ACUTE TOXICITY OF HELIOTRINE IN MALE HAN WISTAR RATS

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Abstract- There is a lack of published information on the oral toxicity of the pyrrolizidine alkaloid heliotrine. The acute toxicity of heliotrine was investigated in male Han Wistar rats, using OECD Test Guideline 425. Lesions consistent with those seen in monocrotaline-induced acute pulmonary hypertension were found in rats dosed with ≥ 160 mg/kg heliotrine. The ability of heliotrine to induce these pulmonary lesions in the rat has not previously been reported and has not been observed in human toxicosis attributed to heliotrine. At doses ≥ 510 mg/kg, a single oral gavage dose of heliotrine caused overt but nonspecific clinical signs and lesions of haemorrhage and centrilobular necrosis in the liver. The median lethal dose was calculated to be 510 mg/kg heliotrine in male Han Wistar rats, a value substantially higher than the range previously reported in the literature. At 1600 mg/kg, toxicosis may be fatal before liver lesions can be discerned by light microscopy.
Keywords- Heliotrine; pyrrolizidine alkaloid; oral toxicity; acute toxicity; rat; pulmonary toxicity; hepatotoxicity

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Introduction

Pyrrolizidine alkaloids (PAs) are a class of naturally-occurring toxins produced by over 6000 plants around the world. More than 660 PAs and PA *N*-oxides have been identified. PAs have caused numerous cases of human hepatotoxicity, including cases resulting from ingestion of herbal preparations and some large-scale epidemics, through contamination of staple foods [1]. All reported cases of human toxicosis due to PAs have been due to ingestion of the PA directly or, in the case of neonatal toxicosis, by intrauterine exposure to PAs ingested by the mother. It is therefore surprising that for the vast majority of PAs, the oral toxicity has not been investigated in experimental animals. For most PAs for which any experimental animal toxicity data exist, only intraperitoneal toxicity data are available, which are not useful to assess the hazard of PAs from oral dosing. Improved characterisation of the toxicological profile of individual PAs administered by the route of exposure most relevant to humans is an important contribution to understanding the risk of PA exposure to human health. Heliotrine has been incriminated as the predominant PA responsible for 'Gulran disease', an epidemic of PA-induced hepatic veno-occlusive disease that affected an estimated 7800 people in Gulran District of Afghanistan between 1974 and 1976, leading to approximately 1600 deaths. Additional outbreaks occurred between 1999 and 2001, with over 100 deaths and again in 2008. Gulran disease was attributed to consumption of bread made from wheat that had been contaminated with seeds of *Heliotropium popovii* subsp. *gillianum.* This plant grows as a weed in the area and is locally known as 'charmac". Gulran disease is considered to occur as a result of regular ingestion of contaminated bread over several weeks [2]. While these incidences associated with PA contaminated grain have been documented in the scientific and medical literature, there is a lack of robust information on the actual toxic dose of heliotrine to human beings.

Reports in the literature note that there are substantial differences in the toxicity of different PAs within a species and of the same PA in different species. Acute toxicity data allows for some comparison of toxicity between related compounds, such as different PAs. In addition, information from acute oral toxicity studies may be used to select doses for subsequent short-term and subchronic toxicity tests when no other toxicology information is available [3].

There is very little information in the literature concerning the short-term oral toxicity of heliotrine in any species, including the laboratory rat. Only one study in the English language was found and attempts to obtain some Russian studies by standard retrieval methods were unsuccessful. The English language study reported that 9/20 Wistar rats were fatally poisoned within 60 days by single oral doses of 150-300 mg/kg heliotrine, with another 3 rats dying between 2 and 12 months after dosing[4]. However, that study had several design limitations. These limitations included: (1) The rats used were pups weighing only 30-60 g when dosed. (2) Both sexes were used but results were presented combined rather than separated by gender, although a number of subsequent studies with other PAs have shown marked gender differences in susceptibility to PA toxicity in the rat. (3) The fatalities were reported in intervals of 1 - 10 days, 11- 60 days and 2-12 months, with individual days of death not reported. (4) Not all rats were treated the same way, in that some but not all rats were group-housed.

The purposes of the present study was to determine the median lethal dose of heliotrine by the oral route in Han Wistar male rats, to identify target organs of toxicity and to obtain dose-rangefinding information for subsequent studies from which to determine a No Observed Adverse Effect Level (NOAEL).

The design of this study was based on OECD Test Guideline 425 [5], with the following three exceptions:

- The gender of the test rats. The guideline recommends the use of female animals because for most toxicants, females are more susceptible than males, but this is not generally the case for PAs. Male rats have been reported to be more susceptible than female rats to a number of PAs, including monocrotaline, riddelliine, senecionine, seneciphilline and retrorsine [6], although female rats are more susceptible than males to lasiocarpine N-oxide toxicity [7]. Male rats are more susceptible than females to heliotrine toxicity by the intraperitoneal route [7].

- The time-point at which the animals were considered to have survived or died. The guideline specifies that the acute oral toxicity is determined at 24 hours post-dosing, but for this study the deadline was extended to 72 hours on the basis of a study in which liver lesions induced by a single oral dose of monocrotaline were not obvious by light microscopy at 48 hours post-dosing, but were severe at 72 hours post-dosing [8].

- The omission of one downward dose step in order to reduce the number of rats used and to conserve test article, because the proposed dose, 160 mg/kg, appeared unlikely to cause fatal toxicosis.

Materials and Methods

The study was conducted at Estendart Ltd., Palmerston North, New Zealand. The study plan complied with the New Zealand *Animal Welfare Act 1999* and the final study design was approved (Approval number AEC 013/10) by the Kaiawhina Animal Ethics Committee.

Male Han Wistar rats were obtained from Otago University, New Zealand. Rats were 8 weeks old at the time of receipt. Rats were acclimated for at least 16 days prior to the start of the study to the study conditions of individual housing in polycarbonate cages, environmental temperature of 22 (±3) °C, relative humidity of 50-60% and artificial lighting set to 12 hours light:12 hours dark. Rat chow, manufactured by the Massey University Food Production Unit and water were provided *ad libitum* when rats were in their cages, with the exception of 4.4 to 6.5 hours prior to dose administration, when food was withheld to ensure that the dose was administered on an empty stomach. Food and water consumption were determined daily. Health observations were made at least once daily prior to dosing and at least twice daily after dosing. After it had been dosed, each rat was weighed daily. All rats were dosed with heliotrine, because OECD Test Guideline 425 does not include the use of control animals.

Heliotrine, 98%, was supplied by Latoxan, Valence, France. Stock dispersions of heliotrine were prepared by Grayson Wagner Co. Ltd., Penrose, Auckland, New Zealand. The first diluent used, a citrate-buffered diluent at pH 5.4, contained 0.3% tetra sodium citrate, 0.1% citric acid, 0.1% methyl paraben and 0.1% xanthan gum, all as w/w in water. A stable dispersion of heliotrine in citrate-buffered diluent was formed by wet grinding heliotrine using 6mm diameter 316 S/S bars in a glass rod mill, with addition of food grade wetting/deaeration surfactants, Tergitol L62, Tergitol L64 and Defoamer RD at 0.5 %, 0.1% and 0.01% w/w respectively to prevent frothing. Subsequently it was found that the use of phosphate buffer resulted in less foaming. The diluent was therefore changed to phosphate-buffered diluent, pH 6.1, containing tetra potassium pyrophosphate 0.2%, 68% phosphoric acid 0.07%, methyl paraben 0.1%, xanthan gum 0.1 - 0.2% and Tergitol L 64 2.0%, all as w/w in water. A stable dispersion of heliotrine in phosphate-buffered diluent was formed by wet grinding for 4 hours using 6mm diameter 316 S/S bars in a glass rod mill. Suspensions were diluted to the appropriate concentration on the day of dose administration, using the citrate-buffered diluent. Animals 1 through 4 inclusive received formulation dispersed with citrate-buffered diluent and the remaining rats received formulation dispersed with phosphate-buffered diluent.

Each rat was weighed on the day it was dosed and heliotrine suspension was administered at a dose volume of 10 mL/kg bodyweight using a flexible 18 gauge gavage tube. The first rat was dosed with 50 mg/kg heliotrine, on the basis of the lower end of the single-dose toxic range of 150-300 mg/kg for heliotrine previously reported [4, 9]. Subsequent rats were dosed on the basis of the outcome for the previous rat as determined at 72 hours after dosing, using the dose calculation specified by OECD Test Guideline 425 [5], with the exception of Animal 8. The recommended dose for Animal 8 was 160 mg/kg (Table 1) but the rat was administered 510 mg/kg, because previous results indicated that 160 mg/kg was unlikely to be fatal. By administering 510 mg/kg to this rat, the total number of rats and the total amount of test article used were both minimised.

Rats found moribund prior to Day 7 and those which survived to Day 7 following dose administration on Day 1 were humanely terminated by using carbon dioxide.

All rats were subject to gross necropsy. Liver was collected from all rats and liver weight determined for rats that were terminated. The right lobe of each liver was preserved in saline-buffered formalin. Lung, kidney and heart were also collected from all but one of the rats (Animal 5). Lungs were gently perfused with buffered formalin via the trachea.

Abbreviations

PA   pyrrolizidine alkaloid
LD50   median lethal dose
OECD   Organisation for Economic and Cooperative Development

Results

The change of diluent buffer had no discernible effect on any results.

Median lethal dose determination

Doses and 72-hour outcomes are shown in Table 1.

Table 1- 72-hour single-dose acute oral toxicity determination of heliotrine by the up-and-down procedure according to OECD Test Guideline 425.

Rat	Recommended Dose (mg/kg)	Administered Dose (mg/kg)	Outcome at 72 hours
1	50	50	Alive
2	160	160	Alive
3	510	510	Alive
4	1600	1600	Dead
5	510	510	Alive
6	1600	1600	Dead
7	510	510	Dead
8	160	510	Alive
9	1600	1600	Dead

The calculated median lethal dose (LD50 value) at completion of the study was 510 mg/kg, with upper and lower confidence limits of 1142 and 405 mg/kg respectively. A dose averaging estimate performed prior to the calculation reached a value of 677 mg/kg. The study was stopped when at least 4 animals had followed the first reversal and the specified likelihood-ratios exceeded the critical value (Criterion C; OECD Test Guideline 425 [5]).

Mortality

Both the rat dosed with 50 mg/kg and the rat dosed with 160 mg/kg survived to scheduled termination on Day 7. Three of the four rats dosed with 510 mg/kg also survived to scheduled termination on Day 7, but the exception, Animal 7, was terminated in moribund condition on Day 3, just over 48 hours after dosing. All three rats dosed with 1600 mg/kg died before the 72-hour time point. Animal 4 was found dead on Day 2, Animal 6 was terminated moribund on Day 2 and Animal 9 was terminated moribund on Day 3.

Clinical observations

Clinical observations for rats dosed with ≥ 510 mg/kg are summarized in Table 2, with the exception of Animal 4, which was found dead on the morning of Day 2.
No abnormal clinical signs were observed at any time in Animal 1, the rat dosed with 50 mg/kg. The only abnormal clinical sign observed in Animal 2, the rat dosed with 160 mg/kg, was pallor of the mucous membranes on Day 7.
Most of the clinical signs observed were non-specific signs of ill-health. Yellow discolouration of mucous membranes, consistent with icterus secondary to liver damage, was recorded in only two rats, 510 mg/kg Animal 8 and 1600 mg/kg Animal 9.
The severity of the effects of dosing with 510 mg/kg was quite variable. Clinical abnormality in Animal 5 was limited to dehydra-

tion on Days 3 and 4. Rats 3 and 8 were more severely affected, but both appeared to be improving by Day 7. Animal 7, on the other hand, was moribund on Day 3.
The dose level of 1600 mg/kg caused rapid deterioration in all three rats to which it was given.

Body weights, body weight changes, food consumption and water intake

Body weights at dosing and on the day of death are summarized in Table 3. Body weight changes, food consumption and water intake are summarized in Table 4.

Table 3- Initial and final body weights of rats dosed with heliotrine on Day 1.

Rat	Day of death[1]	Nature of death	Body weight change between Day 1 and day of death (g)
		50 mg/kg	
1	7	Scheduled termination	+18
		160 mg/kg	
2	7	Scheduled termination	+7
		510 mg/kg	
3	7	Scheduled termination	-33
5	7	Scheduled termination	-35
7	3	Moribund termination	-39
8	7	Scheduled termination	-80
		1600 mg/kg	
4	2	Found dead	-9
6	2	Moribund termination	-40
9	3	Moribund termination	-28
		[1]Relative to dosing on Day 1.	

Table 4- Body weight changes, food consumption and water intake of rats dosed with heliotrine

Rat	Day[1]	Body weight change (g)	Food consumption (g)	Water consumption (g)
		50mg/kg		
1	2	1.4	31.0	42.9
	3	-0.8	27.7	50.6
	4	5.7	31.9	38.7
	5	5.8	29.1	46.9
	6	3.7	30.0	44.1
	7	5.6	30.0	40.3
		160 mg/kg		
2	2	2.4	25.7	34.7
	3	0.1	30.8	40.2
	4	2.8	31.5	42.0
	5	3.3	27.4	38.5
	6	3.7	29.9	42.3
	7	6.2	29.8	49.9
		510 mg/kg		
3	2	-4.2	20.8	56.7
	3	-3.7	19.2	54.4
	4	-14.0	12.9	31.5
	5	-24.8	9.4	No data
	6	9.7	17.2	59.5
	7	6.0	23.6	45.4
5	2	-8.0	No data	49.3
	3	-8.6	18.4	35.1
	4	-11.6	10.0	37.6
	5	-8.6	20.5	29.2
	6	5.9	21.8	43.2
	7	-1.4	25.2	33.8
7	2	-9.1	12.6	30.6
	3	-27.4	5.3	13.4

Table 4- Continue

8	2	-7.3	19.5	30.1
	3	-17.6	3.2	20.2
	4	-19.7	0.6	1.9
	5	-13.9	1.4	4.8
	6	-7.2	2.1	10.1
	7	-9.8	5.1	4.7
600 mg/kg				
4	2	-8.9	Not recorded	Not recorded
6	2	-35.2	11.52	1.9
9	2	-8.7	15.4	12.7
	3	-18.2	2.4	0.8

Both rats dosed with ≤ 160 mg/kg heliotrine gained weight between dosing and termination, although Animal 1 lost a small amount of weight between Day 2 and Day 3 and Animal 2 gained very little weight over the same interval. Daily food and water consumption by these rats showed little change between dosing and termination. All the rats dosed with ≥ 510 mg/kg heliotrine lost weight between dosing and death. For the three rats dosed with 510 mg/kg that survived to scheduled termination, daily weight loss was most severe mid-week. Animals 3 and 5 showed a reduction in daily food consumption mid-week with subsequent recovery and water consumption did not appear to be affected. In contrast, Animal 8 exhibited a persistent loss of appetite and reduction in water consumption and lost a total of 80 g, the greatest body weight loss of any of the rats in the study. Animal 7, the 510 mg/kg rat that required termination on Day 3, showed substantial weight loss coinciding with decreased intake of food and water. Of the rats dosed with 1600 mg/kg, Animal 4 died so rapidly that 24-hour food and water consumption data were not available, while the other two rats showed low food and water intake prior to death.

Gross necropsy and histopathology

Liver findings on gross necropsy and liver weight relative to body weight, are presented in Table 5.

Table 5- Liver findings on gross necropsy of rats dosed with heliotrine on Day 1.

Rat	Day of death[1]	Nature of death[2]	Appearance of liver at gross necropsy	Liver weight as % of body weight
50 mg/kg				
1	7	T	No abnormality observed	5.4
160 mg/kg				
2	7	T	No abnormality observed	4.6
510 mg/kg				
3	7	T	No abnormality observed	4.5
5	7	T	No abnormality observed	4.3
7	3	M	Liver enlarged and slightly darkened.	4.8
8	7	T	Liver firm, moderate diffuse enhancement of reticular pattern	4.7
1600 mg/kg				
4	2	FD	No abnormality observed	-
6	2	M	No abnormality observed	4.4
9	3	M	No abnormality observed	4.3

[1]Relative to dosing on Day 1. [2] T = Scheduled termination. M = moribund termination. FD = found dead

Gross changes to the liver were apparent in only two rats, Animals 7 and 8, both of which were dosed with 510 mg/kg. The dose of heliotrine had no apparent effect on liver weight relative to body weight.

Two of the three rats dosed with 1600 mg/kg, Animals 6 and 9, were found to have stomachs abnormally distended with large quantities of dried, pasty contents and some gas. These findings were considered by the Board-certified veterinary pathologist who conducted the necropsies to be suggestive with delayed gastric emptying. Animal 9 had consumed only 2.4 g of food over the preceding 24 hours. The gas was not a post-mortem artefact because both rats were necropsied promptly after termination.

Histopathology

Liver was preserved for microscopic examination from all rats. Lung, heart and kidney were also preserved from all rats with the inadvertent exception of Animal 5.

No abnormalities were discovered in the livers of the rats dosed with ≤ 160 mg/kg heliotrine, Animals 1 and 2. Severity of liver pathology was variable in rats dosed with 510 mg/kg. The liver of Animal 3 was moderately congested, with multiple foci of primarily centrilobular necrosis, although not all central veins were affected and in some lobules the distribution was mixed centrilobular/midzonal. Necrotic areas in some lobules extended into the midzonal area. There was mild infiltration of necrotic tissue with by macrophages and plasma cells. The endothelial cells of intact central veins had prominent nuclei. Similar lesions were found in the liver of Animal 5, with the addition that macrophages were frequently present around the periphery of central veins. Animal 7 had more severe liver lesions, with extensive haemorrhage and necrosis, of a centrilobular distribution with bridging between lobules. Apoptotic hepatocytes with small, dense and sometimes fragmented nuclei were very frequently observed. The sinusoidal architecture was obliterated in and near the centre of many lobules. Surviving hepatocytes in periportal islands of tissue showed moderate to severe macrovesicular degenerative changes. Some central veins appeared to have no endothelial cell layer, while others had prominent endothelial nuclei. Many central veins were lined with marginated macrophages. The liver pathology in Animal 8 was similar to that of Animal 7, with the difference that fatty change in degenerating hepatocytes tended to be microvesicular and clumping of cytoplasm was also a common feature in degenerating hepatocytes. Occasional mitotic figures were noted among surviving hepatocytes. Degeneration of the endothelium of central veins, with margination of macrophages, was also observed in this rat (Fig. 1).
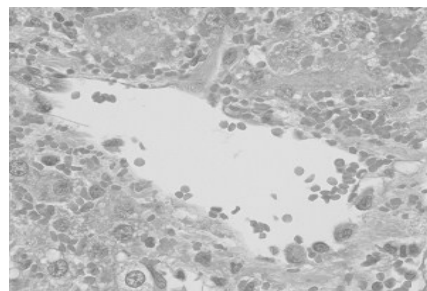

Fig. 1- Central vein in the liver of Animal 8, showing margination of macrophages and degenerative changes in endothelial cell layer. This rat was dosed with 510 mg/kg heliotrine on Day 1 and survived to scheduled termination on Day 7. HE x 40.

Liver pathology was also variable between 1600 mg/kg rats, possibly related to survival time. The liver of Animal 4, found dead on the morning of Day 2, was congested and slightly autolysed, but showed no obvious necrosis. In contrast, the liver of Animal 6, terminated moribund on Day 2, contained extensive zones of haemorrhage and necrosis, centrilobular and midzonal in distribution but with some central veins spared. Apoptotic hepatocytes were very common. Animal 9, which survived to Day 3, had the most severe liver pathology of the rats at this dose level, with extensive severe haemorrhage and necrosis, primarily centrilobular but with bridging between lobules. Marginated macrophages were noted in some central veins and other veins, although in many lobules the sinusoidal architecture was obliterated to such an extent that the original limits of the central vein could not be discerned. In some large veins there were strands of fibrinous material in the lumen roughly parallel to the vein wall, which may represent sloughed endothelial tissue (Fig.2). This was not considered to be a post-mortem change because the rat was necropsied immediately after termination.
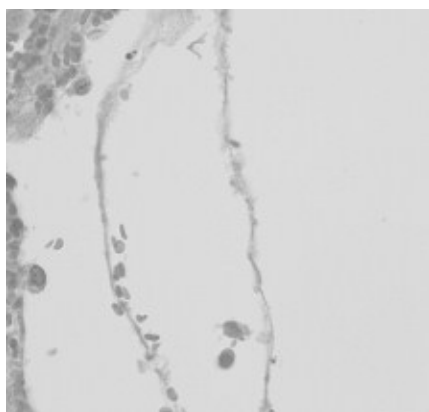

Fig. 2- Strands of fibrinous material in the lumen of a central vein in the liver of Animal 9, which may represent necrotic endothelial tissue. This rat was dosed with 1600 mg/kg heliotrine on Day 1 and terminated in moribund condition on Day 3. The strands of material are not considered to be post-mortem artefact because the rat was necropsied immediately after termination. HE x 40.

Multinucleate, megalocytic hepatocytes were not observed in the liver of any rats, regardless of dose or survival time.
Lesions consistent with those reported in rats dosed with monocrotaline [10-13] but not observed in rats dosed with riddelliine [13], were found in lung sections of rats dosed with ≥ 160 mg/kg heliotrine. Such lesions in monocrotaline-dosed rats are attributed to acute pulmonary hypertension. The lung tissue of 160 mg/mg Animal 2 showed slight to mild oedema between the adventitia and muscular tunic and sometimes between the muscular tunic and the endothelium, in arterioles. Similar changes were present in lung tissue from 510 mg/kg Animal 3. The lung tissue of 510 mg/kg Animal 7 likewise featured periarteriolar oedema and in addition, some small arterioles showed hypertrophy of the muscular tunic. Bronchiolar epithelium showed hypertrophy and hyperplasia. There was diffuse increase in the thickness of alveolar septa through much of the lung and some areas of collapse, although the lung had been manually perfused with buffered formalin after death. In the lung section of 510 mg/kg Animal 8, periarterio-

lar oedema was moderate to severe (Fig.**3**). The muscular tunic of several small arterioles was hypertrophied. Bronchiolar epithelium was moderately hypertrophied and hyperplastic. Alveolar septa appeared slightly thickened. Perivascular oedema of arterioles was moderate to severe in the lung tissue of 1600 mg/kg Animal 4 and hypertrophy of the muscular tunic was evident in some arterioles. The lung was congested and alveolar septal thickness was moderately increased in this rat. Perivascular oedema of arterioles was also evident in the lung tissue of 1600 mg/kg Animal 6, although it was less congested. The lung tissue of 1600 mg/kg Animal 9 was very similar to that of 510 mg/kg Animal 8, with moderate to marked periarteriolar oedema, mild to moderate hypertrophy of the muscular tunic of small arterioles, congestion and increased thickness of alveolar septa and hypertrophy and some hypertrophy of bronchiolar epithelium.
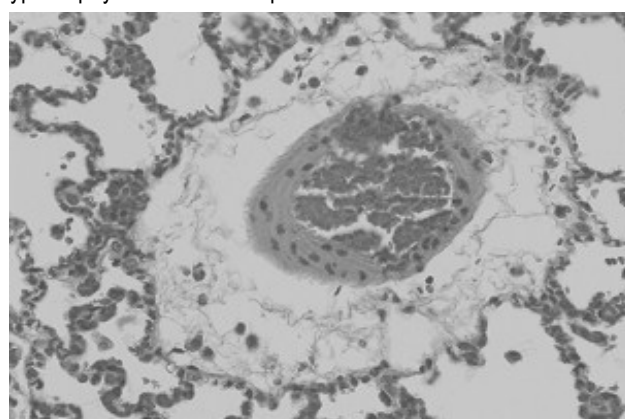

Fig. 3- Arteriole from the peripheral lung of Animal 9, dosed with 510 mg/kg heliotrine, showing perivascular oedema and mild hypertrophy of the muscular tunic. HE x 20

Sections of heart from most rats were normal or slightly congested, but the section from 510 mg/kg Animal 7 contained a large area of haemorrhage in the myocardium, largely that of the wall of the left ventricle close to the heart valves, with blood dissecting between muscle fibres. The haemorrhage appeared to be very recent.
The kidney tissue of some rats was mildly to moderately congested, but no other treatment-related changes were found. However 510 mg/kg Animal 7 was found to have chronic degenerative changes in the kidneys, with tubule dilatation and loss in both cortex and medulla and atrophic changes in some glomeruli. The kidney pathology was considered to pre-date heliotrine administration.

Discussion
The median lethal dose of 510 mg/kg identified in this study is considerably higher than the range of 150-300 mg/kg previously reported [4]. This difference may in part reflect the use of older rats rather than pups of 30 - 60 g, the weight range of the rats used in the earlier study. In addition, in this study considered fatalities only to 72 hours, whereas the earlier study [4] included fatalities up to 60 days after dosing.
The single-dose oral median lethal dose established in this study is also substantially higher than the single-dose intraperitoneal LD50 of 296 mg/kg for heliotrine in rats of 'a hooded strain', which

was also determined at three days post-dosing [7]. This is consistent with our measurement of the acute oral median lethal dose of monocrotaline [13], which was more than three times the reported intraperitoneal LD50 for monocrotaline in rats. Thus, these PAs are less toxic to rats by the oral route than by intraperitoneal administration. This is contrary to the assertion by the International Programme on Chemical Safety (IPCS) [14] that 'the hepatotoxicity of PAs in rats does not differ very much, irrespective of the route of administration' and suggests that acute oral toxicity cannot be reliably extrapolated from an intraperitoneal LD50. The IPCS statement appears to be based on the comparison of IP LD50 values reported by one author [15] with oral toxicity values reported by another team [4], but it should be noted that the study design by which the oral toxicity values were obtained would not provide an LD50.

The results of this study suggest that 160 mg/kg may be suitable as a middle dose for a subsequent single-dose study or as a high dose for a repeat-dose study. Animal 2, dosed with 160 mg/kg, did not develop any clinical signs or liver lesions, but did have lung lesions.

Effects on body weight gain at doses ≤ 160 mg/kg were considered equivocal, particularly because OECD Test Guideline 425 does not include the use of control animals for comparison. However, all rats administered 510 mg/kg showed body weight loss 24 hours after dosing. Of the three 510 mg/kg rats that survived to scheduled termination on Day 7, all three showed the greatest body weight loss on Day 4 or 5 after which Animals 3 and 5 began to regain body weight whereas Animal 8, which had more severe liver pathology, continued to lose body weight, although less rapidly. All three 510 mg/kg rats that survived to elective termination showed some evidence of improvement in the form of a reduction in the number of clinical signs. The liver pathology in Animals 3 and 5 was considered to be survivable.

Animal 4, dosed at 1600 mg/kg, had no liver lesions apparent on light microscopy, presumably because it died before they became evident. The other two rats at 1600 mg/kg developed severe liver lesions. Thus, a sufficiently high dose of heliotrine may be fatal to the rat before hepatic lesions visible by light microscopy can develop. It has been reported that animals may die in less than 1 hour following a massive dose of PA, although the cause of death in such cases has not been determined [16]. Rapid death in rats following injection, to an unspecified site, of large doses of heliotrine or lasiocarpine has been attributed to neuromuscular block, which was demonstrated in isolated nerve/muscle preparations [17].

There was some evidence in this study to suggest inhibition of gastric emptying. Our previous study using monocrotaline also found evidence of delayed gastric emptying in male Han Wistar rats following high doses [13]. This may be a reflection of the anticholinergic action of heliotrine previously demonstrated in isolated guinea pig ileum [18].

The absence of multinucleate, megalocytic hepatocytes in livers of rats dosed with heliotrine, even if they survived to Day 7, is in contrast to our previous study [13] in which a rat dosed with 160 mg/kg monocrotaline that survived to Day 7 with liver lesions had multiple foci of megalocytic hepatocytes.

In previous up-and-down studies with two other PAs, monocrotaline and riddelliine, in Han Wistar rats, this research team did not observe lesions in the lungs in riddelliine-treated rats. However lesions similar to those found in this study, including periarteriolar oedema, hypertrophy of muscular tunics of arterioles and thickened alveolar septa and were observed in the lungs of monocrotaline-treated rats and showed a dose-response relationship [13]. Administration of monocrotaline to the rat is a well-established model of acute pulmonary hypertension and the lesions have been extensively described [10-12, 19, 20]. Similar lesions have been described in rats dosed with two other PAs, fulvine [21] and seneciphilline [22], but these lesions do not appear to have been previously been described in toxicosis due to pure heliotrine in the rat. Medial thickening of pulmonary arteries and arterioles in rats fed a diet containing *Heliotropium circinatum* [23] may have been due to heliotrine, but the plant material contained a mix of PAs, identified as europine (67.33%), heliotrine (16.34%), lasiocarpine (8.12%), heleurine (4.18%), echinatine (1.56%), 7-angelylheliotrine (1.19%) and an unidentified alkaloid (1.28%) [23]. It has been claimed stated that with the exception of monocrotaline and fulvine, PAs do not cause lung lesions in rats except when death from liver toxicity is prevented by other treatments [20], but the results of the current study contradict this. Pulmonary lesions were found at 160 mg/kg, a dose at which no liver lesions were noted. Thus, the threshold for acute toxic effect of heliotrine on rat lung may be lower than that for acute toxic effect on the liver, although further study with more rats will be required to confirm this. In addition, lung lesions were present in conjunction with mild to moderate liver lesions 510 mg/kg Animals 3 and 5. Lung lesions at 510 mg/kg and 1600 mg/kg heliotrine were less severe than those observed by this team at the same doses of monocrotaline [13]. Pulmonary lesions were more severe in the 510 mg/kg rats than in the 160 mg/kg rat, but a clear dose-response relationship over the three doses at which pulmonary lesions were observed (160, 510 and 1600 mg/kg) could not be confirmed because the 1600 mg/kg rats had a much shorter survival time after dosing than most of the 510 mg/kg rats. Further study with more rats, a common day of termination and a control group for comparison, would be necessary to confirm the presence of a dose-response relationship.

Pulmonary hypertension as a result of heliotrine ingestion has not been reported in humans, although large-scale epidemics of heliotrine poisoning have occurred. The literature contains only one case of possible PA-induced pulmonary hypertension in a human being, that of a woman who had severe pulmonary hypertension and a history of habitual ingestion of herbal tea that contained, among other herbs, comfrey [24]. Comfrey (*Symphytum officinale*) is known to contain pyrrolizidine alkaloids including lycopsamine, echimidine, symviridine and lasiocarpine [24, 25]. However, it was not established that the PAs in comfrey were the cause of the pulmonary hypertension in that case. Pulmonary hypertension has never been described in human cases of hepatic veno-occlusive disease resulting from PA ingestion [26].

The relationship to heliotrine administration of the myocardial haemorrhage in 510 mg/kg Animal 7 is considered to be equivocal. Although pulmonary hypertension does cause secondary cardiac disease, this is typically in the form of dilatation or hypertrophy of the right ventricle. A direct toxic effect of monocrotaline on the myocardium of both ventricles in the rat has been described previously, but the histological lesions at 7 days after

dosing that they reported were those of diffuse mononuclear infiltration of the myocardium [27], rather than haemorrhage. It may be relevant that Animal 7 was found post-mortem to have chronic degenerative changes in the kidneys, which may have resulted in impaired excretion of heliotrine and metabolites, with higher plasma levels as a result. Additionally, chronic kidney disease may cause hypertension. Thus, there is a possibility that the pre-existing renal lesions may have predisposed Animal 7 to myocardial haemorrhage in the face of acute heliotrine poisoning. Animal 7, the only one on the study found to have significant pre-existing disease at necropsy, was also the only 510 mg/kg rat that did not survive to 72 hours. Had Animal 7 survived to 72 hours and a subsequent rat dosed with 1600 mg/kg died, the study would have been completed using 8 rats and yielded a calculated LD50 of 903 mg/kg. It is possible that the pre-existing subclinical kidney disease in Animal 7 resulted in an underestimation of the LD50. Although the up-and-down method specified in OECD Test Guideline 425 is intended to minimise the number of animals required to obtain an estimate of the median lethal dose, this does mean that one animal with particular susceptibility to a toxic substance may distort the result. On the other hand, Animal 7 did not show any signs of ill-health prior to dosing.

In conclusion, the 72-hour single-dose oral median lethal dose for heliotrine in male Han Wistar rats established by this study was 510 mg/kg, with upper and lower confidence limits of 1142 and 405 mg/kg respectively. Transient impairment of body weight gain was observed at doses of 50 mg/kg and 160 mg/kg and lesions similar to those seen in monocrotaline-induced pulmonary hypertension were also observed at 160 mg/kg, but rats survived to Day 7. Pulmonary lesions have not previously been reported in heliotrine toxicosis in the rat. At doses ≥ 510 mg/kg, lesions of haemorrhage and centrilobular necrosis were found in the liver, rats showed overt but generally nonspecific signs of ill-health and mortalities occurred.

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Table 2- Clinical observations in rats after oral gavage administration of a single dose of 510 mg/kg or greater of heliotrine

Rat	Day¹	Skin fold test²	Rough coat	Red eye or nose discharge	Mucous membrane colour change³	Reduced activity	Dyspnoea	Hunched posture
					510 mg/kg			
3	2		√					
	3		√			√		
	4		√		P	√		
	5		√		P	√		
	6		√		P	√		
	7				P			
5	2							
	3	√						
	4	√						
	5							
	6							
	7							
7	2		√					
	3		√		P	√	√	√
8	2					√		
	3		√	√	P	√		√
	4	√	√	√	P			
	5	√	√	√	P & Y	√		
	6	√	√	√	P	√		
	7	√	√	√	P			
					1600 mg/kg			
4	Found dead on the morning of Day 2, therefore no clinical signs recorded							
6	2	√	√		P	√	√	
9	2	√		√	P			
	3	√	√		P & Y	√	√	√

¹*Relative to dosing on Day 1.* ²*A positive skin fold test was indicative of dehydration.* ³*P = pale; Y = yellow*