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Effects of horminone on liver mixed function mono-oxygenases and glutathione enzyme activities of Wistar Rat

Rui Ferreira [a,*], Fátima Candeias [a], Fátima Simões [b], José Nascimento [b], J. Cruz Morais [a]

[a] *Department of Chemistry, University of Évora, Apartado 94, 7001 Évora Codex, Portugal*
[b] *Faculty of Pharmacy, University of Lisbon, Avenida das Forças Armadas, 1600 Lisbon, Portugal*

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Abstract

The present study reports on the effects of horminone on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, on hepatic cytochrome P450 (P450) and cytochrome b_5 (cyt b_5) contents and on the activities of NADPH-cytochrome P450 reductase (NR), mixed function mono-oxygenases (MFO), glutathione-*S*-transferase (GST) and glutathione reductase (GR) of Wistar male rat. Horminone is a diterpenoid quinone (7,12-dihydroxyabiet-8,12-diene-11,14-dione) present in several species of the Labiatae family and used as medicinal plants in folk medicine. In this study, horminone was administered by the intraperitoneal route (i.p.) at a concentration of 1 or 10 mg/kg to each group of six mice, using water as a vehicle. On the one hand, results showed that horminone increased serum ALT and AST levels and cyt b_5 content and induced the activities of ethylmorphine *N*-demethylase (EMD). On the other hand, horminone decreased P450 content and inhibited the activities of 7-ethoxyresorufin *O*-deethylase (ERD), 7-ethoxycoumarin *O*-deethylase (ECD), aniline 4-hydroxylase (AH) and NR. Based on these results, the possibility of toxic effects occurring after administration of plant extracts containing horminone must be considered. © 1997 Elsevier Science Ireland Ltd.

Keywords: Horminone; Drug metabolizing enzymes; Liver

1. Introduction

Horminone is a diterpenoid quinone (7,12-dihydroxyabiet-8,12-diene-11,14-dione) found in several species of the Labiatae (Jonathan et al., 1989; De la Torre et al., 1992). Species from this family have been used for medicinal purposes in folk medicine, in gastrointestinal disorders, for treating respiratory problems, in bacterial diseases and for inflammation treatment (Cousins, 1994).

* Corresponding author.

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Several species of *Plectranthus* have been reported to have an anti-inflammatory action (Muzaffer et al., 1990), antimicrobial effect (Vera et al., 1993; Dellar et al., 1996) activities on gastrointestinal and respiratory tracts (Kubo et al., 1984; Mehrotra et al., 1989; Tandon et al., 1991; Githinji and Kokwaro, 1993) and toxic effects (Pages et al., 1991). However, nothing was reported on the pharmacological activities of *Plectranthus hereroensis* Engl. (Labiatae). The roots of this plant were used as an infusion by indigenous people in the Northern part of Mozambique for the treatment of 'liver disorders'. In a preliminary study, we have observed that adult male Wistar rats fed ad libitum with an infusion of *P. hereroensis* roots (10 g powder/200 ml water) showed an 10% increase in liver weight, a 40% increase of serum aspartate aminotransferase (AST) level and a 50% increase of serum alanine aminotransferase (ALT) level, compared with controls fed with pure water (unpublished results). These results suggesting a possible cellular liver damage induced by this plant extract (Cornelius, 1980; Anand et al., 1992).

In a previous study, horminone was isolated from the roots of *Plectranthus hereroensis* Engl. (Labiatae) and was analysed for antimicrobial activity by Batista et al. (1994). The antimicrobial and cytotoxic activities of this compound had already been reported by Goijman et al. (1985) and Jonathan et al. (1989).

The molecule of this compound shows two hydroxyl and one *p*-benzoquinone group (Fig. 1) (Jonathan et al., 1989; Batista et al., 1994).

A number of secondary alcohols and phenols undergo cytochrome P450 and NADPH-cytochrome P450 reductase catalyzed oxidations with formation of ketone and quinones (Koymans et al., 1989; Hoffman et al., 1990; Roy et al., 1992).

Quinones represent an important group of natural compounds of significance in biology and toxicity (O'Brien, 1991; Monks et al., 1992). The ability of these compounds to form covalent adducts with endogenous thiol groups can be an important way of quinones detoxification by conjugation with glutathione, but several quinones are toxic products due to their ability to form covalent adducts with macromolecules (Flowers-Geary et al., 1993). The cytotoxic and antitumor activity of quinones require reductive bioactivation of these compounds by a one-electron mechanism to the corresponding semiquinone free radicals and these reactions are mediated by several enzymes, including P450 (Vromans et al., 1990; Goeptar et al., 1992), particularly the CYP2B1 (Goeptar et al., 1993), NADPH-cytochrome P450 reductase (NR) (Bartoszek and Wolf, 1992; Cummings et al., 1992; Butler and Hoey, 1993) and NADH-cytochrome b_5 reductase (Ncb_5R) (Powis, 1987).

Because P450 and the enzymes of glutathione cycle play an important role in drug metabolism and quinones are related with these enzymatic systems, it is of great interest to know if horminone affects the activity of those enzymes and may account for the decrease of their activity in drug metabolization. In this study, we have evaluated the effects of horminone on AST and ALT serum levels, on hepatic P450 and cyt b_5 contents, on NR, aniline 4-hydroxylase (AH), 7-ethoxycoumarin *O*-deethylase (ECD), 7-ethoxyresorufin *O*-deethylase (ERD) and ethylmorphine *N*-demethylase (EMD) activities and the effects on glutathione-*S*-transferase (GST) and glutathione reductase (GR) activities of Wistar male rat, in order to determine if horminone accounts for the in vivo rat liver damage produced by *P.*

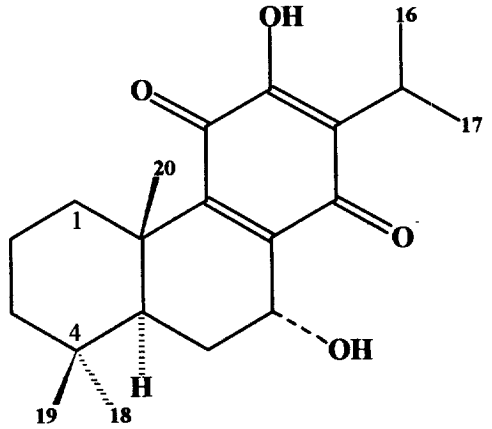

Fig. 1. Horminone, the 7,12-dihydroxyabiet-8,12-diene-11, 14-dione.

hereroensis roots infusion and if this compound affects the liver drug metabolizing enzyme activities.

2. Materials and methods

2.1. Drug source and chemicals

Horminone was isolated from a ketonic extract of *P. hereroensis* Engl. (Labiatae) roots. Specimens of this plant were produced and cultivated in the garden of Faculty of Pharmacy of Lisbon (FPL) from authentic seeds of *P. hereroensis*. The plant material was authenticated by Dr Belo Correia and a voucher specimen was collected by Dr Alexandra and deposited in the Herbarium of Botanic Institute of the University of Lisbon with the number 160382. Hominone was identified by Fátima Simões of FPL by RMN spectrum. NADPH and cytochrome *c* were purchased from Sigma, St Louis, Mo. BSA and other reagents were purchased from E. Merck, Darmstadt, Germany.

2.2. Horminone preparation and identification

An acetone extract (4 g) of dried and powdered roots of *P. hereroensis* (100g) was subjected to column chromatography using Si gel, Merck no. 7734, deactivated with 15% H_2O, w/v, 100 g. Elution with petroleum ether-EtOAc (9:1) gave impure horminone (102 mg). Final TLC purification yielded pure horminone (12 mg). ^1H NMR spectrum was measured on a Varian XL-300 instrument. Data obtained from this measurement were identical to those previously reported by Batista et al. (1994).

2.3. Animals

The experiments were performed with male Wistar rats (weight range 180–200 g) obtained from the Animalarium of Gulbenkian Institute of Science in Oeiras, Portugal. The animals were housed in standard cages placed in a controlled temperature room (25°C) on a 12 h light-dark cycle and allowed free access to rat chow and tap water ad libitum 24 h prior to the onset of the assay. Horminone was dissolved in distilled water and i.p. administered in single doses of 1 or 10 mg/kg to each group of six mice. The control group received i.p. destilled water. The animals were sacrificed at 12 h of assay by decapitation and exsanguinated. The liver was immediately removed and perfused with ice cold 0.154 M KCl, 50 mM Tris–HCl and weighted.

2.4. Serum analysis

The serum was prepared by blood centrifugation at $10\,000 \times g$ for 3 min, at 2–4°C, in a high speed refrigerated centrifuge Hermle Z 382K. The AST and ALT levels were determined using Sclavo kits for in vitro diagnostic use.

2.5. Enzymatic assays

The following procedures were carried out at 0–4°C. Liver microsomes were prepared from a small amount of the organs (≈ 3 g). Each tissue was homogenized with two volumes of ice-cold 0.154 M KCl, 50 mM Tris–HCl buffer pH 7.4, at 5.5 r.p.m., six impulses with homogeneizer Potter type, TRI Instruments, Model K41. Homogenates were centrifuged at $10\,000 \times g$ for 20 min, at 2–4°C, in a high speed refrigerated centrifuge Hermle Z 382K. Supernatants were removed and the pellets were homogenized again in adequate volume of the solution and recentrifuged under the above conditions. Each supernatant was combined with the corresponding one from the first centrifugation. Microsomal fraction was precipitated by centrifuging the post-mitochondrial supernatant at $105\,000 \times g$ for 60 min in an ultracentrifuge Sorvall Ultra 80 and Combi Plus. The supernatant (cytosolic fraction) was frozen in liquid nitrogen. Microsomes were resuspended in ice cold 0.154 M KCl, 50 mM Tris–HCl and subsequently sedimented at $105\,000 \times g$ for 60 min as described in Lake (1987). The washed microsomes were resuspended in ice cold 0.154 M KCl, 50 mM Tris–HCl buffer pH 7.4 solution and used to evaluate P450 and cyt b_5 contents (Omura and Sato, 1964) and the activity of NR, AH, ECD (Lake, 1987) and EMD (Nash, 1953).

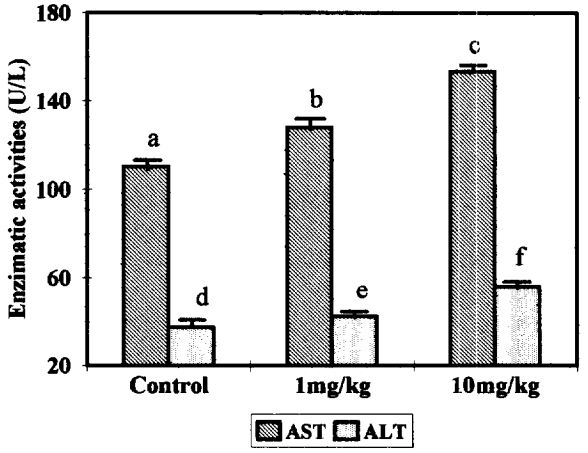

Fig. 2. Acute effect of horminone on aspartate aminotransferase and alanine amino transferase serum levels. Values are expressed as mean ± S.D. from six animals. Groups bearing different letters are significantly different ($P < 0.05$).

Cytosolic fraction was used to estimate GST (Habig et al., 1974) and GR (Bergmeyer et al., 1987).

Protein concentration was determined by the method of Lowry et al. (1951).

All spectrophotometric assays were carried out in a double-beam spectrophotometer Hitachi U2000 with a thermostat cell compartment and a Grant W6 water circulating thermostatic bath and all fluorimetric assays were carried out in a spectrofluoro- photometer Shimadzu RF-5001PC. All enzymatic assays were performed in triplicate.

2.6. Statistical analysis

Data were expressed as means ± S.D. of six animals. Significance was calculated using ANOVA-one way test, where $P < 0.05$ was taken as significant (Sokal and Rohlf, 1981).

3. Results

3.1. Alanine aminotransferase and aspartate aminotransferase

Results in Fig. 2. showed that horminone administered in single doses of 1 and 10 mg/kg increased significantly the serum level ALT to 42.83 ± 2.72 (16%) and 55.80 ± 2.43 (51%) and AST serum level to 127.74 ± 4.02 (16%) and 153.66 ± 2.79 (40%), respectively, compared with controls (37.04 ± 2.78 and 110.17 ± 2.46) and this effect was dose-dependent.

3.2. Cytochrome P450 and cytochrome b_5 contents

The hepatic content of cytochrome P450 decreased significantly with the dose of 10 mg/kg of horminone to 0.286 ± 0.012 (16%) compared with control (0.339 ± 0.014). But the decrease to 0.326 ± 0.009 (4%) was not significant with the dose of 1 mg/kg.

Both doses significantly increased the cyt b_5 content to 0.117 ± 0.005 (7%) and 0.120 ± 0.002 (10%), respectively, compared with control (0.109 ± 0.003), but without a significant difference between the cyt b_5 content produced by each one of these doses (see Fig. 3).

3.3. NADPH cytochrome P450 reductase

Hepatic NR activity decreased significantly with the doses of 1 and 10 mg/kg of horminone to 111.11 ± 6.28 (26%) and 118.45 ± 6.69 (21%), respectively, compared with control (149.45 ± 7.84). There was not a significant difference in enzyme activity of the different doses (see Fig. 4).

3.4. 7-Aniline 4-hydroxylase, 7-ethoxycoumarin O-deethylase, ethoxyresorufin O-deethylase and ethylmorphine N-demethylase activities

Results in Fig. 5 showed that single doses of 1 and 10 mg/kg of horminone produced a significant decrease of the liver activity of AH to 0.716 ± 0.025 (14%) and 0.610 ± 0.034 (27%), ECD to 0.352 ± 0.028 (34%) and 0.321 ± 0.042 (40%) and ERD to 5.118 ± 0.156 (9%) and 3.049 ± 0.239 (46%), respectively, compared with controls (0.834 ± 0.041, 0.536 ± 0.027 and 5.632 ± 0.287). The effect of horminone on AH and ERD was dose-dependent, but in the case of ECD there was not a significant difference in enzyme activity at each tested dose.

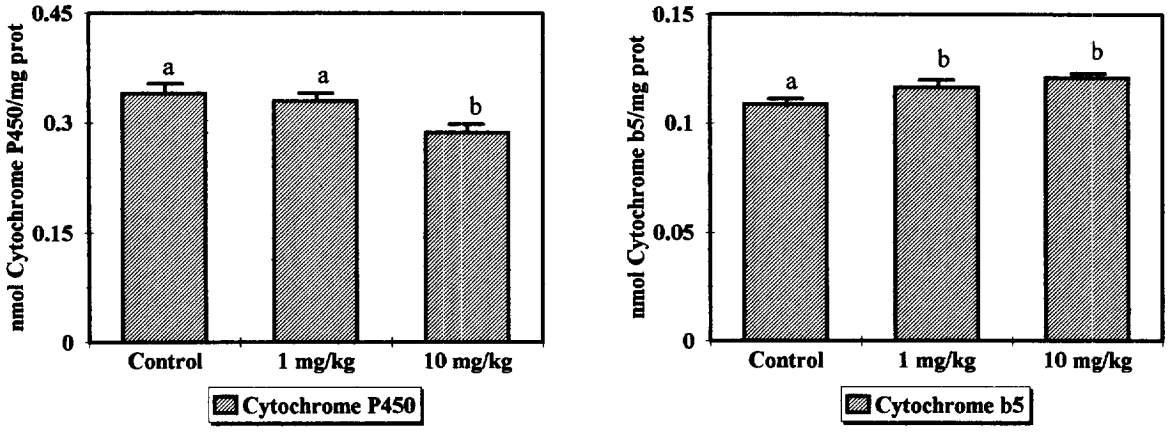

Fig. 3. Acute effect of horminone one liver microsomal cytochrome P450 and cytochrome b_5 contents. Values are expressed as mean ± S.D. from six animals. Groups bearing different letters are significantly different ($P < 0.05$).

Conversely, the EMD activity increased significantly with the same doses to 0.572 ± 0.030 (28%) and 0.559 ± 0.029 (25%), respectively, compared with control (0.448 ± 0.030), there was not a significant difference in enzyme activity at each tested dose.

3.5. Glutathione-S-transferase and glutathione reductase

Results in Fig. 6 revealed that a dose of 1 mg/kg of horminone produced a significant increase of GST activity to 0.910 ± 0.031 (18%) and a not significant decrease to 0.726 ± 0.029 (6%), respectively, compared with control (0.773 ± 0.044).

Conversely, both of the doses of horminone increased significantly glutathione reductase activity to 0.132 ± 0.004 (11%) and 0.131 ± 0.004 (10%), respectively, compared with control (0.119 ± 0.004), but this result was dose-independent.

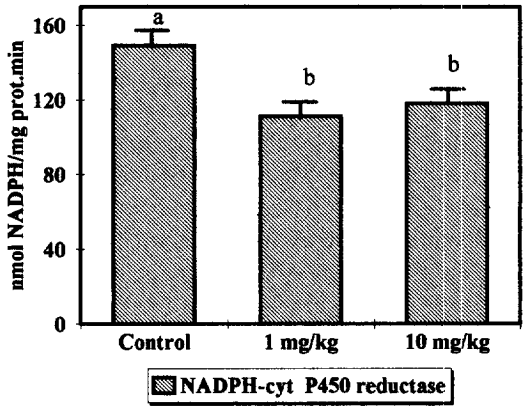

Fig. 4. Acute effect of horminone on liver microsomal NADPH-cytochrome P450 reductase. Values are expressed as mean ± S.D. from six animals. Groups bearing different letters are significantly different ($P < 0.05$).

4. Discussion

In the current work we study the effects of horminone on liver Wistar rat, mixed function mono-oxygenases (MFO) and glutathione enzyme activities. Results of this study showed that horminone induced an significant increase of ALT and AST serum levels in a dose-dependent manner.

AST is an enzyme present in liver, nervous tissue, skeletic muscle and heart and catalyses the conversion of aspartate to oxalacetate and glutamate in mitochondrion and cytosol. Membrane damages or necrosis releases this enzyme into circulation. High levels of AST in serum indicate liver damages due to toxicity and viral hepatitis as well as cardiac infections and muscle injury. ALT is also a cytosolic and mitochondrial enzyme but

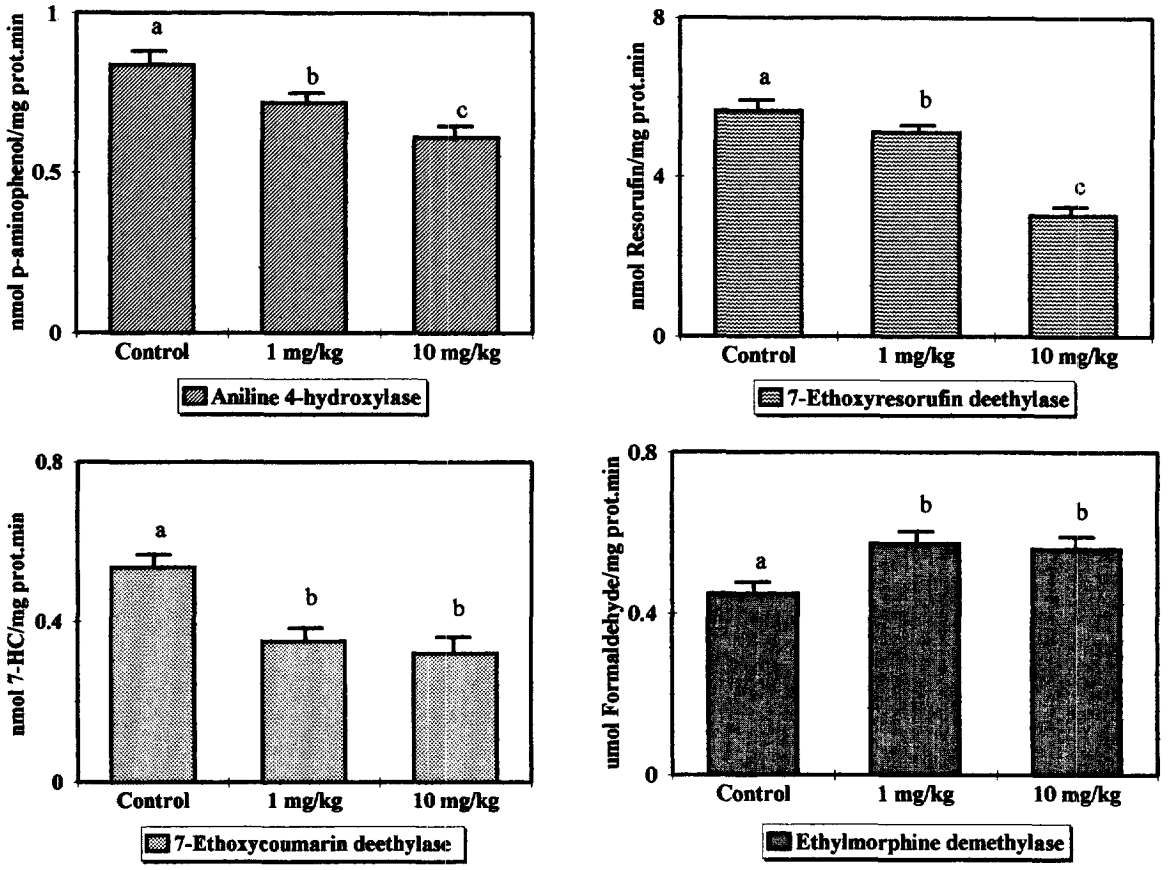

Fig. 5. Acute effect of horminone on liver microsomal aniline 4-hydroxylase, 7-ethoxycoumarin *O*-deethylase, 7-ethoxyresorufin *O*-deethylase and ethylmorphine *N*-demethylase. Values are expressed as mean ± S.D. from six animals. Groups bearing different letters are significantly different ($P < 0.05$).

more specific to the liver and catalyses the conversion of alanine to pyruvate and glutamate. This enzyme is released into circulation in a similar manner and high levels indicate hepatitis or liver damage (Cornelius, 1980).

Our results, showing that i.p. horminone administration induced an increase of ALT and AST serum levels, suggest that this compound produces a rat liver damage in a dose-dependent manner. Since horminone is water soluble, this result shows that horminone may account for the liver damage produced by *P. hereorensis* roots' infusion.

We also investigated the effects of horminone on P450 and cyt b_5 contents and the effects on some MFO activities. Results of this study showed that P450 content decreased at the dose of 10 mg/kg, only, but cyt b_5 content increased with both the doses. These are small effects and they do not have a biological significance.

Liver is the main organ for the metabolization of xenobiotics and its capacity to carry out the several oxidative metabolism is associated with its high cellular content of cytochrome P450 (Coon et al., 1992; Guengerich, 1992, 1993). Nevertheless, in face of a large diversity of isomorphic forms of cytochrome P450, the decrease of its content is not always associated with the diminishing of liver drug metabolization capacity and profiles of cytochrome P450 isozyme activities may change in the absence of measurable changes in total cytochrome content (Okey et al., 1986).

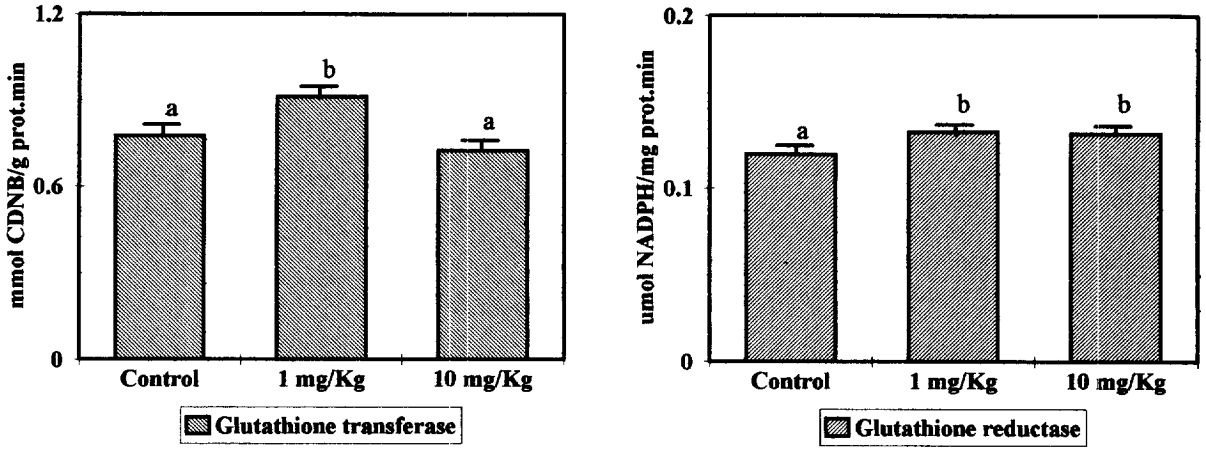

Fig. 6. Acute effect of horminone on liver cytosolic glutathione transferase and glutathione reductase activities. Values are expressed as mean ± S.D. from six animals. Groups bearing different letters are significantly different ($P < 0.05$).

CYP1A1, CYP1A2 and CYP3A are important rat liver enzymes involved in the metabolism of several xenobiotics (Nelson et al., 1996). CYP1A isozymes are inducible by polycyclic aromatic hydrocarbons (PAH)-type inducers. CYP1A1 is produced after enzyme induction and is very active in the aryl hydrocarbons hydroxylation and in the enzymatic conversion of other substrates, including the 7-ethoxyresorufin *O*-deethylation and 7-ethoxycoumarin *O*-deethylation, a reaction also catalysed by CYP2B1. By contrast, CYP1A2 is expressed in normal liver cells and is associated with arylamine metabolism. It participates in the aniline hydroxylation, a reaction also catalysed by the ethanol-inducible CYP2E1 (Wirkner and Poelchen, 1996) and in the 7-ethoxyresorufin and 7-ethoxycoumarin *O*-deethylation. The CYP3A gene family codes for enzymes which catalyse the biotransformation of steroids and several xenobiotics and participates in the ethylmorphine *N*-demethylation, a reaction also catalysed by CYP2D1 (Xu et al., 1997).

In the current study, horminone produced a decrease in hepatic activity of AH, ERD and ECD, but only the effects on AH and ERD activities were dose-dependent. These results suggested that horminone may have inhibited the CYP1A2 activity, in consequence of the absence of CYP1A1 in noninduced rat liver, but only the effects on ERD and ECD activities were significantly decreased (ERD 46% and ECD 40% at 10 mg/kg). Furthermore, horminone induced a significant, but low increase of the EMD liver activity, suggesting that horminone may have induced the CYP3A subfamily.

In cytochrome P450 system, the electron flow from NADPH or cytochrome b_5 through a flavoprotein, NR or NCb_5R, to the different isomorphic forms of P450 (Ortiz, 1995). Inhibitors of the activity or expression of these reductases or chemicals that produce a decrease in cytochrome b_5 content may account for diminishing the activity of the cytochrome P450 isozymes.

In this paper we also investigated the effect of horminone on NR activity. Results of this study showed that NR activity was significantly reduced by horminone, in a dose-independent manner. Although this effect was low and not dose-dependent, we could speculate if the decrease of NR activity (26%) would not be related with the observed inhibition of the CYP1A2 activities.

This study also demonstrated that i.p. administration of horminone in rat increased cyt b_5 content and it induced the activities of ethylmorphine *N*-demethylase, this induction suggesting that horminone may have induced CYP3A.

The effects of horminone on cytosolic GST and GR activities were also investigated in this study.

Glutathione *S*-transferase—a family of isozymes located in both cytosol and endoplasmic

reticulum of the liver (Aniya et al., 1993; Nijhof and Peters, 1993; Gebhardt et al., 1994), kidney (Olivier et al., 1990), testis (Di Biasio et al., 1991) and brain (Strömstedt et al., 1993; Lowndes et al., 1994) of mammals- participates in the conjugation of glutathione (GSH) with the products of the metabolization of endogenous and xenobiotics substances through the cytochrome P450 system, increasing their elimination from the organism in the form of mercapturates (Vermeulen et al., 1996).

Results of current study showed that only the smallest dose of horminone produced a significant activator effect on GST activity, but a favorable effect was not evident because the results were very low and without biological significance.

Glutathione reductase—a family of homologous proteins whose members are at least dimeric, NADPH dependent, FAD containing enzymes—maintains the cellular levels of GSH by reduction of the GSSG, protecting the cellular membranes from peroxides attack (Lalitha et al., 1990; López-Torres et al., 1990; Zarida et al., 1993).

In this study, both doses of horminone increased significantly the GR activity. Nevertheless, the effects were very low and they were without a biological significance.

Although most of the related effects of horminone on liver metabolizing enzymes were very low and some of them had not biological significance, the results reported on this study showed that horminone significantly increased the ALT and AST serum level and the EMD and it inhibited the AH, ECD and ERD activities.

This results cannot justify the pharmacological value of *P. hereorensis* roots infusion, but they showed that horminone isolated from this plant may account for producing liver damage and affecting the liver mixed function mono-oxygenases activities, with a very low effect on liver glutathione enzymes activities. Therefore, the possibility of toxic effects occurring after administration of extract of medicinal plants containing horminone must be considered and more studies with larger ranges of horminone concentrations must be performed, in order to determine more in deep the toxicological significance of this compound.

Acknowledgements

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