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Title page

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Abstract

Semaglutide is a human glucagon-like peptide-1 analogue in clinical development for the treatment of type 2 diabetes. The absorption, metabolism and excretion of a single 0.5 mg/450 µCi [16.7 MBq] subcutaneous dose of [^{3}H]-radiolabelled semaglutide was investigated in healthy human subjects and compared with data from nonclinical studies.

Radioactivity in blood, plasma, urine and faeces was determined in humans, rats and monkeys; radioactivity in expired air was determined in humans and rats. Metabolites in plasma, urine and faeces were quantified following profiling and radiodetection. The blood-to-plasma ratio and pharmacokinetics of both radiolabelled semaglutide-related material and of semaglutide (in humans only) were assessed.

Intact semaglutide was the primary component circulating in plasma for humans and both nonclinical species, accounting for 69–83% of the total amount of semaglutide-related material, and was metabolised prior to excretion. Recovery of excreted radioactivity was 75.1% in humans, 72.1% in rats and 58.2% in monkeys. Urine and faeces were shown to be important routes of excretion, with urine as the primary route in both humans and animals. Semaglutide was metabolised through proteolytic cleavage of the peptide backbone and sequential beta-oxidation of the fatty acid sidechain, and metabolism was not confined to specific organs. Intact semaglutide in urine accounted for 3.1% of the administered dose in humans and less than 1% in rats; it was not detected in urine in monkeys.

The metabolite profiles of semaglutide in humans appear to be similar to the profiles from the nonclinical species investigated.

Keywords (max 6):
Semaglutide, AME, metabolism, GLP-1 analog, clinical, nonclinical

1. Introduction

Native human glucagon-like peptide-1 (GLP-1), a gut-derived incretin hormone, stimulates insulin and inhibits glucagon secretion in a glucose-dependent manner, reduces appetite and energy intake, and also delays gastric emptying (Drucker *et al.*, 2006; Flint et al., 1998; Holst, 2007; Naslund *et al.*, 1999; Willms *et al.*, 1996). The therapeutic potential of GLP-1 in the treatment of type 2 diabetes (T2D) is, however, hindered by its short half-life ($t_{½}$) in the circulation (1–2 minutes), a result of proteolytic degradation by the enzymes dipeptidyl peptidase-4 (DPP-4) and neutral endopeptidase (NEP) (Drucker *et al.*, 2006; Flint et al., 1998; Holst, 2007; Willms *et al.*, 1996).

The first generation GLP-1 analogues, such as once-daily liraglutide, were designed to have a reduced susceptibility to enzymatic degradation, while providing effective glucose control, improved beta-cell function, body weight loss and decreased systolic blood pressure in patients with T2D (Meier, 2012). The primary feature responsible for the longer half-life of liraglutide is acylation and binding to albumin (Madsen, *et al.*, 2007). Recently new GLP-1 products, such as albiglutide and dulaglutide, have longer $t_{½}$ than first-generation therapies, allowing for once-weekly administration. On-going approaches to optimising GLP-1-based therapy should target the development of analogues suitable for once-weekly dosing, with no compromise in – or improved – efficacy.

Semaglutide is a human GLP-1 analogue in clinical development for the treatment of T2D. It shares 94% structural homology with native human GLP-1, with three important modifications: amino-acid substitutions at position 8 (alanine to alpha-aminoisobutyric acid) and position 34 (lysine to arginine), and acylation of the lysine in position 26 with a spacer consisting of two 8-amino-3,6-dioxaoctanoic acid (ADO) moieties, a glutamic acid moiety, and a C-18 fatty di-acid side chain (Lau *et al.*, 2015). The fatty di-acid side chain and spacer mediate strong binding with albumin, while

the amino-acid substitution at position 34 limits the options for acylation to the one remaining lysine in the sequence; the substitution at position 8 reduces the susceptibility of semaglutide to degradation by DPP-4 (Lau *et al.*, 2015). Together, these modifications prolong the $t_{½}$ of semaglutide; a previous pharmacokinetic (PK) trial with semaglutide showed the geometric means (coefficient of variation in %) of $t_{1/2}$ was 165 (14.1) hours (approximately 1 week). Additionally, the dose dependent increase of the C_{trough} values following 4 weeks of treatment with semaglutide 0.25 mg, 0.5 mg and 1.0 mg was 4.4 (31.5), 11.7 (20.2) and 21.2 (19.7) nmol/L, respectively (Kapitza *et al.*, 2015).

Semaglutide 0.5 mg was investigated in this clinical trial, as this was the lower of the two maintenance doses investigated in the phase 3 clinical trial programme for semaglutide, and known to provide sufficient exposure to estimate relevant PK endpoints following single dosing. Semaglutide was radiolabelled in the octadecanedioic acid moiety in the side chain of lysine 26 in order to characterise the metabolism of the most modified part of the molecule. Metabolism of the semaglutide peptide sequence was considered to be more predictable (proteolysis with formation of smaller peptides and amino acids) and therefore not in scope for radiolabelling. The primary objective was to investigate the absorption, metabolism and excretion (AME) of semaglutide after a single subcutaneous (s.c.) injection of 0.5 mg [^{3}H]-semaglutide (450 µCi [16.7 MBq]) in healthy adult men, and to compare these data with those from nonclinical studies used in the safety evaluations of semaglutide. Secondary objectives included assessing the PK, safety and tolerability of semaglutide.

2. Materials and methods

2.1 Tritium-labelled semaglutide

The synthesis of radiolabelled semaglutide was designed to provide a tritium labelling, specifically located α, β to the carbonyl in the amide bond of the octadecanedioic acid moiety in the semaglutide sidechain (Figure 1). [^{3}H]-semaglutide was synthesised in two chemical steps from the two non-radioactive starting materials NNC0113-3747 and NNC0113-0236 (Appendix A, Figure 1) (Lau *et al.*, 2015).

2.1.1 Synthetic procedure for the radioactive building block [^{3}H]-NNC0113-0857

The starting material, NNC0113-3747 (6.6 mg, 6.5 µmol), was dissolved in a solution of N-methyl-2-pyrrolidone (NMP) (1.0 mL) and trifluoroacetic acid (TFA) (5 µL) before 10 wt% Pd/C (6.5 mg) was added. The mixture was subjected to tritium gas (6.9 Ci) and stirred at room temperature. After 4 hours, Pd/C was filtered out and the residue co-evaporated (3 x 1.0 mL) with a 1000:1 solution of EtOH/TFA. The residue was dissolved with NMP (1.0 mL) and the radioactive content was determined (180 mCi/mL, 1.6 mL). High-performance liquid chromatography (HPLC) analysis showed that [^{3}H]-NNC0113-0857 had radiochemical purity of 61%.

2.1.2 Synthetic procedure for [^{3}H]-semaglutide

The peptide backbone (20.4 mg, 6.0 µmol), was dissolved in H_2O (5.0 mL) and the pH adjusted to 11 via the addition of NEt_3. The solution of [^{3}H]-NNC0113-0857 in NMP (180 mCi/mL, 1.6 mL) was slowly added over 15 minutes. After 2 hours, the reaction was quenched and neutralised to pH 7 by addition of 1 M CH_3COOH (120 µL). The reaction mixture was purified by HPLC (column: Luna® reversed phase C18; A-eluent [0.1 M Tris−HCl, 0.05 M H_3PO_4, H_2O]/EtOH 8:2, pH 7.4; B-eluent [0.1 M Tris-HCl, 0.05 M H_3PO_4, H_2O]/EtOH 55:45, pH 7.4) and yielded a [^{3}H]-semaglutide solution of 98% purity. [^{3}H]-semaglutide was desalted on a C18 Sep-Pak® filter and eluted with H_2O/EtOH (3:7) into a solution with a radioactive content of 16.7 mCi/mL. The solution was concentrated to dryness on a

rotary evaporator and then reconstituted in the following vehicle/API solution of semaglutide (1.34 mg/mL, 1.0 mg/mL or 0 mg/mL), propylene glycol (14 mg/mL), Na_2HPO_4 $2H_2O$ (1.42 mg/mL), phenol (5.5 mg/mL), pH 7.4. HPLC analysis of formulated [^{3}H]-semaglutide gave a radiochemical purity of >98%.

2.1.3 Stability of [^{3}H]-semaglutide

[^{3}H]-semaglutide stability in vehicle was assessed over a 15-day period at 5°C and 25°C and confirmed. Radiochemical purity was determined by HPLC. Prestudy stability tests revealed that the tritium tracer showed a satisfactory stability. The amount of tritiated water formed after 15 days' storage at 25°C was low (<0.1%), as was the amount of impurities (~2% at 5°C and ~5% at 25°C).

2.1.4 Stability of semaglutide in human material

Stability of the tracer was also assessed in plasma and urine and showed that the [^{3}H]-semaglutide tracer was stable in human blood, plasma and urine for up to at least 4 hours at room temperature and up to at least 48 hours at nominal +4, −20 and −70°C. The stability of the [^{3}H]-semaglutide radiotracer was, therefore, considered to be adequate for conducting metabolism studies.

2.2 Clinical Trial

2.2.1 Trial design

The clinical trial with [^{3}H]-semaglutide was an open-label clinical pharmacology trial conducted at the PRA Health Sciences Clinical Unit (Zuidlaren, the Netherlands). The protocol and informed consent form were approved by an independent ethics committee, according to the ICH Good Clinical Practice guidelines (European Medicines Agency, 2002) and the EU Clinical Trials Directive as laid down in local law. All subjects provided written informed consent at Visit 1, prior to any trial activities. The trial was performed in accordance with the Declaration of Helsinki (1964) and its amendments (World Medical Association, 2000). Seven healthy male subjects, with a median age of 56 years (range 48–64 years), weight of 73.4 kg (range 62.6–107.3 kg) and mean body mass index of 24.5 kg/m^2 (range 21.7–29.7 kg/m^2), received the trial drug and completed the trial.

The clinical trial design is shown in Appendix B Figure 1. On Day 1, all non-fasted subjects received a single s.c. dose of 0.5 mg [^{3}H]-semaglutide (450 µCi [16.7 MBq]) and subsequently stayed in the clinical unit for 14 days after dosing. Thereafter, subjects attended once-weekly clinic visits, each of 24-hour duration, for a maximum of 56 days after dosing (last subject finalised by Day 57). An end criterion was predefined between Days 36 and 64; this was either 1) >95% recovery of [^{3}H]-labelled drug material in excreta or 2) total [^{3}H]-excretion in urine and faeces of ≤0.5% of the administered dose in 24-hour samples during two consecutive weeks.

2.2.2 Preparation of the clinical trial product

The final formulation of the trial product (1.0 mg/mL [^{3}H]-semaglutide; 900 µCi/mL) was manufactured at the trial site by diluting the radiolabelled stock solution with the unlabelled vehicle, followed by sterile filtration of the mixture. Product preparation was in accordance with Good Manufacturing Practice (World Health Organisation, 2003). The exact drug and radiochemical concentrations of [^{3}H]-semaglutide were measured before the trial product was released. The final trial product was stored in a refrigerator (2–8^{o}C), protected from sunlight and administered by a standard syringe and needle no later than 72 hours after final preparation.

2.2.3 Sample collection and handling

Blood, plasma, urine, faeces and expired air samples were collected during in-house visits. After collection, samples, excluding expired air samples, were frozen prior to analysis. Samples were collected based on dosing taking place on Day 1.

Blood samples were to be collected in ethylenediaminetetraacetic acid (EDTA) tubes at predose and from 8–1512 hours (63 days) postdose. Plasma was prepared from blood by centrifugation. For sampling of urine and faeces, a predose sample was collected up to 12 hours (urine) or up to 48 hours (faeces) prior to dosing; thereafter, samples were to be collected daily in 24-hour intervals until Day 15, and in 24-hour intervals every 7 days up until Day 64. Samples collected over a 24-hour interval were pooled. Within 10 minutes of urine collection, Tween 20 was added to the sample to a final concentration of 0.05% to minimise adsorption of semaglutide-related material to container surfaces.

Samples of expired air were collected for 3 minutes on Days −1, 3, 6, 9, 12, 15, 22, 29 and 36. Subjects exhaled through a plastic tube in a preweighed 20 mL glass vial in dry ice, to facilitate condensation. This procedure was expected to yield between 0.5 and 1.0 mL of water after 3 minutes of expiration. The vial was capped immediately for weighing and counting.

For further information, relating to sampling, refer to Appendix B.

2.3 Nonclinical studies

2.3.1 Laboratory animals

All protocols for the nonclinical *in vivo* studies were reviewed and approved by the ethical review council at Novo Nordisk A/S and at Covance Inc. (Princeton, NJ, USA) prior to study start. The studies were conducted at Covance Laboratories (Harrogate, UK) under the UK Home Office license, and laboratory procedures fully complied with the international standards of Good Laboratory Practices. Animals were killed by a UK Home Office-approved method.

2.3.2 Study design and sample collection – rats and monkeys

The semaglutide doses in non-clinical species were selected to be at the no observed adverse effect level. Non-fasted male Wistar Hannover rats (predose weight: 261–336 g) were administered with a single s.c. dose of 0.3 mg/kg (270 µCi/kg [10 MBq/kg]) [^{3}H]-semaglutide. Of these, three rats were used in the excretion study, three were used in the PK analysis of total radioactivity and five were used to collect plasma for metabolic profiling. Rats used for the excretion study were singly housed in glass cages, otherwise rats were housed (<5 per cage) in solid-floor polypropylene cages containing Aspen wood-chips.

Three non-fasted male Cynomolgus monkeys (predose weight: 2.5–2.7 kg) received a single s.c. dose of 0.03 mg/kg (378 µCi/kg [14 MBq/kg]) [^{3}H]-semaglutide. Prior to the study, monkeys were housed in groups in solid-floor pens conforming to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (Home Office, London, 1989). For further information, please refer to Appendix C. During the study, monkeys were housed, singly, in 'metabolism cages' – air-conditioned rooms where they received 12-hour-per-day light exposure. Blood samples from rats and monkeys were collected in EDTA capillary tubes between 2 and 168 hours and 0.5–168 hours postdose, respectively. Plasma was subsequently prepared by centrifugation. Rat urine and faeces were collected up to 168 hours postdose; expired air up to 24 hours postdose. Monkey urine and faeces was collected over the following time intervals: 0–12 hours (urine only), 12–24 hours (urine only) and in 24-hour intervals between 24–336 hours postdose. For further information, please see Appendix C.

2.4 Sample analysis

2.4.1. Analysis of total radioactivity (human, rat and monkey)

Total [^{3}H]-radioactivity was determined in blood, plasma, urine and faeces using liquid scintillation counting (LSC). Expired air (humans) and cage debris and washings (aqueous and organic; rats and

monkeys) were also assessed for radioactivity using LSC. All samples in rat, monkeys and humans were analysed as intact and dry samples, except for expired air samples. As tritiated water can be formed as a consequence of metabolism (e.g. beta-oxidation), this was monitored during the trial and animal studies. Both intact and dry samples were measured to assess the amount of total radioactivity that corresponded both to volatile compounds (tritiated water) and to semaglutide and its metabolites. Intact samples were those which were unaltered post-collection; dry samples were freeze-dried and subsequently re-constituted in the same volume of water.

2.4.2 Bioanalysis of semaglutide in plasma (human)

Quantification of semaglutide in human plasma samples was performed using liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described previously (Kapitza *et al.*, 2015) and validated according to bioanalytical guidelines (U.S. Department of Health and Human Services, 2001).

2.4.3 Pharmacokinetics (human)

For the intact and dry human plasma samples, AUC_{0-last} was calculated for semaglutide-related material as the sum of the area under the curve (AUC) from time zero to the last quantifiable observation, by using standard non-compartmental methods, employing the linear trapezoidal method on the observed concentrations and using actual timepoints. The $t_{½}$ was determined using $t_{½}=\log(2)/\lambda_z$, where λ_z was estimated by log-linear regression on the terminal part of the concentration–time curve.

2.5 Profiling

2.5.1 Samples selected for metabolite profiling (human, rat and monkey)

In humans, the first sample was taken predose; with regards to the metabolite profiling, this was 'Day 1'. Metabolite profiles of plasma were performed at 11 timepoints (predose [Day 1], Days 2, 3, 4, 5, 8, 11, 15, 22, 29 and 36); urine at 10 time intervals (predose, days after dosing: 0–2, 2–5, 5–8, 8–11, 11–14, 14–21, 21–28, 28–35 and 35–42) and faeces at 7 time intervals (predose, days after dosing 2–5, 5–8, 8–10, 10–12, 12–14 and 14–21). See Appendix B for an overview of included samples. Plasma samples were pooled from all subjects using equal amounts of plasma per timepoint. Urine and faeces from all subjects were pooled by taking a fixed proportion (0.05% for urine and 0.1% for faeces) of each individual sample or homogenate weight, for each time interval.

In rats, plasma metabolite profiles were conducted at 5 timepoints: 2, 12, 24, 48 and 72 hours postdose. For urine, 6 pools (3 per sex) were generated: 0–24, 24–72 and 72–120 hours postdose; for faeces, 4 pools (2 per sex) were profiled: 0–72 and 72–120 hours postdose. Sample pools were prepared based on a fixed proportion (10%) of each individual sample, for each time interval. Profiling was conducted in both male and female rats, the results of which were similar, however, only data collected from male rats are described in this manuscript.

In monkeys, plasma metabolite profiles were conducted at 7 timepoints: 2, 12, 24, 48, 72, 96 and 168 hours postdose. Urine pools were collected at 0–24, 24–72, 72–120, 120–168 and 168–216 hours postdose (5 total); faeces pools were collected at 0–72, 72–120, 120–168 and 168–216 hours postdose (4 total). Urine and faeces samples were pooled based on timepoint, using a fixed proportion (10%) of individual samples.

2.5.2 Sample preparation prior to metabolite profiling

2.5.2.1 Plasma

In humans, pooled samples were mixed with water and acetonitrile in a 4:3:1 ratio, prior to HPLC analysis. Samples were centrifuged for 15 minutes at approximately 16,000 x *g*, and the supernatant

was transferred to an HPLC vial. For rats and monkeys, 10 volumes of plasma sample were mixed with 1 volume of 0.5% Tween 80 in water and 10 volumes of acetonitrile. The samples were centrifuged at 5000 x *g* for 5 minutes. For all samples, the amount of radioactivity was determined following extraction using LSC. For human, rat and monkey, the extraction recoveries were 109–130%, 89–117% and 64–81%, respectively.

2.5.2.2 Urine

In humans, pooled samples were centrifuged for 15 minutes at approximately 16,000 x *g* and the supernatant was transferred to an HPLC vial. In rats and monkeys, urine pools were centrifuged at 1500 x *g* for 5 minutes.

2.5.2.3 Faeces

In humans, 4000 µL of water/acetonitrile (75/25) was added to ~1 g of pooled sample and sonicated for 1 hour at room temperature. Sample extracts were centrifuged for 15 minutes at 1600 x *g* and the supernatant transferred to 8 mL vials. Prior to analysis, 1000 µL was transferred to a microcentrifuge tube, centrifuged for 15 minutes at approximately 16,000 x *g* and the supernatant transferred to an HPLC vial. In rats and monkeys, the pools of faeces samples (n=3) based on sex and timepoints were extracted three times with methanol (0.50 g homogenate: 1.5 mL methanol). Vortex mixing and sonication or rotation for 3 minutes was applied, followed by centrifugation at 2000 x *g* for 10 minutes. Following this, for monkeys, the samples were diluted 1:1 with Milli-Q water prior to HPLC analysis. For all samples the amount of radioactivity was determined following extraction using LSC. For humans, rats and monkeys, the recovery after sample preparation was 76–81%, 63–106% and 59–81%, respectively.

2.5.3 HPLC methods

2.5.3.1 Metabolite profiling

Metabolite profiles were prepared from urine and faeces samples using an Ultimate® 3000 RSLC HPLC system (Thermo Scientific-Dionex, Sunnyvale, CA, USA). Sample extracts were analysed by radiodetection following separation on a Jupiter C-12, 250 x 4.6 mm, 4 µm Proteo 90 Å column (Phenomenex, Torrance, CA, USA). The column temperature was 30°C and the flow rate was 1.0 mL/minute. Samples collected from rats and monkeys were analysed using online detection by a Radiomatic 150TR radioactivity detector (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). Samples collected from human subjects were fractionated (250 µL portions of each fraction) into 96-well Deepwell Lumaplates (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) and evaporated using a vacuum centrifuge. The radioactivity in each fraction was determined using the Top count NXT (TopCount NXT/HTS Microplate Scintillation and Luminescence Analyser System, Perkin Elmer Inc., Downers Grove, IL, USA).

2.5.3.2 Metabolite identification

Metabolite identification was performed in human urine and plasma samples.

Sample preparation and HPLC fractioning of metabolites. Plasma samples from human blood collected at 96 hours postdose were pooled (n=6) and plasma proteins precipitated by mixing 3.0 mL with 6.0 mL acetonitrile:methanol 3:1 (v/v) and centrifuged for 5 minutes at 5000 x *g* (4^{o}C). The 96-hour postdose timepoint was selected for metabolite identification based on the high levels of the two plasma metabolites with the highest area under the curve (P2 and P3). The precipitation procedure was repeated once and the supernatants from both preparations were mixed and evaporated to 1.1 mL under a nitrogen stream before being centrifuged with 5000 x *g* for 5 minutes

to remove precipitated material. For urine, 192–216 hours post-dose was selected for metabolite profiling based on the high level of the three most abundant urine components – U6, U7 and semaglutide. The individual urine samples collected at 192–216 hours postdosing were pooled (n=6) and two 25 mL fractions were freeze-dried. Each urine residue was re-dissolved in 1.3 mL water, vortex-mixed for 5 minutes and centrifuged at 5000 x *g* for 2 minutes to remove undissolved material.

After preparation, the plasma and urine samples were fractionated using the same HPLC system as used for profiling analysis. The HPLC fractions from specific plasma and urine peaks were selected for ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) analysis.

*UPLC-MS analysis of metabolites***.** The UPLC-MS analyses of the HPLC fractions with plasma and urine components were conducted with a Synapt G2-S system equipped with an Acquity I-Class UPLC instrument and a high resolution time of flight mass spectrometer (Waters, Milford, MA, USA). In addition, UPLC chromatography of the metabolites was also conducted with fraction collection of UPLC eluent followed by radioactivity monitoring of the fractions (UPLC-RAM) with a TopCount NXT scintillation counter (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). See Appendix E & F for details of sample preparation.

3. Results: Clinical data

3.1 Pharmacokinetics

3.1.1 Pharmacokinetics of semaglutide

The mean plasma concentration−time profile for 0.5 mg s.c. semaglutide in humans measured by LC-MS/MS, is shown in Figure 2. The median t_{max} was 56 hours, the geometric mean half-life ($t_{½}$) was 168 hours and the $AUC_{0–\infty}$ was 3123.4 nmol*h/L (Table 1).

3.1.2 Pharmacokinetics of [^{3}H]-semaglutide-related material

[^{3}H]-semaglutide-related material refers to radiolabelled material originating from the labelled compound. It includes intact semaglutide and its metabolites; it also includes volatile components such as tritiated water.

PK endpoints and geometric mean concentration–time profiles for intact and dry [^{3}H]-semaglutide-related material was similar indicating a limited amount of volatile tritiated compounds (water) in the plasma (Figure 2 and Table 1). For both profiles t_{max} was at 56 hours. Mean concentrations of [^{3}H]-semaglutide-related material in plasma subsequently declined until the last sampling timepoint (1344 hours [56 days]), at which time radioactivity was still detectable (Figure 2).

3.1.3 Pharmacokinetics of [^{3}H]-semaglutide

PK endpoints were also obtained for [^{3}H]-semaglutide based on the metabolite data (Figure 3). The area under the curve ($AUC_{0–last}$) and $t_{½}$ of [^{3}H]-semaglutide (P4) in intact samples was 2940 nmol*h/L and 166 hours, respectively.

3.2 Blood-to-plasma ratio

The average blood-to-plasma ratio of total [^{3}H]-semaglutide-related material determined for dry samples remained relatively constant over time (0.51−0.57).

3.3 Excretion of [^{3}H]-semaglutide related material

The mean total cumulative recovery of [^{3}H]-semaglutide-related material was 75.1% of the administered dose (for urine and faeces, after Day 14 data are interpolated for the time between the 24-hour collection intervals; Figure 4 and Table 2). The variation of total recovery of excreted

radioactivity was low in the human data (69.3–81.7%) and considered to be fairly consistent between individuals. Total cumulative excretion in intact urine was 53.0% compared with 39.6% in dry urine; total cumulative excretion in intact faeces was 18.6% compared with 16.8% in dry faeces. Excretion of [^{3}H]-semaglutide-related material was close to complete by Day 56.

3.4 Metabolite profiling and identification

In the following text, 'P', 'U' and 'F' followed by a number indicate whether the metabolite was detected in human plasma, urine or faeces, respectively, and the peak number of the metabolite in the chromatogram.

3.4.1 Plasma

Seven components were detected in plasma (P1–3, [^{3}H-semaglutide (P4) and P5–P7; Figure 3 and Figure 5) following profiling. [^{3}H]-semaglutide was the primary component in plasma at all timepoints analysed, with a retention time (t_R) of 39.4–39.9 minutes and accounting for 82.6% of the total amount of semaglutide-related material, based on the $AUC_{0–last}$ (Figure 5). In addition, six metabolites (P1–P3 and P5–P7) were detected in plasma; P3 accounting for 7.7%, and the remaining metabolites each accounting for between 0.4% and 3.9% of the total amount of radioactivity, based on AUC_{last} (Table 3). Exposure of all metabolites declined over time and in samples collected after Day 28 postdose, [^{3}H]-semaglutide was the only component detected in plasma (Figure 3).

More advanced chromatographic methods (UPLC) followed by MS detection showed that P3 contained 3 components (P3A, P3B and P3C) (Table 4). The structure of P3A could not be characterised, and only the sum of P3A, P3B and P3C could be quantified. The molecular weight of P3B obtained from MS data matched that of a peptide sequence from semaglutide with truncation of its first 13 amino acids (des[His^7-Tyr^{19}]) (Figure 7). The retention time and MS data matched that from a synthetic reference for P3B (semaglutide-des[His^7-Tyr^{19}]) and the molecular mass (obtained from MS data) was <20 parts per million (ppm) from its theoretical mass. Three peak components were detected in the same retention time range as the radio-peak of P3C (retention times 36.43, 36.97 and 37.75 minutes). The mass accuracies of P3C were in the range 0.6–4.9 ppm compared with the theoretical molecular mass of semaglutide. The closely matching mass accuracies (<20 ppm), but different retention times to the data from semaglutide, indicate that P3C contains semaglutide isomers.

3.4.2 Urine

In human urine, the amount of radioactivity profiled Day 0 to Day 42 accounted for 97.4% of the radioactivity excreted in total (Day 0 to Day 56). In total, 22 components (U1–U21; [^{3}H]-semaglutide) were detected. [^{3}H]-semaglutide accounted for 3.1% of the administered dose with a t_R of 39.9 minutes; this was verified by MS. In addition, 21 metabolites were detected in urine; the two most abundant (U6 and U7) each comprised approximately 14% of the administered dose (all other metabolites were each ≤1.8%) (Figure 6). The structure of the metabolites U6 and U7 were identified (Figure 7; Table 4).

Using MS detection, the urine metabolite U6 was identified as the free Lys^{26} amino acid with a butyric (C4) di-acid group in the side chain. The retention time and MS data matched a synthetic reference of U6 and the molecular mass was <20 ppm from its theoretical mass. Furthermore, product ions from collision-induced dissociation of U6 and the reference were identical.

U7 was also identified by MS to have a free Lys^{26} amino acid with a hexanoic (C6) di-acid group in the side chain. The retention time and MS data matched a synthetic reference of U7 and the molecular

mass was <20 ppm from the theoretical mass of U7. Furthermore, product ions from collision induced dissociation of U7 and the reference were identical.

3.4.3 Faeces

In faeces, the amount of radioactivity profiled predose to Day 21 accounted for 72% of the radioactivity excreted in total (predose to Day 56). After this time the concentration of radioactivity was very low and technical barriers prevented sampling at later timepoints. In total seven metabolites, each accounting for ≤1.5% of the administered dose, were detected in faeces. Intact [^{3}H]-semaglutide was not detected in faeces. Metabolite identification was not performed.

3.5 Safety assessment

All seven subjects completed the trial. No serious adverse events were reported. Seven subjects reported 22 adverse events; the most frequent were gastrointestinal disorders (10 events reported by 4 subjects). All events were mild or moderate.

4. Results: Nonclinical data

4.1 Blood-to-plasma ratio

The blood and plasma samples collected up to 24 hours in rats were used for assessment of the blood-to-plasma ratio, as the primary component within this timeframe was semaglutide and not metabolites or tritiated water. The average blood-to-plasma ratio for rats was found to be 0.44– 0.46. For monkeys, the average blood-to-plasma ratio was in the range 0.54– 0.60 for samples collected up to 48 hours postdose with no, or a low amount, of tritiated water.

4.2 Excretion of [^{3}H]-semaglutide related material

In rats, the total excretion recovery of [^{3}H]-semaglutide-related material after 168 hours showed that urine and faeces were important routes of excretion (Table 2 and Appendix C, Figure 1). Radioactivity was still detectable in urine 168 hours after dose administration, and 22.4% was recovered in the carcasses. Less than <0.5% of radioactivity was detected in expired air.

For monkeys, the total recovery of [^{3}H]-semaglutide-related material after 336 hours showed that, similar to rats, urine and faeces were important routes of excretion (Table 2; Appendix C, Figure 1). Recovery in urine and faeces was not complete when collections were halted, which is visible from the slightly positive slope of the cumulative excretion versus time profile (Appendix C, Figure 1). Unlike for rats, radioactivity in the carcasses was not determined. The difference in total recovery between intact and dry urine and faeces samples was 12.6% and 7.0%, respectively.

4.3 Metabolite profiling

4.3.1 Plasma

In rats, 12 components were detected in plasma following profiling. [^{3}H]-semaglutide was the primary component in plasma at all timepoints analysed, with the retention time of the [^{3}H]-semaglutide reference substance being similar to the major peak in the plasma chromatogram (Figure 5); [^{3}H]-semaglutide accounted for 69% of the total amount of semaglutide-related material, based on $AUC_{0–last}$. In addition, a further 10 metabolites were detected in plasma each accounting for <1–7% of the total AUC_{last} (Table 3). The retention time for the first eluting peak was characteristic of tritiated water, and this was confirmed with freeze-dried data.

In monkeys, 6 components were detected in plasma; these were identified as peak regions as some of them were very broad (apparently containing several poorly separated components). [^{3}H]-semaglutide was the primary component in plasma at all timepoints analysed (Figure 5,

representative sample shown), accounting for 71% of the total amount of semaglutide-related material, based on $AUC_{0\text{-last}}$. Four of the other peak regions, containing 4 metabolites, eluted close to semaglutide (Figure 5) and each accounted for <1–9% of the total $AUC_{0\text{–last}}$ (Table 3). The retention time for the first eluting peak was characteristic of tritiated water, and this was confirmed with freeze-dried data.

4.3.2 Urine

For rats, the amount of radioactivity profiled in urine 0–120 hours postdose accounted for approximately 89% of the radioactivity excreted in total (0–168 hours). Six components were detected in total. The first eluting peak was the primary component in urine at all timepoints analysed, accounting for 26% of the administered dose; this peak was characteristic of tritiated water, as it was not detected after freeze drying. The other components each accounted for <4% of the administered dose.

In monkeys, the amount of radioactivity profiled in urine 0–216 hours postdose accounted for 63% of the radioactivity excreted in total (0–336 hours). Nine components were detected in total; no component had a t_r similar to [^{3}H]-semaglutide. The first eluting peak accounted for 12.6% of the administered dose and was expected to be tritiated water. The other components each accounted for between 0.1–4.9% of the administered dose.

4.3.3 Faeces

In rats, the amount of radioactivity profiled in faeces 0–120 hours postdose accounted for 89% of the radioactivity excreted in total (0–168 hours). Fourteen components were detected; the first eluting peak, expected to be tritiated water, accounted for 4.0% of the dose. The other components each accounted for between 0.7–3.2% of the administered dose. After comparison with a faeces sample that had been spiked with a [^{3}H]-semaglutide reference solution, one peak had a similar LC retention time to intact semaglutide, and was expected to be semaglutide; this was excreted in low amounts and comprised 1.4% of the administered dose in rats. However, its identity was not confirmed by MS.

In monkeys, the amount of radioactivity profiled in faeces 0–216 hours postdose accounted for 74% of the radioactivity excreted in total (0–336 hours). Fifteen components were detected in monkeys; the first eluting peak, expected to be tritiated water, was the most abundant, accounting for 7% of the administered dose. One peak had a similar t_R to [^{3}H]-semaglutide and was expected to be unchanged semaglutide; it constituted 0.6% of the total dose.

5. Discussion

The absorption, metabolism and excretion of a single s.c. dose of semaglutide were investigated in healthy human subjects as well as in rats and monkeys using radiolabelled semaglutide. Radiolabelling the fatty di-acid moiety allowed tracking of the fatty acid side chain in the human trial and all study species; tritium labelling was selected for this, in favour of carbon-14, due to its greater specific radioactivity. While random loss of tritium tracers through hydrogen exchange is a concern, the labelled position on semaglutide – a covalent and non-labile C-H bond – is specific and chemically stable at physiological pH. The high stability of the [^{3}H]-semaglutide tracer, and low amounts of tritiated water formed during stability investigations (during both formulation and in the biological matrices), confirmed its suitability for use in metabolism studies.

The human blood-to-plasma ratio for radioactivity analysis from dosed subjects was 0.51–0.57 and the haematocrit was 0.45 at baseline, meaning that the plasma fraction was 0.55. Similar observations were obtained for nonclinical species (ratios were 0.44–0.60), verifying that red blood

cell partitioning of [^{3}H]-semaglutide material is negligible and that plasma is a relevant matrix for assessing PK parameters and metabolism profiles. Semaglutide was shown to be the primary component circulating in plasma in both humans (83%) and nonclinical species (58–72%); it was slowly but extensively metabolised prior to excretion – primarily in urine – with metabolite profiles that appear to be similar across species. Semaglutide PK was similar to that observed in previous clinical studies (Kapitza *et al*., 2015).

In addition to semaglutide, six metabolites were identified in human plasma. The most abundant metabolite (P3) accounted for 7.7% of all [^{3}H]-semaglutide-related material; the other metabolites each accounted for between 0.4% and 3.9%. P3 was found to consist of several co-eluting components; three peaks were resolved and two of these three peaks were structurally characterised as a metabolite with truncated His^7-Tyr^{19} sequence (with an intact fatty di-acid and ADO-linker) and three semaglutide isomers (which co-eluted in one peak). Profiling confirmed that the metabolites in human plasma were formed following proteolytic cleavage of the peptide backbone and beta-oxidation of the fatty acid side-chain. The human plasma exposure of metabolite peak P3 was assessed as the total exposure of P3A, P3B, P3C-I, P3C-II, P3C-III, which corresponds to 7.7 % of total AUC, where the individual exposure of each component is far below the relative exposure limit (<10 %) addressed for safety assessments in guidelines (FDA-MIST and ICH M3 R2). Similar to humans, ten and four metabolites were identified in the plasma of rats and monkey, respectively, each accounting for less than 9% of all [^{3}H]-semaglutide-related material.

As expected for a compound with long $t_{½}$ (approximately one week/168 hours in humans), the excretion rate was slow and the dosed radioactivity was not completely recovered in excreta. The mean total recovery of [^{3}H]-semaglutide and its metabolites was 75.1% in humans; 53% in urine and 18.6% in faeces, with a minor amount (3.2%) in expired air. In addition to the PK of semaglutide, other factors such as non-specific adsorption to containers and the formation of tritiated water (with $t_{½}$ of 4–18 days in humans) can influence the recovery (Foy, 1964). To prevent semaglutide and metabolite adsorption to the containers Tween detergent was added to all containers used for handling of urine in the clinical trial. This method was developed using [^{3}H]-semaglutide prior to initiation of the clinical trial, but it is not known whether it was adequate in preventing adsorption of the formed metabolites present in the urine. Considering factors such as PK, possible adsorption and the relatively long collection periods, the obtained radioactive recovery was considered acceptable and the primary excretion route was comparable with that from a similar clinical trial with the GLP-1 analogue liraglutide, where the majority of dosed radioactivity was also excreted in urine (Malm-Erjefält *et al.*, 2010). Drug retention in human tissues was considered low, which is supported by quantitative whole-body autoradiography studies in rats (data not shown) showing that the amount of radioactivity declines over time in all tissues, and in sampling times later than 168 h the content of radioactivity was below LLOQ in the majority of tissues. The total recovery for rats was 72.1% in excreta (36% in urine) and expired air, while the carcasses contained 22.4% of administered radioactivity. The total recovery in excreta was 58.2% (30% in urine) for monkey. Neither expired air nor carcass details were assessed in the monkey study and the use of Tween was not applied in the nonclinical studies. The lower recovery in the monkey study was also due to the study being terminated while semaglutide-related material was still being excreted after 14 days. This was to avoid long-term housing of monkeys in the metabolism cages.

In the current trial, 22 components of [^{3}H]-semaglutide were detected in human urine and seven components in faeces; the level of intact [^{3}H]-semaglutide in urine was only 3.1% of the administered dose and semaglutide was not detected in faeces. All the metabolites in urine and faeces showed shorter retention times than semaglutide in the reversed phase chromatography; this

was as would be expected from degraded products with higher hydrophilicity than intact semaglutide. Tritiated water was also present in increasing amounts over time in both urine and faeces suggesting metabolic degradation of the fatty acid part of the molecule. Structural elucidation of the two most abundant metabolites (U6 and U7) in human urine revealed that the structures were formed after proteolytic cleavage of the peptide backbone and beta-oxidation of the fatty di-acid side chain. The two metabolites were identified as the free Lys^{26} amino acid with butyric (C4) and hexanoic (C6) di-acid side chains. The ADO-linker remained intact in these metabolites throughout excretion. Six and nine components, with greater polarity than semaglutide, were detected in rat and monkey urine, respectively. Fourteen and 15 components were detected in rat and monkey faeces, respectively, the majority of which had greater polarity than semaglutide. Based on comparison of the chromatograms and retention times, unchanged semaglutide was detected in both urine and faeces (<1% of the dose) in rats and in faeces (<1% of dose) in monkeys, and the metabolite profiles appear to be similar across species (Figure 6).

Peptide degradation and beta-oxidation are common metabolic pathways both in animals and humans, and are not confined to specific organs. Two enzymes are known to be involved in native GLP-1 degradation: DPP-4 and NEP (Deacon *et al.*, 1995; Hupe-Sodmann et al., 1995). [1, 2] DPP-4, which is found in plasma as a soluble enzyme and within various body compartments, is responsible for the inactivation of GLP-1 via truncation of the N-terminal dipeptide sequence, His-Ala. NEP, a membrane-bound enzyme, is less specific and known to hydrolyse peptide bonds on the N-terminal side of aromatic or hydrophobic amino acids (Roques et al., 1993). NEP is primarily present in kidneys, but it is also found in the brain, lung, lymph nodes and intestine (Roques *et al.*, 1993). Both enzymes have also been shown to degrade the GLP-1 analogue liraglutide in vitro (Malm-Erjefält *et al.*, 2010) though at a significantly slower rate than native GLP-1 (human $t_{½}$ values: liraglutide, 13 hours; GLP-1, 1–2 minutes) (Jacobsen *et al.*, 2016). Exploratory *in vitro* incubations of these enzymes with semaglutide indicate that NEP can degrade semaglutide, but the rate at which it is degraded is even slower than for liraglutide (data not shown). The cleavage position for the most abundant human metabolite detected (cleaved at the N-terminal side of the hydrophobic amino acid, Leu) correlates well with the specificity of NEP; in another study, a similar metabolite was also detected following *in vitro* incubation with NEP (data not shown). Degradation of semaglutide by DPP-4 has not been investigated, but this is expected to be minimal as the non-coding amino acid (alpha-aminoisobuturic acid) at position number 8 has been shown to decrease enzymatic degradation (Lau *et al.*, 2015).

When taking dosing regimens into consideration, the PK properties of semaglutide reported in this clinical trial were comparable with those of two recent studies: one single-dose trial in subjects with different degrees of renal impairment, and the other a multiple-dose trial in postmenopausal women with T2D (publication in preparation; Kapitza *et al.*, 2015). The PK human plasma-versus-time profile of semaglutide in this trial was as follows: total exposure 2940 nmol*h/L (based on metabolite profiling) and 3123 nmol*h/L (based on bioanalysis); and $t_{1/2}$ was 166 hours (based on metabolite profiling) and 168 hours (based on bioanalysis). The similarities between the profiles produced by both techniques validate each dataset.

6. Conclusions

In AME studies, the comparison between the metabolite profile in humans and nonclinical species is of key interest; major differences may indicate the need for additional toxicity studies to verify the safety profile of the investigational drug. Similarly, metabolites comprising a large proportion (>10%) of the total drug exposure may give cause for further investigation. In the trial and animal studies

presented here, no major between-species differences were observed with semaglutide, and metabolites comprised only a small proportion of all [^{3}H]-semaglutide-related material.

Semaglutide was the primary component circulating in plasma for humans and both nonclinical species. The metabolite profiles were comparable between species. Results from the human trial suggest that semaglutide is slowly, but extensively, metabolised across tissues via proteolytic cleavage of the peptide backbone and sequential beta-oxidation of the fatty di-acid chain, and that degradation products are excreted via urine and faeces. Only a minimal amount of intact semaglutide (3%) was excreted in the urine.

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7.1 Authorship contributions

Lene Jensen, Hans Helleberg, Ad Roffel, Jan Jaap van Lier, Inga Bjørnsdottir, Palle Jacob Pedersen, Everton Rowe, Julie Derving Karsbøl, and Mette Lund Pedersen all contributed to data interpretation and preparation of the manuscript.

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8. Ethical approval

All procedures performed in studies involving human participants were in accordance with ethical standards, and all applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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Tables

Table 1: Human pharmacokinetic endpoints in plasma following single s.c. dose administration of [^{3}H-semaglutide]

Pharmacokinetic parameter	Pharmacokinetic endpoint		
	Semaglutide	[^{3}H]-semaglutide-related material	
		Intact[b]	Dry[c]
$AUC_{0–last}$, nmol*h/L	3123.4 (12.0)[a]	4047.3 (13.3)	3928.6 (14.0)
C_{max}, nmol/L	10.9 (18.2)	11.5 (18.9)	11.6 (19.0)
$t_{½}$, h	168.3 (6.3)	201.2 (5.8)	180.5 (6.0)
$t_{max,}$ h	56	56	56
CL/F, L/h	0.039 (12.021)	NA	NA
Vz/F, L	9.4 (15.3)	NA	NA

Pharmacokinetic values are geometric means (CV%) with the exception of t_{max} values which are medians. Values are obtained using LC-MS/MS analysis. [a]For semaglutide, $AUC_{0–\infty}$, not $AUC_{0\text{-last}}$ is given. $AUC_{0–\infty}$ is from time 0 until infinity after a single dose; [b]The originally collected samples; [c]Freeze-dried samples. The time interval for $AUC_{0\text{-last}}$ measured via LC-MS/MS is predose to Day 36. The time interval for the two other $AUC_{0\text{-last}}$ values is predose to Day 49. AUC, area under the concentration–time curve; C_{max}, maximum concentration; CV, coefficient of variation; h, hour; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NA, not available; s.c., subcutaneous; $t_{½}$, half-life; t_{max}, time to maximum concentration.

Table 2: Excretion of radioactivity following single s.c. dose administration of [^{3}H]-semaglutide

Species	**Human[a] (% of dose [%CV]) n=7**	**Rat (% of dose [%CV]) n=3**	**Monkey (% of dose [%CV]) n=3**
Dose	0.5 mg 16.7 MBq	0.3 mg/kg 10 MBq/kg	0.03 mg/kg 14 MBq/kg
Time	0–64[b] days	0–1 week	0–2 weeks
Gender	M	M	M
Urine	53.0 (8.2)	35.6 (27.7)	30.3 (25.3)
Faeces	18.6 (19.9)	32.6 (9.7)	20.7 (3.0)
Expired air	3.2 (9.0)	<0.2	NA
Carcass	NA	22.4 (27.0)	NA
Cage wash and debris	NA	3.7 (22.2)	7.2 (28.4)
Total excretion	75.1 (5.2)	72.1 (8.8)	58.2 (10.2)
Total recovery	75.1	94.5 (0.5)	58.2

[a]Mean body weight was 79.1 kg, corresponding to a dose of 0.0063 mg/kg (0.21 MBq/kg). [b]Maximum planned duration; measurement was stopped after 57 days. %CV, coefficient of variation in %; NA, not applicable; s.c., subcutaneous

Table 3: Exposure of semaglutide and metabolites in plasma across species

AUC_{total}	**Timepoint of samples profiled**	**% semaglutide (% of AUC_{total})**	**Total number of metabolites**	**% metabolites (% of AUC_{total})**
Human	0–35 days	83	6	<1-7.7
Rat	2–72 h	69	10	<1–7
Monkey	0.5–168 h	71	4	<1–9

AUC_{total}, area under the concentration–time curve.

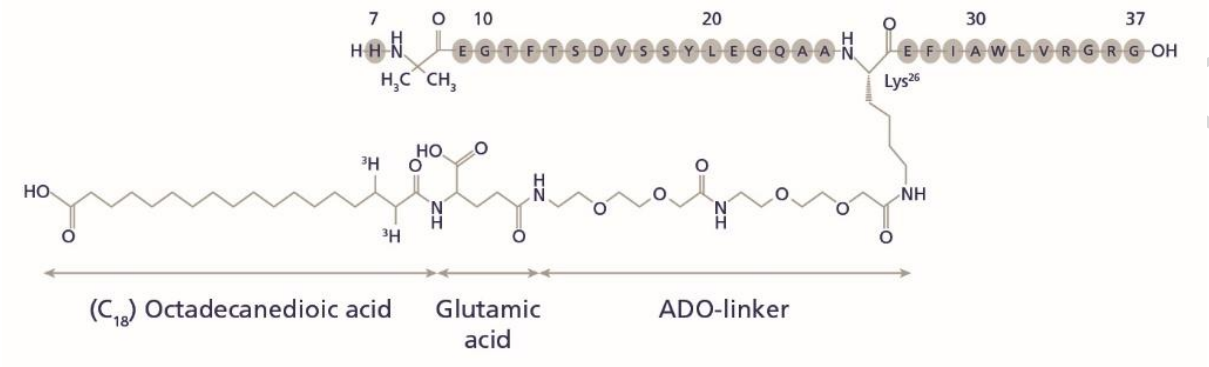
Table 4: Human metabolite and isomer structure identification in plasma and urine

HPLC peak	Metabolite /isomer	[a]MS-peak retention time (min)	[a]Radio-peak retention time (min)	Mass of protonated ion (g/mol)	Type of ion	Obtained mass in neutral state (g/mol)	Mass of proposed structure (g/mol)	Mass accuracy (ppm)[b]
P3	P3B	34.03	34.75	2716.5317[c]	$[M+H^+]^+$	2715.5244	2715.5211	1.2
	P3C-I	36.43	37.00	4112.1187[c]	$[M+H^+]^+$	4111.1114	4111.1154	–1.0
	P3C-II	36.97	[d](35.75–38.25)	4112.1167[c]	$[M+H^+]^+$	4111.1094	4111.1154	–1.5
	P3C-III	37.76		4112.1250[c]	$[M+H^+]^+$	4111.1177	4111.1154	0.6
Semaglutide (plasma)		40.24	40.50	4112.1328 4165.0454	$[M+H^+]^+$	4111.1255	4111.1154	2.5
U6	U6	16.75	17.25	666.3265[e]	$[M+H^+]^+$	665.3192	665.3120	10.8
U7	U7	20.86	21.25	694.3484[e]	$[M+H^+]^+$	693.3411	693.3432	–3.0
Semaglutide (urine)		25.69	25.75	4112.1191	$[M+H^+]^+$	4111.1118	4111.1118	–0.9

[a]Matching retention times from radio- and MS-peaks; [b]Difference between obtained mass and mass of proposed structure divided by mass of proposed structure (structures are shown in Figure 7. [c]Monoisotopic masses from deconvolution of multiply charged positive ions; [d]Retention time range at peak base; [e]Monoisotopic masses from singly charged positive ions. HPLC, high-performance liquid chromatography.

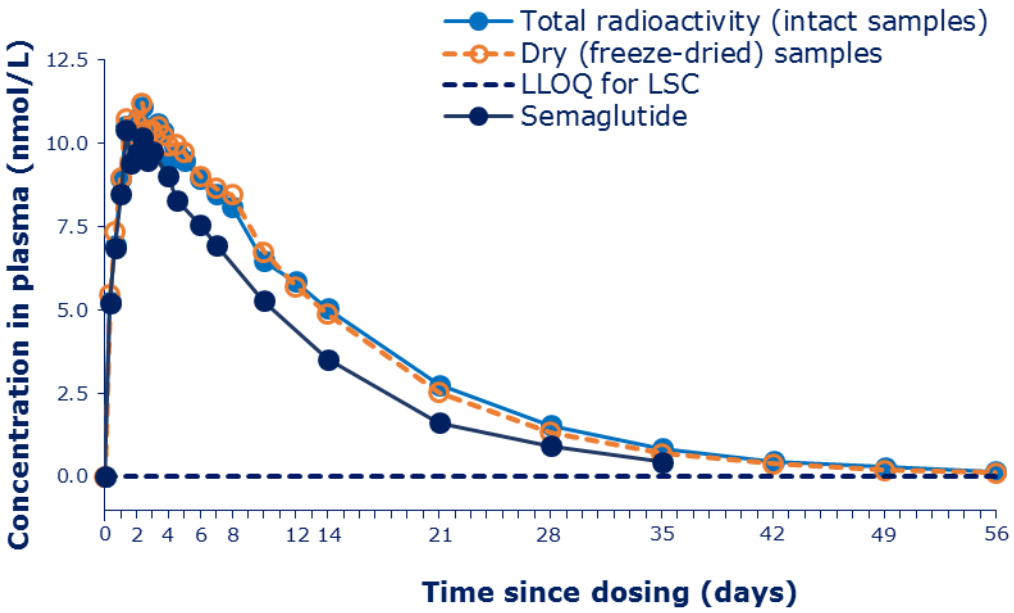
Figures

Figure 1: Radiolabelled semaglutide test substance with marked positions for tritium atoms (^{3}H) and side chain with ADO-linker, glutamic acid moiety and octadecanedioic acid



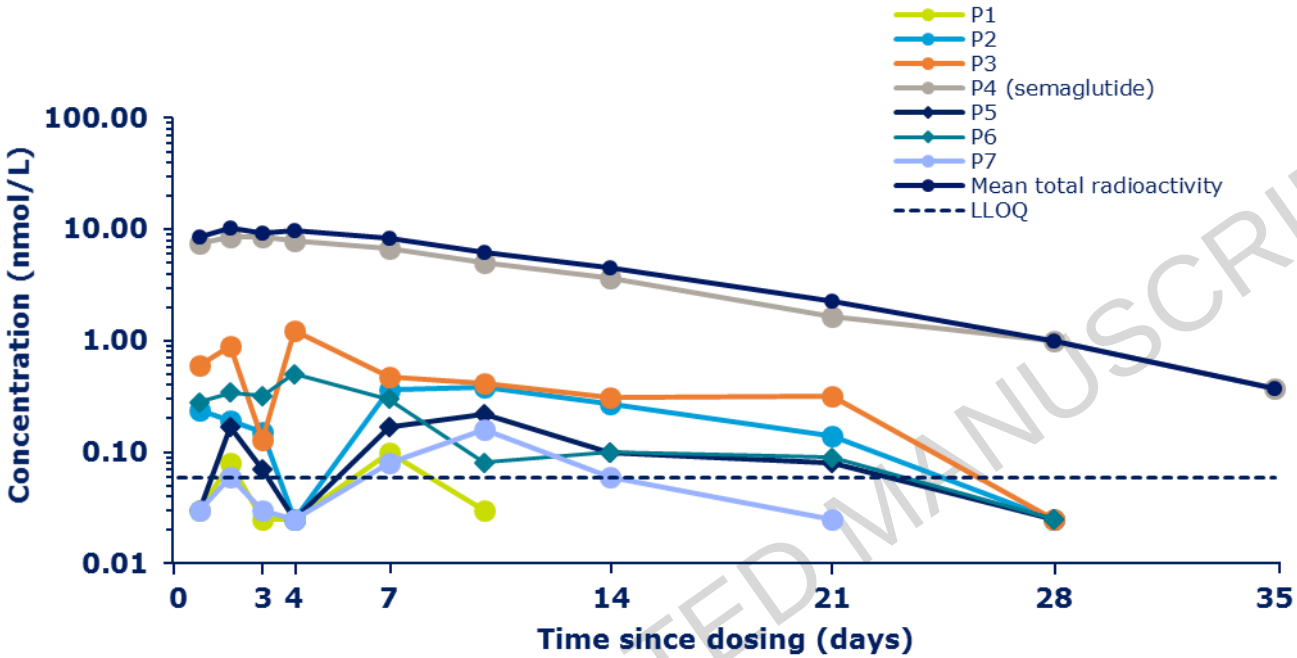
Changes to semaglutide compared with native human GLP-1 are: amino-acid substitutions at position 8 (alanine to alpha-aminoisobutyric acid) and position 34 (lysine to arginine), and acylation of the lysine in position 26 with two ADO moieties followed by a glutamic acid moiety (the 'spacer') and a C-18 fatty di-acid side chain (Lau *et al.*, 2015). ADO, 8-amino-3,6-dioxaoctanoic acid.

Figure 2: Pharmacokinetics in plasma in humans: Mean concentration–time profile for [^{3}H]-semaglutide-related material in intact and dry plasma samples, and for semaglutide in intact plasma samples in humans



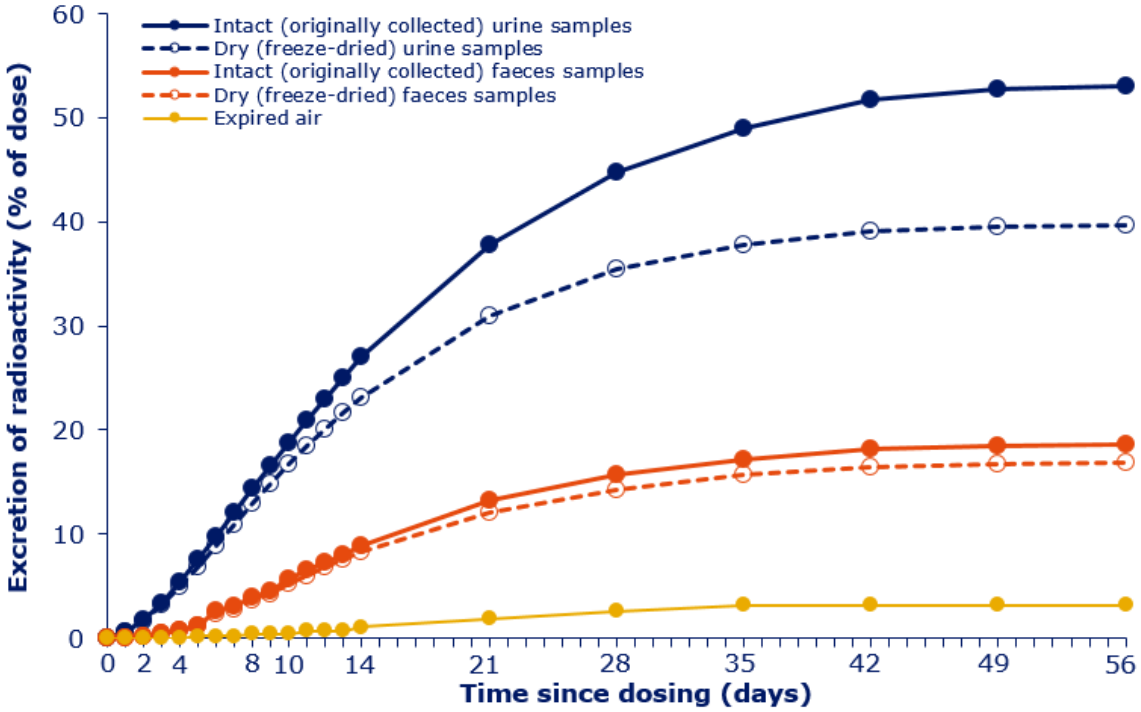
Mean is the geometric mean for all subjects (n=7) after a single, s.c. dose of 0.5 mg/450 µCi (16.7 MBq) [^{3}H]-semaglutide. Values below LLOQ are imputed. Semaglutide concentrations were obtained using LC-MS/MS analysis. Concentrations of [^{3}H]-semaglutide-related material (intact and dry samples) were obtained using LSC. LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantification; LSC, liquid scintillation counting; s.c., subcutaneous.

Figure 3: Human pharmacokinetics of [^{3}H]-semaglutide and metabolites detected in plasma



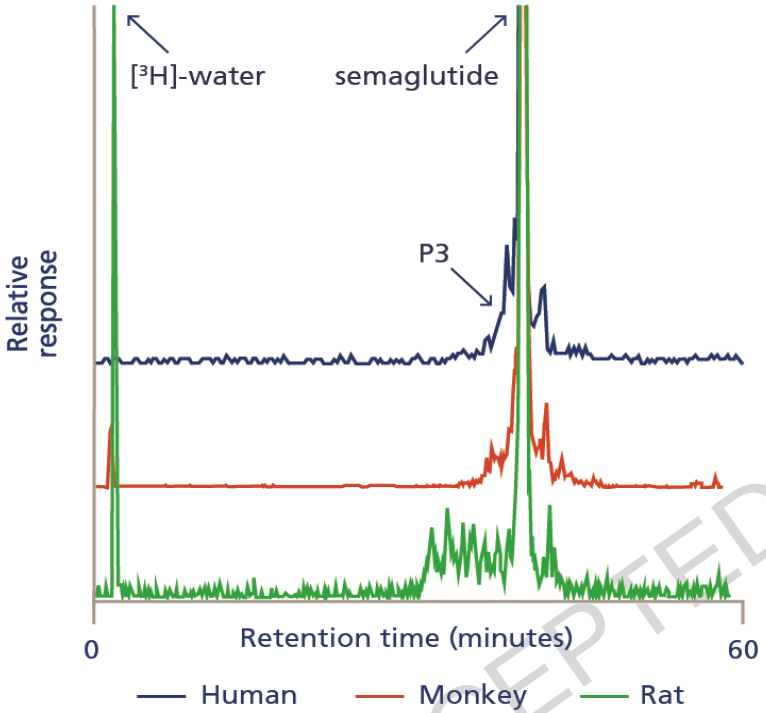
---Reference line for LLOQ for all analytes. Values below LLOQ are imputed. At Day 28 no metabolites were present in the samples analysed. For the metabolites the value included at Day 28 in the figure is ½ LLOQ. Metabolite profiling was performed on pooled samples from 7 subjects. LLOQ, lower limit of quantification.

Figure 4: Cumulative excretion of radioactivity in human urine, faeces and expired air



Mean for all subjects (n=7) after a single, s.c. dose of 0.5 mg/450 µCi (16.7 MBq) [^{3}H]-semaglutide. Urine and faeces were collected up to Day 64; expired air was collected up to Day 36. Recoveries stated for urine, faeces and expired air are the geometric means. S.c., subcutaneous.

Figure 5: Chromatograms from HPLC analysis with radioactive detection showing the metabolite profile in plasma from rat, monkey and human samples at 24, 24 and 96 hours, respectively, following s.c. administration of [^{3}H]-semaglutide



Note, integrations of metabolites are not shown. The relative response is shown in % of the maximum response of semaglutide in each chromatogram. Background levels are different for each species due to different radiodetection techniques (on-line vs. off-line). Human samples were evaporated to dryness prior to detection of the amount of radioactivity, while rat and monkey samples were not; therefore, tritiated water is not seen in the human profile. S.c., subcutaneous.

Figure 6: Structure of semaglutide and human metabolites

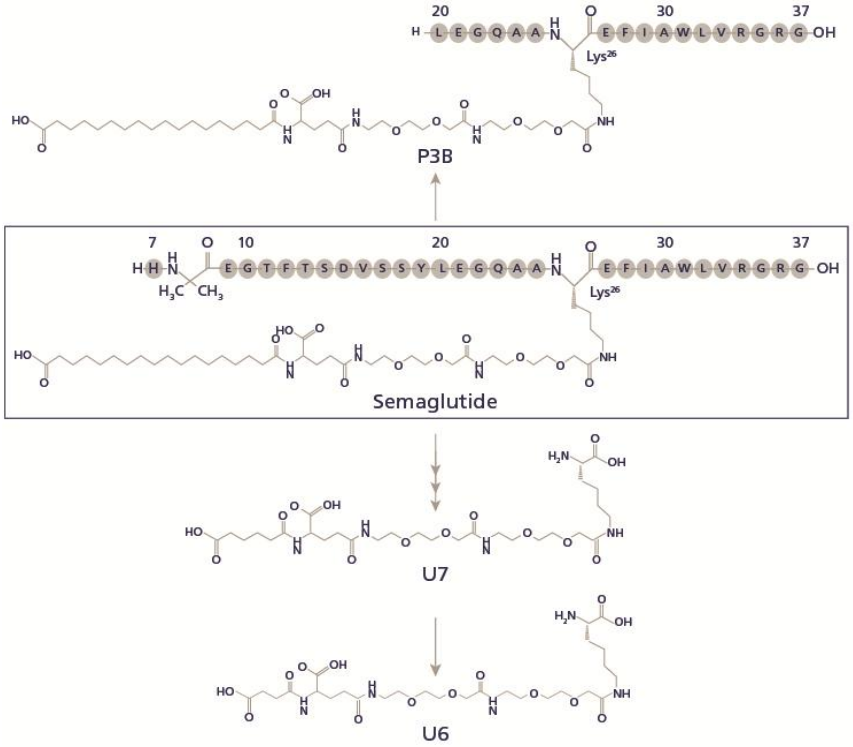
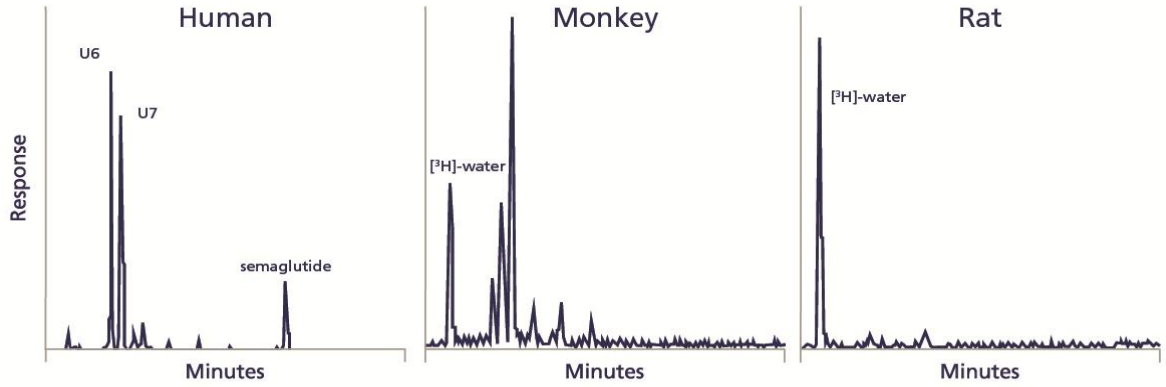
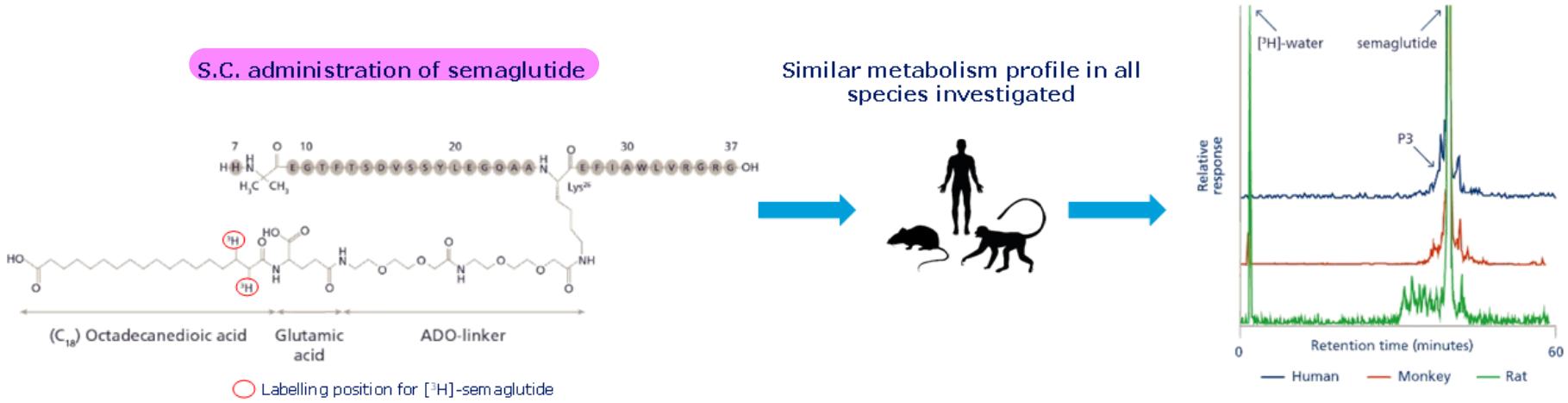


Figure 7: Metabolite profiles from urine at 48–120 (human) and 24–72 hours (monkey and rat) after s.c. administration of [^{3}H]-semaglutide



Human samples were evaporated to dryness prior to detection of the amount of radioactivity, while rat and monkey samples were not; therefore, tritiated water is not seen in the human profile. The number of metabolites detected in human, rat and monkey were not the same due to the different radiodetection techniques used (on-line vs off-line). The human metabolites U6 and U7 were also expected to be seen in rat and monkey, as peaks with similar retention times were detected. S.c., subcutaneous.



Graphical abstract