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First-pass Metabolism of Peptide Drugs in Rat Perfused Liver

YOKO TAKI, TOSHIYASU SAKANE, TANEKAZU NADAI, HITOSHI SEZAKI, GORDON L. AMIDON*, PETER LANGGUTH† AND SHINJI YAMASHITA

*Faculty of Pharmaceutical Sciences, Setsunan University, Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan, *The University of Michigan, College of Pharmacy, Ann Arbor, MI 48109, USA, and †Astra Hassle AB, Karragatan 5, S-431 83 Molndal, Sweden*

Abstract

To elucidate the extent and mechanisms of the first-pass metabolism of peptide drugs in the liver after oral administration, a liver perfusion study was performed in rats using metkephamid, a stable analogue of methionine enkephalin, and thyrotropin-releasing hormone (TRH), as model peptides.

The fraction of intact metkephamid recovered after single-pass constant perfusion through rat liver reached steady-state very quickly, and it was concluded that metkephamid was hydrolysed enzymatically at the surface of hepatocytes or endothelial cells of microvessels, or both, rather than being taken up by hepatocytes. The fraction of metkephamid recovered intact was approximately 40% under protein-free conditions but increased to 70–75% on addition of bovine serum albumin (BSA) to the perfusate. The fraction of metkephamid bound to BSA was approximately 50% under these conditions, implying that only the free fraction of metkephamid in the plasma was metabolized in the liver. Calculations based on the tube model showed that approximately 30–35% of metkephamid absorbed from the intestine undergoes first-pass metabolism before entering the systemic circulation in-vivo. In contrast, the fraction of TRH metabolized in the liver was less than 10%, indicating a remarkably low contribution of first-pass metabolism to the bioavailability of TRH.

These results show that hepatic first-pass metabolism of metkephamid contributes to its low systemic bioavailability. After intestinal absorption free metkephamid is rapidly hydrolysed on the surface of hepatocytes or endothelial cells, rather than being taken up by hepatocytes. This information has important implications in the oral delivery of many kinds of peptide.

The systemic bioavailability of peptide drugs after oral administration is primary restricted by their extremely low absorption from the gastrointestinal tract, because of their low permeation through intestinal membrane and their high affinity for proteolytic enzymes. To elucidate the contributions of these factors to peptide absorption in-vivo we have already studied the intestinal absorption of metkephamid, an analogue of natural [Met]enkephalin, by means of vascular perfusion of the rat small intestine (Yamashita et al 1994a; Taki et al 1995). Absorption of metkephamid was estimated quantitatively by calculating its clearance by degradation and permeation. Because the degradation clearance of metkephamid is much greater than its permeation clearance, suppression of enzymatic hydrolysis in the intestine might be a promising means of enhancing the bioavailability of such peptide drugs. Several relevant studies have been reported, including inhibition of peptidase activity with appropriate inhibitors (Langguth et al 1994a, b), chemical modification of peptide molecules (Tenma et al 1993; Asada et al 1994) and the delivery of peptides to the colon (Friend 1991; Tozaki et al 1997).

It is suspected that after absorption most peptide drugs undergo first-pass metabolism in the liver before entering the systemic circulation. Hepatic metabolism of peptides is usually investigated in-vitro by use of isolated hepatocytes, or liver homogenates or its microsomal fraction. Although those in-vitro studies reveal the mechanisms and corresponding enzymes of hepatic metabolism,

Correspondence: Y. Taki, Faculty of Pharmaceutical Sciences, Setsunan University, Nagaotoge-cho, Hirakata, Osaka 573-01, Japan.

they furnish little information about the extent of first-pass metabolism of these peptide drugs in-vivo.

In this study we have determined the first-pass hepatic metabolism of metkephamid and thyrotropin-releasing hormone (TRH) by means of the single-pass liver perfusion technique. With this method, metabolism in the liver can be detected under conditions similar to those in-vivo. The total bioavailability of metkephamid after oral administration was also evaluated by taking into account results from our previous work (Taki et al 1995).

Materials and Methods

Chemicals

Metkephamid (Tyr-D-Ala-Gly-Phe-*N*-CH_3-Met-NH_2.CH_3COOH, MW 660·8) was donated by Eli Lilly (Indianapolis, IN). Bovine serum albumin (BSA, fraction V) was obtained from Wako (Osaka, Japan), phenol red from Kanto (Tokyo, Japan) and thyrotropin-releasing hormone (TRH) from Sigma (St Louis, MO). Other chemicals were commercially available and of analytical grade.

Liver-perfusion study

Male Wistar rats, approximately 200–250 g, were allowed free access to food and water. The surgical procedure for liver perfusion was performed by the method described by Nishida et al (1989). Briefly, the portal vein and the inferior vena cava were cannulated with polyethylene tubing and the liver was perfused from the portal vein with Krebs–Henseleit bicarbonate buffer (KHBB, pH 7·4) containing D-glucose (10 mM), BSA (0 or 3% w/v) and the drug (metkephamid, 1 or 10 μM; TRH, 1 or 10 μM; phenol red, 10 mg mL^{-1}) after a preperfusion period of 20 min. Effluent was collected from the inferior vena cava. Perfusate was delivered by use of a peristaltic pump at a flow rate of approximately 14 mL min^{-1}. The bile duct was cannulated with polyethylene tubing and bile was collected every 10 min throughout the experiment to check the viability of the liver.

Protein-binding study

An in-vitro protein-binding study of metkephamid and TRH was performed at 37°C by equilibrium dialysis (Yamashita et al 1994b). A defined concentration of metkephamid or TRH was dissolved in KHBB containing 3% w/v BSA and dialysed against an equal volume of KHBB for 8 h, employing a cellulose dialysis membrane (Spectropor, MW cut-off 1000; Spectrum Medical Industries, LA) with a dialysis chamber (Abe Science, Chiba, Japan). After dialysis, the concentrations of metkephamid or TRH on both sides of the membrane were determined to calculate the extent of binding to BSA.

Drug analysis

Intact metkephamid and TRH in the sample were determined by high-performance liquid chromatography (HPLC). The system (LC-10AT; Shimadzu, Japan) was equipped with a variable wavelength UV-detector (SPD-10AV, Shimadzu). The conditions used for HPLC analysis of metkephamid have been described elsewhere (Taki et al 1995). After precipitation of BSA with 10% trichloroacetic acid, TRH was analysed on a 25 cm × 4 mm i.d. LiChrospher 100 RP-18 column (E. Merck, Darmstadt, Germany). The mobile phase was 50 mM phosphate buffer (containing sodium heptane sulfonate (0·01 M) and adjusted to pH 4 by the addition of 85% phosphoric acid)–acetonitrile–methanol, 1800 : 92 : 57·5 (v/v). The flow rate was 1·0 mL min^{-1}, the column temperature 45°C, and the detection wavelength 210 nm (Yamada et al 1992). Phenol red was detected spectrophotometrically at 550 nm (UV-1200, Shimadzu).

Results

First-pass hepatic metabolism of metkephamid and TRH under BSA-free conditions

Figure 1 shows the time-course of the recovered fractions (%) of intact metkephamid and TRH after single-pass constant perfusion through rat liver under BSA-free conditions. For both drugs, 1 and 10 μM concentrations were employed because the concentration of metkephamid in the portal vein in the intestinal vascular perfusion study (Taki et al 1995) was found to lie between these values. Outflow patterns for both drugs reached the steady state very quickly (within 1 min); under steady-state conditions approximately 40% of the infused metkephamid and > 90% TRH were recovered intact in the effluent, implying that metkephamid flowing into the liver from the portal vein undergoes extensive (ca 60%) first-pass metabolism whereas only a few percent of TRH, not significantly different from zero, were metabolized. No significant differences were observed between the results from 1 and 10-μM concentrations of the drugs. The outflow pattern of phenol red, known to be taken up by the hepatocytes and secreted into the bile, is depicted in Figure 2 for reference. Compared with the patterns for metkephamid and TRH, the fraction of phenol red recovered increased gradually, taking longer to reach steady state, when approximately 80% of phenol red was recovered.

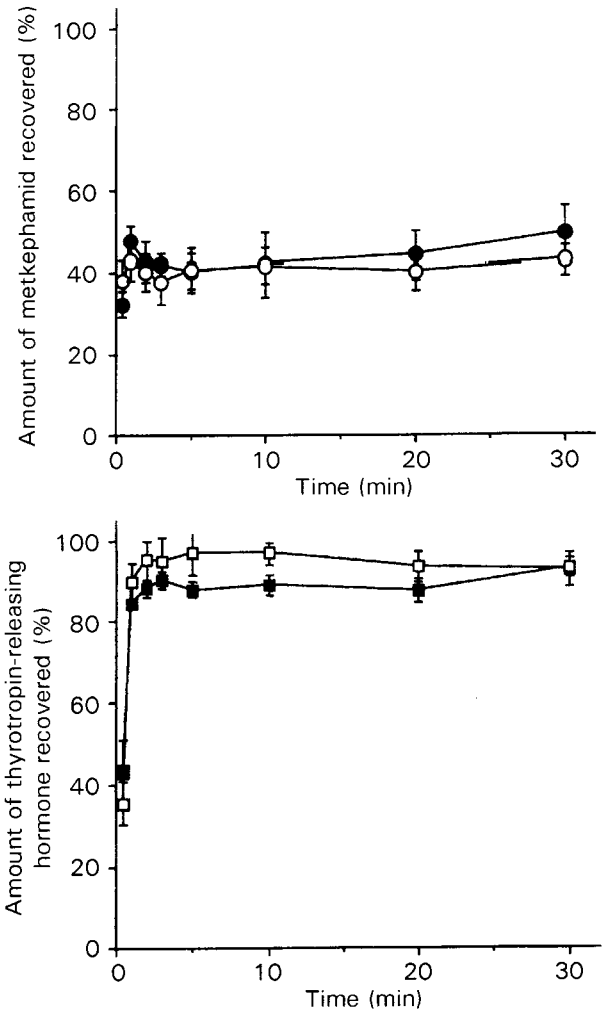

Figure 1. Time-course of the fraction of intact metkephamid and thyrotropin-releasing hormone recovered after single-pass liver perfusion under bovine serum albumin-free conditions. Krebs–Henseleit bicarbonate buffer containing metkephamid (●, 1 μM; ○, 10 μM) or thyrotropin-releasing hormone (■, 1 μM; □, 10 μM) was perfused through the rat liver from the portal vein and the concentration of intact drug in the effluent solution was measured to calculate the percentage recovered. Each point represents the mean ± s.e. of results from at least four experiments.

Effect of BSA on hepatic metabolism

Under in-vivo conditions different proteins, e.g. albumin, present in blood might interact with the peptides and affect their hepatic metabolism. The effect of BSA on the first-pass metabolism of metkephamid is shown in Figure 3. Addition of BSA (3% w/v) to the perfusate increased the fraction of metkephamid recovered at the steady state to approximately 75% and 70% for concentrations of 1 and 10 μM, respectively. The corresponding fraction metabolized, 25–30%, is thus approximately one half that without BSA. This result suggests that BSA protects metkephamid from metabolism in the liver. Figure 4 shows the amount

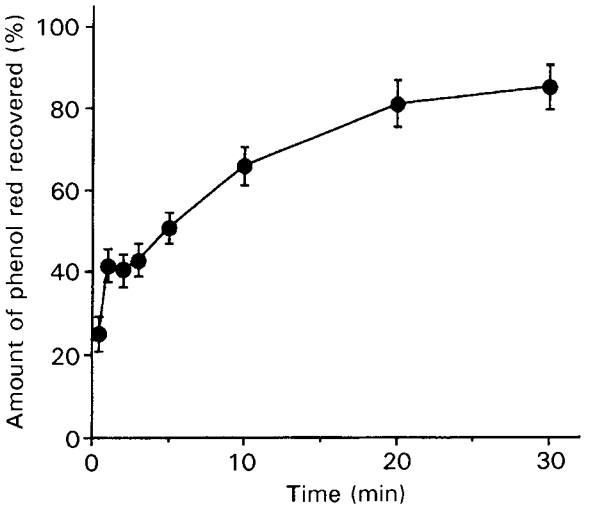

Figure 2. Time-course of the fraction of phenol red recovered after single-pass perfusion through the liver under bovine serum albumin-free conditions. Krebs–Henseleit bicarbonate buffer containing phenol red (10 mg mL^{-1}) was perfused through the rat liver from the portal vein and the concentration of phenol red in the effluent solution was measured to calculate the percentage recovered. Each point represents the mean ± s.e. of results from four experiments.

(%) of metkephamid bound to BSA (3% w/v), as estimated by equilibrium dialysis. The amount of bound metkephamid decreased with increasing metkephamid concentration, indicative of saturation of protein binding. At 1 and 10 μM concentrations the amounts of metkephamid bound to BSA were 48·6% and 43·8%, respectively. Under the same conditions the amount of TRH bound to BSA was not detectable (data not shown).

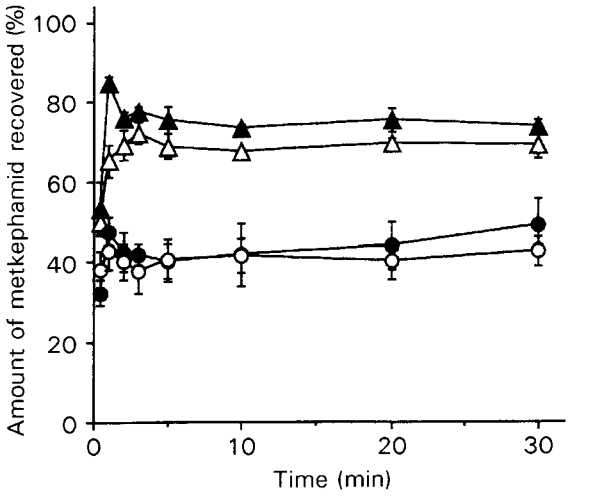

Figure 3. Effect of bovine serum albumin (3% w/v) on the fraction of intact metkephamid recovered after single-pass perfusion through the liver. Recovered fraction (%) of metkephamid (△ and ○, 10 μM; ▲ and ●, 1 μM) after single-pass perfusion through the rat liver was measured using bovine serum albumin-free (○, ●) or bovine serum albumin-containing (△, ▲) Krebs–Henseleit bicarbonate buffer. Each point represents the mean ± s.e. of results from four experiments.

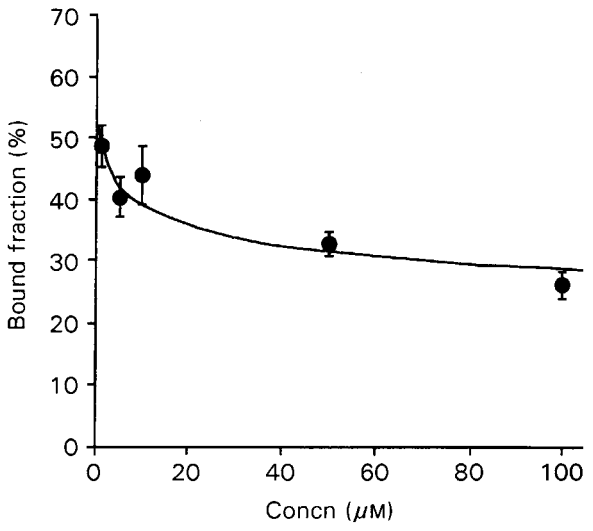

Figure 4. Binding of metkephamid to bovine serum albumin as a function of metkephamid concentration. The fraction (%) of metkephamid bound to bovine serum albumin (3% w/v) was measured by equilibrium dialysis. Each point represents the mean ± s.e. of results from six experiments.

Discussion

As underlying causes of the low oral availability of some drugs two major problems must be considered—low absorption from the gastrointestinal tract and high first-pass extraction by the liver before the drug enters the systemic circulation. We have previously investigated the intestinal absorption of metkephamid, which undergoes extensive hydrolysis by aminopeptidase N at the surface of the intestinal epithelial cells (Taki et al 1995). By use of the enzyme inhibitor puromycin the amount absorbed could be enhanced by approximately 6–7% (Taki et al 1995). To predict the bioavailability of metkephamid, information about the extent of first-pass hepatic metabolism is necessary.

In this study, liver perfusion was initially performed under the simplest conditions—with blood component-free perfusate. The viability of the liver was checked by measuring the bile flow throughout the experiment and, in all experiments, the perfused livers remained viable during the course of study under these conditions, as has been reported by Nishida et al (1991).

Under albumin-free conditions approximately 60% of the infused metkephamid was metabolized in the liver, although the outflow pattern for metkephamid was quite different from that for phenol red. The time required to reach the steady state is much longer for phenol red than for metkephamid, suggesting differences in the mechanism of hepatic extraction of the drugs (Nishida et al 1992). After diffusion into the Disse space across the wall of microvessels phenol red is taken up by hepatocytes and then excreted into the bile. The gradual increase in the fraction of phenol red recovered, shown in Figure 2, indicates that these complex processes and the large distribution volume of phenol red in the liver mean that steady state is reached only after a considerable time. In contrast, the outflow pattern of metkephamid indicates that its metabolism is a comparatively simple process. In the intestine metkephamid is hydrolysed mainly by aminopeptidase N, the membrane-associated aminopeptidase located at the surface of enterocytes.

Because the same types of enzyme are widely distributed on cell membranes of many other organs also, metkephamid is considered to be hydrolysed quickly at the surface of the hepatocytes rather than being taken up into the cells. The contribution of microvessel endothelial cells is also possible, such that a significant fraction of the infused metkephamid is degraded at the surface of the endothelial cells. Consequently, the enzymatic hydrolysis of metkephamid by the blood-vessel endothelium does not seem to be classic hepatic metabolism, although it must nevertheless be regarded as first-pass extraction because the liver is an organ with a high density of microcapillaries.

In this study, no significant degradation of TRH was observed during single-pass liver perfusion. This result is in good agreement with that reported by Yokohama et al (1984) who obtained the same blood concentration–time curves for TRH after intra-antecubital and intra-portal administration of TRH to dogs and to rats. They concluded that the liver is not a principal site of TRH degradation.

The presence of BSA in the perfusate reduced the extraction of metkephamid to half that under BSA-free conditions. Because the protein binding of metkephamid is approximately 50%, it was suggested that only the free fraction of metkephamid undergoes hydrolysis. Listed in Table 1 are the observed and predicted values of the recovered fraction (Fh) of metkephamid in the presence of 3% BSA. The values were predicted using the intrinsic hepatic clearance of metkephamid (CL_{int}) estimated from BSA-free experiments (Figure 1), the unbound fraction in the perfusate (f) measured by equilibrium dialysis (Figure 4), and the hepatic blood-flow (Qh) used in experiments incorporating BSA (Figure 3). Because the metkephamid that flows into the liver from the portal vein undergoes rapid hydrolysis on the surface of hepatocytes or the endothelial cells of blood vessels, or both, the degradation of metkephamid should be faster than its diffusion along with the direction of blood flow. Because these are not well-stirred conditions, the predicted Fh was calculated on the basis of the tube

model assuming a concentration gradient of metkephamid in the blood capillary from the inlet to the outlet. At both concentrations, predicted and observed values seemed comparable, although values of Fh 8–10% higher were obtained in experiments incorporating BSA. Addition of BSA to the perfusate increased the viscosity, which could reduce the diffusivity of metkephamid in the solution. In the rapid hydrolysis process the reduced rate of diffusion of metkephamid on to the cellular surface might reduce the fraction degraded, yielding values of Fh higher than that predicted from BSA-free experiments. Inhibition of enzymatic hydrolysis of metkephamid by the small fragment peptides of BSA contained in the reagent itself might also contribute to the higher values of Fh. From these results, therefore, it would seem reasonable to assume that plasma proteins protect against enzymatic degradation of metkephamid. If the affinity of metkephamid for rat serum albumin is similar to that for BSA, the first-pass metabolism of metkephamid in the rat in-vivo would be slightly less than the value reported in Table 1, because the concentration of serum albumin is usually 4–4·5% w/v and other proteins might also interact with the metabolism of metkephamid. Finally, we can assume that the extent of first-pass metabolism of metkephamid in rats totals approximately 30–35% of the amount absorbed in-vivo. This is in good agreement with previous studies in which we have shown that the total absorption of metkephamid from rat small intestine can be enhanced by up to 6–7% of the dose by co-administration with an enzyme inhibitor, and in studies that demonstrated that the systemic bioavailability of metkephamid is in the range of 4–5% of the given dose. Langguth et al (1994b) have monitored the blood concentration of metkephamid in the rat after intra-ileal administration with puromycin in-vivo and calculated the systemic bioavailability of metkephamid to be 4·6% (± 1·66%) of the dose. This agreement in respect of bioavailability strongly supports the validity of our estimate from both intestinal and liver perfusion studies. However, for more precise estimation of the in-vivo oral bioavailability of peptides from the intestinal and liver perfusion experiments, we must take into account the effects of several factors such as inter-species differences in protein binding and enzyme distribution and partition of peptides to the blood cells. Further studies of those factors are now under investigation.

Table 1. Observed and predicted amounts of metkephamid recovered in the presence of 3% BSA after single-pass liver perfusion.

Concentration of metkephamid (μM)	Fraction of metkephamid recovered (%)	
	Observed	Predicted
1	75·08 ± 1·99	65·02
10	68·78 ± 1·13	60·14

The fraction of metkephamid recovered was predicted on the basis of the parallel tube model using the parameters: free fraction of metkephamid (f) measured by equilibrium dialysis (Figure 4); intrinsic clearance in the liver (CL_{int}), estimated from bovine serum albumin-free experiments (Figure 1), and the hepatic blood-flow (Qh) used in the experiments with bovine serum albumin (Figure 3). Values used were: 1 μM concentration: f = 0·514, CL_{int} = 11·69 mL min^{-1}, Qh = 13·43 mL min^{-1}; 10 μM concentration: f = 0·562, CL_{int} = 12·27 mL min^{-1}, Qh = 13·63 mL min^{-1}.

In conclusion, we have successfully demonstrated the contribution of the hepatic first-pass metabolism of metkephamid to its low systemic bioavailability. After absorption from the intestine, the free fraction of metkephamid in blood is considered to be hydrolysed quickly on the surface of hepatocytes or endothelial cells, or both, rather than being taken up by hepatocytes. This information is quite new and is of importance in considering the system of oral delivery of many kinds of peptide.

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