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Public statement on the use of herbal medicinal products containing toxic, unsaturated pyrrolizidine alkaloids (PAs)

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Table of contents

1. Introduction (Problem statement)

It became apparent during assessment of *Symphytum officinale* (monograph EMEA/HMPC/572844/2009) that the risk assessment of pyrrolizidine alkaloids (PAs) poses considerable difficulties, with several PAs being regarded as both hepatotoxic and carcinogenic. Considering that PAs are natural constituents of a number of plants used for medicinal purposes and that PAs might be part of the food chain, the HMPC decided to prepare a public statement on the use of herbal preparations containing PAs.

1.1. Pyrrolizidine alkaloids (PAs)

Pyrrolizidine alkaloids are heterocyclic organic compounds. They occur in nature in more than 6,000 plants (in excess of 300 plant species of up to 13 families, mainly in the families of Boraginaceae (all genera), Asteraceae (tribes Senecioneae and Eupatorieae) and Fabaceae (genus *Crotalaria*)) [PRAKASH *et al*. 1999]. More than 350 different PAs, excluding the N-Oxides, were described up to now and it is assumed that about half of them are hepatotoxic [FU *et al*. 2004].
Furthermore, both the composition and concentration of PAs may fluctuate according to climatic and environmental conditions, the age and part of the plant and the variety (genotype/chemotype) [HOOGENBOOM *et al*. 2011]. Thus, all known PAs of a PA-containing plant are not necessarily found together at the same time. Furthermore, the same species growing in different locations or in different seasons may contain different alkaloids [MATTOCKS 1986].

1.2. Chemistry of pyrrolizidine alkaloids

Most PAs are esters of hydroxylated 1-methylpyrrolizidines. The basic components, called necines, are derived from bicyclic amino alcohols which, in turn, are derived from 1-hydroxypyrrolizidine. The acids with which the necines are esterified are called necic acids.

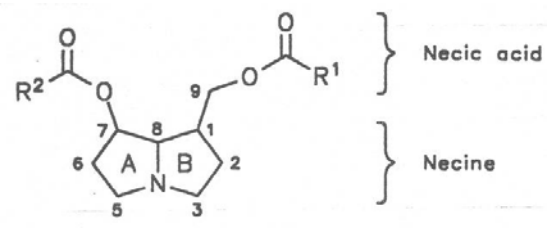


Fig. 1: general structure of PAs [ROEDER 2000]

Necines
In PAs of the retronecine- and heliotridine type, the necine base is made up of two five membered rings, inclined towards each other and sharing a common nitrogen at position 4. The necine can either be saturated or possess a double bond in the 1,2-position (ring (b), Fig. 2). In almost all cases the necine has a hydroxymethyl group at C-1 and usually a hydroxyl group at C-7 as well. Esterification can take place in this position. In addition, the necine may have one or two hydroxy groups at C-2 or C-6 resulting in the formation of stereoisomers [ROEDER 2000].

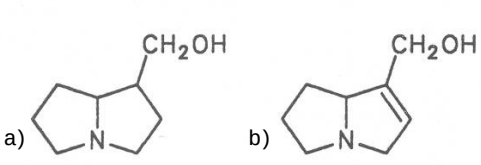


Fig. 2: structure of necines (retronecin type) [ROEDER 2000]

Otonecine-type PAs do not contain genuine bicyclic five-membered ring systems. They may act as a pyrrolizidine ring system due to transannular interactions. The PAs derived from these structures constitute a subgroup of the otonecine alkaloids (OPAs).

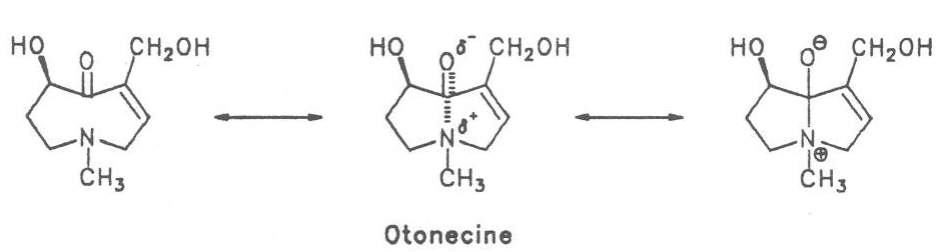


Fig. 3: otonecine: the binding between the N atom and the CO group is widened to such an extent that the indicated resonance structures result [ROEDER 2000]

Necic acids

Apart from acetic acid, the necic acids, possess 5 to 10 C atoms and differ from each other in their structure. They include mono- and dicarboxylic acids with branched carbon chains. Substituents may be hydroxy, methoxy, epoxy, carboxy, acetoxy or other alkoxy groups besides methoxy substituents. Thus numerous structural, stereo- and diastereoisomers may be derived. Double esterification may lead to 11- to 14-membered ring systems (macrocyclic diesters). The most widely known PAs are 11-membered monocrotaline, 12-membered alkaloids senecionine and senkirkine, 13-membered doronenine, and 14-membered parsonsine [ROEDER 2000].

N-Oxides

Excluding otonecine alkaloids, which cannot form N-oxides, together with the N-oxides of the other alkaloids more than 660 alkaloids are known [ROEDER 2000]. Metabolised products (free bases) of N-oxides are toxic.

Biosynthesis of PAs takes place in the roots where the alkaloids occur as N-oxides. The N-oxides are very polar compounds which are readily soluble in water and insoluble in most organic solvents. Unlike typical tertiary alkaloids, they are not able to non-specifically permeate biological membranes in their unprotonated form. Due to their properties, N-oxidated PAs can easily be translocated to the target organ(s) within the plant. They are taken up via membrane transporter molecules and stored in the vacuoles [HARTMANN & TOPPEL 1987]. N-oxides can easily be reduced to the corresponding tertiary alkaloids, not only in the alimentary tract or in experimental conditions but also within the plants (e.g. by enzymatic reactions).

Structural requirements for toxicity

The minimum structural requirements for toxicity of PAs are:

(1) a double bond in 1,2 position of a pyrrolizidine moiety

(2) a hydroxymethyl substituent (C-1 position) in the pyrrolizidine moiety, preferably with a second hydroxyl group in the C-7 position

(3) esterification of the primary hydroxymethyl group with a branched mono- or dicarbolic acid containing at lease 5 C-atoms (necid acid).

[PRAKASH *et al*. 1999, FSANZ 2001, TEUSCHER & LINDEQUIST 1994].

2. Discussion

The relevant literature on PAs and PA-containing preparations was searched principally via PubMed. The cut-off date was July 2011.

2.1. Regulatory/legal status of PAs or PA-containing products

Only few regulatory guidance documents concerning limits of intake of PAs exist either in the field of medicinal products or in the field of food/food supplements, for instance in Germany, Belgium or Austria. In Germany in 1992, a graduated plan concerning medicinal products containing PAs with a necine system unsaturated in 1,2 position came into force. The maximum daily dose of PA for internal use is set at 1 µg for a duration of maximum 6 weeks/year and 0.1 µg without any limitation in the duration. The maximal daily dose of PAs in case of cutaneous application is 100 µg for a duration of maximum 6 weeks/year and 10 µg without any limitation in the duration of use [BUNDESANZEIGER 1992]. In Belgium medicinal products for internal use containing PAs are not allowed to be marketed [ALBERT 2000] and in Austria it has to be proven that the medicinal product which contains herbal preparations from PA-containing plants has no PA in the final product [BUNDESGESETZBLATT 1994]. Several other countries refer to the CPMP document “Herbal drugs with serious risks - Listing of herbs and herbal derivatives withdrawn for safety reasons” [CPMP 1992].

Some regulatory data are also available for foodstuffs, even though uniform regulations are missing in this field as well. In 1988, WHO recommended that the exposure to PAs should be minimised as far as possible [IPSC 1988]. In 2001 the FDA advised all dietary food supplement manufacturers to remove products containing *Symphytum* (and also all other of PA-containing material) from the market, due to the lack of data for a limit which could guarantee a safe intake (FDA 2001). EFSA recommended that feed material which can be contaminated with PA should be monitored and considered that more data are needed to assess human PA exposure resulting from feed and carry-over into animal products [EFSA 2007]. EFSA concluded that more data should be made available on the potential carry-over from PAs into milk, considering that infants have a relatively high consumption per kg body weight (BW). Moreover, it was claimed that more data would be needed to quantitatively assess the contribution of honey to human exposure, as the latter is regularly found to contain residual amounts of PA metabolites. In the meantime, the so-called ‘zero-tolerance principle’ can be applied. This principle is used in cases where either no safe or tolerable level can be determined based on available, valid scientific data, or if insufficient toxicological data are available. The same recommendation was given by the Bundesamt für Risikobewertung (BfR) in Germany [BfR 2007]. The Committee on Toxicity (COT) in UK stated that more information is needed concerning the levels of PAs in grain to enable assessment of exposure and risk to consumers from this source [COT 2008]. The Dutch Institute for Food Safety (RIKILT) recommended extending the monitoring of additional PAs in animal forage. Furthermore, to assess the potential risk of PAs entering the food chain through transfer to milk, the monitoring data should be combined with *in vitro* and *in vivo* experiments because the data currently available on milk transfer is rather limited. So the transfer ratios of individual PAs (in their tertiary as well as N-oxide form) from feed to milk should be investigated, as it can be expected that differences in polarity and chemical reactivity may affect metabolism and result in different transfer ratios [MULDER *et al*. 2010].

In 2011 EFSA and BfR published opinions on PAs in food [EFSA 2011, BfR 2011] which focus mainly on the occurrence of PAs in honey. EFSA pointed out that on the basis of the genotoxic and carcinogenic

properties of 1,2-unsaturated PAs, it was not appropriate to establish a Tolerable Daily Intake (TDI), and decided to apply the Margin of Exposure (MOE) approach instead. A $BMDL_{10}$ for excess cancer risk of 70 µg/kg bw per day was calculated for induction of liver haemangiosarcomas by lasiocarpine in male rats and used as the reference point for comparison with the estimated dietary exposure. Whilst the MOEs for adults (calculated on consumption data) were seen to be of low concern (MOE of 10,000 or higher), it was concluded that there is a risk for those juveniles who are high consumers of honey. The BfR identified that for 1,2-unsaturated PAs, a daily intake of 0.007 µg/kg (0.42 µg/60 kg adult) should not be exceeded. It was also pointed out that children in particular can be exposed to amounts of PAs that exceed this limit. Both publications indicate that there is a need for research (e.g. defined performance criteria for the analysis of PAs in feed and food, collection of analytical data, data on the occurrence of PAs in other possibly relevant foods and a need for toxicological data relating to the PAs most commonly found in honey).

Until now no limits for PAs in food exist within the EU, with the exception of refined echium oil for which the PA limit was given with 4 µg/kg [COMMISSION DECISION 2008/558/EG 2008].

2.2. Mechanism of toxic action of PAs

PAs themselves are chemically un-reactive. As ester alkaloids, they may be partially saponified by nonspecific hydrolases to the corresponding necines and necic acids both in the intestinal tract and during transit to the liver. Like the parent alkaloids, the fission products are non-toxic and are excreted via the renal system [ROEDER 2000]. Bioactivation (similar to aflatoxins) is necessary for toxic actions of PAs.

The cyclic diesters are thought to be the most toxic alkaloids and the noncyclic diesters are of intermediate toxicity, whilst the monoesters are the least toxic. Saturated PAs are non-toxic according to the literature. The extent of toxicity depends on the structure and the resulting metabolic pathways and detoxification rates. Furthermore many other factors such as species, age, sex or biochemical, physiologic and nutrition status might influence bioactivation. Highly reactive electrophilic pyrroles are short lived. They quickly bind with and damage nearby hepatic molecules. Some PAs or their metabolites are more stable. So they may circulate and damage extra-hepatic tissues.

Cellular mechanisms lead to pyrrole adducts, which are rapidly excreted. However, some pyrrole-tissue adducts may persist for months and years as well. It is thought, that pyrrolic adducts may be recycled, reacting with new nucleophiles and stimulating further cellular damage.

PA exposition over longer periods of time is mainly known to damage the liver (due to the liver being the main production site), lung or the blood vessels. Kidney, GI tract, pancreas and bone marrow are damaged to a lesser extent. Venous occlusions in the liver and lung, megalocystosis, inhibition of cell division (mitosis) and liver cirrhosis are all signs of PA toxicity. Genotoxic effects are seen as well [MATTOCKS 1986, FU *et al*. 2004].

2.3. Pharmacokinetics of PAs

Bioactivation occurs primarily in the liver by the action of several different mixed function oxidases. Metabolism steps which either lead to activation or detoxification are described in the literature. The non-toxic metabolites are quickly excreted. Toxification occurs via oxidation, to didehydropyrrolizidine derivatives (DHP, pyrroles). These pyrrolic alkaloids possess an allylic structure which promotes an increase in their reactivity. Once formed, the pyrroles can rapidly bind with DNA, protein, amino acids and glutathion [STEGELMEIER *et al*. 1999, KEMPF *et al*. 2010b]. Protein binding can alter cell functions and cause cell damage and death while cross-linking to DNA may initiate carcinogenesis.

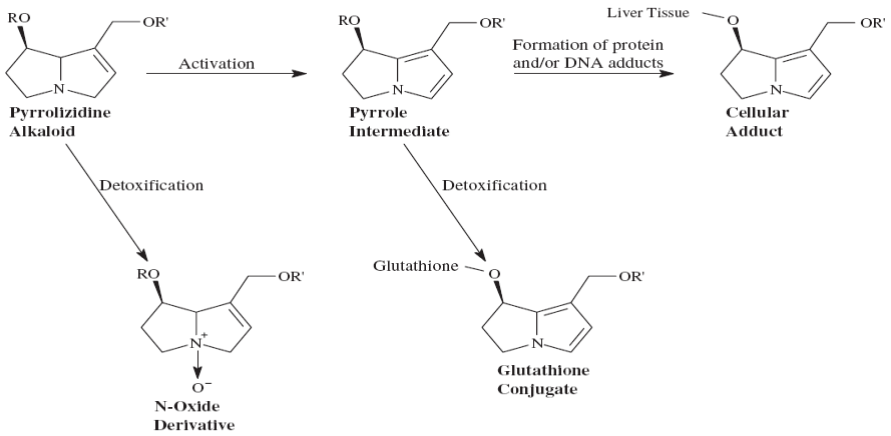


Fig. 4: activation and biotransformation of pyrrolizidine alkaloids [BARCELOUX 2008]

N-Oxides cannot be directly converted into pyrroles. However, on oral ingestion they are reduced either by the gut enzymes or the liver microsomes and NADP or NADPH to the free bases which are toxic [WIEDENFELD 2011].

Absorption

Different PAs are transferred across the ileum and jejunum, but not the stomach, as measured by SWICK *et al*. (1982) in rabbits. In rats, both oral and i.v. administration of senecionine and adonifoline resulted in fast absorption with lower bioavailability and quick metabolism to PA N-oxides and hydroxylation products of PAs or their N-oxides. It could be seen that the plasma concentration ratio of senecionine N-oxide to senecionine was significantly larger than that for adonifoline N-oxide and adonifoline [WANG *et al*. 2011]. Riddelliine was completely absorbed from the gavage dose within 30 min in all rats and mice [WILLIAMS *et al*. 2002].

The oral and percutaneous absorption of a crude alkaloid mixture obtained from *Symphytum officinale* in rats was investigated by BRAUCHLI *et al*. [1982]. A dose of 194 mg/kg was either given by gavage, or was applied to the shaved skin and left for 44 h. After the dermal application, the excreted N-oxides in urine (up to 48 h) amounted to 0.1-0.4% of the dose. After oral dosage the excreted level of N-oxides and alkaloid bases was quoted as being 20-50 times greater.

Metabolism to toxic metabolites

The metabolic pattern and DNA adduct profiles produced by human liver microsomes are similar to those formed in rat liver *in vitro* and *in vivo*, indicating that the results of mechanistic studies with experimental rodents are highly relevant to humans [YAN *et al*. 2008]. Conversion of PAs to reactive pyrrolic metabolites occurs by C- and N-oxidation catalysed by cytochrome P450 monooxygenases [PRAKASH *et al*. 1999; FU *et al*. 2004] while flavin-containing monooxygenases and carboxylesterases are considered to be involved in detoxification pathways [FU *et al*. 2004]. The most commonly identified isoforms catalysing bioactivations are isoforms of the CYP3A subfamily, but CYP2B and CYP2D isoforms also have this activity. Strong evidence exist that CYP3A4 plays a major role in toxification of several PAs [PRAKASH *et al*. 1999, HUAN *et al*. 1998, FU *et al*. 2004]. The abundance of this enzyme in liver varies over a 30-fold range between individuals which suggest an individual variation in toxification of PAs.

DHP may undergo hydrolysis with the formation of the corresponding pyrrolic alcohol [FSANZ 2001].

A rapid and extensive conversion of riddelliine to the N-oxide was shown, with the exception that female rats produced lower serum concentrations of the N-oxide. All rodents produced small amounts of retronecine. The elimination half-times increased in the following order: riddelliine<retronecine<N-

oxide consistent with metabolism of parent compound. Internal exposures ($AUC_{0-\infty}$) increased in the order: retronecine<riddelliine<N-oxide, with male rats as the exception [WILLIAMS *et al*. 2002].

Distribution

Heliotrine (i.p.) was present in the liver after 2 min (3.7% of total dose), the level peaking at 5 min (6.3%), and dropping to 2.2% at 1 h and 0.5% at 2.5 h. In adult rats, the level in the liver at 5 h was 0.07% of the total dose. Five minutes after i.p. dosing, 30-40% of the initial dose remained in the peritoneal cavity, and the blood level of heliotrine was 60 mg/l, dropping to 3 mg/l at 1 h. Blood levels of senecionine in rats (i.p.) were 0.38, 0.32, and 0.14 mg/l at 0.5, 1, and 2 h after injection, respectively [IPCS 1988].

Concerning distribution of radioactivity from a tritiated PA analogue (i.v.); in rats the highest concentrations of radioactivity were seen in the liver, lungs, kidneys, and spleen (respectively, 3.9%, 0.19%, 0.18%, and 0.27% of the dose given). Radioactivity in the expired air was negligible. The binding of radioactivity in the liver, and especially the lungs, was more persistent than in other organs [MATTOCKS 1977]. When tritium-labelled indicine N-oxide was given i.v. to mice or monkeys, at 2 h the highest concentrations of radioactivity were in the kidneys, liver, and intestines [EL DAREER *et al*. 1982].

Studying the distribution of the uniformly ^{14}C-labelled senecionine in lactating mice, after 16 h, 0.04% of the radioactivity had been recovered in the milk; the liver contained 1.92%. [IPCS 1988].

Excretion

The urinary excretion of monocrotaline in rats was 50-70% within the first day [IPCS 1988]. Similar results were reported by MATTOCKS [1977] and WHITE [1977]. Excretion of pyrroles continued for a little longer. In rats given retrorsine, the urine in the first 24 h contained 10.6% unchanged alkaloid, 13.3% N-oxide, and 13.4% pyrrolic metabolites. During the second day, only 0.1% alkaloid, 0.2% N-oxide, and 1.8% pyrroles were excreted. Biliary excretion also occurred. About one-quarter of an i.v. dose of retrorsine in rats was excreted in the bile as pyrrolic metabolites, and 4% as unchanged alkaloid; most of this excretion occurred during the first hour after the injection [WHITE 1977]. The proportion of urinary excretion of unchanged base increases with the hydrophilicity of the alkaloid, e.g. being 62% for heliotrine N-oxide, 30% for heliotrine, and only 1-1.5% for lasiocarpine [IPCS 1988]. After small doses of tritiated senecionine or seneciphylline (0.3-3.3 mg/kg) given to rats, most radioactivity was eliminated in the urine and faeces within 4 days.

Giving uniformly ^{14}C-labelled senecionine in lactating mice, after 16 h, 75% of the radioactivity had been recovered in the urine and 14% in the faeces.

Indicine N-oxide is very rapidly excreted, either unchanged or conjugated. Thus, indicine N-oxide given i.v. to mice, monkeys, or rabbits disappeared from the serum with initial half-lives ranging from 3 to 20 min. Over 80% of tritium-labelled indicine N-oxide given i.v. was excreted in the urine of mice or monkeys within 24 h. Urinary excretion of indicine N-oxide was also rapid in rabbits, but somewhat slower in human beings [POWIS *et al*. 1979; EL DAREER *et al*. 1982].

To summarise, the available evidence suggests that ingested PAs are rapidly metabolised and that the excretion of unchanged alkaloid and of most metabolites is rapid as well. Thus, within a few hours, only a relatively small proportion of the dose remains in the body, much of it in the form of metabolites bound to tissue constituents. It is unlikely that a significant amount of unchanged alkaloid will remain in the body after the first day.

2.4. Single and repeat dose toxicity in animals

There is conclusive evidence from studies on experimental animals that the effects of a single exposure to PAs may progress relentlessly to advanced chronic liver disease and cirrhosis, following a long

interval of apparent well-being, and without any other latent or provocative factor. The lowest levels of such alkaloids administered thus far to experimental animals, e.g., 1-4 mg/kg diet, have produced chronic liver disease and tumours [IPCS 1988].

The acute toxicity of PAs varies widely. The rat LD_{50} of most alkaloids known to be significant for human health is in the range of 34-300 mg/kg. The toxicity of N-oxides is similar of that of the parent alkaloid [IPCS 1988].

In addition the relative toxicity of PAs varies between mammalian species; the differences probably arising from different toxicokinetics. Nevertheless, the fundamental metabolic and cytotoxic processes are common to all species [MOLYNEUX *et al*. 2011]. Pigs and poultry are most susceptible, while horses and cattle are less so and sheep and goats are relatively resistant to PA toxicity [PRAKASH *et al*. 1999].

In acute poisoning, death occurs within about 7 days. Chronic liver disease including cirrhosis has been shown to develop in the rat following administration of a single dose of a PA [IPCS 1988]. While in most cases the liver is the principal target organ, in a number of animal species, the lungs develop vascular lesions characteristic of primary pulmonary hypertension with secondary hypertrophy of the right ventricle of the heart. The central nervous system is the target organ of the toxic PAs contained in *Trichodesma*, which produce spongy degeneration of the brain.

In small laboratory animals, doses approaching a lethal dose produce a confluent, strictly zonal haemorrhagic necrosis in the liver lobule, within 12-48 h of administration of PAs. At about the same time in non-human primates, or after a short time in the rat, chicken and pig, changes begin to occur, and later become organised in the subintima of the central or sublobular veins in the liver resulting in their occlusion. The reticulin framework in the central zone of the lobule collapses following necrosis leading to scarring. Repeated administration of suitable doses leads to chronic liver lesion characterised by megalocytosis (the presence of enlarged hepatocytes containing large, hyper-chromatic nuclei), and increasing fibrosis, which may result in cirrhosis [IPCS 1988]. The enlarged hepatocytes arise through the powerful antimitotic action of the pyrrole metabolites of PAs. In experimental animals, protein-rich and sucrose-only diets have given some measure of protection against the effects of the alkaloids, as has pre-treatment with thiols, anti-oxidants, or zinc chloride. On the other hand, PAs have been shown to act synergistically with aflatoxin in causing cirrhosis and hepatoma in primates [LIN *et al*. 1974].

In Big Blue transgenic rats receiving riddelliine for 12 weeks a number of genes involved in liver injury and abnormalities were altered. Significantly changes were seen in genes which are linked to cell death, cellular growth and proliferation, oxidative stress and liver morphology. Liver endothelial cells were more involved than liver parenchymal cells [MEI *et al*. 2007].

Alkaloids/toxic metabolites have been shown to be secreted in the milk of lactating dairy cattle and rats, and both male and female young have been shown to suffer toxic damage, even when suckled by retrorsine-treated mothers, who apparently are not affected themselves [SCHOENTAL 1959]. Such suckling animals may also be in apparent good health while the livers show toxic effects. Protein-deficient and young suckling animals are particularly vulnerable [SCHOENTAL 1959]. Heliotrine at doses of 50 mg/kg body weight or more, administered to rats during the second week of gestation, has been shown to induce several abnormalities in the fetus. Doses of 200 mg/kg bw resulted in intrauterine deaths or resorption of fetuses. Dehydroheliotridine, the metabolic pyrrole derivative of heliotrine, was 2.5 times more effective on a molar basis than its parent PA in inducing teratogenic effects. The ability of PAs to cross the placental barrier in the rat and to induce premature delivery or death of litters has been demonstrated. The embryo *in utero* appears to be more resistant to the toxic effects of PAs than the neonate [IPCS 1988].

PAs are noted mainly for the poisoning of livestock due to the animals grazing on PA-containing toxic weeds, and large-scale outbreaks have been recorded from most parts of the world. Most commonly,

clinical signs such as sluggishness. weakness, loss of appetite, wasting, ascites, jaundice, photosensitisation and behavioural abnormalities relate to hepatic insufficiency [FSANZ 2001].

Toxic Actions of DHP

Pyrrolic derivatives prepared chemically from PAs, as well as some analogous compounds, have been tested in experimental animals and *in vitro* systems, and showed a variety of toxic actions.

DHP

DHPs are very reactive and their effects *in vivo* are largely confined to the first tissues they encounter. When given orally to rats, they are destroyed almost immediately in the aqueous acid of the stomach and show no toxic action. When given i.p., they cause severe local irritation and peritonitis; s.c. injection leads to skin lesions. After i.v. injection of pyrroles into the tail veins of rats, toxic injuries appear principally in the lungs. Depending on the dose, these include vascular lesions and pulmonary oedema; a progressive alveolar proliferation similar to that produced by very much larger doses of the parent alkaloid. Injections of DHPs or synthetic analogues into mesenteric veins of rats lead to liver damage after smaller doses than the alkaloids themselves [IPCS 1988].

Pyrrolic alcohols (dehydro-necines)

These alcohols are much less reactive than the pyrrolic esters but far more persistent. They are seen as secondary toxic metabolites which are not acute toxicants but can cause extensive extrahepatic injury, involving almost all rapidly developing tissues, especially in young animals [FSANZ 2001].

Dehydroheliotridine is less acutely toxic than its parent alkaloids; it has an LD_{50} (7 days) of about 250 mg/kg bw in mice. Its effects on 14-day-old rats were studied. All rats given i.p. doses of 0.4 mmol/kg bw survived, but a dose of 0.6 mmol/kg killed most animals within 10 days. Toxic effects were mainly found in rapidly developing tissues. In young rats, it caused fur loss, tooth defects, and atrophy of hair follicles, gut mucosa, spleen, thymus, testis, and bone marrow. The lungs were not affected. Pathological effects in the liver were confined to necrosis of isolated cells and antimitotic action, which was manifested as a mild megalocytosis in rats surviving 4 weeks or more. The persistent antimitotic action of dehydroheliotridine and of its parent alkaloid lasiocarpine in the liver of rats was investigated and the mitotic block was located as being either late in the DNA synthetic (S) phase or early in the post synthetic (G2) phase of the cell cycle. Dehydroheliotridine is also carcinogenic. It could be shown that rats given 9 i.p. injections of this compound (60-76.5 mg/kg bw) over 23 weeks had a shorter life span and suffered a significantly higher incidence of tumours than control rats. It was concluded that dehydroheliotridine is responsible for some, or possibly all, of the carcinogenicity of its parent alkaloids. Dehydroheliotridine was found to be teratogenic when given i.p. to female hooded rats on gestation day 14. A dose of 40 mg/kg bw produced effects similar to those produced by the alkaloid heliotrine at a dose of 200 mg/kg [IPCS 1988].

The persistent antimitotic action on the liver that leads to the formation of giant hepatocytes can be produced both by pyrrolic ester metabolites [HSU *et al*. 1973a, b], and by pyrrolic alcohols [PETERSON *et al*. 1972]. Both kinds of metabolites can lead to similar alkylation products. The antimitotic action must be accompanied or followed by a stimulus of cell division to be sufficient. Such a stimulus may be provided by the acute necrotic effect of primary pyrrolic metabolites or by any other cause of acute liver injury that leads to tissue regeneration. In very young animals, the stimulus can be the enhanced rate of replication that already exists in them.

2.5. Acute and chronic toxicity in humans

In man, PA poisoning is usually manifested as acute veno-occlusive disease (VOD) characterised by a dull dragging ache in the right upper abdomen, rapidly filling ascites resulting in marked distension of the abdomen and sometimes associated with oliguria, swelling feet and massive pleural effusion. There might be vomiting of blood in advanced stages of the disease. Acute liver damage includes

centrilobular haemorrhagic necrosis and hepatomegaly with accompanying ascites. It can also manifest as subacute disease with vague symptoms and persistent hepatomegaly, in which the small hepatic veins become occluded by endothelial proliferation and medial hypertrophy leading to restricted blood flow, necrosis of surrounding tissue, fibrosis, nodular regeneration and in many cases, cirrhosis [PRAKASH *et al*. 1999]. In some cases, a single episode of acute disease has been demonstrated to progress to cirrhosis (even in a period as short as 3 months from the acute phase), in spite of the fact that the patient has been removed from the source of toxic exposure and has been given symptomatic treatment [TANDON *et al*. 1977, STUART & BRAS 1957]. Tissue-bound DHP adducts are considered to be a source of ongoing alkylation either by releasing 6,7-dihydropyrrolizine carbonium ions capable of forming new adducts directly, or via the hydrolytic release of dihydropyrrolizine alcohols [MATTOCKS 1986]. Thus, following dietary exposure to PAs, *in vivo* alkylation continues until the reservoir of labile tissue-bound adducts is eliminated, mainly as soluble conjugates (e.g. with GSH) in urine and bile. This may take many months so that even a single dietary exposure to PAs continues to produce silently progressing chronic diseases, which are unlikely to be attributed to PAs in food [EDGAR *et al*. 2011].

Mortality to PA can be high with death due to hepatic failure in the acute phase or due to haematemesis resulting from ruptured oesophageal varices caused by cirrhosis. Less severely affected cases may show clinical, or even apparently complete, recovery. It was reported that after acute poisoning in man with significant acute toxicity, approx. 20% will die rapidly and 50% of patients will recover completely. Of the survivors, about 20% appear to recover clinically but may go on to develop cirrhosis and liver failure years later. Others may develop subacute liver pathological changes, which will either eventually resolve or go on to cirrhosis and liver failure [FSANZ 2001]. In several publications the mortality of VOD is given with approx. 50% [STICKEL & SEITZ 2000].

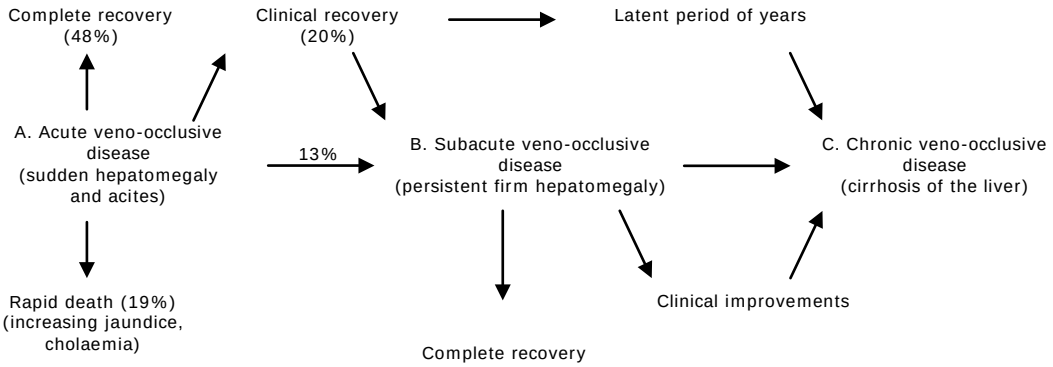


Fig. 5: clinical natural history of VOD of the liver. B and C may be present with no clinical history of preceding illness [STUART & BRAS 1957]

Furthermore the possibility of the development of toxic pulmonary disease in man cannot be ruled out. It is possible that the greater capacity of the liver to repair damage would lead to the situation where at some low levels and rates of exposure to PAs, liver damage may be minimal while lung damage continues to develop. In this scenario sporadic small doses of PAs over an extended period, expected from current levels of dietary exposure, may produce cancer and pulmonary hypertension rather than liver damage [EDGAR *et al*. 2011]. There is a report of an outbreak of *Trichodesma* poisoning in the former USSR in which the symptoms were mainly neurological [IPCS 1988].

In the 1970s and 1980s, studies from Hong Kong, the United Kingdom and the USA reported instances of human disease that have been caused by the use of medicinal products containing PAs, resulting in fatality or the development of cirrhosis, even in countries with well-developed health services and among the higher economic and educated strata of society [IPCS 1988].

Liver damaging agents, e.g. viruses, bacterial endotoxins, aflatoxins and environmental copper, can act synergistically and increase liver damage and cancer caused by PAs [YEE et al. 2000; IPCS 1988]. Although all age groups might be affected by PA poisoning, children are particularly vulnerable to the effects of PA. One of the explanations therefore might be, that in neonates and foetuses, liver copper levels are naturally high [RIORDAN & RICHARDS 1980, EDGAR *et al*. 2011] which could potentiate the effects of PAs.

2.6. Genotoxicity and Carcinogenicity of PAs

Genotoxicity

Several PAs, PA-derivatives, and related compounds have been shown to produce genotoxic effects (mutations, sister chromatid exchanges, chromosomal aberrations) in plants and several cell culture systems after metabolic activation [KRAUS *et al*. 1985, FU *et al*. 2004, MEI *et al*. 2010]. Some PAs induce micronuclei formation in erythrocytes in the bone marrow and foetal liver in mice [IPCS 1988].

Several DHPs were shown to have an inhibitory action in cultures of human KB cells, cultured rat liver cells and to cause chromosome breaks and sister chromatid exchange. Cell death was preceded, first by the swelling and disruption of organelles, including mitochondria, and then by the rupture of plasma membranes with the release of cell components [IPCS 1988].

Chromosomal aberrations have been demonstrated in rats and humans with VOD. In humans, this is believed to have been caused by fulvine [MARTIN *et al*. 1972].

DNA-adduct formation may play a role in the genotoxicity of riddelliine. Riddelliine induced a higher frequency of mutations in non-neoplastic endothelial cells (but not in parenchymal cells) in the cII gene mutation assay in transgenic Big Blue rats. The predominant mutations observed were G:C to T:A transversions, which are consistent with riddelliine-induced formation of DNA adducts involving G:C base pairs [MEI *et al*. 2007].

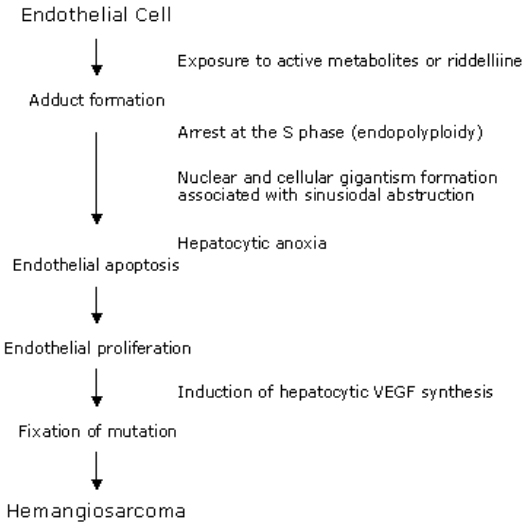
Carcinogenicity

The carcinogenic activity of PAs appears to parallel their mutagenic behaviour, but not their hepatotoxicity. In rats, appropriately low repeated doses of several alkaloids have been shown to induce tumours. In some studies, a single dose has been carcinogenic. It is notable that dose rates that have been effective in inducing tumours in rats are mostly equivalent to 0.2–6 mg/kg bw/day for the initial period and 0.2-3 mg/kg bw/day for the 12 month period. These dosages are roughly similar in magnitude to estimated intake rates (0.01-10 mg/kg bw/day) in several episodes of human toxicity. Comparison of the total intakes resulting in human toxicity with the total doses to death observed in the chronic toxicity studies on rats indicates that human beings are more susceptible and suggests that human beings may survive for sufficient time to develop cancer after only a brief exposure at this level or a longer exposure at a markedly lower level [CULVENOR 1983, IPCS 1988].

A 2-year study carried out as part of the National Toxicology Program showed that riddelliine induced liver hemangiosarcomas in both male and female rats and male mice, hepatocellular adenomas and carcinomas in male and female rats, and lung alveolar adenomas in female mice. Riddelliine was classified as “reasonably anticipated to be a human carcinogen” [NTP 2008]. The DHP derived DNA adducts are responsible for liver tumour induction. Mechanistic studies with retrorsine, monocrotaline, clivorine, lasiocarpine, riddelliine N-oxide, retrorsine N-oxide and monocrotaline N-oxide generated the same set of DHP derived DNA adducts [YAN *et al*. 2008].

The proposed mechanism for the induction of liver hemangiosarcoma suggests that the active metabolite of riddelliine interacts with endothelial DNA, causing damage, including karyomegaly, cytomegaly, and apoptosis, to endothelial cells of the liver. The enlarged endothelial cells obstruct the blood vessels causing local hypoxia. Hepatic hypoxia was shown to induce VEGF (Vascular Endothelial Growth Factor) production by hepatocytes. Increases in VEGF then induce increases in endothelial cell

441 replication. The increased replication enhances the probability that DNA damage, either spontaneous or
442 drug-induced, will escape repair and become fixed as mutations that eventually lead to
443 hemangiosarcomas. It was suggested that hypoxia also triggers replication in the endothelial cells.
444 [NYSKA *et al*. 2002, SMITH *et al*. 2004].



445

446 Fig. 6: proposed mechanism for the induction of liver heamnagiosarcoma by riddelliine in rats [NYSKA *et al*.
447 2002]

448 Carcinogenesis related gene expression patterns resulting from the treatment of comfrey and
449 riddelliine are found to be very similar, even though the number of genes altered by comfrey was
450 much higher, possible due to the fact that comfrey is a complex mixture compared to the isolated
451 substance [GUO *et al*. 2007].

452 No information is available on the long-term follow-up of the human population, to ascertain whether
453 the exposure to PAs could have resulted in an increased incidence of liver cancer or other types of
454 cancer. However, various PAs have been shown to be carcinogenic for experimental animals, which
455 implies that a potential cancer risk for human beings should be seriously considered.

456 *2.7. Human exposure to PA by food*

457 Episodic and catastrophic, acute and chronic poisonings have been documented particularly in
458 developing countries. Thousands of people might be affected, as in India in 1972, Tadjikistan in 1992
459 or in Afghanistan in the 1970s and 1990s, 2000, 2007 and 2008 [MOLYNEUX *et al*. 2011]. Such
460 problems are typically triggered by environmental factors.

461 In developed countries levels of PA intake are mostly low. Beside the direct intake of PAs via herbal
462 medicinal products secondary contamination of food with PAs was observed: e.g. in foods of animal
463 origin (as milk, eggs, honey, pollen products), in grain and in packed lettuce boxes as recently
464 detected in Germany [MOLYNEUX *et al*. 2011]. So depending on the individual preference in food
465 selection, great variability of PA exposure in humans is expected.

466 Globalisation of markets also leads to situations where previously localised toxins are shipped around
467 the world in contaminated products. During the past few years it appears that, because of the lack of
468 natural control factors, the expansion of certain invasive plants e.g. *Senecio madagascariensis*
469 (Australia, Hawaii) and *Senecio jacobaea* (Germany, UK, USA, New Zealand) creates serious problems
470 for animals and via animal products, for humans as well.

471 Several independent risk assessments have proposed tolerable levels of exposure for unsaturated PAs
472 and their N-Oxides:

473 Table 1: Proposed tolerable levels of exposure for unsaturated PAs and their N-Oxides

Authority	TDI for unsaturated PAs and their N-Oxides
Bundesanzeiger (1992)	1 µg/day (max. 6 weeks per year) 0.1 µg/day (no restriction) (for medicinal products only)
BfR (2011)	0.007 µg/kg/day
Food Standards Australia New Zealand (FSANZ) (2001)	1 µg/kg bw/day (TDI based on avoidance of VOD, cancer risk considered not proven)
Rijksinstituut voor Volksgezondheid en Milieu (RIVM) (2007) [KEMPF *et al*. 2010b]	0.1 µg/kg bw/day (based on virtual safe dose of 0.43 ng/kg bw/day)
Committee on Toxicity (COT) (2008)	0.1 µg/kg bw/day (non-cancer unlikely) 0.007 µg/kg bw/day (cancer unlikely)

Honey, Pollen

The levels of PAs and N-oxides found in many honeys could, according to published risk assessments (Table 1), cause chronic diseases such as liver cirrhosis, pulmonary hypertension and cancer if these honeys are regularly consumed at the recommended serving sizes of 15–25 g. PA levels up to 3900 µg/kg honey were found. In the United Kingdom the highest honey consumers are infants eating up to 32 g/day of honey, school children consuming up to 60 g/day and adults eating as much as 92 g/day [EDGAR *et al*. 2011]. If honey contains ~2500 µg/kg of PAs with two average serving sizes of 40 g a person would be exposed to 100 µg PAs/day. This would exceed the recommended doses. It has been reported that a woman who consumed 20–30 µg of PAs/day during her pregnancy gave birth to a child suffering fatal liver damage [RASENACK *et al*. 2003].

KEMPF *et al*. [2010a] reported that 17 (31%) of 55 commercial bee pollen products purchased in Europe have been found to contain 1080–16350 µg PA/kg. The authors have calculated, based on a 30% probability of PA occurrence, that consumption of the recommended daily amount of 10 g of bee pollen would expose an average consumer to 15 µg (retronecine equivalents) of PAs.

Grain, Milk, Eggs, Meat

There are many examples of acute poisonings in humans by PA contaminants in grain. All foreign seeds in grain, including those containing PAs, are removed normally prior to milling. These measures may be the reasons that large-scale, acute PA poisoning incidents seen in some developing countries have not been seen in developed countries. However, chronic PA poisoning is still conceivable because it has been shown that complete removal of seeds containing PA from heavily contaminated grain leaves readily detectable levels of PAs in the 'cleaned' grain.

In the only experiment with radiolabelled PAs in cows, a single oral dose of 1 mg of [^{3}H] seneciphylline/kg bw resulted in >102 ng equivalents/l of seneciphylline in the milk after 16 h, decreasing to 5 ng/l after 64 h. The total of radiolabel excreted in the milk was 0.16% of the original dose. Measured at 2 and 27 h post-dosing, the level of N-oxides detected in the milk increased from 2.9% to 11.2% of the radiolabel present at that time. HOOGENBOOM *et al*. [2011] showed that the overall transfer of PA from *Senecio jacobaea* and *Senecio inaequidens* was rather low (0.1%), but that for specific PAs this number might be higher (4-7%). By feeding cows with 200 g *Senecio* per day milk with PA content up to 10 µg/l was quickly produced. The intake of 10 ml and 35 ml of such milk would lead to the permitted 0.1 µg and 0.007 µg/kg PA/day (for a human of 50 kg bw), respectively [BUNDESANZEIGER 1992, COT 2008)]. These and other results from rats and mice show that only low levels of PAs seem to be transferred into milk. Whether water-soluble dihydropyrrolizine alcohols are transferred into milk needs to be determined.

Levels of 5–168 µg PA/kg in eggs (layer hens had been inadvertently poisoned by *Heliotropium europaeum* and *Echium plantagineum* contamination in the grain) have been reported while in other tests (e.g. hens were fed with *Senecio vernalis*) no PAs were detected in eggs.

It has been shown that oral dosing of animals with radiolabelled PAs results in most of the radiolabel being eliminated within 24 h, however small amounts of radiolabelled dihydropyrrolizine adducts remain detectable for many months in edible tissues, particularly in the liver. When puppies were fed cooked meat (or milk) from animals poisoned by a PA-producing species of Trichodesma, it resulted in death or production of irreversible pathological changes within 3-4 months. A recent study reported the presence of the 'pyrrolic' adducts and free PAs up to 250 µg/kg in muscle and 2500 µg/kg in the liver of animals consuming levels of PA-producing plants that failed to cause overt poisoning.

Salads, teas, spices

Some leafy PA-producing plants, e.g., species of Borago and Symphytum are recommended as salads. The leaves of the common weed Senecio vulgaris accidently co-occurred with salad leaves of similar appearance being sold in supermarkets in Germany. PA-producing plants are also recommended for making teas, e.g., Symphytum spp. and sauces, e.g., traditional "Fränkische Grüne Sosse" contains borage (Borago officinalis). PAs have also occurred in a cooking spice that was implicated in the death of a late-term foetus that died of liver failure.

Whilst for honey and pollen fairly recent data concerning PA content exist, for other food products, the possibility of contamination with PA can only be assumed. More data on the levels of PAs in grain and flour, and foods incorporating these, are desirable before the contribution of PAs in grain-based products can be assessed as a potential cause of slowly progressing chronic poisoning of humans. The same applies for milk (which might be the dominant nutritional source for many infants), eggs and meat (PAs contained in meat and milk are not destroyed by cooking).
It seems important to accept that relative low and sometimes sporadic amounts of PA might be taken in by food. However even those amounts can be a potential cause of slowly progressing chronic diseases in human consumers.

3. Conclusions and recommendations

Hepatotoxicity following the intake of PAs is established. However, the dose-effect relationship remains unclear and inter-individual differences in susceptibility are large. The intoxications with PAs were described as an "iceberg disease". That means that only a very few apparent cases (except for sporadic epidemic situations) with many subclinical manifestations are known. However, most of the cases will remain unrecognised. Since the alkaloids are eliminated within 24 h, suspicion could not be confirmed, as the symptoms may take several days or months to appear. Furthermore, hepatotoxicity caused by PA may easily be misinterpreted as the result of other aetiologic factors, such as alcohol abuse for example [Stickel & Seitz 2000, Edgar *et al*. 2011].

However, there are no substantial, long-term follow-up data to assess whether exposure to PAs results in increased incidence of chronic liver disease or cancer in man. Available clinical and experimental data suggest that a single episode of PA toxicity and possibly also a long-term low level exposure may lead to cirrhosis of the liver. PAs could also be possible carcinogens in man, since a number of them have been demonstrated to induce cancer in experimental animals. In addition, in several instances of human toxicity, the reported daily rates of intake of PAs were in close range of those known to induce tumours in rats. Estimates of intakes causing toxic effects in human beings indicate that they are more sensitive than rats and domestic animals. Rats dosed with lasiocarpine at a rate equivalent to 0.2 mg/kg bw/day developed tumours. Pigs fed monocratoline equivalent to about 0.08 mg/kg bw/day developed chronic liver damage in several months. The lowest intake rate causing VOD in a human

being was estimated to be 0.015 mg/kg bw/day, and was a result of a self medication with a comfrey preparation.

The International Agency for Research on Cancer (IARC) evaluated several PAs for carcinogenicity in 1976 and 1983. It was concluded that there was in experimental animals "sufficient or limited evidence" for the carcinogenicity of monocrotaline, retrorsine, isatidine, lasiocarpine, petasitenine, senkirkine, and of extracts of the PA-containing plants *Petasites japonicum*, *Tussilago farfara*, *Symphytum officinale*, *Senecio longilobus*, *Senecio numorensis*, *Farfugium japonicum* and *Senecio cannabifolius*. The main target organ is the liver, where liver cell tumours and haemangioendothelial sarcomas were observed. In some instances, tumours in extra-hepatic tissues (lung, pancreas, intestine) were also observed, namely with monocrotaline, retrorsine, and lasiocarpine. Some PAs, for example, retrorsine, have been shown to be carcinogenic after a single dose. The pyrrolic metabolites have also been shown to be carcinogenic for rats. However, IARC concluded that the compounds are not classifiable as carcinogenic for humans. Due to the NTP data on riddelliine carcinogenicity, IARC changed the classification into “possibly carcinogenic to humans”, while NTP itself concluded that riddelliine is “reasonably anticipated to be a human carcinogen” [IARC 2002, NTP 2008].

In some countries and in some areas of usage, limits for the PA intake were set (see also table 1). The basis for the calculations is often not known.

Low level, intermittent dietary exposure to PAs can be expected, so that slowly progressing chronic diseases such as cancer, cirrhosis and pulmonary hypertension are possible outcomes from eating foods sometimes containing relatively low levels of PAs. Hepatotoxicity may not always be the most prominent effect. P450 enzymes are also subject to induction by many (herbal) medicinal products and their use could significantly enhance the toxicity of PAs in the diet. The extended time period of progressive chronic disease development adds to the difficulty in identifying dietary sources of PAs. It has to be considered that honey-containing products as mead, candy etc. may also contain PAs, as shown by KEMPF *et al*. [2011]. Familial susceptibility to PAs toxicity can also be expected. It should not be forgotten that anti-mutagenic compounds will also be ingested from food plants so that the impact of both mutagenic and anti-mutagenic compounds will be modulated by polymorphisms in genes associated with nutrient or xenobiotoc uptake, distribution and metabolism [FERGUSON & PHILPOTT 2008].

Because of their known involvement in human poisoning and their possible carcinogenicity, exposure to PAs should be kept as low as practically achievable, as pointed out by IPCS 1988, EFSA 2007, BfR 2007. According to the published literature, it is possible that the average dietary daily intake might already be more than the amounts of PA which are seen to be safe. According to KEMPF *et al*. 2010b and EDGAR *et al*. 2011 the daily amount of PA-intake via honey can easily reach 10-100 µg PA/day. Other sources of PA containing food (e.g. milk, convenience products, which may contain PA-traces, and meat) are known so that the actual exposure cannot be assessed.

Recommendations

Because of their known involvement in human poisoning and their putative carcinogenicity, exposure to PAs should be kept as low as practically achievable, as recommended by IPCS 1988, EFSA 2007, BfR 2007.

Oral use

The potential daily intake of PAs via food cannot be ignored especially as consumers/patients are not able to avoid them. On the basis of the available kinetic data, it seems clear that ingested PAs will be absorbed and metabolised. Herbal medicinal products containing herbal preparations with toxic, unsaturated PAs (even in very low amounts) should not be used orally.

Cutaneous use

Until now only rudimentary data concerning absorption of PAs through the skin exist. The study by BRAUCHLI et al. (1982) suggests that at least in rats, the dermal absorption could be 20-50 times less than absorption via the intestinal route. The used test model (rat) is not sufficient for the risk assessment in humans. More data, especially in animal species which are more comparable to human beings in relation to the skin or in vitro human skin preparations, generated with modern analytical techniques, are required before a final assessment can be made.

Content and absorption rates considering the limit of quantification should be investigated and discussed within a benefit/risk assessment.

Use in children and pregnant woman

Children (including also foetuses) and adolescents are especially vulnerable to the effects of PA. The population of pregnant and nursing woman and children/adolescents should therefore be excluded from the usage of products containing toxic, unsaturated PAs (even in very low amounts).

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