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Microbial Cultures as Models to Predict Drug Metabolism : Studies with Phenytoin

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It is known that microbial model systems can be employed as good predictors of mammalian drug metabolism. Metabolites of phenytoin, a model drug for aromatic hydroxylation were isolated and characterized using HPLC andTLC after pre-incubation with some microbial cultures. Among different organisms screened, *pseudomonas aeruginosa* and *Proteus vulgaris* were found to metabolize phenytoin. Both produced a phydroxylated derivative of phenytoin which was the main metabolite observed in rabbits. *Pseudomonas aeruginosa* also produced one unknown metabolite of phenytoin which was not observed in mammalian systems. These two organisms could be used as microbial models of metabolism of other drugs whose metabolism is expected to be similar to that of phenytoin.

PHENYTOIN is an hydantoin derivative used in the treatment of epilepsy and cardiac arrhythmias[2]. It is metabolised in the body through aromatic hydroxylation by CYP2D6, a specific hepatic microsomal enzyme.[1,2]. The major metabolite is 5-*p*-hydroxyphenyl-5-phenylhydantoin. Generally, drug metabolism studies can be performed either on whole animal systems *(in vivo)* usually utilising small laboratory animal models or *in vivo* using tissue culture, microbial preparations, or perfused organ systems[3,4].

Microorganisms such as bacteria and fungi have recently been successfully used as *in vitro* models for the prediction of mammalian drug metabolism and successful applications have been recently reviewed.[3,4]. In the present investigations, phenytoin, a model drug for aromatic hydroxylation, is used for evaluating the ability of different bacteria to metabolise phenytoin. The aim of this investigation is to identify the microorganisma that can be used to study metabolism of newer drug moities. The use of microbial syatems allows for the collection of metabolites in larger amounts for further characterisation as well as pharmacological and toxicological evaluation.

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EXPERIMENTAL

Microorganisms :

Bacterial cultures were obtained from the microbial type culture collection (MTCC), Chandigarh, India. The bacterial cultures used in the present work were : *Bacillus subtilis* (MTCC 619), *Escherichia coli* (MTCC 531), *Staphylococus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 647), *Klebsiella Pneumoniae* (MTCC 109), *Proteus vulgaris* (MTCC 426) and *Agrobacterium rhizogenes* (MTCC 530).

Fermentation conditions

The preliminary screening screening and large scale experiments were carried out using a culture medium consisting the following composition : Peptone-5 g, sodium chloride - 5 g, Beef extract: 1.5 g, Yeast extract - 1.5 g, Dextrose - 20 g, distilled water-1000 ml. Stock cultures of bacteria were stored on slants prepared according to the following compositions; Peptone - 5g, Sodium chloride - 5g, Yeast extract - 1.5 g, Agar - 15 g, distilled water - 1000 ml (Himedia Pvt. Ltd., Bombay, India) at 4°. Media were sterilized in an autoclave for 15 min at 121° and 151bs/ sq.in.

Microbial metabolism studies were carried out by shake-flask culture on a model G-24 Gyratory Shaker (New Brunswick Scientific Co., New Jersey), operating at 250 rpm at 37°. Preliminary screening experiments were carried out in 250 ml glass conical flasks containing 40 ml of medium. Fermentations were carried out according to a standard protocol[5]. In brief, the substrate (phenytoin) was prepared as a 1% (W/v) solution in 0.1 N NaOH solution and added to the 24 hold stage II culture medium of the microorganism at a concentration of 25 mg/ml of medium. Substrate controls comprised of sterile medium to which the substrate was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without the substrate. The containers meant for sterilization were kept in an autoclave and sterilized for 15 min at 121° and 15 lbs/Sq.in.

Analysis of culture extracts of microbial samples and serum samples [6] : The TLC chromatographic analyses were carried out on pre-coated silica G-25UV $_{254}$ plates (Macherey-Nagel Duren, USA). The TLC plates were visualized in a UV chamber. Chloroform and methanol in 8:2 ratio was used as mobile phase.

HPLC ANALYSIS :

Photodiode-array HPLC analysis of phenytoin and metabolites was carried out using the method of Soto-otero *et al*[7],. using column, Lichrosphere 100 RP 18, Merck (300x 3.9 mm); solvent, methanol: water (1:4), 1.2 ml/min flow rate using a Beckmann 110 A Pump, a Gilson 231 sample injector equipped with a 20 µl loop, a Pye unicam LC - UV detector set at 216nm, and a Shimadzu LC 10 As recorder. Column temperature was maintained at 45°

The samples (along with bacterial mass) was centrifuged at 3000 rpm for 15 min (G24 Remi centrifuge Pvt. Ltd, India). The clear supernatant liquid was collected and extracted by dichloromethane (HPLC grade, Ranbaxy fine chemical Ltd. Delhi, India) at a ratio of 1:3. This extract was evaporated on a water bath and reconstituted with 100 µl of methanol (HPLC grade, Ranbaxy fine Chemical Ltd, Delhi, India), and 20 µl of portions were injected into the HPLC system.

Animal Studies[8]

Four male rabbits (New Zealand), weighing 1.5-2 kg were used in the study. Phenytoin tablet (Eptoin, Knoll Pharmaceuticals Pvt. Ltd., India) at a dose of 100 mg, was administered orally to each rabbit. Before and after 6 h of phenytoin administration, 2 ml blood samples were collected from the marginal ear vein of rabbit. The samples were allowed to clot, centrifuged at 3000 rpm for 15 min and 1 ml of clean serum was collected for extraction. The extraction protocol was similar to that of microbial cultures.

RESULTS AND DISCUSSION

In TLC analysis (Table-1) it was found that the spot P with R_f value 0.51 would represent phenytoin, the spots M_7 and M_8 with R_f value 0.11 represent a common constituent of rabbit's serum. The spots M_4, M_6 and M_{11} with R_f value 0.29 were identical and were present in cultures of both *Pseudomonas aeruginosa* and *Proteus vulgaris* and in the 6 h serum sample of rabbit. The spot M_3 with R_f value 0.43 was observed only in *pseudomonas aeruginosa* culture.

In the HPLC analysis of the culture extract of *Bacillus subtilis* (Table-2), 3 peaks were observed with the retention time 2.53, 3.76 and 12.06 min By comparing these peaks with those of control and blank samples, it was found that the peaks at 2.53 min and 3.76 min represent solvent and both constituents whereas the peak at 12.06 min was due to phenytoin.

In case of other organisms like *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Agrobacterium rhizogenes* the peaks obtained were almost identical with those seen with *Bacillus subtilis*. No detectable metabolites have been found using all these 4 organisms.

Interestingly, the culture of *Proteus vulagris* (Table-2) produced the peaks at 2.53, 3.83 and 5.3 min and 12.63 min. The peak at 12.63 min was due to phenytoin and the one at 5.3 min is considered to be 5-p-hydroxy phenyl-5-phenyl hydantoin, basing on retention time and spectral analysis.

The culture of *Pseudomonas aeruginosa* (Table-2) produced the peaks at 2.53, 3.83, 5.3, 8.0 and 12.63 min. As in the case of *Proteus vulgaris* the peak at 5.3 min is due to 5-p-hydroxy phenyl-5-phenyl hydantoin. The peak at 8.0 min appears to be a new metabolite of phenytoin,

Table-1 : TLC Analysis of Microbial and Rabbit Serum Samples

Ps. aeruginosa Spot (R_f)	*P. vulgaris* Spot (R_f)	O serum sample Spot (R_f)	6th serum sample Spot (R_f)	Control Spot (R_f)	Blank-I Spot (R_f)
M_2 (0.51)	M_{10} (0.52)	M_8 (0.11)	M_5 (0.51)	P (0.52)	M_1 (0.51)
M_3 (0.43)	M_{11} (0.29)		M_6 (0.29)		
M_4 (0.29)			M_7 (0.12)		

Blank- I contains medium + Substrate (No Culture) and Control consists of only phenytoin in 0.1 N NaOH, without medium and culture.

Table-2 : HPLC Analysis of Microbial Samples

Blank-I	Blank-II	Control	Sample-I *(B.subtilis)*	Sample-II *(P.vulgaris)*	Sample-III *(Ps.aeruginosa)*
2.53	2.53	2.40	2.53	2.50	2.53
3.80	3.80	3.93	3.76	3.70	3.83
—	—	—	—	5.30	5.30
—	—	—	—	—	8.00
12.26	—	12.10	12.63	12.06	12.06

Retention time is in minutes. Blank-I contains medium + Substrate (No culture) Blank-II contains medium + Culture (No substrate) Control consists of only phenytoin in 0.1 N NaOH, without medium and culture and Sample consists of medium + culture + substrate

Table-3 : HPLC Analysis of Rabbit Serum Samples

Retention time

Control	0 h serum sample	6 h serum sample
2.40	2.50	2.50
3.93	3.89	3.90
—	—	5.3
12.26	—	12.26

Retention time in minutes. Control consists of only phenytoin in 0.1 N NaOH.

since it has a spectral pattern similar to that of phenytoin, but has retention time which is different from that of phenytoin and the p-hydroxy metabolite. Its characterization, however requires mass spectral studies which are in progress.

The HPLC analysis of animal studies (Table-3) revealed 4 peaks at 2.53, 3.83, 5.3 and 12.63 min. The peak at 12.63 min was due to phenytoin and the peak at 5.3 min was due to a p-hydroxy derivative of phenytoin.

It is therefore possible to conclude from our investigation that *Proteus vulgaris* and *Pseudomonas aerugonosa* produce an isoenzyme of cytochrome p450 monooxygenase system which is specifically responsible for phenytoin aromatic hydroxylations similar to animal systems, and these cultures can therefore be used in studies of metabolism of drugs to predict the metaboliic patterns in humans. This oxidising enzyme does not seem to be present in the other species of bacteria employed in our study.

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