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RESEARCH ARTICLE

Optimization Study of Coumarin 7-Hydroxylase Enzyme Activity in Mouse Brain and Liver

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Summary

In this study, the optimum conditions (pH, amount of protein, substrate concentration, incubation time) of a cytochrome P450 dependent enzyme, coumarin 7-hydroxylase (COH), activity were determined in microsomal fractions of mouse liver and brain. It's indicated that different reaction conditions are required for this enzyme activity in mouse liver and brain microsomes. The results obtained, reveal the requirement of optimization studies on COH activity for different tissues of mouse.

Key Words: *Mouse, liver, brain, CYP450, coumarin 7-hydroxylase.*

Fare Karaciğer ve Beyin Mikrozomlarında Kumarin 7-Hidroksilaz Aktivitesi Optimizasyon Çalışması

Özet

Bu çalışmada fare karaciğer ve beyin mikrozomal fraksiyonlarında sitokrom P450'ye bağımlı bir enzim olan kumarin 7-hidroksilaz (COH) için pH, protein miktarı, substrat konsantrasyonu ve inkübasyon süresi göz önüne alınarak optimizasyon çalışmaları yapılmıştır. Yapılan optimizasyon çalışmaları sonucunda, fare karaciğer ve beyin mikrozomlarına ait COH aktivitelerinde farklı reaksiyon şartlarına gereksinim duyulduğu görülmüştür. Elde edilen sonuçlar, COH aktivitesine ilişkin optimizasyon çalışmalarının farenin farklı dokularında yapılmasının gerekliliğini ortaya koymaktadır.

Anahtar Kelimeler: *Fare, karaciğer, beyin, sitokrom P450, kumarin 7-hidroksilaz*

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INTRODUCTION

Due to rapid industrialization in recent years, exposure to lipophilic compounds has been increased. These compounds are absorbed easily however their elimination is limited. Biotransformation of lipophilic xenobiotics is carried out by xenobiotic metabolizing enzymes. These are known as Phase I and Phase II enzymes. Phase I enzymes are responsible for the functionalization of xenobiotics whereas phase II enzymes are responsible for conjugation (1,2). These reactions convert xenobiotics to more polar species and are frequently achieved by the cytochrome P450-dependent enzyme system.

The CYP450-dependent xenobiotic metabolizing enzyme present in hepatic and extrahepatic organ and tissues is involved in the oxidative, reductive, peroxidative metabolism of many endogenous substances such as steoroids, bile acids, fatty acids, prostaglandins, leucotrienes, vitamins A and D, biogenic amines, retinoids, lipid hydroperoxidases and phytoalexines. Many of these enzymes also metabolize exogenous substances like synthetic chemicals including drugs, environmental pollutants, polycyclic aromatic hydrocarbons and aromatic amines (2-7).

This enzyme system also changes the therapeutic activity of the drugs, causes carcinogenicity and mutagenicity by activating and inactivating the substance under concern. However, this enzyme system and different forms of P450 are inhibited and stimulated by various xenobiotics. Stimulation and inhibition of monooxygenases on the enzyme system and isozymes of P450 forms can alter the toxicity and/or therapeutic activity of drugs. P450 system has many forms and each of them is encoded and synthesized by a different gene and each form has different substrate selectivity.

As it is known, liver is the major xenobiotic metabolizing organ. Phase I and Phase II enzymes are included in metabolic reactions and it's determined that these enzymes are also expressed in extrahepatic tissues and organs such as lung (8), kidney (9), intestine, skin, nasal epithelium (10), breast (11,12), brain (13-16). Brain is as substantial as liver over the enzyme system and acts in metabolism of drugs and other xenobiotics (14,17).

CYP2A is one of the six CYP2 families known to be present in human. Metabolic characteristics of CYP2A have been intensively studied in mouse, rabbit, rat, monkey, hamster and human tissues and subcellular organelles. It became clear that these proteins differ substantially in catalytic specificity and inducibility between species and even strains. CYP2A6 is the best characterized enzyme in this sub-family. Like most other CYPs, CYP2A6 is the most abundant in liver, and its levels in extrahepatic tissues are very low. It has been shown to catalyze the metabolic activation of several procarcinogens including the liver specific carcinogen aflatoxin B1, several nitrosamines and 1,3 butadiene. Clinically used drugs metabolized by CYP2A6 include coumarin, methoxyflurane, and novel platelet-activating factor antagonist SM-12502. CYP2A6 is also a major catalyst in the oxidative metabolism of nicotine and cotinine, as well as several pharmaceuticals (18,19,20). CYP2A6 converts nitrosamines (especially N-nitrosodiethylamine) which are potential carcinogens (procarcinogens) found in cigarette smoke, to cytotoxic and mutagenic species. Individuals with deficient activity of the enzyme may generate fewer carcinogens from the procarcinogens in cigarette smoke (21).

Cyp2a5, the mouse orthologue of CYP2A6, is a major P450 isoform in mouse olfactory mucosa and is also expressed in liver, kidney and lung. Studies on Cyp2a5 have shown that Cyp2a5 is inducible by a number of xenobiotic compounds which are structually dissimilar, including phenobarbital, pyrazole, heavy metals such as stannus chloride and cobaltous chloride (18,22).

Coumarin is a naturally occurring compound found in a variety of plants, microorganisms, some animal species and can be synthetically produced as well (23). Because of having a characteristic odor it is used as a food additive and an ingredient in perfume. When ingested, coumarin acts as an anticoagulant. Coumarin is 7-hydroxylated by the CYP450 isoform Cyp2a5 in mouse and CYP2A6 in human. Various drugs, endogenous substances, plant substances

and carcinogens, altogether about 90 chemicals were evaluated as possible inhibitors of COH activity in mouse microsomes (24). The coumarin-7 hydroxylation seems to be one of the most specific probe activities available for any CYP enzyme, since no other human CYP form has the capacity to catalyze coumarin 7-hydroxylation to a significant degree (25).

Although there have been many studies about COH activity and protein level in different species and tissues there haven't been sufficient brain-related studies. Considering the increasing importance of brain on the enzyme system which plays a substantial role in the metabolism of xenobiotics and drugs, studies on activity and protein level of COH become crucial. As a concequence, this optimization study is meant to be a pre-study to measure and compare COH activities in mouse brain and liver. According to these findings it's thought that, such pre-studies will initiate the studies about the COH activity, especially its role in brain xenobiotic metabolism, in the future.

MATERIALS and METHODS

Chemicals

D-glucose–6 phosphate monosodium salt, D-glucose-6-phosphate dehydrogenase, and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were purchased from (Sigma-USA), 7-hydroxy coumarin and coumarin were purchased from (Aldrich-Belgium) and (Merck-Germany) respectively. Crystallized bovine albumin (BSA) was obtained from (BDH-UK). All the chemicals used were of analytical grade.

Animals

Male Swiss Albino mice (25–30 g) were used in this experiment. They were obtained from Başkent University Test Animals Production Unit (Ankara, TURKEY). The animals were housed in polystyrene cages with cellulose bedding at 22–25°C and fed with standard laboratory rat chow pellet and tap water *ad libitum*. Lighting was on a 12 h light-12 h dark cycle for 24 hours. The animals were starved for 24h prior to sacrifice. After decapitation liver and brain tissues were removed immediately and washed in ice-cold distilled water. Then they were blotted with paper and weighed with the sensitivity of 0,001 g. Brain tissues were studied as whole brain. All these processes were carried out at +4°C.

Sample Preparation

Mouse Brain and Liver Microsomes

Brain and liver tissues were chopped and homogenized separatelly in a solution of 1.15% KCl which was added 5 ml and 3.5 ml respectively for 1 gram of brain and liver tissue, prior to subcellular fractionation. Tissue homogenates were centrifuged at 10.000 × g for 20 min. to remove mitochondria and nuclei.

The supernatant was centrifuged at 108.000 × g for 90 min. Microsomal pellets were suspended in 20% (v/v in glycerol) for 1 gram tissue. Microsomal fraction was used for protein determination and the rest was stored at –80°C until the process. All these processes were carried out at +4°C.

Protein Determination

Protein contents of brain and liver microsomes were determined by the method of Lowry et al. with the use of bovine serum albumin used as a standard (26).

Determination of coumarin-7-hydroxylase activity

Coumarin-7-hydroxylase is the enzyme which achieves the transformation of coumarin to 7-hydroxy coumarin. The method used to determine the COH activity is based on the spectroflourimetric measurement of the 7-hydroxy coumarin. NADPH producing system was used as a cofactor. Coumarin–7-hydroxylase activity was measured as described by Aitio (27).

Reaction Medium for Maximum Enzyme Activity in Optimum Conditions

Standard

7-hydroxy coumarin solution was used as a standard in order to determine the COH activity. 1 mM 7-hydroxy coumarin solution was freshly prepared just before use. Standards were prepared at four different concentrations (20, 40, 80, 160, 320 nM)

Reaction Medium for Mouse Brain and Liver

0.5 mL reaction medium of mouse brain and liver contained 1.5 and 1.0 mg microsomal protein

respectively, 0.05 mM coumarin, as substrate, 20 mM pH 7.4 potassium phosphate buffer; 5 mM glucose-6-phosphate as cofactor, 0.5 mM $NADP^+$, 1 U glucose 6-phosphate dehydrogenase, 5 mM $MgCl_2$, 59.8 mM pH 7.8 potassium phosphate buffer. Cofactor was prepared just before use.

Procedure

Test tubes containing all substances –except substrate– in the reaction medium were preincubated in a shaking water bath at 37°C for 2 min. Reaction was started with the addition of 1mM 0,05 mL coumarin to each test tube and incubation was continued for 30 min. at the same temperature in a shaking water bath. After 30 min. the reaction was stopped by the addition of 0.5 mL of TCA to each test tube. Denatured protein was centrifuged and 0.5 mL of supernatant was transferred to another test tube. 2 ml of 1.6 M pH 10.4 glycine buffer was added and fluorescence intensity was measured spectroflourimetrically (Exc. 365 nm and Em. 454 nm). The process is the same for mouse liver as indicated in that of mouse brain however; incubation time is considered as 45 minutes.

RESULT and DISCUSSION

In this study, optimum conditions for COH activity in mouse liver and brain microsomes were investigated with respect to pH, protein and coumarin concentrations and incubation time.

Effect of Protein Amount on COH Activity

The effect of protein amount on COH activity in mouse brain microsome was examined by adding 0.5, 1.0, 1.5 and 2.0 mg of protein to the 0.5 mL incubation medium and maximum activity was observed at 1.5 mg. The effect of protein amount on COH activity in mouse liver was examined by adding 0.25, 0.5, 0.75 and 0,1 mg of protein to the 0.5 mL incubation medium and maximum activity was observed at 1.0 mg. (Table 1, Figure 1).

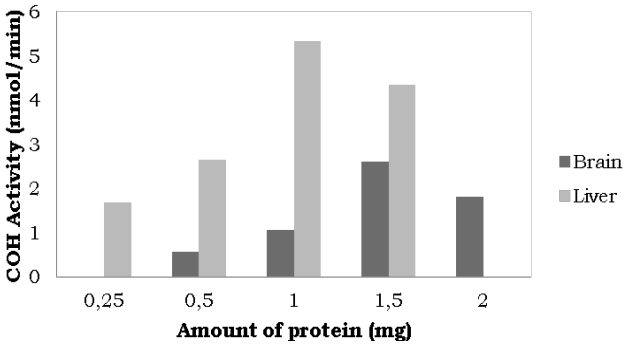

Figure 1. Effect of protein amount on COH activity in mouse brain and liver

Table 1. Protein amount and COH activity in mouse brain and liver*

	COH Activity (nmol/min)	
Amount of Protein (mg)	Brain	Liver
0,25	–	1,67 ± 0,07
0,5	0,55 ± 0,06	2,65 ± 0,11
1,0	1,06 ± 0,13	5,34 ± 0,23
1,5	2,60 ± 0,23	4,35 ± 0,11
2,0	1,81 ± 0,11	–

* Each result indicates mean of 3 different experimental outcomes (± S.D.) Each experiment was conducted triplicate.

Effect of pH on COH Activity

The effect of pH on COH activity in mouse brain and liver microsomes was examined with the use of 20 mM of potassium phosphate buffer having a pH interval 7.2-7.8 in 0.5 mL reaction medium and maximum activities were observed at pH 7.4. (Table 2, Figure 2).

Effect of Incubation Time on COH Activity

The effect of incubation time on COH activity in mouse brain and liver microsomes was examined by incubation of 0.5 mL reaction medium between a time

Table 2. pH and COH activity in mouse brain and liver*

	COH Activity (nmol/mg/min)	
pH	Brain	Liver
7,2	0,95 ± 0,06	4,08 ± 0,03
7,4	1,73 ± 0,14	5,34 ± 0,23
7,6	0,67 ± 0,03	2,36 ± 0,23
7,8	0,88 ± 0,07	3,14 ± 0,28

* Each result indicates mean of 3 different experimental outcomes (± S.D.) Each experiment was conducted triplicate

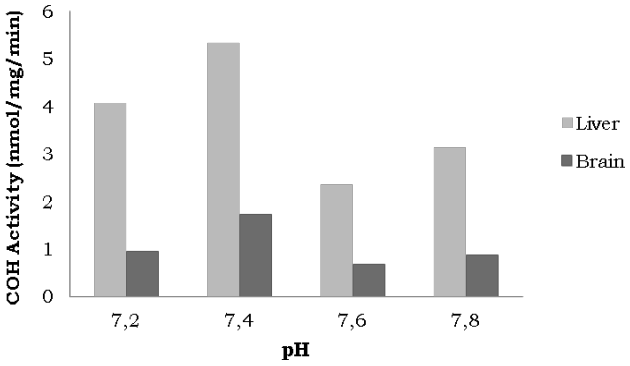

Figure 2. Effect of pH on COH activity in mouse brain and liver

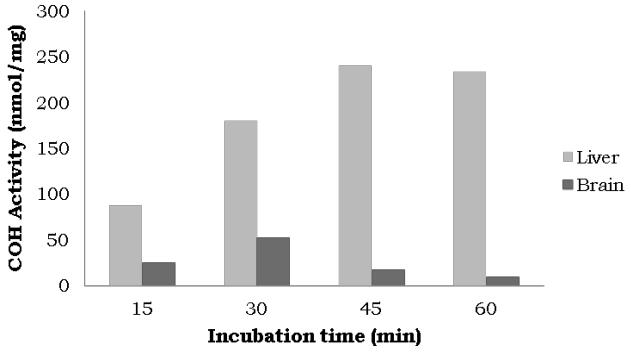

Figure 3. Effect of incubation time on COH activity in mouse brain and liver

interval of 15–60 minute and maximum activities were observed at 30 and 45 minutes, respectively (Table 3).

Effect of Substrat Concentration on COH Activity

The effect of coumarin concentration on COH activity in mouse brain and liver microsomes was examined by adding 0.025, 0.05 and 0.1 mM of coumarin to the 0.5 mL reaction medium and maximum activities were observed at of 0.05 mM. (Table 4, Figure 4).

As a result, in this optimization study it is determined that different reaction conditions are required in order to measure one of the cytochrome P450-dependent enzymes, Coumarin 7-hydroxylase (COH), activity in mouse brain and liver microsomes. pH, protein, substrate concentration and incubation time are considered and different responses of brain and liver are explained as a result of various

Table 3. Incubation time and COH activity in mouse brain and liver*

	COH Activity (nmol/mg)	
Incubation time (min)	**Brain**	**Liver**
15	25,12 ± 2,36	87,80 ± 8,41
30	51,90 ± 4,23	179,71 ± 0,28
45	16,78 ± 1,28	240,37 ± 5,75
60	9,23 ± 0,76	233,10 ± 4,09

* Each result indicates mean of 3 different experimental outcomes (± S.D.) Each experiment was conducted triplicate

Table 4. Coumarin concentration and COH activity in mouse brain and liver*

	COH Activity (nmol/mg/min)	
Coumarin concentration (mM)	**Brain**	**Liver**
0,025	0,81 ± 0,07	3,19 ± 0,35
0,05	1,73 ± 0,14	5,34 ± 0,23
0,10	1,04 ± 0,09	3,65 ± 0,41

* Each result indicates mean of 3 different experimental outcomes (± S.D.) Each experiment was conducted triplicate

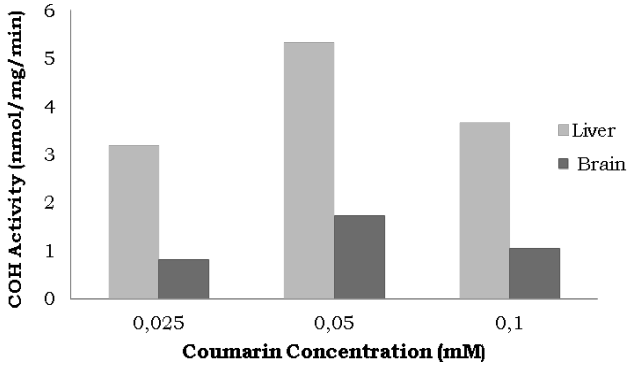

Figure 4. Effect of coumarin concentration on COH activity in mouse brain and liver

levels of enzyme in these tissues. As mentioned above, protein amount is determined to be 1.0 mg for mouse liver and 1.5 mg for mouse brain. The obtained results reveal the requirement of optimisation studies on COH activity for different tissues of mouse. These findings also provide the basis for investigating the effects of xenobiotics such as drugs, pesticides, polycyclic aromatic hydrocarbons, aromatic amines and organic solvents as well as endogenous substances like steroids, fatty acids and prostaglandins on mouse liver and brain coumarin 7-hydroxylase activity.

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