Hepatic Extraction of Hexarelin, a New Peptidic GH Secretagogue, in the Isolated Perfused Rat Liver

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Received March 19, 1997; accepted May 5, 1997

Purpose. To assess the hepatic extraction of hexarelin (HEX), a novel peptidyl GH secretagogue, in the isolated perfused rat model and document the *in vitro* binding of HEX to plasma proteins using plasma from rats, dogs, pigs, and humans.

Methods. Rat liver was perfused *in situ* using a recirculating system. The recirculating perfusate consisted of a Krebs Henseleit buffer containing 20% (v/v) prewashed bovine red blood cells, 1% albumin, and 1g/L dextrose. Three HEX concentrations of 5, 50, and 500 ng/ml were examined. *In vitro* plasma binding was determined by the ultrafiltration method.

Results. The disappearance rate constant (K), half-life ($t_{1/2}$), clearance (Cl), and hepatic extraction ratio (E) were: K = 0.013–0.014 min^{-1}, $t_{1/2}$ = 45–55 min, Cl = 0.345–0.401 ml/min/g liver, and E = 19-21% for the different concentrations of HEX. A linear increase in AUC (270-24334 min pmol/ml) was observed with increasing concentrations. Binding of HEX to plasma proteins of rats, dogs, pigs, and humans was 68.7 ± 0.8%, 78.7 ± 0.6%, 67.3 ± 0.7%, and 65.2 ± 0.6% respectively. Plasma binding was concentration-independent in the range between 0.003–3 μM for the four species examined.

Conclusions. These results show that 1) the hepatic extraction of HEX is low, 2) the hepatic clearance is concentration independent up to 500 ng HEX/ml of perfusate, and 3) the plasma protein binding of HEX is significant over the dose range studied. HEX exhibits a low hepatic extraction ratio, allowing us to predict that its hepatic clearance may be limited upon HEX protein binding.

KEY WORDS: hexarelin; growth hormone releasing peptides; isolated perfused rat liver; hepatic extraction; *in vitro* plasma protein binding.

INTRODUCTION

The pulsatile secretion of growth hormone (GH) is under the regulation of two hypothalamic hormones: GH-releasing hormone (GHRH) which stimulates the secretion of GH, and somatostatin which inhibits its release. Recently, the existence of a third neuroendocrine pathway for the episodic release of growth hormone, induced by GH secretagogues, has been confirmed following the cloning of a heterotrimeric GTP-binding protein coupled receptor from human pituitary and hypothalamus (1). This protein appears to be the target of the peptidyl Growth Hormone Releasing Peptides (GHRPs) (2) and the non-peptidyl benzolactam derivative GH secretagogues (3).

Among the GH releasing class, a novel and potent GHRP analog hexarelin [His-D-Trp(2Me)Ala-Trp-D-Phe-Lys-NH_2] has recently been developed. Pre-clinical studies of hexarelin in rats and dogs following intravenous and subcutaneous administration have demonstrated that the peptide elicits a long-lasting GH release and is more effective than GHRP-6 (4,5). In humans, clinical studies have shown that intravenous, subcutaneous, intranasal, and oral administration of hexarelin results in substantial dose-dependent increases in plasma GH concentrations (6).

Despite the low bioavailability of hexarelin, satisfactory GH-releasing activity is observed following a 20 mg oral dose of this peptide in man (6). The results are comparable with a GH response similar to that observed after i.v. administration of 1 μg/kg hexarelin. The low bioavailability of hexarelin stems from either two possible sources: 1) an elevated hepatic extraction and/or 2) a limitation in drug absorption from the gastrointestinal (GI) tract.

Since little is known about the disappearance rate of hexarelin in the liver, the objective of this study was to assess the hepatic extraction on the clearance of hexarelin at 5, 50, and 500 ng/ml by an isolated perfused rat liver model. The pharmacokinetic parameters, dose-dependency, and extraction ratio of the peptide were documented. In addition, the binding of hexarelin to plasma proteins was documented *in vitro* using the plasma of rats, dogs, pigs, and humans.

MATERIALS & METHODS

Chemicals

Hexarelin (EP 23905) was provided by Europeptides (Argenteuil, France). [^{3}H]Hexarelin (9.4 Ci/mmol, 348 GBq/mmol), code TRQ7484, was custom synthesized by Amersham International (Buckinghamshire, England). Bovine serum albumin (BSA) fraction V, and polyethyleneimine were obtained from Sigma Chemicals (St. Louis. MO). The O_2/CO_2 gas mixture was purchased from CryoGas (Montreal, Quebec). Bovine blood was supplied by the Department of Veterinary Medicine, University of Montreal (St. Hyacinthe, Quebec) in Citrate Phosphate Dextrose Adenine Solution USP (CPDA-1) blood pack units. The erythrocytes were washed five times with approximately three volumes of 0.9% NaCl and used immediately. Unless otherwise specified, all the reagents and materials used were of analytical grade and were purchased from Fisher Scientific (Montreal, Quebec).

Isolated Perfused Liver

Male Sprague Dawley rats (Charles River, St. Constant, Quebec), weighing 210–230 g, were used as liver donors. The animals were maintained on standard laboratory chow pellets and water *ad libitum*. Rats were anesthesized by intraperitoneal injection of sodium pentobarbital (1 ml/kg). The portal vein, the common bile duct, and the vena cava above the hepatic veins were cannulated as described by Ross (7). The rat was then transferred to the perfusion system where the liver, maintained at a temperature of 37°C, was perfused *in situ* in a recirculating circuit using an MX/AMBEC two/ten perfuser (MX International Aurora, CO). The perfusion system included

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0724-8741/97/0800-1008$12.50/0

a Masterflex pump (Cole-Palmer Instrument, Chicago, Il) and two distinct reservoirs.

The perfusion medium consisted of a Krebs-Henseleit buffer containing 20% (v/v) washed bovine erythrocytes, 1% BSA, and 1 g/L dextrose. The perfusate was adjusted to pH 7.4, saturated with a mixture of 95% oxygen and 5% carbon dioxide, and thermostatically controlled at a temperature of 37°C. The recirculating perfusate volume was 200 mL. The perfusion rate was fixed at 16 ml/min and verified volumetrically following diversion of venous outflow at the end of each experiment. The first reservoir contained the perfusion medium without drug, and was used to equilibrate the liver during a period of approximately twenty minutes.

Following equilibrium, the circuit was switched to the reservoir containing a fixed final concentration of hexarelin. Samples of the medium, 0.5 ml from the reservoir (C_{in}) and 0.5 ml from a catheter of the caval vein (C_{out}), were taken at 0, 1, 3, 5, 7, 10, 15, 20, 30, 60, 90, 120 min and kept on ice during the study. Bile samples were collected at 10 min intervals until the end of the perfusion and the volumes measured. Perfusate samples were centrifuged and the plasma obtained was stored at −20°C until analysis.

Experimental Design

Three sets of experiments were conducted: 1) liver perfusion (n = 4) at three hexarelin dose levels (5 ng/ml, 50 ng/ml, 500 ng/ml); 2) control liver perfusion (n = 5) without hexarelin; 3) control perfusion (n = 4) without the liver in the perfusion circuit at 5 ng/ml concentration.

Liver Viability

Blood gas electrolytes as pH, pO_2, pCO_2, and HCO_3 were monitored throughout the perfusion experiment by a blood gas autoanalyzer (Ciba Corning, Model 288) and adjusted by the addition of small amounts of a 7.5% sodium bicarbonate solution or by varying the carbogen levels in order to maintain physiological parameters. The viability of the liver was assessed by 1) gross appearance, 2) oxygen consumption, 3) bile production, and 4) measurement of biochemical enzyme activity such as lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) at 30, 60, 90, and 120 min during the study.

In Vitro Binding Studies

Purification of [^{3}H]hexarelin

[^{3}H]hexarelin was loaded on a reverse-phase HPLC column (Vydac C18, 4.6 × 50 mm, 5 μm, 300 Å). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (A), and acetonitrile in 0.1% TFA (B). Separation was achieved using a Waters automated gradient controller equipped with M 510 pumps (Waters Assoc., Milford, MA). The radiolabelled peptide was purified by running a linear gradient of B from 15% to 45% in 60 min at a flow rate of 1 ml/min. The purified peptide, which eluted at 25% B, was collected and lyophilized immediately. The sample was reconstituted in 0.9% NaCl. Ascorbic acid and n-acetylcysteine, having final concentrations of 0.1% and 10^{-4} M respectively, were added as antioxidants.

Binding to Plasma Proteins

The protein binding of hexarelin in plasma was determined by the method of ultrafiltration. Frozen plasma samples from rats, pigs, and dogs were pooled from eight, two, and three animals respectively. Human plasma was pooled from three volunteers. Stock solutions consisting of a fixed amount of [^{3}H]hexarelin and various concentrations of unlabelled compound, diluted in a 0.054 M phosphate buffer (pH 7.4), were mixed with plasma to yield final concentrations of 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 μM. The samples (in triplicate) were capped and incubated at 37°C for 30 min. One ml aliquots were transferred to the sample reservoir of micropartition centrifuge tubes (Centrifree Micropartition Centrifuge Tube, Amicon, Beverly, MA) which had been pre-treated with 2% polyethyleneimine for 36 hours. Aliquots (50 μl) were withdrawn and analyzed by liquid scintillation counting. The remainder was centrifuged at 37°C in a Sorvall RC-28S Refrigerated Centrifuge (Dupont, Mississauga, Ontario) for 20 min at 1000 × g. An aliquot of each ultrafiltrate (50 μl) was analyzed for radioactivity.

Non-specific Binding to Centrifree Tubes

The effect of non-specific adsorption of hexarelin to the micropartition tubes was investigated. The lowest (0.003 μM) and highest concentration (3 μM) points in the range studied were diluted in protein free plasma.

Hexarelin Protein Binding in Fresh Versus Frozen Plasma

Since the binding studies were conducted using frozen plasma, the effects of freezing on the extent of plasma binding was examined. Fresh plasma was obtained from rats (n = 4), and the percentage of hexarelin bound to plasma proteins was determined as described above. The unused portion of plasma was frozen and stored at −20°C for 48 hours, at which point the experiment was repeated.

Binding to Red Blood Cells

Quadruplicate determinations of the hematocrit were made from fresh pooled rat blood. Stock solutions consisting of a fixed amount of [^{3}H]hexarelin and various concentrations of unlabelled compound, diluted in a 0.054 M phosphate buffer (pH 7.4), were mixed with whole blood to yield final concentrations of 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 μM. Following the 30 min incubation period, aliquots of blood containing [^{3}H]hexarelin were pipetted for radioactivity determination. The remaining blood was centrifuged at 1000 × g for 15 min, and the resulting plasma was analyzed for radioactivity.

Analytical Methods

Assay of Hexarelin in the Perfusion Medium

Hexarelin in the perfusate samples (C_{out}) was measured using a hexarelin radioimmunoassay method as described by Roumi et al. (8).

Radiometric Analysis

Radioactivity was measured in an LKB Wallac 1217 liquid scintillation counter (Fisher Scientific, Montreal, Quebec). For

Table I. Viability of the Isolated Rat Liver Perfused with a Krebs-Henseleit Buffer Containing 20% RBC, 1% Albumin, and 1 g/L Dextrose

Dose (ng/ml)	N	pH	Hematocrit (%)	Perfusion flow rate (ml/min/g)	Bile flow rate (μl/min/g)	O_2 consumption (μmol/min/g)	LDH (IU/min)	AST (IU/min)
Control	6	7.36 ± 0.04	22.4 ± 2.45	1.83 ± 0.14	1.17 ± 0.17	2.39 ± 0.17	0.11 ± 0.04	0.08 ± 0.03
5	4	7.36 ± 0.01	22.9 ± 2.05	1.72 ± 0.07	1.08 ± 0.25	2.48 ± 0.25	0.15 ± 0.06	0.08 ± 0.02
50	4	7.33 ± 0.00	22.0 ± 0.58	1.91 ± 0.04	1.12 ± 0.55	2.26 ± 0.14	0.12 ± 0.01	0.06 ± 0.01
500	4	7.33 ± 0.04	23.4 ± 0.98	1.97 ± 0.34	1.17 ± 0.15	2.32 ± 0.26	0.16 ± 0.10	0.05 ± 0.03

the binding study with plasma proteins, 50 μl aliquots of plasma and ultrafiltrates (in triplicate) were mixed with 4 ml of a liquid scintillant (Ecolite, ICN, Aurora, OH) in a vial for direct counting. For the binding with red blood cells, blood samples (20 μl) were solubilized with 0.2 ml hyamine hydroxide (ICN, Costa Mesa, CA) and incubated for 24 hours at 50°C. The samples were then decoloured with 0.5 ml hydrogen peroxide (VWR Scientific, Mont-Royal, Quebec) and further incubated for 3 hours before counting in 15 ml of a scintillation liquid (Cytoscint, ICN, Costa Mesa, CA).

Data Analysis

Calculation of Pharmacokinetic Parameters

The medium concentration data were analyzed by a non linear least squares program (PCNONLIN, Scientific Consulting Inc., Apex, NC) and by model independent equations (9).

Hepatic clearance (Cl) was estimated from the ratio of the hexarelin initial dose (C_0) measured just before the beginning of the perfusion per volume of perfusate (V_R), to the calculated $AUC_{0-\infty}$:

$$Cl = (C_0 \cdot V_R)/AUC_{0-\infty} \quad (1)$$

The clearance values were expressed as Cl/gram of liver. The hepatic extraction ratio (E) was determined by dividing Cl by the perfusion flow rate (Q)

$$E = Cl/Q \quad (2)$$

Linearity of the three doses (D) was verified by determining the ratio $AUC_{0-\infty}/D$ for each concentration.

Calculation of Drug Binding

The fraction of hexarelin bound (β) to plasma proteins was calculated according to equation (3)

$$\beta = [T - U/(1 - NSB)]/T \quad (3)$$

where T is the concentration of total radioactivity, U is the radioactivity found in the ultrafiltrate, and NSB is the amount of non-specific binding of hexarelin to the micropartition tube or membrane.

The fraction of drug distributed in red blood cells (f_{RBC}) was determined from

$$f_{RBC} = 1 - [(1 - H) \cdot (C_p/C_b)] \quad (4)$$

where H is the hematocrit, and C_p/C_b is the ratio of drug concentration found in plasma and blood.

Statistical Methods

Statistical analysis of clearance, half-life, and extraction ratio parameters were conducted using one way ANOVA in order to verify difference between dose groups ($p < 0.05$).

RESULTS

Liver Perfusion

Metabolic Viability

For all the studies, livers appeared macroscopically intact. They were not swollen nor did they contain patchy areas that may suggest malperfusion or congestion. Liver function parameters monitored throughout the experiment for the control group and the three hexarelin concentrations are presented in Table I. Because no choleretic agents were added in the medium, bile production was high the first hour, then gradually decreased during the remainder of the study. LDH and AST during perfusion did not significantly differ at the three hexarelin concentrations compared with the control perfusions.

Extraction by the Perfused Liver

To determine whether or not hexarelin adsorbs to the tubing used, perfusions of the lowest hexarelin concentration (5 ng/ml) were conducted with and without the liver. As demonstrated in Figure 1, in the absence of the liver, perfusate levels of

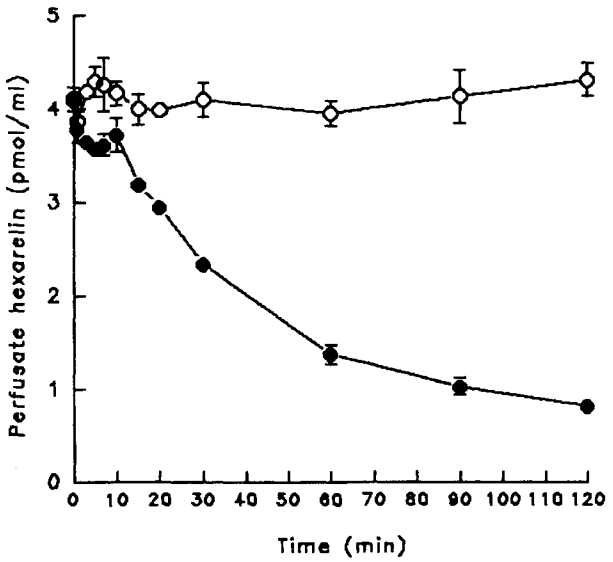

Fig. 1. Disappearance of hexarelin (5 ng/ml) from the recirculating perfusion medium with (●) and without (○) the liver [n = 4].

Table II. Pharmacokinetic Parameters of Hexarelin Following Administration of Single Doses of 5 ng/ml, 50 ng/ml or 500 ng/ml in the Perfusion Medium [n = 4]

Parameter	5 ng/ml	50 ng/ml	500 ng/ml
K (min^{-1})	0.013 ± 0.000	0.016 ± 0.002	0.014 ± 0.000
$t_{\frac{1}{2}}$ (min)	55.20 ± 1.90	45.06 ± 5.71	49.93 ± 0.81
$AUC_{0-\infty}$ (min·pmol/ml)	269.85 ± 12.55	1992.62 ± 116.64	24334.35 ± 1492.75
Cl (ml/min/g liver)	0.34 ± 0.01	0.40 ± 0.04	0.37 ± 0.02
E (%)	20.14 ± 0.88	20.95 ± 1.93	18.86 ± 0.67
AUC/D (min/ml)	0.33 ± 0.02	0.31 ± 0.03	0.32 ± 0.02

Note: Abbreviations of mean (±SEM) pharmacokinetic parameters: K, disappearance rate constant; $t_{\frac{1}{2}}$, terminal half-life; $AUC_{0-\infty}$, area under the curve from time zero to infinity; Cl, total clearance per gram of liver; E, hepatic extraction ratio.

hexarelin remained constant (4.028 ± 0.05 pmol/ml) over the two hour study.

Figure 2 illustrates the profiles of the disappearance of hexarelin from the recirculating perfusate over time following the addition of 5, 50, and 500 ng/ml hexarelin to the perfusion medium. As presented in Table II, the pharmacokinetics of hexarelin are concentration-independent. This conclusion is attributed to the fact that a linear increase in AUC was observed when increasing doses of the peptide were added to the perfusion medium. Consequently, the clearance values are not significantly different over the dose range studied, as are the half-life values. The determination of the extraction ratio indicates that hexarelin displays a hepatic extraction coefficient of 20%.

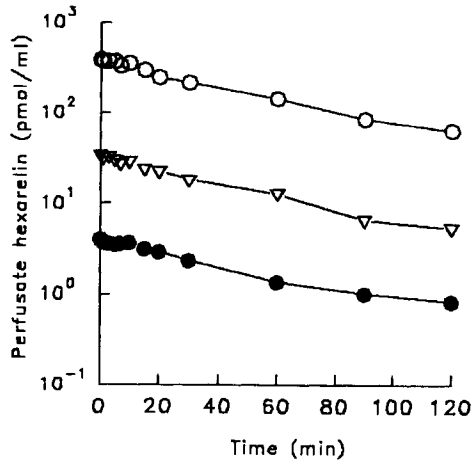

Fig. 2. Disappearance of hexarelin from the recirculating perfusate (mean ± SEM) following the addition of 5 ng/ml (●), 50 ng/ml (▽), and 500 ng/ml (○) to the medium [n = 4].

In Vitro Binding Studies

Binding to Plasma Proteins

To reduce the nonspecific adsorption of hexarelin to the micropartition tubes, an optimal concentration of 2% polyethyleneimine (PEI) was added to the Centrifree tubes and the filtrate cups. After a 36 hour incubation period, the tubes were centrifuged for 10 min at 1000 × g, and the PEI discarded. This pre-treatment step reduced nonspecific adsorption of the peptide by more than 87%. The optimal equilibrium time was determined by incubating hexarelin at a concentration of 0.03 μM in dog plasma for 0, 15, 30, 60, 90, and 120 min. It was observed that maximum binding of the peptide to plasma proteins was achieved almost instantly. Studies however, were conducted at 30 min incubation time periods. The extent of binding of hexarelin to plasma proteins is summarized in Table III. There are no appreciable differences found between rat, pig, and human plasma, all of which exhibited a significant degree of binding ($\beta \geq 64\%$). Binding of hexarelin to dog plasma protein was slightly higher ($\beta \approx 79\%$). For the concentration range studied, all species demonstrated a virtually concentration-independent degree of binding. The suitability of the ultrafiltration method using frozen plasma samples indicated that there was approximately a 6% increase in β when fresh rat plasma was used.

Binding to Red Blood Cells

The hematocrit value obtained for rat blood was 46.6% ± 0.1%. As shown in Table IV, the plasma to blood ratio (C_p/

Table III. Percent Fraction of [^{3}H]Hexarelin Bound to Plasma Proteins (β) [n = 3]

Plasma concentration (μM)	Rat[a]	Dog	Pig	Human
0.003	70.73 ± 0.72	80.73 ± 0.62	64.70 ± 0.98	65.67 ± 0.40
0.01	68.22 ± 0.97	77.99 ± 0.30	67.70 ± 0.91	63.20 ± 1.18
0.03	70.65 ± 0.55	80.78 ± 0.61	65.29 ± 0.46	65.20 ± 1.06
0.1	68.93 ± 1.25	78.63 ± 1.02	66.68 ± 2.07	63.10 ± 1.97
0.3	67.92 ± 0.74	79.22 ± 1.17	68.28 ± 1.51	65.47 ± 0.38
1	64.33 ± 0.45	76.08 ± 1.13	69.34 ± 0.79	67.33 ± 0.48
3	69.86 ± 0.98	77.63 ± 0.05	68.86 ± 0.62	66.35 ± 1.24

[a] There is approximately a 6% increase in (β) for *fresh* rat plasma versus *frozen* plasma.

Table IV. Plasma: Blood Ratio (C_p/C_b) and Fraction of [^{3}H]-Hexarelin Distributed to Rat Red Blood Cells (f_{RBC})

Blood concentration (μM)	C_p/C_b	f_{RBC}
0.003	1.54	0.17
0.01	1.62	0.14
0.03	1.72	0.08
0.1	1.54	0.18
0.3	1.65	0.12
1	1.69	0.10
3	1.66	0.11

C_b) was greater than 1.5 for the concentration range studied. This indicates that the bulk of hexarelin was present in the plasma, and there was no uptake of the peptide by red blood cells. The distribution of the peptide in blood appeared to be independent of the hexarelin blood concentration.

DISCUSSION

In the present study, a recirculating isolated perfused rat liver model was used to evaluate the hepatic extraction of hexarelin. The optimal perfusion conditions established allowed analysis with livers that displayed good overall viability parameters. All of the liver function parameters obtained from the groups treated with hexarelin were comparable with those of the control group, indicating that not only the metabolic integrity of the organ was retained, but that the presence of hexarelin at the higher doses (500 ng/ml) had no toxic effect on the liver.

Consequently, two main conclusive findings on the elimination of hexarelin by the liver have been made. First, at physiological pH and temperature, hepatic clearance of hexarelin obeys first-order kinetics over the concentration range studied because the area under the curve (AUC) increases in a first-order fashion, and clearance values remain constant independent of dose. Secondly, according to the present *in situ* model, hexarelin displays a low hepatic extraction ratio (20%). Unlike other perfusion studies whereby the use of hemoglobin-free buffers necessitate very high flow rates, and as a result, underestimate the extraction ratio, the livers in the present analysis were perfused at a rate ranging between 1.7–1.9 ml/min/g liver. According to literature, normal perfusion of rat blood flow is 1.25 ml/min/g liver (10). Hence, it would be feasible to assume that the low extraction ratio determined in this model is quite comparable, if not slightly lower, than that found *in vivo*.

Although there is no data on the hepatic extraction of GHRP analogs in the literature, the clearance of hexarelin may be compared with other neuropeptides, as cholecystokinin (CCK-8) and somatostatin (SS), whose hepatic extraction have recently been documented (11,12). Whereas the two forms of somatostatin, SS-14 and SS-28, display first-order kinetics, CCK-8 exhibits concentration-dependent kinetics. The hepatic extraction of CCK-8, in the concentration range of 5–20 ng/ml, is significantly high (40–60%) following a single-pass perfusion. These values contrast with the low extraction ratio of hexarelin documented after a two hour recirculating perfusion study. The hepatic extraction ratio of the active form of somatostatin, SS-14, was reported to be 35% following a recirculating perfusion study.

Among the several implications associated with a poorly extracted drug, the relationship between protein binding and drug clearance becomes notably important as protein binding may be a rate-limiting factor. Hence, plasma binding studies were conducted in order to assess the degree of binding of hexarelin to plasma proteins. Although the method of ultrafiltration has been extensively used to evaluate protein binding of synthetic compounds, it is not frequently employed for peptidic drugs. One major obstacle encountered when using the micropartition tubes is the high protein adsorption, a key problem common to most peptides. In order to minimize adsorption to the styrene acrylonitrile surface of the sample reservoirs, several pre-treatment agents were tested such as 0.1% bacitracin, and various concentrations of polyethyleneimine (PEI). Coating the tubes with the hydrophilic, positively charged PEI solution, at a concentration of 2%(w/v), best reduced adsorption of hexarelin to the micropartition surfaces. Thus, use of the ultrafiltration method should no longer be a deterrent for the evaluation of plasma protein binding with peptides as this successful pretreatment technique appears to be promising in reducing the non specific adsorption of small peptides to surfaces.

It has been observed that the binding of hexarelin to plasma proteins is concentration independent, and is found to be somewhat species dependent, as a slightly greater binding in dog ($\beta \approx 79\%$) is observed. Only a minor portion of the peptide in blood is taken up by blood cells. Hence, in view of the above findings, one may conclude that the hepatic clearance of hexarelin could be limited by its binding to plasma proteins and may suggest that the extraction of hexarelin might be dependent on plasma protein binding.

In conclusion, it has been demonstrated that the isolated perfused rat liver clears hexarelin in its unbound state from the perfusate by a first-order kinetic process up to a 500 ng/ml dose. The hepatic extraction ratio of hexarelin, being low, may be limited by its binding to plasma proteins. As a clinical implication, even though the contribution of intestinal metabolism on the effect of first-pass merits investigation, the low hepatic extraction of hexarelin is an advantage for the potential oral administration of the peptide.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Barré, Dr. Huet, and Dr. Varin for their expert advice in the field of liver perfusions and allowing the use of their perfusion equipment and laboratory facilities. Hexarelin was kindly provided by Dr. Deghenghi of Europeptides. This work was supported by Pharmacia Upjohn.

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