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

Laura Honeycutt,1 Jeff Wang,1 Hossein Ekrami,1 and Wei-Chiang Shen1,2

Received May 1, 1996; accepted June 21, 1996

Purpose. The alteration of the pharmacokinetic parameters of the polypeptide BBI through conjugation with palmitic acid was examined.

Methods. 125I-BBI or 125I-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 125I-BBI was rapidly cleared from the kidneys, 125I-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 125I-BBI and 125I-Pal-BBI were conducted in CF-1 mice and were compliant with the
“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 125I-BBI label or unlabeled Pal-BBI spiked with a 125I-Pal-BBI label, respectively, at a dose of 3 mg/kg and with a specific radioactivity of 6.44 μCi/mg for both compounds, corresponding to approximately $1 \times 10^6$ 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 125I-BBI and 125I-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 (Vss) were calculated with the parameters from RSTRIP using the following equations:

\[ Cl = \frac{Dose}{AUC} \]
\[ V_{ss} = Cl \times MRT \]

**Binding of BBI and Pal-BBI to Serum Proteins**

To determine the in vitro binding of BBI and Pal-BBI to serum, 125I-BBI or 125I-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 125I-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 125I-BBI or 125I-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 125I-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 125I-BBI and 125I-Pal-BBI. The amount of radioactivity in the last two time point from the 125I-BBI dose was insufficient to quantify on the Sephadex G-50 column. At 4 h, 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-t1/2 for 125I-BBI and 125I-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 125I-BBI and 125I-Pal-BBI treatment.

The distribution half-life (α-t1/2) for 125I-BBI is approximately 5.4 min, while for 125I-BBI it is approximately 11.1 min. Once equilibrium is established, the elimination half lives (β-t1/2) are 105.4 min and 176.0 min, respectively. The mean

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>BBI</th>
<th>Pal-BBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>80.1</td>
<td>96.0</td>
</tr>
<tr>
<td>10</td>
<td>61.3</td>
<td>92.5</td>
</tr>
<tr>
<td>20</td>
<td>27.9</td>
<td>83.9</td>
</tr>
<tr>
<td>60</td>
<td>14.3</td>
<td>69.5</td>
</tr>
<tr>
<td>120</td>
<td>8.6</td>
<td>69.4</td>
</tr>
<tr>
<td>240</td>
<td>6.8</td>
<td>58.4</td>
</tr>
<tr>
<td>360</td>
<td>—b</td>
<td>61.6</td>
</tr>
<tr>
<td>480</td>
<td>—b</td>
<td>45.8</td>
</tr>
</tbody>
</table>

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.