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Characterization of Pyribenzoxim Metabolizing Enzymes in Rat Liver Microsomes

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ABSTRACT. The primary metabolism of pyribenzoxim was studied in rat liver microsomes in order to identify the cytochrome P450 (CYP) isoform(s) and esterases involved in the metabolism of pyribenzoxim. Chemical inhibition using CYP isoform-selective inhibitors such as α-naphthoflavone, tolbutamide, quinine, chlorzoxazone, troleandomycin, and undecynoic acid indicated that CYP1A and CYP2D are responsible for the oxidative metabolism of pyribenzoxim. And inhibitory studies using eserine, bis-nitrophenol phosphate, dibucaine, and mercuric chloride indicated pyribenzoxim hydrolysis involved in microsomal carboxylesterases containing an SH group (cysteine) at the active center.

Keywords: Pyribenzoxim, Metabolism, Microsomes, Esterases, Cytochrome P450.

INTRODUCTION

Pyribenzoxim, benzophenone *O*-[2,6-bis[(4,6-dimethoxy-2-pyrimidinyl)oxy]benzoyl]oxime, is a new post-emergence herbicide providing broad-spectrum weed control in rice fields (Koo *et al.*, 1997, 1998). As do the sulfonylurea and imidazolinone herbicides, this pyrimidinyloxybenzoate is known to inhibit acetolactate synthase (ALS), the enzyme involved in the biosynthesis of the branched amino acids in plants (Bae *et al.*, 1997). Seo *et al.* (2002) suggested that the possibility of pyribenzoxim bioconcentration is not likely to occur in the aquatic environment. There was also no significant maternal or embryonic toxicity (Shin *et al.*, 1998), and no phytotoxicity was observed (Koo *et al.*, 1997). The bioavailabilty was negligible in rats by the elimination of radioactivity with feces (~88%) and urine (~8%) after 7 days of treatment (Liu *et al.*, 2001).

In a study of the *in vitro* metabolism of pyribenzoxim using rat liver microsomes (Kim *et al.*, 2000), four metabolites, BDB, benzophenone oxime, hydroxypyribenzoxim, and dihydroxypyribenzoxim were identified using tandem mass spectrometer. Kim *et al.* (2000) suggested that BDB and benzophenone oxime are produced by non-oxidative reactions, possibly from enzymatic hydrolysis of pyribenzoxim by microsomal esterases, whereas hydroxypyribenzoxim and dihydroxypyribenzoxim are formed with oxidative reactions by cytochrome P450. However, the specific CYP isoforms and esterases responsible for the metabolic transformations of the pyribenzoxim have not been identified definitely.

This report presents the effects of chemical inhibitors selective for various CYP isoforms and esterases on these reactions. All experiments were performed in rat liver microsomes.

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MATERIALS AND METHODS

Materials

Pyribenzoxim (purity 98.3%) was obtained from LG Chem Investment (Daejeon, Korea). Benzophenone, dimethylformamide, metyrapone, bis-p-nitrophenylphosphate,

and octylamine were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). β-Nicotinamide adenine dinucleotide phosphate (β-NADP), D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase (Bakers yeast dry ammonium sulfate cake type V), chlorzoxazone, coumarin, dibucaine, eserine, α-naphthoflavone, naringenine, quinidine, quinine, SKF-525A, sulfaphenazole, and tolbutamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). p-Nitrophenol, troleandomycin, and 10-undecynoic acid were obtained from Fluka Chemie GmbH (Buchs, Switzerland). Benzophenone oxime was prepared according to the method reported previously (Liu *et al.*, 2001). All solvents used for HPLC analysis were glass distilled (Duksan, Korea). All the other reagents and common chemicals were reagent grade or better.

Instruments

HPLC was performed using an HP 1100 system (Hewlett-Packard, CA, USA) with an HP Eclipse XDB-C18 column (4.6 × 150 mm, 3.5 μm, Hewlett-Packard). The mobile phase consisted of water-acetonitrile containing 0.1% acetic acid. A one-step linear gradient [A = water + 0.1% acetic acid, B = acetonitrile + 0.1% acetic acid: 60% A, 40% B at 0 min; 60% A, 40% B at 3 min; 30% A, 70% B at 7 min] was employed over 30 min with flow rate of 1.0 ml/min. UV detection (244 nm) was performed with a variable wavelength detector (HP 1100 series).

Preparation of microsomes

Preparation of rat liver microsomes was based on the method of Kim *et al.* (2000) using specific pathogen-free Sprague-Dawley rats which was obtained from SamTako Bio Korea (Osan, Korea). Rats (190~210 g) were sacrificed by cervical dislocation and the livers were immediately excised into beakers containing ice-cold saline to remove excess blood followed by homogenization with 4 volumes of ice-cold 1.15% potassium chloride solution (pH 7.4). The liver homogenates were centrifuged at 9,000 ×g for 10 min at 4°C, and the resulting post-mitochondrial supernatants were centrifuged again at 105,000 ×g for 60 min at 4°C. The microsomal pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA. Aliquots of liver microsomes were stored at -70°C until use. The microsomal protein content and cytochrome P450 (CYP) contents were determined using the bicinchoninic acid procedure (Smith *et al.*, 1985) and according to Omura and Sato (1985), respectively.

Rat liver microsomal reactions of pyribenzoxim

Rat liver microsomes (0.3 mg) were preincubated in 50 mM potassium phosphate buffer (1.0 ml, pH 7.4) in the presence of NADPH-generating system containing $NADP^+$ (0.8 mM), glucose-6-phosphate (10 mM) and 1 unit glucose-6-phosphate dehydrogenase for 10 min at 37°C in shaking water bath. The control incubations were conducted with heat-denatured microsomal preparations (80°C for 10 min) or with sodium fluoride (120 mM) or in the absence of NADPH-generating system. The reaction was initiated by the addition of 30 mM pyribenzoxim and proceeded for 30 min at 37°C. Reaction rates were linear with incubation time under these conditions.

Reactions were terminated at 30 min after treatment by adding 85% phosphoric acid (50 ml). The microsomes samples were extracted with ethyl acetate (2 ml) by vortexing (1 min), and centrifuged (4,000 ×g, 5 min). This procedure was repeated once more to increase extraction efficiency. The combined organic phase was concentrated by purging with nitrogen gas, the residue was dissolved in methanol (200 ml), and an aliquot (20 ml) was analyzed by HPLC.

Cytochrome P450 (CYP) inhibition study

The selective effects of inhibitors or substrates (potential competitive inhibitors) to various CYP isozymes in pyribenzoxim metabolic pathways were studied. The CYP isozyme selective inhibitors or alternative substrates used were SKF-525A (CYP) (Lubet *et al.*, 1985), α-naphthoflavone (CYP1A) (Nnane *et al.*, 1999), chlorzoxazone (CYP2E) and coumarin (CYP2A) (Teramura *et al.*, 1997), metyrapone (CYP) (Murray and Reidy, 1990), naringenine (CYP1A) and p-nitrophenol (CYP2E) (Teramura *et al.*, 1997), *n*-octylamine (flavin-containing monooxygenase (FMO) activator) (Ziegler, 1980), quinidine (CYP2D) and quinine (CYP2D) (Kobayashi *et al.*, 1989), sulfaphenazole (CYP2C), tolbutamide (CYP2C), and troleandomycin (CYP3A) (Teramura *et al.*, 1997), and undecynoic acid (CYP4A) (Murray and Reidy, 1990), Methanol (0.5% final incubation concentration) was used to dissolve various inhibitors except for water soluble quinidine and quinine.

Other solvents (acetone, dimethylsulfoxide (DMSO), and methanol) considered to be the potential inhibitors were tested in liver microsomes with respect to their influence on pyribenzoxim metabolism. All solvents were studied at a concentration of 0.5% (v/v). In all cases, inhibited activities were compared with activities in control, which contained 5 mL water as appropriate.

Inhibitors were preincubated with microsomes and the NADPH-generating system for 10 min at 37°C before the reaction was started by addition of the substrate, pyribenzoxim, followed by 30 min of incubation as above.

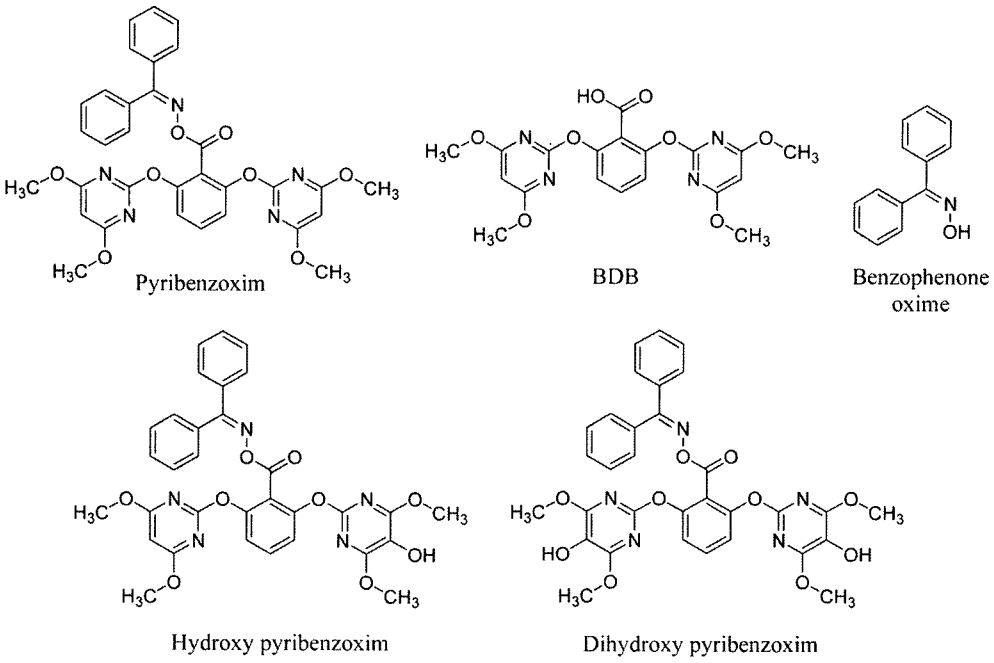

Fig. 1. Structure of pyribenzoxim, BDB, benzophenone, benzophenone oxime, hydroxy pyribenzoxim and dihydroxy pyribenzoxim.

Control incubations were conducted without any inhibitors in parallel. Duplicate samples were used in all inhibition experiments.

Esterase inhibition study

To determine the identity of esterases responsible for the formation of the hydrolysis products such as benzophenone oxime, microsomes were preincubated (10 min at 37°C) with various esterases inhibitors (4-bromophenylboronic acid (BPBA), calcium chloride, cobalt chloride, dibucaine, EDTA, eserine, magnesium chloride, mercuric chloride, sodium fluoride, or quinidine) prior to the addition of pyribenzoxim, followed by 30 min of incubation as above in the absence of NADPH-generating system. Control incubations without any inhibitors in the incubation were carried out in parallel.

Statistical analysis

Statistical analyses were performed using Student's *t*-test (JMP Statistical Software, SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Metabolism of pyribenzoxim by rat liver microsomes

In the rat liver microsomes reaction with pyribenzoxim, four metabolites (BDB, benzophenone oxime, monohydroxy pyribenzoxim, and dihydroxy pyribenzoxim, Fig. 1) were observed as reported by Kim *et al.* (2000). It was observed that the oxidation reaction of pyribenzoxim was inhibited by cytochrome P450 inhibitors, metyrapone and SKF-525A, indicative of cytochrome P450 mediation in the reaction (Table 1). To investigate the role of flavine-containing monooxygenase (FMO) in oxidative metabolism of pyribenzoxim, *n*-octylamine was treated in microsomes fraction. However, *n*-octylamine did not significantly activate pyribenzoxim metabolism ($p < 0.05$), indicating that FMO was not involved on the oxidative pyribenzoxim metabolism (Table 1).

Characterization of pyribenzoxim metabolizing CYP isozymes. As many cytochrome P450 (CYP) inhibitors are poorly soluble in water or buffer, thus, organic solvents are frequently used to aid inhibitorsa solubility in *in vitro* experiments. The presence of an organic solvent can strongly affect the reliability and interpretation of *in vitro* data. These effects can be associated with solvation properties or competitive metabolism of the solvents for the enzyme in question (Chauret *et al.*, 1998). For example, it has been reported, using specific cytochrome P450 substrates, that DMSO can inhibit the activity of CYP2E1 and that several organic solvents can affect the activity of CYP2A6 (Draper and Parkinson, 1997; Yoo *et al.*, 1987). Therefore, the effects of several solvents for pyribenzoxim metabolism were studied.

Table 1. Effect of FMO activator and CYP inhibitors on the oxidative metabolism of pyribenzoxim in rat liver microsomes

Inhibitor	Inhibitor concentration (μM)	Percentage of control activity[a]		
		Dihydroxy pyribenzoxim	Hydroxy pyribenzxoxim	Pyribenzoxim
Control	100	100	100	100
Octylamine	100	95.9	103.4	104.3
SKF-525A*	100	24.9	80.6	124.3
Metyrapone*	100	0	21.5	136.9

*Significant difference compared with control activity ($p < 0.05$).
[a]Each value are represented as mean ($n = 2$ independent experiments performed in triplicate).

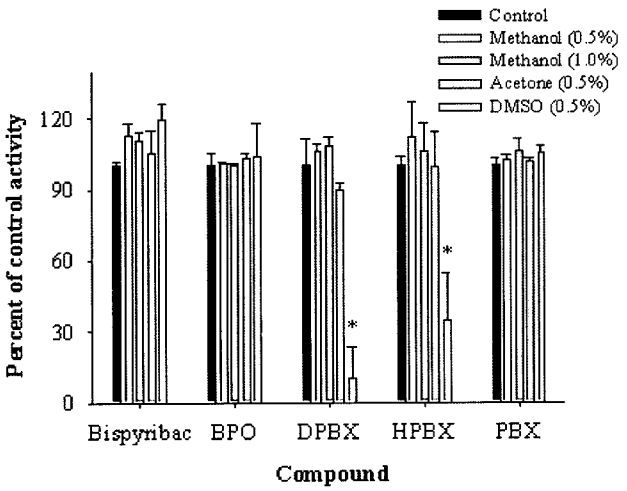

Fig. 2. Effect of organic solvents on the oxidative pyribenzoxim metabolism with rat liver microsomes (*significant difference at $\alpha = 0.05$; dimethylsulfoxide (DMSO), benzophenone oxime (BPO), dihydroxy pyribenzoxim (DPBX), hydroxy pyribenzoxim (HPBX), pyribenzoxim (PBX)).

The effect of methanol, DMSO, and acetone on the oxidative metabolism of pyribenzoxim are summarized in Fig. 2. In all cases, the solvents did not affect the enzymatic hydrolysis of pyribenzoxim. Also methanol (≤ 1%) and acetone (≤ 0.5%) did not affect the oxidative metabolism of pyribenzoxim, but DMSO inhibited the production of oxidative metabolites at a solvent concentration of 0.5% ($p < 0.05$). This is consistent with the fact that DMSO can inhibit the activity of various CYPs even at low levels (0.2%) (Chauret *et al.*, 1998). In general, methanol and acetonitrile represent better alternatives as long as the content is kept at a relatively low level.

CYP, a superfamily of haem-containing monooxygenases, catalyzes the metabolism of xenobiotics, such as drugs, pesticides and endogeneous substrates with broad overlapping specificities (Guengerich, 1991). CYPs are microsomal enzymes that exist mainly in liver as well as in extrahepatic tissues such as intestines, lungs and kidneys (Emoto *et al.*, 2000). Rat CYPs have been characterized over the years, and using immunochemical methods it has been shown that CYP 1A, 2A, 2B, 2C, 2D, 2E, 3A, and 4A constitute the majority of the CYPs present in the liver. Also, it has been widely shown that some chemicals can inhibit, in a very spe-

Table 2. Effect of CYP isozyme inhibitors on the oxidative metabolism of pyribenzoxim in rat liver microsomes

Inhibitor	Target CYP isozyme	Inhibitor concentration (μM)	Percentage of control activity[a]		
			Dihydroxy pyribenzoxim	Hydroxy pyribenzxoxim	Pyribenzoxim
Control		100	100	100	100
α-Naphthoflavone*	CYP1A	100	8.0	34.4	143.7
Naringenine*	CYP1A	100	54.2	88.3	151.8
Coumarin	CYP2A	100	92.2	111.4	112.9
Tolbutamide	CYP2C	100	90.9	103	116.1
Sulfaphenazole	CYP2C	100	-[b]	112.3	116.0
Quinidine*	CYP2D	100	64.5	91.9	126.6
Quinine*	CYP2D	100	57.0	63.5	99.9
Chlorzoxazone	CYP2E	100	105.2	111.8	106.4
p-Nitrophenol	CYP2E	500	105.2	108.8	109.3
Troleandomycin	CYP3A	100	102	104.8	102.4
Undecynoic acid	CYP4A	100	119.2	106.5	101.4

*Significant difference compared with control activity ($p < 0.05$).
[a]Each value are represented as mean ($n = 2$ independent experiments performed in triplicate).
[b]Not determined due to overlapping with inhibitor-oriented peaks.

cific way, the *in vitro* activity of CYP mediated enzymatic reactions (Chauret *et al.*, 1997).

Catalytic analysis might have higher sensitivity than immunoblot analysis to characterize microsomal CYP enzymes (Emoto *et al.*, 2000). Effects of various CYP inhibitors on the metabolism of pyribenzoxim were examined (Table 2). Methanol was selected to dissolve water insoluble inhibitors based on the results obtained through the solvent inhibition study. Inhibitors selective for CYP2A (coumarin 100 μM), CYP2C (tolbutamide or sulfaphenazole 100 μM), CYP2E (chlorzoxazone 100 μM or p-nitrophenol 500 μM), CYP3A (troleandomycin 100 μM), or CYP4A (undecynoic acid 100 μM) did not significantly inhibit pyribenzoxim metabolism ($p < 0.05$). Both hydroxy pyribenzoxim and dihydroxy pyribenzoxim formation were significantly inhibited by CYP1A inhibitors (α-naphthoflavone or naringenine) and CYP2D inhibitors (quinidine or quinine) (Fig. 3). More inhibition by those inhibitors was observed at higher concentrations giving higher inhibition of dihydroxy pyribenzoxim formation than that of hydroxy pyribenzoxim formation. Quinine inhibited hydroxy pyribenzoxim formation more potently than quinidine because quinine is a potent inhibitor of rat CYP2D, while its stereoisomer quinidine is not (Kobayashi *et al.*, 1989).

In summary, the effects of putative inhibitors of hydroxy pyribenzoxims formation suggested that at least two CYP isozymes (CYP1A and CYP2D) are involved in oxidative reaction. Selective inhibitors of CYP2A, CYP2C, CYP2E, CYP3A and CYP4A had little or no effect.

This chemical inhibitors have a number of advantages in that they are simple to use and readily available. But it is important to note that nonspecific inhibition of CYPs can be observed at high concentrations of specific inhibitors. That fact should be taken into consideration when inhibition is only observed at relatively high inhibitor concentrations (Chauret *et al.*, 1997). For example, recent accounts have shown that many of the specific inhibitors (diethyldithiocarbamate, furafylline, ketoconazole, quinidine, and troleandomycin) can cause up to 20% inhibition of other CYP activities at the high range of concentration used (Newton *et al.*, 1995). Therefore, other experiments such as immunoblot analysis were needed to identify pyribenzoxim metabolizing CYP isozymes more precisely. Also the use of mono-

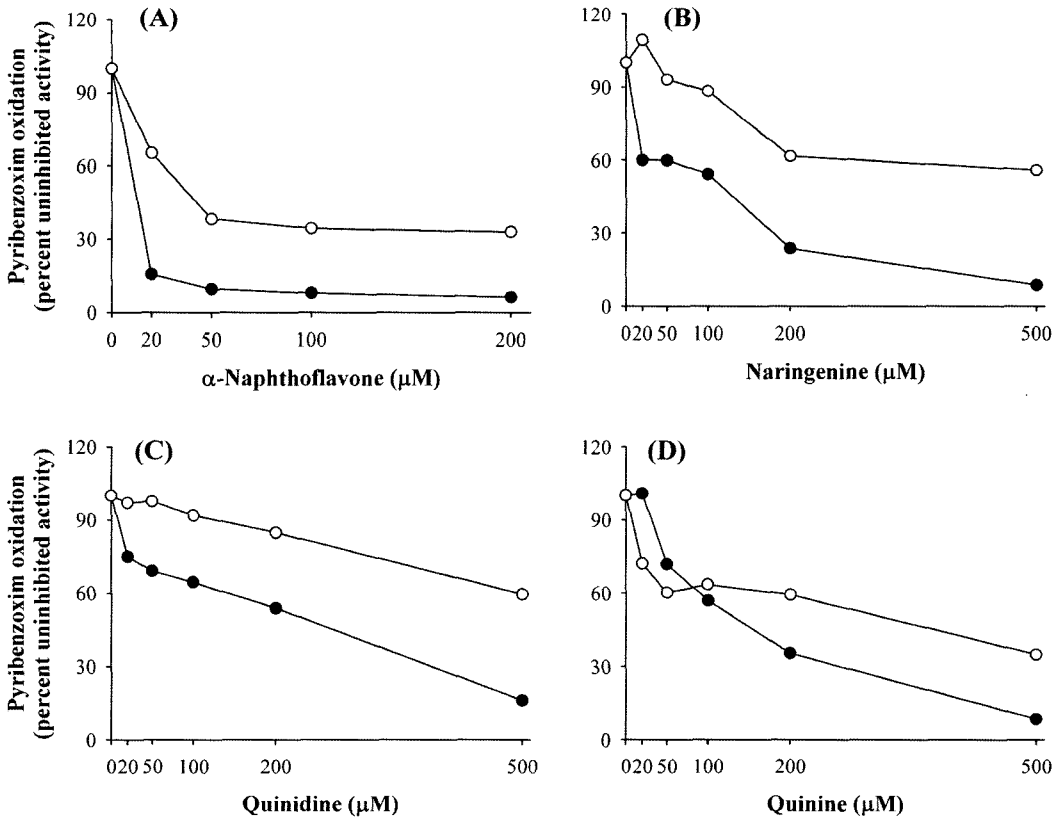

Fig. 3. Inhibition of oxidative metabolism of pyribenzoxim by various CYP isozyme inhibitors ((A) α-naphthoflavone, (B) naringenine, (C) quinidine, and (D) quinine) in rat liver microsomes (Dihydroxy pyribenzoxim: ●; Hydroxy pyribenzoxim: ○).

clonal antibodies directed against specific CYP isozymes and expressed specific CYP isozymes provided further confirmation of the involvement of specific isozymes in the oxidative metabolism of pyribenzoxim.

Characterization of pyribenzoxim esterases

Humans are exposed to a range of xenobiotic esters used as pesticides both occupationally and in the general environment. Hydrolysis by esterases, present in liver microsomes, cytosol and blood has been shown to limit the activity of many esterified chemicals and drugs. Xenobiotic metabolizing esterases include: A-esterases which hydrolizing organophosphates, B-esterases [cholinesterases (EC 3.1.1.8) and carboxylesterases (EC 3.1.1.1)] which are inhibited by organophosphates, and C-esterases which do not interact. A-esterases include plasma paraoxonases now classified as phosphoric triester hydrolases (EC 3.1.8.1) as a separate enzyme from arylesterases (EC 3.1.1.2) (McCracken *et al.*, 1993). Many criteria have been employed to differentiate these classes of enzyme but, because of their widely overlapping substrate specificities, the best criterion for classfication is their sensitivity to various activators and inhibitors (Ecobichon , 1970).

To characterize the enzymes involved in hydrolysis of pyribenzoxim, inhibition studies were carried out using a variety of inhibitors (Table 3). Addition of mercuric chloride into the incubations of pyribenzoxim with liver microsomes completely inhibited the production of BDB and benzophenone oxime, thereby showing that enzymes containing an SH group at the active center (including

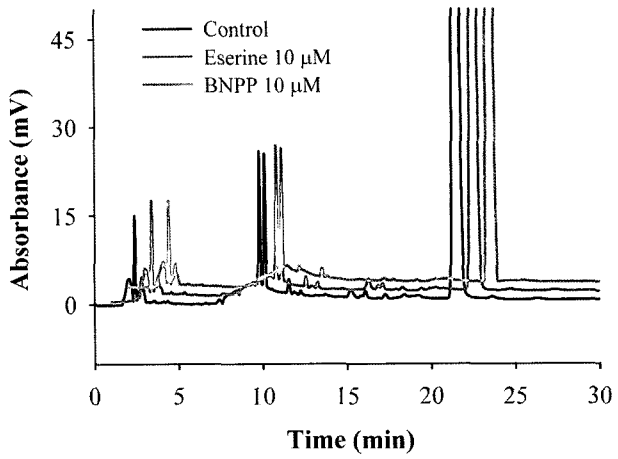

Fig. 4. HPLC elution profiles of rat liver microsomal reation mixture with pyribenzoxim and esterases inhibitors (eserine, bis-nitrophenylphosphate (BNPP)).

arylesterases) are involved in the catalysis of this reaction (Iatsimirskaia *et al.*, 1997). But, the activity of microsomal esterases was not affected by Ca^{2+} (A-esterases activator) (Erdos *et al.*, 1959), Mg^{2+} (A-esterases inhibitor) (Erdos *et al.*, 1959), Co^{2+} (A-esterases inhibitor) (Erdos *et al.*, 1959), or EDTA (A-esterases inhibitor due to chelation of calcium ions) (Tang and Chambers, 1999) suggesting that the esterases were not A-esterases. Micromolar BNPP concentrations caused progressive inhibition of many carboxylesterases, while cholinesterases were unaffected even at about 100 μM BNPP (Reiner *et al.*, 1998; Simeon *et al.*, 1988). BNPP (0.1, 0.01 mM) completely suppressed the hydrolysis of

Table 3. Effects of various esterases inhibitors on the hydrolysis of pyribenzoxim by rat liver microsomes

Inhibitor	Target esterases	Concentration (μM)	Specific activity (mM/min/mg protein)[a]		
			BDB	Benzophenone oxime	Pyribenzoxim
Control	-	-	0.79 ± 0.03	0.58 ± 0.02	14.83 ± 0.23
$CaCl_2$**	A-esterases	100	0.74 ± 0.03	0.54 ± 0.01	14.67 ± 0.37
EDTA	A-esterases	3000	0.77 ± 0.01	0.57 ± 0.02	14.77 ± 0.25
$CoCl_2$	A-esterases	1000	0.76 ± 0.02	0.55 ± 0.02	14.57 ± 0.34
$MgCl_2$	A-esterases	1000	0.75 ± 0.03	0.55 ± 0.02	15.22 ± 0.39
$HgCl_2$*	Arylesterases	100	0	0	15.91 ± 0.19
NaF*	General esterases	10	0.28 ± 0.02	0.19 ± 0.01	15.92 ± 0.33
		50	0.09 ± 0.01	0.07 ± 0.01	16.06 ± 0.27
Eserine	Cholin-esterases	10	0.76 ± 0.04	0.57 ± 0.01	15.02 ± 0.04
		100*	0.30 ± 0.01	0.20 ± 0.00	15.73 ± 0.04
Bis-nitrophenyl phosphate*	Carboxyl-esterases	10	0	0	16.23 ± 0.36
		100	0	0	16.54 ± 0.49
Dibucaine	Cholin-esterases	10	0.78 ± 0.02	0.59 ± 0.00	15.01 ± 0.39
		100	0.79 ± 0.04	0.58 ± 0.04	14.81 ± 0.33
Quinidine	Cholin-esterases	10	0.79 ± 0.02	0.56 ± 0.02	14.95 ± 0.76
		100	0.79 ± 0.01	0.55 ± 0.03	15.02 ± 0.05

*Significant difference compared with control activity ($p < 0.05$).
**A-esterases activator.
[a]Each value are represented as mean ($n = 2$ independent experiments performed in triplicate).

pyribenzoxim in microsomal incubations (Fig. 4). Inhibition of hydrolysis by eserine, a specific inhibitor of cholinesterases (10 μM, 100% inhibition, Fig. 3) (Ecobichon, 1970; Augustinsson, 1958), was not observed at low concentration (10 μM), but observed at high level (100 μM) probably due to inhibition of carboxylesterases (Ecobichon and Kalow, 1963; Krisch, 1971). Dibucaine (Nigg *et al.*, 1996), and quinidine (Whittaker, 1984), other inhibitors of cholinesterases, did not significantly inhibit the hydrolysis of pyribenzoxim by the liver microsomes. These data indicate that the production of BDB and benzophenone oxime in rat is catalyzed by microsomal carboxylesterases containing an SH group (cysteine) at the active center. Ali *et al.* (1985) reported that carboxylesterases hydrolyzing hydrocortisone esters containing an SH group at the active site exists in liver microsomes.

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