

Research Article

Microbial Metabolism and Inhibition Studies of Phenobarbital

Kummarigunta Kavitha, Maravajhala Vidyavathi*, Sepuri Asha and TVL Hima Bindu

Institute of Pharmaceutical Technology, Sri Padmavathi Mahila Viswa Vidyalayam, Tirupathi – 517 502, A.P., India

Abstract

Purpose: Screening scale studies were performed with eight cultures for their ability to metabolize phenobarbital, an antiepileptic, sedative, hypnotic and substrate for CYP 2C9 and 2C19.

Methods: The transformation of phenobarbital was confirmed and characterized by fermentation techniques, high performance liquid chromatography (HPLC), mass spectrometry (MS) and metabolism inhibition studies.

Results: Among the different cultures screened, a fungus, *Rhizopus stolonifer* NCIM 880, transformed phenobarbital to its metabolite, the N-glucuronide of p- hydroxy phenobarbital. HPLC data show a solvent peak at 2.4 min, culture components peaks at 4.0 and 5.4 min, respectively, and phenobarbital peak at 10.3 min, for both controls and test samples, but only the sample of *Rhizopus stolonifer* showed an additional peak at 3.1 min, indicating formation of a metabolite.

Conclusion: Microbial metabolism of phenobarbital was similar to the metabolism of the drug in mammals. Therefore, *Rhizopus stolonifer* can be used as a suitable in vitro model to mimic CYP 2C9 metabolism and to synthesize metabolites required for further pharmacological and toxicological studies.

Keywords: Microbial metabolism, Phenobarbital, Inhibition studies, *Rhizopus stolonifer*, CYP 2C9, Fenofibrate

Received: 1 February 2011

Revised accepted: 2 December 2011

*Corresponding author: **Email:** vidyasur@rediffmail.com

INTRODUCTION

An important factor in the evaluation of safety and efficacy of any drug is the knowledge about its metabolism. A better understanding of the metabolism of drug is essential to know about drug action, distribution, toxicity, excretion and storage in the body. Drug metabolism is a series of enzymatic biotransformation of chemical leading to formation of relatively polar substances, which are easily excreted. Drug metabolism studies are also important for studying induction/inhibition of metabolism of several drugs. Cytochrome P450 enzyme plays a major role in the metabolism of the drugs [1,2]. To underline the importance of CYP450 and its isoforms, significant progress has been made in the use of *in vitro* methods to characterize the pharmacokinetics of potential drugs in man. This type of work requires selective substrates for each isoform of P450 system, to allow detailed metabolism experiments using microbes [3,4].

Drug metabolism studies have relied on the use of model systems to produce expected human metabolites of drugs. Traditionally, drug metabolism studies were conducted on small animal models, perfused organs, tissue culture, microsomal preparation [5,6] *in vitro* enzyme systems and *in vitro* cell cultures. Microbial model was developed as one of the *in vitro* model to overcome the disadvantages of other models. Nowadays microbial model is used as a complementary tool to mimic mammalian metabolism by reducing the usage of animals in the drug development process. Microorganisms such as fungi, bacteria and yeast have been successfully used as *in vitro* models for the prediction of mammalian drug metabolism with successful applications [7-10].

Phenobarbital is mainly metabolized to p-hydroxy phenobarbital and glucuronide conjugate of p-hydroxy phenobarbital in man, rats [11-13] and mice [14]. The present study was aimed at developing a microbial model to use as tool for the study of metabolism of

drugs by CYP 2C9, as phenobarbital is a probe for CYP 2C9. The involvement of these enzymes in microbial metabolism would be confirmed by inhibition studies using CYP 2C9 and 2C19 inhibitors.

EXPERIMENTAL

Microorganisms

Cultures were obtained from National Chemical Laboratory (NCL), Pune and Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. The cultures used in the present work were *Streptomyces griseus* (NCIM 2622), *Nocardia hydrocarboxydans* (NCIM 2386), *Rhizopus stolonifer* (NCIM 880), *Cunninghamella blakesleeana* (MTCC 3729), *Cunninghamella elegans* (NCIM 689), *Cunninghamella echinulata* (MTCC 4279), *Aspergillus terreus* (NCIM 657) and *Saccharomyces cerevisiae* (NCIM 3090).

Chemicals

Phenobarbital and fluoxetine were purchased from Sigma, Mumbai, India. Fenofibrate was a gift from Therdose Company, Hyderabad, India. All the reagents used in the analysis were of HPLC, including methanol and water (deionized and glass-distilled) which were obtained from SD Fine Chemicals Ltd, Mumbai, and ethylacetate from Merck, Mumbai, India. Culture media components were purchased from Qualigens, and SD Fine Chemicals, Mumbai, India.

Cultures

All the cultures were maintained on their respective agar slants at 4 °C and transferred every 6 months to maintain viability. The experiments were carried out using their respective growth media for the different microorganisms. The composition of the medium for bacteria was: beef extract, 1 g; sodium chloride, 0.5 g; peptone, 1 g; distilled water, to 100 ml; final pH 7-7.5. For fungi, the composition was as follows: potato chips, 20 g/100 ml (steamed for 30min.); dextrose, 2 g ;

yeast extract, 10 mg; distilled water, to 100 ml; final pH 5.6. The medium for yeast was: malt extract, 0.3 g; glucose, 1 g; yeast extract, 0.3 g; peptone, 0.5 g; distilled water, to 100 ml; final pH 6.4 - 6.8 for yeast. These media were sterilized in individual Erlenmeyer flasks by autoclave at 121 °C at 104 kPa for 30 min. prior to fermentation.

Fermentation

Metabolism

Fermentation for microbial metabolism was carried out in 250 ml Erlenmeyer flasks containing 50 ml medium. The culture flasks were incubated in a rotary shaker, operating at 120 rpm at 37 °C for 24 - 48 h. Fermentation was carried out according to standard protocol. The substrate (phenobarbital) was prepared by dissolving 20 mg of the drug in 10 ml of methanol, and 20 µg/ml was added to the culture medium of the selected organism and incubated in a shaker. For each biotransformation study, one sample and two controls were used. The study included the substrate control to which substrate was added and incubated without microorganism. Culture control consisted of fermentation blanks in which the microorganisms were grown under identical conditions without the substrate to test whether phenobarbital would be chemically decomposed or transformed during the incubation period.

Inhibition studies

The inhibition studies were carried out to confirm the involvement of specific CYP450 isoenzyme in phenobarbital metabolism. One sample and four controls were used. These include a substrate control, inhibitor control and inhibitor and substrate control to check the interference of substrate, inhibitor alone and in combination with the media. Culture control consisted of fermentation blanks in which the microorganisms were grown under identical conditions without the substrate.

The test was performed in two stages; in the first stage, drug control, culture control and inhibitor control were incubated in orbital shaker for 72 h. Inhibitor and substrate control and samples were incubated under the same conditions for 24 h by adding 20 µg/ml of inhibitor. In second stage, i.e., after 24 h of incubation 0.5 ml of drug solution was added to the inhibitor and substrate control and to the sample for a further 48-h incubation. The contents of the flask were extracted and analyzed by HPLC to determine the extent of inhibition of metabolism.

The experiments were carried out in culture flasks (250 ml), each containing 50 ml of broth, and incubated in a rotary shaker operated under similar conditions. The substrate (phenobarbital), CYP 2C9 inhibitor, fenofibrate, and CYP 2C9 and 2C19 inhibitor, fluoxetine stock solutions, were prepared separately by dissolving 20 mg in 10 ml of ethanol.

Extraction

The incubated medium was heated on a water bath at 50 °C for 30 min and centrifuged at 3000 rpm for 10 min at 37 °C (Remi Instruments Pvt Ltd, Mumbai, India) A clear supernatant liquid was collected, 8ml of which was extracted with 5 ml of ethyl acetate [15]. The upper organic layer was separated and dried in vacuum dryer. During HPLC analysis, it was reconstituted with 0.5 ml of mobile phase and centrifuged at 13000 rpm for 10 min. Aliquots (25 µl each) were injected into the HPLC facility for analysis. Controls were also prepared similarly to provide suitable blanks.

High pressure liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) analysis was carried out using a HPLC system (Waters, USA) which consisted of Waters 515 solvent delivery module, Waters 2489 UV- detector and a Wakosil II SC-18 rs-100 a. 5 µl, 4.6 x 250 mm stainless

steel column. Sensitivity was set at 0.001a.u.f.s. The mobile phase consisted of ammonium acetate buffer (0.025 M) of pH 5.5 and methanol in a ratio of 60:40 v/v and was used at a flow rate of 1 ml/min. Elution was monitored using a UV-detector set at 220 nm.

Mass spectrometry (MS)

The metabolite found in the sample of *Rhizopus stolonifer* by HPLC analysis was collected from the elute for further analysis by mass spectrometry. MS/MS analysis was carried out using model API 3000, MS/MS operated with an ion trap mass spectrometer (IIC, Hyderabad) in positive electron spray ionization (ESI) source system. The interface was adjusted to the following conditions: ion mode, positive: spray voltage, 3.5 KV; capillary temperature, 325°C; and auxiliary

gas (nitrogen), 40 Psi. A mass spectrum was collected to obtain the protonated molecules (M+H) of drug and metabolite. MS/MS spectra were obtained by collision induced dissociation (CID) using helium (He) as the collision gas.

Statistical analysis

The results of inhibition studies are presented as the mean \pm SD. The data were evaluated by paired Student's 't'-test using Graph Pad Prism, version 4.0 program and statistical significance was set at $p < 0.001$

RESULTS

The results of HPLC analysis of phenobarbital and its metabolite in different cultures are given in Table 1. The peaks at

Table 1: HPLC data for phenobarbital and its metabolite obtained from a microbial culture extracts

Name of organism	Retention time (min)			Sample
	(Blank I) Drug control	(Blank II) Culture control	(Control) Pure phenobarbital	
<i>Rhizopus stolonifer</i> (NCIM 880)	2.5	2.4	2.5	2.6
	-	-	-	3.11*
	-	4.0	-	4.1
	10.37	-	10.37	10.37
<i>Aspergillus terreus</i> (NCIM 657)	2.6	2.5	2.5	2.5
	-	4.0	-	4.0
	10.3	-	10.37	10.37
<i>Cunninghamella</i> <i>elegans</i> (NCIM 689)	2.3	2.4	2.3	2.3
	-	4.7	-	4.7
	10.3	-	10.37	10.37
<i>Cunninghamella</i> <i>echinulate</i> (MTCC 4279)	3.6	3.6	-	3.6
	10.37	-	10.37	10.37
<i>Cunninghamella</i> <i>blakesleeana</i> (MTCC 3729)	2.42	2.53	2.4	2.57
	-	5.4	-	5.5
	10.37	-	10.37	10.37
<i>Nocardia</i> <i>hydrocarboxydans</i> (NCIM 2386)	2.6	2.6	2.5	2.6
	5.23	5.2	-	5.2
	10.3	-	10.37	10.37
<i>Streptomyces griseus</i> (NCIM 2622)	2.65	2.64	2.5	2.65
	-	6	-	6.1
	10.37	-	10.37	10.37
<i>Saccharomyces</i> <i>cerevisiae</i> (NCIM 3090)	4.02	4.1	-	4.07
	-	6.23	-	6.23
	10.37	-	10.37	10.37

* Metabolite peak

the retention time of 2.4 min. represents solvent peak while the peaks at 4.0 and 5.4 min represent culture components; the peak at 10.3 min represents phenobarbital. *Rhizopus stolonifer* showed an extra peak at 3.1 min, as indicated in Fig 1.

The mass spectrum of phenobarbital showed a molecular ion peak at m/z 231 while that of the metabolite was at m/z 425 and fragment ion peaks occurred at 395, 379, 359, 351, 343, 295, 269, 263, 177 and 174 (Fig 2). The mass fragmentation pattern is shown in Fig 3, based on which the metabolic pathway of phenobarbital by *Rhizopus stolonifer* was proposed.

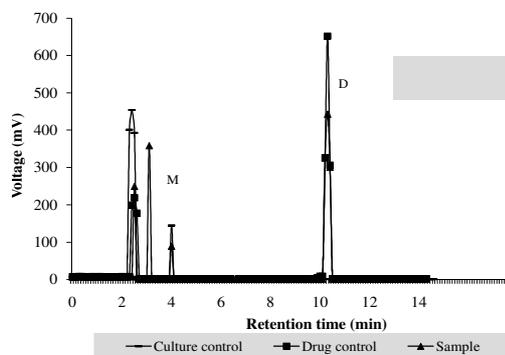


Fig 1: HPLC chromatogram of phenobarbital from culture extracts of *Rhizopus stolonifer*. **Key:** D = drug; M = metabolite; - = culture control; ■ = drug control; ▲ = *R. stolonifer* sample

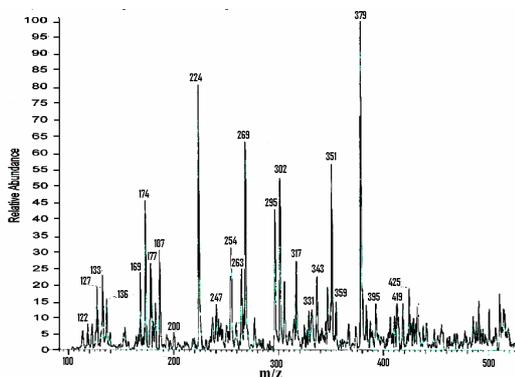


Fig 2: Mass spectrum of phenobarbital metabolite produced by *Rhizopus stolonifer*

Table 2: Data of metabolite formation and inhibition in inhibition studies

Drug	Metabolite formation (% , ±SD)	Metabolism inhibition (% , ±SD)
Phenobarbital (substrate)	38.17±0.02*	0
Fluoxetine (inhibitor)	21.60±0.01*	43.50±0.01*
Fenofibrate (inhibitor)	10.05±0.04*	73.60±0.02*

* $p < 0.001$ compared to substrate (i.e., without inhibition)

DISCUSSION

Eight microorganisms were screened in the present study. The chromatograms of the culture controls did not show any peak for phenobarbital or its metabolite; however, the substrate control showed only the presence of phenobarbital. The samples of all cultures except the sample of *Rhizopus stolonifer* showed identical peaks in sample and controls which indicated that the organisms tested could not metabolize the drug, but interestingly an extra peak was detected in the sample of *Rhizopus stolonifer*, which represents the formation of phenobarbital metabolite, thus indicating the biotransformation of phenobarbital by *Rhizopus stolonifer*.

The metabolite formed by *Rhizopus stolonifer* was isolated from elute of HPLC and its structure was identified by MS/MS analysis. The mass spectrum of phenobarbital exhibited a molecular ion peak at m/z 233 (M+1). The molecular ion of m/z 425 (M+1) in mass spectrum of metabolite of phenobarbital represents the N-glucuronide of p-hydroxy phenobarbital. Formation of the above metabolite was supported by fragment ion peaks at m/z 395, 379, 359, 351, 343, 295, 269, 263, 177 and 174 (Fig 3). Phenobarbital is mainly metabolized to p-hydroxy phenobarbital and glucuronide conjugate of p-hydroxy phenobarbital in man and rats while N-glucuronide of p-hydroxy phenobarbital is found in mice [14].

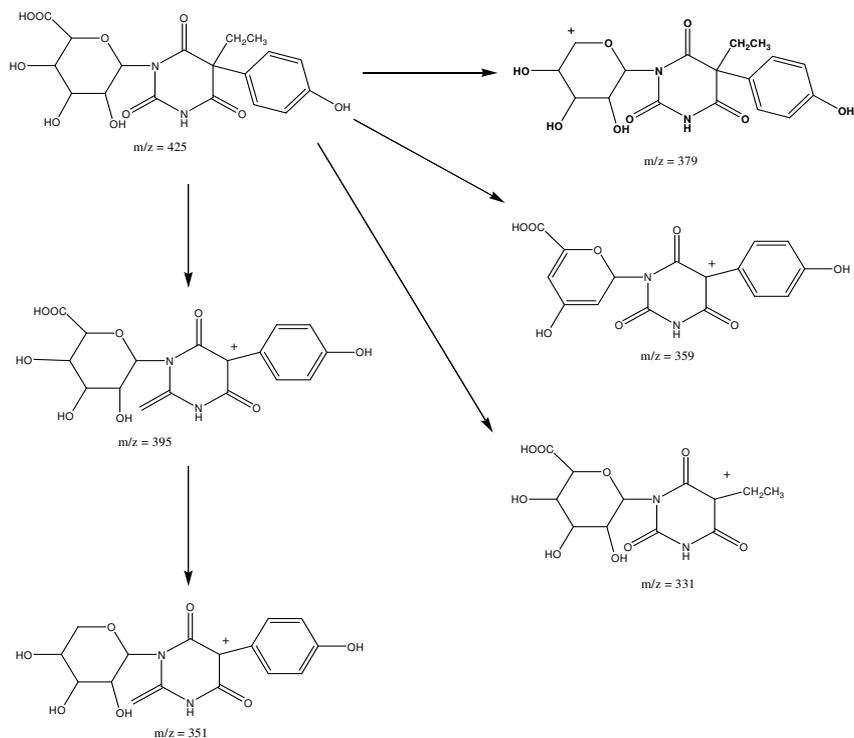


Fig 3: Mass fragmentation pattern of phenobarbital metabolite produced by *Rhizopus stolonifer*. The inhibition are shown in Table 2., Fenofibrate inhibited 73.6 % of metabolite formation, with metabolite production decreasing from 38.17 to 10.05 % whereas fluoxetine inhibited 43.5 % of metabolite formation, decreasing formation of metabolite from 38.17 to 21.6 %. Percent metabolite formation or inhibition was calculated by taking the sum of drug and metabolite peak areas as 100%.

The fungus, *Rhizopus stolonifer*, is of importance in medical mycology. It converted phenobarbital to N-glucuronide of p-hydroxy phenobarbital, indicating similarity with the metabolism of phenobarbital in mammals. The formation of the metabolite was confirmed by mass spectroscopy and inhibition studies. It has been documented that *Rhizopus stolonifer* metabolizes various drugs, including diclofenac [16], sclareol [17] and α -agarofuran [18] to their respective metabolites similar to those formed in mammals. Based on the foregoing, the metabolic pathway of phenobarbital due to the action of *Rhizopus stolonifer*, is proposed as hydroxylation followed by glucuronidation.

In the present study, a CYP 2C9 inhibitor, fenofibrate, and CYP 2C9 and 2C19 inhibitor, fluoxetine, were used, in the inhibition studies

to confirm the involvement of a specific CYP enzyme in metabolite formation by *Rhizopus stolonifer*. Inhibition of metabolite formation was 73.6 % by fenofibrate and 43.5 % by fluoxetine. Thus, the two inhibitors not only demonstrated significant inhibition ($p < 0.001$) of phenobarbital metabolism by *Rhizopus stolonifer* but also showed that there was a difference in the capacity of the two inhibitors ($p < 0.001$) to inhibit phenobarbital.

CONCLUSION

The present study revealed the evidence for the functional role of the enzymes in *Rhizopus stolonifer* in the metabolism of phenobarbital in a manner similar to CYP 2C9 and 2C19. Inhibition by CYP 2C9 inhibitor (fenofibrate) was significantly higher than by CYP 2C9 and 2C19 inhibitor

(fluoxetine), thus indicating the significant involvement of CYP 2C9 in the biotransformation of phenobarbital by *Rhizopus stolonifer*. Therefore, it can be concluded that *Rhizopus stolonifer* metabolizes phenobarbital to its N-glucuronide of p-hydroxy phenobarbital by CYP 2C9 as reported in mammals.

ACKNOWLEDGEMENT

The authors are thankful to University Grants Commission of India for providing funds to conduct this work.

REFERENCES

1. McGinnity DF, Parker AJ, Soars M, Riley RJ Automated Definition of the Enzymology of Drug Oxidation by the Major Human Drug Metabolizing Cytochrome P450. *Drug Metab. Dispos* 2000; 28: 1327-1334.
2. Prasanthi S, Vidyavathi M, Prasad KVSRG, Krishna DR O - Demethylation of dextromethorphan using microbial cultures. *Drug. Metab. Letters* 2009; 3(1): 10-14.
3. Ono S, Hatanaka T, Hotta H, Satoh T, Gonzalez FJ , Tsutsui M Specificity of substrate expressed human P450s and human liver microsomes. *Xenobiotica* 1996; 26: 681-93.
4. Smith DA, Jones BC , Walker DK. Design of drugs involving concepts and theories of drug metabolism and pharmacokinetics. *Med. Res.* 1996; Rev 16: 243-266.
5. Clark AM, McChesney JD, Hufford CD The use of microorganisms for the study of drug metabolism. *Med. Res.* 1985; Rev 5: 231-253.
6. Clark AM, Hufford CD. Use of microorganisms for the study of drug metabolism – an update. *Med. Res.* 1991; Rev 11: 473-501.
7. Beukers R, Marx AF, Zuidweg MHJ Microbial conversion as a tool in the preparation of drugs. In: Ariens EJ, Editor. Vol.3, *Drug Design*, New York: 1972; Academic Press, p. 1.
8. Smith RV, Rosazza JP Microbial models of mammalian metabolism. *J. Pharm. Sci.* 1975; 64: 1737-1758.
9. Smith RV, Rosazza JP Microbial models of mammalian metabolism. *J.Nat. Prod*; 1983; 46: 79-91.
10. Ratna kumari T, Vidyavathi M, Asha S , Prasad KVSRG Biotransformation of paracetamol by *Cunninghamella echinulata*. *J.Pharm. Res.* 2009; 8(1): 1-5.
11. Whyte MP , Dekaban AS Metabolic fate of phenobarbital. A quantitative study of p-hydroxyphenobarbital elimination in man. *Drug. Metab. Dispos* 1977; 5(1): 63-70.
12. Levin SS, Vars HM, Schleyer H, Cooper DY. The metabolism and excretion of enzyme inducing doses of phenobarbital by rats with bile fistules. *Xenobiotica* 1986; 16(3): 213-224.
13. Horning MG, Butler CM, Nowlin J , Hill RM Drug metabolism in the human neonate. *Life. Sci* 1975; 16: 651-671.
14. Suzanne MN , Soine WH Identification of phenobarbital N- glucuronides as urinary metabolites of phenobarbital in mice. *Drug. Metab. Dispos.* 1995; 23(5): 548-552.
15. Bernus I, Dickinson RG, Hooper WD , Eadie MJ Inhibition of phenobarbitone N- glucosidation by valproate. *Br. J. Clin. Pharmac.* 1994; 38: 411-416.
16. Ibrahim ARS, El-Ferally FS Microbial transformation of diclofenac. *Saudi. Pharm. J.* 1996; 4: 165-169.
17. Diez D, Sanchez JM, Rodilla JM, Rocha PM, Mendes RS, Paulino C, Marcos IS, Basabe P, Urones JG. Microbial hydroxylation of sclarelol by *Rhizopus stolonifer*. *Molecules.* 2005; 10(8): 1005-1009.
18. Alarcon AJ, Aguila JB, Peter S Regio and stereoselective hydroxylation of alpha-agarofuran by biotransformation of *Rhizopus* . *J.Chil. Chem. Soc* 2005; 50(4): 715-718.