Stereoselective Metabolism of Benalaxyl in Liver Microsomes from Rat and Rabbit

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ABSTRACT Benalaxyl (BX), methyl-*N*-phenylacetyl-*N*-2,6-xylyl alaninate, is a potent acylanilide fungicide and consist of a pair of enantiomers. The stereoselective metabolism of BX was investigated in rat and rabbit microsomes in vitro. The degradation kinetics and the enantiomer fraction (EF) were determined using normal high-performance liquid chromatography with diode array detection and a cellulose-tris-(3,5-dimethylphenylcarbamate)-based chiral stationary phase (CDMPC-CSP). The $t_{1/2}$ of (−)-R-BX and (+)-S-BX in rat liver microsomes were 22.35 and 10.66 min of *rac*-BX and 5.42 and 4.03 of BX enantiomers. However, the $t_{1/2}$ of (−)-R-BX and (+)-S-BX in rabbit liver microsomes were 11.75 and 15.26 min of *rac*-BX and 5.66 and 9.63 of BX enantiomers. The consequence was consistent with the stereoselective toxicokinetics of BX in vitro. There was no chiral inversion from the (−)-R-BX to (+)-S-BX or inversion from (+)-S-BX to (−)-R-BX in both rabbit and rat microsomes. These results suggested metabolism of BX enantiomers was stereoselective in rat and rabbit liver microsomes. *Chirality 23:93–98, 2011.*

KEY WORDS: benalaxyl; stereoselective metabolism; liver microsomes; rat; rabbit

INTRODUCTION

Nowadays, a great variety of the most widely used pesticides have optical isomers. About 25% among the frequently used pesticides have optical enantiomers.[1] Most of them are released into the environment in racemic form. It is well known that the enantiomers of a chiral pesticide usually show not only different biological activities but also metabolic process in organisms in the environment.[2–4] In general, the passive processes in absorption, distribution, and excretion do not differentiate between enantiomers of a chiral drug. When a chiral drug interacts with optically active biological macromolecules, such as enzymes, then the discrimination and high degree of stereoselectivity, most commonly in metabolism and protein binding.[5,6] Therefore, growing concern about the side effects of chiral agrochemicals on nontarget organisms and natural resources has promoted the use of enantiomerically pure or stereochemically enriched compounds.[7] So, chiral analysis of chiral pesticides will help to improve our understanding of the pesticide's safety to humans, animals, and the environment.[8]

Benalaxyl (BX), methyl-*N*-phenylacetyl-*N*-2,6-xylyl alaninate, is a systemic fungicide belonging to the acylalanine family(see Fig. 1).[9] In agriculture, it was used to control late blights of potatoes and tomatoes, downy mildew of hops, vines, lettuce, onions, soya beans, and tobacco and many diseases of flowers and ornamentals.[10] BX has a chiral carbon and consists of two enantiomers. The activity of BX is mainly attributed to the (−)-R-enantiomer.[11] A number of methods have been reported for the determination of BX residues in various samples, such as enzyme-linked immunosorbent assay in water and wine[12,13] and gas chromatography in vegetables and fruits.[14] The stereoselective degradation of BX in rabbit plasma,[11] soils, and cucumber plants[15] were investigated by chiral high-performance liquid chromatography.

However, to our knowledge, the stereoselective metabolism of BX in liver microsomes from rat and rabbit has not been reported. In this study, we investigated the behavior of the two enantiomers of BX in liver microsomes from rat and rabbit. For the metabolites were complicated, we apply intrinsic clearance to calculate enzyme kinetics parameter. This study was attributed to understand their different metabolism behavior in animals. The results may have some implication for the environmental and ecological risks assessment for chiral pesticides.

MATERIALS AND METHODS

Chemicals and Reagents

rac-BX (>99% purity) was provided by the China Ministry of Agriculture's Institute for Control of Agrochemicals. (−)-R-BX and (+)-S-BX were prepared on an Agilent

Abbreviations: CDMPC-CSP, cellulose-tris-(3,5-dimethylphenylcarbamate)-based chiral stationary phase; CSP, chiral stationary phase; CYP, cytochrome P-450; EF, enantiomer fraction; HPLC, high performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; NADPH, β-Nicotinamide adenine dinucleotide phosphate.

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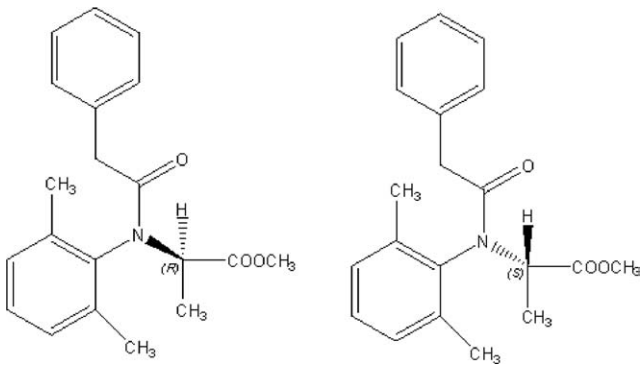

Fig. 1. Chemical structure of benalaxyl (BX) enantiomers.

HPLC with a preparative chiral column (cellulose-tris-(3,5-dimethylphenylcarbamate)-based chiral stationary phase; CDMPC-CSP, provided by the Department of Applied Chemistry, China Agricultural University, Beijing). Water was purified by a Milli-Q system. Stock solution of racemic standard was prepared in 2-propanol and was stored at −20°C. Working standard solutions were obtained by dilutions of the stock solution in 2-propanol. β-Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Sigma-Aldrich (St Louis, MO, USA). *n*-propanol, *n*-hexane, and ethyl acetate (analytical grade) were from Yili Fine Chemicals (Beijing, China), distilled and filtered through a 0.45 μm filter membrane before use. All other chemicals and solvents were analytical grade and purchased from commercial sources.

Preparation of Rat and Rabbit Liver Microsomes

Animals were sacrificed and the liver was quickly removed, blotted, weighed, and placed in ice-cold 1.15% KCl solution. Tissue was minced with scissors and washed with 1.15% KCl solution to remove blood. After draining the 1.15% KCl solution, individual liver was homogenized in ice-cold SET solution (1 mM ethylenediamine tetra-acetic acid and 50 mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 10,000*g* for 20 min at 4°C and the pellet was discarded. The supernatant was centrifuged at 108,000*g* for 60 min at 4°C. The supernatant (cytosol) was decanted. The pellet was washed with 50 mM Tris-HCl and the homogenate was centrifuged at 108000*g* for 60 min at 4°C again. The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol. This procedure was used for the preparation of microsomes from Sprague-Dawley rat (male, $n = 6$) and Japanese white rabbits (male, $n = 3$). Protein concentration was determined by the method of Bradford with BSA as the standard,[8] and microsomes were stored at −80°C until used.

HPLC-DAD Analysis

Chromatography was performed using an Agilent 1100 series HPLC equipped with a G1311A pump, G1322A degasser, G1328A injector, a 20-ml sample loop, and G1315A DAD. The signal was received and processed by an Agilent Chemstation for 3D LC (Agilent Technologies, Palo Alto, CA).

Enantiomers were separated on a cellulose tris-(3,5-dimethylphenylcarbamate) (CDMPC)-based chiral stationary phase (CSP, provided by the Department of Applied Chemistry, China Agricultural University, Beijing) under normal-phase conditions. The CSP was prepared according to the procedure described in the literature.[16,17] CSP was packed into a 250 × 4.6 mm (i.d.) stainless steel column. The mobile phase was a mixture of 95% *n*-hexane and 5% *n*-propanol with a flow rate of 1 ml/min. Chromatographic separation was conducted at 20°C and UV detection at 220 nm.

Sample Preparations

Substrate-depletion studies in vitro were performed by incubation of BX (80 μM) or its enantiomers (40 μM) for rat liver microsomes and BX (60 μM) or its enantiomers (30 μM) for rabbit liver microsomes with 1 mg microsomal protein in 50 mM Tris-HCl buffer (pH 7.4) with 5.0 mM $MgCl_2$. BX or its enantiomers were prepared in alcohol and added to incubations. The volume of alcohol added to each incubate was less than 1.0% v/v. All reaction mixtures were preincubated in a heated water bath at 37°C for 5 min before initiation of the reaction with the addition of NADPH at a final reaction concentration of 1.0 mM, the final total reaction volume was 1.0 mL. After incubation in a water bath (37°C) for 5–40 min, the reactions were terminated by adding 5 mL of ice-cold ethyl acetate, the sample was vortexed for 5 min. After centrifugation at 3500 rpm for 5 min, the clear solution was decanted into a test tube. The extraction and centrifuge steps were repeated with another 5 mL of ethyl acetate. The organic phase was combined and evaporated to dryness under a stream of nitrogen at 50°C, the resulting residue was redissolved in 200 μl 2-propanol for HPLC analysis.

Data Analysis

The enantiomer fraction (EF) was used to measure the enantioselectivity of the BX enantiomers. EF of (−)-R-BX is defined by eq. 1.EF =Peak areas of the

$$\text{EF} = \text{Peak areas of the } \frac{(-)}{[(+)+(-)]} \tag{1}$$

where (−) and (+) are the first and second enantiomers determined by the result of polarimeter in previous works.[11] The EF for racemate is 0.50, whereas preferential degradation of the (+) or (−) yields EF <0.50 and >0.50, respectively.

The metabolism of *rac*-BX or its enantiomers appeared to follow a first-order kinetic reaction, and the degradation rate constants were derived from "$\ln(C_0/C)$ versus t" plots by regression analysis for experiment (Excel 2003, Microsoft®). The starting point was the maximum concentration. The in vitro elimination half-life ($t_{1/2}$) was determined by the following eq. 2:

$$t_{1/2} = \frac{0.693}{k} \tag{2}$$

Nonlinear regression of substrate concentration versus reaction velocity curves were analyzed using Origin 7.5

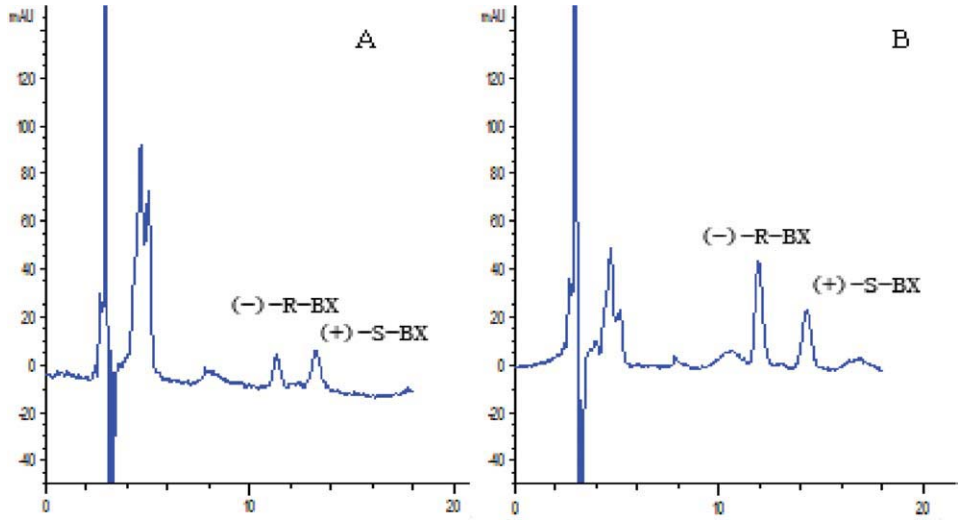

Fig. 2. Representative HPLC chromatograms of (**A**) extract from rabbit microsomes sample after incubation 15 min with *rac*-BX 60 μM; (**B**) extract from rat microsomes sample after incubation 20 min with *rac*-BX 80 μM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

software by fitting experimental data to the Michaelis–Menten equation. The degradation of BX enantiomers by rat and rabbit liver microsomes were fitted to eq. 3, and the K_m and V_{max} values were calculated by the following equation:

$$V = \frac{V_{max} \times S}{K_m + S} \quad (3)$$

where V, S, V_{max}, and K_m represent the rate of metabolism, substrate concentration, maximum rate of metabolism, and Michaelis constant, respectively. Intrinsic metabolic clearance (CL_{int}) was calculated by the following eq. 4:

$$CL_{int} = \frac{V_{max}}{K_m} \quad (4)$$

RESULTS AND DISSCUSSION

Calibration Curves and Assay Validation

On CDMPC, the (−)-R-BX elutes first in *n*-hexane/2-propanol mobile phase.[18] In this study, it was found that the elution order of the two enantiomers in *n*-hexane/*n*-propanol was same with that in *n*-hexane/2-propanol. Thus, the first eluted stereoisomer in *n*-hexane/*n*-propanol mobile phase was confirmed as (−)-R-BX, while the second one was (+)-S-BX.

(−)-R-BX and (+)-S-BX were baseline separated (Fig. 2). There were no endogenous interference peaks eluted at retention times in all samples. Linear calibration curves were obtained over the enantiomer concentration range of 0.5–200 μM/l in microsomes for (−)-R-BX ($y = 24.63x + 139.39$, $R^2 = 0.9995$) and (+)-S-BX ($y = 24.725x = 116.59$, $R^2 = 0.9998$). The accuracy and precision of the assay, for both enantiomers, ranged from 1 to 6% (RSD) and 87 to 101% (accuracy) over the entire calibration range (Table 1). Method recovery data for rat and rabbit liver microsomes are presented in Table 2. The lowest recovery was over 85%. The LOD was 0.03 μM/l and the LOQ was 0.1 μM/l in rat and rabbit liver microsomes.

Kinetic Degradation in Liver Microsomes

Concentration-time curves of (−)-R-and (+)-S-BX after incubation of rat liver microsomes with *rac*-BX at 80 μM

TABLE 1. Accuracy (%) and precision (RSD %) of the chiral HPLC method for measurement of benalaxyl enantiomers ($n = 6$)

	(−)-R-benalaxyl			(+)-S-benalaxyl		
Concentration (μm/l)	Concentration found	Accuracy (%)	RSD (%)	Concentration found	Accuracy (%)	RSD (%)
Within-day						
5	4.71 ± 0.14	94.20	2.94	4.63 ± 0.11	92.60	2.41
25	22.48 ± 0.63	89.91	2.80	21.79 ± 0.52	87.16	2.37
100	91.54 ± 4.86	91.54	5.31	91.03 ± 4.69	91.03	5.15
Day-to-day						
5	4.97 ± 0.09	99.45	1.74	4.90 ± 0.07	97.95	1.34
25	23.43 ± 0.54	93.70	2.32	23.97 ± 0.55	95.90	2.29
100	98.52 ± 5.10	98.52	5.18	100.15 ± 4.60	100.15	4.59

TABLE 2. Summary of method recovery data for both benalaxyl enantionmers from rat and rabbit liver microsomes

	(−)-R-benalaxyl		(+)-S-benalaxyl	
Concentration added (μm/l)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Rat				
0.5	91.25 ± 2.97	3.25	90.97 ± 1.71	1.89
25	98.69 ± 1.79	1.81	97.42 ± 1.49	1.53
200	90.57 ± 3.39	3.74	92.56 ± 3.74	3.42
Rabbit				
0.5	94.59 ± 0.79	0.84	92.08 ± 2.37	2.57
25	94.54 ± 3.72	3.94	92.67 ± 4.33	4.67
200	87.43 ± 1.46	1.67	86.20 ± 1.49	1.73

and rabbit liver microsomes at 60 μM were shown in Figure 3.The mean concentrations of two enantiomers were different from each other at each time point. In rat liver microsomes, the $t_{1/2}$ of (−)-R-BX and (+)-S-BX were 22.35 min and 10.66 min, whereas in rabbit microsomes, the $t_{1/2}$ of (−)-R-BX and (+)-S-BX were 11.75 min and 15.26 min, respectively.

Results of the incubation with (−)-R-and (+)-S-BX at 40 μM in rat liver microsomes indicated that similar to *rac*-BX, the (+)-S-BX degraded faster than its antipode, the $t_{1/2}$ of (−)-R-BX and (+)-S-BX were 5.42 min and 4.03 min, separately. After incubation with (−)-R-and (+)-S-BX at 30 μM in rabbit liver microsomes, the result showed that (−)-R-BX also degraded faster than its antipode. The $t_{1/2}$ of (−)-R-BX and (+)-S-BX were 5.66 min and 9.63 min in rabbit liver microsomes.

The EF was 0.50 after incubation of *rac*-BX in liver microsomes without NADPH, but the EF not only exceeded 0.50 but also increased with time in the rat liver microsomes with NADPH. Unlike the rat liver microsomes, the EF fell below 0.50 and decreased with time in rabbit liver microsomes with NADPH (see Fig. 4).

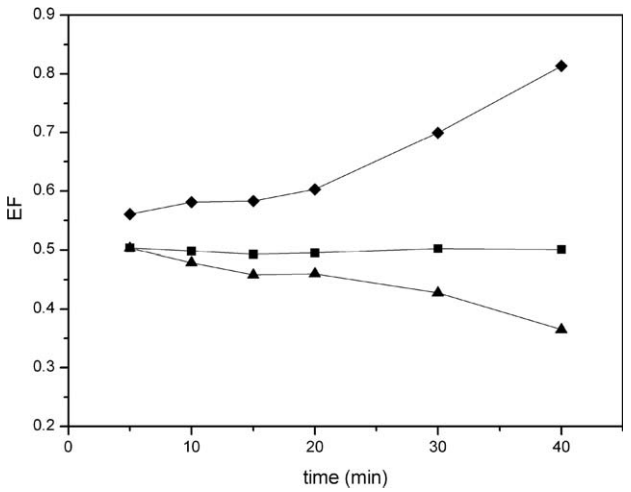

Fig. 4. Enantiomer fraction (EF) of BX enantiomers in liver microsomes after incubation of *rac*-BX administration at 80 μM for rat liver microsomes and 60 μM for rabbit liver microsomes: □, rat liver microsomes; ▲, rabbit liver microsomes; and ■, rabbit liver microsomes without NADPH.

Enzyme Kinetic Analysis

Both BX enantiomers were degraded by rat and rabbit liver microsomes and the degradation was NADPH-dependent. Metabolic rate constants (apparent K_m and V_{max}) were determined after a 10 min incubation period in rat and rabbit microsomes and Michaelis–Menten plots are shown in Figure 5. The apparent kinetic constants of BX degradation in rat and rabbit liver microsomes are shown in (Table 3)

According to the previous studies, *rac*-BX was ideally separated on a CDMPC-CSP under normal phase condition.[9] The selectivity factor and retention time increased when the percentage of *n*-propanol in the mobile phase (*n*-propanol + *n*-hexane) decreased. In this work, the HPLC method was developed to separate enantiomers of

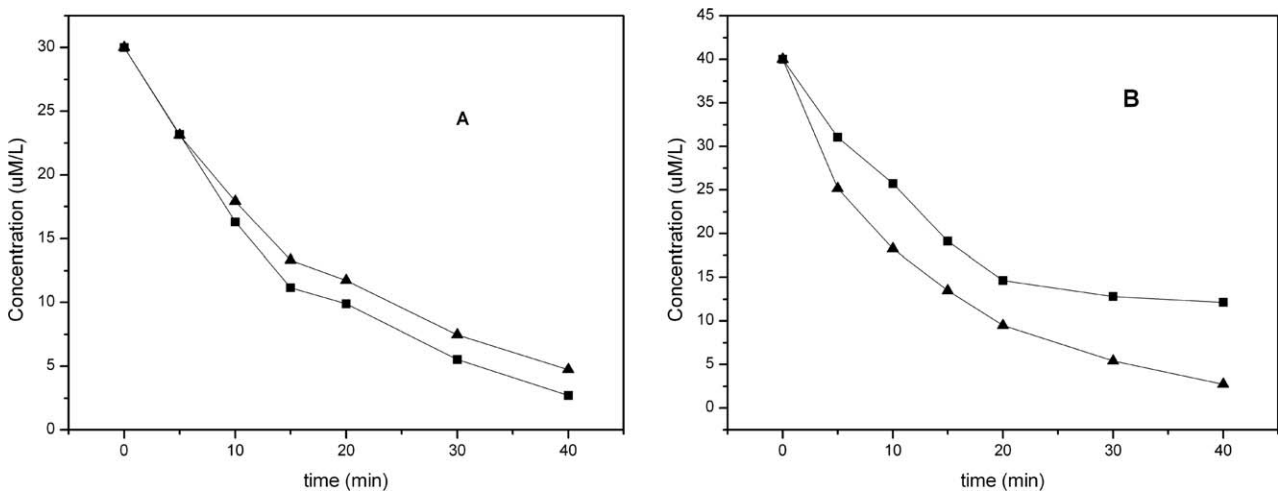

Fig. 3. Concentration-time curves of BX enantiomers after 40 min incubation of (**A**) *rac*-BX (60 μM) in rabbit liver microsomes, (**B**) *rac*-BX (80 μM) in rat liver microsomes. ■, (−)-R-BX and ▲, (+)-S-BX.

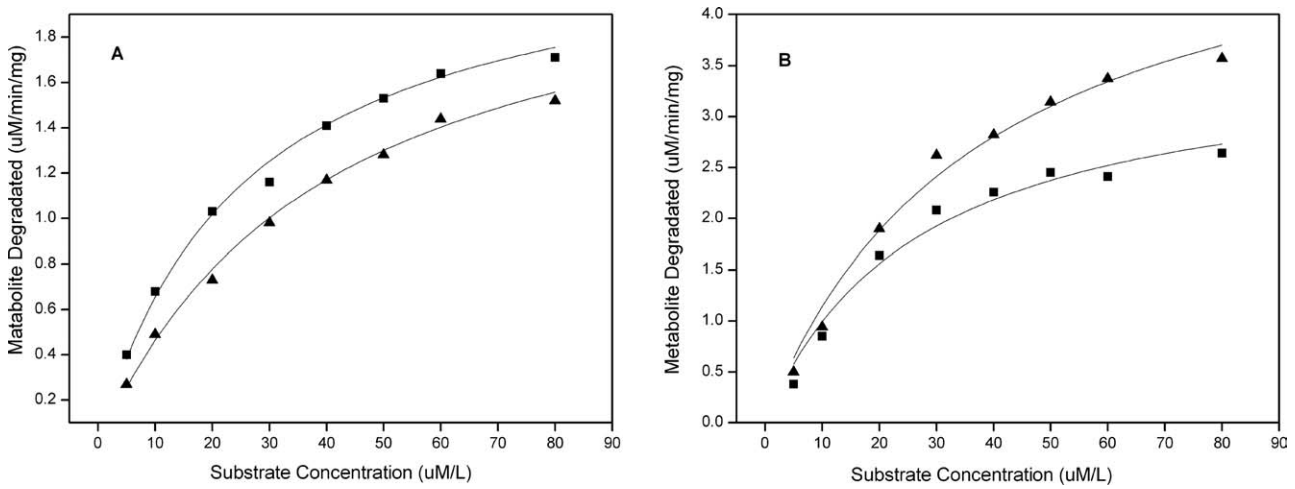

Fig. 5. Degradation rate of BX in rabbit (**A**) and rat (**B**) liver microsomes after 10min incubation of (+) and (−)-BX enantiomer. ■, (−)-R-BX and ▲, (+)-S-BX.

BX in rat and rabbit liver microsomes samples. The sample preparation procedure for liver microsomes was proved to be reproducible and precise with mean recovery above 86%. The extraction procedure did not cause epimerization of BX enantiomers.

In this study, it is noteworthy that these in vitro studies showed there is great difference in the $t_{1/2}$ of enantiomers between rat and rabbit liver microsomes. In rat liver microsomes (+)-S-BX was degraded faster than its antipode, on the contrary, (−)-R-BX was degraded faster than (+)-S-BX in rabbit liver microsomes. These results indicated there was stereoselectivity on metabolism of BX and its enantiomers in rat and rabbits liver microsomes. Even more importantly, stereoselective metabolism and pharmacokinetics of chiral drugs in rat and rabbit liver microsomes should also be helpful in the effective extrapolation of safety data to humans.

To elucidate the mechanism of stereoselective toxicokinetics of BX, we evaluated the possibility of stereoselective metabolism of BX using rat liver microsomes as an example of explaining stereoselective toxicokinetics. The $t_{1/2}$ of (−)-R-BX was two-fold of (+)-S-BX after incubation of *rac*-BX in rat liver microsomes. The intrinsic clearance of the (+)-S-BX was also higher than the (−)-R-BX in rat microsomes. In this context, significant differences were found for the stereoselective degradation of BX. Enzyme kinetic study using also show stereoselective differences on BX metabolism.

TABLE 3. Apparent kinetic constants of benalaxyl degradation in rabbit and rat liver microsomes

Sample	V_{max}(nmol min^{-1} mg^{-1} protein)	K_m (μm)	CL_{int} (ml min^{-1} mg^{-1} protein)
Rat			
(−)-R-benalaxyl	3641.1	26.71	136.32
(+)-S-benalaxyl	5446.3	37.77	144.20
Rabbit			
(−)-R-benalaxyl	2310.4	25.34	91.18
(+)-S-benalaxyl	2299.5	38.62	59.54

According to previous study, Wang et al. [19] reported the stereoselective degradation kinetics of θ-cypermethrin in rats after i.v. injection. The results showed the conversion of (+)-enantiomer to (−)-enantiomer in plasma after injection of (−)-and (+)-θ-cypermethrin separately. However, in this study, after incubation the single enantiomer of BX in rat or rabbit liver microsomes, all the results clearly indicated that there was no chiral conversion from the (+)-S-BX to (−)-R-BX or conversion from (−)-R-BX to (+)-S-BX.

CONCLUSIONS

The chiral HPLC method described in this investigation was validated for the study of the stereoselective behavior of BX enantiomers in rat and rabbits liver microsomes. This study demonstrated the stereoselective degradation of the BX and its enantiomers by rabbit and rat liver microsomal protein. These data may be important relative to elucidate the mechanism of stereoselective toxicokinetics of BX in liver microsomes and contribute to public health. A future study will be focused on the isoforms of cytochrome P-450 (CYP) that contribute to its stereoselectivity.

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