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Development and Validation of Liquid Chromatography Tandem Mass Spectrometry Method and Its Application in Pharmacokinetic Study of Acetamiprid in Sprague-Dawley Rat

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ABSTRACT

Present study was aimed to develop and validate a liquid chromatography spectrometry method and pharmacokinetic analysis of acetamiprid in Sprague Dawley rat. For the development of pharmacokinetic method, ChemT was used as internal standard. Extraction and recovery of plasma analyte were done using 0.1% formic acid and acetonitrile solution. An eleven point standard curve was prepared by spiking 2µl of working stock of concentration 2ng/ml. Three precision accuracy samples were processed on three separate days. The apparent maximum plasma concentration and the time at which it was achieved were determined. The area under the plasma concentration-time curve was also measured. The elimination rate constant was 0.14±0.06 per hour and the terminal half-life after oral administration were found to be 6.58±1.85h^{-1}. LC-MS/MS method proved to be sensitive and simple tools for PK/TK parameters of any new chemical entities.

INTRODUCTION

For food security and agricultural productivity, pest control is an essential component. As herbivorous pests, weeds and pathogens can cause significant losses in staple food crops unless control measures are in place (Nakasu *et al*., 2014). Among all the insecticides used in agricultural field, neonicotinoids group is most important (Swenson and Casidaas, 2013) as resistant strains of insects are developing to carbamate, organophosphate, organochlorine, pyrethroid compound, which is impetus to its development (Alizadeh *et al.,* 2014). The neonicotinoids, the newest major class of insecticides, have outstanding potency and systemic action for crop protection against piercing-sucking pests, and they are highly effective for flea control on cats and dogs (Tomizawa and Casida, 2005). Acetamiprid, a member of the neonicotinoid insecticide family, is a fairly new insecticide that has recently entered into the marketplace but it is having some non-target effect on mammals (Nakasu et al., 2014).

Dissemination of acetamiprid into the environment has necessitated accurate identification of their potential hazards to animal and human health. Pharmacokinetic studies constitute an important phase in the process of designing a toxicity study of a molecule in order to select the most appropriate route of administration and the best dose regimen (Uys et al., 2010; Liu et al., 2010).

Quite a number of techniques have already been reported by investigators for the quantification of acetamiprid in rat plasma by high-performance liquid chromatography (HPLC) (Pinthong et al., 1991) or liquid chromatography tandem mass spectrometry (LCMS/MS) (Yang et al., 2012) and solid phase extraction (SPE) for sample preparation which is very costly as well as having longer runtime for analysis (Wangboonskul et al., 2006).

The objective of the present work was to develop a simple, accurate, sensitive method and validate the LC-MS/MS method for the determination and quantification of acetamiprid in rat plasma. The total run time for analysis was short and easier process of sample preparation i.e., protein precipitation. This gives an advantage compared with the previously published method. The method was also validated for selectivity, sensitivity, recovery, linearity, accuracy, precision, and stability according to the USFDA guidelines, 2001. There is no such report about a pharmacokinetic study of acetamiprid in this method in rats. Therefore the present study has

been taken to understand the pharmacokinetic profile as well as to validate the tandem mass spectrometry in acetamiprid exposure. The method was successfully applied to a single dose oral pharmacokinetic study at 10 mg/kg dose.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade water (resistivity of 18 M.cm) generated from Milli-Q water purification system, methanol (9093-68), acetonitrile (9017-03) from JT Baker, acetamiprid (33674-100MG-R) from sigma and acetamiprid technical grade from JU Pesticides and Chemical Pvt. Ltd were purchased. Blank rat plasma with EDTA-K_3 anticoagulant used throughout the analysis.

Instrumentation and chromatographic conditions

The liquid chromatography (LC) system consisting of the LC-20ADvp pump (Shimadzu, Kyoto, Japan), CTC PAL (HTS) autosampler, and the mass spectrometer composed of turbo ion spray with atmospheric pressure ionization source (API-4000, AB Sciex Instruments, Foster CA). Detection and quantification were performed using Analyst 1.4.2.

LC-MS/MS analysis was performed on a C18 column (2x30 mm, 5 µm) from phenomenex (CA, USA) with a flow rate of 0.8 ml/min at room temperature. The mobile phases were 0.1% formic acid in water (A) and a mixture of acetonitrile and water with 0.1% formic acid i. e. solvant A (80:20, v/v) (B). The gradient elution program was as follows, first 60 sec only A for washing and then 30 sec for gradient up to 100% B and it was continued for next 60 sec and total run time was 3.0 min. The column eluent was split and approximately 350 µl was introduced in the mass spectrometer.

Preparation of stock and working solutions

The primary stock solution of acetamiprid was prepared by dissolving the accurately weighed standard compound in DMSO to obtain 2 mg/ml. The working stock solutions for calibration control were 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.488, 0.244, 0.122, 0.061 µg/ml and 0.183, 23.44 and 46.88 µg/ml for LQC, MQC and HQC respectively, made by serial dilution and all stock solutions were stored in polypropylene vials at -20^{0}C.

Preparation of calibration standards and quality control (QC) samples

An eleven point standard curve was prepared by spiking 2 µl from working stock to 98 µl blank plasma to get final concentration 1250.00, 937.50, 625.00, 312.50, 156.25, 78.13, 39.06, 9.80, 4.90, 2.44, 1.22 ng/ml and final concentration of ChemT as internal standard (IS) in plasma was 300 ng/ml. Three levels of QC samples along with a lower limit of quantitation (LLOQ) at a concentration of 3.66 ng/ml, 468.75 ng/ml (medium quality control, MQC) and 833.33 ng/ml (high-quality control, HQC) for acetamiprid were also prepared following the appropriate dilution method in DMSO. QC samples were bulk spiked and then aliquoted into pre-labeled 2-ml polypropylene vials and stored at -20°C.

Sample preparation

The aliquot of 0.02 ml of plasma was taken into 96 well MT plate and direct precipitation of matrix was done by adding ice cold acetonitrile (4x of sample volume). 10 µl of IS solution was mixed with the sample. The mixture was vortexed in a thermomixer and centrifuged at 5000 rpm for 10 min at 10^0C. 60 µl of clear supernatant was transferred to a 96 deep well plate and diluted with 60 µl of water. After thoroughly mixing the final plate was loaded into the CTC PAL autosampler cabinet and 10 µl aliquots was injected to LC-MS/MS.

Method validation

The bioanalytical method was validated for selectivity, linearity, precision, accuracy, recovery, matrix effect (ME), and stability according to the principles of Food and Drug Administration (FDA) industry guide. Three precision accuracy batches were processed on three separate days. Each batch included two sets of calibration standards and six replicates of LLOQ, LQC, MQC and HQC samples to evaluate the accuracy and precision of the method.

Selectivity and specificity

The selectivity and specificity were carried out by analyzing 6 different blank plasma samples from rats, to demonstrate the lack of chromatographic interference from endogenous plasma at the retention time of the analyte and IS.

Lower Limit of quantitation, linearity

LLOQ was determined from the analyte peak signal and baseline noise level. Calibration curves were acquired by plotting the peak area ratio of the analyte to that of IS against the nominal

concentration of calibration standards. Analyte concentration of different CC and QC samples were prepared as mentioned above. Results were fitted to the linear regression analysis to determine the linearity of the calibration curve. Acceptance criterion for each back calculated standard concentration was ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20% (U.S. F.D.A., 2001).

Accuracy and precision

Within-run and between-run precision and accuracy was determined by analyzing six replicates at three QC levels (LQC, MQC, and HQC) and LLOQ on three different days. Criteria for acceptability of the data included accuracy within ± 15% coefficient of variation (CV), except for LLOQ, where it should not exceed ± 20% of SD (U.S. F.D.A., 2001).

Extraction recovery and matrix effect

The extraction recovery of acetamiprid at three different QC levels (n=6) was evaluated by comparing the spiked analyte response and with the response from post-extracted matrix standard sample at equivalent concentration and matrix effect on analyte was also determined.

The matrix effect of acetamiprid at three different QC levels was evaluated by comparing the spiked plasma or tissues response and particular standard working solution response.

Recovery (%) = (peak area of extracted analyte x 100/peak area of non extracted analyte). ME (%) = {1-(response for post-extraction spiked drug) / (response in pure solvent)} x100

Stability

Stability study was evaluated as part of the method validation. The processed sample stability was evaluated by comparing the precipitated samples that were injected immediately (time = 0 h), with the samples that were re-injected after loading into the autosampler at 4^0C for 12 h. Stability of analyte in the biomatrix after 24 h exposure on bench-top at two QC levels in six replicates was determined and analyzed against freshly spiked standard curve and QC samples for short time stability. The long-term stability of spiked rat plasma was stored at -20^0C and evaluated by analyzing all QC samples that were stored at -20^0C for 30 days together with freshly spiked standard curve and QC samples. The freeze-thaw stability was conducted by comparing the stability samples that had been frozen and thawed thrice, with the plasma thawed once (U.S. F.D.A., 2001).

In-vivo experiment

Animals were kept in open cage system in the separate experimental room and allowed 7 days to be acclimatized. Sterilised husk was used as bedding material. Changing frequency of bedding material was twice in a week. Rats were provided standard feed and water *ad libitum.*

A day before experiment the caudal portion of the rat was cleaned by using auto trimmer (NL9206AD-4, Philips, Indonesia). In a pharmacokinetic study (n=5), acetamiprid was administered orally at 10 mg/kg dose to determine the PK parameters. On the day of sampling vaseline, gel was applied to the region of saphenous vein course. A pressure was applied to thigh muscle to engorge the vein. By using 26 gauge needle, a single prick was done on the vein. Collection of blood was done initially in capillary tube and then into the endorf. Approximately 150 µl of blood was collected with K_3EDTA via saphenous vein puncture method (Beeton et al, 2007) at each time point (0, 0.25, 0.5, 1, 2, 4, 6, 8, 10 and 24hrs). Every time before collection either scab formed at prick site was removed or a fresh prick was given for recollection of blood. The blood sample was centrifuged at 3500 rpm for 5 min at 15^0C to harvest plasma and stored at -20^0C until bioanalysis.

Pharmacokinetic analysis

The concentrations of acetamiprid in plasma were determined at each time point (Fig. 5). The pharmacokinetic parameters, like area under the plasma-concentration-time curve from zero to last measurable plasma sample time and to infinity (AUC_{0-24} and AUC_{0-inf}), maximum concentration (C_{max}), time to reach maximum concentration (T_{max}), elimination rate constant (K_{el}), and elimination half-life ($t_{1/2}$) were determined by non-compartmental method. The area under the plasma concentration-time curve, $AUC_{0-tlast}$, was calculated from time 0 to the last quantifiable time point, using linear trapezoidal method. The area from the last to infinity ($AUC_{tlast-\alpha}$) was estimated as $C_{est(last)}/K_el$, where $C_{est(last)}$ represents the quantifiable concentration at the last time point. The total area under the curve ($AUC_{0-\alpha}$) was estimated as the sum of AUC_{0-last} and $AUC_{tlast-\alpha}$.

RESULTS

Optimization of chromatography

Chromatographic conditions were optimized to obtain high sensitivity, reproducibility, and sample throughput. A silica-based C8 and phenyl-hexyl and C18 column remain a good starting point because of their high efficiency and stability. The analyte was retained on the C18 column which benefited the retention time of the analyte due to its hydrophobicity. Mobile phase systems consisted of 0.1% v/v acetic acid or formic acid in water or 2 mM ammonium acetate buffer or acetonitrile or methanol or in various proportions of the following were tested whereas flow rate, loop size was fixed at 0.8 ml/min and 25 µl respectively. Adjustment of pH was also investigated for proper retention, peak shape as well as improved response. Finally, optimal separation as well as with good sensitivity and peak shape was achieved with solvent A=0.1% formic acid in water and solvent B = acetonitrile: water A (80:20 v/v) with 0.1% formic acid at a flow rate of 0.8 ml/min in C18 column.

Specificity and Selectivity

Positive electrospray mass spectra of acetamiprid showed an intense $[M+H]^+$ ion at *m/z* 223.20 ion and for IS $[M+H]^+$ ion at *m/z* 687.20. After fragmentation of these parents (Q1) ions produce most intense daughter ions at *m/z* 126.2 and 320.20 respectively. The collision energy and other optimized parameters for analyte and IS are presented (Table 1).

Separation and specificity

No interference peak was found in the MRM profile for double blank plasma and matrix blank (Fig. 1, Fig. 2) at the retention time for both IS and analyte. A representative chromatogram at LLOQ concentration and test sample were depicted in Fig. 3 and Fig. 4. The total chromatographic run time was 3.0 min.

Limit of quantitation, linearity

Lower limit of quantitation was established as 1.22 ng/ml for plasma. The equation of the calibration curve was obtained by linear regression analysis of the peak area ratio of analyte to internal standard versus concentration. The curve was linear in the concentration range 1.22 to 1250.00 ng/ml with an average regression coefficient of 0.9947 ± 0.0011. The calibration curve

shows with the average slope and intercept 0.01008 (±0.006), and 0.0110 (±0.003) respectively. All back calculated values indicate excellent accuracy and precision.

Accuracy and precision

The back-calculated concentration values for QCs run in six triplicate at each concentration level on six different occasions, were used to assess the accuracy and precision of the assay. The between-run and within-run precision for the various concentrations ranged from 2.84 to 7.32% and 3.88 to 8.88% and accuracy was between 88.53%–110.72% and 92.85% to 109.59% respectively (Table 2 and Table 3).

Recovery & Matrix effect

Six replicates of LQC and HQC samples were prepared for recovery as well as matrix effect determination. Mean extraction recovery and matrix effect were at LQC, MQC and HQC levels were ranged from 87.57% - 92.25% and 1.62% - 4.57% (Table 4).

Pharmacokinetic application

The apparent maximum plasma concentration, $C_{max,}$ (9.83±0.94 µg/ml) and the time at which it was achieved, $T_{max,}$ (3.33±0.67h) were determined. The area under the plasma concentration-time curve, i.e., $AUC_{0\text{-}tlast,}$ (140.98 ± 20.36 µg.h/ml) and $AUC_{0\text{-}\alpha,}$ (158.87±28.09 µg.h/ml) were also measured. The elimination rate constant, K_{el} was 0.14±0.06 per hour and the terminal half life, $T_{1/2}$ after oral administration were found to be 6.58±1.85h^{-1} (Table 5).

DISCUSSION

In this study the pharmacokinetic parameters of acetamiprid were determined and the apparent Cmax reported, was 9.83 µg /ml and it was achieved within 3.33 hrs post dose. The elimination half life was found to be 6.58hrs while the total exposure (AUC0–α) was 158.87 µg.hr/ml. Taira et al (2013) also noted qualitative profiling and quantification of neonicotinoid metabolites in human urine by liquid chromatography coupled with mass spectrometry. European food safety authority panel on plant protection products and their residues (PPR) in scientific opinion on the developmental neurotoxicity potential of acetamiprid revealed that acetamiprid absorbed rapidly and Cmax reached within 0.5-7 hrs post oral dosing. The present study of the pharmacokinetic profile of acetamiprid is in agreements with the panel's observation.

The LC-MS/MS method described here has significant advantages over the other technique already described in the literature. The method has proved to be sensitive, simple and it was successfully applied for pharmacokinetics study of acetamiprid in experimental rats and could be used for plasma analysis of toxicokinetic study.

Conflict of interest

The authors declare that there is no conflict of interest.

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Table 1: MS parameters of acetamiprid and internal standard

Compound	Q1	Q3	DP	EP	CE	CAD	IS
Acetamiprid	223	126	85	10	32	5	5000
ChemT	687	320	80	10	35	5	5000

Table 2: Intra day and interday accuacy and precission of acetamiprid.

Matrix		Intra-day accuacy and precision			Inter-day accuacy and precision		
	Quality Contol	Concentration of acetamiprid (ng/ml)					
		Mean	%CV	%accuracy	Mean	%CV	%accuracy
Plasma	LLOQ	1.24	5.57	101.64	1.21	5.12	99.18
	LQC	3.59	5.41	98.087	3.45	3.86	94.262
	MQC	472.85	4.73	100.87	482.11	3.31	102.85
	HQC	859.81	1.65	103.18	799.51	2.41	95.942

Table 3 Stability study data of acetamiprid

Matrix	Stability test data of acetamiprid				
	Quality Contol	Stability	Measured concentration (ng/ml)		
			Mean	%CV	% accuracy
Plasma	LQC	0 h	3.69	6.72	100.82
		24 hrs autosampler	3.72	5.88	101.64
		8 hrs bench top	3.55	6.11	96.99
		third freeze-thaw	3.49	6.72	95.35
		30 days at -20^{0}C	3.65	4.65	99.73
	HQC	0 hr	840.51	3.28	100.86
		24 hrs autosampler	812.12	3.76	97.45
		8 hrs bench top	860.5	3.68	103.26
		third freeze-thaw	810.21	3.19	97.22
		30 days at -20^{0}C	795.5	3.47	95.46

Table 4: Extraction recovery and matrix effect of acetamiprid

Matrix	Spiked concentration (ng/ml)					
	3.66		468.75		833.33	
	Recovery (%)	Matrix Effect (%)	Recovery (%)	Matrix Effect (%)	Recovery (%)	Matrix Effect (%)
Plasma	87.57±6.56	4.57± 0.25	89.45±3.32	2.82±0.16	92.25±3.09	1.62±0.12

Table 5: Pharmacokinetic parameter of oral acetamiprid in SD rat

PK parameter	Mean±SEM
AUC0-24(hrs *ug/mL)	140.98±20.37
AUC0-inf(hrs *ug/mL)	158.87±28.09
Cmax(ug/mL)	9.84±0.94
Tmax(hrs)	3.34±0.67
Kel(1/hr)	0.14±0.06
T1/2 (Terminal)(hrs)	6.58±1.85
Cl(mL/ hrs /kg)	67.3±12.51
Vd(mL/kg)	577.52±118.13
MRT(hrs)	7.37±0.88

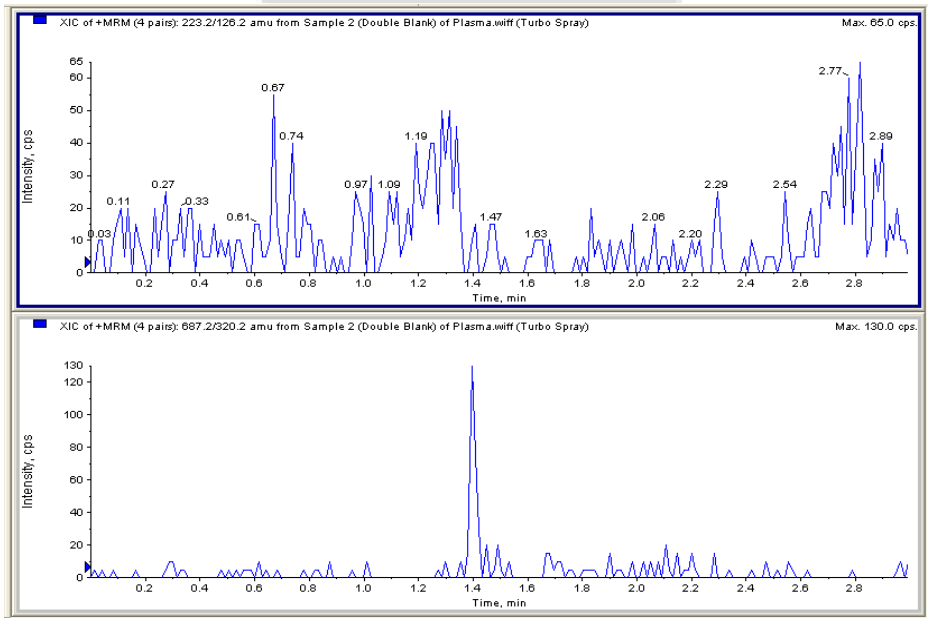

Fig. 1 Chromatogram of double blank plasma (without analyte and internal standard).

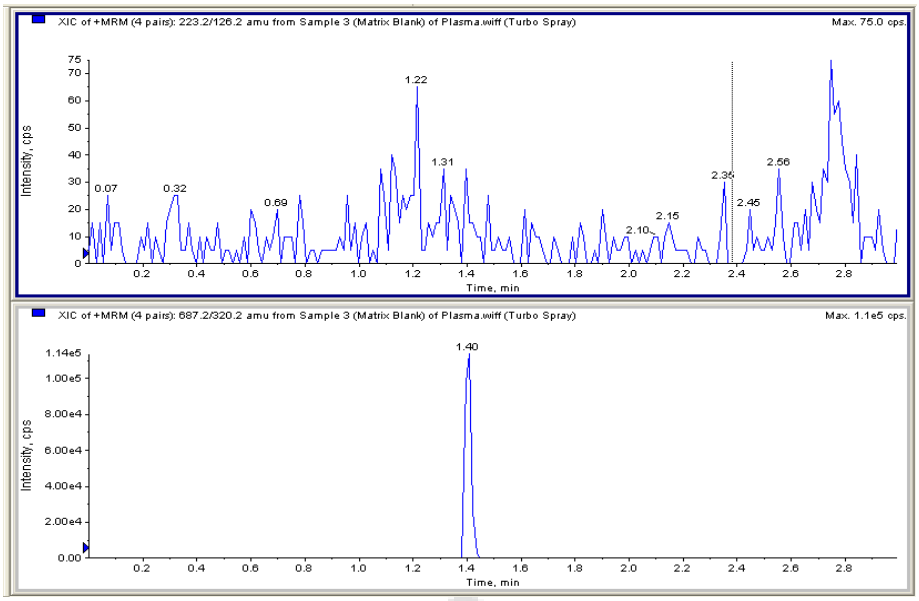

Fig. 2 Chromatogram of matrix blank (with internal standard).

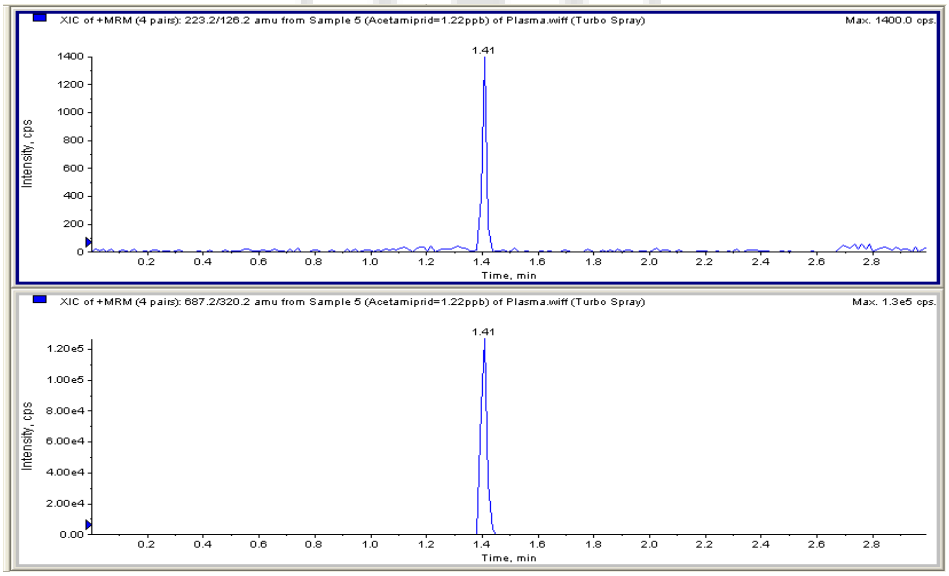

Fig. 3 Chromatogram of LLOQ.

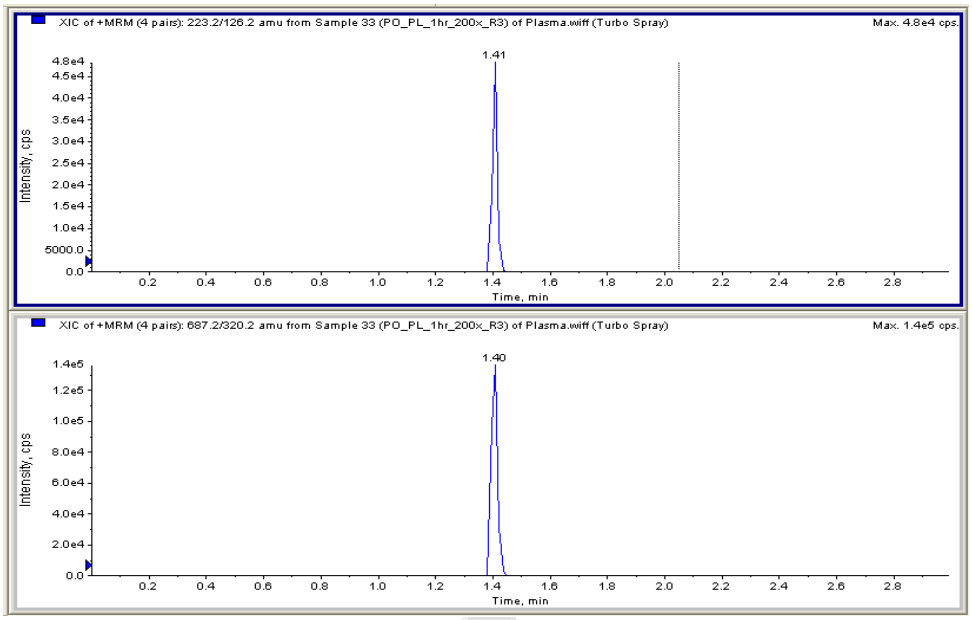

Fig. 4 Specimen chromatogram of test sample.

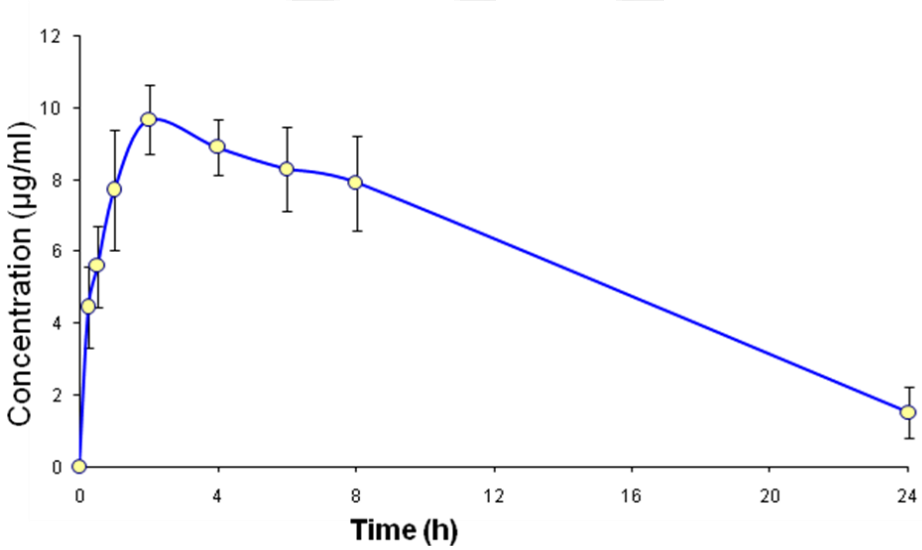

Fig. 5 Mean (± SEM) concentration of plasma exposure of acetamiprid after oral exposure at 10mg/kg.