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HUMAN XENOBIOTIC METABOLIZING ESTERASES IN LIVER AND BLOOD

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Abstract—Esterases in human liver microsomes hydrolysed fluazifop-butyl (V_{max} 9.8 ± 1.6 μmol/min/g tissue), paraoxon (V_{max} 47.4 ± 7.5 nmol/min/g tissue) and phenylacetate (V_{max} 57 ± 8 μmol/min/g tissue), whereas esterases found in the human liver cytosol hydrolysed fluazifop-butyl (V_{max} 10.0 ± 0.5 μmol/min/g tissue) and phenylacetate (V_{max} 37 ± 2.9 μmol/min/g tissue) but not paraoxon. Human plasma esterase hydrolysed fluazifop-butyl (V_{max} 0.09 ± 0.006 μmol/min/mL), paraoxon (V_{max} 210 ± 14 nmol/min/mL) and phenylacetate (V_{max} 250 ± 17 μmol/min/mL). Inhibitory studies using paraoxon, bis-nitrophenol phosphate and mercuric chloride indicated fluazifop-butyl hydrolysis involved carboxylesterase in liver microsomes and cytosol, and cholinesterase and carboxylesterase in plasma. Phenylacetate hydrolysis involved arylesterase in plasma, both arylesterase and carboxylesterase in liver microsomes and carboxylesterase in liver cytosol. Plasma hydrolysis is less important and overall esterase activity is lower in humans than in the rat which is therefore a poor model.

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 [1]. In a previous study the tissue distribution of xenobiotic metabolizing esterases was studied in the rat [2].

Xenobiotic metabolizing esterases include: "A" esterases which hydrolyse organophosphates, "B" esterases which are inhibited by organophosphates and include cholinesterase (EC 3.1.1.8) and carboxylesterase (EC 3.1.1.1) and "C" esterases which do not interact [3]. "A" esterases include plasma paraoxonase now classified as phosphoric triester hydrolase (EC 3.1.8.1) as a separate enzyme from arylesterase (EC 3.1.1.2) [4].

Human plasma contains cholinesterase but little carboxylesterase in contrast to the rat plasma which has high carboxylesterase levels. Plasma cholinesterase contributes significantly to hydrolysis of many ester drugs as well as liver [5].

Paraoxonase present in the human plasma has been shown to be under genetic control, with high and low activities. Variation in plasma paraoxonase activity may contribute to inter-individual variation in susceptibility [6]. It is, therefore, important to assess plasma and red blood cell hydrolysis and correlate this to liver hydrolysis.

In this study hydrolysis of fluazifop-butyl, paraoxon and phenylacetate by human plasma, red blood cells, liver microsomes and cytosol was determined. Inhibitory studies were carried out to distinguish the esterases involved in hydrolysis of the three substrates. The esterases involved in human tissue could be compared to the previous study in the rat in which these substrates were also used [2].

Studies in man have found that no unchanged fluazifop-butyl is seen in the blood after oral or dermal dosing to human volunteers [7, 8] and only fluazifop acid, the major metabolite, is excreted in the urine. Parathion is activated by microsomal enzymes to paraoxon [*o*,*o*-diethyl-*o*-(4-nitrophenyl)phosphate] [9] which is hydrolysed by paraoxonase to diethylphosphoric acid and *p*-nitrophenol.

METHODS AND MATERIALS

Chemicals. Fluazifop-butyl and fluazifop were gifts from Zeneca plc (Alderley Park, Cheshire, U.K.). All other chemicals were obtained from the Sigma Chemical Co. (Poole, U.K.).

Blood and liver samples. Samples of human liver were obtained from kidney transplant donors from a number of hospitals in the North East of England. Liver samples were placed into liquid nitrogen as soon as possible after removal and stored at −80° until analysis. Patient details can be seen in Table 1. Samples of venous blood (10 mL) were collected into heparinized tubes from 10 healthy male volunteers (aged 20–36 years) who were drawn from scientific staff, University of Newcastle upon Tyne.

Separation of plasma and red blood cells. Plasma and red blood cells were separated by centrifugation using a Mistral 3000 centrifuge, at 880 *g* for 5 min. The plasma was removed and stored at −80° before analysis. The red blood cells were washed using an equal volume of isotonic saline. After centrifugation at 880 *g* for 10 min the upper layer was removed and discarded. This procedure was again repeated. The red blood cells were then stored at −80° before analysis. Thawing prior to assay lysed the red blood cells.

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Table 1. Human liver samples

Liver sample number	Sex	Age	Liver function test	Cause of death
1	Male	55	Normal	Head injury
2	Female	63	Normal	Cerebral haemorrhage
3	Female	47	—	Subarachnoid haemorrhage
4	Male	35	Normal	Subarachnoid haemorrhage
5	Male	55	Normal	Head injury
6	Female	45	Normal	Subarachnoid haemorrhage
7	Female	47	Normal	Subarachnoid haemorrhage

Table 2. Enzyme kinetics for hydrolysis of fluazifop-butyl by human liver subcellular fractions, plasma and red blood cells

	V_{max} (μmol/min/g(mL) of tissue)	K_m (μM)
Liver microsomes	9.8 ± 1.6	36 ± 4.2
Liver cytosol	10 ± 0.52	32 ± 3.7
Plasma†	0.088 ± 0.006	88 ± 7.8*
Red blood cells†	0.005 ± 0.0002	ND

* $P < 0.05$ between tissues.
Mean ± SEM (N = 7).
† Mean ± SEM (N = 10).
ND = not detectable.

Preparation of liver subcellular fractions. Subcellular fractions of liver were prepared as described previously [2].

Incubations. Fluazifop-butyl hydrolysis. Liver microsomes or cytosol equivalent to 0.8 mg wet weight liver, 50 μL plasma or 50 μL lysed red blood cells were incubated with fluazifop-butyl (final concentration of 0.02–1.0 mM). Incubations were described previously [2]. HPLC conditions were as described previously [2].

Paraoxon hydrolysis. An aliquot of the liver microsomal or cytosolic fraction equivalent to 10 mg original wet weight was incubated with paraoxon (final concentration of 0.1–1.0 mM). Incubations and HPLC conditions were as described previously [2]. The hydrolysis of paraoxon to *p*-nitrophenol in the plasma was measured using a modification of the method of Eckerson *et al.* [10]. Plasma (50 μL) was incubated with paraoxon (1 mM) in a final volume of 3 mL 50 mM glycine buffer pH 10.5, containing 1 mM calcium chloride at 30°. Reactions were started by the addition of paraoxon. Formation of *p*-nitrophenol was continuously monitored at 412 nm in a spectrophotometer. Control incubations containing 50 mM glycine/1 mM calcium chloride buffer pH 10.5 alone were conducted in parallel.

Phenylacetate hydrolysis. Microsomal or cytosolic protein equivalent to 3 mg liver 50 μL plasma or 50 μL lysed red blood cells were incubated with a final concentration of 0.5–4 mM phenylacetate. Incubations were as described previously with spectrophotometric detection [2].

Enzyme kinetics. Conditions of linearity for protein and time had been established at high and low substrate concentrations. Enzyme activities were expressed as μmol product formed/min/g (mL) tissue. Median values for V_{max} and apparent K_m were calculated and results expressed as mean ± SEM.

Inhibition studies. Microsomal or cytosolic protein equivalent to 0.8 mg or 50 μL of plasma was preincubated at 37° with 0.1 mM paraoxon, 0.1 mM bis-nitrophenol phosphate (BNPP), 0.1 mM physostigmine or 0.1 mM mercuric chloride for 5 min prior to the addition of fluazifop-butyl (final concentration 25 μM). Control incubations without any inhibitors in the incubation were carried out in parallel.

Similarly microsomal and cytosolic protein equivalent to 3 mg or 30 μL plasma was preincubated with 0.1 mM paraoxon, 0.1 mM mercuric chloride, 0.1 mM BNPP or 0.1 mM physostigmine for 5 min before phenylacetate (2 mM) was added. Control incubations with no inhibitor were carried out in parallel.

RESULTS

Fluazifop-butyl esterase

Fluazifop-butyl was hydrolysed to fluazifop by microsomal and cytosolic fractions from human liver as well as by plasma and red blood cells. Hydrolysis by all tissues followed Michaelis–Menten kinetics and V_{max} and K_m values are shown in Table 2. Hydrolysis was equally distributed in the microsomes and cytosol fraction of the liver. Plasma esterase activity was approximately one hundredth the liver microsomes and cytosol with little activity in the red

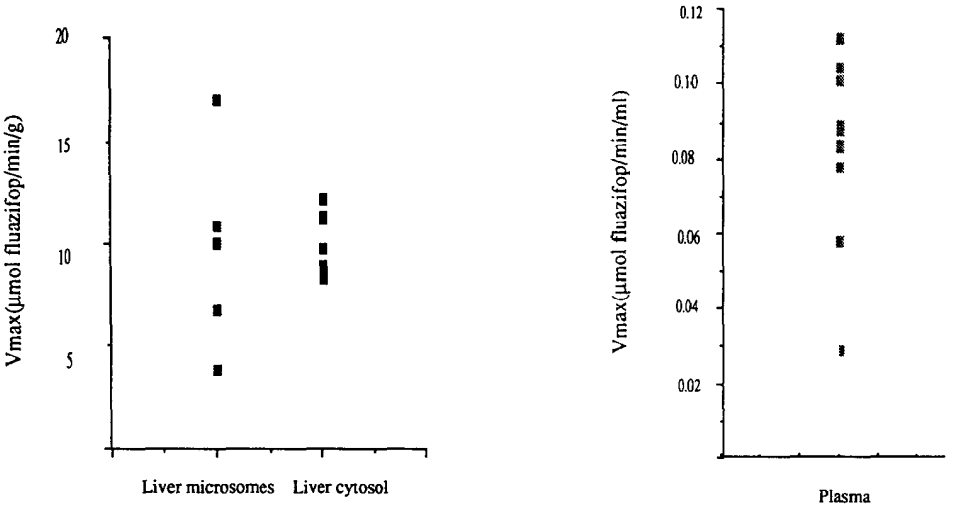

Fig. 1. Hydrolysis of fluazifop-butyl by human liver microsomes and cytosol (N = 7) and by plasma (N = 10).

Table 3. Inhibition of fluazifop-butyl hydrolysis to fluazifop in human liver subcellular fractions and plasma by 0.1 mM BNPP, physostigmine, mercuric chloride and paraoxon

		Remaining activity (%)				
	Substrate (μM)	Control	BNPP	Physostigmine	Mercuric chloride	Paraoxon
Liver microsomes	25	100	14 ± 1.3	86 ± 5.2	97 ± 1.8	0 ± 0
Liver cytosol	25	100	8 ± 0.52	89 ± 3.1	97 ± 2.4	4 ± 1.0
Plasma*	25	100	44 ± 2.4	59 ± 4.2	—	0 ± 0

Results were expressed as % of control.
Mean ± SEM (N = 7).
* Mean ± SEM (N = 10).

blood cells. There was greater inter-individual variation in the liver microsomal and plasma samples compared to the liver cytosolic fraction (Fig. 1).

Apparent K_ms for microsomal and cytosolic fluazifop-butyl esterase were similar (Table 2). The apparent K_m for plasma esterase was greater than that of liver fractions indicating a lower affinity of the plasma enzyme for fluazifop-butyl.

Paraoxon completely inhibited fluazifop-butyl hydrolysis in microsomes, cytosol and plasma (Table 3). Inhibition of hydrolysis of fluazifop-butyl by BNPP, with liver microsomal and cytosolic fractions was greater than 80%, but lower in the plasma. Inhibition of hydrolysis by physostigmine was less (11% liver cytosol, 14% liver microsomes and 31% plasma). Mercuric chloride did not significantly inhibit the hydrolysis of fluazifop-butyl by the liver microsomal and cytosolic fractions.

Paraoxonase

Paraoxonase activity was detected only in the liver microsomes and the plasma, no paraoxonase activity was found in the cytosolic fraction of the liver or in the red blood cells [limit of detection >0.3 nmol/min/g(mL)]. The specific activity of plasma paraoxonase (expressed /mL of plasma) was four times greater than liver paraoxonase (expressed /g tissue). Values for V_{max} can be seen on Table 4. There was significant interindividual variation in both the liver microsomal and plasma samples (Fig. 2).

The apparent K_ms for liver microsomal and plasma paraoxonase were similar (Table 4) suggesting the same enzyme.

Phenylacetate esterase

Phenylacetate was hydrolysed by both microsomes and cytosol from liver, as well as by plasma (V_{max} values are shown in Table 4). Hydrolysis was equally distributed in the microsomes and cytosol fraction of the liver. Plasma esterase activity was greater than the liver microsomes and cytosol with no activity found in the red blood cells. Inter-individual variation in liver (microsomal and cytosolic) and plasma samples was small.

Table 4. Enzyme kinetics of the hydrolysis of paraoxon and phenylacetate by human liver subcellular fractions, plasma and red blood cells

	Paraoxon		Phenylacetate	
	V_{max} (nmol/min/g(mL) of tissue)	K_m (μM)	V_{max} (μmol/min/g(mL) of tissue)	K_m (μM)
Liver microsomes	47.4 ± 7.5	280 ± 23	57 ± 8.0	280 ± 35
Liver cytosol	ND	ND	37 ± 2.9	190 ± 25
Plasma†	210 ± 14	240 ± 31	250 ± 17	1480 ± 90*
Red blood cells†	ND	ND	ND	ND

* $P < 0.05$ between tissues, within substrate.
Mean ± SEM (N = 7) (pH 8, 37°).
† Mean ± SEM (N = 10) (pH 10.5, 30°).
ND = not detectable.

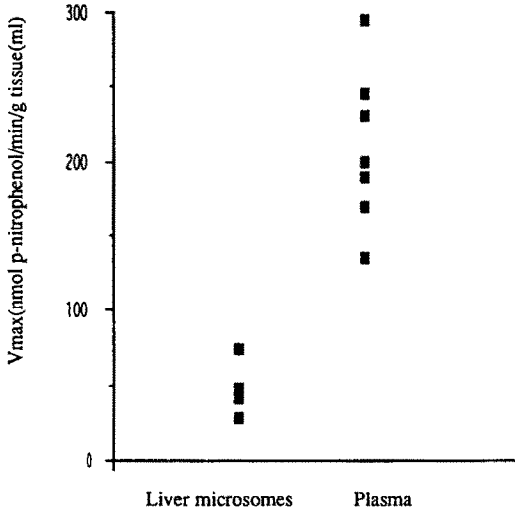

Fig. 2. Hydrolysis of paraoxon by human liver microsomes and by plasma.

The apparent K_ms for microsomal and cytosolic phenylacetate esterase were similar. The apparent K_m for plasma was significantly higher than liver microsomal or cytosolic fractions (Table 4).

Inhibition of hydrolysis of phenylacetate by paraoxon was similar for liver microsomal and cytosolic fractions but less for the plasma. BNPP inhibition was similar to that of paraoxon with higher values seen in the liver compared to the plasma. Inhibition by physostigmine was less than that due to BNPP in the liver cytosol (26%) and plasma (1%) but the same in the liver microsomes (52%). Inhibition due to mercuric chloride was greater in the liver microsomes than in the cytosol (Table 5).

DISCUSSION

Both fluazifop-butyl and phenylacetate were hydrolysed by microsomes and cytosol from human liver. This differed from paraoxon which was hydrolysed by the liver microsomes but not in the cytosol. In the human plasma all three substrates were hydrolysed, however, only fluazifop-butyl was detectably hydrolysed by esterases found in the red blood cells. The apparent K_m values for hydrolysis of paraoxon and phenylacetate were similar suggesting the involvement of similar esterases, whereas apparent K_m values for fluazifop-butyl hydrolysis were lower.

Paraoxonase and arylesterase have recently been classified separately [4], however, there has been controversy as to whether phenylacetate and paraoxon are hydrolysed by the same enzyme in human plasma [11]. Esterases responsible for both paraoxon and phenylacetate hydrolysis were detected in the human liver microsomes and plasma. There

Table 5. Inhibition of phenylacetate hydrolysis to phenol in human liver subcellular fractions and plasma by 0.1 mM BNPP, physostigmine, mercuric chloride and paraoxon

	Substrate (mM)	Remaining activity (%)				
		Control	BNPP	Physostigmine	Mercuric chloride	Paraoxon
Liver microsomes	2	100	48 ± 2.9	48 ± 2.9	59 ± 6.3	38 ± 10
Liver cytosol	2	100	24 ± 1.4	74 ± 1.4	90 ± 3.1	32 ± 4.7
Plasma*	2	100	97 ± 2.6	99 ± 1.0	—	94 ± 2.1

Results were expressed as % of control.
Mean ± SEM (N = 7).
* Mean ± SEM (N = 10).

was however phenylacetate hydrolysis in the cytosolic fractions although no paraoxon hydrolysis was detected. The difference in paraoxonase activity between liver microsomes and plasma may be explained by the differing assay conditions: pH and incubation temperature. Recent findings (Mutch *et al.*, unpublished results) have indicated that paraoxonase activity in human liver microsomes showed a pH maximum at pH 9.0 with an 8-fold decrease in activities at pH 8.0. The effect of increasing incubation temperature, 30° to 37° increases the enzyme activity by 1.4-fold [12]. Taking assay conditions into account maximal activities for plasma and liver microsomal paraoxonase are similar. K_m values were similar suggesting the involvement of a similar enzyme in the plasma and liver. In contrast, phenylacetate esterase activity was higher in the plasma than liver (microsomes and cytosol together), but with a very much lower affinity.

To characterize the enzymes involved, inhibitory studies were carried out using differential inhibitors. Paraoxon completely inhibits cholinesterase and carboxylesterase (B esterases) BNPP has been shown to be a differential inhibitor of carboxylesterase isoenzymes in the liver [13] and lung [14] and physostigmine, an anticholinesterase, inhibits cholinesterase. Mercuric chloride inhibits enzymes which contain an -SH group (cysteine) at the active centre such as "A" esterases and arylesterases.

Inhibition indicated the involvement of carboxylesterases in liver microsomes and cytosol in fluazifop-butyl hydrolysis. Similarly, "B" esterases are involved in plasma; probably cholinesterase with a lower affinity (K_m) for fluazifop-butyl than the liver carboxylesterases. Hydrolysis of phenylacetate by liver appears to involve both "A" and "B" esterases in contrast to the plasma where arylesterases were involved.

The distribution of esterases in the human liver and plasma were similar to those found in the rat [2], although activities in the rat were generally greater than those found in human. These results compare to studies conducted by Clark *et al.* [15] who found a 5-fold range in fluazifop-butyl esterase activity in the human skin, with mean activity one tenth the fluazifop-butyl activity of the rat skin. Differences in esterase activities between rat and humans could have important implications when predicting pesticide hydrolysis. In man, fluazifop-butyl hydrolysis in the plasma is low due to reduced levels of carboxylesterase and the fact that fluazifop-butyl is a poor substrate for cholinesterase. Therefore, following dermal exposure of fluazifop-butyl the liver and not the plasma would appear to be the major site of metabolism. In contrast in the rat, fluazifop-butyl hydrolysis in the plasma is extensive due to high levels of carboxylesterase, therefore, following dermal absorption the plasma would play a major role in metabolism before reaching the liver [2]. Following exposure to paraoxon, plasma and liver paraoxonase are both major sites of hydrolysis and may play an important role in the detoxification of paraoxon in both humans and rats *in vivo*.

Although it seems that the distribution of esterases in the microsomal and cytosolic fractions of the human liver and the plasma are similar to those in the rat, the importance of the liver and plasma following exposure to different pesticides in human and rat models could be different, therefore extrapolation of rat results would not necessarily be predictive of those in a human model.

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