Disposition of Basal Insulin Peglispro Compared with 20-kDa Polyethylene Glycol in Rats Following a Single Intravenous or Subcutaneous Dose

Mary Pat Knadler, Bernice B. Ellis, Patricia L. Brown-Augusburger, Anthony T. Murphy, Jennifer A. Martin, and Victor J. Wroblewski

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana

Received April 10, 2015; accepted July 14, 2015

ABSTRACT

Basal insulin peglispro (BIL) comprises insulin lispro covalently bound to a 20-kDa polyethylene glycol (PEG) at lysine B28. The biologic fate of BIL and unconjugated PEG were examined in rats given a single 0.5-mg/kg i.v. or 2-mg/kg s.c. dose of BIL with 14C label in 20-kDa PEG or 125I label in lispro. Unconjugated 14C-labeled 20-kDa PEG was dosed at 10 mg/kg i.v. or s.c. Blood, urine, and feces were collected up to 336 hours after dosing. Radioactivity in urine was 20-kDa PEG with lysine attached. The half-lives of 14C after BIL and PEG dosing were similar. The clearance of immunoreactive BIL was 2.4-fold faster than that of 14C and 1.6-fold faster than 125I. After a subcutaneous dose of BIL, immunoreactive BIL accounted for 31% of the circulating 125I and 16% of the circulating 14C, indicating extensive catabolism of BIL. Subcutaneous bioavailability of BIL was 23%–29%; bioavailability for unconjugated PEG was 78%. For unconjugated PEG, most of the 14C dose was recovered in urine. For BIL, ≥86% of 125I was eliminated in urine and 14C was eliminated about equally in urine and feces. The major 14C-labeled catabolism product of BIL in urine was 20-kDa PEG with lysine attached. The attachment of 20-kDa PEG to lispro in BIL led to a different elimination pathway for PEG compared with unconjugated 20-kDa PEG.

Introduction

Basal insulin peglispro (BIL, LY2605541) is a novel, PEGylated insulin lispro that has a large hydrodynamic size (Beals et al., 2009). The compound has a covalently bonded 20-kDa polyethylene glycol (PEG) attached to insulin lispro through a urethane linkage on the lysine at position 28 in the B-chain (Moore, et al., 2014). The hydrodynamic diameter is 7.8 ± 0.4 nm, a diameter 4 times larger than that of insulin lispro and analogous to the size of an ~75-kDa globular protein. In contrast, insulin lispro is a rapid-acting analog of insulin (Humalog, 1996) in which the amino acid proline at B28 has been replaced with lysine and the lysine at B29 has been replaced by proline, yielding a molecular weight of 5.8 kDa.

PEGylation of protein compounds in development as pharmaceutical agents has resulted in novel therapeutic agents with modified pharmacokinetic (PK) profiles that may improve their therapeutic potential (Webster et al., 2007, 2009; Fishburn, 2008). BIL has a prolonged duration of action compared with insulin lispro that is related to a delay in insulin absorption and a reduction in clearance. BIL is currently in phase 3 studies for the treatment of patients with type 1 or type 2 diabetes mellitus.

There are many PEGylated therapeutic compounds either in drug development or currently used in the pharmaceutical drug marketplace; however, little is known about the biologic fate of the PEG moiety associated with PEGylated proteins. The PEG moieties used in currently available drugs range in size from a few hundred daltons to 40-kDa PEG, and some molecules have multiple PEG moieties attached (e.g., pegloticase has 40 10-kDa PEG moieties attached). The size of the PEG moiety has been reported to affect the elimination of the PEG (Webster et al., 2009); however, little information is available on how the drug or protein attached to the PEG moiety can affect the disposition of the different PEGs.

Tracking the metabolic fate of the PEG moiety is challenging because PEG has no UV chromophore, is nonfluorescent, has few high-affinity antibodies, and is difficult to ionize. In addition, high-molecular-weight PEGs (>30 kDa) are refractory to mass spectrometry (MS) analyses because of their polydispersed nature (Wang et al., 2012a). NMR spectroscopy has been used to analyze the biologic fate of a PEGylated protein; however, a full mass balance could not be established with NMR spectroscopy, and these experiments could not tease apart the fates of the intact molecule and metabolites with PEG attached. There are reports of studies using immunoblotting with antibodies to PEG combined with NMR spectroscopy to assess the tissue uptake of a 40-kDa PEG moiety attached to insulin (Elliott et al., 2012). More recently, 3H, 125I, and 14C have been used to assess the disposition of PEGylated proteins such as 40-kDa branched

ABBREVIATIONS: AUC, area under the curve; BIL, basal insulin peglispro; CL, clearance; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; ISF, in-source fragmentation; LC, liquid chromatography; LY2605541, basal insulin lispro; MS, mass spectrometry; PEG, polyethylene glycol; PK, pharmacokinetic; t1/2, half-life; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.
PEGylated $[^{125}I]$Adnectin (Wang et al., 2012a), 2 $\times$ 20-kDa PEGylated $[^{14}C]$ peginesatide (Woodburn et al., 2012, 2013), 46-kDa PEGylated $[^{1}H]$neuromedin-U receptor agonist (Zou et al., 2013), and 30-kDa PEGylated $[^{125}I]$erythropoietin (Wang et al., 2012b).

For this report, we sought to characterize the disposition of BIL with a focus on the PEG component and compare those findings to the fate of unconjugated $[^{14}C]$20-kDa PEG. The fates of the PEG and lispro portions of BIL were examined by analyzing the PK and elimination profiles of $[^{14}C]$20-kDa PEG–labeled BIL and $[^{125}I]$lispro–labeled BIL after subcutaneous or intravenous administration in rats. For comparison, the fate of unconjugated $[^{14}C]$20-kDa PEG was similarly analyzed for PK and elimination profiles in rats in a separate study.

Materials and Methods

Materials. The $[^{14}$-$^{15}$C]-radiolabeled PEG test articles ($[^{14}$-$^{15}$C]$^{20}$-kDa PEG and $[^{14}$-$^{15}$C]BIL) were synthesized by Analytical Bio-Chemistry Laboratories, Inc. (Columbia, MO); the BIL was labeled with $[^{125}$I at Eli Lilly and Company (Lilly Research Laboratories, Indianapolis, IN). The $[^{14}$C]-label was placed on carbons 4 through 8 on the 20-kDa PEG from the urethane bond in the BIL. Lactoperoxidase was used to iodinate the tyrosines in the lispro portion of BIL, and free iodine was removed from the preparation. The specific activity of the $[^{14}$C]-labeled BIL was 7.89 $\mu$Ci/μg, the radiopurity was 97%, and the chemical purity was 95%–99% by high-performance liquid chromatography (HPLC). The specific activity of the $[^{125}$I]-labeled BIL was 0.23 $\mu$Ci/μg, and the radiopurity was 99%. The $[^{14}$C] in the unconjugated PEG was on carbons 4 and 5. The specific activity of the $[^{14}$C]-unconjugated PEG was 4.8 $\mu$Ci/μg, and the radiopurity was 99.5%. $[^{14}$C]-lysine was synthesized at Eli Lilly and Company. Permafluor, Carbo-Sorb, and Ultima Gold XR were obtained from PerkinElmer (Waltham, MA); HPLC scintillation cocktail (Ultima Flo M) was obtained from PerkinElmer (Waltham, MA); and PLRP-S polymeric reversed-phase HPLC column (100 $\times$ 0.3 mm) was obtained from Higgins Analytical, Inc. (Mountain View, CA).

Study Design. The rat studies were conducted at Covance Laboratories, Inc. (Madison, WI). The studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the Covance Animal Care and Use Committee. Male Sprague-Dawley rats, aged 7–9 weeks, were obtained from Harlan Laboratories (Indianapolis, IN). For the investigation of BIL, the rats were assigned to eight groups: two groups received intravenous $[^{14}$-$^{15}$C]BIL, two groups received subcutaneous $[^{14}$-$^{15}$C]BIL, two groups received intravenous $[^{125}$I]BIL, and two groups received subcutaneous $[^{125}$I]BIL. For each dose paradigm, one of the two groups was used for PK sampling and one was used for mass balance determination.

In the study evaluating the biologic fate of unconjugated PEG, male rats were assigned to four groups: two groups received the intravenous dose, and two groups received the subcutaneous dose. For each dose paradigm, one of the two groups was for PK sampling and one was for mass balance.

Dosing and Sample Collection. For both studies, the volume of radiolabeled dose formulation to be administered to each rat was calculated by using a weighting factor of 1/$Y$. The quantitative range for the serum assay was 75–500 pM, and for urine it was 25–500 pM. Samples with higher concentrations were analyzed after dilution of the sample. The percentage relative error, a measure of accuracy, was <1% for serum and <5% for urine. The percentage relative S.D., a measure of precision, was <25% for both serum and urine assays.

Characterization of Serum and Urine PEG-Derived Products Using Liquid Chromatography–MS. The serum and urine profiles of $[^{14}$-$^{15}$C]PEG-derived products after subcutaneous administration of BIL were assessed by using a modified liquid chromatography (LC)–MS method described by Liu et al. (2013) for characterizing PEG and its related products in biologic matrices. This method provided information regarding the identity of the products but was not used to quantitate the products. Serum samples and urine samples from rats in each group were each pooled by time point, and a 50-μl aliquot was mixed with 75 μl of 0.1% trifluoroacetic acid (TFA) in acetonitrile. After centrifugation, a 50-μl aliquot of the supernatant was mixed with 25 μl of
0.1% formic acid in water. The sample was centrifuged at 4000 rpm for 20 minutes at 4°C, and the supernatant was injected onto the chromatographic system.

A combination of intact and in-source fragmentation (ISF) techniques was applied to the MS analyses. Intact mass of the PEGylated products was obtained using a PLRP-S column (100 × 0.3 mm) at 85°C with a binary gradient consisting of mobile phase A (0.01:100, TFA/water) and mobile phase B (0.01:100, TFA/water). The binary gradient was set up for a programmed elution profile of time, %B (0.00, 40) (2.00, 40) (2.05, 50) (16.0, 65) (16.05, 95) (17.00, 95) (17.05, 40) (22.00, 40) at a flow of 15 μl/min. The effluent from the chromatographic system was mixed with a secondary pump delivering 3.5 μl/min of 0.5:50:50 diethylmethylamine/water/acetonitrile to provide charge reduction. The neutralized effluent was connected to a Micromass Synapt Q-TOF mass spectrometer (Waters, Inc., Milford, MA) for detection in the positive ion mode. Post-column neutralization was used for analyses of intact PEGylated products.

ISF MS/MS confirmation of the PEGylated products was obtained by using a similar chromatographic system described above without the post-column neutralization. The chromatographic system was connected directly to the Micromass Synapt Q-TOF mass spectrometer for mass spectral detection in the positive-ion mode. The eluent from the LC was introduced into the Q-TOF using an electrospray source.

For the post-column neutralization analyses and the ISF, data from the Q-TOF mass spectrometer were collected using MassLynx (version 4.1; Waters, Inc.) and MaxEnt1 (Waters, Inc.) deconvolution software.

**Data Analyses.** The key PK parameters reported for radioactivity in serum or plasma and for immunoreactive BIL in serum included maximum concentration (Cmax), the time to reach maximum concentration (Tmax), extrapolated concentration at time zero (C0), half-life (t1/2), area under the concentration-time curve from 0 to the last measurable time point (AUC0–∞), area under the concentration-time curve from 0 to infinity (AUC0–∞), volume of distribution (Vd), total body clearance (CL), and bioavailability([AUC0–∞,inf]/[AUC0–∞,inf]) × (DoseC₀/DoseC₀). Noncompartmental PK parameters were calculated using WinNonlin Professional Edition, version 5.2 (Pharsight Corporation, Princeton, NJ). A composite concentration-time curve was used to calculate the PK parameters due to the sparse sampling paradigm, where there were samples from 3–4 animals per time point. S.D. values were calculated for the individual time points and are shown in the figures, but no further statistical analyses were conducted. Dose tables were compiled with mean and S.D. values calculated with Excel, version 11.0 (Microsoft Corporation, Redmond, WA). Radioanalysis data tables were generated by Debra, version 5.7.8.124 (LabLogic Systems Ltd., Sheffield, UK).

**Results**

**Pharmacokinetics.** Following intravenous administration, the serum CL of immunoreactive BIL was ~2.4-fold greater than the CL of 14C radioequivalents and 1.6-fold greater than the CL of 125I radioequivalents (Table 1). The CLs of immunoreactive BIL after 14C dosing and 125I dosing were similar, although the t1/2 values appeared to differ. Immunoreactive BIL was quantifiable longer after 125I administration and so appears to have a longer elimination phase. The mean Vd of immunoreactive BIL was ~2.4-fold lower than the Vd of total 125I radioactivity in serum and 47-fold lower than that of 14C radioactivity in serum.

Following subcutaneous administration of 14C-BIL, radioactivity was rapidly absorbed, with the same mean Tmax values for both total radioactivity and immunoreactive BIL. AUCs for immunoreactive BIL were 41% and 16% of those for circulating 14C after intravenous and subcutaneous dosing, respectively (Fig. 1; Table 1). AUCs for immunoreactive BIL were 63% and 31% of those for circulating 125I after intravenous and subcutaneous dosing, respectively. The subcutaneous bioavailability of BIL was 23%–29%, and it was determined on the basis of comparison of the AUC of 14C radioactivity after intravenous and subcutaneous administration that ~75% of the dose was absorbed. After subcutaneous dosing, the AUC of 14C was 1.75-fold greater than the AUC of 125I, indicating that during catabolism the protein portion with the 125I- and the 13C-containing PEG moiety was cleaved. After subcutaneous administration of both 14C[BIL] and [125I]BIL, the molecule appeared to be extensively catabolized; some of the catabolism was most likely to have occurred before reaching the systemic circulation. The t1/2 of 14C was 4.4-fold longer than that of 125I, whereas the t1/2 of immunoreactive BIL was longer for the 125I group than for the 14C group, as observed after intravenous administration.

In the unconjugated PEG study, following the subcutaneous dose, the highest mean concentrations of radioactivity in plasma were at 10.5 hours after dosing (Fig. 2; Table 1). Of note is that the first sample was not collected until 6 hours after dosing in this study. After subcutaneous and intravenous administration, radioactivity was eliminated from plasma with a mean t1/2 of 202 ± 27 hours and 165 ± 8 hours, respectively, which was similar to that for 14C after administration of either intravenous or subcutaneous [14C][BIL]. The CLs of 14C radioactivity for unconjugated PEG and PEG from BIL were similar following intravenous dosing. The relative subcutaneous bioavailability of [14C]20-kDa PEG in rats was calculated to be ~78%, which was higher than the bioavailability of immunoreactive BIL but similar to the percentage of the [14C][BIL] dose absorbed.

**Excretion.** Excretion of radioactivity was generally similar after subcutaneous or intravenous dosing of [14C][BIL], although a slightly

**TABLE 1**

Estimated mean PK parameters after SC and IV administration

<table>
<thead>
<tr>
<th>Route</th>
<th>Dose</th>
<th>SC</th>
<th>IV</th>
<th>SC</th>
<th>IV</th>
<th>SC</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>14C</td>
<td>125I</td>
<td>14C</td>
<td>125I</td>
<td>14C</td>
<td>125I</td>
<td>14C</td>
</tr>
<tr>
<td>2 mg/kg (77 nmol/kg)</td>
<td>0.5 mg/kg (19.4 nmol/kg)</td>
<td>2 mg/kg (77 nmol/kg)</td>
<td>0.5 mg/kg (19.4 nmol/kg)</td>
<td>10 mg/kg (500 nmol/kg)</td>
<td>10 mg/kg (500 nmol/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₀ or Cmax (nM or nM eq)</td>
<td>102</td>
<td>95</td>
<td>550</td>
<td>641</td>
<td>93</td>
<td>41.6</td>
<td>371</td>
</tr>
<tr>
<td>T max (h)</td>
<td>1</td>
<td>1</td>
<td>N.A.</td>
<td>N.A.</td>
<td>12</td>
<td>3</td>
<td>N.A.</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>197</td>
<td>6</td>
<td>191</td>
<td>5</td>
<td>45</td>
<td>35</td>
<td>31</td>
</tr>
<tr>
<td>AUC0–∞ (nM eq·h or nM·h)</td>
<td>4530</td>
<td>930</td>
<td>1680</td>
<td>809</td>
<td>3370</td>
<td>1030</td>
<td>1740</td>
</tr>
<tr>
<td>CL (l/h per kg)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>0.00978</td>
<td>0.0239</td>
<td>N.A.</td>
<td>N.A.</td>
<td>0.0111</td>
</tr>
<tr>
<td>Vd (l/kg)</td>
<td>N.A.</td>
<td>N.A.</td>
<td>1.43</td>
<td>0.030</td>
<td>N.A.</td>
<td>N.A.</td>
<td>0.176</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>75</td>
<td>29</td>
<td>N.A.</td>
<td>N.A.</td>
<td>48</td>
<td>23</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

equ. equivalents of [14C][BIL] or [14C][BIL], representing any molecules containing the radioisotope 14C and 125I, although the exact identity is unknown; IV, intravenous; N.A., not applicable; SC, subcutaneous.

aRepresents immunoreactive BIL.

bThe last plasma sampling time was < 2 t1/2; therefore, the estimated mean elimination t1/2 should be interpreted with caution.
A greater percentage of the dose was recovered in feces after intravenous administration than after subcutaneous administration of the $^{14}$C-BIL (Fig. 3). $^{14}$C radioactivity was eliminated slowly in both urine and feces and was still being eliminated in both urine and feces at the end of the 2-week sample collection period after the intravenous and subcutaneous doses. Because sample collection was terminated after 2 weeks (<5 elimination $t_{1/2}$ for $^{14}$C in serum), between 32% and 40% of the $^{14}$C dose was found either in the carcass or in the cage rinses/washes. $^{125}$I radioactivity was eliminated rapidly; after intravenous and subcutaneous administration, $\sim$91%–98% of the administered radioactive dose was recovered by 72 hours after dosing, primarily in urine. Immunoreactive BIL was excreted more rapidly than the $^{14}$C radioactivity in urine and appeared to be excreted in a similar time frame as the $^{125}$I radioactivity. The slow elimination and less than full recovery of $^{14}$C were most likely due to slow elimination of catabolism products of BIL, not slow absorption. The $^{125}$I showed complete recovery of the dose, although comparison of the AUC of $^{125}$I after subcutaneous and intravenous administration indicated lower absorption.

Immunoreactive BIL in urine accounted for 11% of the intravenous dose of $^{14}$C-BIL and 2.5% of the subcutaneous dose of $^{14}$C-BIL. The mean percentages of TCA-precipitable radioactivity in urine after dosing with $^{125}$I-BIL ranged from 7.24%–29.8%, indicating that radioactivity in urine was primarily associated with small peptide fragments or free iodine, rather than with labeled intact BIL or tyrosine-containing peptides attached to the 20-kDa PEG moiety.
For the unconjugated PEG study, urinary excretion was the primary route of elimination for $^{14}\text{C}$ (Fig. 4). Some radioactivity was still being excreted 2 weeks after dosing, although the majority of the elimination occurred within the first 72 hours after dosing. Sample collection was terminated after 2 weeks, which was <5 plasma elimination $t_{1/2}$. At the end of the sample collection period, 62%–86% of the $^{13}\text{C}$ dose of unconjugated PEG had been recovered in urine and feces.

A summary of intravenous and subcutaneous excretion after dosing of $[^{14}\text{C}]20$-kDa PEG, $[^{14}\text{C}]$PEG-BIL, or $[^{125}\text{I}]$lispro-labeled BIL is shown in Fig. 5.

**Catabolism Products.** The catabolism products of BIL in serum and urine were characterized following subcutaneous administration by using MS. BIL was identified using the specific ion trace of 1821.53 amu corresponding to the A-chain + the B-chain B27 ion in the ISF MS/MS spectra. PEG-lysine is 20-kDa PEG covalently linked to lysine and was a potential catabolism product of BIL. Standards of BIL and PEG-Lys were used to establish retention times and spectral differentiation. Urine sample analyses showed that BIL was detectable only in the 0- to 12-hour urine samples even though it was quantifiable at additional time points using the ELISA. This difference was because of the higher detection limit in the MS analyses (15.5 nM) than in the ELISA (30 pM).

In addition to BIL, a second peak at 12.5 minutes was detected in the urine samples through 240 hours. Figure 6 shows zoomed spectra for the 12.5-minute PEGylated peak compared with BIL, PEG-Lys, and 20-kDa PEG. The spectra show that the peak corresponds to PEG-Lys. The intact spectra confirmed the assignment, although the MS/MS spectra of the product were insufficient. No other catabolism product was observed in the urine samples.

Serum samples analyzed by intact analyses showed that BIL was detected at 9–10 minutes but only through 12 hours. BIL was detectable in serum using the ELISA for much longer times. The lower limit of quantitation for the serum ELISA was 75 pM and that for the urine ELISA was 34 pM. ISF data showed the presence of a PEGylated catabolism product; however, identification could not be confirmed because of low signal relative to BIL. The PEGylated product did not contain the B-chain because of the lack of a distinctive signal at 875.4 amu. No other long-chain amino acids were detected in the serum, most likely indicating that the catabolism product was PEG-Lys.

Exploratory size-exclusion chromatography of urine from the rats receiving unconjugated $[^{14}\text{C}]$PEG indicated that the 20-kDa PEG remained intact, similarly to after BIL dosing (data on file, Eli Lilly and Company).

**Discussion**

BIL is a novel, PEGylated insulin lispro that has a large hydrodynamic size. It has a prolonged duration of action that is related to a delay in insulin lispro absorption and a reduction in CL (Sinha et al., 2014). The fate of therapeutic proteins is generally thought to be via catabolism to small peptides and amino acids, but the biologic fate of PEGylated proteins has not been well studied because of the difficulties in quantifying PEG. The fate of a 40-kDa PEG attached to insulin has been determined by using immunoblotting with antibodies to PEG combined with NMR spectroscopy (Elliott et al., 2012), and the tissue uptake of 40-kDa branched PEG on Adnectin was determined by using a $[^{13}\text{C}]$-labeled cross-linker on 40-kDa branched PEGylated Adnectin (Wang et al., 2012a). The disposition of $[^{13}\text{C}]$peginesatide, a $2 \times 20$-kDa PEGylated erythropoietin, after intravenous administration to rats and monkeys has been reported (Woodburn et al., 2012, 2013); renal excretion of the parent molecule was the primary route of elimination. Zhong et al. (2013) reported that for a 46-kDa PEGylated $[^{3}\text{H}]$neuromedin-U receptor agonist the protein route of elimination in both rats and dogs was urinary excretion of the parent molecule, although recovery of radioactivity ranged from 38%–80%.

In our studies, we administered $[^{14}\text{C}]$PEG-labeled BIL and $[^{125}\text{I}]$lispro-labeled BIL to assess the biologic fate of both the protein and 20-kDa PEG portions of BIL. We also determined the PK of the intact molecule (immunoreactive BIL) with a validated ELISA. By tracking the intact molecule using ELISA, the PEG moiety and its products using $^{14}\text{C}$, and the protein moiety and its products using $^{125}\text{I}$, in addition to MS for PEG products, we were able to determine the disposition of BIL and to determine its catabolism products. Finally, we compared the biologic fate of $^{14}\text{C}$-labeled unconjugated 20-kDa PEG with $[^{14}\text{C}]$PEG-labeled BIL to assess how the disposition of 20-kDa PEG is influenced by the attached protein.

Following administration of labeled BIL, greater systemic exposure and longer $t_{1/2}$ were observed for $^{14}\text{C}$ than for $^{125}\text{I}$, which indicates that the $^{125}\text{I}$ or the protein portion (lispro) of BIL (containing the labeled tyrosine residues) was cleaved from the PEG moiety. MS indicated that the 20-kDa PEG primarily retained a lysine. The resulting PEG-lysine product was cleared more slowly than the protein portion of BIL. The $t_{1/2}$ of $^{14}\text{C}$ after BIL dosing was similar to the $t_{1/2}$ of $^{14}\text{C}$ after unconjugated PEG dosing. The 23%–29% bioavailability of radiolabeled BIL and the >75% absorption of $^{14}\text{C}$ indicate extensive catabolism of BIL. This is also shown by the low percentage of the circulating $^{125}\text{I}$ and $^{14}\text{C}$ that is present as intact BIL. Therapeutic biologic drugs are generally eliminated via catabolism to smaller peptides and amino acids, but little information is available on whether this occurs presystemically or after absorption. In the case of BIL, it appears that catabolism is at least partially presystemic in rats; similar results have been reported for PEGylated erythropoietin in rats (Wang et al., 2012b). The presystemic catabolism in rats appears to be higher than in humans; the subcutaneous bioavailability of BIL in humans was reported to be 75% (Sinha et al., 2014). After administration of $[^{14}\text{C}]$BIL, the radioactivity recovered in the urine was primarily PEG-lysine, indicating that the 20-kDa PEG moiety did not undergo catabolism and that the catabolism was primarily via proteolysis on the protein moiety in BIL. Through a comparison of circulating BIL, $^{125}\text{I}$, and $^{14}\text{C}$, PEG-lysine also appeared to be a major contributor to the circulating radioactivity, although this could not be confirmed with MS because of the high MS detection level of PEGylated products in serum. Unconjugated 20-kDa PEG also did not appear to undergo catabolism but was excreted in urine primarily unchanged (data on file, Eli Lilly and Company). These data are
consistent with the current literature showing that the metabolic CL of PEGs with a molecular weight of >5000 is insignificant (Fishburn, 2008). This appears to be the case whether the PEG is unconjugated or conjugated to a protein such as lispro. Thus, the 14C can be considered to represent the "total PEG" and is the intact BIL and all of the PEG-related products that retain the 14C label, including PEG-lysine.

Following subcutaneous or intravenous dosing of [14C]20-kDa PEG or [14C]BIL, radioactivity declined over time but was still measurable.

Fig. 5. Comparison of the elimination of radioactivity following administration of [14C]20-kDa PEG, [14C]BIL, or [125I]BIL. "Other" includes the residual radioactivity in the carcass at the end of the 2 weeks. Samples were collected for 2 weeks, but urine and feces would need to be collected for ~40 days to obtain full recovery in excreta based on 5 elimination t1/2s.

Fig. 6. Summed (~40 scans) and aligned (vertical lines) positive-ion full scan spectra for 24- to 48-hour urine sample (A), 20-kDa PEG-Lys standard (B), 20-kDa PEG standard (C), and BIL standard (D). The LC eluent was introduced into the Q-TOF using an electrospray source. The region of m/z = 5190–5280 captures the most abundant charge states for BIL.
in serum after 14 days; ~13%–40% of the dose remained in the carcasses at 336 hours after dosing. Termination of the sample collection after <5 elimination t1/2, or at least 40 days, resulted in less than full recovery in urine and feces. Radioactivity was still being eliminated in urine and feces at the end of the 2 weeks, which was not unexpected given the long elimination t1/2 for 14C. The recovery of total radioactivity in other studies with PEGylated molecules has been reported to be similarly low: 38%–80% for 46-kDa PEGylated [14]H-neuromedin-U receptor agonist in rat and dog (Zou et al., 2013) and 53%–67% for 2 × 20-kDa PEGylated [14]C peginesatide in rats and monkeys (Woodburn et al., 2012, 2013). In a study with nonradiolabeled 2 × 20-kDa PEG insulin, Elliott et al. (2012) reported recovery of 35%–47% of the PEG dose in urine after 28 days. These studies and ours demonstrate the difficulty of obtaining complete recovery of the dose when PEGylated molecules are administered.

The dose of the unconjugated 20-kDa PEG was higher than the PEG dose in BIL due to the differences in specific activity of the dose and the difficulty in measuring unconjugated PEG. The use of a higher dose of unconjugated PEG did not appear to alter the elimination of the unconjugated 20-kDa PEG from being primarily urinary. As indicated in the literature, urinary excretion has been reported to be the major elimination route for PEG moieties of <50 kDa (Yamaoka et al., 1994; Webster et al., 2007). In contrast, when the 20-kDa PEG was covalently attached to lispro in BIL, the PEG appeared to be excreted about equally in urine and feces, even though examination of circulating and urinary products did not seem to show that it had been catabolized. In vitro experiments have also shown that the 20-kDa PEG in BIL is recycled out of the cells and is not catabolized (Kiselyov et al., 2014). Fecal and urinary excretion were important routes of elimination for [14]C-BIL-related radioactivity after a single subcutaneous or single intravenous dose in rats. These differences between the excretion of PEG administered alone or as part of BIL indicate that the biologic fate of the PEG is directed, at least in part, by the insulin-receptor interactions and subsequent intracellular protein degradation that occur with BIL but not with unconjugated PEG.

Mass balance studies in humans with [14]C-radiolabeled BIL are not feasible because only low doses (typically <9 nmol/kg) of BIL could be administered without hypoglycemia occurring and because high specific activity would be necessary for detection of radioactivity at such low doses. The dose of 2 mg/kg (77 nmol/kg or 8.6 U/kg) BIL s. c. chosen for the rats was the high dose that was used in toxicology studies and was much higher than the doses that are administered therapeutically. The higher dose in the rats was a dose used in the 6-month toxicity studies demonstrating dose linearity in the exposure of basal insulin peglispro (data on file, Eli Lilly and Company). The excretion observed in the rats is expected to be predictive of the excretion in humans; therefore, in humans, the PEG in BIL would be expected to be eliminated via urine and feces. There are limited data in the literature regarding the excretion profiles of PEG molecules in humans; however, it has been hypothesized that the size of the PEG moiety determines the elimination route, i.e., via urine and/or feces (Webster, et al., 2007). This study in rats demonstrates that the size of the PEG is only one determinant of the excretion and that the protein attached to the PEG moiety may play a more important role in the elimination. Thus, to understand the biologic fate/elimination of PEG in a given PEGylated biopharmaceutical agent, studies specific to the molecule are needed because the polypeptide component may influence the balance between the routes of excretion.

Together, the data suggest that elimination of PEG derived from BIL in rats is by both biliary and renal routes. Although studies with radiolabeled BIL are not feasible in humans, we would anticipate a similar observation: that PEG in BIL has two routes of elimination, nonrenal and renal, and that the lispro portion of BIL would be eliminated renally as small peptides and/or amino acids.

Acknowledgments

The authors acknowledge the assistance of Boris Czeskis of Eli Lilly and Company for radiolabeled synthesis, Jennifer Gluff of Eli Lilly and Company for study monitoring, and Nancy Sheridan of INC Research for writing support.

Authorship Contributions

Participated in research design: Martin, Brown-Augusburger, Wroblewski, Ellis, Knadler.
Conducted experiments: Murphy.
Contributed new reagents or analytic tools: Brown-Augusburger, Murphy, Ellis.
Performed data analysis: Murphy, Knadler.
Wrote or contributed to the writing of the manuscript: Knadler, Ellis, Brown-Augusburger, Murphy, Martin, Wroblewski.

References


Address correspondence to: Dr. Mary Pat Knadler, Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, DC 0710, Indianapolis, IN 46285.
E-mail: knadler_mary_pat@lilly.com