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Metabolism and Excretion of the Once Daily Human GLP-1 Analog liraglutide in Healthy Male Subjects and its In Vitro Degradation by Dipeptidyl Peptidase IV and Neutral Endopeptidase

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**Nonstandard abbreviations**

AUC, area under the curve from zero to infinity

$C_{\text{max}}$, maximum concentration

dpm, disintegrations per minute
DPP-IV, dipeptidyl peptidase IV
ELISA, enzyme-linked immunosorbent assay
EDTA, ethylenediaminetetraacetic acid
GLP-1, glucagon-like peptide-1
HBSS, Hank's buffered saline solution
HPLC, high-performance liquid chromatography
HSA, human serum albumin
LC-MS, liquid chromatography mass spectrometry
LSC, liquid scintillation counting
MS, mass spectrometry
MS/MS, tandem mass spectrometry
NEP, neutral endopeptidase 24.11
$R_t$, relative retention time
$t_R$, retention time
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 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 (AUC₂⁻₂₄ 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-terminal 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 similar manner to 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, 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 antidiabetic 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 (Vilsboll 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 Lys26 position with a glutamate spacer bound to a $^{16}$C fatty
acid (N-ε-(γ-L-glutamyl(N-α-palmitoyl) (Fig. 1) (Knudsen, 2004). This structural change gives a molecular conformation that stabilizes liraglutide against metabolic degradation and enables reversible binding to plasma albumin proteins, leading to a further decreased clearance and protracted pharmacologic activity (Knudsen et al., 2000; Madsen et al., 2007). In addition, slow absorption from the subcutaneous (s.c.) injection site is thought to occur as 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 8–12 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 & 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 show reduced clearance and may thereby increase the risk of exposure-dependent side-effects (Linnebjerg et al., 2007; Johansen and Whitfield, 2008). Renal
dysfunction has however 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) showed that $[^{3}\text{H}]$, $[^{125}\text{I}]$, or $[^{14}\text{C}]$-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 following a single s.c. injection of $[^{3}\text{H}]$-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 $[^{3}\text{H}]$-liraglutide and $[^{3}\text{H}]$-GLP-1(7-37) degradation products and with those reported for native GLP-1.
Materials and Methods

Radiolabeled Test Material

$^3$H-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 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 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 were shown to be 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 $[^3]$H-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 a median age of 57 years (range: 47–60 years), a 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 and food containing methylxanthine within 48 h before dosing. Moreover, subjects were not allowed to perform strenuous exercise or smoke more than five cigarettes per 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 post dose. 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 s.c. injection of $[^{3}H]$-liraglutide (0.75 mg/14.2 MBq). Blood, urine and feces were sampled from Day 1, 2 h post dosing, until Day 10 and safety evaluations including physical examination, vital signs, hematology and clinical chemistry analysis (e.g. plasma glucose) were regularly carried out. The level of total radioactivity was measured in urine and feces from Day 5 onwards 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 post-dose) or until follow up at Day 14 post-dose, whichever came first.

Analysis of liraglutide in Plasma by Enzyme-linked Immunosorbent assay (ELISA)
Venous blood was collected in tubes containing ethylenediaminetetraacetic acid (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 ELISA, as previously described (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 ($C_{\text{max}}$), 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 (2s) 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 (Downers Grove, IL) to detect retained $^3$H-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, 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, 6, 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 (Perkin Elmer Life Sciences, Groningen, The Netherlands) before
dissolution in liquid scintillation fluid (Monophase S, Perkin Elmer) and counted by LSC. Plasma samples (both ‘wet’ and reconstituted ‘dry’ samples) were diluted with blank plasma and dissolved in liquid scintillation fluid (Flo Scint-A, Perkin Elmer) and analyzed by LSC.

Urine Sampling and LSC Analysis

Urine was sampled before dosing (~0 h) and at intervals 0–4 h, 4–8 h, 8–12 h, 12–24 h, and then at 24-h intervals until the end of the study. After recording their weight, 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 LCS Analysis

Feces were sampled at before dosing (~0 h) and at 24-h intervals until the end of the trial. After recording the total weight of each feces sample, 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 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 s.c. 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 liquid scintillation fluid and mixed repeatedly on a vortex mixer for at least 45 sec 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. Briefly, each sample was mixed with 0.5% Tween 20 (Sigma Aldrich, St Louis, MO) and acetonitrile (Merck, Darmstadt, Germany) in 10:1:10 (v:v:v) and centrifuged at 4 °C for 5 min at 2,000 g. The supernatant was collected and the centrifugation repeated, while 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 48–72-h and 120–144-h intervals and a pooled sample of aliquots from urine collected during the first 6 days after dosing (0–4 h, 4–8 h, 8–12 h, 12–24 h, 24–48 h, 48–72 h, 72–96 h, 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 1,500 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, Darmstadt, Germany) (3 ml/g homogenate), sonicated for 5 min, and centrifuged at 4 °C for 5 min at 600 g. The liquid fraction was collected and the pellet extracted twice with ethylacetate (Merck) (2 × 3 ml/g homogenate), sonicated, and then centrifuged. All collected liquid fractions were pooled and the mixture was analyzed by HPLC and Top Count NXT (Perkin Elmer) 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., 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 radiochemical detection was carried out on a Radiomatic 150TR radioactivity detector (Perkin Elmer Life and Analytical Sciences, Boston, MA) equipped with a 500-µl detector cell. The instrument was operating in the homogenous liquid scintillation mode and the HPLC eluent was mixed 1:2 with Ultima Flow M scintillation liquid (Perkin Elmer). The radiochemical detection, in the feces metabolite profiling and plasma profiling reference analyses, was carried out on a Top Count NXT scintillation counter (Perkin Elmer Life and Analytical Sciences) after collection of the HPLC eluent on 96-well Deepwell Lumaplates (Perkin Elmer), 0.25 min/fraction, with a Gilson FC-204 fraction collector (Gilson, Middleton, WI). The plasma profiling reference analysis was conducted in order 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 respective enzyme in Hank’s buffered salt solution (HBSS)/1% human serum albumin (HAS) was pre-incubated at 37 °C for 3–5 min, in a 1.5-ml test tube, before the addition of 30 µl test substance to give final concentrations of 2 µg/ml DPP-IV or 1.6 µg/ml NEP and 0.05 and 1µM [³H]-liraglutide or [³H]-GLP-1 (2 µCi/ml). To test the stability of the test substances under incubation conditions, control samples without DPP-IV or NEP were run in parallel. Samples in triplicates 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 sufficient amount of metabolites for metabolite identification. The reactions were stopped by addition of 75 µl cold acetonitrile followed by thorough mixing. The samples were centrifuged at 4 °C for 15 min at 14,000 g and the supernatant collected. The metabolite profile was immediately analyzed by HPLC and on-line detection of radioactivity, or else 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 time points, DPP-IV and liraglutide were incubated with 30 µl of the DPP-IV specific inhibitor diprotein-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 substances were incubated with 30 µl
of phosphoramidon (25 µM and 125 µM) for 8 h. The reactions were stopped and analyzed as described above.

Metabolite Profiling

Prior to 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 [3H]-liraglutide and [3H]-GLP-1 with DPP-IV or NEP were analyzed by a similar HPLC system and online detection of radiochromatography as described in the section for plasma, urine and feces metabolite profiling.

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 following LC-MS and MS/MS analysis on a QSTAR XL mass spectrometer equipped with an electrospray interface from Sciex (Thornhill, Canada).
The HPLC system was an Agilent 1100 HPLC system (Agilent, Palo Alto, CA). Gradient LC-MS analysis was made on a Jupiter Proteo C-12, 250 × 4.6 mm (4 µm) from Phenomenex (Torrance, CA) 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 a final isocratic phase with 100% B for 10 min. This was 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$_3^{3+}$ or MH$_4^{4+}$ 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 5–10 µl/min using the TurboIonSpray® interface (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. Out 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 10586 pmol/l (SD 3202), followed by a decline towards baseline during the next 48 h with a mean apparent $t_{1/2}$ of 15.6 h (SD 2.1). The recovered plasma profile and calculated mean AUC of 311.1 nmol × h/l (SD 61.8) was comparable with results from previous trials investigating the pharmacokinetics of unlabeled liraglutide (Agersø et al. 2002; Elbrønd et al. 2002).

Plasma Radioactivity

Similarly to the plasma liraglutide pharmacokinetic profile (Fig. 2a), radioactivity in plasma (Fig. 2b) peaked at 12–16 h after dosing with a mean $C_{\text{max}}$ of ~10930 pmol
equiv/l and declined thereafter towards 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 similar profile to that in plasma,
peaking 12–16 h after dosing and thereafter declining towards baseline. As the mean
hematocrit of the subjects was 0.44 and the mean whole blood to plasma ratio
approximately 0.6 (range 0.57–0.68 between 2 and 72 h after dosing), 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 ($t_R$) of 37.0–38.0 min (Fig. 3). In addition, two minor
metabolites that were more lipophilic than the parent compound were detected: P1, with
$t_R= 39.0–40.0$ min (relative retention time; $R_R \sim 1.05–1.06$), was seen at 12, 24, and 48
h in all subjects; P2 ($t_R= 42.7–43.4$ min; $R_R 1.14–1.15$) was seen at 12, 24, and 48 h in
five subjects and at 24 and 48 h, or 24 h only, in the remaining two subjects. P1
represented $\leq 9\%$ of the radioactivity exposure in pooled plasma samples ($\text{AUC}_{2–24\,h}$).
The mean relative peak area was 7.0\% (SD $\pm 1.1$; n=6). P2 represented $\leq 5\%$ of the
radioactivity exposure with a mean relative peak area of 3.2% (SD ± 1.2; n=5). No structural characterization of these minor plasma metabolites was carried out due to 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 pre-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 dosed [³H]-liraglutide leaked out at the s.c. 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<sub>R</sub>=3.3–3.6 min) and U2 (t<sub>R</sub>=3.9–5.0 min) while 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% (SD 1.0; $n=7$) whereas U2 represented 0.55% (SD 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 an 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), F3 only in one subject, while 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. As 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 3–5% of the total administered radioactivity, that is, a similar fraction to that recovered from components excreted in urine. Overall, no structural elucidation of the urine or feces metabolites could be carried out due to the very low amounts. Tritiated water was not detected due to the freeze-drying step in the pre-preparation 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 \( t_R = 40.7 - 40.8 \text{ min} \), \( R_t 1.05 \) than the parent compound liraglutide \( t_R 38.7 \text{ min} \); Fig. 7a). The cleavage site was demonstrated to be exclusively in the Ala8–Glu9 position of the N-terminal 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-terminal between the Tyr19 and Lys26 positions. Continued cleavage lead 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 diprotin A and phosphoramidon.
Discussions

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. As 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), suggests 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 bodyweight 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 where s.c. administered liraglutide labeled with $^{125}$I or tritium in the peptide moiety mainly resulted in excretion of free $^{125}$I or tritiated water, while $^{14}$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, bile and pancreatic ducts) (Mentlein, 1999), and has the potential 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. As N-truncation of liraglutide results in a molecule with higher lipophilicity, this could explain the plasma metabolite with slightly longer chromatographic $t_R$ than the parent compound. When comparing the primary metabolite in plasma, P1 ($R_{tR} \sim 1.05–1.06$), with the single metabolite observed after DPP-IV cleavage *in vitro*, liraglutide(20-37) ($R_{tR} 1.05–1.06$), there was a clear correlation in $R_{tR}$. Thus, our data suggest that liraglutide could be degraded by DPP-IV *in vivo* in a similar manner to 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 comparing the cleavage sites of NEP-truncated liraglutide in this study with the reported sites of native-GLP-1 cleavage (Hupe-Sodmann et al., 1995), the positions correlated well. Similarly 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 ($R_t$ 1.14–1.15) correlated with truncated liraglutide(24-28) ($R_t$ 1.15). The NEP-truncated liraglutide(19-37), (19-27), (20-37) and (24-27), all within $R_t$ 1.04–1.07, also eluting 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.

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, adipose) also contribute to the elimination of GLP-1 from the circulation (Deacon et al., 1996). Conversely, 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). In spite of its similar size to exentide (4.18 kDa) and GLP-1 (3.36 kDa), liraglutide (3.75 kDa) is not thought to be subject to significant renal filtration due to its high binding (of approximately 99%) to serum albumin. This 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 hepatic impaired patients the plasma exposure of liraglutide was not increased from that seen in healthy subjects (Flint et al., 2007). Also, porcine extraction or rat perfusion studies have not shown any significant hepatic extraction of liraglutide from the
circulation (data on file, Novo Nordisk). However, as DPP-IV and NEP are located in hepatocytes and around bile caniculi and the bile duct epithelium (Mentlein, 1999), there is potential scope for the liver to contribute to the overall metabolism of liraglutide. The low amount of recovered feces metabolites in this study could be products from DPP-IV or NEP cleavage as the retention time of F1 (t_R 40.1 min) is very similar to that of P1 and the DPP-IV truncated liraglutide (9-37) or the NEP truncated liraglutide(19-37) or (24-27). The F3 component eluting at the very end of the chromatogram (t_R 82.8 min) might be the labeled 16C fatty acid alone, as it appeared at t_R ~80–82 min. However, as for the urinary metabolites, these components may also be products from tritium labeled recycled parts of liraglutide or tritiated water. Taking all available data together, the liver is a possible player in elimination, but is not the single major organ involved in liraglutide clearance from the body.

In conclusion, we have shown that liraglutide is fully metabolized within the body and that the widely distributed endogenous enzymes, DPP-IV and NEP, are likely to be involved in its degradation. The cleavage sites of liraglutide were similar to those reported for GLP-1, although the degradation was much slower for liraglutide. These observations, taken together with data from previous studies in animals and humans, suggest that liraglutide is cleared by slow but complete degradation within the body in multiple organs and tissues; it is not predominantly eliminated by one single organ.
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References


Footnotes

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Legends for Figures

Figure 1. Structure of [3H]-liraglutide (top) and the GLP-1 receptor agonists liraglutide and exenatide in comparison to native GLP-1.

Figure 2. Mean concentration–time profiles of plasma liraglutide as measured by ELISA (a) or total radioactivity (b) in healthy male subjects (n=7) after a subcutaneous single dose of [3H]-liraglutide (0.75 mg/14.2 MBq). From 48 h and onwards a part of the radioactivity in (b) was volatile, most likely tritiated water, as shown by the difference between ‘wet’ and reconstructed ‘dry’ samples.

Figure 3. Chromatograms from HPLC analysis with radiometric detection showing the metabolite profile in plasma at 6 h (a), 12 h (b), 24 h (c), and 48 h (d) from one subject after a subcutaneous single dose of [3H]-liraglutide (0.75 mg/14.2 MBq).

Figure 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 onwards the majority of recovered radioactivity excreted in urine was volatile (most likely tritiated water), whereas the fecal excretion from Day 6 and onwards only comprised to a minor extent volatile radioactivity as shown by the difference between ‘wet’ and reconstructed ‘dry’ sample analysis.
Figure 5. Chromatograms from HPLC analysis with radiometric detection showing the metabolite profile in urine collected at Day 3 (a), Day 6 (b), and in a pooled sample of Days 1–5 (c) from one subject after a subcutaneous single dose of [³H]-liraglutide. The expected retention time of liraglutide is indicated on the figures by an arrow.

Figure 6. Chromatograms from HPLC analysis with radiometric detection showing the metabolite profile in pooled feces collected on Days 1–6 (0–120 h) from three individual subjects (a–c) after a subcutaneous single dose of [³H]-liraglutide. The expected retention time of liraglutide is indicated on the figures by an arrow.

Figure 7. Chromatograms from HPLC analysis with online radio-detection showing the metabolite profile of 1 µM [³H]-liraglutide following 4, 8, and 24 h incubation with 2.0 µg/ml DPP-IV (a) or 1.6 µg/ml NEP (b).

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 acids residues. The major degradation products which are in common with the GLP-1 degradation products reported in literature are marked with an asterisk.
Figure 9. Chromatograms from radiometric high performance liquid chromatography showing the metabolite profile of 1µM [³H]-GLP-1 following 0.5, 1, and 2 h incubation with 2.0 µg/ml DPP-IV (a) or NEP (b).
Tables

Table 1. Overview of liraglutide degradation products characteristics obtained after liraglutide incubation with DPP-IV and NEP *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 amino acids residues.

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<th>Liraglutide (7-37)</th>
<th>NEP</th>
<th>DDP-IV</th>
<th>Molecular weight (monoisotopic mass)</th>
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<table>
<thead>
<tr>
<th>Product</th>
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<th>Molecular weight</th>
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Figure 4

a) Excretion of radioactivity (% of dose) vs Time (h)

- Wet samples
- Dry samples

b) Excretion of radioactivity (% of dose) vs Time (h)

- Wet samples
- Dry samples
Figure 6

(a) Liraglutide

(b) Liraglutide

(c) Liraglutide
Figure 7

(a) 4 H  |  Liraglutide

(b) 4 H  |  Liraglutide

(c) 8 H  |  Liraglutide

(d) 8 H  |  Liraglutide

(e) 24 H |  Liraglutide

(f) 24 H |  Liraglutide
Figure 9

(a) 0.5 H

(b) 0.5 H

1 H

2 H

GLP-1

mV

Time (min)

0 10 20 30 40 50 60 70 80 90 100

0 100 200 300 400 500 600 700 800 900 1000 1200

mV

Time (min)

0 10 20 30 40 50 60 70 80 90 100

0 100 200 300 400 500 600 700 800 900 1000 1200

mV

Time (min)

0 10 20 30 40 50 60 70 80 90 100

0 100 200 300 400 500 600 700 800 900 1000 1200

mV

Time (min)

0 10 20 30 40 50 60 70 80 90 100

0 100 200 300 400 500 600 700 800 900 1000 1200

mV

Time (min)