CHARACTERISTICS OF IMIDACLOPRID TOXICITY IN TWO APIS MELLIFERA SPECIES

Séverine Suchail, David Guez, Luc P Belzunces

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Séverine SUCHAIL, David GUEZ and Luc P. BELZUNCES

Institut National de la Recherche Agronomique, Laboratoire de Toxicologie Environnementale, Unité de Zoologie et d'Apidologie, Site Agroparc, 84914 Avignon Cedex 09, France.

Running Title: Imidacloprid toxicity to bees

Corresponding author: Luc P. Belzunces - INRA, Laboratoire de Toxicologie Environnementale, Unité de Zoologie et d'Apidologie, Site Agroparc, 84914 Avignon Cedex 09, France.
Tel. +33 490316057 – Fax: +33 490316403
Email: belzunce@avignon.inra.fr

ABSTRACT

Imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) belongs to a new chemical family of chloronicotinyl compounds whose mode of action on the insect nervous system differs from that of traditional neurotoxic products. Imidacloprid, a strong systemic compound, is effective against several sucking and mining pests. The acute toxicity of oral and contact applications on two *Apis mellifera* species, *A. m. mellifera* and *A. m. caucasica*, was investigated. The dose-effect relation revealed important characteristics. With low imidacloprid concentrations, a mortality peak appeared with both application modes, especially with the oral mode. With medium doses, mortality profiles at 24 h and 48 h were different only after oral application. The mortality kinetics showed that the higher the imidacloprid dose, the later the mortality. After oral intoxication, the LD50 values of imidacloprid at 24 h and at 48 h were about 5 ng.bee^{-1} for both *A. m. mellifera* and *A. m. caucasica*. After contact application, the LD50 values at 24 h and at 48 h were approximately 24 ng.bee^{-1} for *A. m. mellifera* and 14 ng.bee^{-1} for *A. m. caucasica*. Imidacloprid ranks among the more potent insecticides when in direct contact with the bees.

Keywords: imidacloprid, *Apis mellifera*, honey bees, toxicity, lethal dose, insecticide

INTRODUCTION

The honey bees, *Apis mellifera*, is widely recognised as an insect of great agronomic, ecological and scientific importance. At the agronomic level, it produces valuable products (honey, pollen, royal jelly, propolis and wax) and plays a major role in crop protection [1]. One of the main problems currently hindering with crop pollination is pollinator losses after the bees contact with pollutants such as pesticides. Hence, because of agronomic and environmental problems efforts have been made to improve the assessment of pesticide-related risks to bees [2,3].

Recently, the chloronicotinyl molecules a novel class of selective insecticides was discovered, its leading first compound is imidacloprid, a nitroguanidine systemic molecule that has a new mode of molecular action by competiting agonistically with the nicotinic acetylcholine receptor (nAChR) of insects [4,5,6,7]. Imidacloprid is extremely effective against sucking insects and some heteroptera, coleoptera, lepidoptera species, whereas vertebrates appear to be relatively insensitive to it [8,9]. In contrast with nicotine, imidacloprid is a striking example of a product with high insecticide activity and low mammalian toxicity; the oral lethal dose 50 (LD50) in rat was about 450 $mg.kg^{-1}$ [10].

Major applications of imidacloprid include seed dressing, spraying, and the use of pills and granules [11]. With seed dressing, insects can be poisoned through the oral way by parent or metabolite compounds. With spraying, nectar can also be contaminated and bees are poisoned either through direct contact with the product or through contact with its residues.

The purpose of this research was to examine the acute effects of imidacloprid to foraging bees. To determinate the intrinsic toxicity of this insecticide to honey bees, studies of laboratory-based dose response were carried out on two *Apis mellifera* species, *A. m. mellifera* and *A. m. caucasica* to provide an estimate of the median lethal dose (LD50) after oral and contact applications. Mortality kinetics were also studied using different imidacloprid doses.

MATERIALS AND METHODS

Materials

The effects of technical grade (98% pure) imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) from Bayer AG (Leverkussen, Germany) was investigated from June to August. Two honey bees species (*A. m. mellifera* and *A. m. caucasica*) mainly foragers were captured from honey and pollen combs in a healthy queen-right colony. Immediately before treatment, bees were anaesthetised with carbon dioxide and kept in cages (10.5 x 7.5 x 11.5 cm) placed in a temperature-controlled chamber at 25 ± 2°C with 60 ± 10% relative humidity. They were fed a 50% sucrose solution *ad libitum* [12]. In each experiment, three cages of twenty bees were used for each dose of treatment. Experiments were conducted at least three times.

Mode of treatment

Oral application

The honey bees were deprived of food for 2 h before administration of imidacloprid. Imidacloprid solutions were prepared in a 1% dimethylsulfoxide solution (DMSO) and then diluted 10-fold in the 50% (w/v) feeding sucrose solution. The final concentration of DMSO in all tests was 0.1%. The contaminated solutions were prepared extemporaneously for each test. Each bee received 10 µl of 50% sucrose solution containing or not (control group) imidacloprid. After consuming this solution, bees were fed 50% straight sucrose solution *ad libitum*. Bee mortality was recorded at 2, 4, 6, 10, 14, 20, 24 and 48 h.

Contact application

One µl of insecticide solution in 100% DMSO was applied with a microsyringe on the dorsal part of the bee's thorax. After the application, all bees were fed 50% sucrose solution *ad libitum*. Control bees received 1 µL of pure solvent. Bee mortality was recorded 24 and 48 h after topical application.

Data analysis

Mortality data were corrected according to Abbott [13]. The usual log-probit representation was not used because the dose-effect relation did not systematically increase throughout the

range of doses tested. A polynomial regression analysis was used to determine oral and contact LD50. One-way analysis of variance (ANOVA) was used to evaluate statistically significant differences between groups.

RESULTS

The toxicity of imidacloprid to foraging bees was investigated with different application modes. For both *A. m. mellifera* or with *A. m. caucasica*, within 24 h following imidacloprid application, regardless of dose, most of the bees exhibited neurotoxic symptoms such as trembling, tumbling and lack of coordination.

The contact toxicity of imidacloprid was studied first. In *A. m. mellifera*, 24-h mortality increased for doses of between 1 and 7 ng.bee^{-1} and then decreased for doses ranging from 7 to 15 ng.bee^{-1} (Fig. 1A). At doses higher than 15 ng.bee^{-1}, the mortality rate increased steadily as the dose increased. This mortality profile was also noted at 48 h; it was less pronounced. Conversely in *A. m. caucasica*, at 24 h and 48 h, the mortality peak was less pronounced and occurred at lower doses (Fig. 1B). In both species, there was no significant difference between the mortality rate at 24 h and 48 h.

Oral application, in contrast with contact application, triggered important differences of mortality between 24 and 48 h at intermediate doses. In *A. m. mellifera*, 24-h and 48-h mortality rates rose with doses up to approximately 50 ng.bee^{-1} (Fig. 2A). At higher doses, the mortality rate decreased slightly and then started climbing again. This phenomenon was more pronounced with *A. m. caucasica* (Fig. 2B). At 24 h, doses of up to approximately 20 ng.bee^{-1} caused a maximum mortality rate of about 90% while doses between 20 and 90 ng.bee^{-1} made the rate fall to about 60%. For doses between 90 and 200 ng.bee^{-1}, the mortality rate remained more or less stable, and gradually rose at doses above 200 ng.bee^{-1}. At 48 h, the mortality profile was different from that obtained at 24 h. There was only a slight decrease in the 48-h mortality rate with doses between 20 and 90 ng.bee^{-1} and then the mortality gradually went up for both species as the doses of imidacloprid were increased.

The kinetics of mortality in both species was studied using the oral mode of intoxication because (i) with treatment by seed-dressing, the main intoxication mode is the ingestion of contaminated nectar, (ii) a mortality difference only existed in the oral mode between 24 and 48 h and (iii) a mortality peak occurs after the oral application. In *A. m. mellifera*, with 1 ng.bee^{-1}, the maximum mortality level was reached within the first ten hours (Fig. 3A). For doses ranging from 5 to 50 ng.bee^{-1}, the mortality kinetics were similar during the first fourteen hours but at 50 ng.bee^{-1} mortality rates subsequently increased. At 200 ng.bee^{-1}, mortality appeared to be delayed since it and was lower than with doses at 5, 10 and 50 ng.bee^{-1} during at least 20 h, but continually increased after 24 h. A mortality delay was

also observed in *A. m. caucasica*, (Fig. 3B). The mortality delay was more pronounced than in *A. m. mellifera* and systematically increased with the doses, throughout the tests. The higher the dose increased, the longer the mortality was delayed.

The LD50 values of imidacloprid in honey bee species obtained with contact and oral tests are summarised in Table 1. The LD50 values of imidacloprid in *Apis mellifera* were very low. In *A. m. mellifera* the LD50 mean at 24 h and 48 h were approximately 4.5 ng.bee^{-1} and 24 ng.bee^{-1} for oral and contact application, respectively. In *A. m. caucasica*, the LD50 means at 24 h and 48 h were approximately 6.5 ng.bee^{-1} and 14 ng.bee^{-1} for oral and contact application, respectively. ANOVA tests ($p<0.05$) indicated a significant difference of sensitivity to imidacloprid between *A. m. mellifera* and *A. m. caucasica* for contact application at 24 h but not at 48 h.

DISCUSSION

Contact and oral intoxication by imidacloprid induces behavioural abnormalities in the two *Apis mellifera* species. Most of the bees showed neurotoxic symptoms such as movement coordination problems, trembling and tumbling. Similar behaviour after imidacloprid application was described in a Coleoptera *Diaprepes abbreviatus* [14]. The biological activity of Heteroptera *Podisus maculiventris* after different imidacloprid applications has also been investigated [15]. After 24 h, all pathways of exposure to imidacloprid caused neurotoxic symptoms in most of individuals.

For a given species, imidacloprid toxicity changes with the application mode. In *P. maculiventris*, the toxicity decreases in the following order: topical exposure > ingestion > residual contact. In *A. mellifera*, imidacloprid unlike most insecticides is more toxic in the oral mode than in the contact mode. The toxicity of organophosphate insecticides, such as chlorpyrifos, for instance, is four times higher by contact application than by oral application (contact LD50 = 59 ng.bee^{-1}, oral LD50 = 250 ng.bee^{-1}). Similarly, contact application of bifenthrin pyrethroid is seven times more potent than oral application (contact LD50 = 15 ng.bee^{-1}, oral LD50 = 100 ng.bee^{-1}).

The LD50 values of imidacloprid obtained in *A. mellifera* (LD50 ranging from 4 to 24 ng.bee^{-1}) are very low compared with those of insecticides from other families with different modes of action. Three of the most toxic insecticides of different families, the triazophos organophosphate (contact LD50 = 55 ng.bee^{-1}), and the cyhalothrin and deltamethrin pyrethroids (contact LD50 = 27 ng.bee^{-1}, LD50 = 51 ng.bee^{-1}), are all less harmful to honey bees than imidacloprid [16]. For these insecticides, the highest toxicity is obtained by contact treatment but not by oral treatment, as it is the case for imidacloprid. Thus, imidacloprid is one of the most potent insecticides to honey bees and should not be applied during the flowering period [11]. Applications by seed dressing and granules could preclude a direct effect on honey bees.

Imidacloprid is very selective towards insect species. Imidacloprid has a low insecticidal activity towards *Heliothis virescens* and *Spodoptera Littoralis*, two polyphagous pests (at 48 h, contact LD50 = 350 ng.mg^{-1} of insect, for *H. virescens*, and LD50 = 650 ng.mg^{-1} of insect, for *S. Littoralis*) [17]. Hence, for this species, imidacloprid is not potent enough to efficiently control cotton insect pest populations in the field. On the other hand, imidacloprid is extremely effective against sucking insects such as *Myzus persicae* (48 h oral LD50 = 3 pg.mg^{-1} insect) [18]. Thus, honey bees have an intermediary sensitivity to

imidacloprid (48 h oral LD50 = 50 pg.mg^{-1} insect) compared with these pest species. The differences in sensitivity between the honey bee and sucking insects is an important feature because in using field rates only toxic to *M. persicae*, it could be possible to protect honey bees life.

One of the most surprising characteristics of imidacloprid toxicity is the unusual mortality profile observed with the two application modes: honey bee mortality rates rise with low doses of imidacloprid, then, with medium doses fall off, but to rise again, an increase with high doses. Furthermore, with contact application, the 24-h and 48-h mortality rates are similar whereas with oral application, the mortality rate is higher at 48 h than at 24 h. Another interesting observation is that mortality kinetics show that the more the dose increases, the more the mortality is delayed. These particular features of imidacloprid toxicity suggest that metabolic pathways might be involved in the imidacloprid toxicity. With low imidacloprid doses, the few toxic metabolites produced together with the parent compound might be responsible for the high mortality during the first ten hours. Medium doses of imidacloprid may trigger an induction of detoxifying enzymes that reduces honey bees mortality. With higher doses, the increase of mortality could be due either to a high amount of toxic metabolites or to the saturation of the pesticide-metabolising enzymes. This is supported by the fact that honey bees, like other insects, possess various enzymes that can be induced by various chemicals (xenobiotic, plant chemicals…) and can metabolise pesticides [19]. Induction of cytochrome P-450 has been noticed in honey bees after 2 days of exposure to fluvalinate pyrethroid, which demonstrates that induction of pesticide-metabolising enzymes can occur rapidly [20]. In polyphagous insects, small quantities of plant substances suffice to induce mixed function oxidases within 30 minutes following initiation of feeding [22]. Hence, it is not possible to rule out the possibility of a rapid metabolic activation of imidacloprid as already demonstrated for organophosphate compounds [23]. Thus, in imidacloprid toxicity, metabolisation might explain not only the shape of the dose-response curve but also the evolution of mortality between 24 h and 48 h.

Studies in rats show that imidacloprid metabolisation leads to the formation of different main metabolites such as mono-hydroxy-imidacloprid, guanidine-compound, olefin and 6-chloro nicotinic acid [22]. It would be interesting to study the toxicity of the main imidacloprid metabolites in *A. mellifera* to explain the occurrence of a mortality peak when doses are low and the mortality difference at 24 h and 48 h. To understand the mechanism of mortality induced by imidacloprid, this study should be expanded to include the kinetics of imidacloprid degradation and the appearance of metabolites.

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