

## Potential Cancerostatic Benfluron is Metabolized by Peroxidase: *In vitro* Biotransformation of Benfluron by Horseradish Peroxidase

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**Abstract.** Horseradish peroxidase (HRP) was used to investigate whether benfluron (a potential cytostatic drug) can be biotransformed extra-hepatically by systems other than flavin-containing monooxygenase and cytochromes P450. Three types of incubation mixtures differing in buffers (Na-phosphate buffer 50 mmol/l, pH 6.8 and 8.4 and Tris-HCl buffer 25 mmol/l, pH 7.5) were tested. The amount of N-demethylated benfluron (demB) formed was significantly higher (up to 4 times in the Na-phosphate buffer, pH 8.4, and 5 times in the Na-phosphate buffer, pH 6.8, and in the Tris-HCl buffer, pH 7.5) compared to control experiments. The highest yields of demB were obtained with the moderately alkaline Na-phosphate buffer (50 mmol/l, pH 8.4). The concentration of demB increased during thirty minutes of incubation, and then remained constant through the end of two-hour incubation. The results support the hypothesis that benfluron can be metabolized extra-hepatically by N-demethylation reaction catalyzed by peroxidases.

**Key words:** Horseradish peroxidase — Dealkylation — Neoplasms — Benfluron — Cytochrome P-450

### Introduction

Benfluron (5-(2-dimethylaminoethoxy)-7H-benzo[c]fluorene hydrochloride) is a prospective cancerostatic agent (Mělka and Křepelka 1987), the biological effects, biodistribution and biotransformation of which have been investigated in depth both *vivo* and *in vitro* (Kvasničková et al 1984, Francová et al 1985, Nobilis et al 1991). The metabolites of benfluron have been identified (Fig. 1), and enzyme systems involved in benfluron biotransformation have also been investigated (Kvas-

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the use of aspirin, an inhibitor of PGHS, is associated with a reduced risk of this disease (Thun et al 1991)

Horseradish peroxidase (HRP) is often used as a tool for modeling extra-hepatic biotransformation of xenobiotics (Josephy et al 1983a,b, Ross et al 1985, Sugiyama et al 1994) It possesses heme b (a ferriprotoporphyryn IX) as the prosthetic group, and nitrogen atom from a histidine residue as the heme iron proximal ligand (Meunier 1987) We used HRP as a model peroxidase to investigate its ability to catalyze (in the presence of hydrogen peroxide) the conversion of benfluron to known metabolites

## Materials and Methods

### *Chemicals*

Benfluron was a generous gift from Research Institute of Pharmacy and Biochemistry in Prague (Czech Republic) HRP (type II) was purchased from Sigma (St Louis, USA) Acetonitrile was purchased from Merck (Darmstadt, Germany), and nonylamine was from Fluka (Buchs, Switzerland) All other chemicals were from Lachema (Brno, Czech Republic), and were of analytical-grade purity

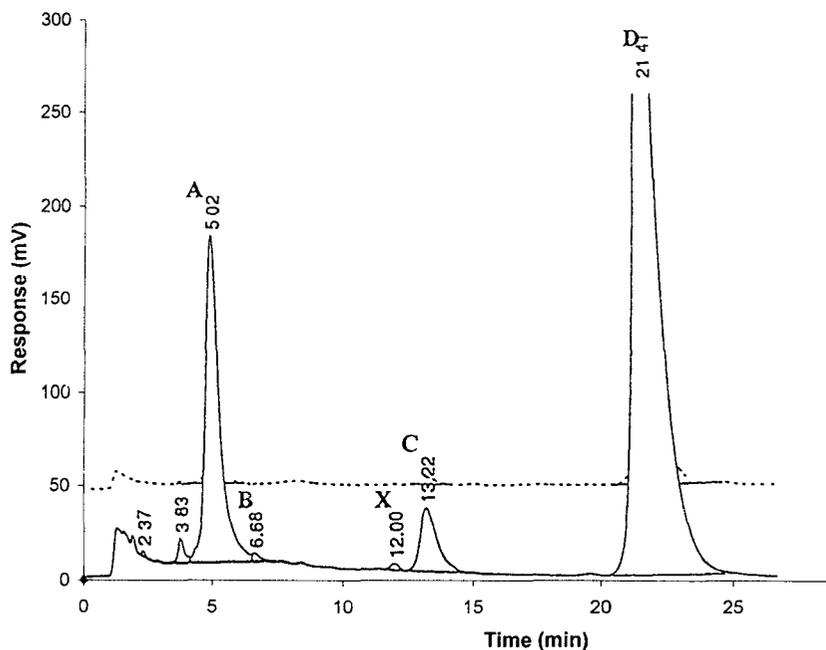
### *Incubation and extraction procedures*

Incubation mixture contained HRP (2.0 mg), hydrogen peroxide (1.5 mmol/l), benfluron (0.2 mmol/l) Three types of buffers were used: Na-phosphate buffer (50 mmol/l, pH 6.8 and 8.4) and Tris-HCl buffer (25 mmol/l, pH 7.5) Total volume of incubation (mixture) was 1.5 ml Samples were preincubated 5 minutes at 37°C, then HRP was added, and the reaction mixture was incubated for 120 minutes at 37°C Incubations were done in triplicate Control incubations without HRP were made simultaneously For the time experiment, the reaction mixture was incubated for 1, 3, 5, 15, 30, 60, and 120 minutes Incubation was stopped by addition of 5 ml 5% (v/v) ammonium hydroxide and cooling The samples were extracted three times with 8 ml of ethylacetate each Extracts were collected and ethylacetate was evaporated under vacuum at 40°C The residue was dissolved in a small volume of methanol, the solvent was then evaporated by a stream of nitrogen at 50°C, and the samples were stored for HPLC

### *HPLC*

Samples were analyzed on a reverse-phase C<sub>18</sub> column (5 μm, 125 × 4 mm) (Merck) with a system consisting of AS3500 autosampler and P4000 ternary pump (Thermo Separation Products) Analyses were done under isocratic conditions with a mobile phase consisting of 40% nonylamine buffer pH 7.41, 40% acetonitrile, 20% isopropanol at the rate of 0.90 ml/min HPLC profile was monitored at 295 and

340 nm using Spectra FOCUS forward optical scanning detector (TSP) Metabolites were quantified by comparing their peak areas with peak areas of external standards This procedure was done using Spectra SYSTEM™ software PC1000 (TSP) Metabolites were identified by their known retention times and by their characteristic spectra taken by the detector



**Figure 2.** HPLC elution profile of benfluron incubation with HRP Peak A, benfluron N-oxide peak B 9-hydroxybenfluron, peak C, N-demethylated benfluron, peak D, benfluron, peak X, unidentified metabolite Thick line, absorbance at 295 nm, dotted line absorbance at 340 nm

## Results and Discussion

HPLC profiles of benfluron and its metabolites are shown in Fig 2 Retention times of peaks A, B, C, D were in accordance with the retention times of known standards Peak A was identified as benfluron N-oxide (N-oxB), peak B as 9-hydroxybenfluron (9-OHB), peak C as N-demethylated benfluron (demB), and peak D as benfluron Furthermore, peak X was detected in extracts from HRP incubations and control incubations Its retention time did not correspond to retention time of any known metabolite

**Table 1.** Effect of various buffers on the concentrations of benfluron metabolites from incubations with HRP\* and from control incubations

Metabolite	Concentration [nmol/ml] <sup>†</sup>		
	N-oxB	9-OHB	demB
Na-phosphate buffer 50 mmol/l, pH 8.4	60.50 ± 13.03	0.73 ± 0.20	6.74 ± 1.37
Control	99.15 ± 3.41	0.87 ± 0.13	1.83 ± 0.46
Na-phosphate buffer 50 mmol/l, pH 6.8	53.51 ± 13.13	0.81 ± 0.25	2.99 ± 0.84
Control	134.69 ± 9.23	n d <sup>‡</sup>	0.71 ± 0.07
Tris-HCl buffer 25 mmol/l, pH 7.5	52.84 ± 8.85	1.49 ± 0.38	3.59 ± 1.74
Control	108.14 ± 18.67	n d	0.80 ± 0.19

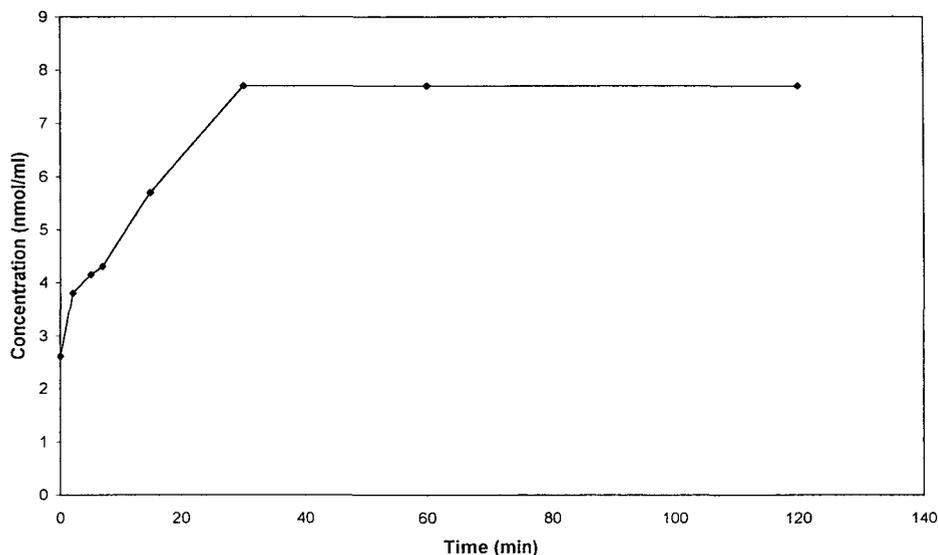
\* Abbreviations used: HRP, horseradish peroxidase; N-oxB, benfluron N-oxide; 9-OHB, 9-hydroxybenfluron; demB, N-demethylated benfluron.

<sup>†</sup> Values are means ± S.D. for three experiments.

<sup>‡</sup> n.d. not detected.

Concentration of demB was significantly increased compared with control experiment. The concentrations of identified metabolites are shown in Table 1. The results show a significant increase of N-demethylated product formation compared to control experiments. On the other hand, yields of N-oxB are significantly diminished in the presence of HRP. This reaction is known to take place spontaneously: benfluron in solutions slowly oxidizes to form this compound. Also, another metabolite, 9-OHB, was formed. However, its yields were relatively low. The results show that this product could also be formed in the absence of HRP in alkaline pH (Table 1). Na-phosphate buffer (pH 8.4) was selected as a medium to study the kinetics of demB formation. The results are shown in Fig. 3. DemB already occurred after preincubation. This is in agreement with the results shown in Table 1: spontaneous formation of demB in the presence of hydrogen peroxide could be an explanation. Nevertheless, there was a significant increase in demB concentration during thirty minutes of incubation with HRP. Thereafter, demB concentration remained constant throughout the end of two-hour incubation.

The metabolism of benfluron has recently been extensively studied. FMOs are known to be involved in benfluron biotransformation yielding an N-oxide, and that hydroxylation and N-demethylation are catalyzed by P450 (Kvasničková et al. 1984). The results of a biodistribution study (Francová et al. 1985) indicated that benfluron could also be a substrate for extra-hepatic biotransformation catalyzed by different systems. Among them, the peroxidases occurring e.g. in the urinary bladder, in the mammary gland, eosinophiles, leukocytes, the uterus or in pul-



**Figure 3.** Formation of N-demethylated benfluron over time. The results shown are means from five experiments.

monary and renal cells (Stiborová et al. 1991) may play a significant role. We used HRP to model extrahepatic biotransformation by peroxidases. This approach has been widely used (Josephy et al. 1983a,b; Sugiyama et al. 1994; Ross et al. 1985; Meunier 1987; Guengerich 1990). Our results clearly show that HRP catalyzed N-demethylation of benfluron as the amounts of demB were significantly increased in the presence of this hemoprotein. It is known that HRP is able to catalyze N-demethylation reactions as well as P450; the reaction mechanisms, however, are most probably different (Okazaki and Guengerich 1993; Anzenbacher et al. 1996). This property seems to be typical of all heme-containing systems (hemoproteins as well as the model ones).

The results show that benfluron could be metabolized by N-demethylation reaction mediated by peroxidases. This fact adds new piece of evidence to suggestions on the importance of extra-hepatic biotransformations and the role of peroxidases in these processes.

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