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Analytical method for urinary metabolites of the fluorine-containing pyrethroids metofluthrin, profluthrin and transfluthrin by gas chromatography/mass spectrometry

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ABSTRACT

An analytical method was developed for measurement of the major urinary metabolites in rats administered fluorine-containing pyrethroids (metofluthrin, profluthrin and transfluthrin) which are widely used recently as mosquito repellents or mothproof repellents. Eight metabolites, 2,3,5,6-tetrafluorobenzoic acid, 4-methyl-2,3,5,6-tetrafluorobenzoic acid, 2,2-dimethyl-3-(1-propenyl)-cyclopropanecarboxylic acid, 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (carboxylic metabolites), 2,3,5,6-tetrafluorobenzyl alcohol, 4-methyl-2,3,5,6-tetrafluorobenzyl alcohol, 4-methoxymethyl-2,3,5,6-tetrafluorobenzyl alcohol and 4-hydroxymethyl-2,3,5,6-tetrafluorobenzyl alcohol (alcoholic metabolites), were extracted from enzymatic hydrolyzed urine using toluene and then concentrated. After transformation to their *tert*-butyldimethylsilyl derivatives for carboxylic metabolites or their trimethylsilyl derivatives for alcoholic metabolites, analysis was conducted by gas chromatography/mass spectrometry in the electron impact ionization mode. The calibration curves for each metabolite were linear over the concentration range of 0–20 μg/ml in urine, and the quantification limits were between 0.009 and 0.03 μg/ml. The relative errors and the relative standard deviations on replicate assays were less than 6% and 5%, respectively, for all concentrations studied. The measurements were accurate and precise. The collected urine samples could be stored for up to 1 month at −20 °C in a freezer. The proposed method was applied to the analysis of several urine samples collected from rats treated with these pyrethroids.

1. Introduction

People are exposed daily to a wide variety of environmental chemicals. Because we spend much time indoors, such as at home, indoor air quality has a strong effect on our health. Previous studies have revealed the presence of many insecticides in indoor air [1–5]. In recent years, the use of synthetic pyrethroids derived from chrysanthemic acid is increasing in place of more toxic insecticides such as organochlorine and organophosphorus insecticides [6]. Pyrethroids are widely used indoors to control household pests, such as flies, mosquitoes, termites, and other harmful insects, and comprise more than 90% of the household insecticides used in Japan. More recently, fluorine-containing pyrethroids (Fig. 1), which have high vaporization properties and high insecticidal activities in comparison with conventional pyrethroids, are being widely used as mosquito repellents that do not use heat sources (metofluthrin: [2,3,5,6-tetrafluoro-4-(methoxymethyl) phenyl]methyl 2,2-dimethyl-3-(prop-1-enyl)cyclopropane-1-carboxylate), mothproof repellents for clothes (profluthrin: (2,3,5,6-tetrafluoro-4-methylphenyl)methyl 2,2-dimethyl-3-(prop-1-enyl)cyclopropane-1-carboxylate) and aerosols that have long-lasting effects against mosquitoes (transfluthrin: (2,3,5,6-tetrafluorophenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate). These pyrethroids were detected from indoor air in houses where mosquito repellents or mothproof repellents containing these compounds were used [7]. However, metofluthrin and profluthrin are known to have adverse effects on the nervous system and liver according to results from subacute and chronic toxicity studies in mammals [8,9]. Exposed rats had tremors, clonic convulsions, increased liver weight and hepatocellular hypertrophy. Metofluthrin produced hepatocellular tumors in rats [10] while transfluthrin induced urinary bladder tumors and necrosis of the urothelial superficial layer in rats given a dosed diet for a long term [11]. Transfluthrin was even suggested to have a genotoxic effect on the epithelial cells of human nasal mucosa [12]. Therefore, knowing the amounts of these pyrethroids absorbed by the body is important for evaluating their adverse effects on humans in indoor environments.

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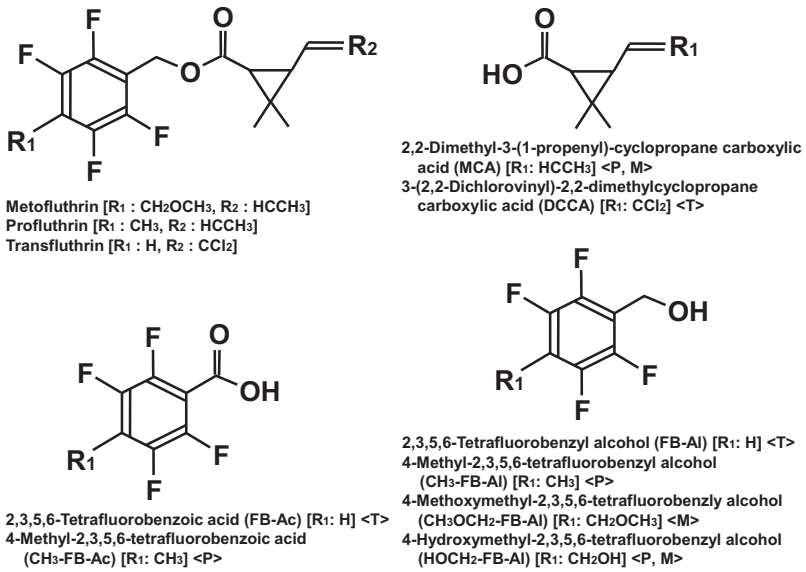


Fig. 1. Chemical structures of the original pyrethroids, metofluthrin, profluthrin and transfluthrin, and 8 urinary metabolites targeted in the present study. ⟨M⟩, ⟨P⟩ and ⟨T⟩ are metabolites for metofluthrin, profluthrin and transfluthrin, respectively, in rats. Abbreviations of the metabolites are shown in parentheses.

Generally, the amounts of compounds absorbed are evaluated using their metabolites excreted *via* the urine. In order to do this, the urinary metabolites that can serve as markers of the absorption amounts of the parent compound must be selected from the metabolites. However, there have been hardly any reports on the human metabolism of the fluorine-containing pyrethroids, and knowledge about their urinary metabolites in animals is also limited [8,9,11,13]. Namely, the biological markers for evaluating their absorption amounts have not been established for humans or animals. In our previous study, the major urinary metabolites of transfluthrin, profluthrin and metofluthrin were identified in rats [14]. The metabolites identified were as follows: 2,3,5,6-tetrafluorobenzyl alcohol (FB-Al), 2,3,5,6-tetrafluorobenzoic acid (FB-Ac) and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (DCCA) for transfluthrin; 4-methyl-2,3,5,6-tetrafluorobenzyl alcohol (CH_3-FB-Al), 4-methyl-2,3,5,6-tetrafluorobenzoic acid (CH_3-FB-Ac), 4-hydroxymethyl-2,3,5,6-tetrafluorobenzyl alcohol ($HOCH_2$-FB-Al) and 2,2-dimethyl-3-(1-propenyl)-cyclopropanecarboxylic acid (MCA) for profluthrin; 4-methoxymethyl-2,3,5,6-tetrafluorobenzyl alcohol (CH_3OCH_2-FB-Al), $HOCH_2$-FB-Al and MCA for metofluthrin (Fig. 1).

Aim of this study was to develop a method for measuring the concentrations of the urinary metabolites in rats dosed with metofluthrin, profluthrin or transfluthrin. Such a method should make it possible to establish suitable biological markers for evaluating the absorption amounts of these pyrethroids in the general population by examining the relationships between their doses and the amounts of their metabolites excreted *via* the urine in animals. Although there have been many reports on quantitative analytical methods for urinary pyrethroid metabolites [15–28], the types of metabolites targeted were limited to compounds such as 3-phenoxybenzoic acid (3-PBA), 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA), DCCA, 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid (DBCA), and chrysanthemumdicarboxylic acid. This is because exposure to conventional pyrethroids, such as cyfluthrin, permethrin, phenothrin, cyphenothrin, cypermethrin and deltamethrin, was assumed in these studies. There are hardly any reports on analytical methods to determine the urinary metabolites, except for DCCA which is one of the metabolites of transfluthrin, in human and animals exposed to metofluthrin, profluthrin or transfluthrin. As DCCA is a common urinary metabolite resulting from many pyrethroids, *e.g.*, cyfluthrin, permethrin and cypermethrin, identification of the parent pyrethroid exposed is not possible from urinary DCCA. On the other hand, metabolites having a tetrafluorobenzene nucleus in their molecules are assumed to be suitable as biological markers for exposure to metofluthrin, profluthrin or transfluthrin because they are characteristic urinary metabolites for the fluorine-containing pyrethroids.

In many previous analytical methods for urinary metabolites of the conventional pyrethroids by gas chromatography/mass spectrometry, the compounds extracted from urine with organic solvents or solid-phase extraction cartridges were determined after derivatization (methylation, hexafluoroisopropylation or *tert*-butyldimethylsilylation) by gas chromatography combined with single quadrupole mass spectrometry (GC/MS) in the electron impact ionization mode (EI) (GC/MS(EI)) [15–20] or in the negative ion chemical ionization mode (NCI) (GC/MS(NCI)) [21–23], or by gas chromatography combined with tandem mass spectrometry (GC/MS/MS) in EI (GC/MS/MS(EI)) [24], or by gas chromatography combined with high resolution mass spectrometry (GC/HRMS) in EI (GC/HRMS(EI)) [25] or in NCI (GC/HRMS(NCI)) [25,26]. In the past, gas chromatography with electron capture detection (GC-ECD) [27,28] has been applied for their analysis. However, the instruments for GC/MS/MS and GC/HRMS, while offering highly sensitive and selective analysis in comparison with GC/MS, are expensive and not readily available in many laboratories. GC/MS(EI) is more cost-effective and a more common analytical method than GC/MS(NCI). In most previous studies on analytical methods for the pyrethroid metabolites containing DCCA by GC/MS(EI), the metabolites hydrolyzed by hydrochloric acid or sulfuric acid were determined after being transformed into their methyl derivatives with methanol and sulfuric acid [15–17] or into *tert*-butyldimethylsilyl derivatives with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTB-STFA) [19,20]. The *tert*-butyldimethylsilyl derivative of DCCA was presumed to have a higher sensitivity than its methyl derivative when comparing the results obtained in these studies. On the other hand, in our previous study [14], the alcoholic metabolites, FB-Al, CH_3-FB-Al, CH_3OCH_2-FB-Al and $HOCH_2$-FB-Al, among the metabolites of the fluorine-containing pyrethroids were not transformed

to their *tert*-butyldimethylsilyl derivatives by MTBSTFA, although the carboxylic metabolites, DCCA, MCA, FB-Ac and CH_3-FB-Ac, were transformed to the corresponding derivatives. In contrast, the carboxylic metabolites were not transformed to their trimethylsilyl derivatives by a mixture of *N*-trimethylsilylimidazole (TMSI) and trimethylchlorosilane (TMCS) although the alcoholic metabolites were transformed. Furthermore, in our preliminary examination, MCA was found to be unstable against acid hydrolysis.

In the present study, a method was developed for analysis of eight urinary metabolites, shown in Fig. 1, in rats dosed with fluorine-containing pyrethroids, *i.e.*, metofluthrin, profluthrin or transfluthrin, in order to identify suitable metabolites as biological markers. The urinary metabolites hydrolyzed using enzymes were extracted with organic sorbents, which were inexpensive compared to solid-phase extraction cartridges. After transformation into their *tert*-butyldimethylsilyl or trimethylsilyl derivatives, they were measured by GC/MS(EI).

2. Experimental

2.1. Chemicals

Authentic standards used to quantify the targeted eight urinary metabolites of the pyrethroids were as given below. FB-Ac, FB-Al, $HOCH_2$-FB-Al and CH_3-FB-Ac were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). DCCA was purchased from Princeton (NJ, USA). MCA, CH_3OCH_2-FB-Al and CH_3-FB-Al, which were not available as reagents, were gifts from Sumitomo Chemical (Tokyo, Japan).

4-Methoxy-2,3,5,6-tetrafluorobenzoic acid (CH_3O-FB-Ac) and 4-methoxy-2,3,5,6-tetrafluorobenzyl alcohol (CH_3O-FB-Al) as internal standards were obtained from ART-CHEM (Berlin, Germany) and AK Scientific (CA, USA), respectively. MTBSTFA from Tokyo Kasei Kogyo and TMSI and TMCS from Wako Pure Chemical (Osaka, Japan) were used as derivatizing reagents for the metabolites and standards. Sulfatase from *Helix pomatia*, Type H-1, lyophilized powder (activity: >10,000 units/g solid, β-glucuronidase activity: >300 units/mg solid, at pH 5.0), was purchased from Sigma–Aldrich (MO, USA). Other chemicals were obtained from Wako Pure Chemical. Toluene and acetone were of a grade for analysis of pesticide residue and polychlorinated biphenyl. Hydrochloric acid (35%, w/w) was of a grade for analysis of hazardous metal, and anhydrous sodium sulfate was for analysis of polychlorinated biphenyl and phthalate. All other chemicals were of analytical-reagent grade. To apply the proposed method, metofluthrin, profluthrin and transfluthrin for dosage to animals were prepared from commercially available household insecticides.

2.2. Instruments

A Shimadzu (Kyoto, Japan) GCMS-QP2010 gas chromatograph mass spectrometer, combined with a Shimadzu AOC-20i auto injector, was controlled with a Fujitsu (Kanagawa, Japan) FM V-6766CL7c computer installed with GCMSsolution software for instrument control and data analysis. A Hitachi (Tokyo, Japan) Model Himac CF7D centrifuge was used. A wave rotor for agitation of samples and a heating block were purchased from Thermonics (Model WR-100, Tokyo, Japan) and Yamato Scientific (Model HF-21, Tokyo, Japan), respectively. A GL Sciences (Tokyo, Japan) Model Soldry SD905 was used to concentrate the sample and standard solutions. A water bath shaker for hydrolysis of conjugates of the urinary metabolites was obtained from Taitec (Model MM-10, Saitama, Japan).

All of the glassware for preparation of the samples and standard solutions used during the experiments were washed with deionized water and acetone and then dried at room temperature.

2.3. Animals and sample collection

Male Sprague–Dawley rats were purchased from Clea Japan (Tokyo, Japan), and were used when 9 weeks old and weighing 340–360 g. Urine samples from rats treated and untreated with the fluorine-containing pyrethroids were collected by using Clea Japan Model CT-10S metabolic cages. Food (Laboratory Chow MF, Oriental Yeast, Tokyo, Japan) and water were available to all animals *ad libitum*. The environment was maintained at a constant temperature (22 °C) and under a light–dark cycle (light period; 08:00–18:00 h) before and during urine sample collection. The samples obtained were stored at −20 °C in a freezer until analysis.

All studies were carried out according to the Guidelines of the Animal Use and Care Committee of the Osaka Prefectural Institute of Public Health.

2.4. Mixed metabolite stock solution and mixed internal standard solution

Each stock standard solution of eight metabolites was prepared with 10.0 mg/ml of each compound by dissolving it in acetone in 10 ml brown high-airtight vials (Kanto Kagaku, Tokyo, Japan). Aliquots of these stock standard solutions were combined, and the mixed metabolite stock solution of eight compounds was prepared at 1000 μg/ml by dilution with acetone in another 10 ml brown high-airtight vial.

A mixed internal standard solution (CH_3O-FB-Ac and CH_3O-FB-Al) was prepared at 5.0 mg/ml by dissolving the compounds in acetone in a 2 ml brown high-airtight vial.

All of these solutions were stored at 4 °C in a refrigerator.

2.5. Sample preparation

A thawed urine sample was centrifuged (3000 rpm × 10 min). The supernatant (2.0 ml) of the sample was decanted into a 10 ml brown screw-capped tube, and was hydrolyzed with 5 mg of sulfatase lyophilized powder at pH 5 for 12 h at 37 °C using the water bath shaker after adding 0.48 ml of 0.35%-hydrochloric acid and 40 μl of acetone to conform to the urine properties of the following standard urine solutions of metabolites. After hydrolysis reaction, 0.1 ml of concentrated hydrochloric acid and 2.4 g of ammonium sulfate were added, and the sample was agitated for 5 min using the wave rotor to sufficiently dissolve ammonium sulfate. Next, 5 μl of the mixed internal standard solution was added to the solution. After 2 ml of toluene had been added to the solution, the sample was shaken with a stirring apparatus for 10 min to extract the metabolites from the urine solution. The organic layer was decanted into another screw-capped tube after centrifugation (3000 rpm × 10 min). The extraction process by toluene was repeated, and the two extracts were combined. After 1 g of anhydrous sodium sulfate had been added to the extract, the supernatant was transferred as 1.5-ml portions to two brown screw-capped tubes to transform the metabolites into their derivatives by using two derivatizing reagents, TMSI and TMCS (TMSI–TMCS), and MTBSTFA. The extracts were concentrated to 1 ml by using the concentration apparatus under a stream of nitrogen gas (flow rate, 150 ml/min per tube; temperature, 60 °C). MTBSTFA at 30 μl was added to the concentrated sample, and 50 μl of TMSI and 10 μl of TMCS were added to the other sample. The metabolites having a carboxylic group in their molecules were transformed into their *tert*-butyldimethylsilyl derivatives by MTBSTFA, and those with an alcoholic hydroxyl group into their

Table 1
Selected target ions and reference ions (*m*/*z*) for GC/MS analysis of metabolites.

Compound	Target	Reference
Derivatization by MTBSTFA		
FB-Ac	251	252
MCA	211	212
CH_3-FB-Ac	265	266
CH_3O-FB-Ac[a]	281	207
DCCA	265	267
Derivatization by TMSI–TMCS		
FB-Al	237	238
CH_3-FB-Al	251	252
CH_3O-FB-Al[a]	267	268
CH_3OCH_2-FB-Al	281	282
$HOCH_2$-FB-Al	339	340

[a] Internal standard.

trimethysilyl derivatives by TMSI–TMCS. The solutions were heated for 30 min at 70 °C using the heating block to form derivatives of the metabolites. After cooling the reaction solutions to room temperature, they were cleaned up twice with 1 ml of purified water. Anhydrous sodium sulfate, 0.7 g, was added to the solutions, and the supernatants were transferred to another 1.5 ml vial for the auto injector as analytical solutions.

2.6. Pooled urine and standard urine solutions of metabolites

Urine samples collected from six rats untreated with the pyrethroids were combined and centrifuged (3000 rpm × 10 min), and the supernatant was used as a pooled urine sample.

The mixed metabolite stock solution was diluted with acetone on each day of analysis at concentrations of 0, 2.5, 5.0, 10, 25, 50, 100, 250, 500 and 1000 μg/ml. Portions of 40 μl of each solution were added to 10 ml brown screw-capped tubes containing 2.0 ml each of the pooled urine, and the working standard urine solutions of metabolites (0–20 μg/ml as the urinary concentrations) were treated in the same manner as above for the samples, except for the addition of 40 μl of acetone to the solutions.

2.7. Gas chromatography/mass spectrometry

The analytical conditions for GC/MS were as follows: injection amount, 1.0 μl; injection mode into the gas chromatograph, splitless; sampling time, 2.0 min; capillary column, DB-5ms (30 m × 0.25 mm I.D., 0.25 μm film thickness, J&W Scientific, CA, USA); column oven temperature, 70 °C (2 min)–12 °C/min–280 °C; injection port temperature, 250 °C; carrier gas, helium; carrier gas pressure, 89.7 kPa; total flow rate, 20.0 ml/min; column flow rate, 1.38 ml/min, interface temperature, 290 °C; ionization method on the mass spectrometer, EI; ionization energy, 70 eV; ionization current, 60 μA; ion source temperature, 200 °C; analytical mode, selected ion monitoring (SIM). The ions selected for SIM quantification of the compounds are listed in Table 1.

2.8. Calibration

The metabolites in the urine samples were determined by the internal standard method. Their concentrations were calculated by interpolation from the linear least-squares regression line of the multi-level standard curve plot of peak-area ratio (area of metabolite/area of internal standard) *versus* the concentration of metabolites in the working standard urine solutions. The internal standards for determining the carboxylic and alcoholic metabolites were CH_3O-FB-Ac and CH_3O-FB-Al, respectively.

2.9. Recovery

Because authentic standards of *tert*-butyldimethylsilyl esters or trimethylsilyl esters of the target metabolites were not available, the efficiency of the derivatizing reaction could not be determined, and therefore, the absolute recoveries of the metabolites during the complete sample preparation could not be evaluated. In this study, the recoveries from the hydrolysis of urine samples until before the derivatization of the metabolites were examined as follows. The pooled urine samples (2.0 ml, $n = 5$) spiked with known amounts (20 μg/ml as the urinary concentrations) of each compound were treated and determined according to the present method. On the other hand, the pooled urine samples non-spiked with the compounds were treated similarly until before the derivatization with MTBSTFA or TMSI–TMCS, and the compounds were spiked to 1.5 ml of the solutions (10 μg/ml as the concentrations in the solutions, $n = 5$). The solutions were concentrated to 1.0 ml, and the compounds were determined after their derivatization. Both quantitative values for each compound were compared.

2.10. Quantification limits

The concentrations of the quantification limits of the metabolites in urine samples were calculated from the quantitative values of the working standard urine solution of the minimum concentration needed to construct the calibration curves. The solution of 2.5 μg/ml of each metabolite concentration was prepared by diluting the mixed metabolite stock solution with acetone. A 40 μl portion of the solution was added to 2.0 ml of the pooled urine sample ($n = 6$) and analyzed according to the present method. The standard deviations of the quantitative values were calculated for each metabolite, and the quantification limits were defined as being ten times the standard deviations [29].

2.11. Precision and accuracy

The precision and accuracy of the method were demonstrated by repeated analysis of the urine samples spiked with each authentic standard. Portions of 40 μl of the mixed metabolite stock solution (1000 μg/ml) were spiked to 2.0 ml of the pooled urine samples ($n = 7$, 20 μg/ml as urinary concentrations), and they were analyzed according to the present method. The precision of this method was evaluated by the relative standard deviations (RSD) in studies with replicate assays ($n = 7$), and the accuracy of the method was evaluated based on the error of the assayed samples relative to their spiked concentrations (RE). Furthermore, the urine samples (2.0 ml) spiked with 40 μl aliquots of the other solutions, which were diluted to the concentrations of 50 and 2.5 μg/ml from the mixed metabolite stock solution, were examined similarly (urinary concentrations: 1.0 and 0.05 μg/ml each of metabolites, respectively).

2.12. Storage stabilities of metabolites in urine samples

The solution of 750 μg/ml of each metabolite concentration was prepared by diluting the mixed metabolite stock solution with acetone. A 1.2 ml of the solution was added to 60 ml of the pooled urine (15 μg/ml as urinary concentrations). The urine solution was transferred in 2 ml portions into 24 brown screw-capped tubes. The metabolites in three tubes were analyzed immediately after preparation. The average concentrations of each compound determined for the tubes were considered to be those of the metabolites at time zero. The remaining tubes were stored at −20 °C in the freezer until analysis. The contents of three samples each were analyzed periodically.

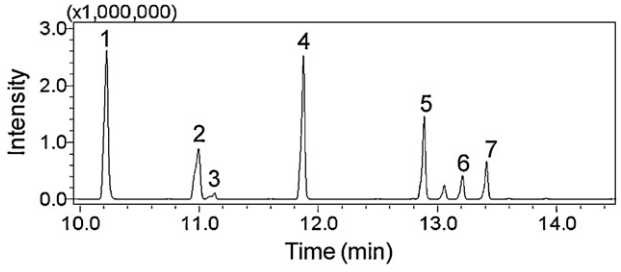
2.13. Application

A single dose of 300 mg/kg body weight of metofluthrin, profluthrin or transfluthrin, diluted in olive oil (100 mg/ml) was injected intraperitoneally to each animal. The pyrethroids and olive oil without the pyrethroids as controls were given to two rats each. The urine from the rats was collected over 48 h after the dosage. The target metabolites in the urine samples were determined by the proposed method.

3. Results and discussion

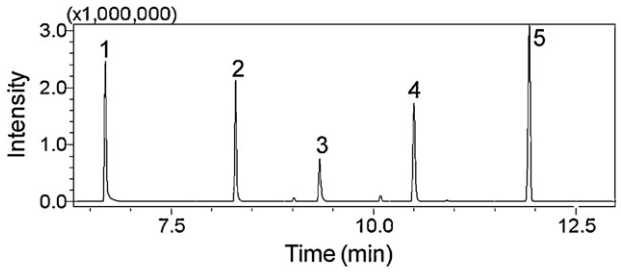
3.1. Chromatography

The chromatograms of the standard urine solutions containing the eight target compounds (concentrations of each metabolite: 20 μg/ml) determined by the present method are shown in Figs. 2 and 3. The carboxylic metabolites were transformed into their *tert*-butyldimethylsilyl derivatives (Fig. 2), and the alcoholic metabolites into their trimethylsilyl derivatives (Fig. 3). All metabolites were resolved within 14 min. The multiple peaks based on the isomers appeared on the chromatogram of MCA and DCCA (Fig. 2). The sections of the chromatograms where the target compounds were detected were free from interfering peaks under both analytical conditions for the metabolites derivatized by MTBSTFA or TMSI–TMCS. Therefore, the present chromatographic method is satisfactory for the separate determination of all of the metabolites in rat urine.



No.	Compound	t_R(min)	No.	Compound	t_R(min)
1	FB-Ac	10.22	5	CH_3O-FB-Ac	12.88
2	MCA (1)	10.98	6	DCCA (1)	13.21
3	MCA (2)	11.12	7	DCCA (2)	13.41
4	CH_3-FB-Ac	11.87			

Fig. 2. Chromatogram of standard urine solution of metabolites derivatized by MTBSTFA (each compound: 20 μg/ml). The figures in parenthesis are to distinguish among the isomers of each compound. CH_3O-FB-Ac: internal standard.



No.	Compound	t_R(min)	No.	Compound	t_R(min)
1	FB-AI	6.69	4	CH_3OCH_2-FB-AI	10.50
2	CH_3-FB-AI	8.30	5	$HOCH_2$-FB-AI	11.93
3	CH_3O-FB-AI	9.34			

Fig. 3. Chromatogram of standard urine solution of metabolites derivatized by TMSI–TMCS (each compound: 20 μg/ml). CH_3O-FB-AI: internal standard.

3.2. Calibration

The slopes and intercepts of the calibration curves obtained by measurement of the standard urine solutions are shown in Table 2. For all compounds, the values of the correlation coefficient of the normal linear regression lines were better than 0.9999 (n = 10), that is, these calibration curves were linear over the concentration range of 0–20 μg/ml in urine.

3.3. Recovery

As shown in Table 2, the recoveries for most metabolites during the sample preparation, namely, hydrolysis of conjugates of the metabolites, extraction from the urine sample by toluene, and dehydration of the extract, were more than 90%, indicating adequate recoveries. On the other hand, the recovery for $HOCH_2$-FB-Al (87%) was low compared to others. Therefore, to accurately quantify the urinary metabolites containing $HOCH_2$-FB-Al, it was necessary to construct calibration curves using the pooled urine spiked with authentic standard materials of the metabolite, and treated in the same manner as the samples.

The recovery of urinary DCCA was 94%, similar to findings in previous studies using a liquid–liquid extraction technique with dichloromethane (91–97%) [22,23] which is more toxic than toluene, or a solid-phase extraction technique (87–104%) [18,21,24] which is more expensive than the liquid–liquid extraction technique, though the recovery was lower with analytical methods using the liquid–liquid extraction with *n*-hexane (77–78%) [19].

3.4. Quantification limits

The quantification limits of the eight urinary metabolites were 0.009–0.03 μg/ml in the urine samples (Table 2). Detection limits of methods for analyzing DCCA in urine samples have been discussed in several previous studies. In the present study, the detection limit of DCCA in the urine samples was defined as being three times [29] the standard deviation that was obtained in the examinations for the quantification limits (Section 2.10). The concentration of the detection limit of DCCA was calculated to be 0.005 μg/ml as the final concentration in the analytical solutions. This value was lower than those (0.008–0.016 μg/ml) calculated from the reports by researchers who analyzed the *tert*-butyldimethylsilyl-derivative of DCCA by GC/MS(EI) [19,20]. Therefore, it was estimated that the detection limits for the other metabolites targeted in the analytical solutions were also sufficiently low in the present study.

3.5. Precision and accuracy

The precision and accuracy of the present method for determination of the urinary metabolites are shown in Table 3. The RSD values for all metabolites were <5%, which indicates good repeatability, for all concentrations of each compound. The RE values for all metabolites were <6% in all samples. These results showed that the present method can offer excellent accuracy and precision, with satisfactorily reliable data.

3.6. Storage stability

The stabilities of the metabolites in urine at −20 °C were examined, and the results are shown in Table 4. For all of the metabolites, no degradation with time was observed over 1 month. The results show that urine samples collected from rats can be stored for up to 1 month at −20 °C protected from light in a freezer until analysis.

Table 2
Calibration graphs, quantification limits and recoveries of urinary metabolites for the present analytical method.

Compound	Calibration graph			Quantification limit (μg/ml)	Recovery[b] (%)
	Slope	Intercept	r^a		
FB-Ac	0.1095	−0.0002	1.0000	0.030	0.99
MCA	0.0482	−0.0029	0.9999	0.021	1.02
CH_3-FB-Ac	0.0968	0.0002	1.0000	0.015	1.00
DCCA	0.0288	−0.0022	0.9999	0.024	0.94
FB-Al	0.1318	0.0033	1.0000	0.009	0.92
CH_3-FB-Al	0.1097	0.0065	0.9999	0.017	0.95
CH_3OCH_2-FB-Al	0.1139	−0.0002	1.0000	0.029	1.05
$HOCH_2$-FB-Al	0.1855	0.0020	0.9999	0.027	0.87

[a] Correlation coefficient ($n = 10$).
[b] Values are means for five samples.

Table 3
Precision and accuracy of the present analytical method.

Compound	20 μg/ml[a]		1.0 μg/ml[a]		0.05 μg/ml[a]	
	RSD[b]	RE[c]	RSD[b]	RE[c]	RSD[b]	RE[c]
FB-Ac	2.4	0.05	4.3	0.6	4.1	2.1
MCA	2.0	4.0	2.8	0.3	3.4	5.6
CH_3-FB-Ac	1.4	1.3	1.7	1.2	1.9	0.06
DCCA	3.0	0.01	3.5	0.7	3.0	1.7
FB-Al	2.8	2.5	2.0	2.2	4.2	0.02
CH_3-FB-Al	3.1	1.1	1.9	1.0	3.2	3.4
CH_3OCH_2-FB-Al	2.3	1.8	1.0	4.5	3.4	5.1
$HOCH_2$-FB-Al	2.0	3.2	1.7	1.3	1.1	4.6

[a] Concentrations of metabolites spiked into pooled urine samples.
[b] RSD values in studies with assays of seven spiked samples (%).
[c] Error of assayed samples relative to their spiked concentrations (%).

3.7. Application

The results of determination of the urinary metabolites in the rats administered metofluthrin, profluthrin or transfluthrin are shown in Fig. 4. None of the targeted metabolites were detected in the urine samples of rats dosed with only olive oil. The concentrations of three metabolites, FB-Ac (225–282 μg/ml), DCCA (253–282 μg/ml) and FB-Al (44–52 μg/ml), which were found in urine samples from rats dosed with transfluthrin, were higher than those of the other metabolites in rats dosed metofluthrin or profluthrin. They were measured after diluting the urine samples and preparing them according to the present procedures. MCA and $HOCH_2$-FB-Al were detected from urine samples of rats administered metofluthrin or profluthrin. The concentrations of CH_3OCH_2-FB-Al (21–24 μg/ml) were highest among the three metabolites identified in the rats dosed with metofluthrin, and the CH_3-FB-Al concentrations (35–43 μg/ml) were the highest among those of the four profluthrin metabolites. Therefore, CH_3OCH_2-FB-Al and CH_3-FB-Al were presumed to be suitable as biological markers to evaluate the absorption amounts of metofluthrin and profluthrin, respectively. On the other hand, FB-Ac or FB-Al might also be useful as a marker for transfluthrin exposure because DCCA is a common urinary metabolite resulting from many conventional pyrethroids such as permethrin, cypermethrin, and cyfluthrin. The analytical method developed in this study should be useful for establishing suitable biological markers for monitoring exposure to fluorine-containing pyrethroids in the general population by examining the relationships between their doses and the amounts of their metabolites excreted into the urine in animal studies.

Table 4
Stabilities of metabolites in urine at −20 °C.

Compound	Storage time (days)						
	1	3	7	10	15	22	30
FB-Ac	104	105	105	97	95	106	105
MCA	108	105	107	94	94	102	104
CH_3-FB-Ac	103	104	102	98	97	102	101
DCCA	100	96	99	100	100	102	103
FB-Al	97	97	97	100	95	97	95
CH_3-FB-Al	98	97	99	101	97	96	98
CH_3OCH_2-FB-Al	101	100	100	99	98	94	96
$HOCH_2$-FB-Al	107	104	103	102	103	92	101

Concentration of each compound just before storage (time: 0) was set at 100% (each compound: 15 μg/ml). The values are means for three samples.

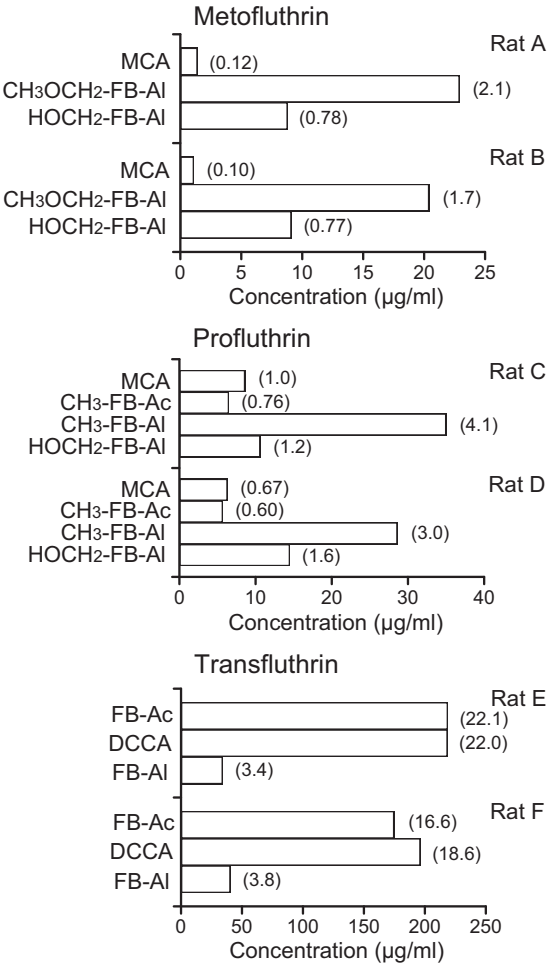


Fig. 4. Concentrations (μg/ml) of metabolites in urine excreted for 48 h after dosage of pyrethroids in rats. A single dose of 300 mg/kg body weight of each pyrethroid was separately administered intraperitoneally to two rats. Figures in parentheses are the urinary excretion amounts (mg) of each metabolite for 48 h after the dosage.

4. Conclusion

To be able to evaluate human exposure to fluorine-containing pyrethroids (transfluthrin, metofluthrin and profluthrin), which are widely used recently as mosquito repellents and mothproof repellents, an analytical method was developed for their eight urinary metabolites (four carboxylic and four alcoholic metabolites) in rats. The urine samples collected were hydrolyzed with sulfatase and β-glucuronidase at pH 5, and concentrated hydrochloric acid and a saturated amount of ammonium sulfate were added to the hydrolysate. The carboxylic and alcoholic metabolites were extracted with toluene and concentrated, then transformed into their *tert*-butyldimethylsilyl derivatives by MTBSTFA and into their trimethylsilyl derivatives by TMSI–TMCS, respectively. They were analyzed by GC/MS(EI) after cleanup of the reaction solutions with purified water. The calibration curves were constructed by preparing and analyzing the standard urine solutions spiked with authentic materials of the metabolites in the same manner as the samples. The metabolites displayed linear calibration curves over the urinary concentration range of 0–20 μg/ml, and could be determined accurately and precisely at the concentrations studied. The quantification limits of the metabolites were 0.009–0.03 μg/ml in urine. In addition, the urine samples collected could be stored for up to 1 month at −20 °C in a freezer until analysis.

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