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ORIGINAL RESEARCH ARTICLE

Impurities and Potential Immunogenicity Associated With Follow-on and Compounded Glucagon-like Peptide-1 Receptor Agonists

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Abstract

Purpose To assess impurity profiles/potential immunogenicity of multiple samples of follow-on or compounded products for two glucagon-like peptide-1 receptor agonists (GLP-1 RAs): semaglutide and liraglutide.

Methods Major histocompatibility complex-II-associated peptide proteomics assay (MAPPs), liquid chromatography-mass spectrometry analysis, photostability testing, and fibrillation assay were performed.

Results For semaglutide and liraglutide, various potentially immunogenic peptides (distinct number/distribution vs originators) were presented on impurity-stimulated monocyte-derived dendritic cells from healthy donors. The follow-on drug substance and follow-on or compounded products had distinct impurity profiles (amino acid deletions/additions and unidentified impurities) versus the originators. Significant disparity in strength, impurity sum, and high-molecular-weight protein level were observed between compounded semaglutide and originator products when exposed to light. The liraglutide follow-ons had reduced physical stability versus the originator.

Conclusions The tested peptide impurities pose increased immunogenicity potential. Follow-on or compounded products also had different impurity profiles, which could be affected by the sourcing of the active pharmaceutical ingredient, manufacturing process, and degradation during storage. The distinct impurity profiles of follow-on and compounded products could lead to an undesirable immune response in patients. These results underscore the importance of *in vitro* immunogenicity assays/clinical immunogenicity studies for GLP-1 RA follow-on or compounded products.

Keywords Compounding · GLP-1 · Impurity · Liraglutide · Semaglutide

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Abbreviations

ADA	Anti-drug antibody
APC	Antigen-presenting cell
API	Active pharmaceutical ingredient
CAD	Charged aerosol detector
DC	Dendritic cell
DCM	Dichloromethane
DDA	Data-dependent acquisition
DDC	Demonstrable difficulties in compounding
DMF	Dimethylformamide
DP	Drug product
DS	Drugs substance
FDR	False discovery rate
GLP-1 RA	Glucagon-like peptide-1 receptor agonist
HLA	Human leukocyte antigen
HMWP	High molecular weight protein
LC-MS	Liquid chromatography-mass spectrometry
LC–MS/MS	Liquid chromatography-tandem mass spectrometry

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MAPPs	Major histocompatibility complex-associated peptide proteomics
MHC	Major histocompatibility complex
moDC	Monocyte-derived dendritic cells
MS	Mass spectrometry
PASEF	Parallel accumulation serial fragmentation
RP-UHPLC	Reversed-phase ultra-high performance liquid chromatography
SPPS	Solid-phase peptide synthesis
T2D	Type 2 diabetes
ThT	Thioflavin T
TIMS	Trapped ion mobility spectrometry
UPLC	Ultra-performance liquid chromatography

Introduction

For any biopharmaceutical product, health authority-approved follow-on products may be mass produced to increase patient access and/or provide alternative treatment options [1]. Although regulatory frameworks are designed to ensure similarity between follow-on products and originators, follow-on products may not have conducted the same kind of clinical testing as the originator product [2–6]. Furthermore, some pharmacies or outsourcing facilities in the United States mass produce compounded medications using different active pharmaceutical ingredients (APIs), compounding methods and processes, dosage forms, administration methods, and packaging from the branded drug, and regulatory approval is not required before marketing these compounded drugs [6]. These compounded medications may be associated with ambiguity in API sourcing and testing, lack of standardized finished product testing and quality control, immunogenicity assessments, and adverse event reporting [7, 8]. Follow-on products include any 'copy' products, regardless of whether regulatory approval is in place, whereas compounded products generally cannot be a 'copy' and lack regulatory approval.

Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) are a class of medication approved for treating type 2 diabetes (T2D) and obesity [9–17]. The efficacy of some GLP-1 RAs in lowering body weight led to an increase in demand, especially for semaglutide injectable medicines, which outpaced manufacturers' supply for a short period of time [18, 19]. As a result, the US Food and Drug Administration (FDA) declared that semaglutide injection products were in shortage in late 2022, which resulted in pharmacies and outsourcing facilities compounding drug products containing semaglutide [20]. These compounded versions continued to be on the market as alternatives to the originators even though the FDA declared that the supply met or exceeded nationwide demand in February 2025 [7, 20, 21]. As of January 21, 2026, the FDA's Adverse Event Reporting System (FAERS) database included 961 reports of adverse events associated with compounded semaglutide products, of which there were 743 serious events, 237 hospitalizations, and 21 deaths [22]. This was over quadruple the number of adverse events for all compounded drugs in fiscal year 2022 [22]. Unlike manufacturers of FDA-approved medicines, which must report all adverse events received to the FDA, compounders are not subject to the same reporting requirements; thus actual numbers of adverse events may be higher, especially considering the large number of 503 A compounding pharmacies in the US [23]. The FDA says that state-licensed pharmacies compounding drugs do not report adverse events to the FDA, while outsourcing facilities compounding drugs are only required to report adverse events that are "serious" and "unexpected." In November 2024, a citizen petition was filed, requesting that the FDA place semaglutide products on a list of drug products (DPs) that present demonstrable difficulties in compounding (DDC) that are reasonably likely to lead to adverse effects on the safety and effectiveness of the DP. This petition suggested safety, effectiveness, and quality issues existed with follow-on semaglutide drugs substance (DS) and compounded DPs [24]. In August 2025, the FDA received a second citizen petition, requesting that the FDA take several actions to address the risks associated with the production and distribution of compounded drugs purporting to contain semaglutide, including asking the FDA to issue an order requiring that facilities compounding semaglutide develop and disseminate a letter to healthcare professionals discussing risks associated with these products, including immunogenicity risks [18].

Drug immunogenicity is an unwanted immune response that patients may develop against pharmaceutical therapies [25, 26]. After administration, a drug can be endocytosed by antigen-presenting cells (APCs), which process the drug into peptide fragments that can be presented on human leukocyte antigen (HLA) class II molecules on the surface of APCs [27]. These peptides contain sequences (epitopes) that can potentially be recognized by activated T-cells. Any activated T-cell can, in turn, activate drug-specific B-cells to differentiate into plasma cells, producing anti-drug antibodies (ADAs) [27]. ADAs are a potential safety risk for the patient, as they can lead to hypersensitivity reactions, as well as have an impact upon pharmacokinetics, pharmacodynamics, and efficacy of the compounded drug. If the drug shares amino acid sequences with an endogenous protein, ADAs have the potential to be cross-reactive to the endogenous counterpart [27]. Additionally, process- and product-related factors such as aggregation, fibril formation, degradation during storage, and impurities introduced during production of the API could contribute to undesirable immunogenic potential [8, 26, 28].

Whereas actual immunogenicity in humans can only be assessed in clinical trials, immunogenicity potential can be assessed *in silico* and *in vitro* by assays such as major histocompatibility complex (MHC)-associated peptide proteomics (MAPPs) and dendritic cell (DC) or T-cell activation assays [29–32]. MAPPs is a mass spectrometry-based method to assess the differential HLA (MHC) class II presentation of exogenous proteins and peptides, thereby identifying potential T-cell epitopes [29, 30]. It is generally robust and highly reproducible across different laboratories [29, 30].

Although these assays cannot predict clinical outcomes, they can assess immunogenic potential [29–32]. High numbers of presented non-human (foreign) peptides in MAPPs indicate high theoretical potential for immunogenic risk. It is worth noting that only clinical trials can assess patient safety. The clinical immunogenicity of marketed, discontinued, or investigational originator GLP-1 RAs has been well described and there is a wide spectrum of ADA levels in different originator GLP-1 RAs (Supplementary Table I) [9, 10, 33–38]. ADAs to GLP-1 RAs are often observed; however, the incidence and clinical consequences have a broad range and need to be assessed in clinical trials. Whereas often there are no consequences, some GLP-1 RAs, such as taspoglutide, had to have their clinical trials halted partly due to immunogenicity [39]. In contrast, immunogenicity of follow-on GLP-1 RA DSs and follow-on or compounded DPs from various pharmacies and outsourcing facilities has not been assessed in clinical trials and their level of immunogenicity is unknown. They may contain unidentified impurities that were not previously described or existing impurities with different levels compared with the originators. In previously published papers, an *in silico* analysis indicated that peptide-related impurities with amino acid additions and deletions present in the semaglutide and liraglutide bulk DSs used in follow-on DPs had the potential to be immunogenic [6, 28]. Therefore, the impurity profiles of these follow-on and compounded GLP-1 RAs warranted further study to understand whether and what peptide-related impurities would have an impact upon APCs and may be associated with immunogenicity risk. It is worth noting that it is not only the impurity content but also different new species of aggregates, such as dimer and trimer forms, that can pose an increased risk in immunogenicity [6, 28, 40].

Another factor that impacts upon the safety and efficacy of GLP-1 RAs is exposure to light, which can reduce the chemical and physical stability of the polypeptide due to its sensitivity to light [41]. For example, the FDA-approved originator semaglutide injections are required to be enclosed in a complex container system with an injection pen cap on to adequately protect the solution from light [12]. However, some compounded semaglutide injections are contained in clear glass multidose vials or prefilled syringes without any guidance on light protection, and their stability under light exposure is unknown.

To extend previously published results [6, 28], this study involved two main parts, namely: 1) using the MAPPs assay to measure the immunogenicity potential of upscaled peptide-related impurities derived from compounded and follow-on semaglutide batches, as well as relevant upscaled liraglutide-derived peptide impurities with amino acid additions and deletions; and 2) the assessment of a sampling of follow-on DSs and follow-on or compounded DPs of semaglutide and liraglutide to understand the impurity profiles and stability of compounds marketed as GLP-1 RAs.

Materials and Methods

MAPPs Assay

Upscaling of Peptide Impurities

Solid-phase Peptide Synthesis (SPPS) Peptides were, in general, synthesized on a Symphony® X peptide synthesizer (Gyros Protein Technologies Inc., Tucson, AZ, United States) according to automated solid-phase peptide synthetic procedures using Fmoc-N-a-amino-protected amino acids and suitable common protection groups for sidechain functionalities. On the Symphony® X peptide synthesizer, the following steps 2–6 were repeated, adding one new amino acid to the growing peptide chain in each cycle starting from the C-terminal. 1) Suitable pre-loaded solid phase resin with the first 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acid was swelled in dimethylformamide (DMF) for 30 min; 2) Fmoc deprotection was carried out by 2-times addition of 20% piperidine in DMF (2 × 10 min); 3) resin was washed by DMF and dichloromethane (DCM); 4) The subsequent Fmoc-amino acid with oxyma (0.3 M solution in DMF) was added to the resin followed by *N,N'*-diisopropylcarbodiimide (DIC, 1.5 M solution in DMF) and collidine (1.5 M solution in DMF); 3 to 10 molar equivalents of amino acids and reagents were used; 5) the mixture was allowed to react from 1 to 3 h under nitrogen mixing; 6) the sample was washed with DMF and DCM.

Sidechain Attachment A suitable orthogonally protected lysine (with 4-methyltrityl [Mtt] or allyloxycarbonyl [Alloc] protection) was used for attachment of the sidechain. After the peptide backbone was synthesized using the general SPPS method, the final N-terminal amino acid was protected by tert-butyloxycarbonyl (Boc). Mtt or Alloc were deprotected using 20% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in DCM or borane-dimethylamine complex with catalyst Pd $(PPh_3)_4$, respectively, and sidechains were synthesized

from individual Fmoc-protected building blocks via SPPS methodology. Final washing was achieved by DMF, DCM, MeOH, and Et_2O, and the resin was dried using a high vacuum.

Cleavage From Resin The cleavage mixture consisted of trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H_2O (92.5:5.0:2.5). If cysteine or methionine was present in the sequence, 2.5% dithiothreitol (DTT) was added. The mixture was stirred for 90 min then filtered, and the filtrate was stirred for another 90 min. Cold diethyl ether was added to precipitate the peptide. The suspension was filtered through a Millipore™ filter (0.45 µm polyvinylidene fluoride [PVDF], Merck Millipore, Burlington, MA, USA) under vacuum, and the precipitate collected and dried, giving the crude peptide.

Purification via Preparative HPLC The crude peptide was dissolved in a suitable mixture of water and acetonitrile, and at a suitable pH to prevent precipitation. The mixture was filtered and loaded on C18 or C8 reverse phase column and purified using gradient elution of solvent A – water with 0.1% TFA and solvent B – acetonitrile with 0.1% TFA. All fractions containing peptide products were analyzed on an ultra-performance liquid chromatography (UPLC; Waters Corporation, Milford, MA, United States) and the ones above purity limit were collected, combined, and freeze-dried to give the final peptide as white lyophilizate.

Content Analysis and Aliquoting The final pure peptides were dissolved in a water and acetonitrile mixture and analyzed on a UPLC/charged aerosol detector (CAD) for content of peptide using an insulin aspart certified reference material as the external standard, and the area under the curve values in the charged aerosol detector trace were used to calculate the molar content. The solution of the peptide was aliquoted by pipetting based on the content to create specific molar amounts in individual vials, which were then freeze-dried.

Donor Selection and Monocyte-Derived Dendritic Cells (moDC) Generation

Whole blood and leukapheresis samples were collected from healthy donors (Supplementary Table II) following the ethical protocol/amendment IXP-009_V1 (Belgium; reg. nr. B0792020000009) and license number DE_TH_01H_MIA_2022_0012 (Germany; ref. nr. 24–2528.03–003). Peripheral blood mononuclear cells were obtained from 15 healthy donors covering the most common HLA-DRB1 alleles. Monocytes were isolated using CD14 magnetic selection (Miltenyi Biotec, Leiden, NL, United States) and differentiated into immature moDCs over 5 days in the presence of interleukin-4 and granulocyte–macrophage colony-stimulating factor. On day 5, moDCs were pulsed with 0.33 µM of the different test articles and matured by addition of lipopolysaccharide (10 µg/mL) overnight. On day 6, cells were harvested, washed twice with phosphate-buffered saline, and stored at –80 °C.

HLA-II Peptide Isolation

For each sample, 1 million mature moDCs were lysed in a non-denaturing lysis buffer supplemented with protease inhibitors (Roche, Basel, Switzerland). Lysates were cleared by centrifugation and pre-cleared using control beads. HLA class II peptide complexes were immunoprecipitated using magnetic beads conjugated with a mixture of monoclonal antibodies against HLA-DR (clone L243), HLA-DP (clone B7.21), and HLA-DQ (clone 1a3) (pan-HLA class II capture) in a KingFisher Apex magnetic robot (Thermo Fisher, Massachusetts, United States). Following extensive washing, bound peptides were eluted with mild acid and purified by C18-based solid-phase extraction.

Liquid Chromatography-tandem Mass Spectrometry (LC–MS/MS) Analysis

Peptides were analyzed on a TimsTOF ULTRA2 mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled with an Evosep One liquid chromatography (LC) system (Evosep Biosystems, Odense, Denmark). LC–MS/MS runs were performed using an EV1064 8 cm × 100 µm Endurance column (Evosep Biosystems, Odense, Denmark), with a 20 µm CaptiveSpray 2 Emitter (Bruker Daltonics, Bremen, Germany). The LC column was heated to 40 °C using a column toaster. Samples were analyzed twice with different LC methods: once using the standard 60 spd method (Evosep Biosystems, Odense, Denmark) and once using the high organic 100 spd method (Evosep Biosystems, Odense, Denmark), which is better tailored to measure peptides containing non-standard modifications.

The data-dependent acquisition (DDA) parallel accumulation serial fragmentation (PASEF) method was used in the positive ion mode with one trapped ion mobility spectrometry (TIMS)-mass spectrometry (MS) survey scan and 10 PASEF MS/MS scans per cycle. The ion mobility scan range was 0.7–1.4 Vs/cm^2, and precursor mass range was 100–1700 m/z. Ramp time and accumulation time were both set to 166.0 ms, corresponding to a duty cycle of 100%. Mobility-dependent collision energy ramping was enabled from 20 eV at 0.60 Vs/cm^2 to 59 eV at 1.60 Vs/cm^2.

LC–MS/MS Data Analysis

Due to non-standard modifications present on the loaded test articles, data analysis was carried out in two phases. First, peptides were identified based only on the MS analyses from the 300 samples after standard LC separation (standard 60 spd method) using Fragpipe 23.1 with a 1% false discovery rate (FDR) threshold applied. A concatenated FASTA database containing the test article sequences (without modifications), the Universal Protein Resource (UniProt) human proteome and contaminant data including reagent-derived proteins was used. Searches allowed for unspecific cleavage (length 7–45 amino acids) and included methionine oxidation and deamidation of asparagine and glutamine as variable modifications. Additionally, every set of samples loaded with the same test article (both MS analyses following the standard 60 spd LC method and following the high organic 100 spd LC method) was analyzed using a condition-specific database in Fragpipe23.1 using condition-specific concatenated FASTA databases containing both the unmodified and modified test article sequence, the UniProt human proteome, and contaminant data including reagent-derived proteins. Searches allowed for unspecific cleavage and included methionine oxidation and deamidation of asparagine and glutamine as variable modifications. In the modified test article sequence, modified residues were substituted with custom amino acids B, Z and U, corresponding to the mass of the substituted amino acid and its modification. A 1% FDR threshold was applied. Peptides ranging from 9 to 45 amino acids were considered for downstream analysis.

Quality Control and Motif Analysis

Peptide length distribution histograms were generated (for each donor to assess enrichment of HLA class II-presented peptides). Motif deconvolution was performed using the MHC motif deconvolution tool [42], applying NetMHCIIpan-4.3 [43] to assign likely HLA class II restrictions based on each donor's genotype. Only high-confidence peptides mapping to the test article sequences were annotated as drug-derived.

Materials for Remaining Assays

Recombinant and synthetic follow-on semaglutide DSs and follow-on or compounded semaglutide DPs were obtained from 36 commercial suppliers across North and South America, Europe, and Asia (Table I). This included nine semaglutide DS follow-on products (S1 – S9), 13 injectable semaglutide compounded products (CP: CP1 – CP13), and 11 follow-on or compounded semaglutide oral products (OP: OP1 – OP11), as well as four follow-on or compounded injectable liraglutide products (L1 – L4). All follow-on or compounded DPs were commercially available for patient use at the time of testing. Novo Nordisk (Bagsværd, Denmark) provided the originator DSs and DPs for semaglutide and liraglutide, for comparison. All the excipients used for testing were of pharmaceutical quality, and each test was performed on a sampling of follow-on or compounded samples.

Liquid Chromatography-Mass Spectrometry (LC–MS) Analysis

LC–MS methods used in this study were pre-specified analyses used for all samples. The LC–MS analysis of DS samples was performed by reversed-phase ultra-high performance liquid chromatography (RP-UHPLC) coupled with MS. Semaglutide samples were tested using a Waters Acquity® Bio H-Class UPLC system (Waters Corporation, Milford, MA, United States) equipped with a Waters Acquity® UPLC Peptide BEH C18 column (130 Å 1.7 µm, 2.1 × 150 mm; Waters Corporation, Milford, MA, United States) and a mixture of 0.1% trifluoro acetic acid in water (eluent A) and 0.1% trifluoro acetic acid acetonitrile (eluent B), and a Waters Xevo® G2-XS mass spectrometer (Waters Corporation, Milford, MA, USA) was used. The column was eluted with a gradient from 70%:30% A:B to 58%:42% A:B over 10 min, followed by a gradient from 58%:42% A:B to 54%:46% A:B over 15 min, then a gradient from 54%:46% A:B to 36%:64% A:B for 10 min, and finally a gradient from 36%:64% A:B to 20%:80% A:B over 1 min and isocratic elution at 20%:80% A:B for 2 min at a flow rate of 0.3 mL/min and a column temperature of 45 °C. The LC–MS analyses on DP samples (injectable and oral) were performed using the methods previously described by Hach et al. [6].

Photostability Assessment

Photostability testing was performed according to the International Council for Harmonization Q1B guideline [44] on the originator and two injectable semaglutide DPs (CP12 and CP13) in vials compounded and distributed by outsourcing facilities. Details of the methodology used have been previously described [44]. The exaggerated conditions for photostability testing were intended to assess whether the DP was sensitive to light, and whether the container closure system would adequately protect that product from light exposure.

Fibrillation Assay

Similar to the method used in the previous Hach et al. study [6], the thioflavin T (ThT) stress assay was performed to assess the physical stability of a representative selection of the liraglutide follow-on products. A sample volume of

Table I Details of the originator (semaglutide and liraglutide) and follow-on DSs and DPs obtained for use during this series of tests

Sample name/number	Sample used (DS or DP or HA-approved generic)	Location of follow-on or compounded producer	Production method (recombinant or synthetic)	Dosage form	Labelled concentration	Storage/handling information
Semaglutide DS						
Originator	DS and DP	Denmark	Recombinant	White powder	n/a	−18 to −25 °C
S1	DS	China	Synthetic	White powder	n/a	−18 to −25 °C
S2	DS	China	Synthetic	White powder	n/a	−18 to −25 °C
S3	DS	China	Synthetic	White powder	n/a	−18 to −25 °C
S4	DS	China	Synthetic	White powder	n/a	−18 to −25 °C
S5	DS	China	Synthetic	White powder	n/a	−18 to −25 °C
S6	DS	China	Synthetic	White powder	n/a	−18 to −25 °C
S7	DS	China	Synthetic	White powder	n/a	−18 to −25 °C
S8	DS	China	Synthetic	White powder	n/a	−18 to −25 °C
S9	DS	China	Synthetic	White powder	n/a	−18 to −25 °C
Semaglutide (injectable)						
Originator	DP	Denmark	Recombinant	Pen 3 mL	1.34 mg/mL	2 to 8 °C
CP1	DP (compounding)	North America	Synthetic	Vial 2 mL	5 mg/mL	2 to 8 °C
CP2	DP (compounding)	North America	Synthetic	Vial 4 mL	4 mg/mL	2 to 8 °C
CP3	DP (compounding)	North America	Synthetic	Vial 4 mL	4 mg/mL	2 to 8 °C
CP4	DP (compounding)	North America	Synthetic	Vial 2 mL	1 mg/mL	2 to 8 °C
CP5	DP (compounding)	North America	Synthetic	Vial 5 mL	1 mg/mL	2 to 8 °C
CP6	DP (compounding)	North America	Synthetic	Vial 1 mL	1 mg/mL	2 to 8 °C
CP7	DP (compounding)	North America	Synthetic	Vial 1 mL	2.5 mg/mL	2 to 8 °C
CP8	DP (compounding)	North America	Synthetic	Vial 1 mL	1 mg/mL	2 to 8 °C
CP9	DP (compounding)	North America	Synthetic	Pen 3 mL	0.4 mg/mL	2 to 8 °C
CP10	DP (compounding)	Europe	Synthetic	Pen 3 mL	1.34 mg/mL	2 to 8 °C
CP11	DP (compounding)	North America	Synthetic	Vial 1 mL	1 mg/mL	2 to 8 °C
CP12	DP (compounding)	North America	Synthetic	Vial	1 mg/mL	2 to 8 °C
CP13	DP (compounding)	North America	Synthetic	Vial	1 mg/mL	2 to 8 °C
Semaglutide (oral)						
Originator	DP	Denmark	Recombinant	Tablet	3, 7 and 14 mg	15 to 25 °C
OP1	DP (compounding)	North America	Synthetic	Suspension in 5 mL vial	1 mg/mL	15 to 25 °C
OP2	DP (compounding)	North America	Synthetic	Gummies	2 or 6 mg/gummy	15 to 25 °C
OP3	DP (compounding)	North America	Synthetic	Lozenges	0.25 mg/troche	15 to 25 °C
OP4	DP (compounding)	North America	Synthetic	Tablet	4 mg	15 to 25 °C
OP5	DP (compounding)	North America	Synthetic	Suspension in 30 mL bottle	1 mg/mL	15 to 25 °C
OP6	DP (compounding)	North America	Synthetic	Suspension in 30 mL bottle	1 mg/mL	15 to 25 °C
OP7	DP (compounding)	North America	Synthetic	Suspension in 15 mL vial	1 mg/mL	15 to 25 °C
OP8	Local HA-approved generic	Asia	Recombinant	Tablet	7 mg	15 to 25 °C
OP9	Local HA-approved generic	South America	Synthetic	Tablet	3 mg	15 to 25 °C
OP10	Local HA-approved generic	South America	Synthetic	Tablet	7 mg	15 to 25 °C
OP11	DP (compounding)	North America	Synthetic	Tablet	4 mg	15 to 25 °C
Liraglutide						
Originator	DS and DP	Denmark	Recombinant	Pen 3 mL	6 mg/mL	2 to 8 °C
L1	Local HA-approved generic	South America	Synthetic	Pen 3 mL	6 mg/mL	2 to 8 °C
L2	Local HA-approved generic	South America	Synthetic	Pen 3 mL	6 mg/mL	2 to 8 °C
L3	DP (compounding)	North America	Synthetic	Vial 3 mL	15 mg/mL	2 to 8 °C
L4	DP (compounding)	North America	Synthetic	Vial 3 mL	6 mg/mL	2 to 8 °C

CP Compounded injectable semaglutide drug product, *DP* Drug product, *DS* Drug substance, *HA* Health authority, *L* Injectable liraglutide drug product follow-on, *OP* Follow-on or compounded oral semaglutide drug product, *S* Semaglutide drug subtance follow-on

200 µL was placed each in six different wells for each sample with a ThT concentration of 20 µM. Samples were then stressed in a FLUOstar Omega microplate reader (BMG LABTECH, Offenburg, Germany), at 37 °C, 600 rpm in double orbital shaking mode using one 3 mm glass bead in each well to facilitate fibrillation. For ThT excitation and emission, 450 nm and 480 nm filters were used, respectively. Fibrillation lag time was defined as the duration required for the ThT fluorescence value to reach a specific threshold.

Results

The MAPPs assay was performed on peptide-related impurities. Previously, the *in silico* analyses identified potential HLA class-II-binding-T-cell epitopes for peptide sequences with Aib8, Ser14, Ser17, Thr11, Thr13, Trp31, or Tyr19 amino acid deletions, or Gln23, Glu9, Gly21, Phe12, Thr13, Trp31, or Tyr19 amino acid additions [6, 28]. As semaglutide and liraglutide represent analogs of native glucagon like peptide-1 (GLP-1), their amino acid sequences are generally numbered from 7 to 37 – corresponding to the active fragment of native GLP-1. The peptide-related impurities included in the current MAPPs assay were selected based on these *in silico* analyses [6, 28] and on an *in vitro* T-cell assay where several compounded liraglutide peptide-related impurities were shown to activate CD4 + T cells (data not published).

Semaglutide Impurity-derived Peptides

Semaglutide peptide sequences were synthesized with Thr11, Ser17, Tyr19, and Trp31 amino acid deletions (des), and Glu9, Phe12, Tyr19, Gly21, Gln23, and Trp31 amino acid additions (endo) (Table II). These were tested in APCs from a cohort of 15 healthy donors with HLA types reflecting the global population using the MAPPs assay (Supplementary Table II).

Peptides from clinically validated originator semaglutide and peptide impurities from follow-ons and compounded semaglutide were presented on APCs and the degree of presentation was variable depending on the impurity (Fig. 1). In total, seven different peptides matching the originator semaglutide were detected in APCs from four out of 15 healthy donors that were stimulated with impurities, matching one binding core, as determined by motive deconvolution [42] and NetMHCIIpan [43]. Three to 31 peptides matching the tested impurity-related sequences were found in APCs across all the donors. Semaglutide-related sequences with the addition of Phe12, Tyr19, and Trp31 were identified in

Table II Semaglutide/liraglutide impurities (peptides) assessed in the MAPPs assay, synthesized based on selected impurities[a]

Identity	Sequence	Modification
Semaglutide		
Originator semaglutide	HAibEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	Semaglutide sidechain
Des Thr11	HAibEG_FTSDVSSYLEGQAAKEFIAWLVRGRG	Semaglutide sidechain
Des Ser17	HAibEGTFTSDV_SYLEGQAAKEFIAWLVRGRG	Semaglutide sidechain
Des Tyr19	HAibEGTFTSDVSS_LEGQAAKEFIAWLVRGRG	Semaglutide sidechain
Des Trp31	HAibEGTFTSDVSSYLEGQAAKEFIA_LVRGRG	Semaglutide sidechain
Endo Glu9	HAibEEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	Semaglutide sidechain
Endo Phe12	HAibEGTFFTSDVSSYLEGQAAKEFIAWLVRGRG	Semaglutide sidechain
Endo Tyr19	HAibEGTFTSDVSSYYLEGQAAKEFIAWLVRGRG	Semaglutide sidechain
Endo Gly21	HAibEGTFTSDVSSYLEGGQAAKEFIAWLVRGRG	Semaglutide sidechain
Endo Gln23	HAibEGTFTSDVSSYLEGQQAAKEFIAWLVRGRG	Semaglutide sidechain
Endo Trp31	HAibEGTFTSDVSSYLEGQAAKEFIAWWLVRGRG	Semaglutide sidechain
Liraglutide		
Originator liraglutide	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	Liraglutide sidechain
Des Ala8	H_EGTFTSDVSSYLEGQAAKEFIAWLVRGRG	Liraglutide sidechain
Des Glu9	HA_GTFTSDVSSYLEGQAAKEFIAWLVRGRG	Liraglutide sidechain
Des Glu27	HAEGTFTSDVSSYLEGQAAK_FIAWLVRGRG	Liraglutide sidechain
Endo Phe28	HAEGTFTSDVSSYLEGQAAKEFFIAWLVRGRG	Liraglutide sidechain
Endo Arg36	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRRG	Liraglutide sidechain

[a]The peptide-related impurities included in the current MAPPs assay were selected based on previous *in silico* analyses [6, 28] and on an *in vitro* T-cell assay where several follow-on liraglutide peptide-related impurities were shown to activate CD4 + T cells (data not published)

Ala Alanine, *Arg* Arginine, *des* Removed from molecule, *Endo* Added to the molecule, *Gln* Glutamine, *Glu* Glutamic acid, *Gly* Glycine, *MAPPs* Major histocompatibility complex-associated peptide proteomics, *Phe* Phenylalanine, *Ser* Serine, *Thr* Threonine, *Trp* Tryptophan, *Tyr* Tyrosine

all 15 donors, while the other sequences were found in a minimum of two and maximum of 14 donors. One to 12 different binding cores or motifs were identified in the test sequences for semaglutide-derived impurities.

Importantly, for 6 out of 10 of the assessed impurities – desTrp31, endo Phe12, endo Tyr19, endo Gly21, endo Gln23, and endo Trp31 – peptides spanning the region of addition/deletion were detected while there were no or significantly fewer peptides detected in the originator semaglutide in the same region (Fig. 1, red lines indicating the position of the amino acid insertion or deletion). For example, in the originator, seven unique peptides (total number of peptides was 12) spanning the relevant amino acid position were presented in 4/15 donors, whereas, for impurity endo Tyr19, 26 unique peptides (total number of peptides was 109) spanning the position were detected in a total of 15 donors. Similarly, for endo Trp31, detected unique peptides spanning the region of insertion increased from one in the originator to 13 in the endo Trp31 impurity. However, in the originator, peptides in this region were only found in two donors, all 15 donors presented peptides spanning the Trp31 insertion.

Impurity Profiles of Follow-on Semaglutide DSs

All follow-on semaglutide DS source products originated from China (Table I). LC–MS identified 57 impurities above a cut-off level of 0.02% across the DSs (S1–S9) that were not detected in the originator semaglutide (Table III); out of these, S2 and S3 were from manufacturers who claimed to be present on the FDA's Green List under Import Alert 66–80. As of the publication of this paper, the FDA has not approved any semaglutide follow-on products, but generated a green list for APIs where the FDA has inspected/evaluated the facilities producing the APIs and deemed these facilities to meet "FDA rigorous standards"; DPs using these APIs have not undergone FDA regulatory review for safety and quality [45]. The identified impurities included various forms of semaglutide with missing components, including deletions of Thr, 2-(2-(2-aminoethoxy)

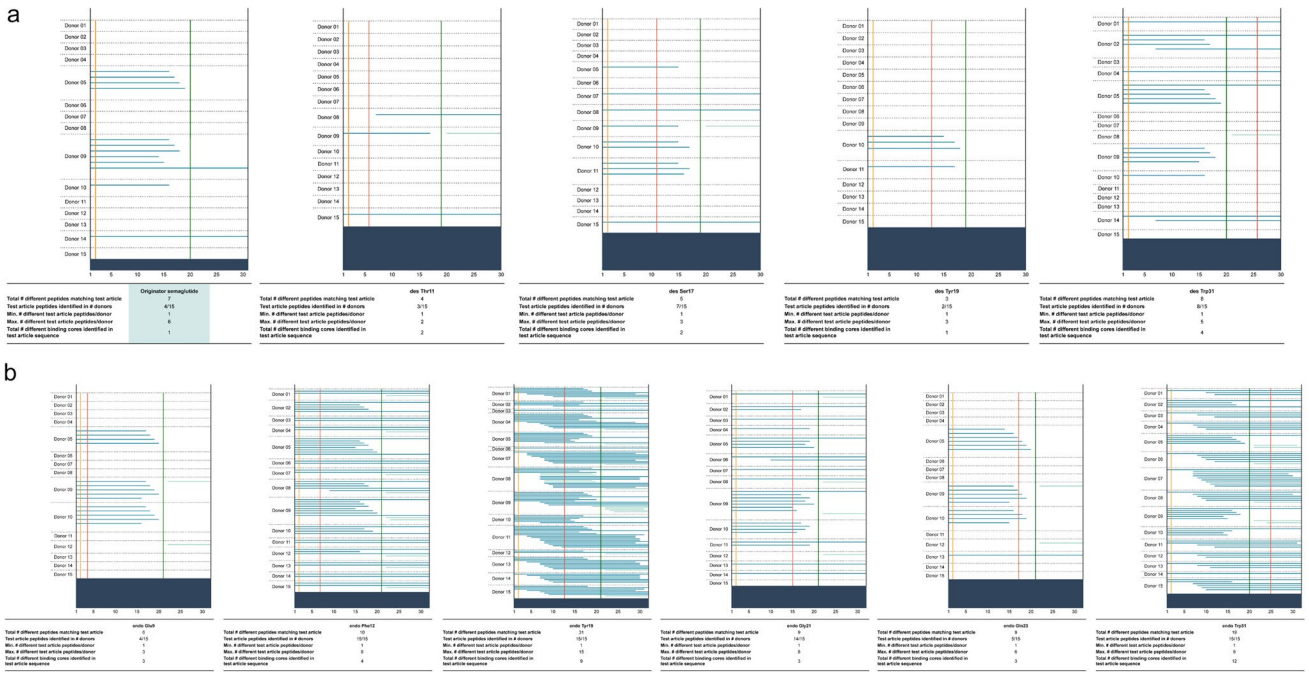

Fig. 1 Impurity-derived peptides detected by MAPPs assay for semaglutide impurities: (**a**) originator semaglutide, des Thr11, des Ser17, des Tyr19 and des Trp31; (**b**) endo Glu9, endo Phe12, endo Tyr19, endo Gly21, endo Gln23, and endo Trp31. Each plot (top) visualizes the detected peptides presented on APCs in individual donors (y-axis, donors 1–15, in between dotted lines) derived from indicated impurities with the x-axis indicating position in the amino acid sequence of the impurity. Each horizontal dark or light green line indicates a detected peptide and its position. Light green lines represent peptides that contain a modification (fatty acid or Aib), dark green lines represent peptides without modification. Vertical orange lines indicate the localization of the AiB modification, vertical green lines indicate localization of fatty acid sidechain. A vertical red line indicates the localization of the impurity-specific amino acid insertion or deletion. The table lists total number of different peptides derived from each indicated impurity, the fraction of donors in which impurity-derived peptides were identified, minimum and maximum number of identified peptides per donor, and total number of distinct binding cores (9-mer sequence) as retrieved from analysis with a MHC motif deconvolution tool [42]. APC, antigen-presenting cell; des, removed from molecule; endo, added to the molecule; Gln, glutamine; Glu, glutamic acid; Gly, glycine; MAPPs, major histocompatibility complex-associated peptide proteomics; max., maximum; MHC, major histocompatibility complex; min., minimum; Phe, phenylalanine; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine

Table III Recorded related impurities in nine semaglutide DS source products, as analyzed by LC–MS

Impurity in DS verified by LC-MS analysis	Semaglutide DS source products								
	S1	S2[a]	S3[a]	S4	S5	S6	S7	S8	S9
Unidentified 1									
Unidentified 2									
Semaglutide backbone									
Semaglutide ox-form 1									
Unidentified 3									
Semaglutide ox-form 2									
Semaglutide isomer form 1									
Unidentified 4									
Unidentified 5									
Semaglutide isomer form 2									
Acylated and diacylated semaglutide backbone									
Acetaldehyde adduct of semaglutide backbone									
Semaglutide diox-form 1									
Semaglutide isomer form 3									
Endo Gly semaglutide form									
Endo Ala semaglutide form 1									
Acylated backbone fragment 16-37									
Semaglutide isomer form 4									
Endo ADO semaglutide 1									
Semaglutide ox-form 3									

ethoxy)-acetic acid (des-ADO), His, and Gly or 'water loss' from semaglutide. Most samples also contained molecules with additions such as acetylated semaglutide. Nine impurities contained in the originator were also found in the follow-on samples, but at increased levels. Unidentified and not clinically qualified impurities were found in all follow-on DS samples. Furthermore, using the LC–MS analysis, the total ion current (counts) was compared between S2

Table III (continued)

Unidentified 6									
Semaglutide diox-form 2									
Endo Ala semaglutide form 2									
Unidentified 7									
Acylated backbone fragment 13-37									
Semaglutide ox-form 4									
Endo ADO semaglutide 2									
Semaglutide isomer form 5									
Endo hexose semaglutide form									
Acylated backbone fragment 14-37									
Semaglutide diox-form 3									
Unidentified 8									
Unidentified 9									
Unidentified 10									
Unidentified 11									
Unidentified 12									
Semaglutide isomer form 6									
Des Thr semaglutide form									
Des ADO semaglutide form 3									
Unidentified 13									
Unidentified 14									
Des His semaglutide form 1									
Formaldehyde adduct of semaglutide									
Acetaldehyde adduct of semaglutide 1									
Semaglutide isomer form 7									
Des Gly semaglutide form									
Unidentified 15									
Semaglutide isomer form 8									

Table III (continued)

Unidentified 16									
Acetaldehyde adduct of semaglutide 2									
Acylated backbone fragment 11-37									
Endo Gly semaglutide form 2									
Unidentified 17									
Semaglutide isomer form 9									
Des His semaglutide form 2									
Semaglutide ox-form 5									
Des His-Aib semaglutide									
Unidentified 18									
Acetaldehyde adduct of semaglutide 3									
Unidentified 19									
Unidentified 20									
Endo Glu semaglutide form									
Des water semaglutide form									
Des 36-37 semaglutide									
Diacylated semaglutide backbone									
Endo ADOADO semaglutide									

Red boxes indicate an impurity not identified in originator semaglutide, and yellow boxes indicate the impurity was found at a higher level in the follow-on than the originator. Impurities are listed in the order they were extracted. S1–S9 identification numbers were assigned to nine different semaglutide DSs tested

[a]Manufacturers claiming to be present on the FDA's Green List under Import Alert 66–80; on September 5, 2025, the FDA published Import Alert 66–80: Detention Without Physical Examination of Glucagon-Like Peptide-1 (GLP-1) Receptor Agonist Bulk Drug Substances [45]. This Import Alert was intended to detain without physical examination any foreign-sourced, GLP-1 bulk drug substances that appear to be adulterated because the methods used in, or the facilities, or controls used for, its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformance with current good manufacturing practice ("CGMP") requirements. Import Alert 66–80 states that the FDA will detain foreign-sourced GLP-1 active pharmaceutical ingredients without physical examination unless the API and firm appear on a "Green List," whose facilities and associated products, based on the FDA's evaluation of "recent evidence" appear to be in compliance with CGMP requirements. As of the publication of this paper, there are no FDA-approved semaglutide follow-on products, so the API manufactured by these manufacturers has not been reviewed in connection with an FDA-approved medicine

ADO 2-(2-(2-aminoethoxy)ethoxy)-acetic acid, *Aib* Aminoisobutyric acid, *Ala* Alanine, *API* Active pharmaceutical ingredient, *des* Removed from molecule, *diox* Dioxidized, *DS* Drug substance, *endo* Added to the molecule, *FDA* US Food and Drug Administration, *GLP-1* Glucagon-like peptide-1, *Glu* Glutamic acid, *Gly* Glycine, *His* Histidine, *LC–MS* Liquid chromatography-mass spectrometry, *ox* Oxidized, *S* Semaglutide drug substance follow-on, *Thr* Threonine

and S3, the samples from manufacturers claiming to be present on the FDA's Green List, and the originator semaglutide DS (Fig. 2). Both S2 (Fig. 2A) and S3 (Fig. 2B) had different profiles for ionizable compounds compared with the originator DS.

Impurity Profiles of Compounded Injectable Semaglutide DPs

All CP source products (except for CP10, which was from Europe) originated from North America (Table I). CP2 and

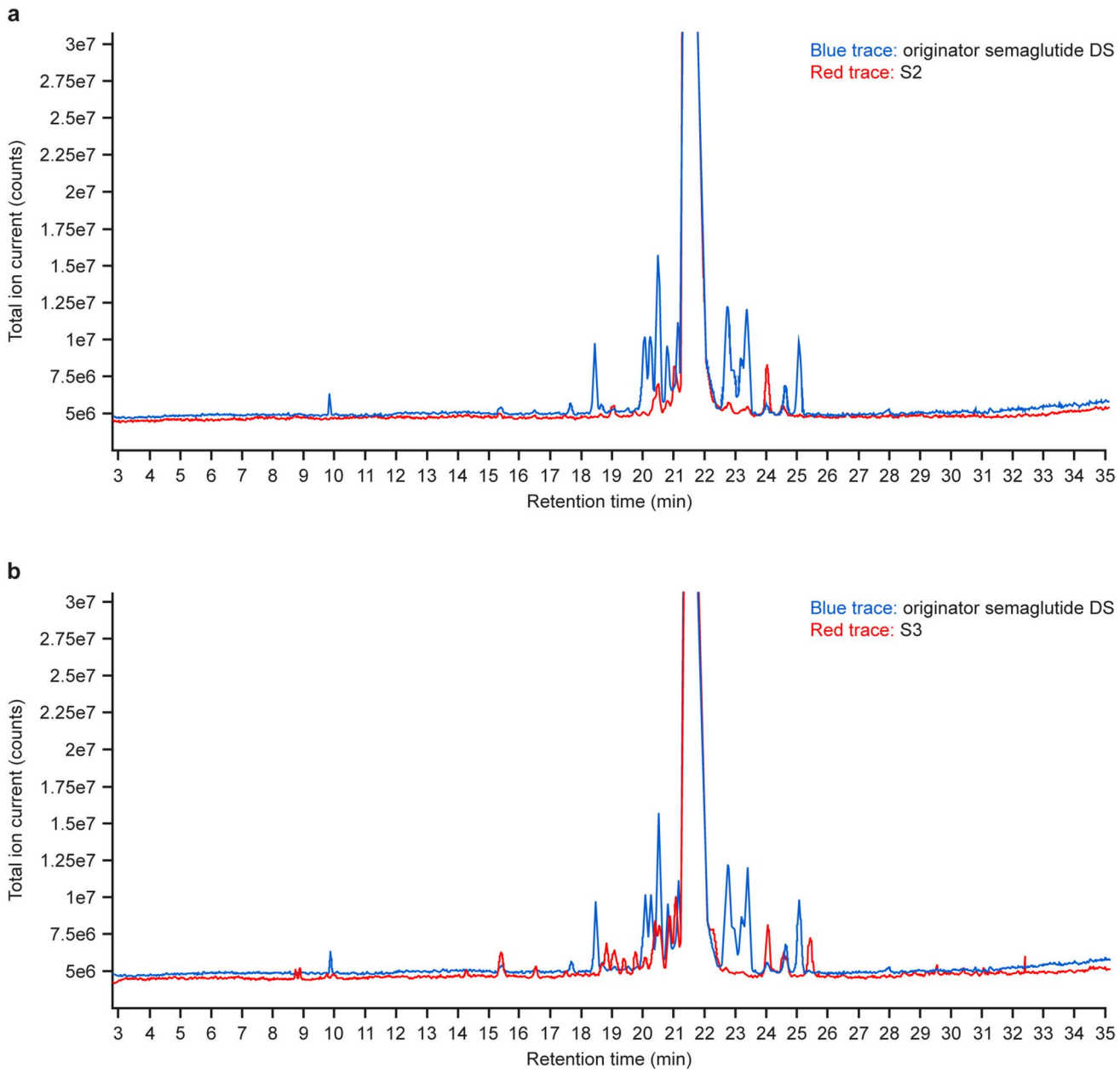

Fig. 2 LC–MS analysis comparing originator semaglutide DS with selected DS samples (**a**) S2, (**b**) S3. DS, drug substance; LC–MS, liquid chromatography-mass spectrometry; S, follow-on semaglutide drug substance

CP3 were compounded by the same pharmacy and were from two different semaglutide DP batches compounded 1 week apart. Similar results to those observed for the follow-on DSs were obtained, whereby 44 impurities were detected in eight CPs assessed using LC–MS. Of these impurities, 34 were not identified in the originator semaglutide and 10 were present at higher levels in the CPs than the originator (Table IV). These impurities included various new forms of semaglutide, with three CPs containing trimer forms never seen in the originator, six CPs with dimer forms not seen in the originator, three CPs with dioxidized forms not seen in the originator, and seven CPs containing an oxidized form found at higher levels than that in the originator. (Table IV).

To further illustrate these impurity profiles, the full LC–MS chromatograms for a sampling of the semaglutide CPs and the originator are provided in Fig. 3. The overlays with originator semaglutide highlighted the presence of new impurities in each of the three CPs (Fig. 3A–C). In particular, the CP8 chromatogram showed covalent dimer

Table IV Recorded related impurities in eight semaglutide CPs, as analyzed by LC–MS

Impurity in DP verified by LC-MS analysis	CP							
	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8
Des 32-37 semaglutide								
Des 31-37 semaglutide								
Semaglutide ox-form 1								
Ethylated semaglutide 1								
Semaglutide ox-form 2								
Ethylated semaglutide 2								
Endo Ser semaglutide form								
Methylated semaglutide 1								
Semaglutide diox-form 1								
Unidentified 1								
Semaglutide isomer form 1								
Semaglutide ox-form 3								
Acylated backbone fragment 18-37								
Unidentified 2								
Semaglutide diox-form 2								
Unidentified 3								
Semaglutide diox-form 3								
Endo Ala semaglutide form 1								
Acylated backbone fragment 13-37								
Semaglutide ox-form 4								
Des Thr semaglutide form								
Formaldehyde adduct of semaglutide 1								
Benzoylation 1								
Acylated backbone fragment 15-37								

Table IV (continued)

Benzoylation 2								
Tert-butyl semaglutide								
Methylated semaglutide 2								
Unidentified 3								
Formaldehyde adduct of semaglutide 2								
Des His-Aib semaglutide								
Des His semaglutide								
Ethylated semaglutide 3								
Methylated semaglutide 3								
Acetaldehyde adduct of semaglutide								
Unidentified 4								
Endo Glu semaglutide form								
Methylated semaglutide 3								
Des 35-37 semaglutide								
Des His-N-Oxalyl semaglutide								
Dimer form 1								
Dimer form 2								
Dimer form 3								
Dimer form 4								
Trimer form								

Red boxes indicate an impurity not identified in originator semaglutide, and yellow boxes indicate the impurity was found at a higher level in the follow-on than the originator. Impurities are listed in the order they were extracted. CP1–CP8 identification numbers were assigned to eight different semaglutide DPs tested

Aib Aminoisobutyric acid, *Ala* Alanine, *CP* Follow-on injectable semaglutide drug products, *des* Removed from molecule, *diox* Dioxidized, *DS* Drug substance, *endo* added to the molecule, *Glu* Glutamic acid, *His* Histidine, *LC–MS* Liquid chromatography-mass spectrometry, *ox* Oxidized, *Ser* Serine, *Thr* Threonine

and trimer forms that were not seen in the originator semaglutide (Fig. 3A). Exceedingly poor purity was seen in CP9, with oxidations, a semaglutide formaldehyde adduct, dimer, and trimer forms, and an undisclosed component with a mass corresponding with tirzepatide detected, indicating possible cross-contamination in the sample or the intentional addition of the undisclosed ingredient (Fig. 3B). Similarly, two unidentified semaglutide-related impurities and a component with mass corresponding to insulin glulisine were detected in CP10 (Fig. 3C).

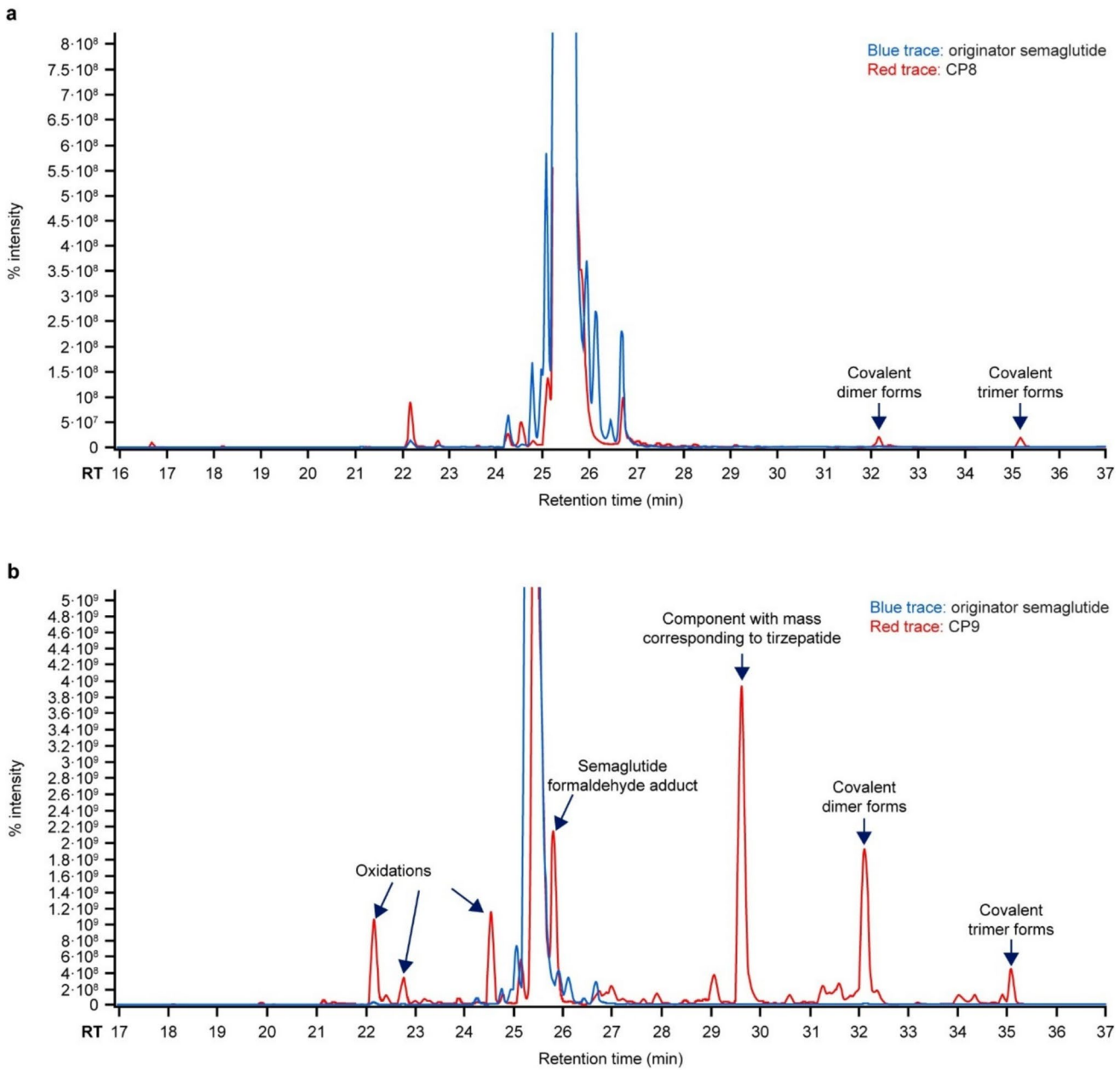

Fig. 3 LC–MS analysis comparing originator semaglutide DP with selected CP samples (**a**) CP8, (**b**) CP9 and (**c**) CP10; and (**d**) comparing CP2 with CP3. Dark blue arrows point to positions with notable differences between the tested drugs. CP, compounded injectable semaglutide drug products; DP, drug product; LC–MS, liquid chromatography-mass spectrometry

Although CP2 and CP3 came from two different batches compounded 1 week apart from the same pharmacy, batch-to-batch variation in the impurity profiles was observed, including semaglutide formaldehyde adduct, and covalent dimer and trimer forms (Fig. 3D).

Impurity Profiles of Follow-on or Compounded Oral Semaglutide DPs

All OP samples originated from North America, South America, or Asia (Table I). In OP5 and OP6, it was not possible to assign the LC–MS impurity profile, due to the interference from excipient signals. In the other five OP samples tested with a cut-off value of 0.02%, there were 38 impurities detected that were different from the originator semaglutide (Table V); out of these, 30 impurities were not detected in the originator semaglutide, and eight were present in higher levels in the OPs than the originator (Table V). Unidentified impurities were found in OP1, OP2, OP3, OP4, and OP7.

When comparing with originator DS using LC–MS, OP8 showed added dihexose, alanine semaglutide, and hexose

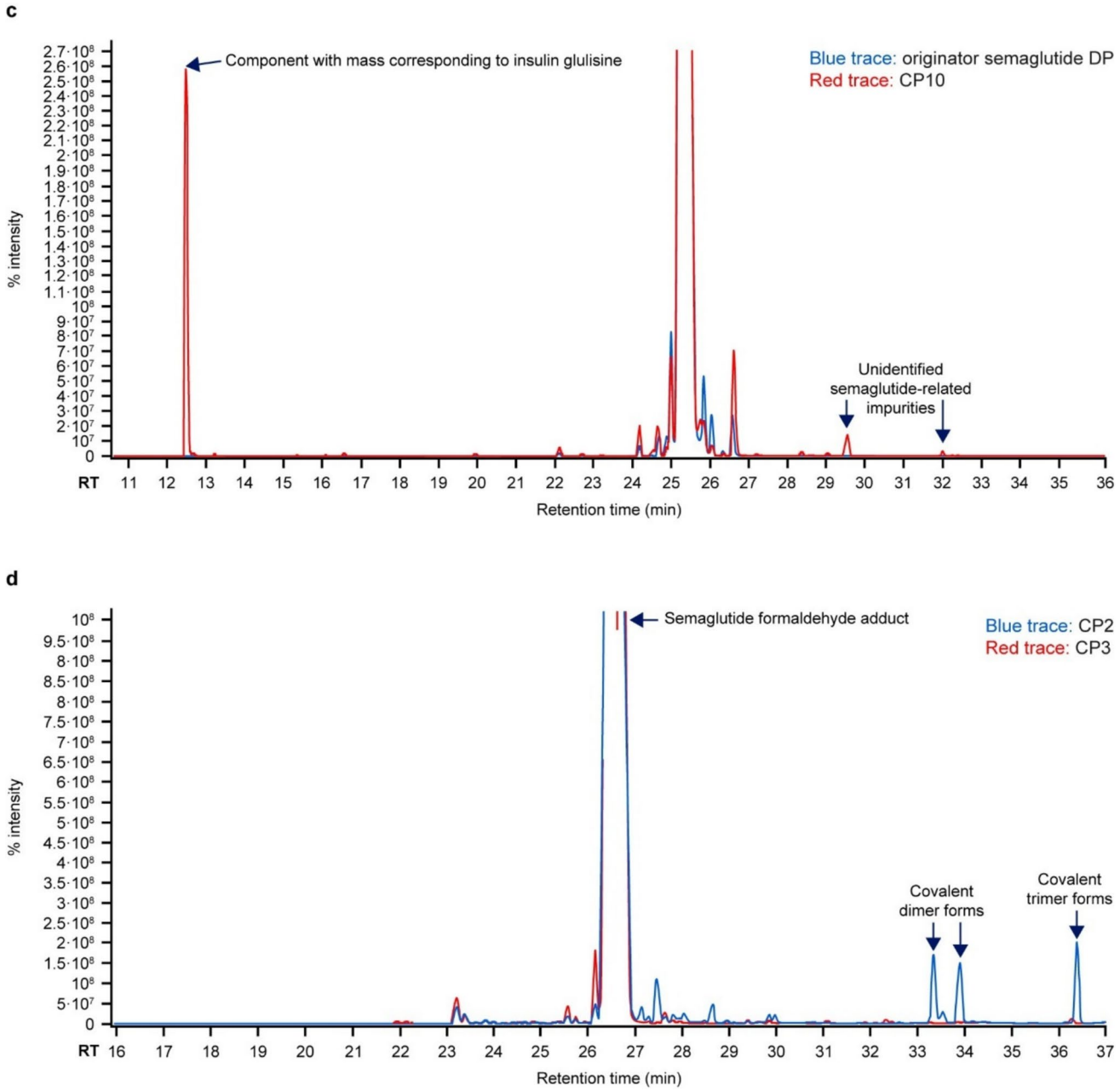

Fig. 3 (continued)

semaglutide forms, even though the recombinant semaglutide API used was approved by local health authorities (Fig. 4A). Similarly, OP2 had several unidentified semaglutide-related impurities and formaldehyde adduct, compared with the originator DS (Fig. 4B). Both OP9 and OP10 used synthetic semaglutide APIs that were in products approved by local health authorities. However, apart from unidentified semaglutide-related impurities, OP9 contained 0.5% semaglutide-sodium N-(8-[2-hydroxybenzoyl] amino) caprylate derivative (Fig. 4C), and OP10 had significant amounts of formaldehyde adduct (Fig. 4D). A comparison in impurity profiles between an injectable (CP11) and an oral form of the compounded semaglutide DPs (OP11) from the same manufacturer was also performed (Fig. 4E). The injectable CP11 presented several impurities, such as oxidation, semaglutide isomers, formaldehyde adduct, des His-Aib semaglutide, and unidentified semaglutide-related impurities, whereas the oral form OP11 had mainly semaglutide formaldehyde adduct. Supplementary Fig. 1 compares the extracts from two compounded semaglutide

Table V Recorded related impurities in seven semaglutide OPs, as analyzed by LC–MS

Impurity in DP verified by LC-MS analysis	OP						
	OP1	OP2	OP3	OP4	OP5[a]	OP6[a]	OP7
Semaglutide ox-form 1							
Semaglutide ox-form 2							
Unidentified 1							
Unidentified 2							
Unidentified 3							
Unidentified 4							
Semaglutide diox-form 1							
Semaglutide diox-form 2							
Endo Ala semaglutide form 1							
Unidentified 5							
Endo dihexose form							
Unidentified 6							
Formaldehyde adduct of semaglutide							
Acetaldehyde adduct of semaglutide							
Acetylated semaglutide form 1							
Endo hexose form 1							
Endo hexose form 2							
Endo hexose form 3							
Endo Ala semaglutide form 2							
Endo Gln semaglutide form 1							
Endo Gln semaglutide form 2							
Des His semaglutide form							
Acylated backbone fragment 11-37							
Methylated semaglutide 1							

Table V (continued)

Ethylated semaglutide							
Unidentified 7							
Butyraldehyde adduct of semaglutide							
Endo Tyr semaglutide form 1							
Acetylated semaglutide form							
Unidentified 8							
Endo Tyr semaglutide form 2							
Des His-N-Oxalyl semaglutide							
Unidentified 9							
Dimer form 1							
Dimer form 2							
Dimer form 3							
Dimer form 4							
Unidentified 10							

Red boxes indicate an impurity not identified in originator semaglutide, and yellow boxes indicate the impurity was found at a higher level in the follow-on than the originator. Impurities are listed in the order they were extracted. OP1–OP7 identification numbers were assigned to seven different oral semaglutide DPs tested

[a]Due to interference from other excipient signals, it was not possible to assign the LC–MS impurity profile; however, the presence of semaglutide within the samples was verified

Ala Alanine, *des* Removed from molecule, *diox* Deoxidized, *DS* Drug substance, *endo* Added to the molecule, *Gln* Glutamine, *His* Histidine, *LC–MS* Liquid chromatography-mass spectrometry, *OP* Follow-on or compounded oral semaglutide drug products, *ox* Oxidized, *Tyr* Tyrosine

suspensions (OP5 vs OP6), where, although the semaglutide signal was seen in both LC–MS chromatograms, there was also the presence of large amounts of unidentified polymers and other impurities.

Photostability of Originator Semaglutide and Two Selected Compounded Injectable DPs

When the FDA-approved originator semaglutide was tested in the original packaging with the injection pen's cap on, the concentration of semaglutide, sum of impurities, and levels of high molecular weight proteins (HMWPs) did not change after exposure to light compared with the control sample (not exposed to light). Specifically, the originator's sum of impurities rose slightly, from 3.4% to 3.5% of the 0.68 mg/mL strength, while the sum of impurities rose slightly for the 1.34 mg/mL strength, from 3.2% to 3.3%. Similarly, the level of HMWPs in originator's 0.68 mg/mL strength remained at 0.6%, while the level of HMWPs in the 1.34 mg/mL strength remained at 0.3%.

In contrast, the strength of compounded semaglutide in CP12 and CP13 decreased from 1.01 mg/mL to 0.95 mg/mL (95%), and from 1.02 mg/mL to 0.86 mg/mL (86%), respectively, after exposure to light. The sum of impurities for CP12 and CP13 climbed from 1.3% to 5.1%, and from 2.5% to 14.2%, respectively, after exposure to light. Furthermore, the HMWPs levels for CP12 and CP13 rose from < 0.3% to 2.6% and from 0.3% to 14.1%, respectively, from baseline to after exposure to light. No light protection measures were available in the pharmacy-provided packaging of CP12 and CP13.

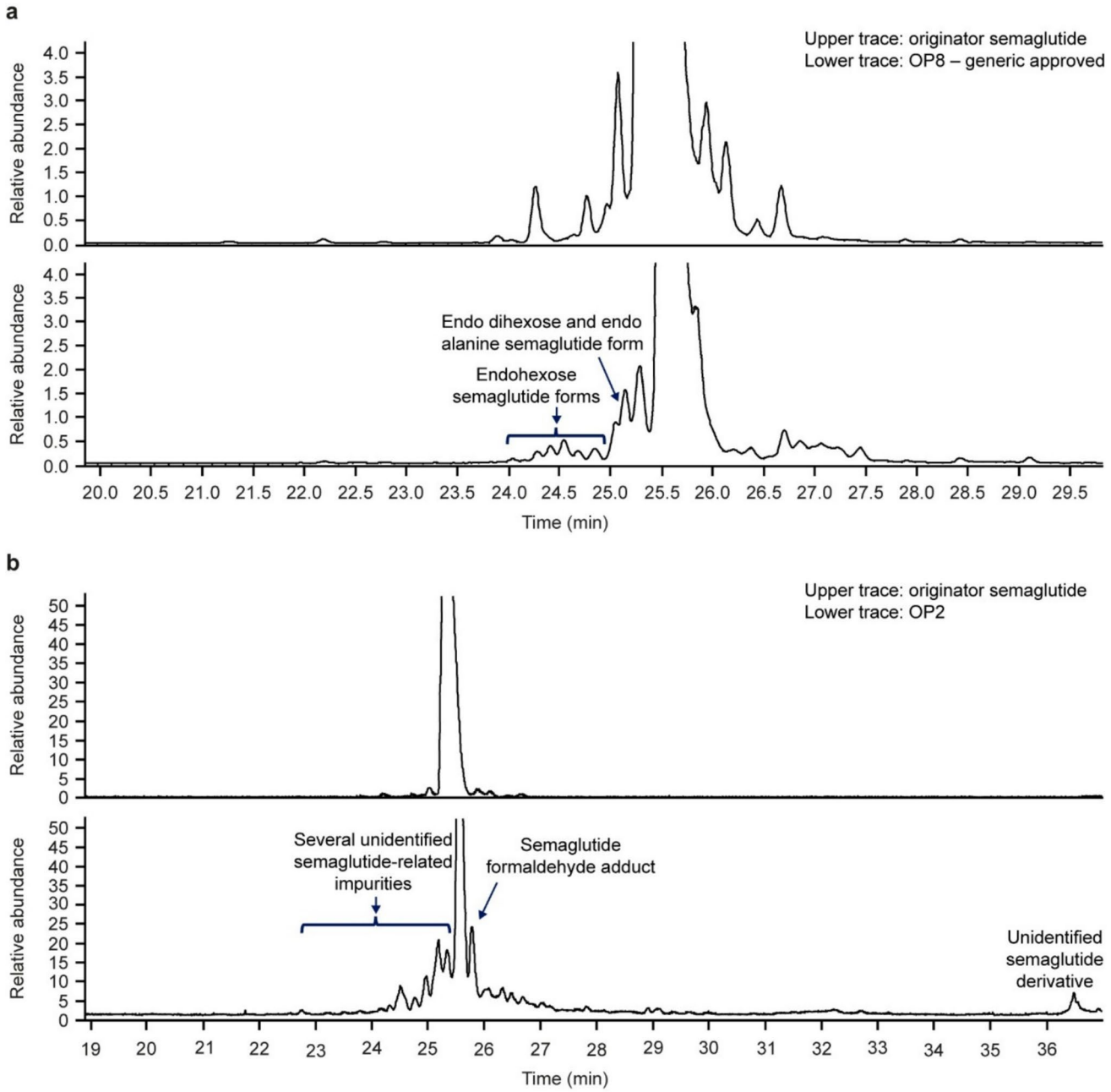

Fig. 4 LC–MS analysis comparing originator semaglutide DS with (**a**) OP8*, (**b**) OP2, comparing originator semaglutide DP with (**c**) OP9[†], (**d**) OP10[‡], and (**e**) comparing CP11 with OP11[§]. *OP8 used recombinant semaglutide API approved by a local health authority. [†]OP9 used synthetic semaglutide API approved by a local health authority. [‡]OP10 used synthetic semaglutide API approved by a local health authority. [§]CP11 and OP11 were from the same pharmacy. Aib, aminoisobutyric acid; API, active pharmaceutical ingredient; CP, compounded injectable semaglutide drug product; des, removed from molecule; DP, drug product; Endo, added to the molecule; His, histidine; LC–MS, liquid chromatography-mass spectrometry; OP, follow-on or compounded oral semaglutide drug product; SNAC, sodium N-(8-[2-hydroxybenzoyl]amino)caprylate

Liraglutide Impurity-derived Peptides

Liraglutide peptide sequences synthesized with Ala8, Glu9, and Glu27 amino acid deletions, and Phe28 and Arg36 amino acid additions were assayed (Table II). Between 52 and 80 peptides matching the tested liraglutide sequences presented on APCs across all donors (Supplementary Fig. 2). The originator liraglutide-derived sequences and the liraglutide-related sequences with deletions of Ala8, Glu9, and Glu27, and the addition of Phe28 was identified in all 15 donor cell cultures; the liraglutide-related sequence with the addition of Arg36 was identified in 12 out of the 15 donor cell cultures. Additionally, seven to 18 different binding cores or motifs were identified in the test sequences for

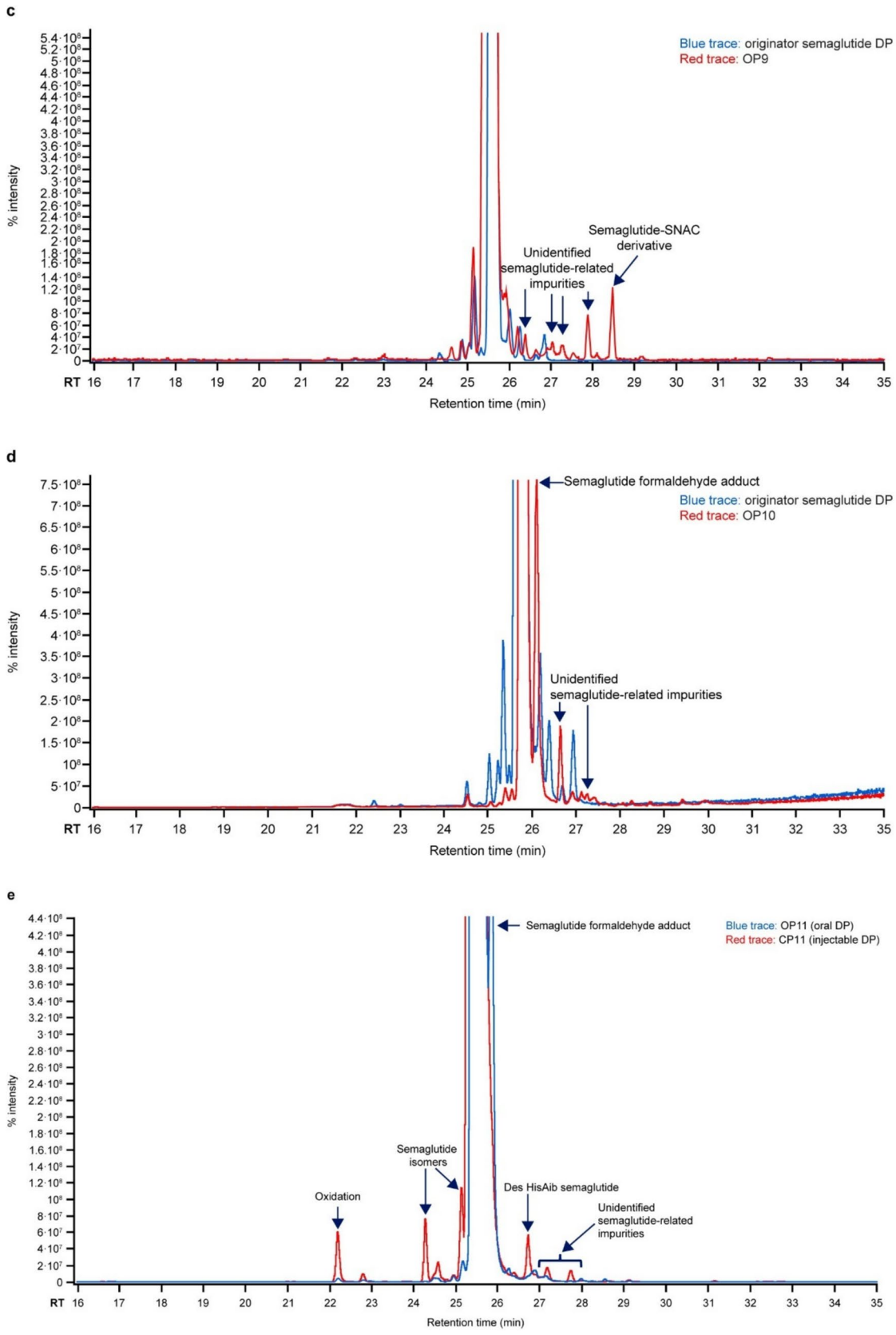

Fig. 4 (continued)

liraglutide, compared to nine different binding cores identified for originator liraglutide. In line with our observations regarding presentation of semaglutide peptide impurities, an increase in presented peptides spanning the specific addition or deletion was detected in several of the liraglutide peptide impurities compared with originator liraglutide. For instance, N-terminal deletion of Glu9 (des Glu9) resulted in a higher number of presented N-terminal peptides in several donors and, similarly, deletion of Glu27 (desGlu27) and insertion of Phe28 (endo Phe28) resulted in a substantial increase of total peptides, including peptides spanning the altered region.

Impurity Profile and Physical Stability of Liraglutide Follow-on and Compounded Products

When assessed using LC–MS analyses, there were notable differences in the impurity profiles between the follow-on and compounded liraglutide DPs and the originator (Fig. 5). Several unidentified liraglutide-related impurities were observed in L3 (Fig. 5a) and L4 (Fig. 5b).

The physical stability of two generic follow-on liraglutide DPs, approved in South America, L1 and L2, were compared with originator liraglutide using a ThT stress test. Both the liraglutide follow-on DPs exhibited a lower fluorescence signal lag time before fibrillation occurred compared with the original product (Fig. 6), demonstrating that the follow-on liraglutide DPs had impaired physical stability compared with the originator.

Discussion

The MAPPs assay demonstrated that various peptides matching the tested impurity-related sequences presented on APCs across all the donors, indicating a risk for immunogenicity. The LC–MS analysis showed that the semaglutide and liraglutide DSs and follow-on and compounded DPs had substantially different impurity profiles compared with the originators. The originator semaglutide and liraglutide medicines have a known safety profile from clinical trials. The amino acid sequence and the impurities at their current and known levels do not pose an appreciable immunogenic risk [9, 35]. In contrast, all the presented peptides with additions and deletions derived from the DSs and follow-on and compounded DPs claiming to contain semaglutide or liraglutide, presented on APCs as well as process-related impurities, which have not been assessed in clinical trials and therefore may pose an immunogenicity risk.

In the current study, the MAPPs assay was chosen as data from this assay are more robust and highly reproducible across different laboratories compared with *in silico* methods or binding assays using synthetic peptides [29, 30]. The peptide sequences identified by the MAPPs assay have the potential to activate a T-cell response, which is a prerequisite to ADA formation [29, 30]. ADAs to the originator semaglutide and liraglutide do not have any reported clinical impact on safety or efficacy [9, 35]. However, ADAs to impurities in the follow-on semaglutide and liraglutide DPs have the potential to lead to a variety of clinically significant adverse outcomes, including hypersensitivity responses, cross-reactivity, and neutralization of GLP-1 RAs, leading to loss of efficacy [12, 18].

In the current study, the MAPPs results indicated that all the upscaled peptide-impurities had the capacity to bind to HLA-II receptors and potentially trigger an immune response [30]. More than half of the tested peptide impurities from follow-ons and compounded semaglutide products were identified in more donors than the originator semaglutide, and all but one of the tested liraglutide-related sequences were identified in as many donors as the originator liraglutide. In addition, all the tested peptide impurities from follow-ons and compounded semaglutide displayed as many or more binding motifs than the originator semaglutide, and all but one of the tested liraglutide-related sequences displayed as many or more binding motifs compared with the originator liraglutide. Importantly, clinical trials have shown that originator semaglutide and liraglutide have an established safety profile, so peptides derived from originator sequences presented on APCs are not considered an immunogenicity risk [9, 10, 33–38]. However, peptides with additions or deletions, identified by MAPPs and derived from follow-on DSs and compounded DPs, have not been tested in clinical trials and may all present an immunogenicity risk. A MAPPs assay could only be the first step in assessing their immunogenicity potential. Although the MAPPs assay can detect APC presentation of impurity-derived peptides, epitope identification based solely on peptide sequences cannot determine if an immunogenic reaction will happen in a patient [30]. This is because the MAPPs assay is unable to assess the functional context of T-cell recognition and it does not account for crucial factors like T-cell receptor interaction, the generation of regulatory T-cell responses [30], or B-cell activation [46]. In the current study, increased numbers of binding motifs were found in essentially all selected impurities, posing a higher immunogenicity risk to patients, compared with the originators. Although the MAPPs assay is a powerful tool to assess potential T-cell epitopes in early research, clinical testing is

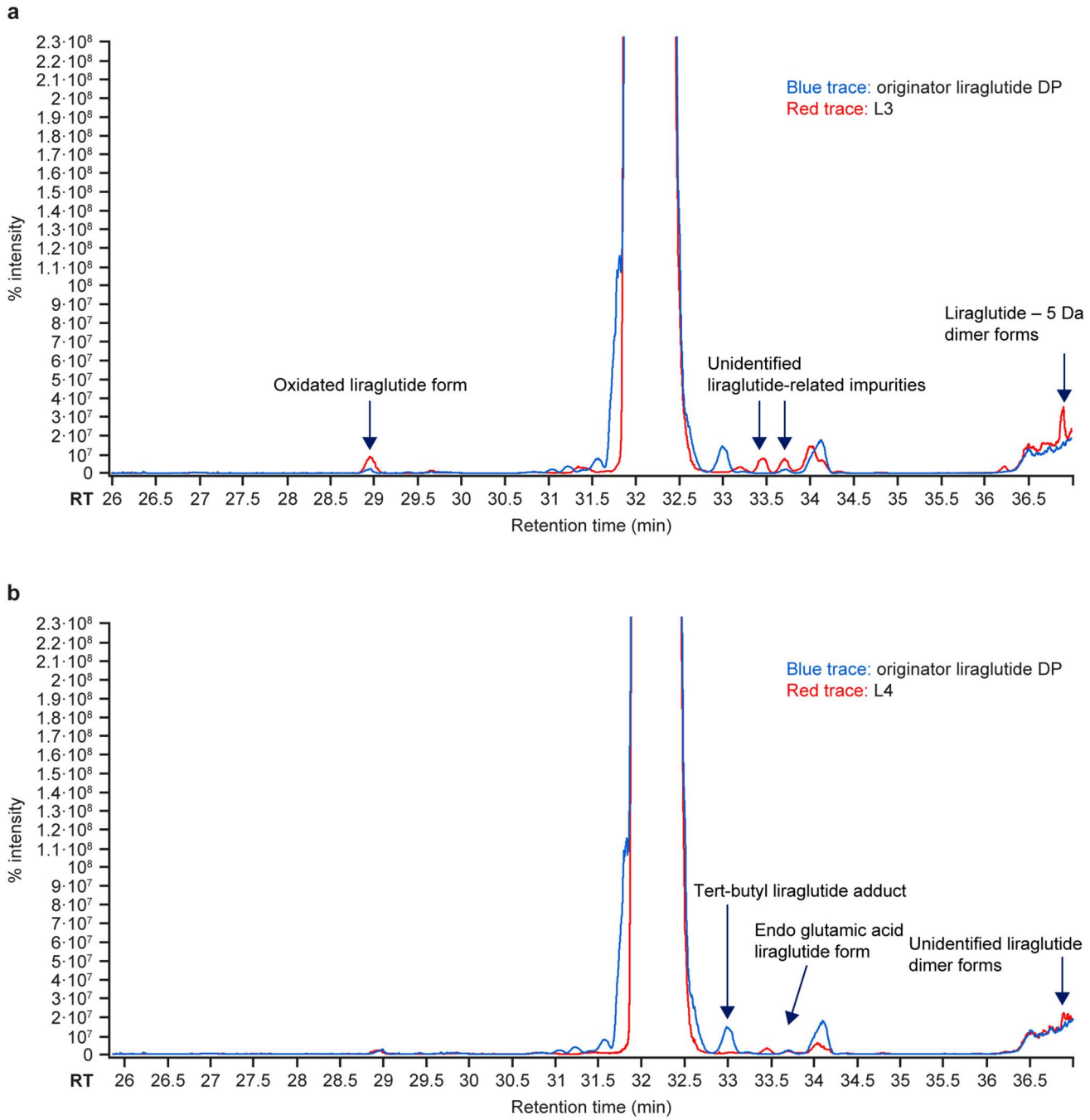

Fig. 5 LC–MS analysis comparing originator liraglutide DP with selected follow-on injectable liraglutide DPs (**a**) L3 and (**b**) L4. Da, Dalton; DP, drug product; Endo, added to the molecule; Glu, glutamic acid; L, follow-on injectable liraglutide drug product; LC–MS, liquid chromatography-mass spectrometry

imperative to fully understand the immunogenicity profile and patient safety of all follow-on or compounded products.

The LC–MS analysis results indicated the extent of the disparity in impurity profiles between semaglutide and liraglutide follow-ons, compounded products and the originators. It is worth noting that both CP9 and CP10 showed components corresponding to other medicines, suggesting potential cross-contamination in these products, which could be for several reasons such as improper cleaning of equipment following production of other products, or the addition of an undisclosed ingredient. Furthermore, significant batch-to-batch variation was noticed between samples CP2 and CP3, both from the same pharmacy and compounded within 1 week, suggesting insufficient quality control and

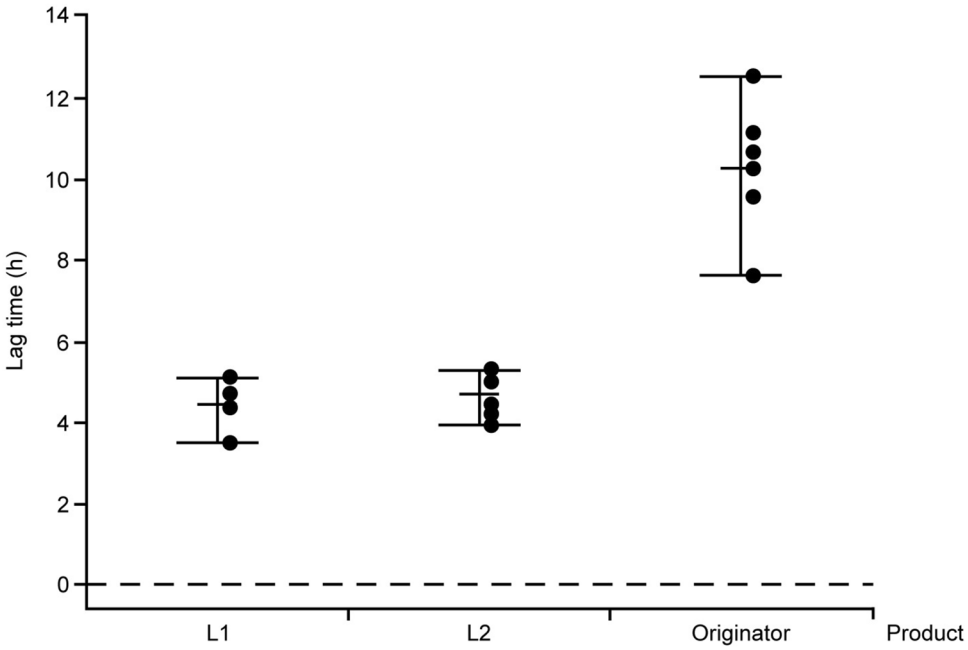

Fig. 6 Fibrillation analysis (ThT assay) of originator liraglutide DP and two follow-on injectable liraglutide DPs, L1 and L2. DP, drug product; L, follow-on injectable liraglutide drug product; ThT, thioflavin T

a lack of manufacturing robustness. A consequence of this finding would be that even where a batch with 'good' quality is produced, quality of other batches produced by the same supplier could vary. Although some of the DSs (S2 and S3) were manufactured by entities claiming to be present on the FDA's Green List for Import Alert 66–80 and some of the follow-on DPs (OP8, OP9, and OP10, which were oral tablets) were approved by other health authorities, distinct differences in impurity profiles were observed between these products and the originator. These results are consistent with previous studies in which impurities with many of the amino acid additions and deletions in the tested semaglutide- and liraglutide-related sequences have been found in DSs and DPs claiming to contain the same active ingredient as the originator [6, 28].

As indicated in the photostability assay results, there was a reduction in strength and a significant increase in the total sum of impurities and the HMWP levels for CP12 and CP13 after exposure to light, indicating poor photostability in its current container. Additionally, it was possible that the HMWPs present in the samples are of different molecular species than the species of HMWPs present in the originator. As mentioned previously, the packaging for compounded semaglutide injection products may not offer adequate protection from light. Instructions on how to properly protect these products from light exposure during storage or use are not necessarily provided for all compounded products. Healthcare professionals and patients who store and use these vials or syringes may risk overexposing the semaglutide DPs to light if the clear multidose vials are not sold with a light-protecting box or container for storage. Such exposure can degrade the peptide [41]. Solutions of compounded semaglutide in glass vials that have not been reviewed in an authority-approved application could also create other quality problems, such as leaching and delamination, metal-catalyzed degradation, and adsorption and potency loss [18]. Proper guidance during storage and use is needed on the label to minimize light exposure.

The physical stability of two injectable liraglutide follow-on DPs that were approved by local health authorities in South America was compared with the originator using the ThT stress assay. The reduced lag time recorded with the liraglutide follow-ons indicated decreased physical stability compared with the originator. This may lead to issues related to safety and efficacy, as these follow-on products do not undergo the same approval process as the approved originators [6, 28, 49].

Various factors could influence the impurity profiles of GLP-1 RAs, including API sourcing, manufacturing processes, and degradation during storage [6]. All the follow-on and compounded samples tested in this study had distinct impurity profiles, compared with the originators, regardless of whether the APIs were approved by local health authorities. For example, in the manufacturing process, the originator semaglutide and liraglutide were produced using recombinant expression [6, 9, 12]. In contrast, most of the follow-on and compounded samples tested in this study (except for OP8 that was recombinant) were synthetic peptides, which were produced via sequential linking of amino acids through various chemical reactions [5, 50]. As a result, peptide sequence alterations, sidechain modification, and reaction biproducts between the API and excipients used in compounding may contribute to their impurity profiles. Furthermore, insufficient photostability and physical stability

during storage and usage lead to a risk of degradation of these products. The impact of these impurity profiles in the follow-on and compounded products on the safety and efficacy profiles of these GLP-1 RAs has not been tested clinically. In contrast to small molecules, the differences in impurity profile of these follow-on and compounded peptides have the potential to increase the immunogenicity risk. Similarity between products is initially determined using relatively simple comparative analytical methods without any clinical testing [2, 3, 51]. Although intended to ensure safety and efficacy, such a similarity comparison appears insufficient for follow-on GLP-1 RAs [8, 18, 22, 28], given the number of distinct impurities measured in our analyses.

The assessed impurities have the potential to be immunogenic, have not been clinically evaluated, and could lead to serious harm to patients [6, 9]. Process-related impurities could also contribute to undesirable immunogenicity, especially for injections, because the impurities quickly enter the circulation and come into direct contact with tissues where immune cells can be readily activated. Furthermore, oral formulations require a complex manufacturing process to overcome insufficient uptake in the gastrointestinal environment and ensure the required bioavailability [48]. The indicated cross-contamination represent failures in manufacturing processes and environmental controls [47]. Variances in different manufacturing methods and quality control in production processes may introduce unidentified impurities and a higher level of already known impurities. All these results support a requirement for more stringent surveillance of marketed products.

Despite bias-mitigation steps such as pre-specified analyses and objective selection criteria such as origin of the API and formulations diverging from the originator formulation, there were limitations to this study. First, a small number of samples were tested, and not all follow-on semaglutide or liraglutide DSs and follow-on or compounded semaglutide or liraglutide DPs available worldwide were obtained to include in the tests. Second, only some samples were tested using specific analytical tools, although results presented here are indicative of the general trend for all the samples. LC–MS analysis was performed on all samples; however, if distinct differences between the follow-on compounded and originator samples were observed, no further testing was carried out.

Conclusion

In conclusion, various peptides matching the tested impurity-related sequences were presented on immune cells across all healthy donors, indicating an immunogenicity potential for these peptide impurities. In addition, the semaglutide and liraglutide follow-on DSs and follow-on or compounded DPs presented distinct impurity profiles, compared with the originators. These distinct impurity profiles, such as additions and deletions in the peptide sequences, and unidentified impurities, in the semaglutide and liraglutide follow-on DSs and follow-on or compounded DPs could lead to an immune response in patients. These results underscore the importance of *in vitro* immunogenicity assays and especially clinical immunogenicity studies for follow-on GLP-1 RA products.

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Data Availability Contact the authors with any questions regarding the data presented.

Declarations

Conflicts of Interest K.L.K., K.L., O.S., A.K.Ø., M.W., J.E.M., H.S.R.-A., C.L.S., and M.H. are employees and shareholders of Novo Nordisk A/S. A.S. is a shareholder of Novo Nordisk A/S and was an employee of Novo Nordisk A/S at the time that this study was undertaken.

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