EFFECTS OF ENCAPSULATION OF PRIMIDONE ON ITS OXIDATIVE METABOLISM IN RATS

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SUMMARY

The aim of this study was to evaluate the influence of primidone (PRM) nanoencapsulation on its metabolism. Suspensions of PRM powder and PRM-loaded poly-ε-caprolactone nanocapsules were administered orally in the same way to rats. Primidone-loaded poly-ε-caprolactone nanocapsules were prepared according to the interfacial deposition technique. Free PRM suspensions were obtained by addition of PRM powder to a suspension of 0.212% carboxymethylcellulose CMC 12H in water. The dose was 20 mg/kg, n = 6, for each experiment. Urinary and faecal levels of PRM and of its three major metabolites, phenylethylmalonamide (PEMA), phenobarbital (PB), and *p*-hydroxyphenobarbital (*p*-HO-PB), were determined. Concentrations were evaluated by high-performance liquid chromatography (HPLC) according to a validated analytical method. After PRM nanocapsule administration, non-metabolised PRM urinary levels were increased compared to those observed after administration of a suspension of primidone powder (43.7 ± 8.8% after PRM-loaded nanocapsule and 37.7 ± 8.1% after free PRM administration). For phenylethylmalonamide, no difference was observed in urinary excretion in the two cases. For two of the oxidised metabolites, PB and *p*-HO-PB, excretion was delayed and shortened. The amount of these oxidised metabolites was lowered from 0.95% after free PRM administration to 0.25% after PRM-loaded nanocapsule adminis-

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tration. No difference was noted in non-metabolised primidone excretion in faeces. These results suggest that primidone-loaded nanocapsules could be used as a vehicle for oral primidone administration in order to minimise the phenobarbital metabolic pathway.

KEY WORDS

nanocapsules, primidone metabolism, phenylethylmalonamide, phenobarbital, *p*-hydroxyphenobarbital

INTRODUCTION

The increase of teratogenesis in treated epileptic mothers has been attributed to antiepileptic drugs rather than to the epilepsy itself /1/. All major antiepileptic drugs (AEDs) have been associated with teratogenesis /2/. Primidone (PRM) was one of the last prescribed drugs in the treatment of pregnant epileptic women because of its low sedative effects compared to all other major antiepileptic drugs.

Nevertheless, in both animals /3/ and man /4/, primidone is metabolised, mainly in the liver, into two major pharmacologically active substances, phenylethylmalonamide (PEMA) and phenobarbital (PB) /5,6/. This specific combination (PRM + PB) produced a higher incidence of congenital malformations compared to both PRM alone and PB alone in humans and animals /7-9/. Offspring of rats exposed to PRM showed significant neurobehavioral teratology resulting in embryological malformation. Because PRM and PEMA were reported to carry less neurotoxicity than PB, the side effects affecting the limbic system were attributed primarily to the biotransformation of PB *in vivo,* and functional teratology is certainly the result of PRM metabolism to PB /10/. In addition, Nagaki *et al.* showed recently that concentration equilibration between the blood and cerebrospinal fluid (CSF) compartments was very rapid, especially for PB /11/. This suggests that PB's action on the central nervous system could be fast. It was thus necessary to find a way to control and if possible to avoid this oxidative metabolism.

Colloidal drug carriers, such as nanoparticles, are used as drug delivery systems /12/ and can enhance the efficacy of drugs, reduce their toxicity /13/, modify their metabolism and play a role in the

absorption /14/ and elimination /15/ of drugs. Nanocapsules improve passage through biological barriers, avoid degradation by acidic medium in the stomach, and protect against enzymatic biodegradation during the distribution phase, because the encapsulated drug is not in contact with the external biological medium. It may be hypothesised that the administration of PRM-loaded nanocapsules could minimise the formation of phenobarbital.

The aim of this study was to evaluate the role of nanoencapsulation on primidone metabolism and to study whether this drug delivery system is able to minimise the oxidative metabolic pathway. PRM powder suspension and PRM-loaded nanoparticles were administered separately to different lots of six rats. This species was chosen because these experiments cannot be carried out in humans for ethical reasons, and because rats are described as more efficient than mice or humans at producing phenobarbital and phenylethylmalonamide from primidone /16/.

Poly-ε-caprolactone, which has proved to be a suitable polymer for the inclusion of drugs with physicochemical properties similar to those of primidone, such as phenobarbital /17/ or phenylbutazone /18/, was used for the preparation of primidone-loaded nanocapsules by an interfacial deposition technique. The nanoparticle suspensions and their *in vitro* release characteristics have been previously assessed /19/. Standard concentrations of primidone and of its three metabolites were determined in urine and faeces using the analytical method previously described /20/.

MATERIALS AND METHODS

Chemicals

The polymer poly-ε-caprolactone (PECL) (MW: 40,000) and benzyl alcohol were purchased from Aldrich-Sigma (France). Synperonic® PE/F68 (non-ionic polyoxyethylene polyoxypropylene copolymer) was provided by I.C.I. (Belgium). All other reagents and solvents were of analytical grade.

Preparation of PECL nanocapsule suspensions

PECL nanocapsules were prepared according to the technique of interfacial deposition of a preformed polymer with slight modifications /21/. PECL (1.5 g) was dissolved in acetone (250 ml). A solution of primidone (60 mg) in benzyl alcohol (12 ml) was then added. This organic phase was poured, under moderate magnetic stirring, into water (500 ml) containing a surfactant, Synperonic® PE/F68 (1 g). The aqueous phase immediately became turbid due to the precipitation of the polymer. Acetone was evaporated under reduced pressure and the colloidal aqueous suspension was concentrated under vacuum to the desired final volume (15 ml).

Physicochemical characterization of nanocapsules

The size of nanocapsules was estimated by photon correlation spectroscopy (Coulter® N4MD submicron particle analyzer, Coultronics, Margency, France) and by scanning electron microscopy (JSM 5400 Scanning Electron Microscope, JEOL-Europe). Samples of nanocapsules were stabilised on a carbon patch with liquid nitrogen at -196°C and were coated with gold for 15 min using an ion-sputter module in a high vacuum evaporator followed by examination at 2.9 kV.

Primidone encapsulation efficiency was assessed for every batch of nanocapsules according to the high-performance liquid chromatography (HPLC) method previously described /19/. Briefly, 1 ml of the suspension was dissolved in acetonitrile (20 ml). After addition of methanol (25 ml), the polymer was eliminated by filtration and the solvents were evaporated under reduced pressure to dryness. The white residue obtained was dissolved in 80 ml of methanol and an aliquot (20 µl) was injected into the HPLC system. Free drug was measured in the clear supernatant following separation of nanocapsules from aqueous medium by centrifugation of 5 ml colloidal suspension at 19,000 rpm for 30 min (Beckman J2-21, USA). 20 µl of this supernatant were analysed directly by HPLC. The drug encapsulation efficiency was calculated as the difference between the total amount of PRM in the nanocapsule suspension and the free amount of PRM measured in the supernatant.

Chromatographic analysis was performed on a Beckman system at 227 nm. This HPLC system consisted of an isocratic pump Beckman

Model 110 A (Beckman, San Ramon, CA, USA), a sample injector with 20-µl loop (Rheodyne, Cotati, CA, USA) and a variable wavelength UV detector (Beckman Model 166).

The data recording system consisted of an IBM personal computer PS/2 Model 555X with system Gold software (Beckman). Separation was achieved using an ODS ultrasphere column C_{18} (Beckman, Germany) (5 µm; 150 x 4.6 mm) at 40°C. Samples were eluted with acetonitrile:methanol:potassium phosphate 0.01 M (pH 7) (50:30:110; v:v:v) at a constant flow rate of 0.4 ml/min.

A calibration curve was obtained from a methanolic PRM working solution over the range 25-200 µg/ml. The curve was linear and passed through the origin (r = 0.999). The validation results were obtained for 3 injections per concentration, and 6 concentrations were studied. The repeatability and reproductibility were 0.40% and 0.45%, respectively. The experimental quantification limit was defined as the lowest concentration of PRM in a PRM metabolic sample which gives rise to a signal able to be quantified by the integrator (signal-to-noise ratio = 10) and was found to be 2 µl/ml. The detection limit of PRM was approx. 0.5 µg/ml (signal-to-noise ratio = 3).

In vitro drug release studies

In vitro release profiles of primidone from nanocapsules were studied at pH 1.25 and pH 7.4 and compared to those obtained with a primidone oily solution in the same conditions. All experiments were performed under "sink" conditions in order to exclude the influence of already released drug. Samples of nanocapsule suspension (12 ml) were introduced into 1 l beakers of a dissolution apparatus (Sotax) containing 363 ml of simulated gastric fluid and intestinal fluid (USP XXIII) without enzyme. 200 µl of the release medium maintained at 37°C under constant stirring (70 rpm) were collected at appropriate time intervals (0, 15′, 30′, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h). A clear supernatant was obtained by a combined ultrafiltration centrifugation technique (11000 *g* for 10 min) using an Ultrafree® MC Unit (30000 MW, Millipore S.A., St Quentin-Yvelines, France). *In vitro* release kinetic measurement of PRM was determined using the HPLC method described elsewhere /19/.

Preparation of primidone suspensions

Primidone (1.197 g) was suspended in a solution of 0.212% (w/v) carboxymethylcellulose CMC 12H in 300 ml of water. The final suspension concentration used in the *in vivo* experiment is presented in Table 1. PRM concentrations were determined according to the amount of PRM powder/volume of suspension.

***In vivo* studies**

Procedures and urine sample collection

Two groups of six female Sprague-Dawley rats (body weight ~350 g) received a daily dose of 20 mg/kg of primidone orally via cannula Carrieri (60-R).

Primidone incorporated in poly-ε-caprolactone nanocapsules was administered to one group of rats intragastrically by force-feeding for five days. Simultaneously, the other group of rats received by force-feeding for five days a suspension of PRM powder (4 mg/ml) prepared with 0.212 % (w/v) carboxymethylcellulose CMC 12H in water.

The rats were housed individually in metallic cages (Pajon®, France) suitable for separate collection of urine and faeces. Urine and faeces samples were collected each day at 09.00 a.m. during the five days of PRM administration and for five days after the administration period. All samples were kept frozen at -20°C until analysed. All animals had free access to pelletted rat chow and tap water. The metabolic cages were washed with a proper amount of water every 24 h and were in a room maintained under a 12 h:12 h light:dark cycle (light on from 8.00 to 20.00 h) and at 22-24°C with constant relative humidity of 60% for the duration of the study.

Analytical methods

The concentrations of primidone and of its three metabolites, PEMA, PB, *p*-HO-PB, were analysed by HPLC. Centrifuged rat urine (5 ml) was transferred into a glass test tube and HCl 12 M (0.5 ml) was added. After acid hydrolysis at 100°C for 2 h at pH 1.5, the mixture was adjusted to pH 4.0 with NaOH 12 M (0.45 ml) and to a volume of 50 ml with distilled water. The resulting solution (10 ml) was extracted from rat urine by fixation on an activated C_8 Bond Elut

LCR cartridge, followed by elution with ethyl acetate:hexane solvent (in different proportions /20/), with good clean-up. The solvent was evaporated to dryness under reduced pressure. The residue was dissolved in 0.5 ml of mobile phase and an aliquot (20 µl) was injected into the HPLC system.

The extracts were chromatographed on a Nucleosil 100-5µm C_{18} (250 × 4.6 mm) reversed phase column (Macherey-Nagel, Düren, Germany) by isocratic elution at 40°C, with UV detection at 227 nm. The HPLC system used for the animal studies was the same Beckman HPLC system previously described for the physicochemical characterization of the PRM-nanocapsule suspension. It consisted of an isocratic pump Beckman Model 110 A (Beckman, San Ramon, CA, USA), a sample injector with 20-µl loop (Rheodyne, Cotati, CA, USA) and a variable wavelength UV detector (Beckman Model 166). The data recording system consisted of an IBM personal computer PS/2 Model 555X with system Gold software (Beckman). The mobile phase was 0.01 M potassium phosphate buffer (pH 4.0):methanol: acetonitrile (270:30:30, v:v:v) circulating at a flow rate of 1.0 ml/min. Validation of this method was recently performed in our laboratory /20/. The experimental quantification limits were defined as the lowest concentrations of PEMA, *p*-HO-PB, PRM and PB in a spiked urine sample which give rise to a signal able to be quantified by the integrator (signal-to-noise ratio = 10) and were found to be 1.5 µl/ml for PEMA and *p*-HO-PB, and 2 µl/ml for PRM and PB. The detection limits of PEMA, *p*-HO-PB, PRM and PB were approx. 0.5 µg/ml (signal-to-noise ratio = 3). The accuracies of this procedure were 64% for PEMA, 67% for *p*-HO-PB, and 98% for PRM and PB, respectively.

Urine standard curves were prepared at concentrations ranges as follows: for PRM: 37.4, 74.8, 149.6, 224.5 and 299.3 µg/ml; for PB: 26.4, 52.8, 105.6, 158.4 and 211.2 µg/ml; for *p*-HO-PB: 12.5, 25.0, 50.0, 75.1 and 100.2 µg/ml; for PEMA: 12.1, 24.2, 48.5, 72.7 and 97.0 µg/ml, and gave the regression line slope equations:

(PRM)	$y = -0.61722 + 7.4063\ x$	$r^2 = 0.999$
(PB)	$y = -0.00332 + 4.9579\ x$	$r^2 = 0.999$
(PEMA)	$y = -0.00212 + 9.6232\ x$	$r^2 = 0.999$
(*p*-HO-PB)	$y = -0.00605 + 5.6494\ x$	$r^2 = 0.999$

where y and x represent area and concentration, respectively.

For each compound, five concentrations were studied (n = 3 for each concentration). Good linearities were observed and the repeatability and reproductibility were in the range of 3.2-10.3% and 6.1-14.3%, respectively.

After acidic hydrolysis and extraction with aqueous solution at 100°C for 2 h at pH 1.5, a similar procedure was used for faeces.

Urinary excretion half-life values of each compound were determined with time corresponding to 50% of the total excretion amount of each product.

Statistical analysis

Daily excretion concentrations of the four compounds after free primidone oral administration and primidone-loaded nanocapsule oral administration were compared using analysis of means (Student's t-test). Differences of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

In order to test the hypothesis that the administration of PRM-loaded nanocapsules could minimise the biotransformation of PB, PRM-loaded nanocapsules were prepared by interfacial deposition of poly-ε-caprolactone. The mean encapsulation efficiency observed was 74.25%, and 25.75% of PRM was free in the colloidal PRM-loaded nanocapsules suspension.

The concentration and the encapsulation efficiencies of the various batches of primidone-loaded nanocapsules, administered to rats, are shown in Table 1. The size distribution of the nanocapsules showed a unimodal profile with a mean diameter of 291 ± 30 nm (n = 7). These results were confirmed by SEM photography (Fig. 1).

In vitro drug release studies showed that 100% of the primidone in oily solution diffused out within 10 h. In contrast, only 80% at pH 1.25 and 70% at pH 7.4 of the encapsulated drug was released during the same period. Despite the important burst effect, a significant difference was observed from the oily control solution after 2 h ($p < 0.01$). With nanocapsules, primidone was released to a lesser extent in comparison with the control. The release rate was slower than when free drug was used /19/. These results show that these nanocapsules are suitable for administration to rats.

TABLE 1

Physicochemical characteristics of primidone (PRM) suspension and PRM-loaded nanocapsules used for the *in vivo* experiments

	Size (nm) (mean ± SD)	**PRM conc.** (mg/ml) (mean + SD)	**Encapsulation efficiency** (%)	**Free PRM** (%)
PRM	–	4.00	–	100
PRM-loaded nanocapsules	299 ± 48	3.96 ± 0.7	74.3	25.7

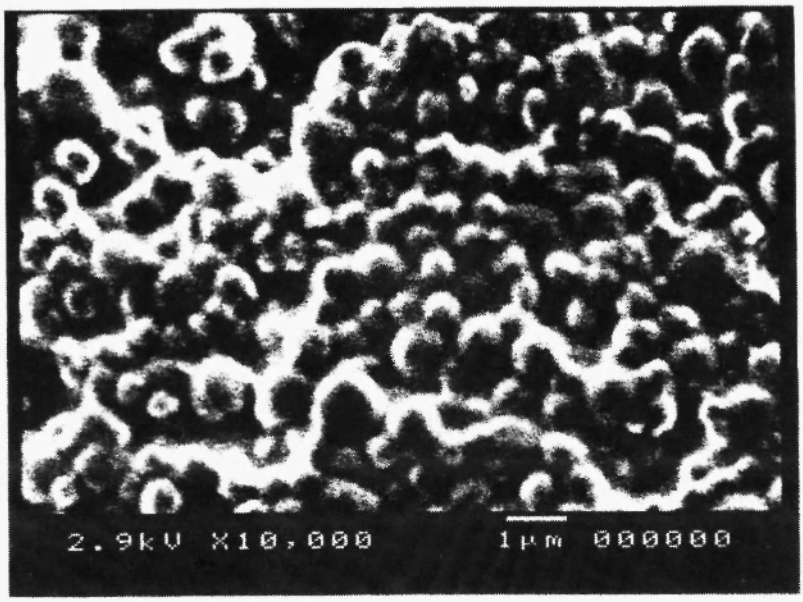

Fig. 1: Scanning electron micrograph of primidone-loaded nanocapsules.

After PRM administration, we observed for the first time that the total amounts of compounds recovered in urine and faeces were 66.8 ± 16.8% and 63.9 ± 20.4% for nanocapsules and for free primidone administration, respectively (Table 2). These results obtained for free primidone were in agreement with previous studies /22/.

Changes in the amounts of metabolites were observed. In the case of nanocapsule administration, the amount of excreted PEMA (8.83 ± 2.42%) was not significantly different from the amount excreted after free primidone administration (9.03 ± 3.64%) (Table 3). However, for PB and *p*-HO-PB, the differences were significant: 0.25% of these oxidised metabolites were obtained in the case of nanocapsule administration compared to 0.94% in the case of free primidone administration. Unchanged primidone excretion was not significantly modified in either urine or faeces (Table 3).

These results show that nanoparticle administration lowers the amount of PRM entering the metabolic pathway leading to the group formed by PB and its own metabolite *p*-HO-PB. Due to the fact that only 75% of primidone was effectively encapsulated, 25% of the drug remained free in suspension. The theoretical quantities of metabolites produced by this 25% of non-entrapped drug are 2.27% for PEMA, 0.13% for PB and 0.11% for *p*-HO-PB (Table 4). The amounts of PB and *p*-HO-PB actually observed were 0.17% and 0.08% for PB and *p*-HO-PB, respectively (Table 4). No significant difference was noted between the theoretical quantities of metabolites produced by 25% of unentrapped primidone and those actually observed. A significant difference was found for PRM and PEMA between the theoretical values and the observed values (Table 4). Thus, one could attribute the formation of the metabolites of the PB group to the degradation of the remaining amounts of free primidone in the nanocapsule suspension and consider that the encapsulation of the PRM protected the drug from oxidative metabolism.

These results are in agreement with those observed by Berrabah *et al.* /17/ who showed that encapsulated phenobarbitone could be protected against oxidative metabolism. Administration of phenobarbitone-loaded nanocapsules to rats suggested that the oxidative metabolism of phenobarbitone could be minimised. Indeed, the quantities of its major oxidative metabolite compound (*p*-HO-PB) were lowered after phenobarbitone-loaded nanocapsule administration compared to those determined after phenobarbitone suspension

TABLE 2

Mean and individual urinary and faecal metabolites excretion (%) following oral administration of primidone (PRM) during 5 days (20 mg/kg/day): (I) primidone suspension and (II) primidone-loaded nanocapsules

Administration I: Primidone suspension				Administration II: Primidone-loaded nanocapsules			
Rat	Total compounds recovered %			Rat	Total compounds recovered %		
	urine	faeces *	urine + faeces		urine	faeces *	urine + faeces
A	39.0	23.0	62.0	A'	39.0	35.3	74.3
B	50.1	46.0	96.1	B'	33.7	10.5	44.2
C	27.8	15.4	43.2	C'	36.7	10.5	47.2
D	36.8	30.5	67.3	D'	44.6	37.3	81.9
E	42.1	31.2	73.3	E'	52.9	18.8	71.7
F	30.5	10.8	41.3	F'	55.2	26.1	81.3
Mean (n=6)	37.7	26.1	63.9	Mean (n=6)	43.7	23.1	66.8
SD	8.1	12.6	20.4	SD	8.8	11.8	16.8

*In faeces, only PRM was excreted and no metabolite was detected.

TABLE 3

The amounts obtained in urine (PRM, PEMA, PB, *p*-HO-PB) were calculated in molar percentage of administrated primidone during 5 days (20 mg/kg/day): (I) primidone suspension and (II) primidone-loaded nanocapsules

	Administration I: Primidone suspension				
Rats	**PEMA**	***p*-HO PB**	**PRM**	**PB**	**Cumulative eliminated amounts**
A	9.06	0	29.94	0	39.0
B	13.45	0.82	35.14	0.70	50.1
C	7.26	0.62	19.57	0.39	27.8
D	7.04	0.68	28.19	0.86	36.8
E	7.38	0	34.22	0.52	42.1
F	8.65	0.52	20.75	0.53	30.5
Mean (n=6)	8.83	0.44	27.97	0.50	37.7
SD	2.42	0.35	6.58	0.29	8.06

Administration II: Primidone-loaded nanocapsules					
Rats	**PEMA**	***p*-HO PB**	**PRM**	**PB**	**Cumulative eliminated amounts**
A'	5.81	0.15	32.73	0.33	39.0
B'	8.34	0.09	25.00	0.25	33.7
C'	9.33	0	27.23	0.19	36.7
D'	6.49	0.08	37.98	0.07	44.6
E'	8.29	0.19	44.26	0.22	52.9
F'	15.91	0	39.34	0	55.2
Mean (n=6)	9.03	0.08	34.42	0.17	43.7
SD	3.61	0.07	7.44	0.12	8.81

PRM = primidone; PEMA = phenylethylmalonamide; PB = phenobarbital; *p*-HO-PB = *p*-hydroxyphenobarbital.

TABLE 4

Comparison between primidone (PRM) metabolism after oral administration of PRM-loaded nanocapsules with theoretical amounts of metabolites assessed after oral administration of free PRM

	Administration I: PRM suspension (%)	Theoretical metabolites of 25% of PRM non entrapped (%)	Administration II: PRM-loaded nanocapsules (%)
PRM	27.97	7.71	34.42
PEMA	8.83	2.27	9.03
PB	0.50	**0.13**	**0.17**
***p*-HO-PB**	0.44	**0.11**	**0.08**

See Table 3 for abbreviations.

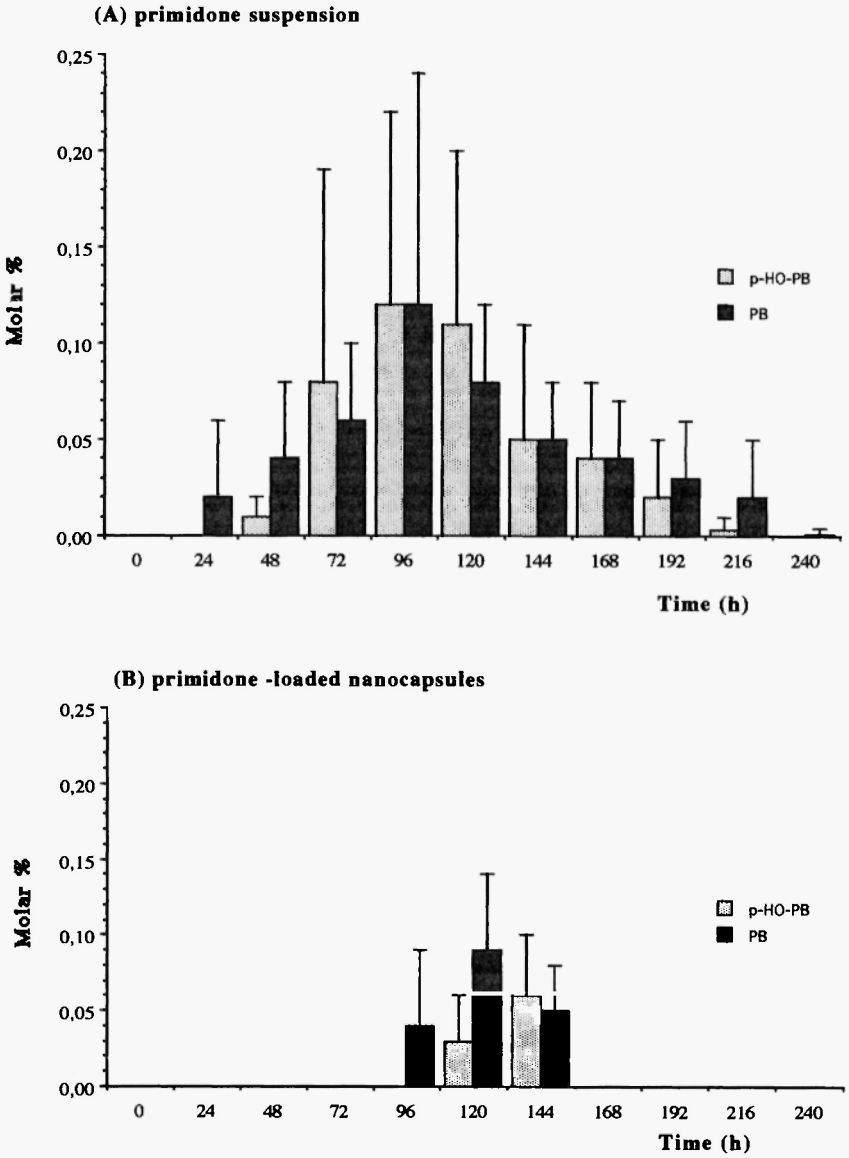

Fig. 2: Urinary excretion of phenobarbital (PB) and *p*-hydroxyphenobarbital (p-HO-PB) after administration of (**A**) primidone suspension and (**B**) primidone-loaded nanocapsules in the rat.

administration to rats. Berrabah *et al.* showed in rats that 10% of phenobarbitone was unchanged and 58.8% of *p*-HO-PB was excreted after phenobarbitone suspension administration, and 15.5% of phenobarbitone was unchanged and only 34.5% of *p*-HO-PB was excreted after phenobarbitone-loaded nanocapsule administration.

No significant difference was observed between PRM and PEMA kinetics of excretion after oral administration of free primidone and primidone-loaded nanocapsules. In the case of PB and *p*-HO-PB, the kinetics of excretion were significantly affected by the administration of primidone-loaded nanocapsules (Fig. 2). Phenobarbital excretion started 96 h after nanoparticle administration instead of 24 h for the suspension. *p*-HO-PB excretion started 120 h after administration instead of 48 h, and *p*-HO-PB urinary excretion half-life was significantly increased (130 h instead of 100 h) (Student's t-test, $p < 0.01$) (Table 5).

TABLE 5

Mean urinary excretion half-life (hours) of primidone and its three major metabolites after administration of (I) primidone suspension and (II) primidone-loaded nanocapsules in rats (n=6)

	I: Primidone suspension (mean ± SD)	**II: Encapsulated primidone** (mean ± SD)
PRM	72 ± 15	62 ± 4.0
PEMA	79 ± 13.0	80 ± 5.0
PB	109 ± 18.0	111 ± 13.0
***p*-HO-PB**	100 ± 16.0 *	130 ± 7.0 *

See Table 3 for abbreviations.
* $p < 0.01$.

CONCLUSION

This study demonstrated the influence of primidone encapsulation on its metabolism. These results confirm our hypothesis that the administration, in rats, of a suspension of primidone-loaded nanocapsules could minimise the formation of the oxidised metabolites which are responsible for the toxicity of this antiepileptic drug in pregnant women. It is important to assess the teratogenic effect of such drug carriers and to investigate further whether encapsulation could circumvent this deleterious effect of PRM administration.

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