in foodstuffs

Daily exposure	**Comments**	**Reference**
4.3 mg	European data	SCF 2001
1 mg	approximate estimate, total intake from all sources	CoE 2005
166 µg 400-600 µg/day	US population from spice and spice oils estimate	JECFA 2009

EFSA (2009) calculated the intake of estragole from bitter fennel fruits. The exposure to estragole from bitter fennel fruits can be estimated based on the assumption that 4.5 to 7.5 g (3 times 1.5 to 2.5 g) of fennel fruits per day would be used for the preparation of fennel tea. Assuming that fruits contain 5% essential oil, that the extraction efficiency of the essential oil is 25-35%, and that there is 3.5-12% estragole in the oil, this would imply an intake of 1.9 to 15.8 mg estragole per day. For a 60 kg person this amounts to an intake of 33 to 263 µg estragole/kg bw/day.

Presence of estragole in actual preparations has been estimated in two studies. In a study of Bilia *et al.* (2002), fennel teas were prepared by classical infusion or microwave decoction of unbroken and crushed fruits, pre-packaged teabags and instant teas and estragole was analysed by gas chromatography/mass spectrometry (GC–MS). Estragole was present in teas as a minor component, 0.8–4.1% of the total volatiles, but it is not possible to estimate the extraction percentage from the original preparation. A recent study of van den Berg *et al*. (2014) described the analysis of estragole content in dry fennel preparations and in infusions prepared from them with a special emphasis on extraction efficiency. Estragole levels demonstrated a wide range of 0.15-13.3 mg/g in starting dry fennel preparations, whereas the estragole content in infusions was considerably lower ranging between 0.4 and 133.4 µg/25 ml infusion prepared from 1 g dry material. Extraction efficiency varied between <0.1 to 2.5% in a sample of 37 fennel-based preparations. Also the nature of the starting material proved important, because infusions prepared from whole fennel fruits contained about 3-fold less estragole compared to infusions prepared from fine cut fennel material. It seems obvious that the assumption of EFSA (2009) about extraction efficiency regarding infusions, 25-35%, is probably at least 10-fold higher than the actual extraction into infusion.

1.3. Regulatory status

There are currently no limits for estragole in the area of medicinal products.

In 2000 the Committee of Experts on Flavouring substances of the Council of Europe evaluated estragole and recommended a limit of 0.05 mg/kg (detection limit). Whether this limit is of intake or of content in herbal substance is not clear.

SCF (2001) concluded that estragole is both genotoxic and carcinogenic and on this basis recommended reduction in exposure levels and restrictions on use.

The expert panel of the Flavor and Extract Manufacturers Association concluded in 2002 that dietary exposure to estragole from spice consumption does not pose a significant cancer risk to humans because several studies clearly established that profiles of metabolism, metabolic activation and covalent binding were dose dependent at high levels but diminished markedly at lower levels of exposure (Smith *et al*., 2002).

EFSA (2009) used the study of Miller *et al*., (1983) as a basis for the derivation of margin of exposure (MOE) values for estragole. Groups of 50 CD-1 female mice, approximately 8 weeks old, were maintained for 12 months on grain diets containing 2300 or 4600 mg/kg estragole and the incidence of hepatomas was quantified (Miller *et al*., 1983). Incidences of hepatomas in female mice were 56 and 71%, respectively. Calculations on the basis of the worst-case scenario concluded that the $BMDL_{10}$ values vary between 9 and 33 mg/kg bw/day for female mice. The exposure to estragole from bitter fennel fruits estimated based on the assumption that 4.5 to 7.5 g of fennel fruits per day would be used for the preparation of fennel tea, amounts to 33 to 263 µg estragole/day for a 60 kg person. Using the $BMDL_{10}$ values of 9 to 33 mg/kg bw/day for female mice as derived from the Miller *et al*. study one can calculate a MOE of about 34 to 1000 which indicates that use of bitter fennel fruits for preparation of fennel tea could be considered a high priority for risk management.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) recently evaluated a group of allyl alkoxybenzenes, including estragole, present in foods and essential oils and used as flavouring agents (JECFA, 2009). The Committee concluded that the data reviewed on the six alkoxy-substituted allylbenzenes provide evidence of toxicity and carcinogenicity to rodents given high doses for several of these substances. A mechanistic understanding of these effects and their implications for human risk have yet to be fully explored and will have a significant impact on the assessment of health risks from alkoxy-substituted allylbenzenes at the concentrations at which they occur in food. Further research is needed to assess the potential risk to human health from low-level dietary exposure to alkoxy-substituted allylbenzenes present in foods and essential oils and used as flavouring agents.

2. Discussion

Since 2005, a large number of significant publications on estragole (and of various alkenylbenzenes) have appeared in the scientific literature and prompted HMPC to reassess the toxicology of estragole and of preparations containing these constituents.

2.1. Pharmaco-/toxicokinetics, ADME characteristics

The major metabolic pathways of estragole have well characterised in rats and mice *in vitro* and *in vivo* and studies have been published on *in vitro* metabolism of estragole in human hepatic preparations (Fig. 2). Three major metabolic pathways have been established:

1. O-demethylation resulting 4-allylphenol and more distal metabolites (and ultimate formation of CO_2). O-demethylation represents a detoxication pathway.
2. 1'-hydroxylation, which is a proximal active metabolite undergoing sulfoconjugation to 1'-sulfooxyestragole capable of binding to DNA and protein. 1'-Hydroxyestragole undergoes also further oxidation to 1'oxoestragole and glucuronidation to 1'-O-glucuronide. The principal enzymes in the bioactivation pathway are CYP1A2 (Jeurissen *et al*., 2007, human and mouse enzymes) and SULT1A1 (Suzuki *et al*., 2012, mouse enzyme).
3. Epoxidation of the allyl side chain leading to estragole-2',3'-epoxide, which is rapidly metabolised by epoxide hydrolase and glutathione transferase to detoxified metabolites (Guenthner *et al*., 2001). This pathway is also regarded as a detoxification route.

There is also the side chain terminal hydroxylation to 4-methoxy-cinnamyl alcohol, but it is not known what is the exact pathway for the formation of this metabolite, i.e whether it is formed via 2,3-epoxidation.

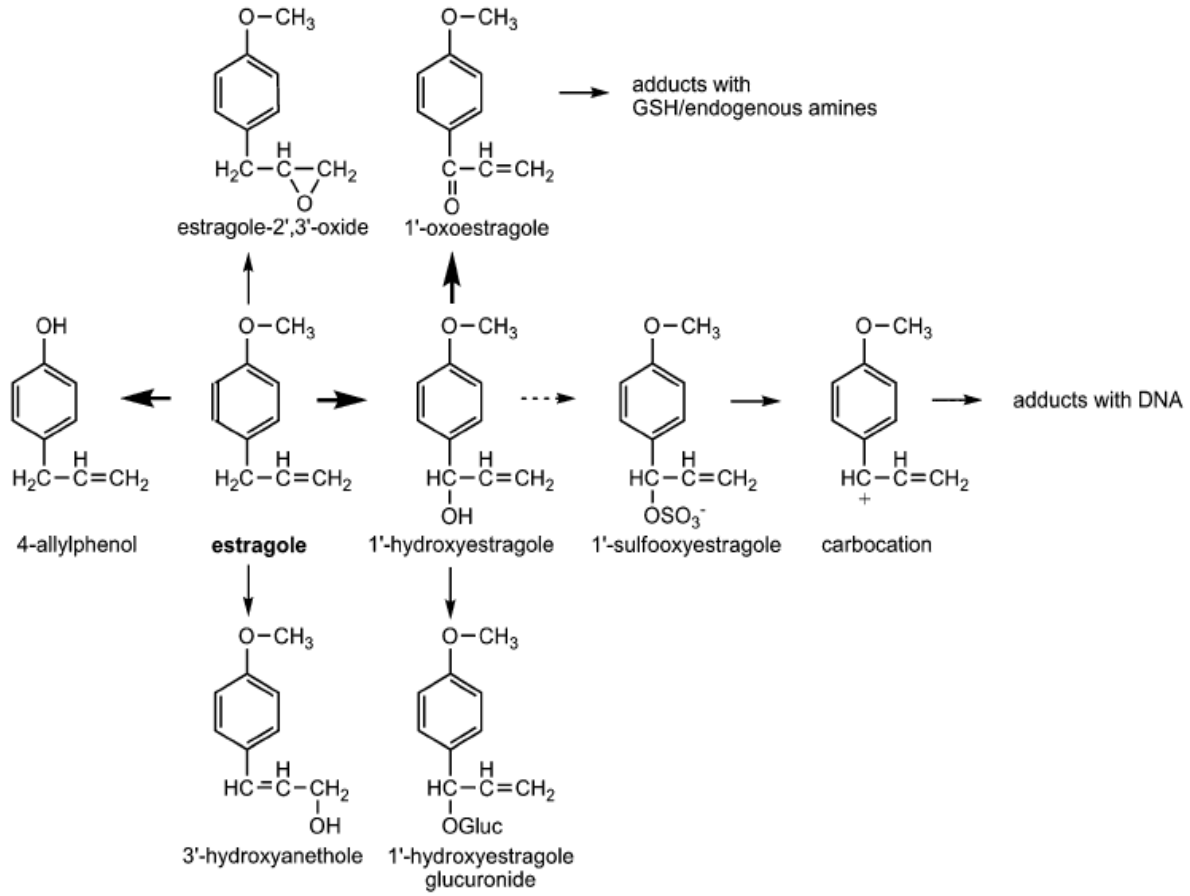

Fig. 2: Metabolic pathways of estragole (from Paini *et al*., 2012).

Proportions of individual metabolites of different pathways have been proposed to change as a function of dose (Anthony *et al*., 1987). At low doses (in the range of 0.05 to 50 mg/kg bw) O-demethylation predominates, whereas at higher doses (500 and 1000 mg/kg bw) urinary 1'-hydroxyestragole increases relatively. However, urinary concentrations of any single metabolite such as 1'-hydroxyestragole are dependent on both the formation and further biotransformations (and, naturally, other significant pharmacokinetic processes of importance for this particular metabolite) and do not necessarily reflect the concentration of the metabolite available for, say, adduct formation. Thus, a more distal marker for activation, e.g. adducts in target molecules, are more reliable evidence for potential dose-dependent change.

Concerning humans it has been reported that after oral administration of estragole to two volunteers (100 µg/day for 6 months) the excretion of 1'-hydroxyestragole in the urine amounted to 0.2 and 0.4% of the administered dose. Other metabolites detected were 4-methylhippuric acid 12%, 4-methoxyphenyllactic acid 4%, 4-methoxycinnamoylglycine 0.8% and 4-methoxyphenylacetic acid 0.5% (Sangster *et al*., 1987).

Rietjens's group has developed a physiologically-based biokinetic (PBK) model defined by apparent Vmax and Km values obtained in *in vitro* microsomal studies for the different phase I conversions of estragole and also for the phase II conversion of 1'-hydroxyestragole (Punt *et al*., 2008, 2009, Rietjens *et al*., 2010, Punt *et al.,* 2010). The performance of the model was analyzed based on existing *in vivo* animal and human data. The PBK model was extended into physiologically-based dynamic (PBD) model which would predict the formation of DNA adducts in the liver of male rats on the basis of *in vitro* incubations with rat hepatocytes exposed to estragole (Paini *et al*., 2010). The model was validated

using *in vivo* DNA adduct formation in the liver of mice exposed to estragole (Randerath *et al*., 1984). These models predict that the formation of the principal adduct in rat liver is linear up to at least 100 mg/kg bw, allowing for the estimation of adduct yields at realistic (human) exposures under certain set of assumptions.

For further validation of the model, Paini *et al*. (2012) quantified the dose-dependent estragole-DNA adduct formation in rat liver and the urinary excretion of 1'-hydroxyestragole glucuronide in male outbred Sprague Dawley rats (n = 10, per group), which were administered estragole once by oral gavage at dose levels of 0 (vehicle control), 5, 30, 75, 150, and 300 mg estragole/kg bw and sacrificed after 48 h. A dose-dependent increase in DNA adduct formation in the liver was observed. The increase in DNA adduct formation was statistically significant at a dose of 30 mg/kg and interindividual variability was high. In lungs and kidneys DNA adducts were detected at lower levels and mainly at higher concentrations (>150 mg/kg) than in the liver confirming the occurrence of DNA adducts preferably in the target organ, the liver. The results obtained showed that the PBD model predictions for both urinary excretion of 1'-hydroxyestragole glucuronide and the guanosine adduct formation in the liver were comparable within one order of magnitude to the values actually observed *in vivo*.

2.2. Acute and sub-acute toxicity

Rats given 4 daily doses of 605 mg estragole/kg bw displayed liver injury as observed on gross examination (Taylor *et al*., 1964). In the NTP study (Bristol, 2011) female mice administered 600 mg estragole/kg body weight died during week 1 because of liver necrosis.

2.3. Sub-chronic toxicity

In connection with the NTP program (Bristol, 2011), male and female F344/N rats and B6C3F1 mice were given estragole (greater than 99% pure) in corn oil by gavage for 3 months. Core and special study (rats only) groups of 10 male and 10 female rats and mice were administered 37.5, 75, 150, 300, or 600 mg estragole/kg bw in corn oil by gavage, 5 days per week. The core study groups were given estragole for 3 months and the special study groups for 30 days.

Rat study

All core study rats survived the 3-month exposure period. Toxicologically the most important findings were observed in serum (increase in ALT, SDH and bile salt) and liver (hepatocellular hypertrophy, bile duct hyperplasia, chronic periportal inflammation). Findings were generally dose-dependent and some responses were observed even at the lowest dose (37.5 mg/kg). Additionally, two 600 mg/kg male rats had multiple cholangiocarcinomas in the liver and a third had a hepatocellular adenoma.

Other toxicologically significant findings were observed in the erythron (anemia, decrease in total iron binding capacity, reactive thrombocytosis), bone marrow (hyperplasia), kidney (increased weight, tubular histology), the olfactory epithelium (degeneration at 2 highest doses), the pars distalis of the pituitary gland (chromofobied cells), submandibular salivary gland (cytoplasmic alterations), gastric glands in the stomach (atrophy), testes and epididymic (degeneration, hypospermia).

In the special study, serum gastrin concentration and stomach pH were significantly increased in rats exposed to 600 mg/kg for 30 days. Gastric gland atrophy was significantly increased in the stomach of 300 and 600 mg/kg rats. Hepatic 7-pentoxyresorufin-O-deethylase activity was significantly increased in all exposed groups except 37.5 mg/kg females, and the increases were generally dose related.

Mouse study

In the mouse core study, a 600 mg/kg male died during week 9, and all 600 mg/kg female mice died during week 1; the female deaths were attributed to liver necrosis caused by estragole exposure. In the mouse, liver was the principal target organ based on increased weights, hepatocellular hypertrophy and hepatocellular degeneration, oval cell hyperplasia, and necrosis (all 600 mg/kg female mice). NOAEL level was 37.5 mg/kg bw daily, based on increased liver weights in males and incidences of oval cell hyperplasia in females at 75 mg/kg.

Other significant findings were in the gastric glands of the glandular stomach (degeneration), the forestomach (squamous hyperplasia, mineralization, and ulcer), and olfactory epithelium (degeneration). These findings were statistically significant at the one of two highest doses.

On the basis of acute and sub-chronic studies, liver is the principal target organ in both rats and mice.

2.4. Chronic toxicity

No animal or human studies have been identified in the literature. Estragole is included into the NTP program.

2.5. Genotoxicity

Prokaryotic tests

Earlier studies have been assessed and summarized by Tice (1999), EMEA (2005), CoE (2005) and EFSA (2009).

Results of mutagenicity testing of estragole in *Salmonella typhimurium* were generally negative, likely due to the complex metabolism required for bioactivation *in vivo*. In the NTP study (Bristol, 2011) estragole was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 when tested in the presence or absence of exogenous metabolic activation enzymes.

Positive results were reported for estragole in strain TA1535 with the addition of the sulphation cofactor 3'-phospho-adenosine-5'-phosphosulphate (PAPS). The putative toxic metabolites of estragole, namely 1'-hydroxyestragole and allyl epoxides of estragole, were generally positive in mutagenicity assays with or without exogenous activation.

Estragole produced mixed results in a DNA repair test, exhibiting dose-related DNA damage in *Bacillus subtilis* in one study and exhibiting negative results in *Bacillus subtilis* and *Escherichia coli* in another.

Eukaryotic in vitro tests

Estragole and its metabolites induced unscheduled DNA synthesis (UDS) in several studies in human and rat cell lines or *ex vivo* in the livers of rats treated orally with estragole.

Martins *et al*. (2012) evaluated the genotoxicity of estragole in V79 cells using the sister chromatid exchange (SCE) assay and the alkaline comet assay and in in two CHO cell lines using the Comet assay. An increase in SCE without the S9 mix was observed. A positive result was also observed in the alkaline comet assay without S9, indicating DNA strand breakage. In V79 cells a dose-dependent formation of DNA adducts by use of the (32)P-postlabelling assay was observed. Comet assay in two CHO cell lines was positive without biotransformation. The results suggest that estragole, besides being metabolized to genotoxic metabolites, may also be a weak direct-acting genotoxin that forms DNA adducts.

In vivo tests

In the *in vivo* rat study (Nesslany *et al*., 2010), the UDS assay in rat liver was positive, but a bone-marrow micronucleus test was negative.

In the *in vivo* mouse micronucleus test (Bristol, 2011), no increases in the frequencies of micronucleated normochromatic erythrocytes were observed in peripheral blood samples from male and female mice in the 3-month study.

Estragole is clearly genotoxic in transgenic mouse and rat strains (Suzuki *et al*., 2012a, b). For details of these studies, see below.

2.6. Carcinogenicity

No human studies are available.

Mouse studies

In the early studies of the Millers' laboratory (Drinkwater *et al*., 1976; Miller *et al*., 1983, Wiseman *et al*., 1987) estragole or its natural metabolites including 1'-hydroxyestragole or synthetic derivatives administered to adult or newborn mice of different strains (CD-1, B6C3F1, CeH/HeJ, or C57B1/6J) through different routes of administration (diet, oral intubation, ip or sc injection), produced hepatocellular carcinomas. For the carcinogenic potency of estragole in female mice a TD_{50} of 50-100 mg/kg bw resulted from the above studies (CoE, 2005).

Rat studies

A sc injection study of derivatives of estragole in male rats did not observe any treatment-related increases in tumours.

In the above mentioned 3-month NTP study (Bristol, 2011), two 600 mg/kg male rats out of 10 animals had multiple cholangiocarcinomas in the liver and a third had an hepatocellular adenoma.

Further evidence for carcinogenicity of estragole are provided by a recent ToxCast toxicogenomics-based modelling study of Auerbach *et al*. (2010). An ensemble of support vector machine classification models based on male F344 rat liver gene expression following 2, 14 or 90 days of exposure to a collection of hepatocarcinogens (aflatoxin B1, 1-amino-2,4-dibromoanthraquinone, N-nitrosodimethylamine, methyleugenol) and non-hepatocarcinogens (acetaminophen, ascorbic acid, tryptophan) was developed. Independent validation was performed using expression data from the liver of rats exposed at 2 dose levels to a collection of alkenylbenzene flavoring agents. The models differentiated between hepatocarcinogenic (estragole and safrole) and non-hepatocarcinogenic (anethole, eugenol and isoeugenol) alkenylbenzenes previously studied in a carcinogenicity bioassay. The models predict that two alkenylbenzenes not previously assessed in a carcinogenicity bioassay, myristicin and isosafrole, would be weakly hepatocarcinogenic if studied at a dose level of 2 mmol/kg bw/day for 2 years in male F344 rats.

2.7. Reproductive toxicity

No data on reproductive toxicity and teratogenicity are available.

2.8. Mode-of-action (MoA) considerations

The best evidence for a genotoxic mechanism comes from metabolic activation studies: CYP enzymes, especially CYP1A2 (but also others) catalyze the formation of 1'-hydroxyestragole, which, via

sulfoconjugation by SULT1A1 and the spontaneous formation of reactive carbocation, binds readily to DNA. Adducts have been characterized both in mice and rats also after *in vivo* exposure to estragole.

On the basis of the above consideration, estragole is a genotoxic hepatocarcinogen and DNA adduct(s) is (are) the first pre-initiation step.

Even if there have been no convincing reports regarding estragole hepatocarcinogenicity in rats, a recent study of Suzuki *et al.* (2012a) suggests a possible involvement of genotoxic mechanisms. They examined hepatocarcinogenicity (GST-P, glutathione S-transferase placental type) and proliferation (PCNA, proliferating cell nuclear antigeb) biomarkers, DNA adduct formation and *in vivo* genotoxicity of estragole in the livers of wild and reporter gene-carrying F344 rats. Males were administered 600 mg/kg bw estragole by gavage and sequentially sacrificed at weeks 4, 8 and 16 for GST-P and PCNA immunohistochemistry and measurement of estragole-specific DNA adducts by LC-MS/MS in the livers. GST-P-positive foci increased with time in estragole-treated rats from week 4, PCNA-labeling indices being similarly elevated at both weeks 4 and 8. estragole-specific DNA adducts such as estragole-3'-N(2)-dG, 3'-8-dG and 3'-N(6)-dA were consistently detected, particularly at week 4. In a second study, male F344 gpt delta rats were administered 0, 22, 66, 200 or 600 mg/kg bw estragole for 4 weeks. Gpt (guanine phosphoribosyltransferase) mutant frequency in the liver was increased in a dose-dependent manner, with significance at 200 and 600 mg/kg bw in good correlation with PCNA-labeling indices. Mutation spectra analysis showed A:T to G:C transitions to be predominantly increased in line with the formation of ES-3'-N(6)-dA or 3'-8-dG. These results indicate that estragole could be a possible genotoxic hepatocarcinogen in the rat, at least when given at high doses.

Suzuki *et al*. (2012b) studied the role of SULT1A1 in the potential carcinogenicity of estragole in mice, by assessing the frequency of micronuclei in polychromatic erythrocytes and the mutant frequency of reporter genes in male and female gpt delta mice treated with estragole at doses of 0 (corn oil), 37.5, 75, 150 or 300 (250 in females) mg/kg bw by gavage for 13 weeks. There is a large sex difference in SULT1A1 activity in the mouse liver, higher in females. In this study the mRNA levels of Sult1a1 in female gpt delta mice were 3- to 6-fold higher than those in the males. The levels of estragole-specific DNA adducts in the females were higher than those in the males at all doses except the highest dose. In addition, mutation frequencies of the gpt gene were significantly increased from doses of 75 mg/kg bw of females, but the increment was observed only at the highest dose in males. There were no changes in the micronucleus test among the groups. The authors suggest that specific DNA modifications by the SULT1A1-mediated carbocation formation and the resultant genotoxicity are key events in the early stage of estragole-induced hepatocarcinogenesis of mice. This finding is in line with earlier studies in which a potent inhibitor of SULT activity pentachlorophenol inhibited estragole-induced hepatocarcinogenicity as well as DNA adduct formation (Fennell *et al*., 1985, Wiseman *et al.,* 1987).

2.9. Estragole alone or in plant-derived complex mixtures

One of the basic question concerning estragole toxicity is the following: does the matrix (i.e. phytochemical or formulary environment) affect the toxicity of estragole? Recently, Gori *et al.* (2012) analyzed the factors and conditions affecting the carcinogenicity of estragole and concluded that the studies performed thus far give a toxicological profile of estragole as an isolated compound and not the profile risk of the entire complex phytochemical mixture. In their analysis of literature, a multitude of substances in preparations affect the fate and effects of estragole, and probably to the direction that carcinogenic risk is greatly reduced, if not completely disappeared.

Rietjens *et al*. (2011) have speculated the existence of several concepts which may lead to reassessment of risk analysis of complex herbal mixtures. 1) Reactive electrophilic metabolites may

have beneficial effects, because they may induce the protective gene expression via the electrophile responsive element (EpRE)-mediated pathways, including Nrf-2 pathway. Especially electrophilic quinone/quinone methide type metabolites are implicated in this respect (see Boerboom *et al.,* 2006, Lee-Hilz *et al*., 2007). 2) Inhibition of dissolution, uptake, or activation of alkenylbenzenes by flavonoids, an effect conceptualized as a matrix effect.

Rietjens's group has also some *in vitro* evidence for the inhibition of sulfoconjugation of 1'-hydroxyestragole by constituents of the basic extract, the most potent of which was nevadensin (Ki for SULT inhibition 4 nM) (Jeurissen et al., 2008; Alhusainy *et al*., 2010). By employing the recently developed PBK model (Paini *et al*., 2010) they predicted that co-administration of estragole at a level inducing hepatic tumours *in vivo* (50 mg/kg bw) with nevadensin results in a considerable inhibition of formation of the ultimate carcinogen 1'-sulfooxyestragole. To validate this finding, estragole and nevadensin were co-administered orally to Sprague-Dawley rats, at a ratio reflecting their presence in basil (Alhusainy *et al.*, 2013). Given the role of the SULT-mediated DNA adduct formation in the hepatocarcinogenicity of estragole, these *in vivo* results suggest that the likelihood of bioactivation and subsequent adverse effects in rodent bioassays may be lower when estragole is dosed with nevadensin compared to dosing of pure estragole. In contrast to the above findings, Müller *et al*. (1994) showed that the genotoxic potential of estragole is not masked by ingredients of basil oil. The genotoxic potentials of basil oil and estragole were compared in the UDS test, using basil oil with an estragole content of 88%, and it was concluded that basil oil induced UDS in the same dose range as estragole (Müller *et al.,* 1994). Obviously basil oil contains a high concentration of estragole and the outcome in herbal products with a lower concentration of estragole could be different regarding attenuation of genotoxicity. Consequently, the matrix effect regarding estragole in various herbal preparations remains somewhat debatable.

In conclusion, it seems that there are credible mechanisms or processes which may affect the manifest toxicity of compounds in the phytochemical matrix. However, clear evidence that these mechanisms are operative also in appropriate long-term cancer bioassay conditions, save *in vivo* human situation, may be desirable.

3. Conclusions and Recommendations

3.1. Relevance of experimental toxicities for human risk assessment

Are the tumours observed in animal experiments relevant for human risk assessment?

Hepatocellular tumours, especially adenomas, are often regarded rodent-specific tumours especially if a rodent-specific mechanism of action (liver enzyme induction) could be elicited. There is some preliminary findings of liver enzyme induction in rats, but on the other hand, there is a lot of evidence for genotoxic mechanism, which on the balance may not be equally rodent-specific and seems more significance or at least better investigated. Consequently, genotoxicity-initiated tumours in animals are probably relevant for human risk assessment.

Is the mode of action for tumour formation relevant for human risk assessment?

For estragole, metabolic activation pathway and DNA adduct formation are amply demonstrated in animals and the same pathway is operative in human *in vitro* systems. There is general consensus that adduct formation is causally related to tumorigenesis, unless there are specific and biologically persuasive reasons to the contrary. Consequently, the mode of action for tumour formation is relevant for humans. Furthermore, several closely related alkenylbenzenes such as methyleugenol and safrole display similar characteristics regarding model of action and tumour formation.

Are toxicokinetic data (metabolic behaviour, activation etc) conducive to extrapolation of animal data to humans?

Although toxicokinetics and metabolism of estragole have not been thoroughly studied in humans, there is evidence that under *in vivo* administration of estragole to humans, the liver is exposed to the compound and the first step in metabolic activation, the formation of 1'-hydroxyestragole, takes place. Thus it is probable that toxicokinetic processes in humans are sufficiently similar to those in rodents in which carcinogenicity has been observed, that extrapolation can be regarded adequately reliable. Further *in vitro* and *in vivo* human studies are needed, but it is anticipated that with the help of a refined PB-toxicokinetic/dynamic model scientifically satisfactory view of estragole toxicokinetics and related dynamics could be developed to help human risk assessment.

3.2. Summary of weight of evidence toxicity risk assessment of estragole

A modified weight-of-evidence (WoE) assessment is formally presented in table 3 taking into account the findings and argumentations above.

Table 3: Summary of WoE evaluation of genotoxicity and carcinogenicity of estragole

Structure/grouping	Closely related alkenylbenzenes are animal genotoxins and carcinogens (safrole, methyleugenol: IARC class 2B), which provide additional albeit indirect evidence for estragole assessment
Computational models	Structural alert models: no information Machine learning models based on toxicogenomics of a set of hepatocarcinogens and non-carcinogens suggest that estragole is hepatocarcinogenic.
Metabolic activation	Convincing evidence for the activation pathway via hydroxylation and sulphoconjugation in rodent and human *in vitro* systems and in rodents *in vivo.*
DNA binding *in vitro*	Identified adducts in rodent and human hepatocytes.
DNA binding *in vivo*	Identified and measurable adducts in livers of mice and rats.
Genotoxicity *in vitro*	Difficult to demonstrate in conventional prokaryotic assays probably because of special activation pathway; generally low mutagenicity without S-9 mix. Some evidence in eukaryotic systems.
Genotoxicity *in vivo*	Demonstrated in rats and mice by transgene mutation techniques. Micronucleus tests consistently negative.
Carcinogenicity	Clear evidence of carcinogenicity in mice. Suggestive, but indirect evidence in rats.
Human information	Metabolic activation pathway present and operative also *in vivo*.
Non-linearity in metabolic activation	Some evidence of dose-dependent non-linearity of metabolic activation and adduct formation. Biokinetic modelling based on *in vitro* and *in vivo* parameters suggests dose-dependent activation.

Potential matrix effects	Some evidence for potential effects of activation inhibitors in herbal and botanical mixtures (nevadensin). Herbal mixtures may contain antigenotoxic and anticarcinogenic substances.
WoE conclusions	Estragole is a genotoxic carcinogen in rodents. The MoA seems to be similar in humans as far as it has been possible to study. Processes resulting in a threshold for genotoxic and carcinogenic actions are possible, but ultimately need further investigations. Exposure to estragole may be assessed as if it is "reasonably anticipated to be a human carcinogen", i.e. risk assessment paradigm should follow other proven carcinogens (however, 'officially' no such evaluation and conclusion by IARC or NTP has been made).

3.3. Recommendations

Because of the generally accepted evidence of genotoxic carcinogenicity, exposure to estragole should be kept as low as practically achievable. In the evaluation of herbal medicinal products containing estragole Member States should take steps to ensure that the public are protected from exposure and the following thresholds should be applied.

The existence of mechanisms leading to a dose response that is non-linear or has a practical threshold is increasingly recognized, also for DNA-reactive compounds, whose effects may be modulated by, for example, rapid detoxification before coming into contact with DNA, or by effective repair of induced damage, all factors mentioned in the recently endorsed guideline (ICH M7). With respect to complex herbal preparations, it is of importance to consider that the actual exposure situation possibly creates practical thresholds. There are several factors which interfere the absorption and bioavailability of other components, inhibit the bioactivation of potential toxicants, scavenge reactive intermediates or provide protection against toxic mechanisms by rapid detoxication, antioxidation or antimutagenesis (see section 2.9 for further details concerning estragole). Consequence of these protecting mechanisms may be the existence of a practical threshold. In individual cases these mechanisms may be difficult to quantify, but if there are experimental results to point to such factors, as is the case with estragole (see section 2.9), a conservative estimate is that they may provide at least 10-fold increase in a limit value. The regulatory approach to such compounds can be based on the identification of a No-Observed Effect Level (NOEL) and use of uncertainty factors to calculate acceptable limits. However, until now, no such data are available for estragole.

Oral use

In the case of estragole, the $BMDL_{10}$ value 10 mg/kg bw/day - based on induction of hepatomas by estragole in female mice (EFSA, 2009) – is taken as a measure of potency. Because the value is the statistical lower boundary value for 10% response, it is an effect value and consequently the NOAEL value is lower. This fact can be taken into consideration by using a higher uncertainty factor of 1000 to provide an acceptable level of protection.

To derive an acceptable dose, divide by 1000: 10 mg/kg/day ÷ 1000 = 10 µg/kg/day

Generally for adults the calculation is done with a body weight of 50 kg[1]. Therefore the daily dosage would be: 10 µg/kg/day x 50 kg body weight = 0.5 mg/person/day

Thus, the acceptable daily dose is 0.5 mg/person/day.

It is also of importance to take into consideration the duration of treatment by a herbal medicinal product, especially when potentially genotoxic carcinogens are dealt with. The intake of 0.5 mg/person/day (even if the limit presents the overall intake from all sources) can be accepted for herbal medicinal products as short-term (maximum 14 days) intake.

Dietary background

The potential daily intake of estragole via food cannot be ignored especially as consumers/patients are not able to avoid them. Although rigorous and comprehensive estimates of estragole intake via food are not available, values of 0.5–5 mg daily have been presented by various authorities in the EU and the USA (see Table 2). The dietary intake estimates are thus up to 10-fold higher that the above limit value of 0.5 mg/person/day. However, the extraction efficiency of estragole from food items may be considerable less than 25-35%, assumed by EFSA (2009). Assuming the maximum extraction value of 2.5% taken from Van den Berg *et al*. (2014) and the maximum intake of 5 mg via food items, the calculated "real" intake is 0.125 mg/person/day and probably much less. This theoretical calculation demonstrates that it is very important to investigate extraction efficiencies of estragole from various commodities and products.

Sensitive groups: Children

If children are included in the usage of certain products the daily amount of estragole has to be adjusted to the body weight of the age group: e.g. body weight of 20 kg would lead to an acceptable daily intake of 0.2 mg estragole/day.

Pregnant and breast feeding woman

Sensitive groups such as pregnant and breast feeding woman are also covered by the limit calculated above. If these limits are complied with, the chapter 4.6 of the SmPC of the products concerned should be phrased according to the 'Guideline on risk assessment of medicinal products on human reproduction and lactation: from data to labelling' (EMEA/CHMP/203927/2005).

Cutaneous use

No data concerning absorption of estragole through the skin exist. It is to ensure that the amount of estragole within the daily dose is <0.5 mg for adults (maximum 14 days). The use is restricted to intact skin.

Higher contents of estragole within the products would be possible if for the relevant product (means the relevant matrix, because absorption might be greatly influenced by the excipients, for instance essential oils as enhancers) low absorption rates can be shown, not exceeding the daily intake of 0.5 mg estragole for adults.

[1] For ~18% (average) of the European population the body weight is given with less than 60 kg [EUROPEAN COMMISSION 2006]. These numbers would increase to up to 30%, if only taking into account woman. Therefore the calculation is linked to a body weight of 50 kg.

Sensitive groups: Children

If children are included in the usage of certain products the daily amount of estragole has to be adjusted to the body weight of the age group: e.g. body weight of 20 kg would lead to an acceptable daily intake of 0.2 mg estragole/day.

Pregnant and breast feeding woman

Sensitive groups such as pregnant and breast feeding woman are also covered by the limit calculated above. If these limits are complied with, the chapter 4.6 of the SmPC of the products concerned should be phrased according to the 'Guideline on risk assessment of medicinal products on human reproduction and lactation: from data to labelling' (EMEA/CHMP/203927/2005).

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