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In vivo metabolic fate of [^{14}C]-acetamiprid in six biological compartments of the honeybee, *Apis mellifera* L

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Abstract: The *in vivo* metabolism of acetamiprid was studied in the honeybee, *Apis mellifera* L. The distribution of acetamiprid and its metabolites was monitored over a 72-h period in six biological compartments: head, thorax, abdomen, haemolymph, midgut and rectum. Honeybees were treated orally with 100 μg [^{14}C]-acetamiprid kg^{-1} bee, a dose which is about 1500 times lower than the median lethal dose. After 72 h, only 40% of the total radioactivity was eliminated, suggesting that acetamiprid and its metabolites tended to persist in the honeybee. Acetamiprid was rapidly distributed in all compartments and metabolized. Just after administration, radioactivity was mainly localized in the abdomen and subsequently in the rectum. After 72 h, the maximum amount of radioactivity (about 20% of the ingested dose) was detected again in the abdomen, whereas the lowest level of total radioactivity was detected in the haemolymph. Radioactivity in the head did not exceed 7.6% of total ingested radioactivity. More than 50% of acetamiprid was metabolised in less than 30 min, indicating a very short half-life for the compound. During the first hours, acetamiprid was mainly detected in nicotinic acetylcholine receptor-rich tissues: abdomen, thorax and head. Of the seven metabolites detected, the major ones were 6-choronicotinic acid and an unknown metabolite called U1, which was present mainly in the rectum, the thorax and the head. Our results indicate that the low toxicity of acetamiprid may reflect its rapid metabolism.

Keywords: acetamiprid; metabolite; honeybee; metabolism; biodistribution; neonicotinoid

1 INTRODUCTION

Acetamiprid is an insecticide belonging to the neonicotinoids, a family of insecticides which was introduced at the beginning of the 1990s. This systemic insecticide is widely used, both in agriculture and domestically, against numerous varieties of insects.[1–7] Neonicotinoids are agonists of nicotinic acetylcholine receptors (nAChR), which are ligand-gated ion channels responsible for rapid neurotransmission.[8] Neonicotinoids exhibit selective toxicity for insects rather than for mammals, because of the stronger affinity of neonicotinoids for insect than for mammal nAChR.[9,10] Differences in subtypes and in the binding sites of nAChR are responsible for this differential affinity.[11–14] Nevertheless, Tomizawa *et al*[12] indicated the possibility that some neonicotinoid insecticides or their metabolites, following accidental human exposure or when they are used to control dog fleas, may also up-regulate nAChR expression in mammals. It has also been reported that chronic exposure of mouse N1E-115 cells to imidacloprid or desnitro derivatives causes activation of the nicotinic receptor-transmitted intracellular signal-regulated kinase cascade and intracellular calcium mobilization.[15] Similar results have been observed following the exposure of rat PC12 cells to nicotine.[16] Acute poisoning with imidacloprid has been reported in humans after ingestion of a formulation containing 9.7% insecticide. The symptoms of this poisoning were the same as those seen with nicotine, ie drowsiness, disorientation, dizziness, oral and gastroeosophageal erosions, haemorrhagic gastritis, cough, fever, leukocytosis and hyperglycaemia.[17]

The metabolism of imidacloprid has been studied in mammals,[18,19] insects,[20] plants and soils.[21–23] The metabolism of acetamiprid has only been studied in plants and soils.[24–28] In order to understand the mode of action of acetamiprid in the honeybee, we first characterized the pharmacokinetics of this insecticide following oral exposure to a low dose. We detected seven metabolites and followed their distribution in six biological compartments, as had previously been done for imidacloprid.[20] No other studies in honeybees or

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other insects have been reported so far. Any in-depth studies of the metabolic fate of pesticides performed in honeybees have so far been limited to the whole animal and the faeces.[29,30]

2 MATERIALS AND METHODS

2.1 Materials

[*pyridinyl*-^{14}C-*methylene*]-Acetamiprid (50 mCi mmol^{-1}), and the low-energy screen (35 × 43 cm) for radioactive compound detection and quantification by Phosphor Imaging with a Storm 820 (Molecular Dynamics), were purchased from Amersham-Pharmacia Biotech (Buckinghamshire, UK). The radiochemical purity (99% pure) was determined by thin-layer chromatography (TLC) and high-performance liquid chromatography using ethyl acetate as solvent. Non-radioactive acetamiprid and its metabolites N^1-(6-chloro-3-pyridyl)methyl-N^2-cyanoacetamidine (IM 2-1), N^1-(6-chloro-3-pyridyl) methyl-N^1-methylacetamidine (IM 1–3), *N*-methyl-(6-chloro-3-pyridyl)methylamine (IM 1–4), (6-chloro-3-pyridyl)methanol (IM-0) and 6-chloronicotinic acid (IC 0) were supplied by Bayer CropScience (Lyon, France) and all had a purity of at least 98%. Silica gel 60F_{254} TLC plates (0.25 mm thickness, 20 × 20 cm) with concentrating zone (2.5 × 20 cm) and organic solvents of analytical grade (Prolabo) were purchased from VWR International SAS (Fontenay-sous-Bois, France). Other chemicals were obtained from Sigma (Saint-Quentin-Fallavier, France). Ultima Gold™ MV high-flash-point LSC came from Packard (Rungis, France).

2.2 Biological materials

For all bioassays, worker honeybees (*Apis mellifera* L), mainly foragers, were collected from honey and pollen combs in a healthy queen-right colony (drones were discarded). To avoid inter-colony and seasonal variations, all bees were taken from the same colony in the summer.

2.3 Bee storage

After collection, the bees were anaesthetized with carbon dioxide and then distributed into cages (10.5 × 7.5 × 11.5 cm) by groups of 20 bees. They were stored in a temperature-controlled chamber at 25 (±1.5)°C and 65% relative humidity in the dark. Under these conditions, they were protected from stress-induced biochemical changes and remained quiet. The bees were fed with a 500 g litre^{-1} sucrose solution *ad libitum*.[31] Experiments were performed in triplicate and repeated at least three times.

2.4 Oral acetamiprid administration

The bees were deprived of food for 3 h before administration of [^{14}C]-acetamiprid. The radioactive acetamiprid solution was diluted in the 500 g litre^{-1} feed sucrose solution and freshly prepared for each test. Each bee ingested 10 µl of sucrose solution containing 10 ng of radiolabelled acetamiprid (100 µg kg^{-1} bee), a dose which is 1500 times lower than the median lethal dose. After consuming this solution, bees were fed *ad libitum* with the sucrose solution. Metabolic studies were performed on groups of 20 healthy bees analyzed 0.5, 2, 4, 6, 10, 15, 24, 30, 48 and 72 h after oral administration of acetamiprid. Time 0.5 h corresponded to the end of total ingestion of the acetamiprid dose by the bees and was used to determine the total ingestion control.

2.5 Sampling of biological compartments

All tissues and organs were extracted from 20 healthy bees and immediately snap-frozen in liquid nitrogen and stored at −80 °C until analysis. Haemolymph was collected by puncturing the dorsal aorta with a 5-µl Hamilton microsyringe. The mean haemolymph volume recovered was 3.6 (±0.4) µl per bee. The last segment of the abdomen was removed to extract and then separate the midgut and rectum, which had respective mean weights of 16 (±2) and 28.2 (±2.8) mg per bee. After the extraction of the haemolymph, midgut and rectum, the head, thorax and abdomen were dissociated (respective mean weights were 15.4 (±1.3), 40.8 (±0.6) and 24 (±1.9) mg per bee) and plunged into liquid nitrogen prior to storage at −80 °C.

2.6 Residue extraction

Samples of haemolymph were supplemented with 4 volumes of acetonitrile + water (9 + 1 by volume). Midguts and rectums from 20 bees were supplemented with sodium hydroxide solution (1 M; 750 µl) to dissociate the tissues, and then ground at 4 °C with a glass Potter homogenizer. Homogenates were incubated in the dark for 3 h at 37 °C. The resulting medium was neutralized with hydrochloric acid (3 M; 250 µl). In order to reduce the quenching of radioactivity, 1/100 volume of hydrogen peroxide was added and samples were incubated overnight at 37 °C before centrifugation at room temperature for 5 min at 13 000 *g*. The supernatant was supplemented with 4 volumes of acetonitrile + water (9 + 1 by volume) solution, dehydrated and adjusted to 1 ml with acetonitrile + water (9 + 1 by volume) to obtain the final extract. The pellets were resuspended in acetonitrile + water (9 + 1 by volume; 1 ml). Heads and abdomens from 20 bees were ground in acetonitrile + water (9 + 1 by volume; 4 ml) and thoraces in 5 ml. Tissue extracts were centrifuged at 4 °C for 30 min at 25 000 *g* and the resulting supernatant dehydrated and adjusted to 1 ml with acetonitrile + water (9 + 1 by volume) to obtain the final extract. The pellets were suspended in acetonitrile + water (9 + 1 by volume; 4 ml). The radioactivity of final samples from haemolymph, midgut, rectum, head, thorax and abdomen and the radioactivity of their pellets were measured with a scintillation counter (Tri-Carb 2300 TR, Packard).

2.7 Thin-layer chromatography and radioanalysis

Silica gel $60F_{254}$ TLC plates (20×20 cm, 0.25 mm thick) were used for the separation and identification of acetamiprid and its metabolites. Extracts (100 µl) from head, thorax, abdomen, haemolymph, midgut and rectum, were loaded on TLC plates along with reference metabolites. The plates were developed using one-dimensional thin-layer chromatography with a migration solvent composed of ethyl acetate + isopropanol + water (68 + 20 + 12 by volume). Radioactive compounds were detected and quantified using Phosphor Imaging by exposing TLC plates to a low energy screen at 25 °C. Unlabelled reference compounds were visualized under UV light at 254 nm.

2.8 Total radioactivity half-life

The half-life was determined by plotting

$$\ln(A/A_o) = -k/t$$

where A_o and A are initial and residual acetamiprid concentrations, respectively, and k is the apparent elimination constant. The half-life ($t_{1/2}$) was calculated using the equation

$$t_{1/2} = (\ln 2)/k.$$

3 RESULTS

3.1 Distribution of total radioactivity

The kinetics of the cumulative distribution of total radioactivity in the different compartments of the honeybee showed three distribution profiles (Plate 1). In the abdomen, radioactivity reached its maximum 30 min after the oral administration of acetamiprid and corresponded to more than 50% of the ingested dose. Radioactivity levels then gradually declined to 10% of the ingested dose 18 h after acetamiprid administration, but then increased to remain constant at about 20% during the remainder of the experiment. The second kinetic profile was discerned in the rectum where radioactivity levels were low during the first 2 h, then increased to a maximum and remained steady until 72 h. The latter profile of distribution was observed in intestine, thorax, haemolymph and head. In these compartments, radioactivity declined only slightly during the first hours and then remained almost constant for the rest of the experiment. After 72 h, about 60% of radioactivity was still recovered from the honeybee; the remaining 40% of the total radioactivity being recovered from the faeces. These results indicate that the elimination half-life of the radioactivity was longer than 72 h (Plate 1).

3.2 Acetamiprid metabolism and localization in different honeybee compartments

In order to gain a clearer understanding of the biological effects of acetamiprid and its metabolites in the honeybee, quantitative and qualitative analyses of acetamiprid metabolism were performed in the six compartments of the honeybee. A TLC method was used to clearly separate acetamiprid, IM 2-1, IM 1–3, IM 1–4, IM 0 and IC 0 (for structures see Fig 1) and two distinct but unidentified metabolites (U1 and U2). The relative fronts (R_f) of these compounds are summarized in Table 1.

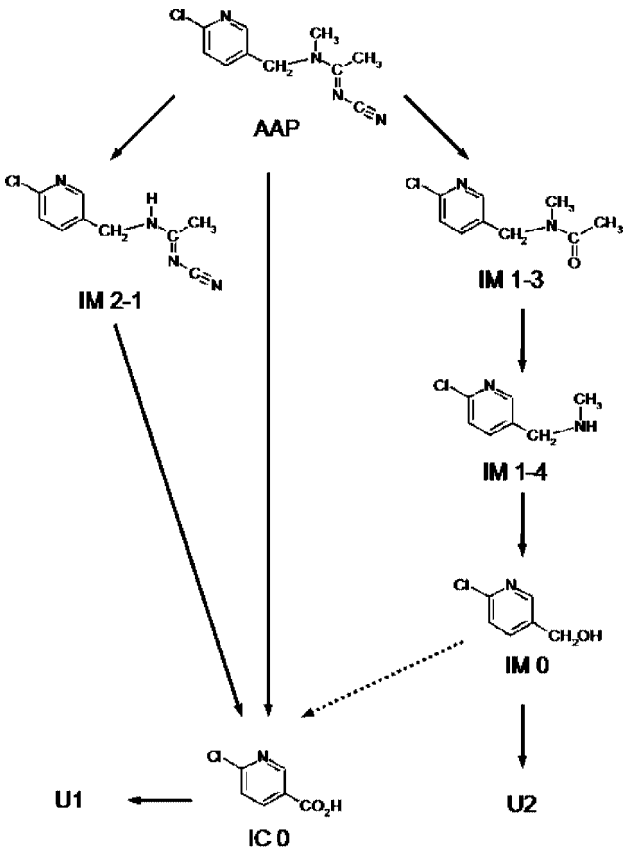

Figure 1. Proposed metabolic pathway for acetamiprid in the honeybee.

Table 1. Relative front (R_f) values of acetamiprid (AAP) and its metabolites separated by one-dimensional TLC

Compound	IM 1–4	U2	U1	IC 0	IM 1–3	AAP	IM 2-1	IM 0
R_f	0.18	0.25	0.36	0.48	0.63	0.69	0.78	0.83

3.2.1 Abdomen

In the abdomen, the metabolic pattern revealed that five major molecules, acetamiprid, IM 1–3, IM 1–4, U1 and U2, could be detected during the observation period (Plate 2A). Acetamiprid appeared rapidly and massively in the abdomen and corresponded to 33% of the dose ingested by the honeybee after 30 min. Acetamiprid levels then fell until 18 h to reach a basal amount of approximately 1% of total ingested dose. Similar results were observed with the IM 1–3 metabolite, which represented 5% of the total ingested dose 30 min after intoxication, declined gradually over time and was almost undetectable 12 h after the oral

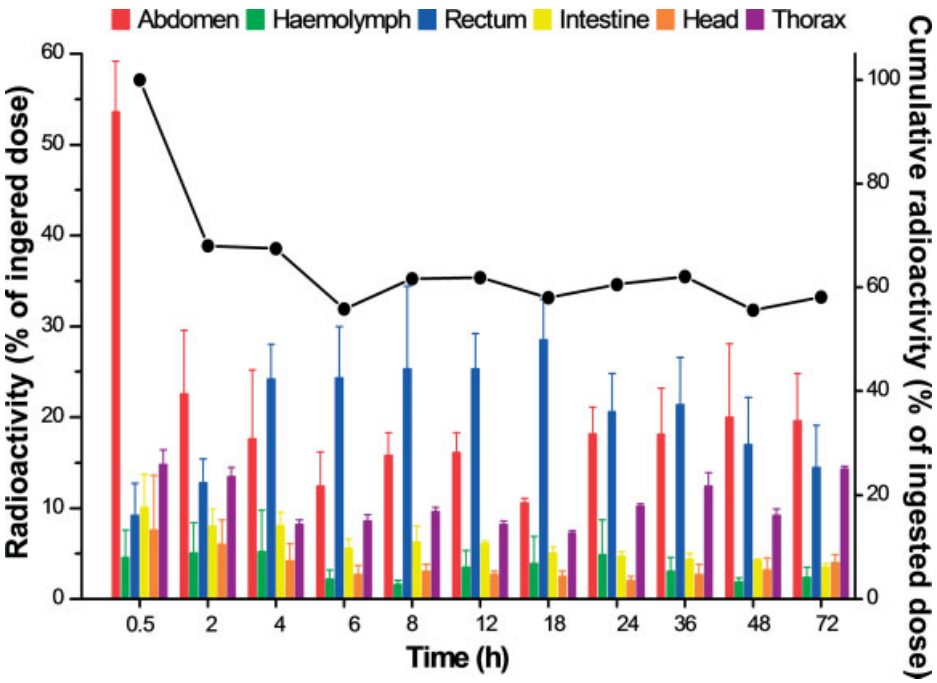

Plate 1. Distribution profile of acetamiprid in the honeybee. Kinetics of the cumulative distribution of total residues were established in six biological compartments of the honeybee over 72 h after oral intoxication with [^{14}C]-acetamiprid (10 ng per bee). (●) Kinetics of cumulative residues in all compartments. The results represent mean values and are expressed as percentages of the ingested dose. Kinetics of total residues of acetamiprid in abdomen, haemolymph, rectum, intestine, head and thorax; mean values ± SD are expressed as percentages of the ingested dose.

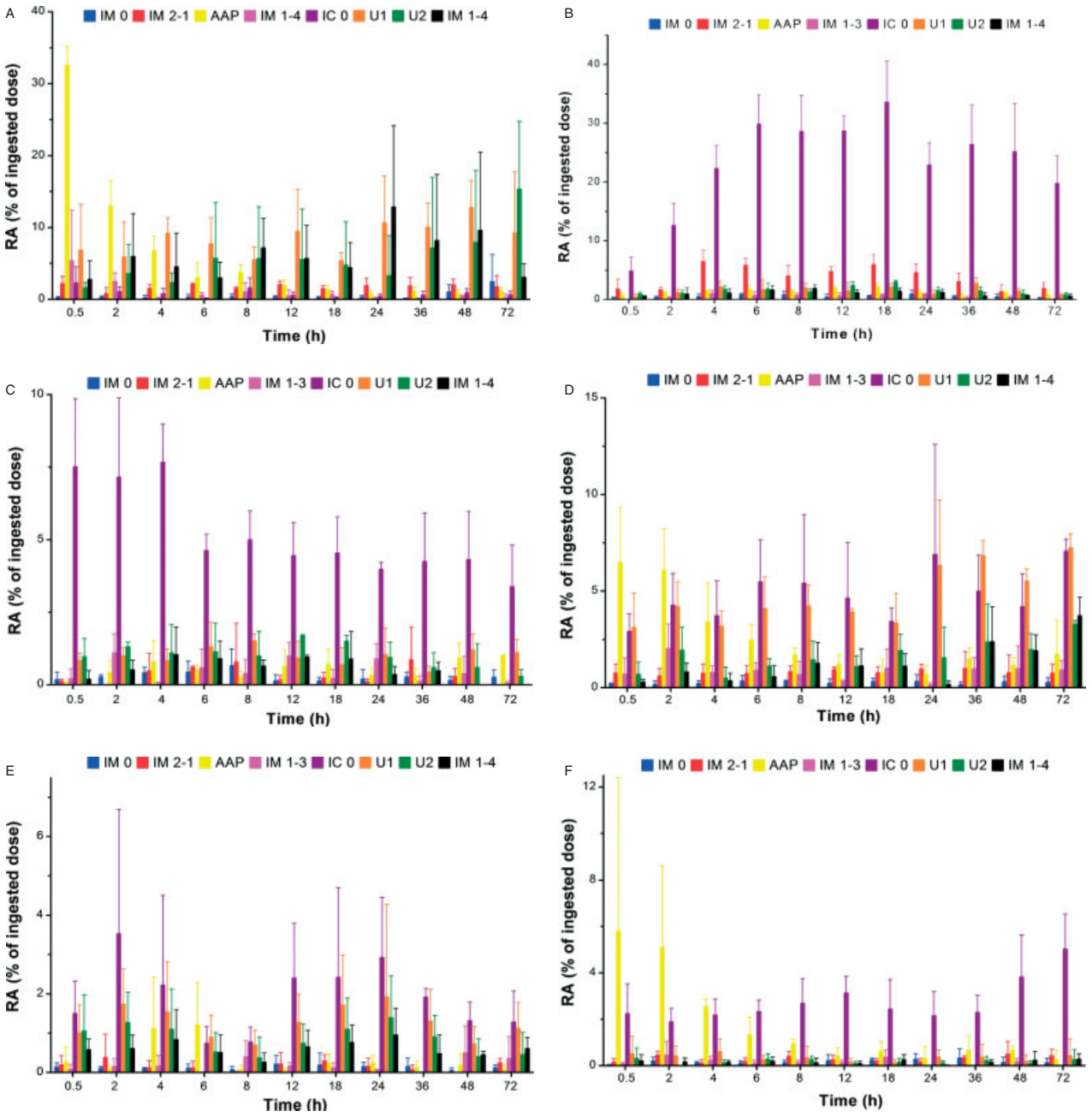

Plate 2. Kinetics of [^{14}C]-acetamiprid metabolism in different compartments of the honeybee. Distribution of acetamiprid and its metabolites in (A) abdomen, (B) rectum, (C) intestine, (D) thorax, (E) haemolymph and (F) head. Radioactive acetamiprid and its metabolites were detected and quantified by phosphor imaging. Acetamiprid (AAP) and its metabolites IM 0, IM 2-1, IM 1–3, IC 0, U1, U2 and IM 1–4 were followed for 72 h after oral treatment. Data correspond to mean values ± SE expressed as percentages of the ingested dose.

exposure. Conversely, levels of U1 and U2 rose slightly to reach about 10% of the total ingested dose up to 48 h after intoxication, with U1 levels always being higher than those of U2. Metabolite IM 1–4 levels rose during the first 24 h to reach 13% of the total ingested dose, and then declined.

3.2.2 Rectum

With the exception of IC 0 and IM 2-1, acetamiprid and all metabolites were present at insignificant levels (Plate 2B). The main metabolite, IC 0, increased to peak at 18 h with a maximum value of 34% of the ingested dose, and then decreased to 20% at 72 h. IM 2-1, which was only weakly present in the other compartments, reached a maximum level of 6.5% 4 h after the ingestion of acetamiprid and then gradually declined. It should be noted that when IC 0 reached its maximum level at 18 h in this compartment, IC 0 was at a minimum in the head and thorax. The amount of the U1 metabolite was low compared with that of the other metabolites and never exceeded 3% of the radioactivity recovered, whereas it was present at higher levels in the other compartments.

3.2.3 Intestine

Acetamiprid and all metabolites were detected in the intestine (Plate 2C). However, IC 0 was the principal metabolite and represented 7.5% of the ingested dose during the first 4 h and about 5% thereafter. Acetamiprid levels in this compartment never exceeded 1% of the ingested dose.

3.2.4 Thorax

Acetamiprid and all metabolites were detected in the thorax (Plate 2D), but the main compounds were acetamiprid, IC 0 and U1. Acetamiprid, which represented 6.5% of the ingested dose at 30 min, was constant during the first 2 h and then declined. IC 0 and U1 presented similar levels. During the first 72 h, their course over time was similar. They exhibited their maximum level of about 7% at 72 h. The other metabolites were present but represented less than 2.5% of the ingested dose, with the exception of IM1-4 and U2 which presented higher levels at 72 h.

3.2.5 Haemolymph

The haemolymph was the compartment exhibiting the lowest level of radioactivity (Plate 2E). Acetamiprid was present only between 2 and 4 h after intoxication. The main metabolites recovered were IC 0 and U1, the distribution kinetics of which were similar to those observed in the thorax. The other metabolites were only detected at low levels in this compartment.

3.2.6 Head

In the head, only acetamiprid and IC 0 were present at significant levels (Plate 2F). Acetamiprid appeared rapidly in the head, reached 6% of the ingested dose after 30 min and then gradually declined. Levels of IC 0 oscillated between 2 and 3% of the ingested dose from 0.5 h to 36 h and then rose to reach 5% at 72 h.

3.3 Acetamiprid metabolism in the whole honeybee

The kinetics of acetamiprid and its metabolites in the whole honeybee were investigated by calculating the total quantity of each compound detected from its residual levels in the different compartments (Plate 3). Acetamiprid represented 44% of the ingested dose at the initial time point (0.5 h) and exhibited an elimination half-life of about 25 min in the whole honeybee. Its metabolism led to four major metabolites appearing sequentially: IC 0, IM 1–4, U1 and U2. During the initial 30 min after exposure, IC 0 was already the main metabolite. It reached its maximum level of 48% 8 h after the oral administration of acetamiprid and then declined gradually to 37% during the next 40 h. U1 was the second main metabolite. U1 levels rose rapidly to 17% of the ingested dose during the first 4 h, and then increased less rapidly to 25%. The kinetic profile of U1 corresponded to an inverted image of the IC 0 profile (Plate 3). IM 1–4 was the third main metabolite, levels of which oscillated around 15% of the ingested dose throughout the observation period, with a maximum of 22% at 24 h. U2 was the last main metabolite recovered in the honeybee. U2 reached a maximum level of 7% of the ingested dose in less than 4 h, and then oscillated around this value up to 48 h, finally reaching 25%. The marked increase of U2 at 72 h suggested that this metabolite might be a terminal product of degradation. IM 2-1 reached its maximum level 8 h after exposure and slowly declined until 72 h. IM 1–3 and IM 0 appeared to be minor metabolites in this study.

4 DISCUSSION

These experiments indicated that acetamiprid was rapidly distributed in the different compartments of the honeybee and just as promptly metabolised into seven metabolites: IM 0, IM 2-1, IC 0, IM 1–3, U1, U2 and IM 1–4. The very rapid absorption of acetamiprid in the honeybee is similar to that of imidacloprid,[20] which is in line with the results observed *in vitro* with the human Caco-2 cell line.[32] This Caco-2 cell line is currently used as a brush border model to study intestinal drug permeability.[33,34] As for imidacloprid,[20] the rapid appearance of metabolites in all compartments suggests that the metabolism of acetamiprid occurs in all compartments and not exclusively in the intestine, which is the principal site of the detoxification system.[35] Again as for imidacloprid, acetamiprid is biotransformed in the honeybee by Phase I enzymes, mainly by mixed function oxidases that convert acetamiprid into more polar metabolites which are more readily excreted. However, it is noteworthy that little is known about the metabolism of acetamiprid in mammals, plants and soils.

During our experiments, the IC 0, U1 and U2 metabolites were recovered in large amounts 72 h after intoxication and represented the majority of the 60% of initially ingested radioactivity. For imidacloprid, at the same time point, most radioactivity has already been excreted.[20] It is probable that the discrepancy between the amounts of radioactivity recovered at 72 h could be explained by a qualitative difference in the metabolites produced, with the urea derivative as the main metabolite for imidacloprid and the IC 0 metabolite for acetamiprid. The increasing levels of U1 and U2 metabolites at 72 h suggest that the IC 0 metabolite might also be converted into either U1 or U2, or both. This putative conversion of IC 0 into U1 and/or U2 metabolites appears to delay the excretion of IC 0 and promotes its persistence in honeybee. The contrasting levels of IC 0, high in the rectum and low in the head and thorax, strongly suggest that IC 0 moves from the anterior to the posterior part of the bee, with excretion as the final step. Considering their profile, U1 and IC 0 appear to be inversely correlated in the whole honeybee, whereas U2 and IC 0 seem to be independent. This suggests that U1 results from IC 0. In any case, IC 0 and U1 were never recovered from the same compartment at high levels, suggesting that either the conversion of IC 0 into U1 or the localization of U1 was tissue-specific. Moreover, the kinetics of IM 1–4 and U2 changed in opposite ways over time, suggesting that their fates may be linked. Thus, IC 0 appears to be converted into U1 and IM 1–4 into U2.

On the basis of the metabolites identified and the kinetics of their appearance, we propose a metabolic pathway for acetamiprid in the honeybee (Fig 1). Three major metabolic routes can be distinguished in the honeybee. The first concerns oxidative cleavage of the nitromethylene bond of acetamiprid and/or its metabolites. The cleavage of acetamiprid is followed by oxidation that results in the appearance of 6-chloronicotinic acid (IC 0). This may then be converted into the U1 metabolite that presents a higher polarity. The second pathway is related to *N*-demethylation and results in IM 2-1. This *N*-demethylation may be followed by oxidation of the nitromethylene bond that converts IM 2-1 into 6-chloronicotinic acid. The third pathway consists of oxidative cleavage of the cyanamine group to form the IM 1–3 ketone derivative. IM 1–3 is converted into IM 1–4 by *N*-deacetylation. *N*-Deacetylation is followed by oxidative cleavage of IM 1–4 to form 6-chloropicolyl alcohol, IM 0. This compound can then follow two routes. First, IM 0 may be oxidized into 6-chloronicotinic acid and, second, it may be converted into a glycoconjugate derivative. Two reasons suggest that the latter route prevails. First, U2 is inversely correlated to IM 1–4 and not to IC 0 and, second, if the IM 0 metabolite resulted in high levels of IC 0, small quantities of U2 should be recovered at 72 h and the disappearance of IM 1–4 should correspond to an increase in U1, which is not the case.

The distribution profile of acetamiprid in the abdomen, thorax and head was similar to that of imidacloprid in the same compartments of the honeybee.[20] Both molecules presented the same kinetics of appearance and disappearance and their radioactivity levels were equivalent in these compartments. This fact is very interesting because imidacloprid is highly toxic to honeybee whereas acetamiprid is less toxic following oral exposure.[36,37] Iwasa *et al*[38] found that, following topical application, imidacloprid is about 400 times more toxic (LD_{50} 17.9 ng per bee) than acetamiprid (LD_{50} 7.1 μg per bee). This difference of toxicity might be explained by a slightly weaker affinity of acetamiprid for nAChR when compared with imidacloprid.[10,12–14,39,40] This differential susceptibility is more likely to be due to difference in metabolism and production of less toxic metabolites of acetamiprid. Following oral exposure, it is interesting to underline that, in the whole honeybee, the half-life of acetamiprid ($t_{1/2} = 25$ min) was much lower than that of imidacloprid, which ranges from 4 to 5 h. Moreover IC 0, the major metabolite, tends to persist in the honeybee, particularly in nAChR-rich tissues such as the head and thorax, even if the present study does not allow a distinction between preferential storage at ganglionic or muscular level. Furthermore, it has been reported that IC 0 was not highly toxic, at least when applied topically (no mortality observed at 50 μg per bee).[38] However, if we consider that IC 0 is much more toxic during oral chronic exposure than following acute exposure (more than 100 000-fold),[41] it would be interesting to verify whether closely reiterated exposures to acetamiprid could induce a higher level of toxicity by gradually increasing the IC 0 concentration *in vivo*. Such higher toxicity at low doses, during chronic exposure, has been reported with organophosphate and pyrethroid insecticides.[42,43] This differential toxicity could be explained either by an inability of low doses to induce detoxifying systems or by the existence of high and low affinity binding sites for the toxic compound. In the case of neonicotinoids, the hypothesis that they could activate nAChRs via two, high and low affinity, binding sites is more probable and is in line with the findings of Suchail *et al*,[41] Nagata *et al*,[44] Lind *et al*[45] and Benson.[46]

5 CONCLUSIONS

In our study, it appeared that acetamiprid was very rapidly metabolised and distributed in the honeybee. The distribution profiles of acetamiprid in the head and thorax were similar to those of imidacloprid.[20] The lower toxicity of acetamiprid, compared with that of imidacloprid, could be explained by the appearance of less toxic metabolites. However, the marked persistence of three metabolites 72 h after exposure could induce a toxicological risk for the honeybee, insofar as the toxicity of some metabolites is greater when bees are subjected to chronic exposure rather than acute exposure. Furthermore, many compounds

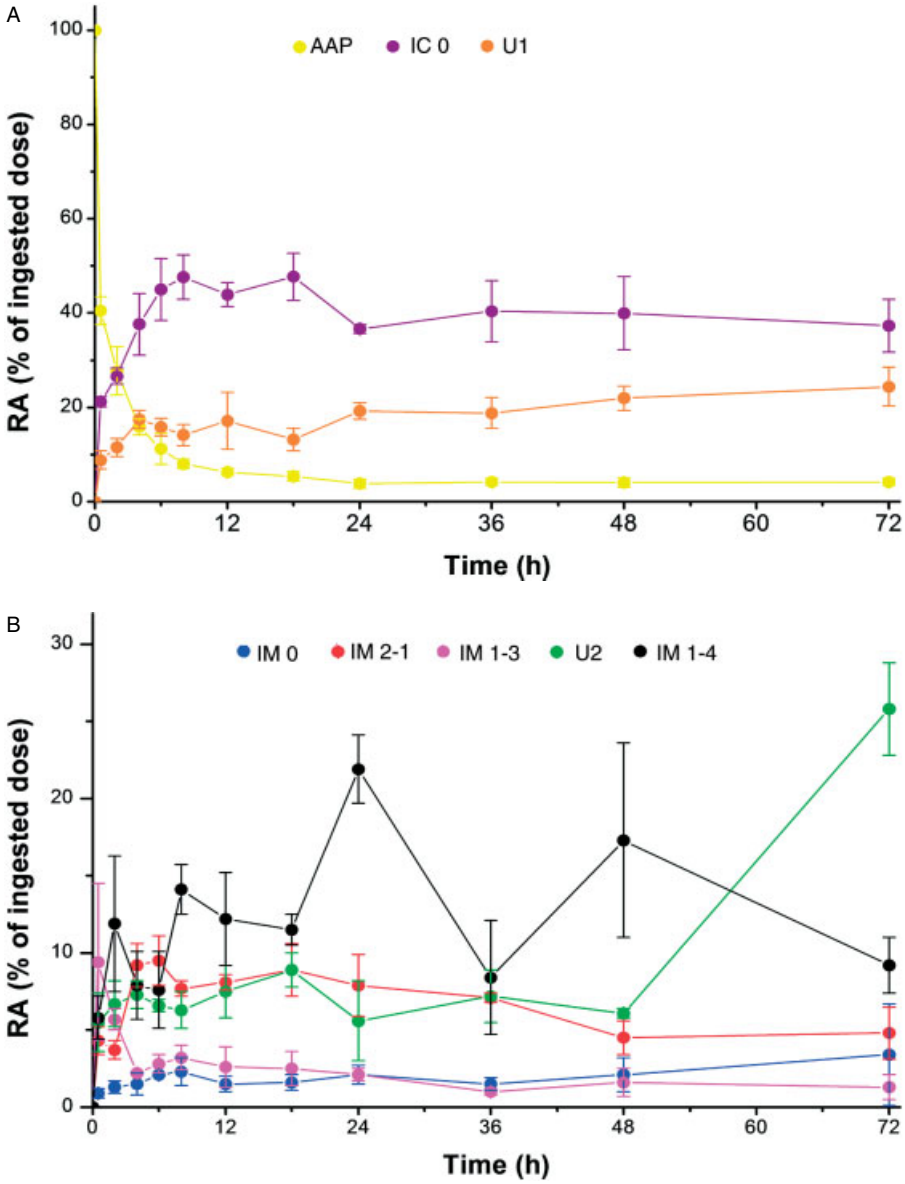

Plate 3. Kinetics of acetamiprid distribution in the whole honeybee. [^{14}C]-Acetamiprid and its metabolites were detected and quantified by phosphor imaging in the honeybee. Acetamiprid (AAP) and its metabolites were followed over 72 h. Data correspond to mean values ± SE expressed as percentages of the ingested dose.

exhibit synergistic action in the honeybee, particularly ergosterol biosynthesis inhibitor fungicides (EBI fungicides) and some neonicotinoids such as thiacloprid[47] and acetamiprid.[38] As these EBI fungicides can inhibit insect cytochrome P450s,[48] it would be interesting to determine the *in vivo* metabolic fate of acetamiprid in six biological compartments of the honeybee after exposure to azole fungicides.

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