PHENOBARBITAL METABOLISM BY HEPATOCYTES ISOLATED FROM RAT

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SUMMARY

In order to test the validity of the use of hepatocytes as an alternative to studies of metabolism in live animals, phenobarbital (PB) was applied at 37°C to isolated rat hepatocytes, which had been previously induced by PB. The metabolites were extracted by ethyl acetate, at various pH, and identified and evaluated by GC-MS. PB was not metabolized to *p*-hydroxyphenobarbital as is usually the case in living animals, but it was transformed into sulphoconjugated 2-phenyl-γ-butyrolactone. This pathway, β-hydroxylation of the ethyl side chain followed by lactonization, previously described as a secondary metabolic pathway occurring during intoxications, was here the only observed biodegradation. These results show that it is not possible to use hepatocytes as an alternative to live animals.

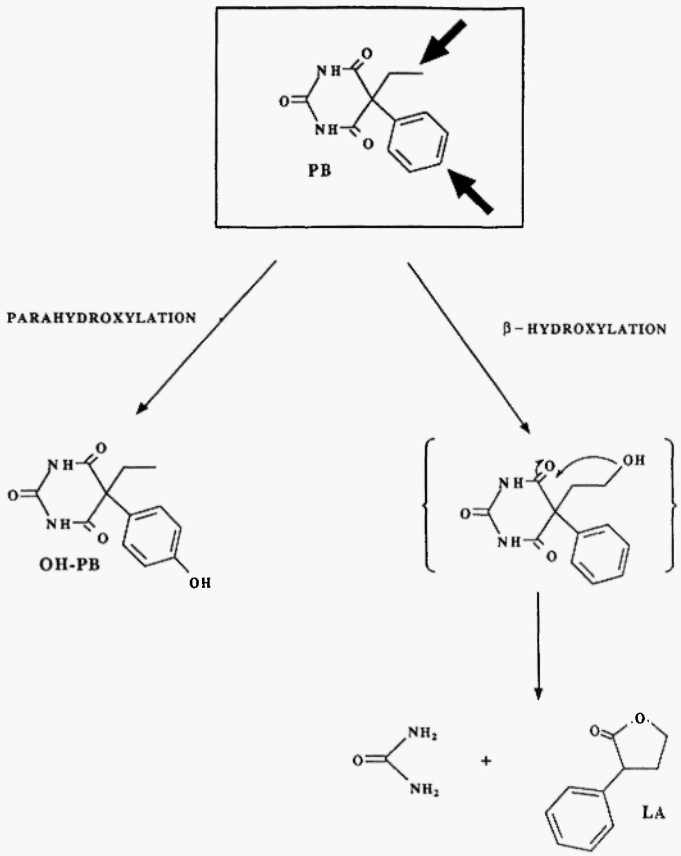
KEY WORDS

phenobarbital, 2-phenyl-γ-butyrolactone, GC-MS, rat hepatocytes

INTRODUCTION

The use of hepatocytes is often considered as an alternative to experiments on live animals for studies of metabolism /1/. In order to test the validity of this method on a compound of which the metabolism is well known in live rats, the degradation of phenobarbital by hepatocytes isolated from rat liver after induction was studied. Induction by phenobarbital usually increases the activity of hepatic cytochromes P-450 /2/.

Phenobarbital (PB) is a compound which is used as a hypnotic and anticonvulsant drug. The metabolism of this barbiturate is by two pathways (Scheme 1):



Scheme 1: The two pathways of phenobarbital metabolism.

1. The main pathway is parahydroxylation of the aromatic nucleus leading to *para*-hydroxyphenobarbital (OH-PB). This compound is further glucurocongjugated in both humans and dogs /3-5/ and sometimes sulphoconjugated /3,6/.
2. The second route is β-hydroxylation of the ethyl chain, which is followed by an intra-molecular alcoholysis leading to 2-phenyl-γ-butyrolactone (LA) /7/. This minor pathway, however, becomes more important at high doses and this metabolite has been isolated in the urine of humans intoxicated by phenobarbital /8/.

We determined whether these two pathways were still observed when PB was applied to isolated rat hepatocytes.

MATERIALS AND METHODS

Materials

Phenobarbital and *p*-hydroxyphenobarbital were purchased from R.P.R.-France and Janssen-Chimica France respectively. 2-Phenyl-γ-butyrolactone was synthesized according to the procedure described in /7/. Its identity was checked by ^{1}H NMR, EI-MS and by CHN microanalysis. Its purity was checked by TLC (stationary phase: Kieselgel 60^{254} F, solvent: chloroform).

Sulphatase S 9754 type VIII, extracted from abalone entrails, and β-glucuronidase G7770 type HP 2S from *Helix pomatia* were purchased from Sigma France.

The chemicals used were high grade analytical preparations.

Animals

Female Sprague-Dawley rats weighing 200 ± 20 g were given phenobarbital as inducer. This compound was administered as a sodium salt (dose 21.9 $mg.kg^{-1}$ = $8.62.10^{-5}$ mol corresponding to PB 20 $mg.kg^{-1}$) for 11 days per os with a cannula Carrieri (60-R). The daily dose was dissolved in 1 ml water. The animals had free access to food and water.

In order to establish the role of enzyme induction, non-induced rats were used as controls; hepatocytes were then submitted to the same treatment as hepatocytes isolated from induced rats.

Hepatocyte isolation

Nine days after induction, hepatocytes were isolated from the rats according to a process summarised in Figure 1, described by Seglen /9/ and adapted by Davy *et al.* /10/.

The compositions of the various solutions indicated in Fig. 1 were the following:

Solution A (calcium-free solution): Sodium chloride (8.9 g = 0.152 mol), potassium chloride (0.5 g = $6.7.10^{-3}$ mol), N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (2.4 g = 10^{-2} mol) were dissolved in water in order to obtain 1000 ml of aqueous solution and pH was adjusted to 7.5 with sodium hydroxide (30%).

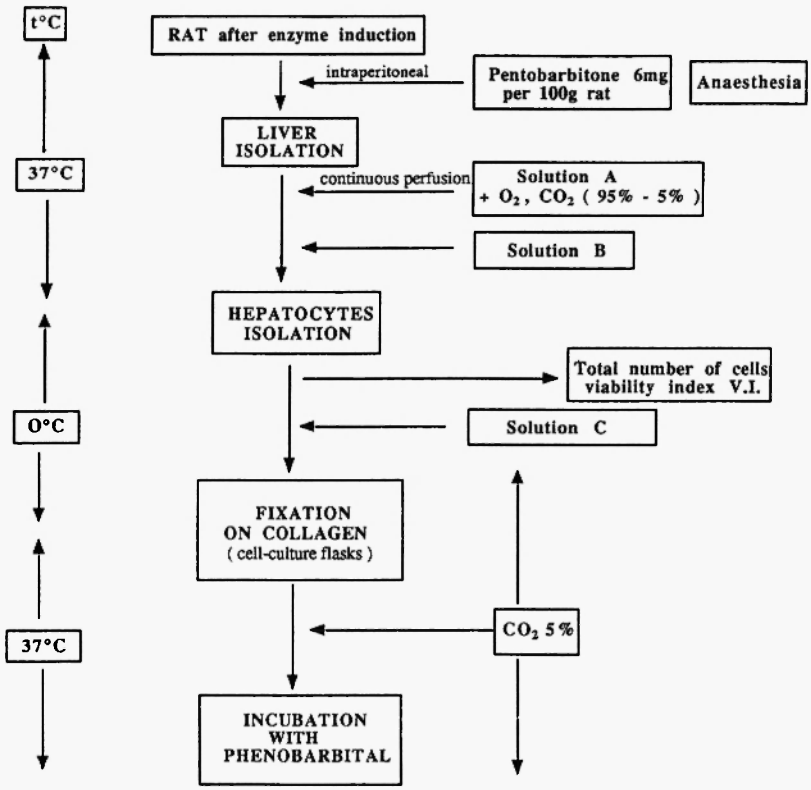


Fig. 1: Summary of hepatocyte isolation and incubation.

Solution B: To 100 ml of solution A were added 20 mg of collagenase and 0.54 ml of a 13% aqueous solution of calcium chloride.

Solution C (nutrient solution): Sodium chloride (5.6 g = $9.58.10^{-2}$ mol), potassium chloride (0.25 g = $3.35.10^{-3}$ mol), magnesium sulphate.$7H_2O$ (0.1 g = $3.78.10^{-4}$ mol), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (1.7 g = $7.13.10^{-3}$ mol), and 1 ml of a 13% aqueous solution of calcium chloride, were dissolved in 700 ml of water. pH was adjusted to 7.45 with N sodium hydroxide.

Incubation and extraction

Methodology for the identification of phase I metabolites

Hepatocytes were incubated with two different doses of PB: 2.10^{-5} mol (4.64 mg)/flask and 2.10^{-4} mol (46.4 mg)/flask for 2, 4 and 24 hours. Control hepatocytes were incubated for 2, 4 and 24 h without addition of PB. Two flasks were prepared for each dose and each incubation time. After incubation, the viable cell number was evaluated. Data concerning incubation are summarised in Table 1.

The contents of the two flasks corresponding to a similar incubation were then pooled, and pH was adjusted to 7.4 with hydrochloric acid. The medium was extracted three times with 50 ml ethyl acetate, and the aqueous phase was adjusted to pH 1 and then extracted again with 50 ml ethyl acetate. The organic phases were dried on sodium sulphate, and evaporated to dryness under partial vacuum. The residue was then studied by gas chromatography - mass spectrometry.

Methodology for the study of conjugation

After incubation of the hepatocytes with 46.4 mg (2.10^{-4} mol) of PB per flask for 24 hours, the contents of 12 flasks were pooled, and then divided into three parts.

The medium of the first lot was adjusted at pH 5 and submitted to the action of sulphatase (802 units) at 37°C for 2 hours. The medium was then extracted at pH 5 with ethyl acetate.

The second lot was adjusted at pH 3.8 and submitted to the action of β-glucuronidase (254-595 units) at 37°C for 2 hours. The medium was then extracted with ethyl acetate.

The third lot was submitted to the treatment described in the preceding section.

TABLE 1

Data concerning incubation of hepatocytes isolated from three different rats and submitted to the action of two different doses of phenobarbital: 4.64 mg or 46.4 mg

		RAT 1						RAT 2						RAT 3					
Weight		217 g						252 g						275 g					
Sex		Female						Female						Female					
Total number of isolated cells		234 10^6						519 10^6						378 10^6					
Cells V.I. (%)		92						91						89					
Number of fixed cells per flask (10^6)		13						12						14					
	Flask	1.1	1.2	1.3	1.4	1.5	1.6	2.1	2.2	2.3	2.4	2.5	2.6	3.1	3.2	3.3	3.4	3.5	3.6
Phenobarbital 4.64 mg	Time (h)	2	2	4	4	24	24	2	2	4	4	24	24	2	2	4	4	24	24
	V.I after incubation	76%	69%	66%	65%	9%	6%	76%	83%	69%	71%	5%	8%	77%	68%	68%	73%	6%	7%
	Number of cells still fixed per box (10^6)	4.8	5.76	9.24	7.92	1.1	0.9	10.16	11.15	5.5	5.04	1.0	1.1	11.76	10.80	4.56	4.56	1.1	1.0
	Flask	1.7	1.8	1.9	1.10	1.11	1.12	2.7	2.8	2.9	2.10	2.11	2.12	3.7	3.8	3.9	3.10	3.11	3.12
Phenobarbital 46.4 mg	Time (h)	2	2	4	4	24	24	2	2	4	4	24	24	2	2	4	4	24	24
	V.I after incubation	83%	79%	71%	64%	5%	7%	67%	76%	64%	65%	4%	3%	65%	73%	69%	68%	7%	7%
	Number of cells still fixed per box (10^6)	6.48	5.83	3.36	3.5	0.7	0.6	10.92	11.01	5.04	5.16	0.8	0.7	10.53	11.20	4.68	4.56	0.9	0.8

All organic phases were dried on sodium sulphate and evaporated to dryness under partial vacuum.

Sample preparation

In order to identify metabolites, residues were dissolved in 5 ml of methanol and then chromatographed. For quantitative evaluation, residues were diluted with a suitable volume of methanol. 2 ml of this solution was added to 2 ml of methanolic solution of internal standard (amobarbital [AMO]) at a concentration of 1 $mg.ml^{-1}$ ($4.42.10^{-6}$ $mol.ml^{-1}$).

Apparatus

A Hewlett Packard 5890 gas chromatograph (Palo Alto, CA, USA) equipped with a capillary column was used. The fused silica column (25 m x 0.2 mm ID x 0.11 μm film thickness) was coated with crosslinked 5% phenyl methyl silicon gum. The carrier gas was helium at an inlet pressure of 62 kPa.

For qualitative analysis, injections were realized in sphtless mode. For quantitative analysis, a Hewlett Packard 7673 A autohquid sampler, operated in fast mode for split injection (split ratio 1:12) was used in conjunction with the gas chromatograph. Before each injection, the 10 μl injection syringe was automatically rinsed out six times with 10 μl of methanol, and then twice with 10 μl of a sample solution. As a rule, 3 μl of the sample solution was injected.

A Hewlett Packard 5970 MSD mass spectrometer operated in electron impact (EI) mode was directly interfaced with the 5890 gas chromatograph by the capillary column and was used either in the full scan mode for identification or in the selected ion monitoring (SIM) mode for assay.

Preparation of calibration curves

Standard solutions of LA in methanol were prepared at 0.078, 0.156, 0.3125, 0.468, 0.625 and 0.9375 $mg.ml^{-1}$ ($4.81.10^{-7}$, $9.63.10^{-7}$, $1.93.10^{-6}$, $2.89.10^{-6}$, $3.86.10^{-6}$, $5.79.10^{-6}$ $mol.ml^{-1}$). In each solution the internal standard (AMO) was incorporated, up to a concentration of 0.5 $mg.ml^{-1}$ = $2.21.10^{-6}$ $mol.ml^{-1}$.

Standard solutions of PB in methanol were prepared at a concentration of 0.03125, 0.125, 0.25, 0.5, 0.75 and 1 $mg.ml^{-1}$

($1.35.10^{-7}$, $5.39.10^{-7}$, $1.08.10^{-6}$, $2.15.10^{-6}$, $3.23.10^{-6}$, $4.31.10^{-6}$ mol), and internal standard (AMO) was incorporated up to a concentration of 0.5 $mg.ml^{-1} = 2.21.10^{-6}$ $mol.ml^{-1}$.

Chromatographic conditions

The initial oven temperature was maintained at 110°C during 1 min, and then allowed to rise at a rate of $10°C.min^{-1}$ up to 270°C for 2 min. Injector and transfer line were maintained at a temperature of 280°C during the experiment. It was checked that no metabolite had a retention time higher than 20 min.

In the full-scan mode, the scan range was selected from m/z 50 to 350. The chromatogram (Fig. 2) obtained after injection of PB, LA, OH-PB and AMO in full scan mode showed four peaks. Corresponding mass spectra are displayed in Fig. 2, and show that the following ions should be monitored in the SIM mode: m/z 232, 204 for PB corresponding to $[M]^{+\cdot}$ = 232, $[M - C_2H_4]^{+\cdot}$ = 204; m/z 162, 117 for LA corresponding to $[M]^{+\cdot}$ = 162, $[M - CO_2 - H]^+$ = 117; m/z 141, 156 for AMO corresponding to $[M - C_5H_{10}]^{+\cdot}$ = 156, $[M - C_5H_{10} - CH_3]^+$ = 141; m/z 248, 219 for OH-PB corresponding to $[M]^{+\cdot}$ = 248, $[M - C_2H_5]^+$ = 219.

Validation of the analytical method

Validation was performed according to Caporal-Gautier *et al.* /11/. Four criteria were studied: repeatability, reproducibility, linearity and sensitivity.

For lactone and phenobarbital, repeatability was studied in solution in methanol for concentrations of 0.3125 and 0.25 $mg.ml^{-1}$ ($1.93.10^{-6}$ and $1.08.10^{6}$ $mol.ml^{-1}$) respectively. Each solution was injected six times. Reproducibility was studied in solution in methanol at the same concentrations. Solutions of both concentrations were prepared on three different days and each one was injected six times. Reproducibility was evaluated for 18 injections. Repeatability and reproducibility results were expressed by the relative standard deviation:

$$RSD\ \% = \frac{S}{\overline{X}} \times 100$$

S = standard deviation
$\overline{X}$ = mean

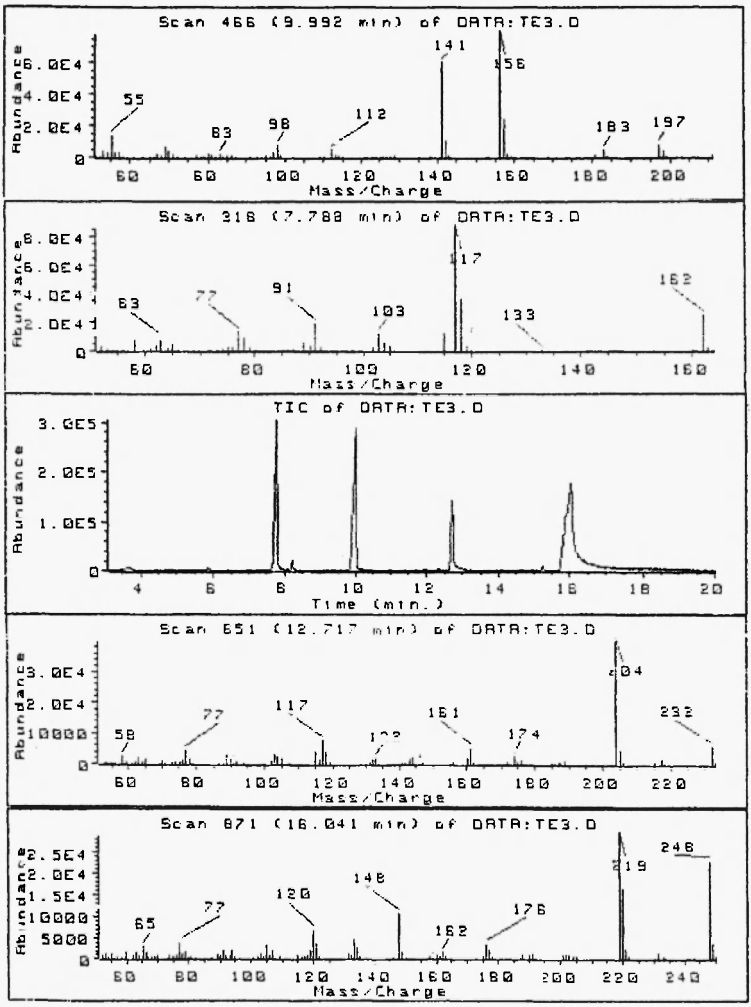


Fig. 2: Chromatogram and spectra of a splitless injection of a methanolic solution containing LA, AMO, PB, OH-PB.

Linearity was established on the standard solutions described above in 'Preparation of Calibration Curves'. Sensitivity was studied on the results of repeatability and expressed according to the following formula:

$$\frac{1.96.S}{\sqrt{n}}$$

S = standard deviation
n = number of injections

Validation results are summarised in Table 2.

TABLE 2

Results of the analytical validations

Compound	PB	LA
Repeatability (n = 6)	RSD: 1.43% (0.25 mg/ml)	RSD: 1.21% (0.3125 mg/ml)
Reproducibility (n = 18)	RSD: 2.28%	RSD: 1.69%
Linearity	y=0.429x - 0.02777, r=0.999	y=1.92x + 0.0180, r=0.999
Linearity range	0.03125 -1 mg/ml	0.078 - 0.9375 mg/ml
Sensitivity	$2.86.10^{-3}$ mg/ml	$3.02.10^{-3}$ mg/ml

RESULTS AND DISCUSSION

This study of the effects of incubation of hepatocytes with PB was performed on three rats for two different doses for each rat. The amounts of the substances found in extracts are shown in Table 3. OH-PB, which is the major metabolite in live animals, was not detected under extraction conditions that usually permit the isolation of this compound when it is present.

When hepatocytes were incubated with 4.10^{-5} mol of PB (9.28 mg), non-modified PB was the only substance found in the extracts. The total amounts found in the extracts (pH 7.4 and pH 1) were 46.55 ± 7.58% of the quantity of PB introduced into the medium after 2 h of incubation, 45.90 ± 1.13% after 4 h, and 45.36 ± 8.44% after 24 h. When hepatocytes were incubated with 4.10^{-4} mol of PB (92.8 mg), the results depended upon time. After incubation for 2 h, 37.64 ± 1.72% of intact PB was found; after incubation for 4 h, 31.18 ± 0.14% of PB was found, and LA appeared in the extracts: 10.46 ± 1.34%.

TABLE 3

Compounds identified in the extracts after incubation of hepatocytes isolated from three different rats, submitted to the action of two doses of phenobarbital: 4.64 mg or 46.4 mg.

Phenobarbital introduced (mg)	Time of incubation (h)	RAT 1			RAT 2			RAT 3		
		PB (mg)	LA (mg)	TOTAL (mg)*	PB (mg)	LA (mg)	TOTAL (mg)*	PB (mg)	LA (mg)	TOTAL (mg)*
9.28	2	2.95	0	2.95	5.3	0	5.3	4.7	0	4.7
9.28	4	4.06	0	4.06	4.42	0	4.42	4.3	0	4.3
9.28	24	4	0	4	5.67	0	5.67	2.98	0	2.98
92.8	2	36.93	0	36.93	31.77	0	31.77	36.08	0	36.08
92.8	4	29.18	8.36	41.15	28.73	8.12	40.35	28.92	5.44	36.71
92.8	24	20.91	26.09	68.27	27.78	32.28	71	25.93	29.78	69.11

PB = phenobarbital, LA = phenyl-γ-butyrolactone

*Total PB + LA is expressed in phenobarbital.

The incubation of hepatocytes with PB during 24 h led to an increase of LA formation: in the extracts, PB (30.58 ± 1.42%) was accompanied by larger amounts of LA (45.34 ± 2.77%).

In the last three experiments (Table 3), PB was extracted at both pH 7.4 and pH 1. The major part of the PB was isolated at pH 7.4. It seems that only a small part of PB was conjugated. On the other hand, LA was exclusively extracted at pH 1, while small free LA can usually be extracted at pH 7.4, at a rate of more than 95%. It is therefore likely that the LA was conjugated.

In order to identify the types of conjugates which were formed, three experiments were performed according to the methodology described above using the action of β-glucuronidase or sulphatase on the medium. The results of these experiments are presented in Table 4.

Hydrolysis of the LA conjugates, present in the medium after incubation for 24 h, took place both after enzyme catalysis by sulphatase (LA concentration in the extracts corresponded to 56.82 ± 8.53%), and in strongly acidic medium (LA concentration was then 53.99 ± 7.2%), but was not observed after the action of β-glucuronidase (LA concentration was then very low: 2.13 ± 0.82%). These results show that LA was sulphoconjugated. In order to verify that a significant amount of phenobarbital or its metabolites was not retained intracellularly, the cells were submitted to ultrasonic sound after the first extraction, and then a second extraction was performed. The results, summarised in Table 4, show that only small amounts of PB were recovered after cell lysis and that very small amounts of LA were present in only one case.

Control hepatocytes isolated from induced rats which were incubated for 2, 4, 24 h, without addition of PB did not produce any PB or any metabolite. These results demonstrate that the metabolism of PB occurs during the incubation and is not a consequence of the induction by phenobarbital.

Hepatocytes isolated from non-induced rats and which were incubated with PB under the same conditions as those used for hepatocytes isolated from induced rats did not lead to any transformation of PB. These results show that the induction is necessary to obtain a biotransformation of PB under the conditions of these experiments.

TABLE 4

Identification of the conjugation in the extracts after incubation of hepatocytes isolated from three different rats, submitted to PB 185.6 mg

		FIRST EXPERIMENT			SECOND EXPERIMENT			THIRD EXPERIMENT		
		PB (mg)	LA (mg)	Total	PB (mg)	LA (mg)	Total	PB (mg)	LA (mg)	Total
Treatment by	Medium	47.4	52.04	125	53.2	88.56	180.43	26.89	79.33	143.72
sulfatase	After cell lysis	4.72	0		0.41	0		1.78	10	
Treatment by	Medium	46.07	0	58.14	37.74	4.68	42.58	23.42	0	26.38
β glucuronidase	After cell lysis	8.36	2.59		0.14	0		1.46	1.05	
Direct extraction	Medium	51.15	0		41.81	0		24.81	0	
pH : 7.4	After cell lysis	2.99	1.37	154.12	0.58	5.33	185.19	2.08	18	154.74
Direct extraction	Medium	25.5	50.22		32.83	67.07		8.73	80.14	
pH : 1	After cell lysis	0.6	0		0.56	4.01		1.77	0	

Extracts were treated by various enzymes or extracted at various pH.
*Total PB + LA is expressed in phenobarbital.

CONCLUSION

The results obtained in these experiments using hepatocytes in order to study the biotransformation of phenobarbital were different from those observed in live animals.

Usually, PB is metabolised by two different metabolic pathways. The major route in live rats is the parahydroxylation of the phenyl nucleus (>50% of the amount of administered PB). The β-hydroxylation of the ethyl side chain is only a minor pathway (<4.5% of the amount of administered PB).

The regiospecificity of this oxidation is related to cytochrome P450 multiplicity and it is obvious that the isoenzyme responsible for β-hydroxylation is not the same as the isoenzyme responsible for para-hydroxylation.

With hepatocytes isolated after induction, the only observed biotransformation corresponded to β-hydroxylation followed by lactonization, the secondary metabolic pathway in live animals. In non-induced hepatocytes, under similar experimental conditions, no biotransformation occured.

These results show that it is not possible to use hepatocytes as an alternative method to live animals to study the metabolism of this kind of compound.

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