Metabolism and Excretion of the Once-Daily Human Glucagon-Like Peptide-1 Analog Liraglutide in Healthy Male Subjects and Its In Vitro Degradation by Dipeptidyl Peptidase IV and Neutral Endopeptidase

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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 (t1/2 = 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 β-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 Lys34 by Arg and derivatization of the GLP-1 protein backbone in the Lys–Arg–Glu (Lys26 position with a glutamate spacer bound to a16C fatty acid ester bound to the aC terminus). This modification resulted in 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 β-cell function with a very low risk of inducing hypoglycemia (Vilsbøll et al., 2008; Garber et al., 2009; Nauck et al., 2009).

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Metabolism and Excretion of Liraglutide in Humans

Materials and Methods

Radiolabeled 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 3H 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 radiolabeled 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 after 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/m² (range 22.7–27.0 kg/m²) 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.

[3H]-labeled GLP-1 and liraglutide were 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.

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).
Analysis of Liraglutide in Plasma by Enzyme-Linked Immunosorbent Assay. Venous blood was collected in tubes containing EDTA as anticoagulant. Blood samples (3.0 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 (Agersø et al., 2002). The ELISA assay is specific to liraglutide and interference from endogenous GLP-1 and truncated versions of liraglutide is very low (Agersø 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 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 (2%) was reached. Duplicate blank samples on a Packard Tri-Carb 3100 TR (PerkinElmer Life and Analytical Sciences, Waltham, MA) were analyzed to check 11C radioactivity. To calculate the content of volatile radioactivity such as tritiated water, another duplicate set of samples was freeze-dried and reconstituted in water (“dry samples”) before LSC analysis.

Blood sampling and LSC analysis. Venous blood was collected in tubes containing EDTA as anticoagulant. Whole blood samples (3.0 ml) were taken at 0 h (before dosing) and at 2, 4, 8, 12, 16, 24, 48, and 72 h after dosing and kept at −20°C until analysis. Blood samples for plasma analysis (6.0 ml) were taken at 0 h (before dosing) and at 2, 4, 8, 12, 16, 24, 48, 72, 120, and 168 h after dosing and centrifuged within 30 min of sampling. The plasma was separated and kept at −20°C until further analysis. Before LSC analysis, the whole blood samples (both wet and reconstituted dry samples) were combusted for 2 min in a Packard 307 Sample Oxidizer (Packard now owned and 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 study. 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 (IKC 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 combusted for 2 min in a Packard 307 Sample Oxidizer before dissolution in Monophase S and LSC analysis.

Analysis of Total Radioactivity in Plaster. The plaster and gauze pads used to cover the subcutaneous injection site (one sample for each dosed subject) were analyzed by LSC to reveal radioactivity content. The plaster and gauze pads were placed in 15 ml of liquid scintillation fluid and mixed repeatedly on a vortex mixer for at least 45 s and then left standing for at least 2 h before analysis by LSC.

Analysis of Metabolite Profiles in Plasma, Urine, and Feces. Plasma sampling and preparation. Individual 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, and 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 (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 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 1500 g. 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 600 g. 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 × 4.6 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 radiodetection was performed on a Radiomatic 150TR radiodetector (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 min in a 1.5-ml test tube, before the addition (100 μl) of 10 μM substrate solution. Final substrates of 2 μM [3H]liraglutide or [3H]GLP-1 (2 μCi/ml). To test the stability of the test substrates under incubation conditions, control samples without DPP-IV or NEP were run in parallel. Samples in triplicate were incubated at 37°C for 4, 8, and 24 h for liraglutide metabolite profiling and 0.5, 1, and 2 h for GLP-1 metabolite profiling. Additional incubations were conducted with [3H]liraglutide and NEP (3.2 μg/ml) and DPP-IV (4 μg/ml) at concentrations of 10 μM liraglutide and 25-h incubation time to generate a sufficient amount of metabolites for metabolite identification. The reactions were stopped by addition of 75 μl of cold acetonitrile followed by thorough mixing. The samples were centrifuged at 4°C for 15 min at 14,000 g, and the supernatant was collected. The metabolite profile was immediately analyzed by HPLC and online detection of radioactivity, or the supernatant was stored at −20°C until analysis with the storage period limited to a few days.

Based on the extent of metabolism observed at the above incubation times, DPP-IV and liraglutide were incubated with 30 μl of the DPP-IV specific inhibitor diprotin A (125 μM, 500 μM, and 500 mM) for 8 h under the same conditions as before. For the NEP inhibition study, NEP and the respective test substrates were incubated with 30 μl of phosphoramidon (25 and 125 μM) for 8 h. The reactions were stopped and analyzed as described above.

Metabolite profiling. Before analysis, incubations were mixed with 0.5% Tween 20 to a final concentration of 0.05% Tween 20. Samples from the in
vitro incubation of [\textsuperscript{3}H]liraglutide and [\textsuperscript{3}H]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 (MSMS) information on the formed metabolites was obtained after LC-MS and MS/MS analysis on a QSTAR XL mass spectrometer equipped with an electrospray 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 100:90:0:30 (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 MH\textsuperscript{3+} or MH\textsuperscript{4+} ions. In the MS/MS mode (product ion scan) the mass spectrometer was scanning in the range m/z 50 to 3000 using high mass 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 C\text{max} of 10,586 pm (S.D. 3202), followed by a decline toward baseline during the next 48 h with a mean apparent t\text{1/2} 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; Elbønd 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 C\text{max} 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.

Plasma Metabolite Profiling. Liraglutide was the major radioactive component in plasma at all time points in all subjects, with a retention time (t\text{R}) of 37.0 to 38.0 min (Fig. 3). In addition, two minor metabolites that were more lipophilic than the parent compound were detected: P1 (t\text{R} = 39.0 to 40.0 min and relative retention time (R\text{t}) ~1.05–1.06), was seen at 12, 24, and 48 h in all subjects; and P2 (t\text{R} = 42.7–43.4 min and R\text{t} 1.14–1.15), was seen at 12, 24, and 48 h in five subjects and at 24 and 48 or 24 h only in the remaining two subjects. P1 represented ≤5% of the radioactivity exposure in pooled plasma samples (AUC\text{2–24 h}). The mean relative peak area was 7.0% (S.D. 1.1; n = 6). P2 represented ≤5% of the radioactivity exposure with a mean relative peak area of 3.2% (S.D. 1.2; n = 5). No structural characterization of these minor plasma metabolites was performed because of the low therapeutic dose levels of liraglutide and the resulting very low quantities of minor metabolites. It should be noted that no tritiated water was present in the plasma metabolite profiles as the preparation of samples included a freeze-drying step that eliminates tritiated water.

Recovery of Radioactivity in Urine, Feces, and Plaster. The cumulative recovery of total radioactivity in urine and feces was 26.3% of the administered dose with 15% of the administered dose shown to be volatile. Thus, 20.1% of total radioactivity was recovered in urine, but only 6.3% of total radioactivity was bound to components other than tritiated water. In feces, 6.2% of total radioactivity was recovered, with 5.1% of total radioactivity bound to components other than tritiated water (Fig. 4). There were only very low levels of radioactivity present in the plasters, within error limits of the administered dose, indicating that no significant amounts of the dose [\textsuperscript{3}H]liraglutide leaked out at the subcutaneous injection site.

Urine and Feces Metabolite Profiling. No intact liraglutide was detected in either urine or feces (Figs. 5 and 6). Two minor radioactive urine...
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 liraglutide suggests the recovery of smaller more hydrophilic products.

In feces there was uneven 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 liraglutide, 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 prepreparation of the samples.

DPP-IV and NEP In Vitro Degradation of Liraglutide and GLP-1. Incubation of liraglutide 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 liraglutide (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 liraglutide (Fig. 8). In contrast, liraglutide 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 liraglutide 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 liraglutide, 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 liraglutide or GLP-1 degradation in the presence of diprotein A and phosphoramidon.

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]liraglutide (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.
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...
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

![Fig. 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)

![Fig. 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 acids residues. The major degradation products that are in common with the GLP-1 degradation products reported in literature are marked with an asterisk.](image)
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 $t_R$ than the parent compound. When the primary metabolite in plasma, P1 ($t_R 1.05–1.06$), is compared with the single metabolite observed after DPP-IV cleavage in vitro, liraglutide(20–37) ($t_R 1.05–1.06$), there was a clear correlation in $t_R$. 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 ($t_R 1.14–1.15$) correlated with truncated liraglutide(24–28) ($t_R 1.15$). The NEP-truncated liraglutide(19–37), (19–27), (20–37), and (24–27), all within $t_R 1.04$ 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</th>
<th>DPP-IV</th>
<th>Molecular Weight (Monoisotopic Mass)</th>
<th>$t_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liraglutide(7–37)</td>
<td>3749.0</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Degradation product</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1 (9–37)</td>
<td>✓</td>
<td>3540.8</td>
<td>1.06</td>
</tr>
<tr>
<td>N1 (20–27)</td>
<td>✓</td>
<td>1211.7</td>
<td>1.03</td>
</tr>
<tr>
<td>N2 (20–37)</td>
<td>✓</td>
<td>2367.3</td>
<td>1.04</td>
</tr>
<tr>
<td>N2 (19–27)</td>
<td>✓</td>
<td>1374.8</td>
<td>1.04</td>
</tr>
<tr>
<td>N3 (19–37)</td>
<td>✓</td>
<td>2530.4</td>
<td>1.07</td>
</tr>
<tr>
<td>N3 (24–27)</td>
<td>✓</td>
<td>794.5</td>
<td>1.07</td>
</tr>
<tr>
<td>N4 (25–27)</td>
<td>✓</td>
<td>713.5</td>
<td>1.08</td>
</tr>
<tr>
<td>N5 (26–27)</td>
<td>✓</td>
<td>642.4</td>
<td>1.11</td>
</tr>
<tr>
<td>N5 (20–28)</td>
<td>✓</td>
<td>1358.7</td>
<td>1.11</td>
</tr>
<tr>
<td>N6 (25–26)</td>
<td>✓</td>
<td>584.4</td>
<td>1.13</td>
</tr>
<tr>
<td>N7 (24–28)</td>
<td>✓</td>
<td>931.6</td>
<td>1.15</td>
</tr>
<tr>
<td>N8 (25–28)</td>
<td>✓</td>
<td>860.5</td>
<td>1.17</td>
</tr>
</tbody>
</table>

![FIG. 9. Chromatograms from radiometric high-performance liquid chromatography showing the metabolite profile of $[^3]$HGLP-1 after 0.5, 1, and 2 h of incubation with 2.0 µg/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 is not thought to be subject to significant renal filtration because of its high binding (of approximately 99%) to serum albumin.

In liver-impaired patients the plasma exposure of liraglutide was not increased from that seen in healthy subjects (Flint et al., 2010). However, because shown any significant hepatic extraction of liraglutide 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 is not thought to be subject to significant renal filtration because of its high binding (of approximately 99%) to serum albumin.

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