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

2nd Draft

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Keywords | Herbal medicinal products; HMPC; pyrrolizidine alkaloids

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

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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 Senecioeae 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-hydroxy-pyrrolizidine. The acids with which the necines are esterified are called necic acids.

![Fig. 1: general structure of PAs](image)

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].

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Public statement on the use of herbal medicinal products containing pyrrolizidine alkaloids (PAs)
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).

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 doronene, 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 dicarboxylic acid containing at least 5 C-atoms (acid).


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. 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 BMDL₁₀ for excess cancer risk of 70 μg/kg bw per day was calculated for induction of liver haemangiosarcomas by lausiarpine 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 [Coulombe 2003].

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 pyroles 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 didehydropyrrolozidine derivatives (DHP, pyroles). These pyrrolic alkaloids possess an allylic structure which promotes an increase in their reactivity. Once formed, the pyroles 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.
N-Oxides cannot be directly converted into pyroles. 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 monoxygenases [Prakash *et al.* 1999; Fu *et al.* 2004] while flavin-containing monoxygenases 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 retronene. The elimination half-times increased in the following order: riddelliine<retronene<N-
oxide consistent with metabolism of parent compound. Internal exposures (AUC\textsubscript{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 senecione 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 \textsuperscript{14}C-labelled senecione 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 pyrroloes continued for a little longer. In rats given retorosine, 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 retorosine 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 senecione 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 \textsuperscript{14}C-labelled senecione 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 LD₉₀ 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 [COULOMBE 2003]. 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 riddelline 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 [SCHOENTHAL 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 [SCHOENTHAL 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 pyroles 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]. Dehydrochelmitridine is less acutely toxic than its parent alkaloids; it has an LD<sub>50</sub> (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 dehydrochelmitridine 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. Dehydrochelmitridine 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 dehydrochelmitridine is responsible for some, or possibly all, of the carcinogenicity of its parent alkaloids. Dehydrochelmitridine 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 described 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 alklylation either by releasing 6,7-dihydropyrrrolizine carbonium ions capable of forming new adducts directly, or via the hydrolytic release of dihydropyrrrolizine alcohols [MATTOCKS 1986]. In literature it was postulated that, following dietary exposure to PAs, in vivo alklylation 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].

![Diagram showing clinical natural history of VOD of the liver](image)

**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]. Results concerning the late onset of changes in the lung after a single exposure to monocrotaline were described in animals [HUXTABLE 1990].

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, RIDKER et al. 1985].

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 riddelline. Riddelline 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 riddelline-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 one study, a single dose has been carcinogenic [CULVENOR 1983]. In the study of Schoental & Magee [1957] a single dose of lasiocarpine provoked after ~13 months changes in the liver which were described as being very similar to those observed in the earlier stages of hepatic carcinogenesis due to several pyrrolizidine alkaloids after multiple dosing.

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

![Fig. 6: proposed mechanism for the induction of liver haemangiosarcoma by riddelliine in rats [NYSKA et al. 2002)](image)

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

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

### 2.7. Human exposure to PA by food

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

In developed countries levels of PA intake are mostly low. Beside the direct intake of PAs via herbal medicinal products secondary contamination of food with PAs was observed: e.g. in foods of animal origin (as milk, eggs, honey, pollen products), in grain and in packed lettuce boxes as recently detected in Germany [MOLYNEUX et al. 2011]. So depending on the individual preference in food selection, great variability of PA exposure in humans is expected.
Globalisation of markets also leads to situations where previously localised toxins are shipped around the world in contaminated products. During the past few years it appears that, because of the lack of natural control factors, the expansion of certain invasive plants e.g. Senecio madagascariensis (Australia, Hawaii) and Senecio jacobaea (Germany, UK, USA, New Zealand) creates serious problems for animals and via animal products, for humans as well.

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

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

**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 [³H] 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%). Feeding cows for 2 weeks with Senecio jacobea
at a dose of 10 g/kg/day (average pyrrolizidine content of 0.16% dry weight) led to jacoline
concentrations in milk ranging from 9.4 to 16.7 µg/100 ml [COULOMBE 2003]. 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 plantaginum 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 contributon 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 monocrotaline 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 numoresens, 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 riddelline carcinogenicity, IARC changed the classification into "possibly carcinogenic to humans", while NTP itself concluded that riddelline 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 meal, candy etc. may also contain PAs, as shown by KEMPFF 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 also 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 KEMPFF 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.
In the evaluation of HMPs/THMPs containing PAs Member States should take steps to ensure that the public are protected from exposure and the following thresholds should be applied.

**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.

In the risk assessment of genotoxic carcinogens the TD₅₀ value (a measure of cancer potency) from the most sensitive species/tumour site is considered an appropriate point of reference for a linear down extrapolation to a “virtually safe dose”, i.e. a dose corresponding to a theoretical excess cancer risk of <1 in 1,000,000 (10⁶) over a lifetime of exposure. Linear extrapolation to a probability of 1 in 1,000,000 is achieved by simply dividing the TD₅₀ by 500,000. This extrapolation scenario would be applied to (traditional) herbal medicinal products mainly because of the background-intake of PAs via food.

The BMDL₁₀ value 70 µg/kg/day - based on induction of liver haemangiosarcomas by lasiocarpine in male rats (EFSA 2011) - could be used instead of the TD₅₀ value. For the calculation of a limit value for acceptable exposure via herbal preparations, this value is the lowest (i.e. most conservative) available, because lasiocarpine is one of the most potent pyrrolizidine alkaloids (e.g. the BMDL₁₀ value of riddelliine is 180 µg/kg/day).

To derive a dose to cause tumours in 1 in 1,000,000 animals, divide by 100,000:

70 µg/kg/day ÷ 100,000 = **0.0007 µg/kg/day**

Generally for adults the calculation is done with a body weight of 50 kg. Therefore the daily dosage would be:

0.0007 µg/kg/day x 50 kg body weight = 0.035 µg/person/day

**Sensitive groups:**

**Children**

If children are included in the usage of certain products the daily amount of PA 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 0.014 µg PA/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**

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.

It is to ensure that the amount of PA within the daily dose is <0.035 µg for adults. The use is restricted to intact skin.

Higher contents of PA 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 (generated with modern analytical techniques; in
animal species which are more comparable to human beings in relation to the skin or in vitro human skin preparations) can be shown, not exceeding the daily intake of 0.035 µg PA for adults.

**Sensitive groups:**

**Children**

If children are included in the usage of certain products the daily amount of PA 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 0.014 µg PA/day.

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