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Running head: Distribution metabolisation of imidacloprid in *Apis mellifera*.

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ABSTRACT

In vivo metabolisation of [^{14}C]-imidacloprid in different compartments of *Apis mellifera*. Séverine Suchail, Georges De Sousa, Roger Rahmani and Luc P. Belzunces. In vivo distribution of imidacloprid, an insecticide that belongs to the neonicotinoid family, was followed during 72 h in 6 biological compartments of *Apis mellifera:* head, thorax, abdomen, haemolymph, midgut and rectum. Honeybees were treated orally with 100 µg of [^{14}C]-imidacloprid per kg of bee, a dose close to the median lethal dose. Elimination half-life of total radioactivity in honeybee was 25 h. Haemolymph was the compartment with the lower amount of total radioactivity whereas rectum had the higher level of total radioactivity during the whole study, with a maximum 24 h after treatment. Elimination half-life of imidacloprid in whole honeybee was 5 h. Imidacloprid was readily distributed and metabolised only by Phase I enzymes into five metabolites: 4/5-hydroxy-imidacloprid, 4,5-dihydroxy-imidacloprid, 6-chloronicotinic acid, and olefin and urea derivatives. Guanidine derivative was not detected. Urea derivative and 6-chloronicotinic acid were the main metabolites and appeared particularly in midgut and rectum. Olefin derivative and 4/5-hydroxy-imidacloprid preferentially occurred in head, thorax and abdomen that are nAChR-rich tissues. Moreover, they presented a peak value around 4 h after imidacloprid ingestion. These results explain persistence of imidacloprid action in bees, and particularly the discrepancy between rapid intoxication symptoms and late mortality.

Keywords: Imidacloprid, Neonicotinoid, Metabolite, Honeybee, Metabolisation, Compartment, Apis.

INTRODUCTION

Imidacloprid is the first active substance of the chemical class of neonicotinoid insecticides. This systemic insecticide is known to be extremely effective against a wide variety of insects (Leicht 1996). The mode of action of imidacloprid was described by Liu and Casida (1993) as an agonistic effect on the cholinergic synapses that resulted in a consequent postsynaptic blockage in the nervous system. Imidacloprid binds to the nicotinic acetylcholine receptors (nAChR) with higher affinity for the insect than the mammalian site (Yamamoto *et al.* 1998). Nevertheless, Tomizawa and Casida (2000) indicated the possibility that some neonicotinoid insecticides or their metabolites, on accidental human exposure or when used for flea control on dogs, may also up-regulate the nAChR expression in mammals. The metabolism of imidacloprid has been studied in mammals, plants and soils, but there is little information about imidacloprid metabolism in insects for which the more in-depth works consist of the study of the metabolic fate in the whole body and the feces (Mota-sanchez *et al.* 2001). In mammalians, Klein (1994) and Thyssen and Machemer (1999) reported the existence of two major metabolic pathways. The first is the oxidative cleavage of the molecule into the imidazolidine part, which is excreted directly via the urine, and the nicotinic moiety, which is subsequently modified by conjugation enzymes. The second important pathway is the hydroxylation of the parent molecule on the imidazolidine ring by the addition of water on the unsaturated metabolite olefin. These corresponding metabolic pathways permit to eliminate as metabolites more than 90 % of the given dose within 24 h and after 48 h imidacloprid was entirely excreted. In plants, biotransformation pathways are qualitatively similar to those of mammals (Araki *et al.* 1994). Main imidacloprid metabolites identified in mammals and plants are the 5-hydroxy-imidacloprid,

4,5-dihydroxy-imidacloprid, 6-chloronicotinic acid, and olefin guanidine and urea derivatives, and all contain the 2-chloropyridine moiety.

Some of imidacloprid metabolites exhibit a toxicity to invertebrates, in particular olefin derivative and 5-hydroxy-imidacloprid. In aphid species and in the cotton whitefly, *Bemisia tabaci*, olefin is about 16 and 10 times more active than imidacloprid, respectively (Nauen *et al*. 1999). In *Apis mellifera*, acute toxicity studies of imidacloprid have revealed important toxicological characteristics (Suchail *et al.* 2000, 2001): *(i)* With oral and contact exposures, an unusual mortality profile is observed: mortality rises with low doses, falls with intermediate doses, and rises again with high doses, *(ii)* the kinetics of mortality is delayed as doses increased, *(iii)* neurotoxicity symptoms appear rapidly whereas mortality occurs 4 h after acute imidacloprid intoxication and is prolonged during more than 96 h. Thus, imidacloprid has either prolonged action or delayed action, but the symptoms are always immediate. As concerns metabolites, out of the 6 imidacloprid metabolites tested (5-hydroxy-imidacloprid, 4,5-dihydroxy-imidacloprid, 6-chloronicotinic acid, and olefin guanidine and urea derivatives) only two, similar in chemical structure to imidacloprid, exhibit an acute toxicity close to that of the parent compound with a higher toxicity for olefin derivative and a lower toxicity for 5-hydroxy-imidacloprid. In contrast, in chronic toxicity studies, all imidacloprid metabolites were found equally toxic to bees, but the total dose ingested by the bees was about 3000-100 000 times lower than the doses needed to produce the same effect after an acute intoxication (Suchail *et al.* 2001). To explain the discrepancy between the kinetics of mortality and the rapid appearance of neurotoxicity symptoms and to discern a possible relation between the metabolite occurrence and the appearance of mortality, we have focused our investigation of imidacloprid biotransformation in whole honeybees into its two toxic metabolites, 5-hydroxy-imidacloprid and olefin derivative (Suchail *et al.* submitted). This study has shown that imidacloprid has a half-life of

approximately 5 h and is rapidly metabolised into 5-hydroxy-imidacloprid and olefin derivative whose presented a peak value 4 h after intoxication. Thus, the appearance of 5-hydroxy-imidacloprid and olefin derivative coincides with the appearance of mortality induced after acute oral intoxication by imidacloprid. These results suggest that 5-hydroxy-imidacloprid and/or olefin derivative take part in extending the action of imidacloprid in honeybees. In contrast to plants, this study has shown that, 5-hydroxy-imidacloprid and olefin derivative could not be the major metabolites in worker bees.

The objective of the present study is a better characterization of the imidacloprid pharmacokinetics after oral exposure to a dose close to the median lethal dose to understand its mode of action in the honeybees. The identification of all imidacloprid metabolites and their distribution in six biological compartments of honeybees were conducted. This study represents an original approach because imidacloprid metabolisation has never been studied in honeybees. In addition, the more in-depth studies performed in insects, and particularly in honeybees, to have taken into account only the metabolic fate of pesticides in the whole animal and in the feces but not in different biological compartments, as in the present study (Mota-Sanchez *et al.* 2001; Pilling *et al.* 1995).

MATERIALS AND METHODS

Chemical Compounds

We used [pyridinyl-^{14}C-methylene]-imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine). The radiolabeled compound had a specific radioactivity of 3.7 MBq/mg (0.1 mCi mg^{-1}), and a radiochemical purity of 99% determined by TLC and HPLC. Radioactive imidacloprid was diluted in 100% dimethylsulfoxide (DMSO) to give a stock solution with a concentration of 5 g a.s. L^{-1}. Unlabelled imidacloprid and its metabolites were of the highest purity available, i.e. at least 97%. Imidacloprid and its metabolites were obtained from Bayer AG (Leverkussen, Germany). All other chemicals and organic solvents used were of analytical grade.

Biological materials

For all bioassays, worker honeybees (*Apis mellifera*) were collected from honey and pollen combs in a healthy queen-right colony (drones were discarded). To avoid intercolony and seasonal variations, bees were taken from the same colony. Immediately before treatment, bees were anaesthetised with carbon dioxide and kept in cages (10.5 x 7.5 x 11.5 cm) by groups of 20. They were stored in a temperature-controlled chamber in the obscurity at 25 ± 1.5°C and 65% relative humidity where they remained quiet and protected from stress-induced biochemical changes. Bees were fed a 50% (w/v) sucrose solution *ad libitum* (EPPO 1992). Experiments were performed in triplicate and repeated at least three times.

Oral application

The honeybees were deprived of food for 2 h before administration of [^{14}C]-imidacloprid. Radioactive imidacloprid solution was diluted in the 50% (w/v) feeding sucrose solution. The final concentration of DMSO in the sucrose solutions of control and assay tests was 0.02% (v/v). The dosing solution was prepared freshly for each test. Each bee received 10 µl of 50% sucrose solution (vehicle) containing 10 ng of radiolabeled imidacloprid (100 µg.kg^{-1} of bee), a dose close to the median lethal dose (Suchail *et al.* 2000) or the dosing vehicle alone (control). After consuming this solution, bees had access to 50% straight sucrose solution *ad libitum*. Metabolic study was performed only on surviving worker bees. Twenty honeybees were sacrificed at specific time: 0, 2, 4, 6, 10, 15, 24, 30, 48 and 72 hours following oral administration. Time 0 corresponded to the end of total ingestion of the imidacloprid dose by honeybees, which took about 20 min.

Extraction of residues from different biological compartments

All tissues and organ were recovered after bees had been anaesthetised with carbon dioxide. After recovery, all tissues and organs were immediately dipped in liquid nitrogen and stored at –80°C, until analysis.

Haemolymph

Haemolymph was collected by puncturing the dorsal aorta with a 5 µL Hamilton microsyringe. The volume removed per bee was 5 ± 0.1 µL(mean ± SE). To each sample of haemolymph from 20 bees, 4 volumes of an acetonitrile/water (2:1, v/v) solution were added.

Midgut and rectum

The last segment of abdomen was pulled out to extract midgut and rectum. Midgut (mean ± SE: 12 ± 0.1 mg) and rectum (20 ± 0.1 mg) were separated. At each time, midguts and rectums from 20 bees were supplemented with 400 µL of 100 mM Phosphate buffer pH 7.4 and immediately grinded at 0°C. Membrane solubilisation were achieved by adding to 1 volume of extract 4 volumes of 1 M NaOH. After stirring, each sample was stored in the dark during 3 hours at 37°C. The medium of resulting sample was neutralised with 3 M HCl and supplemented with 1/100 volume of hydrogen peroxide (110 volumes) to reduce the quenching of radioactivity. After an overnight incubation at 37°C, samples were centrifuged at 13000 g for 5 min at room temperature and the supernatant was supplemented with 4 volumes of an acetonitrile/water (2:1, v/v) solution. The sample was concentrated to obtain 1 mL of final extract.

Head, thorax and abdomen

After extraction of haemolymph, midgut and rectum, the head (mean ± SE:14.4 ± 0.4 mg), thorax (32.4 ± 1 mg) and abdomen (21.9 ± 1.2 mg) were recovered, weighed, plunged in liquid nitrogen and stored at -80°C. Heads and abdomens, and thoraxes of 20 bees were grinded in 4 mL and 5 mL of an acetonitrile/water (2:1, v/v) solution, respectively. Tissue extracts were centrifuged at 25000 g for 30 min at 4°C and the resulting supernatant was concentrated to obtain 1 mL of final extract.

Radioactivity of samples from haemolymph, midgut, rectum, head, thorax and abdomen were measured with a scintillation counter (Tri-Carb 2100 TR, Packard Co.) using scintillation liquid (Ultima Gold, Packard Co.).

Thin-Layer Chromatography and radioanalysis

Silica gel $60F_{254}$ (0.25 mm thickness, Merck) thin layer chromatography (TLC) plates (20x20 cm) were used for analytical separation and identification of imidacloprid and its metabolites. One hundred µL of samples from head, thorax, abdomen, haemolymph, midgut and rectum extracts were loaded on TLC plates along with reference metabolites. The plate was developed by one dimensional thin layer chromatography with the migration solvent: ethyl acetate-isopropanol-water (68:20:12; v/v). Radioactive compounds were detected and quantified by Phosphor Imaging with a Storm 820 (Molecular Dynamics) by exposure of TLC plates to a low energy screen (35 x 43 cm; Amersham Pharmacia Biotech) during 20 days at 25°C. The unlabeled reference compounds were visualised by UV light at 254 nm.

Total radioactivity half-life

Half-life was determined by plotting ln (I/Io) versus time according to the formula $\ln (I/Io) = - k \cdot t$, where Io and I represent initial and residual imidacloprid concentrations, respectively. Half-life was calculated with the equation $t_{1/2} = (\ln 2) / k$, where $t_{1/2}$ is the half-life and k is the apparent elimination constant.

RESULTS

Total radioactivity

The distribution kinetics of total radioactivity in compartments indicated the existence of two different kinetic profiles (figure 1). In thorax and abdomen, total radioactivity was maximum 20 min after the beginning of exposure and then rapidly decreased with time. In contrast, in haemolymph, head, midgut and rectum, the highest total radioactivity was reached only 2, 6, 10 and 24 h after intoxication of bee, respectively. It was observed that radioactivity level in haemolymph was very low, thus showing that haemolymph was rather a transfer compartment than a stocking compartment. Rectum had the highest level of total radioactivity during the whole kinetic with a maximum 24 h after oral treatment. Elimination of total radioactivity in the whole honeybee follows a first-order kinetic ($\rho^2 = 0.8483$) and the elimination half-life of radioactivity, which represents the sum of radioactivity of all detected compounds in all honeybee compartments, was about 25 h.

Distribution and metabolism

To understand the role of the metabolites in imidacloprid toxicity, the quantitative and qualitative analysis of each imidacloprid metabolite was performed in 6 biological compartments of honeybee. Our TLC method permitted to separate distinctly imidacloprid, 4,5-dihydroxy-imidacloprid, mono-hydroxy-imidacloprid, 6-chloronicotinic acid, and olefin, guanidine and urea derivatives. However, in honeybees, guanidine and metabolites different from those cited above were never detected. The retention factors (R_f) of imidacloprid and its metabolites are summarized in Table 1. The 5-hydroxy and 4-hydroxy-imidacloprid will be noted 4/5-hydroxy-imidacloprid because it was not possible to separate 5-hydroxy from 4-hydroxy-imidacloprid.

In head

The metabolic pattern in head showed 5 metabolites that were detected during the 72-h observation period: imidacloprid, 4/5-hydroxy-imidacloprid, olefin derivative, 4,5-dihydroxy-imidacloprid and 6-chloronicotinic acid (figure 2 A). Imidacloprid was distributed very rapidly through the whole bee. Imidacloprid level was about of 5% of the ingested dose during the first 4 hours, 2% between 6 and 24 h, and under the detection threshold after 24 h. Olefin derivative and 4/5-hydroxy-imidacloprid, the two metabolites toxic to honeybees regardless of the dose and mode of treatment, were the main imidacloprid metabolites in this compartment and were detected during 24 and 30 h for 4/5-hydroxy-imidacloprid and olefin derivative, respectively. However, the level of olefin derivative was always higher than that of 4/5-hydroxy-imidacloprid.

In thorax

In thorax, the metabolites detected were the same than those identified in head, but in higher amounts (figure 2 B). In this compartment, kinetic profiles also showed a rapid distribution and metabolisation of imidacloprid and its metabolites. Imidacloprid appeared in the first 20 min, then decreased and was not detected after 30 h, as in the head. Olefin derivative and 4/5-hydroxy-imidacloprid were prevalent during the first 15 h. In contrast with the head, however, 4,5-dihydroxy-imidacloprid and 6-chloronicotinic acid were present in significant amounts. Moreover, 30 h after intoxication of honeybees, metabolites remained in important proportions.

In abdomen

Distribution profile of imidacloprid and its metabolites was similar to that of thorax (figure 2 C). Imidacloprid was the main compound on this compartment and represented 38% of ingested

radioactivity at the initial time (i.e., 20 min after imidacloprid ingestion). Metabolites were in lower proportions than that of imidacloprid but in similar amounts as in head and thorax.

In haemolymph

Haemolymph was the compartment with the lowest quantity of radioactivity and was the only one without detectable amounts of imidacloprid (figure 2 D). Four metabolites were detected: 4/5-hydroxy-imidacloprid, olefin, 4,5-dihydroxy-imidacloprid and 6-chloronicotinic acid. Metabolite amounts fluctuated over time, which suggested that transfers of metabolites occurred between compartments.

In midgut

In midgut, 3 compounds were detected: imidacloprid, 6-chloronicotinic and urea derivative (figure 2 E). Urea derivative was never detected in the other compartments and represented a high proportion of the ingested radioactivity (15% of the ingested dose).

In rectum

In rectum, detected compounds were the same than in the midgut (figure 2 F). Imidacloprid, 6-chloronicotinic acid and urea derivative increased during the first 15 hours with a maximum of radioactivity at 15 h, then they decreased until 48 h and were not detected at 72 h. Urea derivative appeared during the first 20 min, following intoxication whereas 6-chloronicotinic acid was detected only 4 h after intoxication. Urea derivative and 6-chloronicotinic acid occurred in rectum at high amounts and represented approximately 15% and 10% of the ingested radioactivity, respectively 15 h after intoxication.

Metabolites in the whole honeybee

The kinetic of imidacloprid and metabolites in the whole honeybee is shown in figure 3. Values represented the sum of the radioactivity amounts detected for each compound in compartments at each time. Imidacloprid corresponded to 55% of ingested radioactivity at the initial time. The guanidine derivative was never detected in the analysed compartments. Elimination half-life of imidacloprid was about 5 h in the whole honeybee. On the basis of the radiolabeling studies, the major metabolites of imidacloprid identified in the whole honeybee were the 6-chloronicotic acid and the urea derivative with 10 and 20% of the ingested radioactivity, respectively, from 2 h to 24 h after intoxication. Moreover, olefin derivative and 4/5-hydroxy-imidacloprid were at maximum 4 h after intoxication.

DISCUSSION

Results indicate that imidacloprid is readily distributed and metabolised into 5 metabolites: 4/5-hydroxy-imidacloprid, 4,5-dihydroxy-imidacloprid, 6-chloronicotinic acid, and olefin and urea derivatives. The rapid appearance of metabolites in the different biological compartments and the variation of their kinetic profiles suggest the existence of an imidacloprid metabolisation not only in midgut, which is the main place of metabolisation, but also in the other honeybee compartments. Conversely, haemolymph is the compartment with the lowest amount of radioactivity, which confirms that haemolymph is a transfer compartment rather than a storage compartment. It is noteworthy that metabolites detected in these different biological compartments are similar to those found in plants, soils and mammals, but their relative importance are different (Araki *et al*. 1994; Thyssen and Machemer 1999; Robert and Hutson 1999, Rouchaud *et al.* 1996). All metabolites in honeybee result from imidacloprid biotransformation by mixed function oxidases. These enzymes permit to convert imidacloprid into more polar hydrophilic metabolites, which are more readily excreted, by means of several reactions including hydroxylation, epoxidation and other oxidations. In plants and mammals, imidacloprid biodegradation involves conjugation pathways (Araki *et al*. 1994; Thyssen and Machemer 1999). In contrast, in honeybees, no metabolites produced by Phase II enzymes were detected either because they occur at very low levels or are not produced by honeybee, or because imidacloprid is able to inhibit Phase II enzymes. Another difference between plants, mammals and honeybees is the lack of guanidine derivative in honeybee. Guanidine derivative is either not formed or formed but quickly transformed because of a rapid turnover, or is in too low quantity to be detected. The last difference between honeybees and plants is that 6-chloronicotinic acid and urea derivatives are the main metabolites in honeybee instead of 5-hydroxy-imidacloprid and

olefin derivative in plant. The preferential appearance of these two major metabolites in midgut and rectum suggests that they might be late metabolites in honeybee.

On the basis of the metabolites identified and their appearance kinetic, the metabolic pathway of imidacloprid in honeybees was summarised (figure 4). Three major routes of metabolism can be distinguished in honeybee. The first concern the oxidative cleavage of the methylene bond of imidacloprid and/or its metabolites followed by an oxidation to form 6-chloronicotinic acid. The second involves the removal of the nitro group followed by an oxidation to form urea derivative. The third concerns the hydroxylation of the imidazolidine ring to form 4/5-hydroxy-imidacloprid and further 4,5-dihydroxy-imidacloprid. In plants, soils and mammals, it has been proposed that olefin derivative is preferentially formed by the dehydratation of the 4-hydroxy-imidacloprid or 5-hydroxy-imidacloprid (Araki *et al*. 1994; Klein *et al*. 1994). However, the results obtained in bees show that the appearance of olefin derivative and 4/5-hydroxy-imidacloprid coincides but olefin derivative amounts are always higher. This suggests either that olefin results from the dehydratation of 4/5-hydroxy-imidacloprid and that 4/5-hydroxy-imidacloprid has a very high transformation turnover, or that olefin derivative results from dehydrogenation of imidacloprid, which is subsequently transformed into 4/5-hydroxy-imidacloprid after hydratation. In this metabolic scheme, we propose the existence of an intermediary epoxy metabolite that could explain the formation of mono- and dihydroxy-imidacloprid. The formation of such mono- and dihydroxylated compounds from an epoxy derivative formed from desaturated molecules has already been demonstrated for various xenobiotics such as aldrin or halobenzene derivatives (Woolf and Guengerich 1987; Rietjens *et al.* 1997).

The results obtained in this metabolic study could explain in part the action of imidacloprid in bees and particularly the discrepancy between rapid intoxication symptoms and

late mortality, and the reasons of the evolution of mortality during more that 96 h (Suchail *et al*. 2000, 2001). First, the rapid imidacloprid disappearance and its biotransformation into olefin derivative and 5-hydroxy-imidacloprid, during the first 6 h confirm previous results. In fact, study of imidacloprid biotransformation in whole honeybee by means HPLC-MS/MS shows that olefin derivative and 5-hydroxy-imidacloprid present peak values 4 h after oral intoxication (Suchail *et al*. submitted). This time period coincides with the appearance of mortality induced by imidacloprid after acute oral intoxication (Suchail *et al*. 2000). This suggests strongly that olefin derivative and/or 5-hydroxy-imidacloprid might be involved in the toxicity of imidacloprid. However, two experimental results strongly suggest that the involvement of olefin derivative in the imidacloprid toxicity is prevalent. (*i*) Olefin derivative is 2-time more toxic than imidacloprid and 10-time more toxic than 5-hydroxy-imidacloprid (Suchail *et al*. 2000) and (*ii*) in nAChR-rich tissues such as head and thorax, olefin derivative amounts are about to half of imidacloprid amounts during the first 6 h and are higher than that of imidacloprid after 6 h. In addition, in abdomen, olefin derivative and 4/5-hydroxy-imidacloprid were also in high amounts during a relatively long period. Furthermore, in head, thorax and abdomen, olefin derivative presented a long persistence and was in significant amounts after 30 h following intoxication whereas imidacloprid was no longer detected after 24 h. These results are consistent with a prolonged action of imidacloprid due to the appearance of the most toxic metabolite olefin.

The privileged occurrence of olefin and 4/5-hydroxy-imidacloprid in nAChR-rich tissues suggests that they could interact with the nAChR, probably because these metabolites have a chemical structure very close to that of imidacloprid, and induce the honeybee mortality. In honeybees, Nauen *et al*. (2001) have shown that imidacloprid and only metabolites that contain the nitroguanidine pharmacophore (olefin, 5-hydroxy- and 4,5-dihydroxy-imidacloprid) can displace [^{3}H]-imidacloprid from its receptor binding site. In contrast, only olefin derivative and

5-hydroxy-imidacloprid are able to act agonistically on neurons isolated from the antennal lobe of *A. mellifera* and to induce mortality. In the housefly *Musca domestica*, interactions between olefin derivative, 5-hydroxy-imidacloprid and nAChR have been reported by Nauen *et al*. (1998). Compared to imidacloprid, olefin derivative exhibits higher affinity to *M. domestica* head nicotinic receptor, whereas 5-hydroxy-imidacloprid is less specific than imidacloprid but shows high insecticidal activity. Conversely, Liu *et al.* (1993) and Buckingham *et al.* (1995) have shown that the toxicity of imidacloprid and its related compounds correlates closely with their relative affinity for insect nAChRs. Kagabu and Matsuno (1997) have shown that the existence of the 2-chloropyridine moiety in imidacloprid and in metabolites is particularly important and contributes to enhance their affinity for nAChR. Thus, we can assume that the high affinity of olefin and 5-hydroxy-imidacloprid for honeybee nAChR and their strong presence in nAChR-rich tissues confer them an important toxicity to honeybee. Therefore, all results obtained in this present study tend to show that the immediate neurotoxicity symptoms observed in honeybee in our previous toxicological studies are due to the action of imidacloprid, whereas the lethal effect involve 5-hydroxy-imidacloprid and even more so olefin derivative. These two imidacloprid metabolites could act either alone or together with low residual amounts of the parent compound.

The knowledge of *in vivo* metabolisation pathways of imidacloprid in honeybees helps us to better understand the unusual characteristics of imidacloprid toxicity. This study has shown that olefin and 5-hydroxy-imidacloprid, two minor metabolites in honeybee, might be strongly involved in imidacloprid toxicity. Therefore, it is necessary now to consider as pertinent metabolites not only the main metabolites but also minor metabolites to assess the risk of this compound for honeybee and other non-target-insects. Indeed, in the risk assessment methods of future neonicotinoids, it would be important to take into account, in addition to the parent

compound, all metabolites able to induce mortality of non-target-insects. Therefore, the introduction of the new generation of neonicotinoid compounds on the market needs the development of an in-depth understanding of imidacloprid action to better evaluate the potential impact of future neonicotinoids on honeybee colonies as well as on bees in general.

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FIGURE LEGENDS

Figure 1: Distribution kinetic of total radioactivity in different biological compartments of honeybee. Kinetic of total residues is followed in head (◆), thorax (●), abdomen (▲), haemolymph (○), midgut (■) and rectum (▲), during 72 h. Each point represents the mean values of three experiments ± SE.

Figure 2: Distribution kinetic of imidacloprid and its metabolites in head (A), thorax (B), abdomen (C), haemolymph (D), midgut (E) and rectum (F). Radioactive imidacloprid and metabolites were detected and quantified by phospho Imaging. (■) imidacloprid, (■) 4/5 hydroxy-imidacloprid, (■) olefin, (■) 4,5-dihydroxy-imidacloprid, (■) 6-chloronicotinic acid, and (■) urea derivative were followed during 72 h after the imidacloprid oral treatment. Data represent the mean values of three experiments performed in triplicate ± experimentwise SE and are expressed as percent of the radioactivity-ingested dose ratio.

Figure 3: Distribution kinetic of imidacloprid and its metabolites in the whole honeybee. Radioactive imidacloprid and metabolites were detected and quantified by phospho Imaging. (■) imidacloprid, (■) 4/5 hydroxy-imidacloprid, (■) olefin, (■) 4,5-dihydroxy-imidacloprid, (■) 6-chloronicotinic acid, and (■) urea derivative were followed during 72 h after the imidacloprid oral treatment. Data represent the mean values of three experiments performed in triplicate ± experimentwise SE and are expressed as percent of the radioactivity-ingested dose ratio.

Figure 4 : Proposed metabolic pathway of imidacloprid in honeybee. 1: Oxidative cleavage of imidacloprid methylene bridge to form 6-chloronicotinic acid. 2: Reduction of the nitro group to

give urea derivative. 3: Hydroxylation of the imidazolidine ring to form 4/5-hydroxy-imidacloprid and further 4,5-dihydroxy-imidacloprid. 4/5-hydroxy-imidacloprid and 4,5-dihydroxy-imidacloprid can also result from epoxy derivative formed from desaturated molecules. Dehydratation of 4/5-hydroxy-imidacloprid and/or desaturation of the imidazolidine moiety of imidacloprid to form olefin. Asterisks designate positions of carbon labeling.

TABLE

	guanidine	6-CNA	urea	olefin	imid.	4-OH	5-OH	4,5-OH
R_f	0.20	0.43	0.67	0.72	0.78	0.86	0.86	0.93

Table 1 : Retention factor (R_f) values of imidacloprid and its metabolites. Imidacloprid and its metabolites were separated by one-dimensional TLC. Relative migration of compounds were expressed by R_f. 6-CNA, 4-OH, 5-OH, 4,5-OH were 6-chloronicotinic acid, 4-hydroxy-imidacloprid, 5-hydroxy-imidacloprid and 4,5-dihydroxy-imidacloprid, respectively.