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In vitro and *in vivo* studies on degradation of quinalphos in rats

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ABSTRACT

A pharmacokinetic *in vitro* and *in vivo* degradation study has been carried out in rat to evaluate the deleterious effects of exposure to quinalphos on a target population. Degradation of quinalphos in simulated gastric and intestinal phases has been investigated. The metabolic intermediates of quinalphos in serum and urine of albino rats at different time intervals were identified after dosing the animals with 5 mg kg^{-1} body weight. All the samples were lyophilised, extracted and analysed by HPLC and GC–MS. The rate of degradation of quinalphos was accelerated in the presence of the enzymes pepsin and pancreatin contained in the gastric and intestinal simulations, respectively. Quinalphos oxon, *O*-ethyl-*O*-quinoxalin-2-yl phosphoric acid, 2-hydroxy quinoxaline and ethyl phosphoric acid are among the important metabolites identified both in *in vitro* and *in vivo* investigations. In simulated *in vitro* study some isomerised derivatives which were missing in the blood and urine of treated animals were identified. This could possibly be either due to non-formation or faster decay of the isomerised derivatives because of slightly different conditions prevailing in the two cases. The results also indicate that the metabolites, 2-hydroxy quinoxaline and oxon, which are more toxic than the parent compound, seem to persist for a longer time.

1. Introduction

Pesticides are among the few toxic materials deliberately disbursed into the environment to preserve the agricultural produce. Besides the use as agrochemicals, the pesticides are the known homicidal and suicidal agents because of their easy availability [1–3]. According to a WHO estimate as many as three million people per year are poisoned by pesticides resulting in around two hundred thousand deaths. Therefore, the various monitoring programmes need to fulfil the obligation to know as to what is the behaviour of pesticide in the human system. It is important to know the compounds formed during the passage of time and their effects on human systems. The answers to some of these aspects need to be available. Sometimes, because of the social and ethical reasons and delayed response of concerned vigilance authorities, there is a time lag and, invariably, the causative pesticide may be available only in traces in the system. In the absence of a reliable information on the decay products problems are generally encountered by forensic experts to identify the source of poisoning. Thus, the requirement of generating a data bank of metabolites for forensic and epidemiological investigations becomes imperative. By identifying the metabolites in the excreta or stomach wash it may be easier to detect the original pesticide, even if it has decayed substantially. A knowledge of metabolites formed in the human system may be significantly important particularly when the metabolites are more toxic than the parent compound. An analysis of intact pesticides and specific metabolites in body fluids can confirm the absorption and identification of the compound that caused the poisoning [4,5].

A number of studies carried out on various pesticides reveal that exposure to the pesticides produce undesired impacts on enzymes and hormones. Erdoğan et al. [6] reported significantly enhanced expression levels of metallothionein A, metallothionein B and cytochrome P450 1A in rainbow trout muscles. Alterations in gene expressions and enzymatic activity due to pesticide exposure have also been reported [7–9]. Quinalphos (*O*,*O*-diethyl-*O*-quinoxalin-2-yl-phosphorothioate) is a well known organophosphate pesticide which finds a wide spread agricultural usage. It is a systemic pesticide [10] and affects mainly skin, eyes and central nervous system [11–13]. Sarkar et al. [14] concluded that quinalphos decreases fertility efficiency in adult male rats by affecting the pituitary gonadotrophins directly. Vairamuthu and Thanikachalam [15] observed some severe effects on the blood and brain esterase activity in chickens. The effects grew more as the quinalphos dosage increased.

The metabolic fate of quinalphos is well documented in water, soil and plants [16–19]. It is rapidly metabolised in these matrices. Only a few studies are available on the persistence of quinalphos and formation of different metabolites in humans or animals [20,21]. Practically in all the systems 2-hydroxyquinoxaline is the

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main metabolite of quinalphos [17,19]. It is known to photocatalytically destroy antioxidant vitamins and biogenic amines *in vitro* and is also genotoxic. Vassilic et al. [22] detected two phosphorus-containing hydrolytic products in human serum and urine after dosing the humans with quinalphos. According to them, the presence of these metabolites may be taken as an indicator of the exposure to the quinalphos rather than its toxicity [23].

It is evident from the available information that a good number of studies are focused on the toxicological aspects of quinalphos. Some studies are also reported on its degradation under controlled and field conditions. However, no systematic data seem to be available on time dependent metabolic pathway in biological fluids. Therefore, it is planned to carry out a comprehensive study on the *in vitro* and *in vivo* pharmacokinetics of quinalphos in animals. Studies were conducted *in vitro* by simulating digestive system conditions in the laboratory and *in vivo* by administering quinalphos to albino Wistar rats, a species similar to humans. At different time intervals the parent compound and the different metabolites formed were analysed in simulated *in vitro* and blood and urine samples. The simulated studies indicate a time-dependent degradation of the pesticide in the gastric and intestinal phases. The GC–MS data obtained from the *in vitro* and *in vivo* studies suggest the possible pathways of the pharmacokinetics of quinalphos. The results on metabolites may be helpful in assigning possible contributors towards the toxicity of quinalphos in addition to parent.

2. Experimental

2.1. Reagents

Quinalphos standard (99.0% purity) was purchased from Merck (Dr. Ehrenstorfer GmbH Augsburg, Germany) and its purity was checked by HPLC. Methanol used was of chromatographic grade (Rankem, India). Pepsin (1130 U/mg protein), pancreatin (4 × USP activity) and bile extract were procured from Sigma Aldrich (St. Louis, MO, USA). Sodium bicarbonate and hydrochloric acid, used to maintain the pH of the deionised water, were of analytical grade from SRL (India). Deionised water was employed throughout the studies. All the chemicals were used without further purification.

A stock solution of quinalphos with a concentration of 10 mg mL^{-1} was made in dimethyl sulphoxide and stored in dark under refrigeration. It was checked that the stock solution at −4 °C is stable for six months.

2.2. HPLC system

For the decay studies Waters 2489 HPLC system (Waters Corporation, USA) equipped with a UV detector was employed. HPLC system consisting of two high pressure pumps with a flow capability of 9.9 mL min^{-1} and automated gradient controller was used to program the elution system. An octadecyl endcapped RP-C_{18} column (4.6 mm i.d × 25 cm, 5 μm) and Waters universal injector of 100 μL capacity were used. Different mobile phases were tried and the optimum mobile phase composition for quinalphos was methanol in isocratic mode. The UV detector was set at 236 nm. 20 μL sample was injected and a flow rate of 1 mL min^{-1} was maintained. The system was operated at the ambient temperature. Under the specified conditions the retention time for quinalphos is 3.6 min. Calibration curve was constructed by plotting standard peak area *versus* concentration. A linear plot (r^2 = 0.99) was obtained in the concentration range 0.0001–0.5 mg mL^{-1}. Recoveries were calculated as the ratio of peak–area of the analyte from the fortified samples to the corresponding peak–area ratio of standard solutions.

2.3. GC–MS system

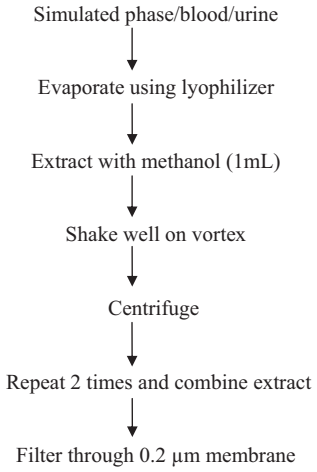
Degradation products of quinalphos were identified with a gas chromatograph interfaced with a mass selective detector (Perkin Elmer-Clarus 500). GC was fitted with an Elite-5 capillary column (Crossbond 5% diphenyl and 95% dimethyl polysiloxane; 30 m × 0.32 mm i.d. × 0.25 μm film thickness, Perkin Elmer™ instrument, Shelton, CT, USA). Helium was used both as the carrier (1 mL min^{-1}) and the make-up gas (40 mL min^{-1}). A split/splitless injector in the splitless mode at 250 °C was used. The transfer line temperature was set at 280 °C and oven temperature was programmed from 60 °C (2 min hold) to 300 °C (5 min hold) at the rate of 10 °C min^{-1}. The injected volume and scan time were 2 μL and 0.2 s, respectively. Chromatographic data was acquired by recording the full scan mass spectra in the range m/z 50–500. Mass spectra corresponding to the identified chromatographic peaks were interpreted directly according to the fragments. Chromatographic data acquisition and processing were carried out with a Turbo mass software (Perkin Elmer-Clarus 500).

2.4. Simulated digestion

For simulation studies method described by Bergqvist et al. [24] was used with a slight modification. In brief, 25 μL of 10 mg mL^{-1} sample of the quinalphos was taken in triplicate for gastric and intestinal simulations along with blank controls in deionised water. The pH of each solution was adjusted to pH 2 with HCl, followed by addition of 0.3 mL of freshly prepared pepsin solution (0.16 g per mL in 0.1 M HCl). The mixture was incubated for simulated gastric digestion on an incubator shaker at 120 rpm at 37 °C. Triplicate samples for time points of 0, 0.5, 1 and 2 h were analysed for gastric digestion. The pH of the remaining gastric digest was brought to 7 by dropwise addition of 1 M $NaHCO_3$. A volume of 1.7 mL pancreatin–bile mixture (0.12 g bile extract and 0.02 g Pancreatin in 5 mL 0.1 M $NaHCO_3$) was mixed with each sample and incubated as described earlier. Triplicate samples were analysed at 0, 0.5, 1 and 2 h intervals for intestinal digestion [25]. The intestinal digests were placed on ice to stop the enzymatic activity.

2.5. Animal groups and treatment

All the experiments were performed as per the guidelines of the Institutional Animal Ethics Committee. The national laws according to the Committee for the Purpose of Control and Supervision of Experiments on Animals were applicable. The project had prior approval from the same committee (registration number: 563/02/a/CPCSEA). Further, "principles of the laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed. Experiments were carried out on pathogen-free albino Wistar rats, *Rattus norvegicus*, of age group 6–8 weeks, purchased from the animal house facility of the National Institute of Pharmaceutical Education and Research (Chandigarh, India). They were housed in a well-ventilated animal house at the Department of Biotechnology, IIT Roorkee in polypropylene cages bedded with sterilised rice husks under a 12 h light/dark cycle. The animals were fed *ad libitum* with a balanced animal feed (Ashirwad Animal Feed Industries, Punjab, India) and had access to the normal drinking water at all the times. The animals were allowed to acclimatize to the laboratory environment for 1 week and then randomly divided into 10(I–X) groups (n = 3). The group I animals were gavaged with the vehicle only (Sham). All the animals were administered with 5 mg kg^{-1} b.wt. quinalphos in DMSO suspended in PBS by oral gavaging and sacrificed after 0.5, 1, 2, 3, 6, 9, 12, 18 and 24 h. It is important to note that all the rats were starved for 12 h before dosing with the quinalphos.



Flowsheet 1. Extraction procedure for *in vitro* and *in vivo* samples.

At definite time points urine was collected using the standard procedures [26,27]. The blood was collected from left ventricle at the same time interval after sacrificing rats under the pentobarbital anaesthesia. The urine and the blood serum samples were stored immediately at −40 °C till the sample processing. All the samples from the same group were pooled together before analysis in order to get a good recovery and statistical representation.

The decay profile of the pesticide was followed in different lyophilised samples of the simulated digestion, blood serum and urine, collected at different time period. Several procedures for the extraction of the pesticide from *in vitro* and *in vivo* samples were tried and judged on the basis of recovery and interferences encountered. Methanol was found as the most suitable extractant. The different steps involved in the extraction procedure are given in the Flowsheet 1. The recovery was 95 ± 3%. The decay was measured for a maximum of 2 h in simulated gastric and intestinal phases and 24 h in rats.

At periodic intervals triplicate samples were removed and analysed by HPLC for the determination of residual quinalphos. The limit of detection for HPLC was 0.0001 mg mL^{-1}. The standard deviation (n = 3) for the measurement of concentration at 0.5 mg mL^{-1} in simulated and biological samples was 1.0 and 1.9, respectively. At periodic intervals duplicate samples were withdrawn and analysed by GC–MS for the identification of metabolites. Controls and blanks were run wherever necessary.

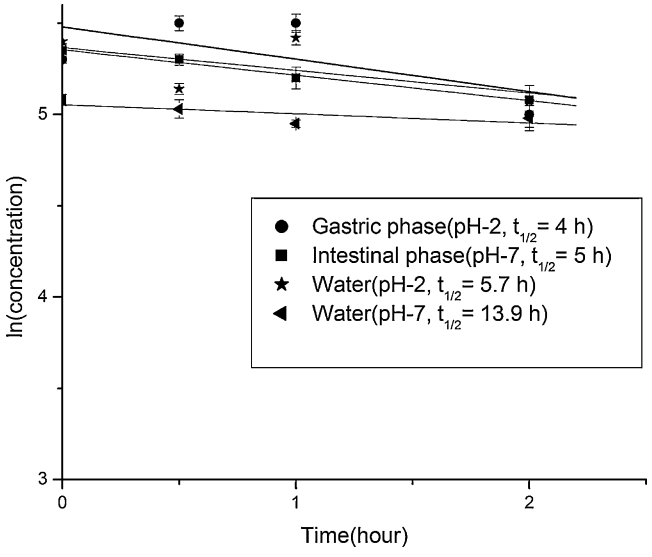


Fig. 1. Persistence of quinalphos in simulated gastric and intestinal phases.

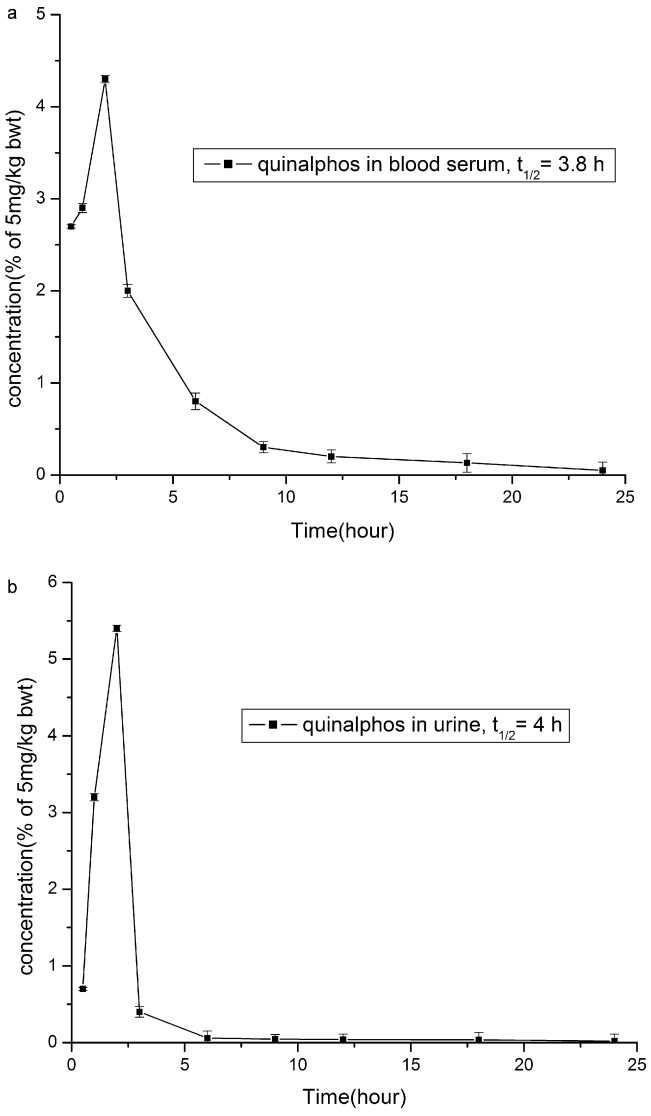


Fig. 2. Persistence of quinalphos in blood serum (A) and urine (B).

3. Results and discussion

3.1. Pharmacokinetics of quinalphos in simulated in vitro

Studies on the degradation of quinalphos in simulated gastric phase (pH 2.0) and intestinal phase (pH 7.0) were carried out at 37 °C at different time intervals (0, 0.5, 1 and 2 h). Simultaneously blank studies were carried out in deionised water adjusted at pH 2.0 and pH 7.0.The data plotted on a natural log scale gave straight lines indicating that the degradation of the pesticide followed a first order kinetics (Fig. 1). The rate of degradation was relatively faster in gastric ($t_{1/2}$ = 4.0 h) and intestinal ($t_{1/2}$ = 5.0 h) phases than that in the blank solution at pH 2.0 ($t_{1/2}$ = 5.7 h) and at pH 7.0 ($t_{1/2}$ = 13.9 h). It is apparent from the control studies that the degradation of quinalphos is faster in acidic conditions. Results further suggest that the presence of pepsin and pancreatin enzymes is leading to a faster decay. This could possibly be due to enzymatic ester hydrolysis. Erah et al. [28] have also observed a faster decay of amoxicillin, clarithromycin and metronidazole in the presence of human gastric. A

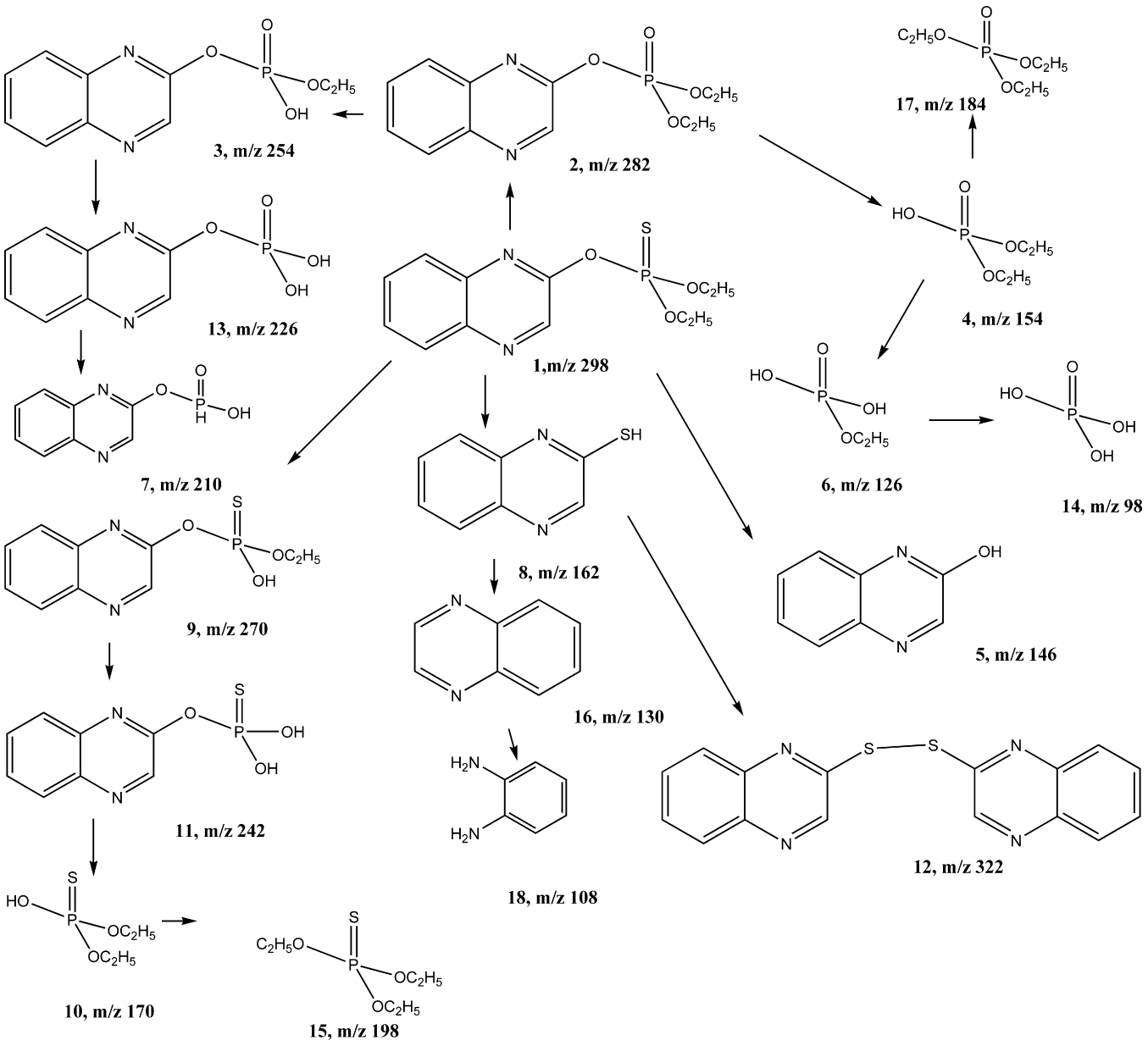


Fig. 3. Metabolic pathways of quinalphos.

similar observation has been reported on cephalosporin prodrug esters in the presence of intestinal juices [29].

3.2. Pharmacokinetics of quinalphos in Wistar albino rats

All the animals survived a single acute oral administration dose of quinalphos (5 mg/kg). The persistence of quinalphos for 24 h in the blood and urine of albino rats is presented in Fig. 2A and B), respectively. The profiles of concentration observed in the biological fluids (Fig. 2A and 2B) suggest that quinalphos is rapidly absorbed and reaches a peak concentration in 2 h after dosing and thereafter it is metabolised with a half life of 3.8 and 4.0 h in the blood and urine, respectively. Similar trend has been reported earlier for chlorpyriphos in rat and humans [30].

3.3. Identification of metabolites of quinalphos

The metabolic pathways of quinalphos in *in vitro* and *in vivo* studies are shown in Fig. 3. In *in vitro* studies, the samples were withdrawn after 0.5, 1 and 2 h interval whereas in *in vivo* investigations the blood and urine samples were collected at intervals of 0.5, 1, 2, 3, 6, 9, 12, 18 and 24 h. After processing the samples were analysed by GC–MS for the identification of metabolites.

3.3.1. In vitro studies

3.3.1.1. Gastric digestion (pH 2). The GC–MS of the 0.5 h sample showed the formation of a product with a molecular ion peak at *m*/*z* 282 (M+) and a fragment ion peak at *m*/*z* 146. On the basis of mass spectrum it was identified as quinalphos oxon (2) formed by the oxidation of P=S bond to P=O. The mass spectrum showed another product with a molecular ion peak at *m*/*z* 254 (M+) and a fragment ion peak at *m*/*z* 146. It was tentatively identified as *O*-ethyl-*O*-quinoxalin-2-yl phosphoric acid (3) formed by the de-alkylation of quinalphos oxon (2).

The GC–MS of 1 h incubated sample showed the formation of (2) and (3) in addition to the other metabolites, identified as diethyl phosphoric acid (4), 2-hydroxy quinoxaline (5), ethyl phosphoric acid (6) and quinoxalin-2-yl hydrogen phosphonate (7) with

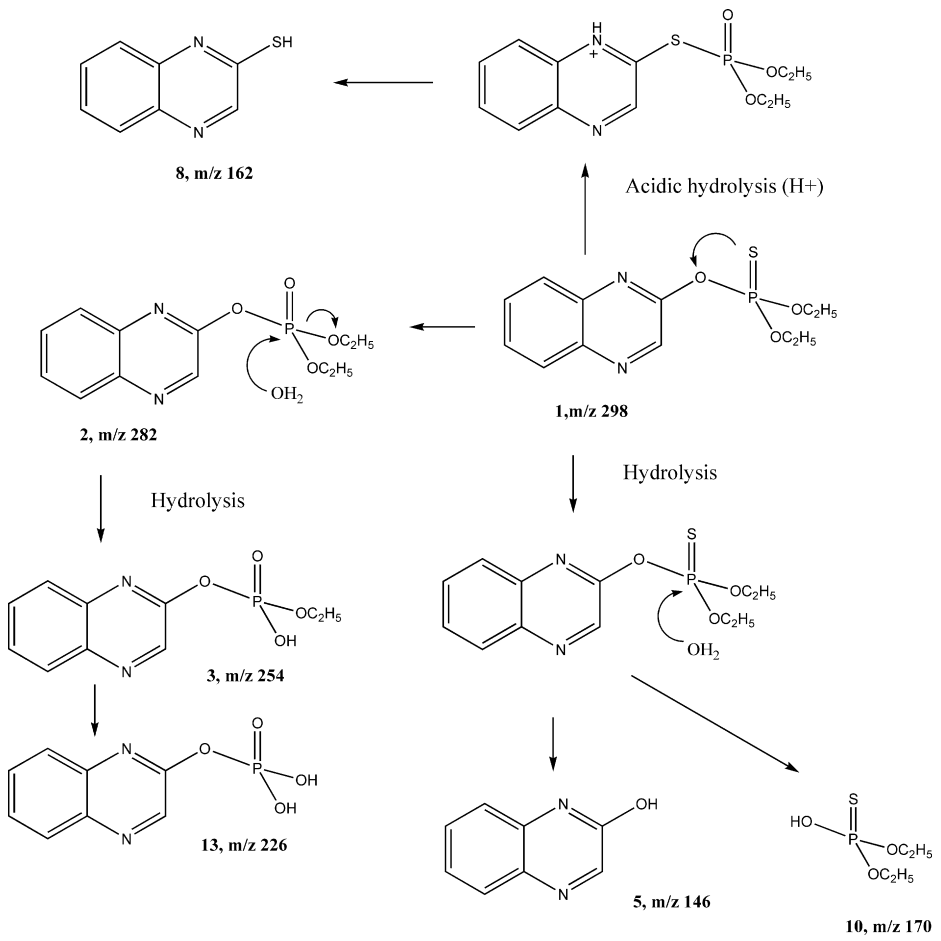


Fig. 4. Mechanism of hydrolysis of quinalphos.

molecular ion peaks at *m*/*z* 154 (M+), *m*/*z* 146(M+), *m*/*z* 126 (M+) and *m*/*z* 210(M+), respectively.

The GC–MS of 2 h incubated sample, besides the peaks of products (2) and (5), showed another metabolite with a molecular ion peak at *m*/*z* 162 (M+). This new product was identified as quinoxaline-2-thiol (8) formed by isomerisation of quinalphos to isoquinalphos followed by hydrolysis.

3.3.1.2. Intestinal digestion (pH 7). The sample collected after 0.5 h besides the formation of metabolites (2), (3), (4), (5) and (8), showed another metabolite with a molecular ion peak at *m*/*z* 270 (M+) along with a fragment ion peak at *m*/*z* 146. It was tentatively assigned to des-ethyl quinalphos (9) formed by de-alkylation process.

The sample withdrawn after 1 h showed the formation of (2), (4), (5), (8), and (9) along with two other metabolites having molecular ion peaks at *m*/*z* 170 (M+) and *m*/*z* 242(M+). The latter two were tentatively attributed to diethyl thio-phosphoric acid (10), a hydrolytic product of quinalphos and *O*-quinoxalin-2 yl- *O*,*O*-dihydrogen phosphorothioate (11) a de-alkylation product.

The 2 h sample showed the formation of metabolites (2), (3), (5), (6), (8) and (10) along with another product, which showed a molecular ion peak at *m*/*z* 322 (M+) and fragment ion peak at *m*/*z* 161. It was tentatively identified as diquinoxalin-2-yl disulfide (12).

3.3.1.3. Blank in vitro. Investigations, carried out in the absence of enzyme in the gastric phase, showed the formation of metabolites (2), (3), (5), (10) along with dihydroxy quinalphos oxon (13, *m*/*z* 226). However, the blank of intestinal phase showed the formation of (2), (5) and phosphoric acid (14, *m*/*z* 98).

3.3.2. In vivo studies

3.3.2.1. Identification of metabolites in blood serum. The blood serum sample collected after 0.5 h showed the formation of (2) and (5). The 1 h sample of blood serum showed the formation of peaks corresponding to structures (2), (5) and (6), whereas samples collected at 2, 3, 6, 9 and 12 h intervals showed the formation of (2), (3), (5) and (6). The sample collected after 18 h showed the formation of (2), (3), (5), (6), (10) and (11). The sample collected after 24 h showed the formation of (8), (9), (11) and triethyl thiophosphate (15, *m*/*z* 198). Most of the metabolites identified in the blood serum are formed with the oxidation and hydrolysis as the main pathway.

3.3.2.2. Identification of metabolites in urine. The sample of urine collected after 0.5 h showed the formation of oxidative products (2) and (3) and a hydrolytic product (5). The urine sample collected after 1 h, showed the presence of (2), (3), (5) and (6), whereas samples collected after 2, 3, 6, 9 and 12 h exhibited the formation of (2), (3), (5), (6), (9) and (10). The sample collected after 18 h prominently indicated the formation of metabolites (9), (11) and (13) along with one more metabolite with a molecular ion peak at *m*/*z* 130(M+), identified as quinoxaline (16). The sample collected after 24 h showed the formation of metabolites (13), (14), triethyl phosphate (17, *m*/*z* 184), and *O*-phenylene diamine (18, *m*/*z* 108). It may be important to mention here that some of the metabolites which appear initially or in earlier samples do not figure in subsequent samples. This may be attributed to the fact that with the time they have decayed to such a low concentration as to respond to the detector.

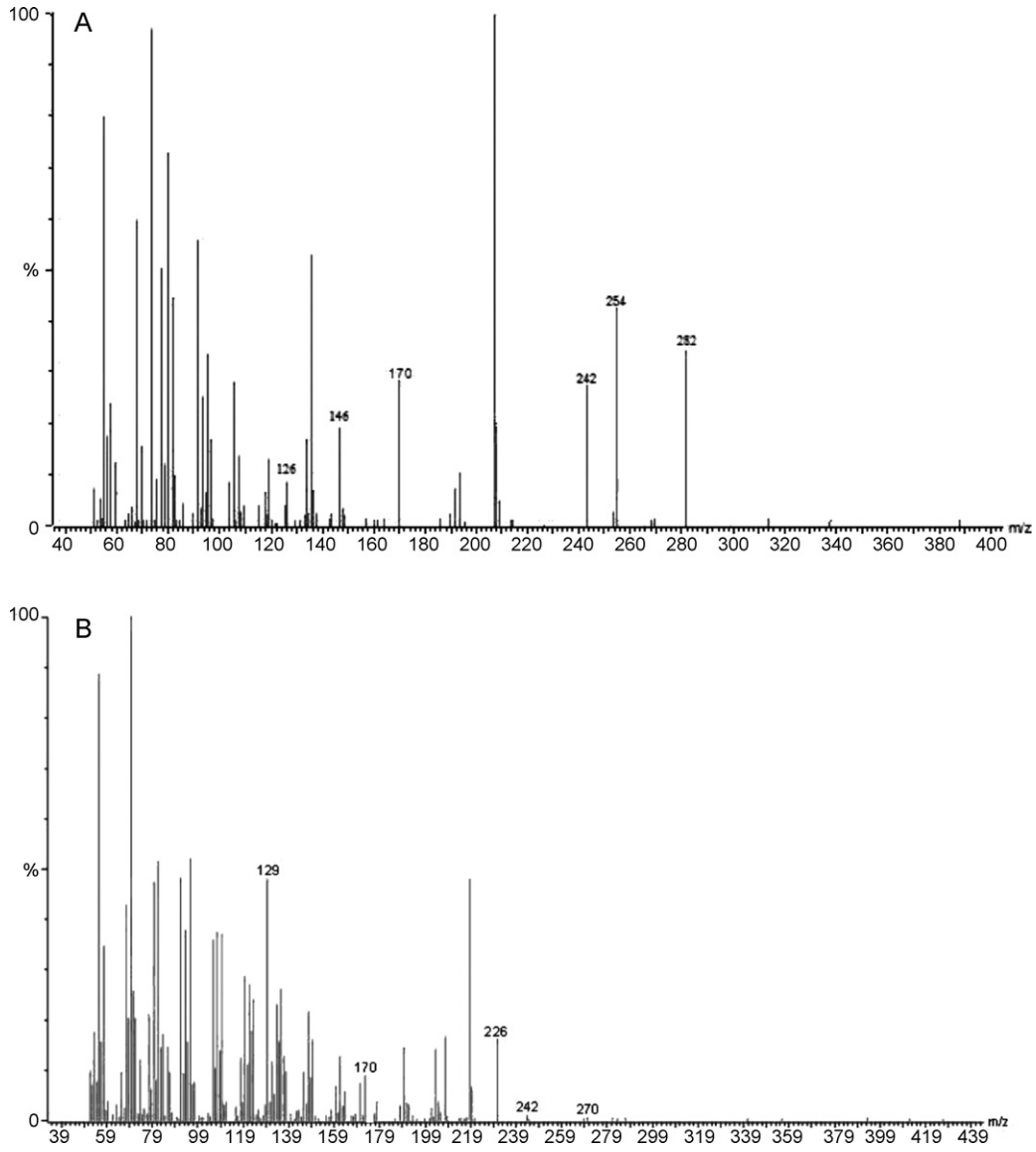


Fig. 5. Mass spectra for the degradation of quinalphos after 18 h dosing in (A) blood and (B) urine.

The possible degradation products of quinalphos identified under simulated gastric and intestinal conditions and in blood serum and urine samples are shown in Fig. 3. The study carried out on the identification of metabolites of quinalphos in *in vitro* and *in vivo* revealed that different metabolites are formed as a result of hydrolysis, S-oxidation, dealkylation and isomerisation. The by-products formed in the presence and absence of enzymes are almost the same except that a fast reaction rate is observed in the presence of enzymes. The dimer (12) is not formed in *in vivo* probably it is unstable in the presence of biological juices. Similar observation has been made earlier by the authors in the case of plants [16]. Conjugated compounds (15) and (17) were observed in *in vivo* study only. It may be suggested that their formation is favoured by the oxidative enzymes (free radical reaction). The results indicate that the metabolites (2) and (5), which are reported to be highly toxic, are reasonably stable in both *in vitro* and *in vivo* conditions. The metabolites (15)–(18) are observed only in *in vivo* investigations.

On the basis of the above results it can be concluded that the metabolite formation at acidic and neutral pH (2.0 and 7.0) is initiated by the hydrolysis (Fig. 4). Further quinalphos is susceptible to oxidation at P=S to form quinalphos oxon (2) as the major metabolite. The nucleophilic attack at phosphorus atom of quinalphos followed by cleavage of POC (aromatic) bond leads to the formation of 2-hydroxyquinoxaline (5) and diethyl thiophosphoric acid (10). A nucleophilic attack at phosphorus atom of quinalphos oxon (2) followed by cleavage of POC (aliphatic) bond results in the formation of (3) followed by (13). The product (8) is formed by the acidic hydrolysis during isomerisation.

A comparison of the metabolites indicated in the blood and urine at different time intervals shows that more or less the same metabolites with the exception of a few are formed. It is apparent that the time of appearance of a certain metabolite in the two types of biological fluids may not be necessarily the same because of the differences in the biological processes involved and their composition. The differences in the mass spectra may be illustrated by taking a typical case of 18th h sample of blood and urine (Fig. 5A and B). At this stage the blood sample has peaks corresponding to (2), (3), (5), (6) and (11) while the urine shows peaks corresponding to (9), (11), (13) and (16). The metabolite (11) appears in both the cases. The formation of 9 (m/z 270) in urine suggests that the direct dealkylation of quinalphos takes place faster in urine. The same

compound, however, appears after 24 h in blood. The phenomenon of ring cleavage is observed only in urine. This is supported by the absence of metabolite (16) in the blood.

4. Conclusions

The pharmacokinetics study carried out on the degradation of quinalphos revealed that a large number of different metabolites are formed at different time intervals. The pattern of metabolites both in blood and urine samples is more or less same with a few deviations. The metabolic pathways seem to be complex consisting of hydrolysis, oxidation de-alkylation and isomerisation processes. The presence of these metabolites in the concerned biological fluids can help in the identification of quinalphos as the source of poisoning. Results of present study and that obtained from investigation on water, soil and plants [16] indicate that dimers are probably unstable in the presence of biological juices as they are not observed in plants, blood and urine. Further, it can be concluded that of the different metabolites formed quinalphos oxon and 2-hydroxy quinoxaline are known to be more toxic than quinalphos and they seem to persist for a longer period in all the investigated matrices namely, water, soil, plants and simulated and biological fluids. They may independently or synergistically enhance the toxicity of the pesticide. Besides these two compounds many other metabolites are formed for which the information on toxicity is not available. The possibility of their contribution towards the toxicity cannot be ruled out.

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