Comparison of Pharmacokinetic Parameters of a Polypeptide, the Bowman-Birk Protease Inhibitor (BBI), and Its Palmitic Acid Conjugate

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Purpose. The alteration of the pharmacokinetic parameters of the polypeptide BBI through conjugation with palmitic acid was examined.
Methods. ^{125}I-BBI or ^{125}I-Pal-BBI was administered iv to 6 week old CF-1 mice at a dose of 3mg/kg. The mice were sacrificed at 5, 10, 20, 60, 120, 240, 360, and 480 min and the total radioactivity was determined for blood and each organ. The blood was analyzed on a Sephadex G-50 size-exclusion column to determine the amount of intact polypeptide present in the blood. From the amount of intact polypeptide at each time point, the pharmacokinetic parameters were determined.
Results. By conjugating three palmitic acids to each BBI molecule, the area under the curve (AUC) and mean residence time (MRT) increase by a factor of 10.8 and 2.8, respectively. There was also a difference in the organ distribution between the two treatments; while ^{125}I-BBI was rapidly cleared from the kidneys, ^{125}I-Pal-BBI was predominantly to the liver. Subsequent studies suggested that the binding of the conjugate to non-albumin serum proteins was most likely the cause of the altered pharmacokinetics.
Conclusions. The residence time in the blood and the lipophilicity of BBI were increased upon conjugation with palmitic acid through a reversible disulfide linkage. Pharmacokinetic studies showed an increase in the AUC and a decrease in kidney clearance in palmitic acid conjugates, indicating a potential increase of the therapeutic efficacy of the polypeptide drug.

KEY WORDS: pharmacokinetics; polypeptide; BBI; palmitic acid; conjugation.

INTRODUCTION

One of the problems with using peptide drugs is that they are frequently subjected to enzymatic or chemical degradation and to rapid kidney elimination, resulting in very short half-lives. One way to overcome the short half-life is to give frequent injections, but when clinical restrictions are considered, this would mean administration by a trained medical professional, leading to higher medical costs and reduced patient compliance. To overcome these problems, one approach is the conjugation of long chain fatty acids to the peptide, which can increase the stability of the peptide and increase the residence time in the blood. Fatty acids have been used in the past as a method of modifying peptides, such as thyroid releasing hormone (1), gastrin (2) and insulin (3), but the emphasis of these studies has been to measure the alteration of activity and/or mucosal absorption. While the pharmacokinetics of a phospholipid-peptide conjugate has been determined (4), the changes in pharmacokinetic parameters of fatty acid-peptide conjugates has not previously been considered.

We have recently developed a novel method for the preparation of fatty acid conjugates of the Bowman-Birk protease inhibitor (BBI), an 8kDa polypeptide isolated from soybean (5), which possesses both trypsin and chymotrypsin inhibitory activities. It has already been shown that BBI can prevent transformation *in vitro* (6) and carcinogenesis *in vivo* (6,7) and has the potential for use in humans as a chemopreventive agent. In studies to improve transport and tissue targeting of BBI, modifications such as lipidization have been used to increase the stability of the polypeptide from proteolysis and to increase its lipophilicity which would increase the amount of transport via passive diffusion. One of the unique characteristics of this fatty acid conjugate is its water solubility, which eases its use *in vivo*. One of the potential side effects of derivitizing peptides and proteins is the loss of biological activity (8,9). Our BBI-palmitic acid conjugate (Pal-BBI, with an average of 3 palmitic acid moieties per BBI molecule) has been screened with an *in vitro* transformation assay which has shown the conjugate to have retained its biological activity (10). The preliminary *in vitro* data showed an increase in uptake in Caco-2 cells (10) and a slower release from the cell surface (data not shown), which could alter the pharmacokinetic parameters. An investigation of this possibility is the focus of this report.

MATERIALS AND METHODS

Synthesis and Radiolabeling of Palmitic Acid-Conjugated BBI

A novel synthetic procedure for the preparation of fatty acid-BBI conjugates was described in detail previously (10). Briefly, the conjugation of BBI to palmitic acid was performed by first synthesizing cysteine 2-pyridine disulfide (CDP) and reacting this with the N-hydroxysuccinimide ester of palmitic acid, making N-palmityl CDP (Pal-CPD). Secondly, BBI was chemically modified with N-succinimidyl propionate pyridine disulfide (SPDP) (Pierce, Rockford, IL) and this pyridine disulfide derivative of BBI was reacted with Pal-CPD, resulting in the final product, a BBI-palmitic acid conjugate with a reversible disulfide linkage (Pal-BBI). The reaction of BBI with SPDP is pH sensitive, allowing the final number of palmitic acid moieties to vary from 1 to 4.5. In this study, a conjugate with an average of 3 modifications was synthesized. After purification, the Pal-BBI conjugate and native BBI were radioiodinated using the chloramine-T method (11).

Pharmacokinetic Study

The pharmacokinetic studies of ^{125}I-BBI and ^{125}I-Pal-BBI were conducted in CF-1 mice and were compliant with the

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ABBREVIATIONS: BBI = Bowman-Birk protease inhibitor; Pal-BBI = palmitic acid conjugate of BBI; CDP = cysteine 2-pyridine disulfide; Pal-CDP = N-palmityl CDP; SPDP = N-succinimidyl propionate pyridine disulfide; BBI-PE = propylene oxide/ethylene oxide conjugate of BBI.

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"Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Two groups of twenty-four mice, 6 weeks old and weighing between 20 and 25 g each, were injected intravenously via the tail vein with unlabeled BBI spiked with a ^{125}I-BBI label or unlabeled Pal-BBI spiked with a ^{125}I-Pal-BBI label, respectively, at a dose of 3mg/kg and with a specific radioactivity of 6.44 μCi/mg for both compounds, corresponding to approximately 1×10^6 CPM per mouse. Three animals from each group were sacrificed at 5, 10, 20, 60, 120, 240, 360, and 480 min. post-injection, respectively. The animals were anesthetized with ethyl ether and blood (0.5–1.0 ml) was collected by heart puncture. Liver, kidneys, lungs, spleen, stomach, intestines, and colon were removed, and the organs were rinsed with fresh, isotonic phosphate-buffered saline (PBS, pH 7). The organ-associated radioactivity was determined in a gamma counter (Packard, Meriden, CT) and the results presented as mean percent injected dose per tissue ± S.D. vs. time (min). A 0.2 ml aliquot of blood was counted for radioactivity to determine the concentration. A value of 2.1 ml was used as the total blood volume per mouse to determine the percent of the injected dose (12).

Analysis of Blood Radioactivity Composition

To each 0.2 ml aliquot blood sample, 0.8 ml of distilled water was added. After vortexing, the blood was incubated in a 37°C water bath for 10 min to lyse the red blood cells. Blood was pooled by combining a 0.33 ml aliquot from the three animals of the same group and same time point. The pooled blood was centrifuged at 200× g for 10 min to remove cell ghosts. To determine the percent of the total radioactivity corresponding to intact protein, a 0.8 ml aliquot of the blood supernatant was applied to a size exclusion Sephadex G-50 column (20 ml) and eluted with PBS, pH 7. One and a half column volumes (30 ml) were collected in 1 ml fractions, and the radioactivity in each fraction was determined, assuming that intact protein eluted at void volume (10 ml), whereas degradation products eluted at column volume (20 ml). The concentration of intact polypeptide was calculated by multiplying the total concentration by the percentage of intact polypeptide.

Determination of Pharmacokinetic Parameters

The pharmacokinetic analysis of the blood concentration vs. time data was performed using a two-compartment model with the RSTRIP program (Micromath, Salt Lake City, Utah). This program determines the mean residence time (MRT) and calculates the area under the concentration vs. time curve (AUC) using the trapezoidal rule and estimates the half-life of ^{125}I-BBI and ^{125}I-Pal-BBI in blood using a nonlinear, weighted, least squares regression. The parameters were determined based on intact protein at each time point for both groups, as described above. The apparent blood clearance (Cl) and steady state volume of distribution (V_{dss}) were calculated with the parameters from RSTRIP using the following equations:

$$Cl = Dose/AUC$$

$$V_{dss} = Cl^* MRT$$

Binding of BBI and Pal-BBI to Serum Proteins

To determine the *in vitro* binding of BBI and Pal-BBI to serum, ^{125}I-BBI or ^{125}I-Pal-BBI was incubated with approximately 0.175 ml of mouse serum at 37°C for 60 min. Subsequently, the serum solution was diluted with 0.6 ml of PBS, and was then analyzed on a Sephacryl S-200 gel-filtration column (40 ml) and eluted with PBS, pH 7 until one and a half column volumes were collected. The recovery of radioactivity from the column was greater than 95% for every sample.

To determine the *in vivo* binding of the polypeptide to plasma proteins, an aliquot of plasma from ^{125}I-Pal-BBI-treated mice was analyzed in the same manner as the above *in vitro* sample.

RESULTS

At the completion of the animal experiment, the isolated organs were counted in a gamma counter. Organs with significant levels of radioactivity are presented as mean percent injected dose per tissue ± S.D. for ^{125}I-BBI or ^{125}I-Pal-BBI treatments (Fig. 1). The spleen, brain, and lungs had less than 2% of the injected dose at any time point, so were not shown in this figure.

In order to properly determine the pharmacokinetic parameters of ^{125}I-BBI and its palmitic acid conjugate, the amount of intact polypeptide had to be determined. In order to do this, the blood was analyzed on a 20 ml size exclusion Sephadex G-50 column to distinguish the intact polypeptide from smaller, degradation products. Table I shows the amount of intact polypeptide as a function of time for ^{125}I-BBI and ^{125}I-Pal-BBI. The amount of radioactivity in the last two time point from the ^{125}I-BBI dose was insufficient to quantify on the Sephadex G-50 column. At 4 hr, there was 6.8% intact polypeptide in the blood, so a conservative estimate of 6.6% intact polypeptide was used for the 6 and 8 hr time points. As this was a conservative estimate, the differences in the AUC and β-$t_{1/2}$ for ^{125}I-BBI and ^{125}I-Pal-BBI are potentially even greater. The amount of intact polypeptide (ng) per ml of blood vs. time (min) was used to determine the pharmacokinetic parameters using the program RSTRIP. Table II shows the final pharmacokinetic parameters for both the ^{125}I-BBI and ^{125}I-Pal-BBI treatment.

The distribution half-life (α-$t_{1/2}$) for ^{125}I-BBI is approximately 5.4 min, while for ^{125}I-BBI it is approximately 11.1 min. Once equilibrium is established, the elimination half lives (β-$t_{1/2}$) are 105.4 min and 176.0 min, respectively. The mean

Table I. IV Study of ^{125}I-BBI and ^{125}I-Pal-BBI in Mice % Intact[a] Polypeptide in Blood

Time (min)	BBI	Pal-BBI
5	80.1	96.0
10	61.3	92.5
20	27.9	83.9
60	14.3	69.5
120	8.6	69.4
240	6.8	58.4
360	—[b]	61.6
480	—[b]	45.8

[a] "Intact" refers to the % of radioactivity found at the Sephadex G-50 column void volume when compared to the total amount of radioactivity in the blood sample analyzed on the column.

[b] The amount of radioactivity of the sample was not sufficient to be analyzed on the Sephadex G-50 column.

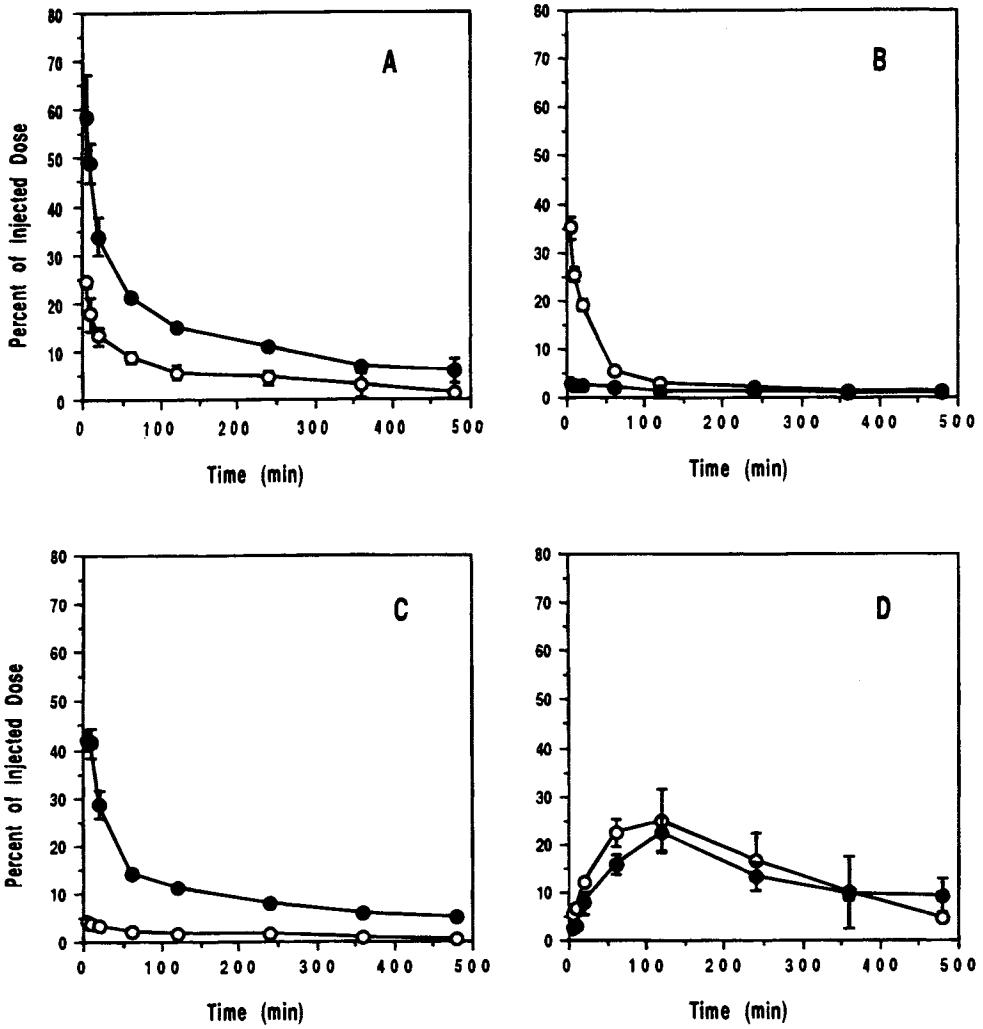

Fig. 1. Total radioactivity per organ as percent of the total injected dose following iv administration of ^{125}I-BBI (○) and ^{125}I-Pal-BBI (●) in mice. The biological matrices with significant levels of radioactivity were the blood (A), kidneys (B), liver (C), and GI tract (D). Each mouse was administered 3 mg/kg, either as the free polypeptide or as the palmitic acid conjugate. Each point represents an average of three animals. The standard deviations are represented with error bars, or are smaller than the symbols.

residence time (MRT) of ^{125}I-Pal-BBI is 218.6 min, which is 2.8-fold higher than that of ^{125}I-BBI, i.e. 77.3 min. The area under the curve (AUC) is the PK parameter with the most dramatic difference. By conjugating three palmitic acids to BBI, the AUC increases by a factor of 10.8.

Table II. Relevant Pharmacokinetic Parameters Following IV Administration of ^{125}I-BBI and ^{125}I-Pal-BBI

	BBI	Pal-BBI
MRT (min)	77.3	218.6
AUC (min.μg/ml)	163.8	1773.5
Cl_T (ml/min.kg)	18.3	1.69
α-$t_{1/2}$ (min)	5.42	11.06
β-$t_{1/2}$ (min)	105.4	176.0
V_d (ml/kg)	1415.0	369.8

For ^{125}I-BBI and ^{125}I-Pal-BBI, the percent of the injected dose was below 2% at all time points in the lungs and spleen. In the blood samples (Fig. 1A) from ^{125}I-BBI-treated mice, the level of radioactivity was 24.3% of the injected dose at 5 min post-dose and declined exponentially to a terminal value of 1.1% at 480 min. In ^{125}I-Pal-BBI-treated mice, blood levels were 58.3% of the injected dose at 5 min post-dose and 5.8% of the injected dose remained at 480 min. When the levels in the kidneys (Fig. 1B) were compared with those in the liver (Fig. 1C), there was a dramatic, yet understandable difference between ^{125}I-BBI and ^{125}I-Pal-BBI. The levels in the kidneys for ^{125}I-BBI started off at 35% of the injected dose, and decreased to 1.4% over the 480 min time course. There was a steady and pronounced clearance during the first 60 min post-injection. In comparison, the levels in the liver started off at 4% as the maximum and decreased gradually over time. On the other hand, for ^{125}I-Pal-BBI the comparison profile was practically reversed. The maximum level in the kidney (2.7%) were at the

first time point and decreased over time. In the liver, the levels were at almost 42% of the injected dose initially. For the first 10 min, the levels were relatively constant, but from 10 to 60 min, there was a rapid clearance followed by a more gradual clearance from the liver over the remainder of the study. For the GI tract (stomach, intestines and colon considered together), there was an increase until 120 min, at which time the maximum levels were reached, then the levels decreased. This trend was seen in both treatments.

For the *in vitro* binding of ^{125}I-BBI or ^{125}I-Pal-BBI in previously-isolated mouse serum, the profile of ^{125}I-BBI indicated that native BBI does not bind to serum proteins, while ^{125}I-Pal-BBI binds to a serum protein with a molecular weight higher than that of albumin (Fig. 2). Similar binding was also observed in plasma which was isolated from ^{125}I-Pal-BBI iv-injected mice (Fig. 2). When ^{125}I-Pal-BBI is incubated with pure bovine albumin, a complex forms that does not significantly change the elution profile of the albumin (13) and these results suggest that the plasma protein has a larger binding affinity for ^{125}I-Pal-BBI than that of albumin.

DISCUSSION

We demonstrated in this paper that by chemically linking palmitic acid to ^{125}I-BBI, more than a 10-fold increase can be achieved in the AUC, an important pharmacokinetic parameter. In a subsequent experiment, ^{125}I-Pal-BBI was shown to bind to a serum protein, most likely to lipoproteins, with a higher molecular weight than that of albumin (Fig. 2). The binding to the plasma proteins explains the increase in AUC of the conjugate over the native ^{125}I-BBI. The half-lives of ^{125}I-BBI and ^{125}I-Pal-BBI are not remarkably different, but that is because only the free form of ^{125}I-Pal-BBI is able to leave the circulation and be eliminated.

The binding of Pal-BBI conjugate to serum protein(s) may explain the increase of conjugate uptake in the liver and the decrease of elimination via the kidneys; consequently, an increase in AUC is observed. Once released from the serum proteins, the unbound form of ^{125}I-Pal-BBI will be less stable and will be eliminated by the kidneys in a similar manner to ^{125}I-BBI because there is not a great difference in size (9kDa versus 8kDa). It is also likely that free ^{125}I-BBI released from the fatty acid conjugate upon reduction of the disulfide linkage in the blood or organs would subsequently be eliminated from the kidneys.

Generally, when the lipophilicity of a drug is increased, the volume of distribution (V_{dss}) increases because the drug will tend to leave the central compartment and enter the periphery. However, in the case of ^{125}I-Pal-BBI, when compared with native ^{125}I-BBI, the V_{dss} is lower. This can be explained by the binding of the conjugate to serum proteins, as described above. Only the unbound form will leave the central compartment.

An amphiphilic block copolymer of ethylene oxide (hydrophilic) and propylene oxide (hydrophobic), and the surface-active polymer proxanol, was used to modify BBI previously (14). Due to a slight increase in hydrophilicity over BBI, their monoaldehyde form of proxanol on BBI (BBI-PE) was found to have reduced interactions with membranes, while our Pal-BBI conjugate demonstrated an increase in uptake of our conjugate in Caco-2 cells (10) as well as a 10.8-fold higher increase in AUC in mice.

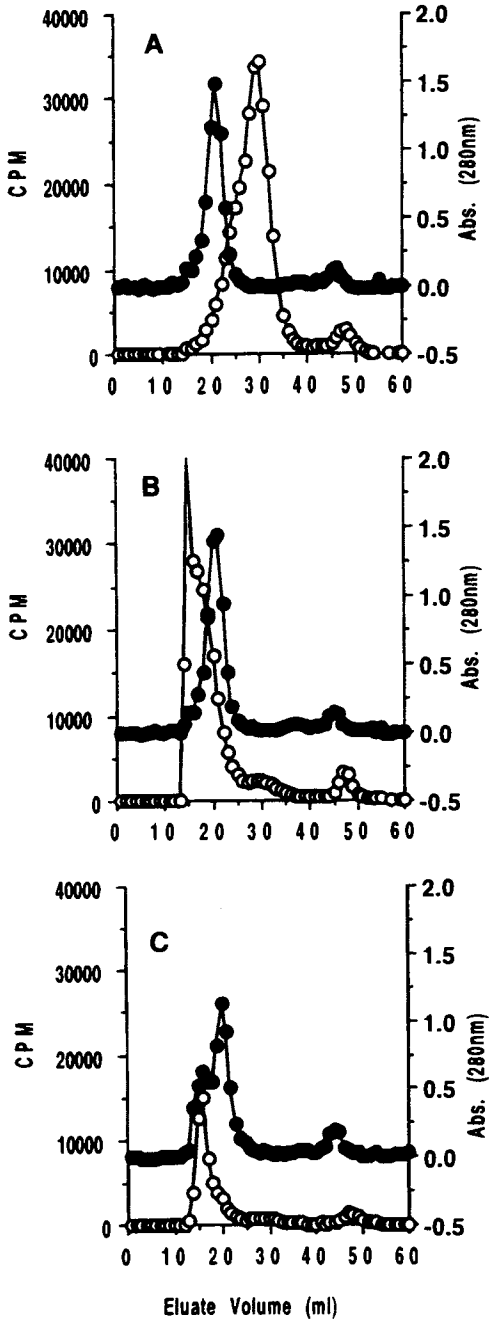

Fig. 2. The *in vitro* binding of ^{125}I-BBI (A) and ^{125}I-Pal-BBI (B) after incubation with mouse serum, compared to the ^{125}I-Pal-BBI plasma profile of ^{125}I-Pal-BBI-treated mice (C). Analysis was on a Sephacryl S-200 column (40 ml). Legend: -●- absorbance (280 nm); -○- radioactivity (CPM/fraction). Standard bovine serum albumin was used as a reference, and emerged from the column at an eluate volume of 20 ml.

In an attempt to alter the pharmacokinetics of a drug or to increase the transport, an analog of the drug can be made by chemical modification of the original drug, such as the covalent addition of an alkyl chain to increase the lipophilicity. Prodrugs are an alternative approach, and our Pal-BBI is an

example. The reversible disulfide linkages in protein conjugate can be reduced at the cell surface after being transcytosed (15). If the conjugate is brought into the cell after association with the membrane, the linkage can be reduced intracelullarly (16). Once the disulfide bonds are reduced, the active form of the drug is present to elicit the therapeutic response, which is the targeted effect of the prodrug approach.

In conclusion, the lipid modification of the polypeptide BBI has increased its lipophilicity, as demonstrated in our previous studies in Caco-2 cells (10), and has increased its AUC, as can be seen in this present report, making Pal-BBI a better drug candidate than its native counterpart, BBI. By derivitizing BBI with palmitic acid, we have changed the pharmacokinetic parameters, and prolonged the blood circulation. For BBI and other peptide or protein drugs, this could mean a longer duration of action and higher tissue exposure, and is a viable approach in drug design.

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