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The enantioselective metabolic mechanism of quizalofop-ethyl and quizalofop-acid enantiomers in animal: protein binding, intestinal absorption, and in vitro metabolism in plasma and the microsome

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Abstract

To investigate the metabolic mechanism and enantioselectivity of the chiral herbicide quizalofop-ethyl and its primary chiral metabolite quizalofop-acid in animals by oral administration, the effects of the processes involved in digestion, absorption, transportation and metabolism, such as protein binding (pepsin, trypsin and serum albumin), intestinal absorption (everted gut sac), and degradation (plasma, liver microsome and cytosol) were studied in vitro. Protein binding experiments showed that quizalofop-ethyl interacted with pepsin, trypsin and serum albumin without enantioselectivity and the proteins did not stimulate metabolic processes that would produce quizalofop-acid. In the everted gut sac, quizalofop-ethyl was degraded rapidly to quizalofop-acid without enantioselectivity and the preferential absorption of (+)-quizalofop-acid was observed throughout the gut. In plasma, the metabolic process of quizalofop-ethyl was enantioselective with preferential degradation of (+)-quizalofop-ethyl and generation of (+)-quizalofop-acid. However there was no enantioselectivity in the degradation of quizalofop-ethyl or formation of quizalofop-acid in liver microsome. The metabolism of quizalofop-acid was noenantioselective by liver microsome, sulfotransferase and glucuronosyltransferase. It could be inferred that the enzymes present in the lumen of intestine, blood and liver might facilitate the rapid transformation of quizalofop-ethyl to quizalofop-acid after

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oral administration, and the enantioselective absorption of quizalofop-acid enantiomers by intestine was the main reason for the enantioselectivity observed in rats in vivo.

KEYWORDS: quizalofop-ethyl; metabolism; enantioselectivity; mechanism

1. Introduction

Pesticides have been widely used in agriculture in the control of pests, weeds and plant diseases, which have drawn attention as environmental pollutants. Chiral pesticides made up about 30% of all pesticides used and the proportion is increasing, with more complex structures coming into use. Enantiomeric pairs of chiral pesticides share identical physical and chemical properties. However, individual enantiomers can interact enantioselectively with enzymes or other naturally occurring chiral molecules [1], so they have different physiological and biochemical properties [2]. Some chiral pesticides are still used as racemates despite the fact that the pesticidal activity of the individual enantiomers may be different. Sometimes only one enantiomer has the desired activity and the other may be less effective or even have adverse effects on non-target species. Enantioselective degradation of the enantiomers has been widely observed in the field and under laboratory conditions [3-9]. Information on stereoselective behaviors of chiral pesticide contributes to minimize environmental risk and improve the evaluation of safety conditions for humans and other animals.

The biological behaviors of chiral pesticides can also be stereoselective in animals. For example, many researchers have reported high enantiomeric ratios for α-hexachlorocyclohexane in wildlife [10-15]. Zhang et al. demonstrated that the degradation of (-)-fenoxaprop-ethyl was much faster than (+)-fenoxaprop-ethyl in rabbit plasma [16]. Brad et al. proved a greater enantioselective biotransformation rate of the (+)-enantiomer than (-)-enantiomer of fipronil in rainbow trout [17]. In most cases, the enantioselectivity of drugs has been mainly attributed to uptake, distribution, metabolism and excretion. Occasionally, this can also result from selective protein binding or tissue transport [18-22]. To investigate the enantioselective behaviors of chiral compounds in animals, in vitro experiments are usually conducted. The liver

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microsome is one of the more widely used in vitro metabolic models because it contains abundant cytochrome P450 enzymes, which plays an important role in drug elimination. Everted gut sacs have a relatively large surface area and mucus layer available for absorption, so they are widely used as an in vitro model to study the kinetics and mechanisms of drug absorption. In general, in vitro experiments can offer more information regarding the mechanisms and reduce the massive use of animals, so they are widely used.

Quizalofop-ethyl (Fig. 1) is a phenoxy propionate herbicide consisting of a pair of enantiomers, with the (+)-enantiomer possessing more herbicidal activity than the (-)-enantiomer. Quizalofop-ethyl can undergo deesterification very quickly in many organisms and its primary metabolite is quizalofop-acid. Quizalofop-ethyl is used for postemergence control of annual and perennial grass weeds in broad-leafed crops such as rape, sunflower, soybeans, cotton, peanuts, flax, alfalfa, and some vegetables. Because of the broad scope of its application, humans and many other animals are exposed to this compound via active or passive processes. The maximum residue limit (MRL) for quizalofop-ethyl was 3 mg/kg in sunflower seeds and 1.5 mg/kg in rapeseed in Canada. In a previous study, quizalofop-ethyl has been proven to affect the development of rats, leading to a significant decrease in neonatal survival rates [2]. Results also showed that (+)-quizalofop-ethyl could induce a mixed cholestatic/hepatocellular liver injury [23]. To assess the potential risk of pesticides to animals, it is important to understand their fate in those animals. For chiral enantiomers, it is also necessary to determine the mechanism underlying stereoselective behaviors.

In our previous work performed in rats[24], quizalofop-ethyl was found to be metabolized very quickly when administered intragastrically, and high concentrations of the metabolite quizalofop-acid was observed in the blood and tissues. In addition, enantioselectivity of quizalofop-ethyl and its chiral metabolite was observed. However, the mechanisms underlying the degradation and enantioselective behaviors are unclear. In most cases, the fate of drugs in animals after intragastric administration might be affected by the enzymes in the stomach and intestine, gut absorption, plasma

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and liver metabolism. For this reason, in this work, the enantioselective protein (pepsin, trypsin and serum albumin) binding, absorption (everted gut sac), and the metabolism of quizalofop-ethyl by plasma， rat liver microsome and cytosol were studied in vitro. The data from in vitro analysis may elucidate the metabolic mechanism and enantioslectivity in vivo and may improve understanding of the fate of chiral pesticides in animals.

2 Materials and methods

2.1 Chemicals and Reagents

Racemic-quizalofop-ethyl (rac-QE, 98%) and racemic-quizalofop-acid (rac-QA, 99%) were obtained from Institute for the Control of Agrichemicals, Ministry of Agriculture of China. Trifluoroacetic acid (TFA), ethyl acetate, ethanol, acetonitrile, isopropanol, porcine pepsin and trypsin were obtained from Alladin Reagent (Shanghai, China). β-Nicotinamide adenine dinucleotide phosphate (NADPH), uridine 5'-diphosphate glucuronic acid (UDPGA), 3'-phos-phoadenosine 5'-phosphosulfate (PAPS), alamethicin and serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Other solvents or chemicals used in this study were of analytical grade.

2.2 Animals

Sprague-Dawley male rats weighing 200-250 g were obtained from Experimental Animal Research Institute of China Agriculture University. Prior to the experiments, the rats were housed in polycarbonate cages placed in well-ventilated house conditions with a 12:12 h light: dark photoperiod. They were allowed free access to water and standard rat food. The rats were allowed to acclimatize in the laboratory for seven days before the experiments started. Before the operation, the rats were fasted overnight. This study and all animal experiments were approved by the local ethics committee (Beijing Association For Laboratory Animal Science) and carried out in strict accordance with the Experimental Animal Regulation Ordinances defined by China National Science and Technology Commission.

2.3 Protein binding of QE with pepsin, trypsin and serum albumin

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Protein binding was studied in vitro by equilibrium dialysis. After 1 mL of pepsin solution (0.08 mol/L HCl containing 10 g/L pepsin, pH=1.5), trypsin solution (0.05 mol/L KH_2PO_4 and Na_2HPO_4 buffer solution containing 22 g/L trypsin, pH 6.8) or albumin solution (50 mmol/L Tris-HCl buffer solution containing 0.1 mol/L NaCl, pH 7.4) were placed in a dialysis tube, the dialysis tube was placed in 20 mL corresponding buffer solution at 37 ℃ and preincubated for 5 min. Rac-QE (dissolved in alcohol, 1% of final volume) was added to the dialysis tube at 10, 30, 50, 70 and 100 mg/L, and the dialysis tube was then incubated at 37 °C. After 24 h, 0.5 mL of the outer dialysate was withdrawn and added to 0.5 mL 10% $HClO_4$. The samples were considered invalid if there was white floc precipitation present. Then 1 mL outer and inner dialysates were collected and 5 mL of ice-cold ethyl acetate was immediately added. The mixture was then stirred for 5 min to terminate the reaction.

2.4 Enantioselective metabolism of QE and QA by rat plasma, liver microsome and cytosol

Rat blank blood was separated from drug-free heart blood and centrifuged at 4000 rpm for 10 min at 4 ℃ to obtain plasma. Then the plasma was diluted by Tris-HCl buffer (pH 7.4) to produce the 10% (v/v) plasma solution. Rac-QE (dissolved in alcohol, 1% of final volume) was added into the 10% plasma solution (preincubated at 37 ℃ for 5 min) at a final concentration of 10 mg/L. Samples (1 mL) were taken out at 0, 5, 15, 30 and 60 min, and 5 mL of ice-cold ethyl acetate was immediately added. The mixture was then stirred for 5 min to terminate the reaction.

Rat liver microsome and cytosol from male Sprague-Dawley rats that used to study the methylated and hydroxylated effects of CYP 450 was prepared according to a previously described method [25]. In brief, after rats were sacrificed, their livers were quickly removed and immediately placed on ice and thoroughly washed with ice-cold 1.15% KCl solution. The pooled livers were minced and immediately homogenized into three volumes of ice-cold SET solution (1 mM ethylenediamine tetra-acetic acid and 50 mM Tris-HCl, pH 7.4). Liver microsomes were used to determine CYP 450 and glucuronosyltransferase activity for QA and cytosols were used for the measurement of sulfotransferase activity for QA. These were prepared using

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differential centrifugation. Protein levels were determined using the Bradford method [26].

The metabolism of QE in the liver microsome was carried out in a reaction Tris-HCl buffer (50 mM, pH 7.4) containing microsomal protein (1 mg/mL). The microsomal suspension was preincubated for 5 min at 37 ℃ and then initiated by addition of rac-QE (10 mg/L, dissolved in alcohol, 1% of final volume). The metabolism of rac-QA by liver microsome was carried out in a reaction mixture containing Tris-HCl buffer (50 mM, pH 7.4), microsomal protein (1 mg/mL), NADPH (1.0 mM) and rac-QA (10 mg/L, dissolved in alcohol, 1% of final volume). After preincubation for 5 min at 37 ℃, the reaction was regenerated by adding NADPH (1.0 mM).

The metabolism of rac-QA by sulfotransferase and glucuronosyltransferase was carried out in this study. The sulfotransferase (SULT) activity for QA was determined in the reaction mixture consisted of hepatic cytosolic protein (0.1 mg/mL), Tris-HCl buffer (50 mM, pH 7.4), $MgCl_2$ (5 mM) and PAPS (0.2 mol/L). After preincubation for 5 min at 37 ℃, the reaction was regenerated by addition of rac-QA (10 mg/L, dissolved in alcohol, 1% of final volume). The glucuronosyltransferase (UGT) activity for QA was studied in the incubation mixture containing Tris-HCl buffer (50 mM, pH 7.4), microsomal protein (0.5 mg/mL), alamethicin (1 mg/10 mg protein) and rac-QA (10 mg/L, dissolved in alcohol, 1% of final volume). The reaction was initiated by the addition of 2 mmol/L UDPGA.

Samples were collected at 0, 10, 20, 40 and 60 min. Then 5 mL of ice-cold ethyl acetate was immediately added. The mixture was then stirred for 5 min to terminate the reaction.

2.5 In vitro everted gut sac absorption

The aim of this study was to assess the stereoselective absorption of QE and QA by intestine in vitro. The Sharma method was used for everted rat gut sac experiments [27]. After an overnight fast, rats were sacrificed by cervical dislocation, and then the intestine was rapidly removed and placed in a beaker containing ice cold Krebs-Ringer (K-R) culture solution (containing 7.8 g NaCl, 0.35 g KCl, 1.37 g

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$NaHCO_3$, 0.02 g $MgCl_2$, 0.32 g NaH_2PO_4 and 1.48 g glucose in 1000 mL distilled water). The jejunal segment (10 cm) was flushed with the K-R culture solution to remove the intestinal contents and to remove the underlying mesenterium. The jejunal segment was then tied at one end, everted with a plastic pipe and removed the mucus carefully. The serosa compartment was filled with 2 mL of K-R culture solution and the everted intestinal sac was suspended in the K-R culture solution (maintained at 37 °C) and continually aerated with 95% O_2 and 5% CO_2. Then rac-QE (dissolved in alcohol, 1% of final volume) was added into the K-R culture solution at a concentration of 10 mg/L. Aliquots of 2 mL serosal solution were taken out from both the mucosal and serosal side at different points in time (10, 30, 60, 90 and 150 min). The serosa compartment was immediately washed three times with K-R culture solution and refilled with 2 mL of fresh K-R culture solution at each sampling point to keep the volume of the serosal solution constant.

The integrity of the gut sac incubated with or without QE was verified according to the previous procedure based on the ratio of the concentration of glucose in the mucosal side to the serosal side [28]. The sacs were incubated in the presence or absence of rac-QE (dissolved in alcohol, 1% of final volume). Samples were taken out exactly as described above and the concentrations of glucose were measured by the glucose assay kit from Nanjing Jiancheng Bioengineering Institute (China).

2.6 Extraction procedure

One milliliter of the samples (rat plasma, liver microsome, buffer solution, pepsin and trypsin solution) was extracted by the addition of 5 mL of ethyl acetate and then acidified with 100 μL of 1 mol/L HCl. The mixtures were vortexed for 5 min and centrifugation at 3500 rpm for 5 min, the organic phase was transferred into a test tube. The extraction was repeated. The combined supernatant extract was evaporated to dryness under nitrogen. Then the residue was redissolved in 0.5 mL of isopropanol, and filtered through a 0.22 μm syringe filter.

2.7 Analytical procedures

HPLC analysis for the determination of QE and QA was performed using an Agilent 1200 series HPLC system consisting of Chiralpak IC column (250 × 4.6 mm,

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Daicel Chemical Industries, Tokyo, Japan) with variable wavelength detector-G1314B. Column temperature was controlled by a column attemperator (Tianjin Automatic Science Instrument Co. Ltd, China). The mobile phase consisted of *n*-hexane: isopropanol: TFA (92: 8: 0.1%, v/v/v). The flow-rate was maintained at 0.6 mL/min, and the detection was performed at a wavelength of 230 nm under constant temperature (15 °C).

2.8 Data analysis

Pesticide protein binding rate was calculated by $P = (C_1\text{-}C_2)/C_1 \times 100\%$, where C_1 and C_2 were the concentrations of pesticide in the inner sideand outer of dialysate. Enantiomeric fraction (EF) was used to denote stereoselectivity, defined as EF= peak areas of (+)/ [(+) + (-)]. The EF of racemate was 0.5, whereas preferential degradation of one enantiomer made EF deviating 0.5.

All values were analyzed by analysis of variance (ANOVA) and expressed as the mean ± SD. P values lower than 0.05 were considered to be of statistical significance.

3. Results and discussion

3.1 Method validation

Fig. 1 showed the typical chromatogram of the separation of the enantiomers of QE and QA. The two pairs of enantiomers could be completely separated on HPLC with Chiralpak IC column using *n*-hexane/isopropanol/TFA (92: 8: 0.1%, v/v/v) at 0.6 mL/min. As shown in Table 1, for all the enantiomers in the concentration range of 0.3-6 mg/L, the correlation coefficients (R^2) were higher than 0.994, indicating a functional linear relationship between the concentration and peak area. The extraction efficiency of (+)-QE, (-)-QE, (+)-QA and (-)-QA throughout the linear range ranged from 88% to 108% with RSD of 3% to 10% (Table S1). The limit of detection (LOD) and quantitation (LOQ) for all enantiomers were 0.1 and 0.3 mg/L respectively.

3.2 Protein binding of QE with pepsin, trypsin and serum albumin

No QE was detected in the blood, kidneys, lungs, livers, brain, urine or feces after intragastric administration of QE to rat in the previous study but high levels of (+)-QA were [24]. Dietary food intake is one major route of pesticide into human body.

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When pesticide enters the digestive system such as stomach or intestine, digestive proteases (pepsin and trypsin) may be the direct binding targets. After being absorbed, pesticide may bind to serum albumin which is the most soluble protein in plasma and can combine with numerous exogenous substances. As the chiral nature and the variety of functional groups presenting at the surface, pepsin, trypsin and serum albumin have the ability to discriminate a chiral molecule[29]. It is therefore important to consider the influences of the protein-pesticide binding effects of pepsin, trypsin and serum albumin on the enantioselectivity. Because QE enters the digestive system mainly in its parent form and is present in the form of QA in the blood, the binding effects of QE with pepsin and trypsin and QA with serum albumin were studied in this article [24]. The binding properties of pepsin, trypsin with QE are shown in Fig. 2A and Fig. 2B. After the incubation of QE with pepsin and trypsin, the protein-binding rate decreased as the concentration of QE increased and the concentrations of the two enantiomers of QE were approximately equal at the end of incubation. Similar results were also found in the incubation of QA with serum albumin (Fig. 2C). No QA was detected during these experiments. Protein binding effects should not be the reason for the enantioselectivity or quick metabolism found in vivo.

3.3 Enantioselective degradation kinetic in rat plasma, liver microsome and cytosol

Xenobiotic metabolism is a biphasic process, comprising phase I (CYP450 and esterase) and phase II (UGT and SULT) enzyme reactions. Many chiral chemicals have demonstrated stereoselective metabolism in animals due to the enzymes like CYP450, esterase, UGT and SULT [30-34].

Concentration-time curves of (+)- and (-)-QE in rat plasma at 10 mg/L are shown in Fig. 3A. The enantioselective metabolic process of QE was very quick in the plasma with preference of (+)-QE and the $t_{1/2}$ of (+)- and (-)-QE were 2.06 and 2.88 min. Along with the metabolism of QE, QA was rapidly generated and reached 10 mg/L within 20 min with preferential formation of (+)-QA. Fig. 3B showed the metabolism of QE and QA in rat microsome in vitro. The degradation of QE in liver microsome could reflect the enantioselective deesterification of QE by esterase in the liver. The results showed that QE was rapidly metabolized to QA with $t_{1/2}$ of 3.13 and

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3.29 min for (+)-QE and (-)-QE, and the degradation was nonenantioselective. The metabolite QA was quickly generated and the concentrations of the two enantiomers were approximately equal with the EF value ranging from 0.496 to 0.508, revealing that the formation of QA in liver microsome was noenantioselective. To further investigate the effects CYP450 enzymes on the enantioselectivity of QA, the metabolism of rac-QA was performed via the NADPH-dependent oxidative metabolism in rat liver microsome. As shown in Fig. 3C, the metabolism of QA by liver microsome was nonenantioselective with the EF values ranging from 0.51 to 0.50. Fig. 3D shows the influence of sulfotransferase on the enantioselectivity of QA in vitro. QA could be sulfate conjugated by SULT and the whole process was nonenantioselective. Similar results were also found in the glucuronidation of QA by UGT (Fig. 3E).

In this way, the esterase present in plasma and liver could be the reason for the quick degradation of QE to QA in vivo and the overwhelming amount of (+)-QA in rat tissues in vivo could not be explained by the preferential metabolism of (-)-enantiomers via the effects of liver microsome, sulfotransferase, glucuronosyltransferase.

3.4 In vitro everted gut sac absorption

Several in vitro methods can be used in the prediction of drug absorption, in which the everted gut sac model is a relatively simple and very reproducible technique. In this study, the intestinal absorptive behavior of QE was studied using the everted gut sac model with K-R culture solution in vitro. QE was added to the donor (mucosal) side of rat jejunum at 10 mg/L. Because glucose is actively transported in the small intestine, intact sacs concentrate glucose in the acceptor side and maintain this gradient. In this way, the effects of QE on the viability and integrity of the gut were examined via the concentration ratio of glucose between the acceptor side and donor side (Fig. 4A). It was obvious that the ratio increased gradually regardless of the presence of QE. Thus, in this study, QE did not induce toxicity and had no obvious influences on the viability or integrity of the gut. Fig. 4B illustrated the distribution of (+) and (-)-QA at the donor and acceptor sides. No QE was detected in the donor side

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since the first time point (10 min). However, a large amount of QA was observed, indicating that QE was metabolized quickly in mucosal side of the jejunum. The rapid metabolism of QE might be caused by the esterases present in the lumen of the intestine, which from enterocytes and pancreatic secretions and could rapidly hydrolyze many kinds of ester-type drugs [35-37]. The two enantiomers of QA were formed in equal amounts in the donor side without enantioselectivity, and great enrichment of (+)-QA was found in the acceptor side with the EF value increasing to 0.88 after 150 min. Stereoselectivity may take place during the processes of absorption and transport because the membrane phospholipids are chiral. Many studies have reported the enantioselective absorption and disposition of chiral drugs [38-40]. In this study, enantioselective absorption by intestine should be the main reason for the observed enrichment of (+)-QA after oral administration in rat in vivo.

In this way, when the oral administrated QE reached the intestine, QE was hydrolyzed rapidly in the lumen of the intestine to QA which was absorbed enantioselectively by intestine reaching into the blood or tissues mainly in the form of (+)-QA.

4. Conclusions

In this study, protein binding, plasma, liver microsome and cytosol degradation, and everted gut sac absorption were performed to investigate the enantioselective metabolism behavior of orally ingested QE in rats. The protein binding process of QE with pepsin, trypsin, and serum albumin was noenantioselective and no degradation to QA was found in this process. The elimination of QE in plasma was quick with slight enantioselectivity, while there was no difference in the formation of the two QA enantiomers. The formation and degradation of QA was noenantioselective in the liver microsome and cytosol, and the metabolism of QE to QA was rapidly. Results showed the metabolism of QE to QA was also very quick in the intestine and the absorption of QA was enantioselective with preference of (+)-QA. The results suggested that the esterase present in the tissues might lead to the quick metabolism of QE to QA and that the absorption of QA by intestine might contribute to the enantioselectivity in

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vivo.

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Table. 1. Validation of QE and QA enantiomer analysis.

Enantiomers	Linear equation	R^2	Linear range (mg/L)	LOD (mg/L)	LOQ (mg/L)
(+)-QE	y=69.48x+15.62	0.9994	0.3-6	0.1	0.3
(-)-QE	y=69.82x+21.83	0.9995	0.3-6	0.1	0.3
(+)-QA	y=130.96x+5.91	0.9999	0.3-6	0.1	0.3
(-)-QA	y=131.12x+4.25	0.9997	0.3-6	0.1	0.3

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Fig. 1. Chemical structures and typical chromatogram of the separation of the enantiomers of QE and QA.

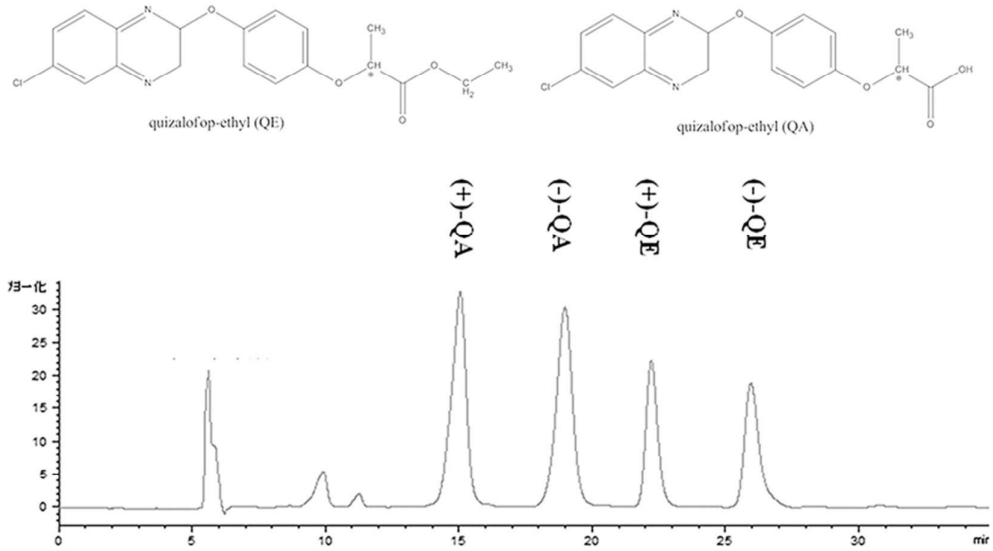

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Fig. 2. The protein binding of QE with pepsin (A), trypsin (A) and albumin (C).

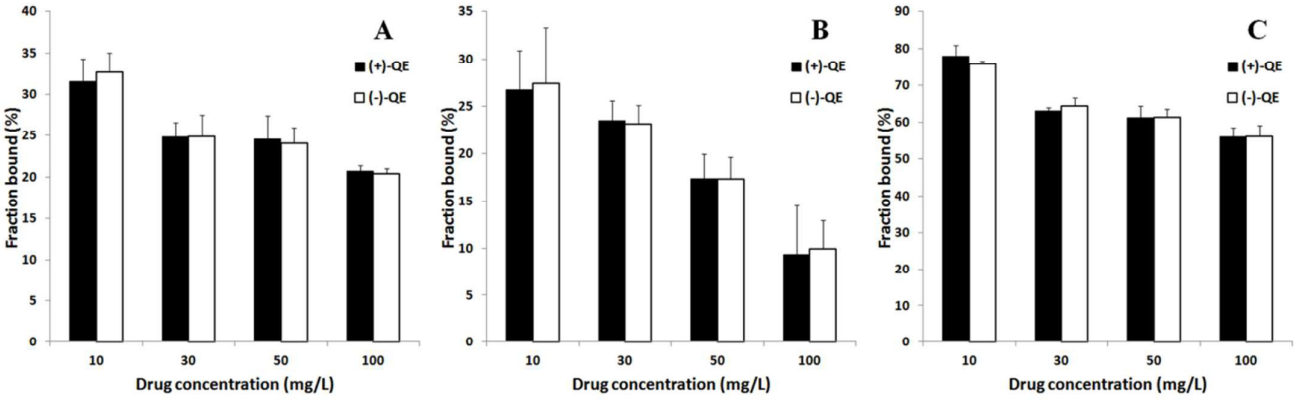

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Fig. 3. Degradation of QE by 10% rat plasma (A), microsome (B), and QA by microsome (C), sulfotransferase (D) and glucuronosyltransferase (E).

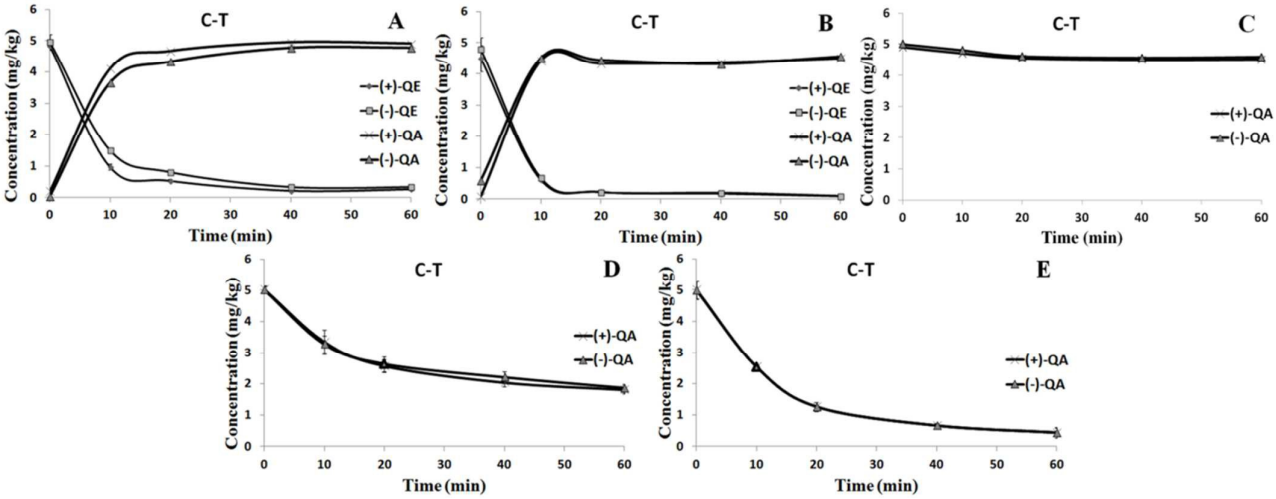

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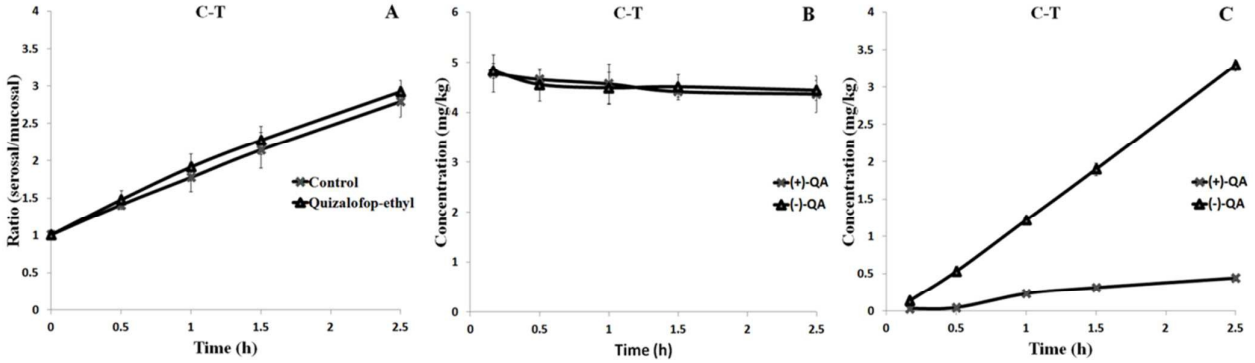
Fig. 4. Glucose transport in everted gut sac (A) and the concentration-time curves of QA in mucosal (B) and serosal side (C).



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