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# A 28-day oral toxicity study of echimidine and lasiocarpine in Wistar rats

Rosalind R. Dalefield [a, *], Michelle A. Gosse [a], Utz Mueller [b]

[a] Food Standards Australia New Zealand, Level 3, 154 Featherston Street, Wellington 6011, 6143, New Zealand
[b] Food Standards Australia New Zealand, 55 Blackall Street, Barton, ACT 2600, Australia

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are a class of naturally-occurring plant toxins. Echimidine is one of the predominant PAs found in honeys produced in Australia and New Zealand. There is a lack of information on the oral toxicity of echimidine on which to base regulatory decisions concerning the risk to humans of these honeys. This GLP study was conducted to assess the subchronic dietary toxicity of echimidine to rats compared to that of lasiocarpine as a positive control. Wistar rats, 10/sex, were fed diets containing 0, 0.6, 1.2 or 2.5 mg/kg bw echimidine. Positive control groups, 10/sex, were fed diets containing 0.6, 1.2 or 2.5 mg/kg bw lasiocarpine.

Neither PA had any effect on survival, food consumption, clinical signs, gross lesions, or histopathology. Consumption of lasiocarpine, but not echimidine, decreased bodyweight gain in males at $\geq$ 1.2 mg/kg bw, and in females at 2.5 mg/kg bw. Slight alterations in white cell counts and serum ALT concentrations at 2.5 mg/kg bw of both PAs were not clinically significant, had no histological correlates, and were considered to be of equivocal relevance.

In conclusion, the subchronic No Observed Adverse Effect Level (NOAEL) for echimidine is 2.5 mg/kg bw/day, whereas, on the basis of a treatment-related decrease in bodyweight gain in males at 1.2 mg/kg bodyweight, the NOAEL for lasiocarpine is 0.6 mg/kg bw/day.



## 1. 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. These compounds have caused numerous cases of human hepatotoxicity, including some large-scale epidemics resulting from contamination of staple foods (Chen et al., 2009). All reported cases of human toxicosis due to PAs found by the authors in a thorough search of the scientific literature have been due either to ingestion or to trans-placental exposure following maternal ingestion. However, 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 acute intraperitoneal toxicity data are available, which are not useful to assess the hazard of ingestion of PAs, particularly chronic repeated ingestion. 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.

Echimidine is a PA synthesised by a number of plants in the genera *Echium* and *Symphytum*, and is the one of the predominant PAs found in honeys produced in Australia and New Zealand. The principal plant source of echimidine in honey in Australia is likely to be *Echium plantagineum* (Skoneczny et al., 2015) whereas in New Zealand the principal source is likely to be *Echium vulgare* (Moar, 1985). Ingestion of echimidine in comfrey (various species in the genus *Symphytum*) consumed as a vegetable or herbal tea has led to several cases of veno-occlusive disease of the liver in human beings (Mei et al., 2010). However, because exposure in these cases was chronic and the level of echimidine in the comfrey products was not determined, there is a lack of robust information on the actual toxic dose of echimidine to human beings. Therefore these human

*Abbreviations:* bw, bodyweight; d, day; NOAEL, No Observed Adverse Effect Level; NTP, National Toxicology Program; PA, pyrrolizidine alkaloid; TBHQ, tert-butylhydroquinone.

* Corresponding author.
*E-mail addresses:* rosalind.dalefield@foodstandards.govt.nz (R.R. Dalefield), michelle.gosse@foodstandards.govt.nz (M.A. Gosse), utz.mueller@foodstandards.gov.au (U. Mueller).

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cases are not useful for the determination of risk to human consumers of echimidine in honey.

The short-term (8 weeks) and chronic (104 weeks) dietary toxicity of lasiocarpine in Fischer 344 rats has been published by the National Toxicology Program (NTP, 1978). Available data in laboratory animals suggests that there are substantial differences in the toxicity of different PAs within a species, and of the same PA in different species. On the basis of median lethal dose studies in male Sprague-Dawley rats the acute oral toxicity of lasiocarpine (110 mg/kg bw) is reported to be approximately 5-fold greater than for echimidine (518 mg/kg bw) in male Wistar rats (Newberne and Rogers, 1970; Dalefield et al., 2015).

The purpose of this study was to compare the short-term repeat dose oral toxicity of echimidine and lasiocarpine in rats. The doses selected were based on those used in the NTP lasiocarpine dose-range finding dietary study which found no appreciable reductions in bodyweight gain at oral doses equivalent to approximately 2 mg/kg bw/day or less (NTP, 1978).

## 2. Material and methods

### 2.1. Compliance with ethical and procedural standards

The study was approved by the Kaiawhina Animal Ethics Committee (approval code AEC 013/14). The study was conducted in line with the applicable guidelines for the repeated dose 28-day oral toxicity study in rodents (OECD Guideline 407) and in accordance with US Code of Federal Regulations Title 21 Part 58 Good Laboratory Practice for Nonclinical Laboratory Studies, OECD Principles of Good Laboratory Practice, and the terms of registration of the International Accreditation New Zealand GLP Compliance Monitoring Programme, with the following exclusions:

- The test site for the in-life phase (Small Animal Production Unit, Massey University)
- Analysis of the nutritional composition of the rat feed
- Analysis of the drinking water
- Allocation of rats to treatment groups
- Statistical analysis

### 2.2. Animals

One hundred and forty 11-week old Wistar rats (70/sex) with males weighing between 362 g and 536 g and females between 221 g and 333 g were obtained from Hercus Taieri Resource Unit, Otago University, New Zealand. Rats were housed individually under environmental conditions of ambient temperature 22 °C ± 3 °C, relative humidity 30–70%, HEPA-filtered air and 12-h light/dark cycle. Drinking water and feed was supplied *ad libitum*. Rats were acclimatised to the study conditions and diet for between 15 and 18 days prior to commencement of treatment. One day prior to commencement of treatment, rats were allocated to a group (10/sex) using a computer-generated randomization scheme.

### 2.3. Test substance and dose preparation

A single batch of echimidine (CAS 520-68-3; Batch XX7-69D; purity 95.5% by HPLC) was prepared by Planta Analytica, Connecticut, USA from an oleoresin derived from *Echium vulgare* (Viper's bugloss) in New Zealand. Impurities were other pyrrolizidine alkaloids. Lasiocarpine (CAS 303-34-3; Batch 14110402; purity 94.1% by HPLC) from *Heliotropium europaeum* was purchased from Phytoplan Diehm & Neuberger GmbH, Heidelberg, Germany. Since echimidine is hygroscopic it was dissolved in reagent grade dichloromethane (Scharlau S.L., Barcelona, Spain) and then the solvent was fully evaporated in the presence of food grade fumed silica (Sipernat® 22, Evonik Industries AG, Germany) to enable reliable weighing. Ground silica concentrates of lasiocarpine and echimidine suspended in food grade rice bran (RB) oil were prepared by Grayson Wagner Co. Ltd, Penrose, Auckland, New Zealand.

Each RB oil/PA mixture was no more than 5% of the weight of the feed. RB oil without PA was added to the diet of the negative control group at 5% (w/w). Fresh diets were prepared weekly, with the quantity of PA added based on predicted bodyweight and feed consumption midweek. The rat feed was produced by the Massey University Food Production Unit.

### 2.4. Diet

Dose analysis of the PAs in the rat feed was performed in triplicate for batches prepared for Weeks 1 and 4 by Eurofins Agroscience Testing New Zealand, Hamilton, New Zealand using a validated method of reverse-phase ultra-fast liquid chromatography coupled with tandem mass spectrometry. Samples from the top, middle and bottom of the batches of rat feed prepared for the first week of the study were sampled for homogeneity of the PAs in the rat feed, and stability over 10 days was also analysed.

### 2.5. Experimental design

Measured parameters during the in-life phase were survival, clinical signs, daily feed consumption, twice weekly bodyweight and bodyweight gain. Rats were fasted overnight prior to scheduled necropsy on day 29, and urine was collected by cage catch on day 29. Parameters measured in urine were appearance, volume, specific gravity, pH, glucose, protein, and sediment microscopy. Rats were anaesthetised prior to termination and blood was collected from the abdominal aorta for haematology and clinical chemistry.

#### 2.5.1. Haematology

Haematological parameters were measured in whole blood with EDTA (1.8 mg $K_2$EDTA/mL blood), using a Sysmex XT haematology analyser. Haematological evaluations included red blood cell count (RBC), haemoglobin concentration (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and a white blood cell count (WBC). Further analysis of white blood cells, differential counts, and RBC morphology was performed by microscopy of the blood smears.

#### 2.5.2. Blood biochemical parameters

Blood samples in plain (serum) and fluoride oxalate (for glucose determination) tubes were centrifuged at 2000g for 15 min; and serum and plasma respectively were collected and stored at −18° C. Analysis was performed on a Hitachi PE Modular chemistry analyser. Measured parameters included alkaline phosphatase (AP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltransferase (GGT), glucose (GLU), urea (BUN), creatinine (CREA), albumin, (ALB), total protein (TP), globulin (GLO; calculated), albumin:globulin ratio (A:G; calculated), triglycerides (TG), cholesterol (CHO), total bilirubin (TBL), sodium (Na), chloride (Cl), calcium (Ca) and phosphate (P).

#### 2.5.3. Histopathological examinations

The following organs of all rats were weighed (paired organs together) and the relative organ weights were calculated on the basis of the final body weight for the adrenals, brain, epididymides, heart, kidneys, liver, prostate, spleen, testes, thymus, seminal vesicles (with coagulating glands), uterus (with cervix) and ovaries.