Short Communication

In vivo metabolic profiling for acetamiprid in rat plasma by liquid chromatography quadrupole time-of-flight mass spectrometry

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Abstract The metabolism of acetamiprid, a neonicotinoid insecticide, in rats was investigated by monitoring time-dependent changes in plasma levels of acetamiprid and its metabolites using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). Acetamiprid was administrated to rats intravenously at 7.1−21.7 mg/kg for analysis of plasma at 0−96 h. Acetamiprid and its four metabolites could be detected and tentatively identified in rat plasma samples. Plasma levels only estimated from peak areas of acetamiprid decreased, with a half-life of about 4 h, and were completely eliminated by 24 h. *N*-[(6-Chloro-3-pyridyl)methyl]-*N′*-cyano-acetamidine was most strongly detected at 4 h and was missing at 48 h. The peak areas of *N*-[(6-chloropyridin-3-yl)methyl]-acetamide increased over time, reached the maximum at 12−24 h, and the metabolite was then completely eliminated at 72 h. *N*-Cyano-*N′*-methylacetamidine and 6-(methylsulfanyl) nicotinic acid showed almost the same behaviors as *N*-[(6-chloro-3-pyridyl)methyl]-*N′*-cyano-acetamidine. These findings suggested that LC-QTOF-MS enabled us to tentatively estimate acetamiprid metabolism in rats and that these four metabolites may also be useful biomarkers for acetamiprid exposure in humans.

Key words: acetamiprid, neonicotinoid, metabolite, liquid chromatography quadrupole time-of-flight mass spectrometry

Introduction

Since the late 1990s, the agricultural use of neonicotinoid insecticides, including acetamiprid, has been increasing worldwide[1−4] because organophosphorus and pyrethroid insecticides are being phased out due to the emergence of insects resistant to conventional pesticides. Acetamiprid is a neonicotinoid insecticide with selective toxicity and is used for the control of sucking-type insects on vegetable, fruits, cotton, and ornamental plants and flowers[1−4]. One of the toxic characteristics of acetamiprid is high mammalian toxicity of neonicotinoids; the oral median lethal dose (LD_{50}) was in the range of 146−217 mg/kg body weight[5,6], similar to those of organophosphorus and pyrethroid insecticides. Homicide or suicide cases using acetamiprid are also expected to increase in the future. Therefore, it is important to monitor the levels of acetamiprid and its metabolites in body fluids, such as blood and urine.

In several reports, the detection of acetamiprid has been

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reported on environmental water[7], soil[8–15], plants[15], insects[16–18], animals[19], and humans[20–25]. Taira et al.[21] reported that *N*-[(6-chloro-3-pyridyl)methyl]-*N*′-cyano-acetamidine (acetamiprid-*N*-desmethyl) is the most dominant urinary metabolite of acetamiprid and that 6-chloronicotinic acid was found in the urine of patients suspected of subacute exposure to neonicotinoid pesticides[20]. Yeter and Aydın[22] reported the results of autopsy samples (blood, liver, stomach contents, and urine) in cases of fatal intoxication. However, available information related to chronological changes in the levels of acetamiprid and its metabolites in body fluids is very limited. Ford and Casida[19] reported the levels of acetamiprid in mouse plasma, but they did not provide detailed results on metabolites.

Recently, liquid chromatography-mass spectrometry (LC-MS) has been used for analysis of complex samples; this powerful analytical technique combines the resolving power of LC with the accuracy of mass separation. LC-quadrupole time-of-flight MS (LC-QTOF-MS) has been used for profiling drug metabolites in various biological samples[26–30]. In this study, we investigated the metabolite profiling for acetamiprid in rats by monitoring time-dependent changes in peak areas of acetamiprid and its metabolites in plasma using LC-QTOF-MS.

Materials and Methods

Materials

Acetamiprid was purchased from Wako (Tokyo, Japan); acetamiprid-*N*-desmethyl(*N*-[(6-chloro-3-pyridyl)methyl]-*N*′-cyano-acetamidine; M1) from Sigma-Aldrich Japan (Tokyo, Japan); isotope-labeled acetamiprid (acetamiprid-d_6) to be used as an internal standard from Hayashi Pure Chemical Ind. (Osaka, Japan). Laboratory distilled water was purified using a Synergy UV (Millipore, Molsheim, France). Other common chemicals used in this study were of the highest purity commercially available.

Animals

Thirteen-week-old male Wistar rats (approximately 300 g) were purchased from Japan SLC (Hamamatsu, Japan). The rats were housed under controlled environmental conditions (temperature of 23±1°C and humidity of 55% ±5%) with a commercial food diet (CLEA Rodent Diet CE-2; CLEA Japan, Tokyo, Japan) and water freely available to animals under a 12-h light/dark cycle (lights on from 08:00 to 20:00 h) for at least 3 days before the experiment. All procedures involving animals and their care conformed to international guidelines, Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985), and Guiding Principles for the Care and Use of Laboratory Animals of Nagoya University.

Animal experiments

One day before the start of the experiments, rats were anaesthetized with an intraperitoneal administration of sodium pentobarbital (25 mg/kg body weight), and the right jugular vein was cannulated with polyethylene tubes (Natsume, Tokyo, Japan) for blood collection and drug administration. The doses of acetamiprid were set at a nonacute symptomatic level (7.1 mg/kg body weight) and one-tenth the LD_{50} (21.7 mg/kg body weight). Blood samples were collected at designated intervals (0.25, 0.5, 1, 2, 4, 6, 12, 24, 48, 72, and 96 h after intravenous injection of each acetamiprid). Plasma samples were immediately obtained by centrifugation at 6000×*g* for 10 min at 4°C. Samples were stored at −80°C until analyses.

Solid-phase extraction procedure

Solid-phase extraction (SPE) of acetamiprid and its metabolites was performed according to our previous report[31] with minor modifications. Briefly, a 100-μL volume of plasma was mixed with 50 ng IS and 4.0 mL of 250 mM sodium bicarbonate solution, vortex-mixed for 1 min, and centrifuged at 1600×*g* for 5 min. For SPE, the supernatant fraction was applied to an Oasis HLB cartridge (60 mg, 3 cc; Waters, Milford, MA, USA) preconditioned with 3 mL methanol and 3 mL distilled water. Each cartridge was washed with 3 mL distilled water and 3 mL of 5% methanol aqueous solution. The cartridges were dried under vacuum for 10 s. The analytes were eluted with 3 mL methanol. The elute was transferred to another tube and evaporated with a centrifugal evaporator (CVE-2000; Tokyo Rikakikai, Tokyo, Japan). The residue was reconstituted in 90 μL of 0.1% formic acid and 10 μL acetonitrile and mixed by vortexing for 1 min.

Instrumental analysis

The LC instrument used in combination with a QTOF-MS detector was a Waters Xevo G2 QTof system, including an Acquity UPLC binary pump and a sample manager (Waters). The column used for chromatographic separation was an Acquity UPLC HSS C_{18} (100×2.1 mm

i.d., 1.8 μm; Waters). The column temperature was maintained at 40°C, and a gradient system was used with mobile phase A (0.1% formic acid aqueous solution) and mobile phase B (0.1% formic acid in acetonitrile) delivered at 0.4 mL/min. The linear gradient program was as follows: 95% A/5% B (0 min) to 5% A/95% B (until 8 min). The final mobile phase was held for 2 min to avoid carry-over from the previous injections. After the elution program, the mobile phase composition was returned to 95% A/5% B within 2 min for the next run. The total run time for each sample analysis was 12 min. The autosampler was maintained at 4°C, and the injection volume was 5 μL.

The LC system was interfaced by electrospray ionization (ESI) to a Waters Xevo G2 QTOF-MS operated in full-scan MS^E mode with positive ionization. The MS^E mode comprised two interleaved full-scan functions, one acquired under low-energy conditions to obtain accurate mass data for intact precursor ions, and one acquired under high-energy conditions to obtain fragment ions and corresponding accurate mass data. No compound-specific tuning of ionization parameters was employed. Data were acquired over the *m/z* range of 50−700 using a capillary voltage of 3.0 kV, sampling cone voltage of 30 V, source temperature of 150°C, and desolvation temperature of 500°C. For the high-energy scan function, a collision energy ramp of 10−30 eV was applied. A continuous lockspray reference compound (leucine enkephalin, *m/z* 555.2693) was sampled at 10-s intervals for centroid data mass correction.

Data processing

Data acquisition was achieved with MassLynx version 4.1 (Waters). Data were processed with the MetaboLynx XS program, a component of MassLynx. Data were initially filtered (±0.03 u) according to accurate mass defects based on substrate elemental compositions using structure-based C-heteroatom dealkylation to construct appropriate mass defect filters for substrate[32]. Filtered sample and control (zero-substrate) data were compared to identify changes potentially associated with drug metabolism and electrophilic trapping using the specific acetamiprid trapping features in the MetaboLynx XS program, and lists of expected and unexpected potential trapped reactive metabolites were populated.

Results and Discussion

Metabolic profile of acetamiprid

We searched for candidate metabolites of acetamiprid using the MetaboLynx XS program and selected the target metabolites in reference to previous studies[32]. We used LC-QTOF-MS to tentatively identify the metabolites of acetamiprid in rat plasma samples collected after intravenous administration of acetamiprid. The metabolites were determined to be *N*-[(6-chloro-3-pyridyl)methyl]-*N*′-cyano-acetamidine (M1), *N*-[(6-chloropyridin-3-yl)methyl]-acetamide (M2), *N*-cyano-*N*′-methylacetamidine (M3), and 6-(methylsulfanyl)nicotinic acid (M4) (Table 1). Extracted ion chromatograms of acetamiprid (*m/z* 223.0750), M1 (*m/z* 209.0594), M2 (*m/z* 185.0481), M3 (*m/z* 98.0718), M4 (*m/z* 170.0275), and acetamiprid-d_6 (*m/z* 229.1127) obtained from plasma samples 12 h after intravenous administration of acetamiprid by LC-QTOF-MS are shown in Fig. 1. The peaks of five analytes and the IS were clearly observable.

We also listed the ranks of the four metabolites at the times of 6 and 24 h after administration according to peak area intensities obtained. The M1 and M3 ranked the top and second at both times, respectively.

Table 1. Identification of acetamiprid and its metabolites in rat plasma using LC-QTOF-MS, together with the ranks of metabolite peak area intensities at 6 and 24 h

Compounds	Formula	Retention time (min)	Measured mass (*m/z*)	Theoretical mass (*m/z*)	Mass error (ppm)	Rank (6 h)	Rank (24 h)
Acetamiprid	$C_{10}H_{11}ClN_4$	3.19	223.0752	223.0750	0.90	—	—
N-[(6-Chloro-3-pyridyl)methyl]-*N*′-cyano-acetamidine (M1)	$C_9H_9ClN_4$	2.88	209.0597	209.0594	1.43	1	1
N-[(6-Chloropyridin-3-yl)methyl]-acetamide (M2)	$C_8H_9ClN_2O$	2.22	185.0478	185.0481	−2.16	4	3
N-Cyano-*N*′-methylacetamidine (M3)	$C_4H_7N_3$	1.28	98.0716	98.0718	−2.04	2	2
6-(Methylsulfanyl)nicotinic acid (M4)	$C_7H_7NO_2S$	3.21	170.0274	170.0275	−0.59	3	4
Acetamiprid-d_6 (IS)	$C_{10}H_5D_6ClN_4$	3.18	229.1124	229.1127	−1.31	—	—

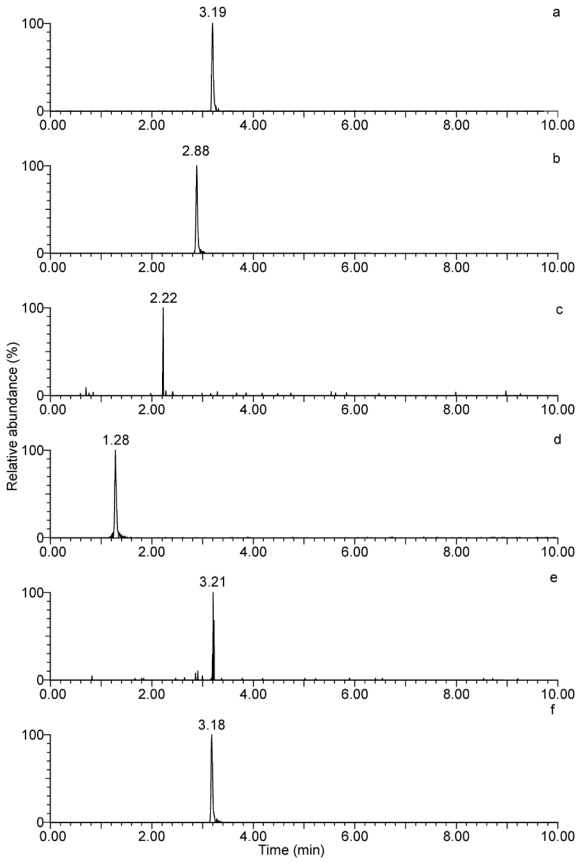

Fig. 1. Extracted ion chromatograms of (a) acetamiprid (*m/z* 223.0750), (b) M1 (*m/z* 209.0594), (c) M2 (*m/z* 185.0481), (d) M3 (*m/z* 98.0718), (e) M4 (*m/z* 170.0275), and (f) acetamiprid-d_6 (*m/z* 229.1127) obtained from plasma samples 12 h after intravenous administration of acetamiprid recorded by liquid chromatography quadrupole time-of-flight mass spectrometry. M1: *N*-[(6-chloro-3-pyridyl)methyl]-*N′*-cyano-acetamidine, M2: *N*-[(6-chloropyridin-3-yl)methyl]-acetamide, M3: *N*-cyano-*N′*-methylacetamidine, M4: 6-(methylsulfanyl)nicotinic acid.

Identification of acetamiprid and its metabolites in rat plasma samples

The LC-QTOF-MS profiles of acetamiprid and its four metabolites are summarized in Table 1. Acetamiprid was eluted at a retention time of 3.19 min. The low-energy mass spectrum showed an $[M+H]^+$ ion at *m/z* 223.0752. The mass error between the measured and theoretical mass values was 0.90 ppm. The high-energy mass spectrum provided a fragment ion at *m/z* 126.0109, which was formed by loss of $C_4H_6N_3$. The mass error between measured and theoretical mass values was −1.59 ppm. These results revealed the high reliability of the identification method.

The metabolite M1, which was the most strongly detected metabolite, was eluted at a retention time of 2.88 min. The low-energy mass spectrum showed an $[M+H]^+$ ion at *m/z* 209.0597. The mass error between measured and theoretical mass values was 1.43 ppm. The high-energy mass spectrum provided a fragment ion at *m/z* 126.0113, which was formed by loss of $C_4H_6N_3$. Acetamiprid was metabolized to M1 by *N*-demethylation. The mass error between measured and theoretical mass values was 1.59 ppm.

Three metabolites, i.e., M2, M3, and M4, were also detected at retention times of 2.22, 1.28, and 3.21 min, respectively, although the intensities were lower than that of M1. The $[M+H]^+$ ions and mass errors were *m/z* 185.0478 and −2.16 ppm for M2, *m/z* 98.0716 and −2.04 ppm for M3, and *m/z* 170.0274 and −0.59 ppm for M4, respectively. The $[M+H]^+$ion of M2 was 24 Da lower than that of M1; therefore, M2 was identified as *N*-[(6-chloropyridin-3-yl) methyl]-acetamide. The $[M+H]^+$ ion of M3 was 125 Da lower than that of acetamiprid. The Metabolynx XS program suggested that M3 was *N*-cyano-*N′*-methylacetamidine by mass defect filtering. The metabolite M4 was 6-(methylsulfanyl)nicotinic acid in reference to a previous study[19] and mass defect filtering. Additionally, the mass spectra of these three metabolites by high-energy collision did not provide structurally informative fragment ions because of low intensities of their $[M+H]^+$ ions. Therefore, further experiments towards attaining enough sensitivity are required. The proposed metabolic pathways of acetamiprid in rats are shown in Fig. 2.

Time-dependent changes in peak areas of acetamiprid and its metabolites in rat plasma

The time-dependent changes in peak areas of acetamiprid and its metabolites in rat plasma are shown in Fig. 3. Acetamiprid was detected in plasma samples taken between 15 min to 24 h after administration of acetamiprid (Fig. 3a). M1, which was most strongly detected, increased over time, reached a maximum at 4−6 h, and was completely eliminated at 48 h (Fig. 3b). The levels of M2 increased over time, reached a maximum at 12−24 h, and were then completely eliminated at 72 h (Fig. 3c). M3 and M4 showed similar behaviors to M1 (Figs. 3d and 3e). Ford and Casida reported that there was an increase from 2.2 ppm acetamiprid at 15 min to approximately 6 ppm at 60−240 min when acetamiprid was administered intraperitoneally at 10 mg/kg to mice[19]. They also found that the levels of M2 peaked at about 30 min[19]. The discrepancy in these results between

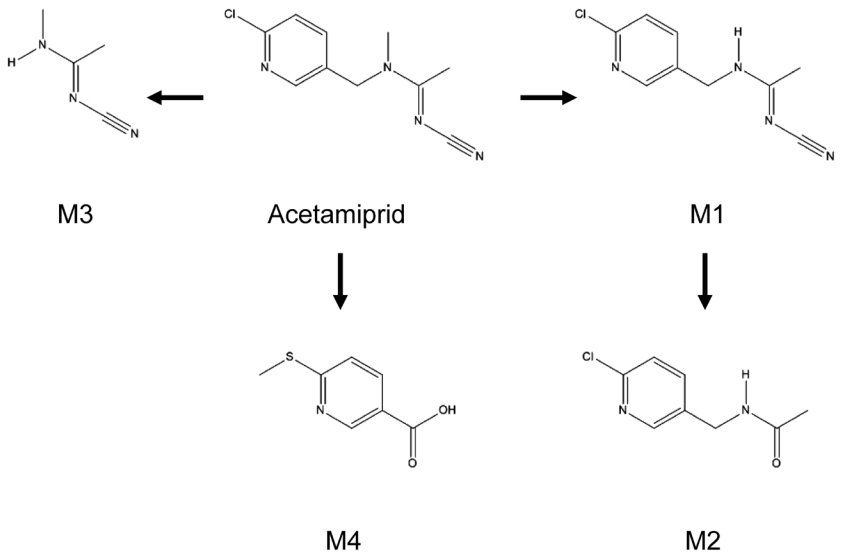

Fig. 2. Proposed metabolic pathways for acetamiprid in rats.

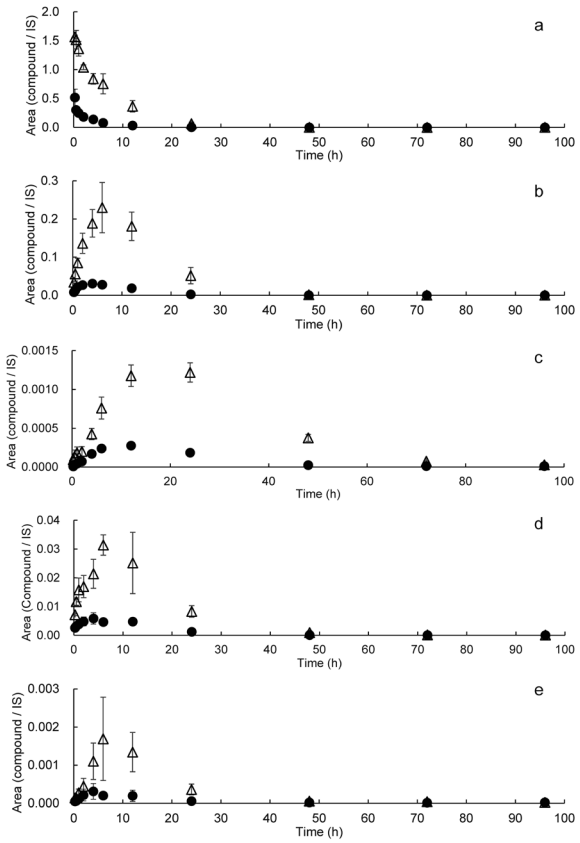

Fig. 3. Time-dependent changes in peak area intensity of (a) acetamiprid, (b) M1, (c) M2, (d) M3, and (e) M4 in rat plasma. The doses of acetamiprid were 7.1 mg/kg (black circle) and 21.7 mg/kg (white up-pointing triangle). Data are expressed as the mean±standard deviation (n=5). *IS* internal standard.

their study[19] and the present study may be due to different administration routes. Yeter and Aydin[22] reported that the metabolite *N*′-carbamoyl-*N*-[(6-chloro-3-pyridyl)methyl]-*N*-methylacetamidine was not detected in decedents' blood, consistent with our findings.

Conclusions

We investigated *in vivo* metabolic profiling for acetamiprid in rats using LC-QTOF-MS. LC-QTOF-MS enabled us to monitor time-dependent changes in plasma levels of acetamiprid and its metabolites. The results from animal experiments showed that the four metabolites detected in the present study may be useful biomarkers for acetamiprid exposure in humans.

Conflict of Interest

The authors declare that we have no conflict of interest.

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