ABSTRACT:

Liraglutide is a novel once-daily human glucagon-like peptide (GLP)-1 analog in clinical use for the treatment of type 2 diabetes. To study metabolism and excretion of [3H]liraglutide, a single subcutaneous dose of 0.75 mg/14.2 MBq was given to healthy males. The recovered radioactivity in blood, urine, and feces was measured, and metabolites were profiled. In addition, [3H]liraglutide and [3H]GLP-1(7–37) were incubated in vitro with dipeptidyl peptidase-IV (DPP-IV) and neutral endopeptidase (NEP) to compare the metabolite profiles and characterize the degradation products of liraglutide. The exposure of radioactivity in plasma (area under the concentration-time curve from 2 to 24 h) was represented by liraglutide (>89%) and two minor metabolites (totaling ≤11%). Similarly to GLP-1, liraglutide was cleaved in vitro by DPP-IV in the Ala8-Glu9 position of the N terminus and degraded by NEP into several metabolites. The chromatographic retention time of DPP-IV-truncated liraglutide correlated well with the primary human plasma metabolite [GLP-1(9–37)], and some of the NEP degradation products eluted very close to both plasma metabolites. Three minor metabolites totaling 6 and 5% of the administered radioactivity were excreted in urine and feces, respectively, but no liraglutide was detected. In conclusion, liraglutide is metabolized in vitro by DPP-IV and NEP in a manner similar to that of native GLP-1, although at a much slower rate. The metabolite profiles suggest that both DPP-IV and NEP are also involved in the in vivo degradation of liraglutide. The lack of intact liraglutide excreted in urine and feces and the low levels of metabolites in plasma indicate that liraglutide is completely degraded within the body.

Introduction

Liraglutide (Victoza; Novo Nordisk A/S, Bagsvaerd, Denmark) is a once-daily glucagon-like peptide-1 (GLP-1) analog that recently has been approved for the treatment of type 2 diabetes. Human GLP-1 is released from the gut in response to meals and has the capacity to regulate insulin secretion, exert extrapancreatic glucoregulatory actions, and affect appetite and food intake (Holst, 2007). However, the very short half-life of GLP-1 in the circulation ($t_{1/2} = 1–2$ min), due to rapid metabolism by the widely distributed enzymes dipeptidyl peptidase-IV (DPP-IV) and neutral endopeptidase 24.11 (NEP) (Holst, 2007), reduces its usefulness for the treatment of type 2 diabetes. Hence, GLP-1 receptor agonists are now being developed as a new class of antidiabetes drugs. In general, these drugs have structures that are highly selective for GLP-1 receptors and are able to activate GLP-1 responses, but they are also much more resistant to enzymatic degradation. In efficacy and safety trials in patients with type 2 diabetes, once-daily liraglutide has provided a significant and sustained improvement in glycemic control, reduction of body weight, and improvement in $\beta$-cell function with a very low risk of inducing hypoglycemia (Vilsbøll et al., 2008; Garber et al., 2009; Nauck et al., 2009).

Liraglutide has 97% amino acid sequence identity to human GLP-1(7–37), but it differs from the native hormone by replacement of Lys26 position with a glutamate spacer bound to a16-C fatty acid.

ABBREVIATIONS: GLP-1, glucagon-like peptide-1; DPP-IV, dipeptidyl peptidase IV; NEP, neutral endopeptidase 24.11; PRA, Pharmaceutical Research Associates; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; AUC, area under the concentration-time curve from zero to infinity; LSC, liquid scintillation counting; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; $C_{\text{max}}$, maximum concentration; $t_{1/2}$, terminal elimination half-life.
FIG. 1. Structure of [3H]liraglutide (top) and the GLP-1 receptor agonists liraglutide and exenatide, in comparison to human GLP-1 (second from top).

[\text{N-\epsilon-\text{(-glutamyl-}(\text{\text{N-\alpha-palmitoyl})})]} \quad \text{(Fig. 1)} \quad \text{(Knudsen, 2004). This structural change gives a molecular conformation that stabilizes liraglutide against metabolic degradation and enables reversable binding to plasma albumin proteins, leading to further decreased clearance and protracted pharmacologic activity (Knudsen et al., 2000; Madsen et al., 2007). In addition, slow absorption from the subcutaneous injection site is thought to occur because liraglutide, in pharmaceutical solutions, forms a micelle-like heptamer, which disassociates at therapeutic plasma levels (Steensgaard et al., 2008). Liraglutide in human subjects shows peak plasma levels after 8 to 12 h with a terminal half-life of 13 h (Agersø et al., 2002; Elbrønd et al., 2002). Hence, the pharmacokinetic profile of liraglutide obtained by slow absorption from the injection site and subsequent protracted activity makes this drug suitable for once-daily administration.

The first GLP-1 receptor agonist to be clinically approved was exenatide (Byetta; Eli Lilly and Co., Indianapolis, IN), which has 53% amino acid sequence identity to human GLP-1 (Fig. 1) (Knudsen, 2004). Exenatide, intended for twice-daily use, is relatively resistant to degradation by DPP-IV and NEP (Hupe-Sodmann et al., 1995; Copley et al., 2006). Preclinical studies have shown that exenatide is eliminated by the kidneys exclusively by glomerular filtration and subsequent tubular catabolism (Parkes et al., 2001; Copley et al., 2006; Simonsen et al., 2006). In patients with renal dysfunction, exenatide showed reduced clearance and may thereby increase the risk of exposure-dependent side effects (Linnebjerg et al., 2007; Johansen and Whitfield, 2008). However, renal dysfunction has not been found to increase the plasma exposure of liraglutide (Jacobsen et al., 2009), indicating a different elimination pathway.

This is the first study assessing liraglutide metabolism and excretion in humans. Preclinical studies in mice, rat, and monkey (data on file, Novo Nordisk A/S) showed that [3H]liraglutide, 125I-labeled liraglutide, or [14C]liraglutide was extensively metabolized in the body. No major route of elimination could be determined, but several minor metabolites were detected in plasma with a similar overall pattern across the species. Thus, the aim of the present study was to elucidate the metabolism and excretion of liraglutide after a single subcutaneous injection of [3H]liraglutide in healthy adult men. Furthermore, the involvement of DPP-IV and NEP was examined by comparing the structures of the plasma and excreta metabolites with the in vitro [3H]liraglutide and [3H]GLP-1(7–37) degradation products and with those reported for native GLP-1.

Materials and Methods

Radioiodinated Test Material. [3H]-labeled GLP-1 and liraglutide were prepared at Novo Nordisk A/S (Novo Nordisk Park, Måløv, Denmark) according to published methods (Larsen et al., 2007). For liraglutide, the specific \(^3\)H labeling was in the palmitic moiety of the side chain (Fig. 1), whereas GLP-1(7–37) was labeled in the Tyr19 position.

Clinical Trial Product Preparation. A stock solution of approximately 38 MBq/ml and 47 μg/ml radioiodolabeled liraglutide was prepared 2 weeks before dosing and stored, protected from light, at −30 to −10°C. The stock solution was diluted with unlabeled 6.0 mg/ml liraglutide and sterile-filtered to produce the final trial product of 1.5 mg/ml and 28.5 MBq/ml in more than 99% chemical and radiochemical purity. The final trial product was produced at the Pharmaceutical Research Associates (PRA) International clinical unit (Zuidlaren, The Netherlands) and analyzed by high-performance liquid chromatography (HPLC) at the Bioanalytical Laboratory of PRA International (Assen, The Netherlands). The trial product was stored in darkness at 5°C until analysis and dosing (within 4 h from preparation). Supplementary tests for sterility and endotoxin were performed at Bactimm BV (Nijmegen, The Netherlands), and results were negative. The radioactivity dose was set according to human dosimetry calculations established by the National Radiation Protection Division (Health Protection Agency, Chilton, UK). Administration of [3H]liraglutide for the radioactive medical product was authorized by the Independent Ethics Committee of the foundation “Evaluation of Ethics in Biomedical Research” (Assen, The Netherlands). The radiation exposure in this trial, approximately 0.65 mSv, fell into the category IIa risk level (0.1–1 mSv) in the International Commission on Radiological Protection Guidelines (1992).

Clinical Study Design. The study was an open-label pharmacologic study conducted at PRA International (Zuidlaren, The Netherlands). The study protocol and informed consent documents were approved by an independent ethics committee according to local regulations. The study was performed in accordance with the Declaration of Helsinki (1964) and its subsequent revisions. All subjects provided written informed consent before the study. Seven healthy male subjects with median age of 57 years (range 47–60 years), median weight of 74.5 kg (range 64.0–88.7 kg), and median body mass index of 24.5 kg/m\(^2\) (range 22.7–27.0 kg/m\(^2\)) were recruited to participate. Subjects were not allowed to take any medication (except paracetamol or topically applied medications) within 2 weeks before dosing or to consume any alcohol or beverages or food containing methylxanthine within 48 h before dosing. Moreover, subjects were not allowed to perform strenuous exercise or smoke more than 5 cigarettes/day within 48 h before dosing. The subjects were admitted to the clinical research unit at dosing day (day 1) and remained at the unit until day 10 after dosing. Standardized meals (breakfast, lunch, evening meal, and snacks) were served all days throughout the in-house period, including day 1. Before bedtime between 9:00 PM and 10:00 PM on day 1, all subjects received a single subcutaneous injection of [3H]liraglutide (0.75 mg/14.2 MBq). Blood, urine, and feces were sampled from day 1, 2 h after dosing, until day 10, and safety evaluations including physical examination, vital signs, and hematology and clinical chemistry analysis (e.g., plasma glucose) were regularly performed. The level of total radioactivity was measured in urine and feces from day 5 onward as quick counts. If the excreted levels of radioactivity had not reached the end criterion (1000 dpm/g in pooled 24-h samples) before discharge at day 10, the subjects were asked to continue sampling urine and feces at home until the end criterion was met (quick counts were measured at an extra visit at day 12 after dosing) or until follow-up at day 14 after dosing, whichever came first.
Analysis of Liraglutide in Plasma by Enzyme-Linked Immunosorbent Assay. Venous blood was collected in tubes containing EDTA as anticoagulant. Blood samples (3 ml) were taken at 0 h (before dosing) and at 2, 4, 6, 8, 10, 11, 12, 13, 14, 16, 24, 36, 48, and 60 h after dosing and kept at −20°C until analysis by enzyme-linked immunosorbent assay (ELISA), as described previously (Agerström et al., 2002). The ELISA assay is specific to liraglutide and interference from endogenous GLP-1 and truncated versions of liraglutide is very low (Agerström et al., 2002). The plasma concentration-time data were analyzed by a noncompartmental statistical model to give the exposure as the area under the concentration-time curve (AUC) from time 0 to infinity, the observed maximum concentration ($C_{\text{max}}$), and the terminal elimination half-life ($t_{1/2}$).

Analysis of Total Radioactivity in Blood, Urine, and Feces. Duplicate samples of whole blood, plasma, urine, and feces (“wet samples”) were analyzed by liquid scintillation counting (LSC) until a statistical error (2σ) of 0.5% was obtained with a maximum counting time of 10 min, together with representative blank samples on a Packard Tri-Carb 3100 TR (PerkinElmer Life and Analytical Sciences, Waltham, MA) to detect retained $^3$H radioactivity. To calculate the content of volatile radioactivity such as tritiated water, representative blank samples on a Packard Tri-Carb 3100 TR (PerkinElmer Life and Analytical Sciences) and counted by LSC. Plasma samples (both wet and reconstituted dry samples) were dissolved with blank plasma, dissolved in liquid scintillation fluid (Monophase S; manufactured by PerkinElmer Life and Analytical Sciences, Groningen, The Netherlands) before dissolution in liquid scintillation fluid (Monophase S; PerkinElmer Life and Analytical Sciences) and counted by LSC. Plasma samples (both wet and reconstituted dry samples) were diluted with blank plasma, dissolved in liquid scintillation fluid (Flo Scint A; PerkinElmer Life and Analytical Sciences), and analyzed by LSC.

Urine sampling and LSC analysis. Urine was sampled before dosing (−0 h) and at intervals 0 to 4, 4 to 8, 8 to 12, and 12 to 24 h and then at 24-h intervals until the end of the trial. After their weight was recorded, the urine samples were kept at −20°C until further analysis. The volume of urine was determined using a standard specific gravity of 1.02 g/ml. The urine samples (both wet and reconstituted dry samples) were dissolved in Flo Scint A and analyzed by LSC.

Feces sampling and LSC analysis. Feces were sampled before dosing (−0 h) and at 24-h intervals until the end of the trial. After the total weight of each feces sample was recorded, a minimum of water (1:2 weight equivalent) was added, and the samples were homogenized with an Ultra Turrax mixer (IKA Labortechnik, Staufen, Germany) for at least 2 min. The homogenized samples were kept at −20°C until further analysis. The homogenized samples (both wet and reconstituted dry samples) were dissolved in Flo Scint A and analyzed by LSC.

Analysis of Total Radioactivity in Plasma. In the same manner, plasma samples collected at 6, 12, 24, and 48 h after dosing and pooled samples of equal aliquots of plasma collected at 2, 6, 8, 12, 16, 24 h were analyzed for each of the seven subjects. In brief, each sample was mixed with 0.5% Tween 20 (Sigma-Aldrich, St. Louis, MO) and acetonitrile (Merck, Darmstadt, Germany) in 10:1:1 (v/v/v) and centrifuged at 4°C for 5 min at 2000 g. The supernatant was collected, and the centrifugation was repeated, whereas the precipitate was discarded. The final recovered supernatant was lyophilized to dryness and reconstituted with hexafluoro-2-propanol (Fluka, Buchs, Switzerland)-water (1:1; v/v) and further analyzed by HPLC and radiochemical detection. Aliquots of the 48-h plasma samples from two subjects were used as reference samples, that is, analyzed without the above preparation.

Urine sampling and preparation. Urine samples collected at the 48 to 72 h and 120 to 144 h intervals and a pooled sample of aliquots from urine collected during the first 6 days after dosing (0–4, 4–8, 8–12, 12–24, 24–48, 48–72, 72–96, and 96–120 h intervals) were analyzed for each of the seven subjects. The weight of each aliquot was 0.25% of the total fraction weight. Each urine sample was centrifuged at 4°C for 5 min at 1500g. The supernatant was collected, lyophilized to dryness and reconstituted in water, and further analyzed by HPLC and radiochemical detection.

Feces sampling and preparation. Aliquots of the homogenized feces from the 24-h intervals collected during the first 6 days (0–120 h after dosing) were pooled and analyzed for each of the seven subjects. The weight of each aliquot was 0.1% of the total fraction weight. The radioactivity was extracted from the feces samples using liquid-liquid extraction. Each sample was mixed with methanol (Merck) (3 ml/g of homogenate), sonicated for 5 min, and centrifuged at 4°C for 5 min at 600g. The liquid fraction was collected, and the pellet was extracted twice with ethyl acetate (Merck) (3 ml/g of homogenate each), sonicated, and then centrifuged. All liquid fractions collected were pooled, and the mixture was analyzed by HPLC and TopCount NXT (PerkinElmer Life and Analytical Sciences) radiochemical detection.

Metabolite profiling. Samples of plasma, urine, and feces were analyzed by HPLC (Merck-Hitachi La Chrom D-7000; Hitachi High Technologies America Inc., Pleasanton, CA) performed with a 4-μm, 90 Å, 250 × 6.6 mm, Jupiter Proteo C-12 column (Phenomenex, Torrance, CA) at a flow rate of 1.0 ml/min. The mobile phase was a binary mixture of 0.01% trifluoroacetic acid in water (solvent A) and 0.01% trifluoroacetic acid in acetonitrile/water (9:1, v/v) (solvent B). The mobile phase gradient started with 5% of solvent B and increased linearly to 48% B over 25 min followed by a linear increase to 100% B over 55 min and a final isocratic phase with 100% B for 10 min. The HPLC radiochemical detection was performed on a Radiomatic 150TR radioactivity detector (PerkinElmer Life and Analytical Sciences) equipped with a 500-μl detector cell. The instrument was operating in the homogeneous liquid scintillation mode, and the HPLC eluent was mixed 1:2 with Ultima Flow M scintillation liquid (PerkinElmer Life and Analytical Sciences). The radiochemical detection in the feces metabolite profiling and plasma profiling reference analyses was performed on a TopCount NXT scintillation counter after collection of the HPLC eluent on 96-Well DeepWell Lumaplates (PerkinElmer Life and Analytical Sciences), 0.25 min/fraction, with an FC-204 fraction collector (Gilson, Inc., Middleton, WI). The plasma profiling reference analysis was conducted to evaluate the sample preparation procedure. The relative levels of drug material were shown to be conserved during preparation.

In Vitro Assays for DPP-IV and NEP Degradation of Liraglutide and GLP-1. For both DPP-IV and NEP incubation, a solution of the respective enzyme in Hanks’ buffered salt solution-1% human serum albumin was preincubated at 37°C for 3 to 5 min, in a 1.5-ml test tube, before the addition (solvent B). The mobile phase gradient started with 5% of solvent B and increased linearly to 48% B over 25 min followed by a linear increase to 100% B over 55 min and a final isocratic phase with 100% B for 10 min. The HPLC radiochemical detection was performed on a Radiomatic 150TR radioactivity detector (PerkinElmer Life and Analytical Sciences) equipped with a 500-μl detector cell. The instrument was operating in the homogeneous liquid scintillation mode, and the HPLC eluent was mixed 1:2 with Ultima Flow M scintillation liquid (PerkinElmer Life and Analytical Sciences). The radiochemical detection in the feces metabolite profiling and plasma profiling reference analyses was performed on a TopCount NXT scintillation counter after collection of the HPLC eluent on 96-well DeepWell Lumaplates (PerkinElmer Life and Analytical Sciences), 0.25 min/fraction, with an FC-204 fraction collector (Gilson, Inc., Middleton, WI). The plasma profiling reference analysis was conducted to evaluate the sample preparation procedure. The relative levels of drug material were shown to be conserved during preparation.
vitro incubation of [3H]liraglutide and [3H]GLP-1 with DPP-IV or NEP were analyzed by an HPLC system and online detection of radiochromatography similar to that described under Analysis of Metabolite Profiles in Plasma, Urine, and Feces. Identification of metabolites. Radioactive peaks were isolated using preparative HPLC. The chromatographic system was identical to the one used for radio-HPLC analysis; however, the radiochemical detector was replaced by a 96-well fraction collector. The radioactive fractions isolated during preparative HPLC were pooled from several injections mixed with water (1 + 1) before liquid chromatography-mass spectrometry (LC-MS) analysis. MS and tandem mass spectrometry (MS/MS) information on the formed metabolites was obtained after LC-MS and MS/MS analysis on a QSTAR XL mass spectrometer equipped with an electro-spray interface from MDS Sciex (Concord, ON, Canada).

An 1100 HPLC system (Agilent Technologies, Santa Clara, CA) was used. Gradient LC-MS analysis was made on a 4-μm, 250 × 4.6 mm Jupiter Proteo C-12 column using acetonitrile, water, and formic acid mixtures in the ratios 100:900:30 (v/v/v) and 900:100:30 (v/v/v) for mobile phase A and B, respectively, at a flow rate of 1.0 ml/min. The mobile phase started with 50% of solvent B for 5 min and increased linearly to 100% B over 30 min and then to a final isocratic phase with 100% B for 10 min, followed by a linear gradient to 50% B and isocratic elution for 5 min before next injection. After the UV detector, the mobile phase was split approximately 1:10; the majority being sent to a fraction collector and the minor part to the mass spectrometer. In the MS mode (full scan), the mass spectrometer was scanning in the range m/z 300 to 3000 using high mass (>10,000) resolution. The instrument settings were optimized with liraglutide to give the most abundant response, typically as the MH3 or MH4 ions. In the MS/MS mode (product ion scan) the mass spectrometer was scanning in the range m/z 50 to 3000 using high resolution. The samples for MS/MS analysis were introduced to the mass spectrometer by continual infusion, typically with a flow rate of 5 to 10 μL/min using the TurboIonSpray interface (MDS Sciex).

Results

Safety Assessment. All seven subjects completed the study. There were no serious events reported, and no subjects were withdrawn from the study because of adverse events. Of seven reported adverse events, occurring in five subjects, only one event was evaluated as possibly related to the trial product (nausea 8 h after dosing). Furthermore, there were no apparent treatment-related clinically relevant changes in other safety parameters including clinical laboratory tests and vital signs.

Liraglutide Pharmacokinetics. After liraglutide dosing, the mean plasma concentration-time profile (Fig. 2a) showed a relatively slow absorption with a maximum concentration at 11.7 h and mean Cmax of 10,586 pM (S.D. 3202), followed by a decline toward baseline during the next 48 h with a mean apparent t1/2 of 15.6 h (S.D. 2.1). The recovered plasma profile and calculated mean AUC of 311.1 nmol × h/l (S.D. 61.8) were comparable with results from previous trials investigating the pharmacokinetics of unlabeled liraglutide (Agersø et al., 2002; Elbrønd et al., 2002).

Plasma Radioactivity. Similar to the plasma liraglutide pharmacokinetic profile (Fig. 2a), radioactivity in plasma (Fig. 2b) peaked at 12 to 16 h after dosing with a mean Cmax of ~10,930 pmol Eq/l and declined thereafter toward baseline, still being detectable 7 days after dosing. The exposure of radioactivity in plasma was slightly larger than the exposure of total liraglutide as measured by ELISA, suggesting the presence of circulating metabolites.

Distribution of Liraglutide between Plasma and Red Blood Cells. The exposure of radioactivity in whole blood had a profile similar to that in plasma, peaking 12 to 16 h after dosing and thereafter declining toward baseline. The mean hematocrit of the subjects was 0.44 and the mean whole blood/plasma ratio was approximately 0.6 (range 0.57–0.68 between 2 and 72 h after dosing); thus, liraglutide was confirmed to be mainly distributed into the plasma compartment.
COMPONENTS WERE DETECTED IN LOW AMOUNTS IN ALL SUBJECTS: U1 ($t_{R} = 3.3–3.6$ min) AND U2 ($t_{R} = 3.9–5.0$ min), WHEREAS ONE SUBJECT SHOWED VERY LOW LEVELS OF AN ADDITIONAL COMPONENT U3 ($t_{R} = 12.9$ min) (FIG. 5). IN POOLED URINE SAMPLES (0–120 h) FROM EACH INDIVIDUAL, THE MEAN FRACTION OF THE RADIOACTIVE DOSE EXCRETED AS U1 WAS 2.9% (S.D. 1.0; $n = 7$), WHEREAS U2 REPRESENTED 0.55% (S.D. 0.22; $n = 4$) AND U3 COULD NOT BE DETECTED. THE MUCH SHORTER RETENTION TIMES FOR THESE COMPONENTS COMPARED WITH THAT OF LIRAGLUETIDE SUGGESTS THE RECOVERY OF SMALLER MORE HYDROPHILIC PRODUCTS.

IN FECES THERE WASUNEVEN RECOVERY OF THREE MINOR RADIOACTIVE COMPONENTS, F1 ($t_{R} = 40.1$ min), F2 ($t_{R} = 45.1$ min), AND F3 ($t_{R} = 82.8$ min), FROM THE DIFFERENT SUBJECTS. F1 AND F3 WERE RECOVERED IN THREE SUBJECTS (FIG. 6a), F1 AND F2 IN ONE SUBJECT (FIG. 6b), F2 AND F3 IN ONE SUBJECT (FIG. 6c), AND F3 ONLY IN ONE SUBJECT, WHEREAS THE LAST SUBJECT DID NOT HAVE ANY PEAKS AT ALL ABOVE THE DETECTION LEVEL. ALL COMPONENTS HAD LONGER RETENTION TIMES THAN LIRAGLUETIDE, SUGGESTING THE RECOVERY OF MORE LIPOPHILIC PRODUCTS. BECAUSE ALL PEAKS WERE CLOSE TO THE DETECTION LIMIT NO INDIVIDUAL QUANTITATIVE DATA WERE GENERATED. HOWEVER, BASED ON THE AMOUNTS EXCRETED IN FECES, THE COMPONENTS WERE ESTIMATED TO COMPRISE IN TOTAL 3 TO 5% OF THE TOTAL ADMINISTERED RADIOACTIVITY; THAT IS, A FRACTION SIMILAR TO THAT RECOVERED FROM COMPONENTS EXCRETED IN URINE. OVERALL, NO STRUCTURAL ELUCIDATION OF THE URINE OR FECES METABOLITES COULD BE PERFORMED BECAUSE OF THE VERY LOW AMOUNTS. TRITIATED WATER WAS NOT DETECTED BECAUSE OF THE FREEZE-DRYING STEP IN THE PREPARATION OF THE SAMPLES.

**FIG. 4.** MEAN CUMULATIVE EXCRETION OF RADIOACTIVITY IN URINE (a) AND FECES (b) OF ALL SUBJECTS ($n = 7$) AFTER A SUBCUTANEOUS SINGLE DOSE OF [3H]LIRAGLUETIDE (0.75 mg/14.2 MBq). FROM DAY 8 AND ONWARD, THE MAJORITY OF RECOVERED RADIOACTIVITY EXCRETED IN URINE WAS VOLATILE (MOST LIKELY TRITIATED WATER), WHEREAS THE FECAL EXCRETION FROM DAY 6 AND ONWARD ONLY COMPRISED TO A MINOR EXTENT VOLATILE RADIOACTIVITY AS SHOWN BY THE DIFFERENCE BETWEEN WET AND RECONSTRUCTED DRY SAMPLE ANALYSIS.

**FIG. 3.** CHROMATOGRAMS FROM HPLC ANALYSIS WITH RADIOMETRIC DETECTION SHOWING THE METABOLITE PROFILE IN PLASMA AT 6 (a), 12 (b), 24 (c), AND 48 h (d) FROM ONE SUBJECT AFTER A SUBCUTANEOUS SINGLE DOSE OF [3H]LIRAGLUETIDE (0.75 mg/14.2 MBq).

**DPP-IV AND NEP IN VITRO DEGRADATION OF LIRAGLUETIDE AND GLP-1.** INCUBATION OF LIRAGLUETIDE IN THE PRESENCE OF DPP-IV RESULTED IN ONE DEGRADATION PRODUCT WITH LONGER RETENTION TIME ($t_{R} = 40.7–40.8$ min AND $R_{t} 1.05$) THAN THE PARENT COMPOUND LIRAGLUETIDE ($t_{R} 38.7$ min) (FIG. 7a). THE CLEAVAGE SITE WAS DEMONSTRATED TO BE EXCLUSIVELY IN THE ALA8-GLU9 POSITION OF THE N TERMINUS IN THE PEPTIDE MOIETY OF LIRAGLUETIDE (FIG. 8). IN CONTRAST, LIRAGLUETIDE WAS EXTENSIVELY DEGRADED BY NEP INTO 11 COMPONENTS, ALSO WITH LONGER RETENTION TIMES THAN THE PARENT COMPOUND (FIG. 7b; TABLE 1). THE INITIAL NEP CLEAVAGE SITE OF LIRAGLUETIDE WAS AT THE SER18-TYR19 AND THE TYR19-LEU20 POSITIONS IN THE PEPTIDE MOIETY. COMPONENTS CLEAVED IN THE GLU27-PHE28 OR PHE28-ILE29 POSITIONS WERE ALSO SEEN, ALL WITH A TRUNCATED N TERMINUS BETWEEN THE TYR19 AND LYS26 POSITIONS. CONTINUED CLEAVAGE LEADS TO SHORTER PEPTIDES, ALL INCLUDING THE SITE OF THE GLUTAMIC ACID LINKER AND FATTY ACID SIDE CHAIN, LYS26 (FIG. 8). OVERALL FOR BOTH ENZYME INCUBATIONS, GLP-1 WAS DEGRADED MUCH FASTER THAN LIRAGLUETIDE, BUT GIVING RISE TO A SIMILAR SET OF DEGRADATION PRODUCTS (FIGS. 8 AND 9). THE DPP-IV AND NEP ACTIVITY WAS CONFIRMED BY THE INHIBITION OF LIRAGLUETIDE OR GLP-1 DEGRADATION IN THE PRESENCE OF DIPROTEIN A AND PHOSPHORAMIDON.
Discussion

This study is the first to elucidate the metabolism and excretion of liraglutide in humans and also provides new information on degradation of liraglutide by DPP-IV and NEP. Because liraglutide is a peptide with a fatty acid attached, it has the structural prerequisite for degradation into peptides, amino acids, and fatty acid fragments. Such degradation products may be recycled into new endogenous proteins and lipids or eliminated by the liver and kidney. If fully degraded, these products could also be excreted from the body as carbon dioxide, urea, and water.

The cumulative recovery of total radioactivity in this study was 26.3% of the administered dose. The low levels of liraglutide-related radioactivity in urine and feces (11.5% of the administered dose) and the presence of volatile radioactivity in urine and plasma (presumably representing tritiated water) suggest full degradation of liraglutide within the body. Accordingly, based on the assumption that the radioactivity excreted in urine at discharge on day 14 (69 Bq/ml) represented tritiated water in equilibrium with whole body water (i.e., approximately 60% of the body weight in male subjects of this age group), 22% of the administered radioactivity was estimated to remain in the body at this time as tritiated water. According to the half-life of total body water (Foy, 1964), elimination of this would be expected to occur within the next 10 days. The remaining 52% of the administered radioactivity was considered to be bound in organic hydrogen-containing compounds likely to be eliminated with a half-life of 40 days, mainly via the urine. The recovery data and assumptions above agree well with unpublished results from animal studies in our laboratory in which subcutaneously administered liraglutide labeled with $^{125}$I or tritium in the peptide moiety mainly resulted in excretion of free $^{125}$I or tritiated water, whereas $^{14}$C labeling in the glutamate spacer mainly resulted in exhaled $^{14}$C carbon dioxide.

Native GLP-1(7–36) amide is inactivated similarly to some other circulating peptide hormones by DPP-IV via the liberation of a

![FIG. 5. Chromatograms from HPLC analysis with radiometric detection showing the metabolite profile in urine collected at day 3 (a) and day 6 (b) and in a pooled sample of days 1 to 5 (c) from one subject after a subcutaneous single dose of $^{3}$Hliraglutide. The expected retention time of liraglutide is indicated on the figures by an arrow.](#)

![FIG. 6. Chromatograms from HPLC analysis with radiometric detection showing the metabolite profile in pooled feces collected on days 1 to 6 (0–120 h) from three individual subjects (a–c) after a subcutaneous single dose of $^{3}$Hliraglutide. The expected retention time of liraglutide is indicated on the figures by an arrow.](#)
Xaa-Ala dipeptide from the N terminus (Mentlein, 1999) resulting in a GLP-1(9–36) amide metabolite (Mentlein et al., 1993; Deacon et al., 1995a,b, 1996). DPP-IV is present as a soluble plasma enzyme and as a membrane-bound enzyme throughout the capillaries and within various body compartments (e.g., kidney, liver, intestine, placenta, and bile and pancreatic ducts) (Mentlein, 1999) and has the potential

![Figure 7. Chromatograms from HPLC analysis with online radiodetection showing the metabolite profile of 1 μM [3H]liraglutide after 4, 8, and 24 h of incubation with 2.0 μg/ml DPP-IV (a) or 1.6 μg/ml NEP (b). Arrows indicate the eluted parent compound liraglutide.](image)

![Figure 8. Overview of identified liraglutide degradation products resulting from DPP-IV and NEP cleavage in vitro. The degradation products are named by a prefix for the enzyme involved, D for DPP-IV and N for NEP, and the number of the amino acid residues. The major degradation products that are in common with the GLP-1 degradation products reported in literature are marked with an asterisk.](image)

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<th>Name</th>
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<tr>
<td>N1 (20–27)</td>
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<tr>
<td>N2 (20–27)</td>
<td>8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37</td>
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<tr>
<td>N3 (19–37)</td>
<td>9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37</td>
</tr>
<tr>
<td>N5 (26–27)</td>
<td>22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37</td>
</tr>
<tr>
<td>N6 (25–26)</td>
<td>23 24 25 26 27 28 29 30 31 32 33 34 35 36 37</td>
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<tr>
<td>N7 (24–28)</td>
<td>24 25 26 27 28 29 30 31 32 33 34 35 36 37</td>
</tr>
<tr>
<td>N8 (25–28)</td>
<td>25 26 27 28 29 30 31 32 33 34 35 36 37</td>
</tr>
<tr>
<td>D1 (9–37)</td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37</td>
</tr>
<tr>
<td>Lira (7–37)</td>
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</table>
to participate in liraglutide metabolism. Our in vitro study demonstrated that DPP-IV can cleave liraglutide in the same position as GLP-1, but at a much slower rate. N-Truncation of liraglutide results in a molecule with higher lipophilicity, which could explain the plasma metabolite with slightly longer chromatographic t\textsubscript{R} than the parent compound. When the primary metabolite in plasma, P1 (R\textsubscript{t} 1.05–1.06), is compared with the single metabolite observed after DPP-IV cleavage in vitro, liraglutide(20–37) (R\textsubscript{t} 1.05–1.06), there was a clear correlation in R\textsubscript{t}. Thus, our data suggest that liraglutide could be degraded by DPP-IV in vivo in a manner similar to that of native GLP-1, but to a much lower extent.

The zinc metalloendopeptidase NEP also contributes to the metabolic instability of GLP-1. NEP is membrane-bound and widely distributed in the body in organs such as kidney, lung, lymph nodes, and intestines, and it has broad substrate specificity (Roques et al., 1993; Turner et al., 2001). It is suggested that up to 50% of GLP-1 entering the circulation may be degraded by NEP and that a combined inhibition of DPP-IV and NEP is superior to DPP-IV inhibition alone in preserving intact GLP-1 (Plamboeck et al., 2005). When the cleavage sites of NEP-truncated liraglutide in this study are compared with the reported sites of native-GLP-1 cleavage (Hupe-Sodmann et al., 1995), the positions correlated well. Similar to DPP-IV cleavage, the NEP degradation of liraglutide occurred at a much slower rate than for GLP-1. Further comparison of the degradation products obtained in vitro with the recovered plasma metabolites showed that P2 (R\textsubscript{t} 1.14–1.15) correlated with truncated liraglutide(24–28) (R\textsubscript{t} 1.07). The NEP-truncated liraglutide(19–37), (19–27), (20–37), and (24–27), all within R\textsubscript{t} 1.04 to 1.07, also eluted close in time to P1. Hence, our data suggest that liraglutide could be susceptible in vivo to NEP degradation similar to that of GLP-1, but again at a much slower rate.

**TABLE 1**

Overview of characteristics of liraglutide degradation products obtained after liraglutide incubation with DPP-IV and NEP in vitro

<table>
<thead>
<tr>
<th>NEP DDP-IV</th>
<th>Molecular Weight (Monoisotopic Mass)</th>
<th>R\textsubscript{t}</th>
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<tr>
<td>Liraglutide(7–37)</td>
<td>3749.0</td>
<td>1.00</td>
</tr>
<tr>
<td>D1 (9–37)</td>
<td>√</td>
<td>3540.8</td>
</tr>
<tr>
<td>N1 (20–27)</td>
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<td>1211.7</td>
</tr>
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<tr>
<td>N2 (19–27)</td>
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<td>1374.8</td>
</tr>
<tr>
<td>N3 (19–37)</td>
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</tr>
<tr>
<td>N3 (24–27)</td>
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</tr>
<tr>
<td>N5 (26–27)</td>
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</tr>
<tr>
<td>N5 (20–28)</td>
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</tr>
<tr>
<td>N6 (25–26)</td>
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</tr>
<tr>
<td>N7 (24–28)</td>
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<td>931.6</td>
</tr>
<tr>
<td>N8 (25–28)</td>
<td>√</td>
<td>860.5</td>
</tr>
</tbody>
</table>

**FIG. 9.** Chromatograms from radiometric high-performance liquid chromatography showing the metabolite profile of \$[^3\text{H}]\text{GLP-1}\$ after 0.5, 1, and 2 h of incubation with 2.0 \$[^3\text{H}]\mu\text{g/ml}\$ DPP-IV (a) or NEP (b).
As for other small regulatory peptides (Carone et al., 1982), GLP-1 is subject to renal filtration and subsequent reabsorption and/or degradation in the proximal tubule (Ruiz-Grande et al., 1990, 1993) where both NEP and DPP-IV are abundant on the luminal membrane (Kettmann et al., 1992; Edwards et al., 1999).

Although important for overall clearance, the kidney is not the prime site of GLP-1 metabolism by DPP-IV, as shown in patients with renal failure (Meier et al., 2004). Porcine studies have demonstrated that liver and peripheral tissues (e.g., muscle, connective, and adipose) also contribute to the elimination of GLP-1 from the circulation (Deacon et al., 1996). In contrast, circulating exenatide is more resistant to enzyme degradation and is thus primarily cleared by glomerular filtration and subsequent proximal tubular degradation (Simonsen et al., 2006). Despite its similarity in size to exendin (4.18 kDa) and GLP-1 (3.36 kDa), liraglutide (3.75 kDa) is not thought to be subject to significant renal filtration because of its high binding (of approximately 99%) to serum albumin. This finding is supported by porcine in vivo studies and rat perfusion studies that show no or very limited renal extraction of circulating liraglutide (data on file, Novo Nordisk) and is likewise supported by the urine excretion pattern observed in this study. The metabolites found in urine are very hydrophilic molecules that are either very small degradation products or small molecules generated by recycling of degradation products from the liraglutide molecule or tritiated water. In addition, there was no increase in plasma exposure of liraglutide when given to patients with renal dysfunction (Jacobsen et al., 2009), suggesting it is very unlikely that the kidneys play a single major role in the elimination of liraglutide.

In liver-impaired patients the plasma exposure of liraglutide was not increased from that seen in healthy subjects (Flint et al., 2010). In porcine excretion or rat perfusion studies have not shown any significant hepatic extraction of liraglutide from the circulation (data on file, Novo Nordisk). However, because DPP-IV and NEP are located in hepatocytes and around bile canaliculi and the bile duct epithelium (Mentlein, 1999), there is a potential for the liver to contribute to the overall metabolism of liraglutide. The low amount of recovered feces metabolites in this study suggests that recycling of degradation products from the liraglutide molecule or tritiated water. In addition, there was no increase in plasma exposure of liraglutide when given to patients with renal dysfunction (Jacobsen et al., 2009), suggesting it is very unlikely that the kidneys play a single major role in the elimination of liraglutide.

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