INDUCTION AND INHIBITION OF CYTOCHROME P450-CATALYSED REDUCTION OF BIOLOGICALLY ACTIVE BENFLURON N-OXIDE

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SUMMARY

Benfluron N-oxide [5-(2-N-oxo-2-N,N'-dimethylaminoethoxy)-7-oxo-7-*H*-benzo[*c*]fluorene] is a biologically active substance which displays a cytostatic effect on several experimental tumour cells. The main metabolic pathway of benfluron N-oxide *in vitro* and *in vivo* - its reduction to the parent tertiary amine benfluron - and the role of cytochrome P450 in this reduction were studied. The value of the benfluron N-oxide/benfluron redox potential as a criterion of suitability of the substrate for cytochrome P450 reductase activity was determined. Results of induction and inhibition studies on rats suggest that cytochromes P4502B and P4502E1 participate in microsomal reduction of benfluron N-oxide. Unlike most cytochrome P450 catalysed reactions, the reduction of benfluron N-oxide also occurs under aerobic conditions. Microsomes induced by phenobarbital, ethanol or β-naphthoflavone showed no significantly greater inhibitory effect of oxygen on benfluron N-oxide reduction.

KEY WORDS

cytochrome P450 reductase activity, N-oxides, microsomal reduction, benfluron, benfluron N-oxide, cytostatic drugs

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INTRODUCTION

Cytochromes P450, generally known as monooxygenase systems, display a significant reductase activity towards some xenobiotics. Under certain conditions, such a xenobiotic replaces oxygen as the acceptor of electrons from the reduced Fe^{2+}-P450 complex in the cytochrome P450 catalytic cycle. This single-electron reduction process during which free radicals are generated may play a significant role in the toxicity of such a xenobiotic. Oxygen participating in the reaction acts as a competitive inhibitor, and thus a major part of such substrates is reduced by cytochrome P450 only under anaerobic conditions. In addition, cytochrome P450 inhibitors which bind to the catalytic site and those that bind to the heme's iron atom in most cases display P450 reductase activity. The exact molecular mechanism of the P450-catalyzed reduction of xenobiotics has not yet been established. Xenobiotics which are reduced by cytochrome P450 share very little structural similarity. The only common property they share seems to be a high redox potential. The most common substrates of P450 reductase activity include halogen-substituted alkanes, azo dyes, nitro-compounds, quinones and benzoquinone imines /1/. Some N-oxide derivatives of tertiary amines are believed to undergo cytochrome P450-catalyzed reduction /2,3/.

The processes of metabolic reduction of N-oxides are interesting from more than one aspect. From the structural point of view, some drugs (e.g. amitriptilinoxide, tirapazamine) belong to the family of N-oxides and their reduction represents one of their main biotransformation mechanisms /4,5/. However, what is of even greater importance is that a number of standard tertiary amine drugs (e.g. imipramine, chloroprothixene, amitryptiline, methadone, promazine, etc.) undergo N-oxidation during their biotransformation, and the N-oxides produced this way may be reduced and converted back to the respective tertiary amines. Differences in physico-chemical properties between tertiary amines and their N-oxides may account for their different pharmacological behaviour. This fact can be made use of in deriving prodrugs, e.g. tramadol N-oxide, intended to serve as the prodrug form of tramadol /6/. Based on the type of biological activity displayed by their respective N-oxides, tertiary amines can be divided into three groups. The first group includes tertiary amines which are rendered pharmacologically inactive as a result of N-oxidation. The second group represents active N-oxides of inactive tertiary amines.

The third group includes bioactive tertiary amines of which the N-oxides display significant biological activity as well. Metabolic interconversion of these tertiary amines and their respective N-oxides then constitutes interconversion of two biologically active substances. This third group includes benfluron [B, 5-(2-N,N′-dimethylaminoethoxy)-7-oxo-7-*H*-benzo[*c*]fluorene] and its N-oxide.

During studies of the metabolism of benfluron, a potential cytostatic, a number of metabolites have been detected (both *in vivo* and *in vitro*) (Fig. 1) /7,8/. One of the main metabolites is the N-oxide, the generation of which is catalyzed by FMO /9/. Studies on the biological properties of B N-oxide have revealed significant anti-neoplastic activity both *in vitro* (inhibition of DNA and protein synthesis in Yoshida tumour cells) and *in vivo* (reduced tumour mass and extended survival period after oral administration of B N-oxide to H mice with Ehrlich or KH tumours and to DBA_2 mice with LH1210 leukaemia) /10/.

Because of the proven biological activity of B N-oxide, its biotransformation was investigated. The main metabolic pathway in rat both *in vivo* and *in vitro* is its reduction back to B /11/. Under *in vitro* conditions, this microsomal reduction is NADPH-dependent and takes place under aerobic conditions. Cytochrome P450 inhibitors (CO, n-octylamine, benzylimidazole) inhibited the reduction by as much as

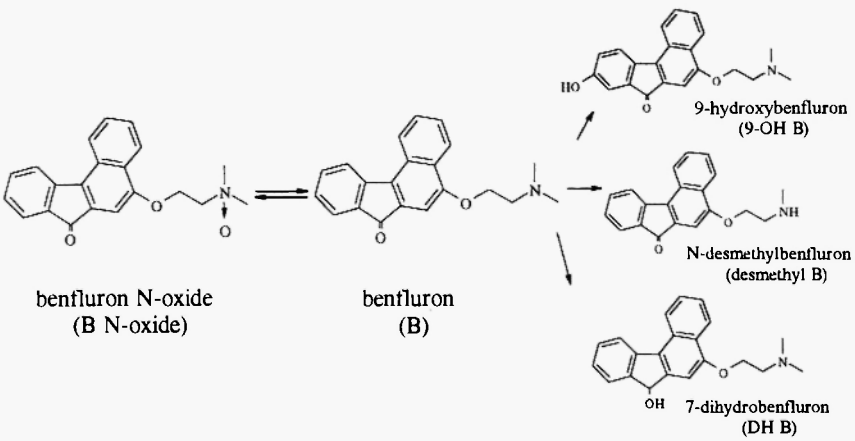

Fig. 1: Microsomal biotransformation of B N-oxide in the rat - the main metabolic pathway /11/.

70% in comparison with controls. The enzyme-generated B is partly converted to other metabolites (Fig. 1) and partly oxidized by monooxygenases back to B N-oxide. In addition to the reduction back to benfluron, the oxo-group of B N-oxide on C7 may also undergo reduction. In the rat, however, this biotransformation pathway is of minor importance (< 2%) /11/.

The aim of these studies was to determine the redox potential value of B N-oxide as a criterion of suitability of the substrate for cytochrome P450 reductase activity and to study the effects of some inducers and specific inhibitors on the activity of the microsomal B N-oxide reductase. Based on the results obtained, the contribution of individual cytochrome P450 isoforms to B N-oxide reduction may be determined.

METHODS

Chemicals

Inhibitors, inducers and specific substrates used in this study were from Sigma Chemicals, NADPH from Boehringer and HPLC chemicals from Merck. Benfluron was obtained from the Research Institute of Pharmacy and Biochemistry, Prague, and B N-oxide was prepared in our laboratory. All other chemicals used were of analytical grade and purchased from Lachema.

Animals and biological material

Male Wistar rats from Velaz (Czech Republic) were fed a standard diet. Subcellular fractions were isolated by fractional ultra-centrifugation.

Redox potential measurement

The redox potential of B N-oxide/B was established by measuring the voltage generated by calomel and platinum electrodes in solutions of 1 mM oxidant and 1 mM reductant.

Induction

Phenobarbital (PB) was administrated p.o. for 5 days (1 ml solution in drinking water daily, 70 mg/kg of body mass), ethanol (EtOH) p.o. for 10 days (1 ml 20% solution in drinking water daily), and β-naphthoflavone (b-NF) i.p. (1 ml solution in oleum helianthi daily, 80 mg/kg) for 3 days, before isolation of microsomes.

Incubation and extraction

Incubation conditions (substrate and coenzyme concentrations, time of incubation) were chosen according to previous experimental results /11/. Microsomal suspension (0.1 ml in 0.1 M Na-phosphate buffer, pH 7.4, i.e. an aliquot corresponding to 0.1 g of original wet liver tissue) was incubated with 0.5 μmol of substrate and 0.6 μmol of coenzyme NADPH in 0.3 ml (total volume) of reaction mixture. Concentration of inhibitors was 1 μmol in the reaction mixture. Incubations were carried out at 37°C for 30 min under aeration or in argon atmosphere. All incubations were terminated by adding 0.1 ml 13% aqueous ammonia solution. Samples were extracted three times with the double their volumes of distilled ethyl acetate and the combined extracts vacuum-evaporated to dryness.

Protein and P450 content determination

Protein was determined according to the method of Lowry *et al.* with 0.1% SDS /12/. The amount of P450 was estimated using the Omura and Sato /13/ method of differential spectrum analysis after CO-reduction.

Enzyme assays:

Aniline hydroxylase activity assay

The assay mixture /14/ contained the following: 10 mg microsomal protein; 5 μmol aniline hydrochloride; 6 μmol NADPH; in 4 ml of 0.1 M phosphate buffer, pH 7.4. Incubations (20 min, 37°C) were terminated and samples deproteinized by 2 ml of trichloracetic acid (20% w/v). Upon centrifugation, 1 ml of Na_2CO_3 (10%, w/v) and 2 ml of phenol (2%, v/v) in 0.1 M phosphate buffer, pH 7.4 were added to 2 ml of supernatant. After 30 min incubation in a water bath (37°C)

and subsequent cooling, optical densities were read at 640 nm against blank (without NADPH) using a Cecil CE 1010 spectrophotometer and p-aminophenol concentrations determined from a calibration curve.

Ethoxyresorufin deethylase (EROD) and pentoxyresorufin deethylase (PROD) activity assays

The assay mixture composition /15/ was as follows: 10 μl of 3 μM 7-ethoxyresorufin or 5 μM pentoxyresorufin dissolved in 1 mM dimethylsulfoxide; 10-200 μg of microsomal protein; 0.5 mM NADPH; 0.1 M Na-phosphate buffer, pH 7.4; in total volume 2 ml. Incubations were carried out for 2 min at 37°C. Increase in reaction product concentration (resorufin) was followed on a Perkin-Elmer luminescence spectrophotometer LS 50B (excitation and emission wavelengths 530 nm and 585 nm, respectively), and EROD and PROD activities calculated on the basis of the addition of a standard amount of the product.

Chromatographic separation and detection

A Spectra Physics chromatograph was used for HPLC detection, separation, quantification and spectral identification /16/. The configuration used was as follows: solvent degasser SCM400, quaternary gradient pump P4000, autosampler AS3500 with 100 μl sample loop, Spectra FOCUS high speed scanning UV detector, system controller, computer with analytical software working under OS-2. Analyses were performed on HPLC column LiChroCART 125 x 4 mm I.D., with precolumn LiChroCART 4 x 4 mm I.D, containing LiChrospher 100 RP-18 (5 μm). Mobile phase consisted of 0.01 M nonylamine buffer, pH = 7.4, acetonitrile and 2-propanol (2:2:1, v/v/v). The flow rate of the mobile phase was 0.9 ml/min. Detection was performed in dual wavelength mode (295 and 365 nm) and in high-speed scanning mode (range 195-365 nm with 5 nm distance). The determination of B and its metabolites was performed using an external standard method at wavelength 295 nm (for 7-oxo-7*H*-benzo(*c*)fluorenes) and 340 nm (for 7-hydroxy-7*H*-benzo(*c*)fluorenes) /8/.

RESULTS

Redox potential measurement

The redox potential of B N-oxide was determined from the voltage of a cell consisting of platinum and calomel electrodes immersed in equimolar solutions of the oxidized and reduced form. The value measured has been recalculated for pH 7. Biochemical standard reduction potential on B N-oxide is:

$$E^{\circ\circ}_{\text{N-oxide B} \leftrightarrow \text{B}} = +0.56 \text{ V}$$

Induction and inhibition of B N-oxide reduction

Phenobarbital (PB) was used to induce cytochrome P4502B. The induction effect of PB was confirmed by assay of PROD activity (Table 1). In order to inhibit P4502B isoforms, metyrapone, a relatively specific inhibitor in the rat /17/, was used. The results presented in Figure 2 show that microsomes isolated from PB-induced rats displayed B N-oxide reductase activity increased by 260% ($p=0.01$) in comparison with microsomes from controls. Metyrapone

TABLE 1

Specific enzyme activities of microsomes prepared from control animals or phenobarbital, ethanol or β-naphthoflavone induced animals

microsomes	aniline hydroxylase activity*	EROD activity*	PROD activity*
control	1.87 ± 0.27	0.18 ± 0.03	0.10 ± 0.03
phenobarbital	1.93 ± 0.18	0.43 ± 0.09	5.49 ± 1.08
ethanol	2.42 ± 0.22	0.11 ± 0.06	0.11 ± 0.09
β-naphoflavone	1.78 ± 0.31	2.43 ± 0.58	0.16 ± 0.10

* (pmol/mg protein/min) + SD

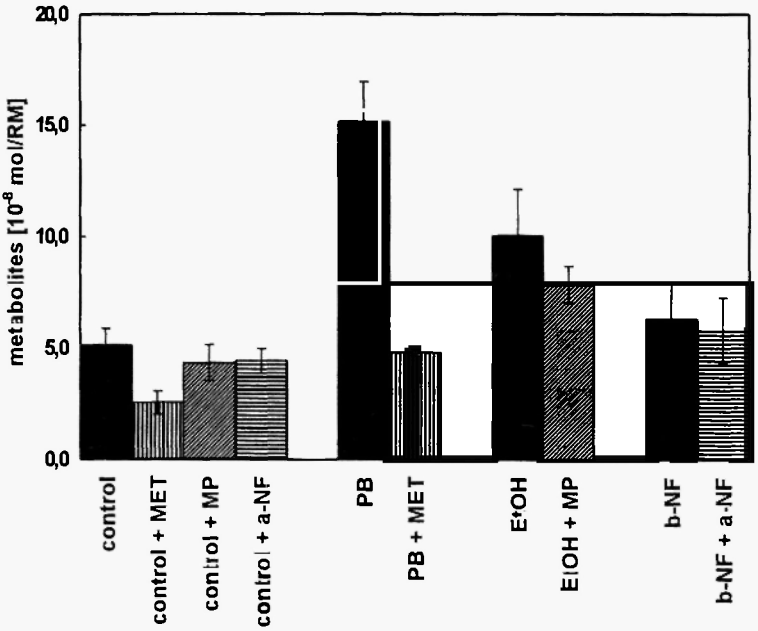

Fig. 2: The effect of inducers (phenobarbital, PB; ethanol, EtOH; β-naphthoflavone, b-NF) and inhibitors (metyrapone, MET; methylpyrazole, MP; α-naphthoflavone, a-NF) on microsomal reduction of B N-oxide. The substrate (B N-oxide) was incubated with NADPH and microsomes under aerobic conditions. The values on the y-axis give the quantities of metabolites in the reaction mixture (RM), i.e. the quantity of B generated by the reduction plus its main metabolites derived subsequently from B (9-OH B, DH B, desmethyl B). Average values and SD from 6 samples.

decreased B N-oxide reduction in microsomes from control and PB-induced animals by 46% and 68% ($p=0.01$) in comparison with samples incubated in the absence of inhibitors. The quantities of metabolites generated upon the addition of metyrapone to the incubation mixture of B N-oxide and PB-induced microsomes were comparable with the quantities obtained during incubations of B N-oxide with control microsomes in the absence of the inhibitors.

Cytochrome P450E1 was induced by ethanol. Ethanol induction was confirmed by assay of aniline hydroxylation (Table 1). Methylpyrazole was used to inhibit cytochrome P4502E1. The results are

presented in Figure 2. Reduction of B N-oxide by microsomes from ethanol-induced animals was increased by 98% (p=0.01) in comparison with the respective controls. Methylpyrazole reduced the induced reductase activity by 22% (p=0.05).

Cytochrome P4501A was induced using β-naphthoflavone. This induction was confirmed by assaying for EROD activity (Table 1). α-Naphthoflavone was employed as an inhibitor of cytochrome P4501A. As shown in Figure 2, neither the inducer nor the inhibitor had any significant effect on the microsomal reductase activity of B N-oxide.

Reduction of B N-oxide under aerobic and anaerobic conditions

Although the reduction of B N-oxide takes place under aerobic conditions, the absence of oxygen increases the reduction /11/. Figure 3 shows the effect of induction on B N-oxide reductase activity under aerobic and anaerobic conditions.

The data show increased B N-oxide reduction under anaerobic conditions by all induced cytochromes (p=0.01). This increased formation of metabolites is approximately the same in both control and induced microsomes irrespective of the inducer employed.

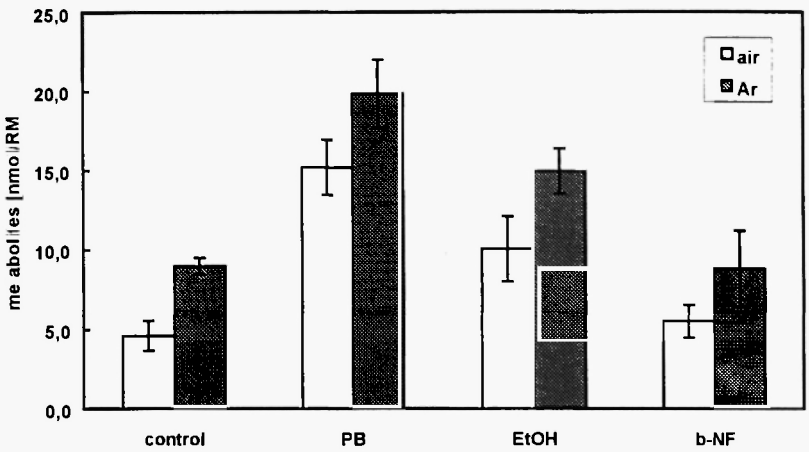

Fig. 3: The effect of aerobic (air) and anaerobic (Ar) conditions on microsomal reduction of B N-oxide. The substrate (B N-oxide) was incubated with NADPH and microsomes from control or phenobarbital (PB), ethanol (EtOH) or β-naphthoflavone (b-NF) induced animals. For other details see the legend to Fig. 2.

DISCUSSION

Our previous results /11/ which suggested that cytochrome P450 might be involved in the reduction of B N-oxide have been supplemented by measurements of the redox potential of the benfluron/B N-oxide redox couple. The redox potential is a criterion to determine whether a particular substance may be a suitable substrate for cytochrome P450 reductase activity. The cytochrome P450 redox potential is -0.35 V and -0.175 V for its low- and high-spin state. Due to the high value of the redox potential of molecular oxygen (+0.81 V) it is believed that only a substrate with a relatively high redox potential value can replace oxygen as electron acceptor in a cytochrome P450 catalytic cycle /1/. Such substrates can therefore be envisaged to be reduced by cytochrome P450. The value +0.56 V of standard biochemical redox potential measured for B N-oxide confirms that this substance is indeed a suitable substrate for cytochrome P450 reductase activity.

Microsomal B N-oxide reduction was also studied from the aspect of the respective contribution by individual cytochrome P450 isoforms to the process. One of the most common approaches employed to identify the role of a certain cytochrome P450 isoform in a biotransformation process is the use of specific inducers, inhibitors and substrates. As cytochromes P4502B, P4502E1 and P4501A are known to account for a major part of the reductase activity in the rat /1/, inducers and inhibitors (as well as substrates) of those cytochrome P450 isoforms were chosen for our experiments.

In the rat, most substrates are reduced by cytochrome P4502B. Its substrates include dimethylaminoazobenzene, tetrachloromethane, chloroform, halothane and some cytostatics (mitomycin C, adriamycin) /1/. Our results show that phenobarbital, which is known to induce cytochromes from the P4502B subfamily, was the most significant inducer of B N-oxide reductase activity in rat microsomes. This phenobarbital-induced activity was significantly reduced ($p = 0.01$) by metyrapone, a relatively specific P4502B inhibitor in the rat /17/. One can thus assume that B N-oxide is predominantly reduced by cytochromes belonging to this subfamily.

Interest in cytochrome P4502E1 reductase activity has recently increased /1/. This isoenzyme, the homologue of which is a significant cytochrome P450 form in man, can also reduce molecular oxygen and some xenobiotics. P4502E1 reductase activity (which also gives rise to

free radicals) occurs mostly under anaerobic conditions. This reaction is believed to have significant toxicological importance in tissues with relatively hypoxic conditions (such as exist, e.g., in central liver lobes) /1/. Ethanol induction of P4502E1 caused a statistically significant increase ($p=0.01$) in microsomal B N-oxide reduction, while methylpyrazole (a specific inhibitor) partially, but significantly ($p=0.05$), inhibited the ethanol-induced activity. Thus, one can assume that cytochrome P4502E1 also plays a role in B N-oxide reduction.

The third subfamily of P450 cytochromes in the rat which displays reductase activity is the P4501A subfamily /1/. β-Naphthoflavone and α-naphthoflavone were used to induce and inhibit these cytochromes, respectively. Neither the inducer nor the inhibitor had any effect on B N-oxide reduction. Therefore, cytochrome P4501A apparently does not reduce B N-oxide in the rat.

During the studies on the B N-oxide reduction processes, the effect of inducers on the course of the reduction was also investigated under aerobic and anaerobic conditions. B N-oxide reduction also takes place significantly under aerobic conditions, although most xenobiotics undergo reduction by cytochrome P450 only under anaerobic conditions. The only substances also known to be reduced by cytochrome P450 under aerobic conditions are some azo dyes /1/. A comparison of azo dyes of which the reduction process is oxygen-insensitive (I-substrates) with those of which the reduction is oxygen-sensitive (S-substrates) has revealed differences in the stability of the intermediates generated by single-electron reduction as well as significant differences in substituents. S-substrates include azo dyes with electrophilic substituents while I-substrates have nucleophilic groups. One cannot exclude the possibility that the nucleophilic group in position 7 of B N-oxide could be one of the reasons why B N-oxide reduction takes place in the presence of oxygen.

Although B N-oxide can be deemed an exceptional substrate which is reduced by P450 cytochromes in the presence of oxygen, a comparison of the quantities of the benfluron formed by microsomal reduction of B N-oxide in the presence and absence of oxygen shows that B N-oxide reduction is higher under anaerobic conditions. Based on previous experiments in which α-naphthylthiourea, a specific FMO inhibitor, increased N-oxide reduction in a comparable manner /11/, we assume that the higher yield of benfluron was caused by the blockage of reoxidation of benfluron to B N-oxide due to the absence of oxygen rather than by oxygen-caused inhibition of B N-oxide reduction. This

hypothesis has been confirmed by our finding that the inducers employed did not cause any significant increase of the reduction under anaerobic conditions. The difference between the yield of the product under aerobic and anaerobic conditions was approximately the same in both control and induced microsomes. Microsomes from both PB- and EtOH-induced animals displayed increased B N-oxide reduction, but no preference for anaerobic conditions. It thus can be assumed that cytochromes P4502B and P450E1 do not significantly prefer anaerobic conditions for the reduction of B N-oxide.

REFERENCES

1. Goeptar RA, Scheerens H, Vermeulen NPE. Oxygen and xenobiotic reductase activities of cytochrome P450. Crit Rev Toxicol 1995; 25: 25-65.
2. Wislocki PG, Miwa GT, Lu AYH. Reactions catalyzed by cytochrome P450 system. In: Jakoby WB, ed. Enzymatic Basis of Detoxication. London: Academic Press, 1980.
3. Testa B. Reduction catalyzed by cytochrome P450 and other oxidoreductases, In: Testa B, Caldwell J, eds. The Metabolism of Drugs and Other Xenobiotics. Biochemistry of Redox Reactions. London: Academic Press, 1995; 398-447.
4. Pfeifer S. Biotransformation von Arzneimitteln. Berlin: Veb Verlag und Gesundheit, 1975.
5. Patterson AV, Barham HM, Chinje EC, Adams GE, Harris AL, Stratford IJ. Importance of P450 reductase activity in determining sensitivity of breast tumour cells to the bioreductive drug, tirapazamine (SR 4233). Br J Cancer 1995; 72: 1144-1150.
6. Raffa RB, Haslego ML, Maryanoff CA, Villani FJ, Codd EE, Connelly CD, Martinez RP, Schupsky JJ, Buben JA, Wu VN, Takacs AN, Mckown LA. Unexpected antinociceptive effect of the N-oxide (RWJ 38705) of tramadol hydrochloride. J. Pharmacol Exp Ther 1996; 278: 1098-1104.
7. Kvasnicková E, Nobilis M, Hais IM. Chromatographic characterization of in vitro metabolites of 5[2-(N,N-dimethylamino)ethoxy]-7-oxo-7*H*-benzo[*c*]-fluorene. J Chromatogr 1984; 295: 201-209.
8. Nobilis M, Kvasničková E, Sroler A, Hais IM. Elimination of benflurone and its metabolites in the faeces and urine of rats. Drug Metab Drug Interact 1991; 9: 225-240.
9. Kvasnicková E, Hais IM, Nobilis M. Relationship between NADPH and NADPH as cosubstrates of N-oxidation of the cytostatic benfluron by FMO. Prog Pharmacol Clin Toxicol 1991; 8: 137-146.
10. Vančurová I, et al. Patent application No. PCT/CS88/239413.

11. Skálová L, Nobilis M, Szotáková B, Trejtnar F, Kvasnicková E. In vitro and in vivo biotransformation of benflurone N-oxide, a biologically active metabolite of a potential cytostatic. Exp Toxic Pathol 1996; 48: 366-370.
12. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin C phenol reagent. J Biol Chem 1951; 193: 165-275.
13. Omura T, Sato R. The carbon-monoxide-binding pigment of liver microsomes. II. Solubilization, purification, properties. J Biol Chem 1964; 239: 2379-2385.
14. Gram TE, Rogers LA, Fouts JR. Further studies on the metabolism of drugs by subfractions of hepatic microsomes. J Pharmacol Exp Ther 1967; 150: 479-493.
15. Lubet RA, Mayer RT, Cameron JW, Nims RW, Burcke MD, Wolf T, Guengerich FP. Dealkylation of penthoxyresorufin: a rapid and sensitive assay for measurig induction of cytochromes P450 by phenobarbital and other xenobiotics in the rat. Arch Biochem Biophys 1985; 83: 43-48.
16. Nobilis M, Anzenbacher P, Pastera J, Svoboda Z, Hruby K, Kvetina J, Ubik K, Trejtnar F. Study of biotransformation of a potential benzo[*c*]fluorene antineoplastic using HPLC with high-speed-scanning UV detection. J Chromatogr B 1996; 681: 143-151.
17. Luu-The V, Cumps J, Dumont P. Metyrapone-reduced cytochrome P450 complex - a specific method for determination of phenobarbital inducible form of rat hepatic microsomal cytochrome P-450. Biochem Biophys Res Comm 1980; 93: 776-781.