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Simultaneous determination of flupyrazofos and its metabolite 1-phenyl-3-trifluoromethyl-5-hydroxypyrazole and flupyrazofos oxon in rat plasma by high-performance liquid chromatography with ultraviolet absorbance detection

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Abstract

An isocratic high-performance liquid chromatography (HPLC) system with UV detection was developed for simultaneous determination of flupyrazofos and its metabolites, 1-phenyl-3-trifluoromethyl-5-hydroxypyrazole and flupyrazofos oxon, in rat plasma. Optimal analytical conditions involved an analytical cartridge column consisting of a phenyl bonded phase, a mobile phase of 50 m*M* phosphate buffer (pH3.0)–acetonitrile (40:60, v/v) and a UV detection wavelength of 232 nm. Under these conditions the peaks of flupyrazofos and its metabolites were all well separated and the total time for complete separation was less than 12 min. The limit of quantitation was 40 ng/ml for flupyrazofos and 20 ng/ml for PTMHP. Recoveries from rat plasma were higher than 90%. Following intravenous administration of flupyrazofos, the method has been successfully applied in a toxicokinetic study in rats involving plasma samples. Therefore, the current method is a valuable analytical tool for investigating the metabolism and toxicokinetics of flupyrazofos.

Keywords: Flupyrazofos; 1-phenyl-3-trifluoromethyl-5-hydroxypyrazole; Flupyrazofos oxon

1. Introduction

A variety of organophosphorus insecticides have been developed since the first one was synthesized in 1937 by a group of German chemists led by Gerhard Schrader [1]. Flupyrazofos (*O,O*-diethyl *O*-(1-phenyl-3-trifluoromethyl-5-pyrazoyl)thiophosphoric acid ester) was recently developed by our institute (Korea Patent No. 36152, U.S. Patent No. 4822779) as an organophosphorus insecticide having a common mechanism of activity, inhibition of acethylcholinesterase, shared by many other organophosporus insecticides [2]. In particular, this agent has been found to be very effective against the diamond-back moth (*Plutella xylostella*) [3]. Critical factors associated with the development of any chemical insecticide include its biological activity in insects and its safety as far as mammals and the environment are concerned. Consequently, strenuous efforts have been made to assess the safety of

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flupyrazofos in animals and the environment using a number of in vivo and in vitro systems [3–6]. Toxicokinetic monitoring of flupyrazofos is an essential component of such research. Therefore, this study was carried out to develop a new method for determining flupyrazofos and its metabolites in plasma using reversed-phase HPLC. The method has been applied to a toxicokinetic study and some of the kinetic parameters obtained are discussed.

2. Experimental

2.1. Materials and reagents

Flupyrazofos (purity >98%), 1-phenyl-3-trifluoromethyl-5-hydroxypyrazole (PTMHP, >99%), *O,O*-diethyl-*O*-(1-phenyl-3-trifluoromethyl-5-pyrazoyl) phosphoric acid ester (flupyrazofos oxon, >97%) and diazinon were kindly supplied from Sung-bo (Ansan, Korea). Sodium phosphate (monohydrate), phosphoric acid and perchloric acid were obtained from Sigma (St. Louis, MO, USA). Potassium fluoride was purchased from Duksan (Yongin, Korea) and heparin sodium from Korea Green Cross (Yongin, Korea). Hydrogenated caster oil was a kind gift from LG Chemical (Taejon, Korea). All the other chemicals and reagents were commercial products of analytical grade. Flupyrazofos and its metabolites, PTMHP and flupyrazofos oxon, were stored at −20°C until use.

2.2. Sample preparation

Sprague–Dawley rats (ca. 250 g body weight, Korea Research Institute of Chemical Technology, Toxicology Center Breeding Facility) were housed under specific pathogen free conditions under a constant 12-h light and dark cycle and given sterilized tap water and a laboratory rodent diet (Jeil Food, Taejon) ad libitum. Blood samples were collected from the tail vein [7] and plasma was prepared by centrifugation (Eppendorf, Centrifuge 5414C). The plasma samples were stored at −20°C until analysis. Plasma proteins were precipitated using a triple volume of methanol, and aliquots (20 μl) of the supernatant were injected into the HPLC column.

2.3. Chromatography

Chromatographic analysis was performed using an HPLC system with ultraviolet detection (UVD). The system consisted of a UV detector (Spectra Focus, Spectra Physics, San Jose, CA, USA), a pump (SP8800, Spectra Physics) and a fixed-loop injector (20 μl, Rheodyne, Cotani, CA, USA). The analytical columns consisted of a phenyl-bonded phase cartridge (PNC, Nova-Pak phenyl, 4 μm, 100×8 mm I.D., Waters Assoc., Milford, MA, USA), a phenyl-bonded column (PN, Capcell-Pak phenyl, 5 μm, 250×4.6 mm I.D., Shiseido, Tokyo, Japan) and C18 (Inertsil ODS, 5 μm, 250×4.6 mm I.D., GL Sciences Inc., Japan). The mobile phase was a mixture of 50 m*M* phosphate buffer (pH 3) and acetonitrile (60:40, v/v) and the flow-rate was 1.0 ml/min. The column temperature was ambient and the UV wavelength selected for detection was 232 nm.

2.4. Selection of column and mobile phase

Both the peak intensity and retention profiles for flupyrazofos and its metabolites, PTMHP and flupyrazofos oxon, were compared using three different types of columns including PNC, PN and ODS. In addition, the retention time profiles for PNC were also examined at various pH values (3, 4.5 and 6) of phosphate buffer, and at various fractions of acetonitrile (50, 60 and 70%) in the mobile phase.

2.5. Linearity

The inter-and intra-day reproducibility of peak linearity were examined over the range 0.1–20 μg/ml by spiking drug-free plasma with known amounts of flupyrazofos and its metabolites. The linear-regression equation and the correlation coefficient were calculated by the least-squares method.

2.6. Precision, accuracy and recovery

The precision and accuracy of the method was determined by replicate analysis of spiked samples at four different concentrations over the range 0.4–20 μg/ml. The accuracy was evaluated as a percent error [(mean of measured–mean of added)/mean of added]×100 [8]. The analytical recovery of the

compounds was determined by comparing the peak area obtained from plasma samples with that measured using equivalent amounts of each compound.

2.7. Limits of quantitation (LOQ) and detection (LOD)

The LOQ or LOD was determined from the signal-to-noise ratio (S/N ratio). The LOQ was defined as the same concentration of the compounds resulting in an S/N ratio of 10 and the LOD was defined as the sample concentration resulting in an S/N ratio of 4.

2.8. Stability

The stability of flupyrazofos, PTMHP and flupyrazofos oxon in methanol (standard solution) was assessed at −20°C. For the stability study in plasma, control rat plasma samples were spiked with 0.4, 2, 10 and 20 μg/ml of flupyrazofos and PTMHP. The short-term stability was assessed after 4 h of storage at both room temperature and 4°C, and the long-term stability was assessed over 4 weeks after storage in a freezer at −20°C freezer. The stability of flupyrazofos oxon was separately examined due to its rapid hydrolysis to PTMHP in plasma. The stability was examined over a 2 h period at room temperature. The stability of flupyrazofos and PTMHP in extract was also examined over 24 h at room temperature and 1 week at −20°C.

2.9. Intravenous kinetics of flupyrazofos

Flupyrazofos was emulsified in 25% ethanol solution containing 0.25% hydrogenated caster oil and administrated intravenously to rats via the jugular vein under light diethylether anesthesia at a dose of 50 mg/kg. Blood samples (ca. 200 μl) were collected at 10 min, 20 min, 40 min, 1 h, 2 h, 4 h, 7 h, 10 h, 14 h, 24 h and 48 h after injection. Pharmacokinetic analysis was performed using a model-independent method [9]. The terminal phase elimination rate constant (k_{el}) was determined by least squares regression analysis of the terminal elimination phase of the plasma concentration–time data, and the terminal phase elimination half-life ($t_{1/2}$) from the ratio $0.693/k_{el}$. The area under the plasma concentration–time curve (AUC) was calculated by the trapezoidal rule. The total body clearance (CL_t) was calculated from dose/AUC and the apparent volume of distribution during the elimination phase ($V_{d,\beta}$) was calculated from CL_t/k_{el}. The maximum plasma concentration (C_{max}) and the time to maximum concentration (t_{max}) for the metabolite PTMHP were directly obtained from the observed values.

3. Results

3.1. Retention characteristics

As shown in Table 1, optimal conditions were obtained using the PNC reversed-phase column, which produced a minimum peak-width and maximum height for the compounds with a total run time of 12 min (Fig. 1). With the ODS column, the retention of flupyrazofos was 44.6 min. In the blank plasma samples there were no peaks that interfered at the retention time of the three compounds when the PNC column was used. Peak retention times and intensity were also examined using various buffer pH values and acetonitrile fractions (Fig. 2). Although PTMHP was not detected at pH 6, no marked changes in retention times and peak areas were observed with increasing buffer pH values. Retention times were significantly reduced and peak areas were slightly increased with an increase in the amount of acetonitrile in the mobile phase.

3.2. Linearity

The standard curves of flupyrazofos and its metabolite PTMHP spiked in plasma were linear over the concentration ranges 0.1–20 μg/ml. Since

Table 1
Elution profiles in different types of columns

Column	Retention time (min)		
	PTMHP	Flupyrazofos oxon	Flupyrazofos
ODS	7.75	13.7	44.6
PN	5.21	7.44	16.5
PNC	4.58	6.15	11.6

The mobile phase was a mixture of 50 m*M* phosphate buffer (pH 3) and acetonitrile (40:60%).

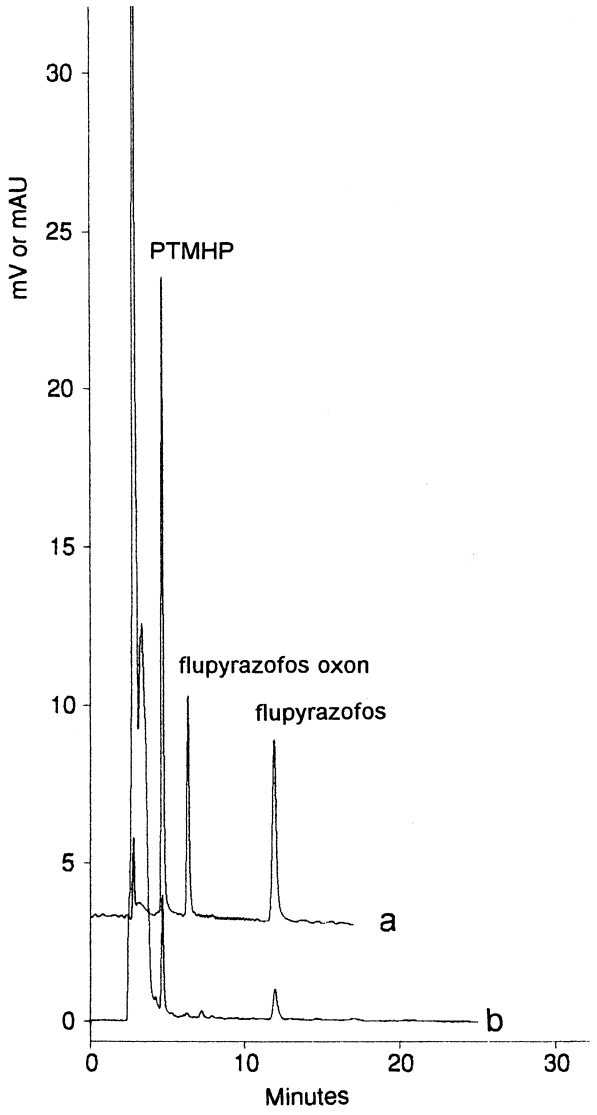

Fig. 1. Chromatogram of a standard solution containing flupyrazofos, PTMHP and flupyrazofos oxon (a), and chromatogram of plasma following administration of flupyrazofos (b). The mobile phase was a mixture of 50 m*M* phosphate buffer (pH 3) and acetonitrile (40:60%). The detection wavelength was 232 nm. The detailed chromatographic conditions and sample preparation are described in Section 2.

flupyrazofos oxon was promptly converted to PTMHP in plasma, its linearity was monitored using a non-spiked standard, where the standard curve was linear over the range 0.1–20 μg/ml. The correlation coefficients were greater than 0.999 for each compound and the coefficients of variation (C.V.) for the slope were smaller than 8%. No significant difference in linear regression was observed between inter- and intra-day assays (Table 2).

3.3. Precision, accuracy and recovery

The results obtained for precision and accuracy, determined by conducting repeated analysis of spiked plasma samples at four different concentrations, are listed in Table 3. The precision, expressed as a mean percentage of the C.V. and accuracy were 6.78% and 9.88% for flupyrazofos, and 4.39% and 6.40% for PTMHP, respectively. The mean recoveries of flupyrazofos and PTMHP from blood plasma were 90.1% and 93.6%, respectively. The values for flupyrazofos oxon were calculated from the value of recovered PTMHP due to its rapid conversion to PTMHP in plasma. The mean percentage C.V., accuracy and recovery were 3.96%, 16.5% and 83.6%, respectively.

3.4. Limits of quantitation and detection

The limit of quantitation was 40 ng/ml for flupyrazofos and 20 ng/ml for PTMHP, while the limit of detection was 20 ng/ml for flupyrazofos and 10 ng/ml for PTMHP, and 20 ng/ml for flupyrazofos oxon.

3.5. Stability

Flupyrazofos and PTMHP were stable in plasma at room temperature, at least up to 4 h. They also remained intact at −20°C for up to 4 weeks. As far as the run-time stability of processed samples after protein precipitation was concerned, no significant loss of flupyrazofos and PTMHP was observed both at room temperature for up to 24 h and at −20°C for up to 1 week. However, flupyrazofos oxon was unstable in plasma (Fig. 3). Most of the flupyrazofos oxon was converted to PTMHP immediately after addition to plasma. On the other hand, flupyrazofos oxon was relatively stable in water and methanol. Esterase inhibitors, including diazinon and potassium fluoride (0.5%), did not block its conversion to PTMHP (Fig. 3).

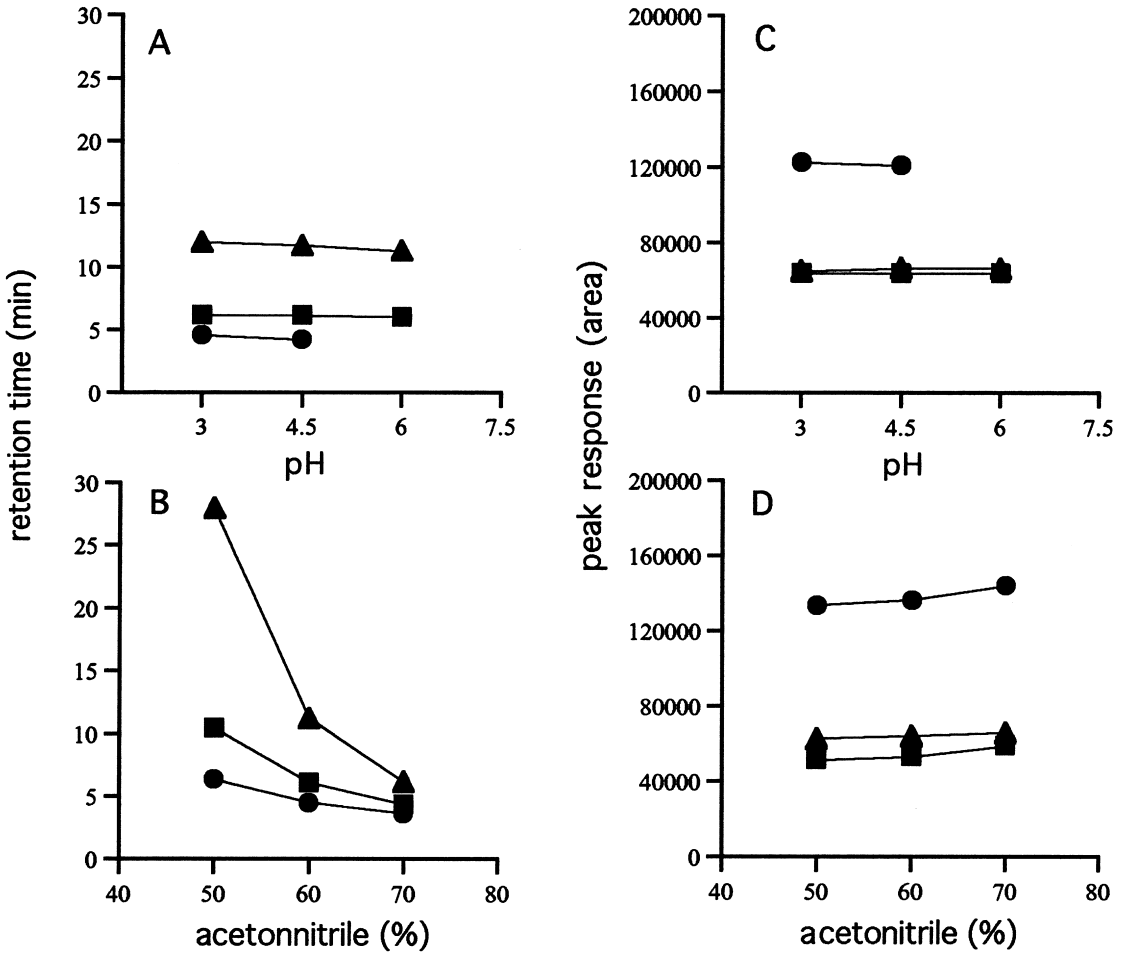

Fig. 2. Retention and peak response characteristics of flupyrazofos (▲) and its metabolites PTMHP (●) and flupyrazofos oxon (■) (30 ng/inj. for each) under various combinations of mobile phase. A and C: The pH value of the phosphate buffer in the mobile phase was changed, but the acetonitrile fraction in the mobile phase was fixed at 60%. B and D: The acetonitrile fraction of the mobile phase was changed, but the pH value of the mobile phase buffer was fixed at 3.0.

3.6. Intravenous kinetics

The proposed method was used for the determination of flupyrazofos and its metabolite PTMHP in rat plasma samples. After a single bolus intravenous administration of 50 mg/kg, plasma concentrations of flupyrazofos declined in a multi-exponential fashion (Fig. 4). PTMHP appeared rapidly in the plasma and then decreased in fashion similar to the parent compound (Fig. 4). The k_{el}, $t_{1/2}$, AUC, CL_t and $V_{d,\beta}$ of flupyrazofos were 0.0368 h^{-1}, 18.8 h, 41.19 h/μg/ml, 1.214 l/h/kg and 32.99 l/kg, respectively. The maximum blood concentration (C_{max}) of 12.44 μg/ml for PTMHP was observed at 10 min post-dosing (t_{max}). The k_{el}, $t_{1/2}$, and AUC of PTMHP were 0.0484 h^{-1}, 14.3 h and 30.17 h.μg/ml, respectively.

4. Discussion

As shown in Fig. 1, flupyrazofos and its metabolite PTMHP are well separated in the chromatogram of plasma, whereas flupyrazofos oxon is not detected due to its rapid hydrolysis to PTMHP in plasma. As a medium for protein precipitation, three volumes of

Table 2
Assay linearity

Reproducibility	Compound	Correlation coefficient of the linear regression[a] (r, mean±S.D.)	Slope (a, mean±S.D.)	Intercept (b, mean±S.D.)
Intra-day (n=4)	PTMHP	0.9998±0.0002 (C.V.=0.02%)	68835±2248 (C.V.=3.27%)	−476±748
	Flupyrazofos oxon[b]	0.9999±0.0001 (C.V.=0.01%)	28848±597 (C.V.=2.07%)	522±804
	Flupyrazofos	0.9996±0.0003 (C.V.=0.03%)	28000±791 (C.V.=2.83%)	−421±682
Inter-day (n=8)	PTMHP	0.9998±0.0002 (C.V.=0.02%)	66681±3831 (C.V.=5.75%)	1034±1354
	Flupyrazofos	0.9996±0.0007 (C.V.=0.07%)	27624±2196 (C.V.=7.95%)	−4±555

[a] Linear unweighted regression, formula: $y=ax+b$.
[b] The linearity for flupyrazofos oxon was obtained as non-spiked standard, because it is promptly converted to PTMHP in plasma.

methanol or acetonitrile, and an equal volume of 0.5 *M* perchloric acid were compared using plasma samples. Methanol was found to be best. There was no significant variation in the linear regression parameters between inter- and intra-day reproducibility studies. The C.V. and accuracy were less than 10% for both flupyrazofos and PTMHP, and the absolute recovery was over 90%. The LOQ and LOD are sufficient for routine toxicokinetic monitoring. Therefore, the present HPLC method is a rapid and simple assay procedure for flupyrazofos and its metabolites in plasma.

Although flupyrazofos and its metabolite PTMHP are stable, its oxon form is unstable in plasma. Most of flupyrazofos oxon is hydrolyzed to PTMHP in rat plasma within a few min after spiking, but this metabolite is relatively stable in water (Fig. 3). Therefore, to check a possible presence of esterase(s)

Table 3
Precision, accuracy and recovery

Compound	Theoretical concentration (μg/ml)	n	Experimental concentration (μg/ml, mean±S.D.)	C.V. (%)	Mean recovery (%)	Accuracy (%)
PTMHP	0.4	8	0.345±0.020	5.80	86.3	13.7
	2.0	8	1.891±0.088	4.65	94.5	5.5
	10.0	8	9.637±0.373	3.87	96.3	3.7
	20.0	8	19.40±0.631	3.25	97.3	2.7
Flupyrazofos oxon	0.5	3	0.415±0.026	6.27	82.9	17.1
	1.0	3	0.891±0.049	5.50	89.1	10.9
	5.0	3	4.504±0.103	2.29	90.2	9.8
	20.0	3	14.38±0.255	1.77	72.6	28.0
Flupyrazofos	0.4	8	0.399±0.051	12.5	99.8	0.2
	2.0	8	1.695±0.084	4.96	84.7	15.3
	10.0	8	8.615±0.342	3.97	86.1	13.9
	20.0	8	17.97±1.020	5.68	89.9	10.1

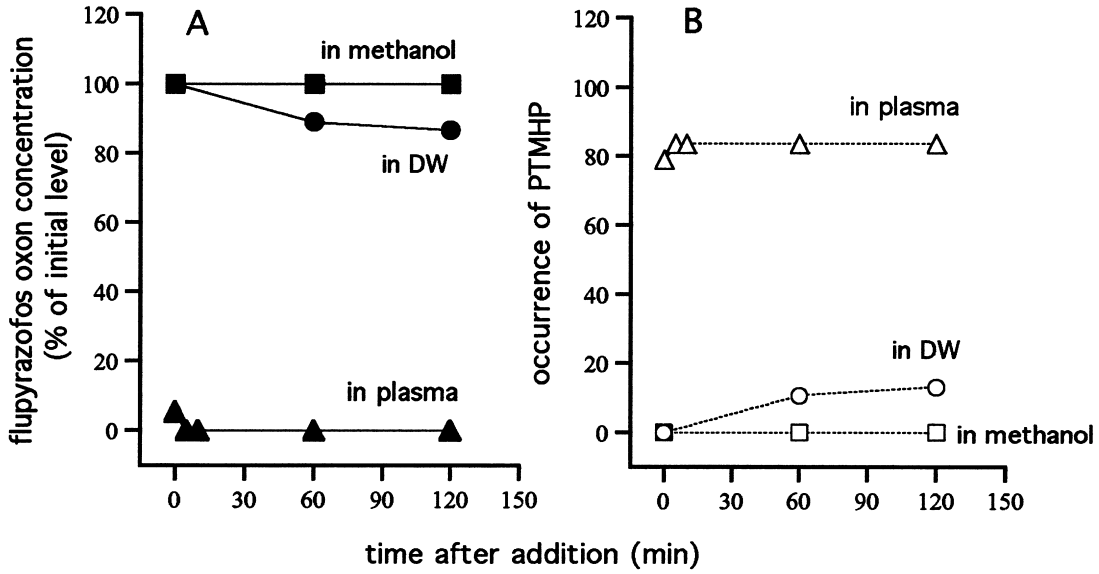

Fig. 3. Stability of flupyrazofos oxon. The stability was examined under three conditions; in distilled water, methanol and rat plasma. A represents the degradation profiles of flupyrazofos oxon and B represents the occurrence of PTMHP resulting from the hydrolysis of flupyrazofos oxon. The initial concentration of flupyrazofos oxon was 5 μg/ml.

in plasma, the well-known esterase inhibitors, diazinon and potassium fluoride, were added to plasma. However, the conversion of flupyrazofos oxon to PTMHP was not blocked. This suggests that conversion is by chemical hydrolysis rather than esterase-mediated enzymatic hydrolysis. Our previous report [10] also noted that the hydrolysis of the oxon form to PTMHP proceeded non-enzymatically in an in vitro study using microsomes.

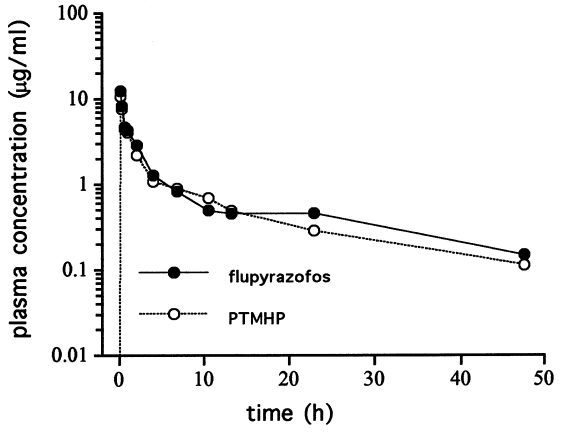

Fig. 4. Plasma concentration–time curve for flupyrazofos and its metabolite PTMHP following bolus intravenous administration of flupyrazofos at a dose 50 mg/kg in rat. The values represent the mean of two rats.

To confirm the suitability of the method, an intravenous kinetic study was performed at a dose of 50 mg/kg. The proposed method accurately characterizes the kinetics of flupyrazofos and its metabolite PTMHP in plasma showing that there is a multi-exponential decline in concentrations. No marked differences in the elimination parameters, including k_{el} and $t_{1/2}$, together with AUC, were observed between the parent compound and metabolite PTMHP. The plasma C_{max} of PTMHP was observed in the first sample collected at 10 min post-dosing. These results indicate that PTMHP occurs in plasma as a major metabolite of flupyrazofos and metabolic conversion is very rapid in vivo. Therefore, although we have no evidences that PTMHP is toxic, it is necessary to consider the toxicokinetics of PTMHP together with that of the parent compound in any future estimation of flupyrazofos toxicity in vivo.

Acknowledgements

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