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In vitro Degradation of L-Phenylalanine Mustard (L-PAM)

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Summary. *L-Phenylalanine mustard (L-PAM), a bis-choroethylamine, is an important drug in the treatment of multiple myeloma and ovarian cancer. It undergoes rapid hydrolysis in vitro and in vivo, forming the mono- and dihydroxy degradation products. L-PAM's first-order disappearance rate in a phosphate-buffered solution did not differ statistically according to the presence or absence of activated rat liver microsomal enzymes. Furthermore, L-PAM's disappearance rate in a rat whole liver perfusion system was not greater than its hydrolysis rate in water. In vitro plasma recovery studies showed that up to 85% of the ^{14}C L-PAM drug equivalents could be recovered as the parent compound and the mono- and dihydroxy degradation products. Thus, L-PAM in in vitro degradation was similar qualitatively and quantitatively to its reported in vivo degradation in animals and man. It is concluded that L-PAM does not undergo important, active in vivo metabolism.*

Introduction

L-PAM has been available for over 27 years since its synthesis by Bergel and Stock [5]. It is now an important drug for the treatment of multiple myeloma and ovarian cancer [4, 15]. L-PAM readily undergoes hydrolysis in vitro, which is characterized by first-order kinetics [17]. The hydrolysis rate is dependent on pH, temperature, bile acids, protein concentration, and chloride ions [7, 10]. Human pharmacokinetic studies have shown that L-PAM has a relatively rapid terminal phase plasma half-life [1]. L-PAM distribution in plasma and excretion in urine after PO administration or IV injection have been described [2, 3, 9]. However, L-PAM metabolism in vivo has not been fully elucidated, although several recent papers have reported biliary excretion in dogs and mice [6, 13]. The purpose of this report is to determine whether L-PAM undergoes active metabolism; rat microsomal enzymes and the perfused whole rat liver were used as test systems.

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Materials and Methods

L-Phenylalanine mustard was obtained from the Division of Cancer Treatment, NCI, Bethesda, MD, USA. ^{14}C-L-PAM (labeled in the chloroethyl moiety) was obtained from Dr. Robert Engle (Head, Chemical Resources Section, Developmental Therapeutics Program, DCT, NCI, Bethesda, MD, USA) through the Stanford Research Institute, Menlo Park, CA, USA. Potassium monophosphate (KH_2PO_4) was purchased from the Sigma Chemical Corp., St. Louis, MO, USA. Medium 199 and fetal calf serum (FCS) were purchased from the Grand Island Biological Company, Grand Island, NY. NADP (oxidized, monosodium salt), glucose-6-phosphate (G6P), and glucose-6-phosphate dehydrogenase (G6PD, 2000 IU/ml) were purchased from Calbiochem, San Diego, CA, USA. All solvents were HPLC pure and purchased from Burdick and Jackson, Muskegan, Michigan, USA. Male Sprague-Dawley rats weighing 200–250 g were purchased from Hilltop Laboratories, Chatsworth, CA, USA.

Methods. L-PAM was analyzed according to the HPLC method reported by Chang et al. [7]. The disappearance rate constant (k) of unchanged drug (L-PAM) was calculated according to $k = 2.3 \log C_0/C_t/T$; where C_0 and C_t are the concentrations of L-PAM at time zero and time T, respectively.

Microsomes were prepared as described by Mazel [16] and their activity was measured in an ethylmorphine *N*-demethylase assay [16]. Whole rat livers were isolated according to the method of de Galdeano et al. [11]. In brief, the perfusion apparatus was filled prior to liver isolation with 200 ml modified medium 199 + 5% FCS and eequilibrated with O_2 at 37° C. Animals were anesthesized with ether and the bile duct cannulated with PE-10 tubing. The portal vein was isolated, and a cannula of the perfusion apparatus (grooved 15-gauge SS needle) inserted and tied at the general locus of the lienal branch. The vena cava was opened at the level of the right kidney and the perfusion started while the animal was still alive. At this time 700 µg L-PAM in 20% DMSO:H_2O were added to the perfusion vessel. The liver was surgically removed, trimmed of extraneous tissue, rinsed out, and placed into

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the perfusion vessel. The cannulated bile was collected outside of the apparatus. In experiments with non-cannulated livers, bile was allowed to flow freely into the perfusion media. Samples (1 ml each) were taken at designated time points and analyzed by HPLC as previously described.

Total ^{14}C-L-PAM drug equivalents were determined by diluting 100 µl whole plasma with scintillation cocktail and counting on a Beckman LS-7000. ^{14}C-protein-bound drug was quantitated by first counting 100 µl methanol-extracted ^{14}C and then subtracting from the total ^{14}C in plasma. Both the mono- and dihydroxy degradation products and the parent L-PAM were collected from the HPLC eluate and counted. The HPLC column was periodically checked for above-background ^{14}C contamination and was negative.

Results

Figure 1 shows the percent recovery of ^{14}C-L-PAM drug equivalents from human plasma following in vitro incubation at 37° C. The disappearance rate constant for L-PAM was 0.41 h^{-1} and followed first-order kinetics. The monohydroxy degradation product (MOH) formed rapidly and its concentration reached a peak within 2 h before declining. Both L-PAM and MOH continued to degrade to the final degradation product dihydroxy L-PAM (DOH). At any time point an average of 85% of the total ^{14}C drug equivalents could be recovered as: MOH; DOH; protein-bound ^{14}C; the unreacted parent drug L-PAM; or a combination of these. By contrast, 98% of the drug could be recovered from incubations in distilled water.

Figure 2 illustrates the rates of degradation of L-PAM from active and heat-denatured microsomal enzymes in a physiological buffer. The disappearance rate constants were 0.70 · h^{-1} and 0.97 · h^{-1} for the heat-denatured and active enzyme systems, respectively. Rapid first-order kinetics for L-PAM disappearance were observed in both systems. L-PAM had increased stability in heat-denatured microsomes compared with its stability in distilled water (0.85 · h^{-1}). L-PAM degradation in rat liver microsomes resulted only in the mono- and dihydroxy degradation products being formed as determined by ^{14}C counting in isolated HPLC peaks. Additionally, the rates of formation of the two degradation products were not changed by exposure to the microsomes in the phosphate-buffered system.

Disappearance curves for L-PAM from the perfused, isolated whole rat liver are shown in Fig. 3. The disappearance rate constants were 0.46 · h^{-1} and 0.68 · h^{-1} for the cannulated and non-cannulated bile duct systems, respectively ($P < 0.05$). The disappearance rate constant for L-PAM in medium alone was 0.30 · h^{-1} ($P < 0.05$ vs. cannulated system, $P < 0.01$ vs non-cannulated system).

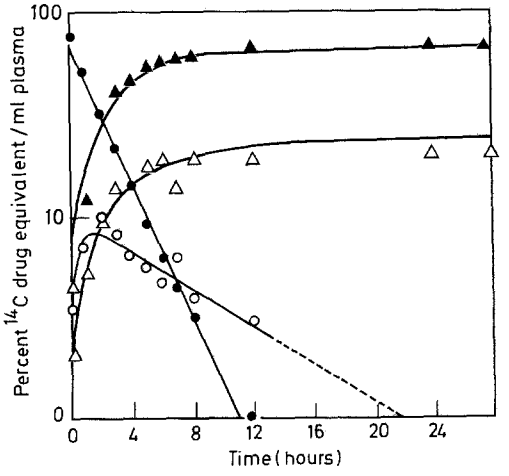

Fig. 1. Percent total ^{14}C-L-PAM drug equivalents per milliliter of human plasma incubated at 37° C in vitro. L-PAM (21 µg/ml, 0.01 mCi) was incubated in human plasma in vitro at 37° C. Samples were taken and counted for total ^{14}C drug equivalents. The HPLC eluate was collected and counted to obtain the percent ^{14}C as protein bound L-PAM (▲——▲); dihydroxy L-PAM (△——△); monohydroxy L-PAM (○——○); and L-PAM (●——●)

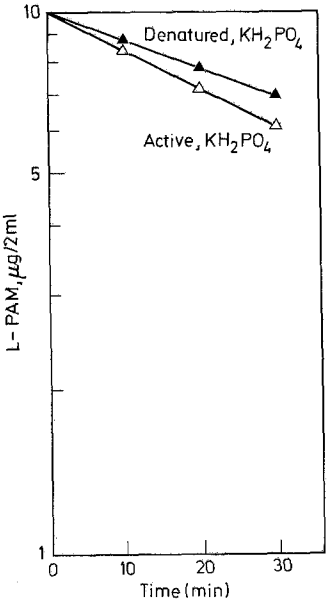

Fig. 2. Recovery of L-PAM from active and heat-denatured microsomes suspended in 0.05 *M* KH_2PO_4 buffer at 37° C. L-PAM (10 µg) was incubated in 1 ml microsomal suspension (6 mg protein/ml) and 1 ml complete medium for 30 min at 37° C. Disappearance rates were: 0.70/h for denatured enzymes in KH_2PO_4 (▲——▲); 0.97/h for active enzymes in KH_2PO_4 (△——△)

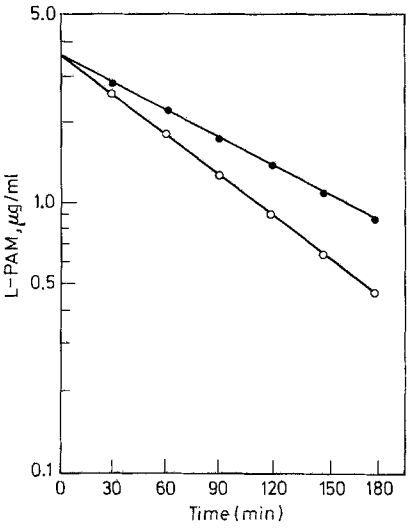

Fig. 3. Disappearance of L-PAM from the perfused whole rat liver in medium 199 plus 5% albumin at 37° C. L-PAM (700 μg in 20% DMSO:H_2O) was added to the perfusion apparatus prior to the addition of the isolated livers. The bile ducts were either cannulated (●——●), in which case the bile was collected outside the apparatus, or not cannulated (△——△), when the bile was allowed to flow freely into the perfusion medium

Discussion

The known in vitro elimination pathways for L-PAM are hydrolysis and alkylation [8, 10, 17]. It is not known whether L-PAM is actively metabolized in vivo. In this study, L-PAM's disappearance rate exhibited rapid first-order kinetics after incubations in various media.

When added to an active microsomal suspension in a physiological buffer, L-PAM's disappearance rate was increased over that in denatured microsomal controls. These data are suggestive of active metabolism of L-PAM by the mixed-function oxidase system and probably by increased hydrolysis. We have not identified any ^{14}C metabolites other than the mono- and dihydroxy L-PAM degradation products in either in vitro and or in vivo samples of plasma and urine. We have not, however, addressed the possibility of metabolism by the soluble enzymes of the liver or other tissues. The increased L-PAM stability in heat-denatured microsomes was probably a result of protein and lipid protection, as reported by Chang et al. [7–9].

The perfusion of L-PAM through the whole rat liver resulted in an increased disappearance rate from simple hydrolysis in buffer alone. This increased drug loss was probably due to alkylation to proteins, increased hydrolysis by enzymes of the liver, and biliary excretion of the parent compound L-PAM. This latter loss has been described by Furner et al. [12, 13], who reported that up to 11% of the total IV dose given to dogs was found in bile fluid within 30 min. Upon analysis, 86% of the total drug equivalent was the parent compound. Biliary excretion of L-PAM has been reported by others [6].

The disappearance of L-PAM was further enhanced in the presence of bile salts (non-cannulated system). There are two possible reasons for this increased drug loss compared with the cannulated system. One, the bile salts, which form polymolecular aggregated of micelles in high concentrations [14, 18], never reach the critical micellar concentration and therefore do not form protective micelles. Secondly, the bile salts themselves become bound to serum proteins and displace L-PAM from drug-binding sites. The effect of high plasma bilirubin levels on L-PAM stability in vivo has been reported [19] and supports these results.

The in vitro recovery of ^{14}C-L-PAM drug equivalents from human plasma averaged 85%. However, the remaining 15% was thought to be protein-bound to small, methanol-soluble peptides, presumably through alkylation. With improved methods of separation by cryoprecipitation and higher-speed centrifugation of later samples, our recovery of ^{14}C drug equivalent was greater than 90%. The in vitro ^{14}C profile of drug equivalents versus ^{14}C-L-PAM incubation time was similar to that observed after IV dosing [3]. Additionally, we have not identified any other degradation products in plasma and urine samples from patients who received PO or IV ^{14}C L-PAM [2, 3]. These close similarities between the results of our in vitro and in vivo studies suggest that L-PAM disappearance in vivo is primarily through hydrolysis and alkylation and not by enzymatic biotransformation.

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