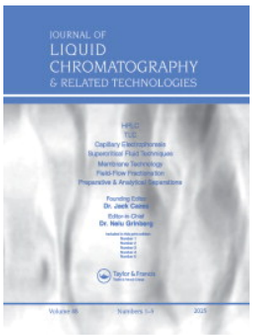


Taylor & Francis
Taylor & Francis Group

Journal of Liquid Chromatography & Related Technologies

ISSN: 1082-6076 (Print) 1520-572X (Online) Journal homepage: www.tandfonline.com/journals/ljlc20

Chromatographic separation and analysis of amidated *vs.* non-amidated novel heterologous produced exenatide using UPLC-QTOF-MS

Lucia Steenkamp & Paul Steenkamp

To cite this article: Lucia Steenkamp & Paul Steenkamp (23 May 2025): Chromatographic separation and analysis of amidated *vs.* non-amidated novel heterologous produced exenatide using UPLC-QTOF-MS, Journal of Liquid Chromatography & Related Technologies, DOI: 10.1080/10826076.2025.2502034

To link to this article: https://doi.org/10.1080/10826076.2025.2502034

Published online: 23 May 2025.

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES
https://doi.org/10.1080/10826076.2025.2502034

Chromatographic separation and analysis of amidated *vs.* non-amidated novel heterologous produced exenatide using UPLC-QTOF-MS

Lucia Steenkamp[a] and Paul Steenkamp[b]

[a]CSIR (Council for Scientific and Industrial Research) Chemicals Cluster, Brummeria, Pretoria, South Africa; [b]Department of Biochemistry, Research Centre for Plant Metabolomics, University of Johannesburg, Johannesburg, South Africa

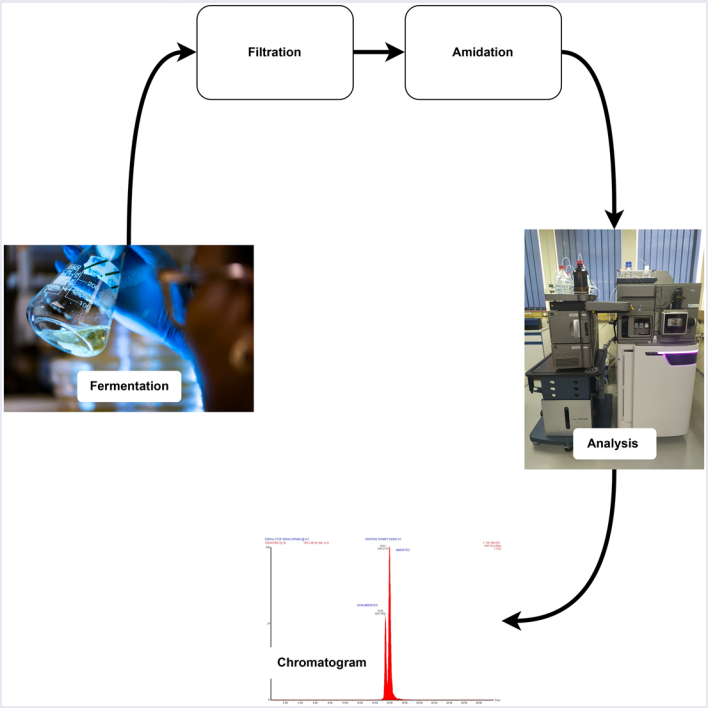
ABSTRACT

Exenatide is used for the treatment of type 2 diabetes. Exenatide is normally produced using solid or liquid phase chemical synthesis which requires protecting side chain groups. The C-terminal is then amidated while the protecting groups are still in place. The novel production of exenatide, using a microorganism with the correct coding sequence, does not require protecting groups. The exenatide is then amidated using a PAM enzyme system. An UPLC-QTOF-MS method was developed to separate and analyze the non-amidated exenatide molecule from the final active amidated exenatide. The non-amidated exenatide was produced using an expression construct comprising a carrier protein open reading frame (ORF) *Yarrowia lipolytica* lipase or truncated *Bacillus halodurans* flagellin cloned in frame with the coding sequence for exenatide. The non-amidated and amidated exenatide differ by 1 Dalton in mass and typically co-elute during analysis. The method developed was able to separate the two compounds and could be used to measure the amidated exenatide produced during the bioconversion. The aim of the study was to investigate the feasibility of producing exenatide and monitoring the amidation during the bioconversion for possible scale-up and commercialization. The results showed complete amidation of the glycine-extended heterologous exenatide. The reproducibility of the analytical method was evaluated and found that the retention times and peaks areas of the detected exenatide were stable, making this analytical method suitable for reaction monitoring.

KEYWORDS

Amidation; accurate mass; exenatide; separation; UPLC-QTOF-MS

GRAPHICAL ABSTRACT



CONTACT Lucia Steenkamp lsteenkamp@csir.co.za CSIR (Council for Scientific and Industrial Research) Chemicals Cluster, Brummeria, Pretoria, South Africa.

Introduction

The increasing use of peptides as pharmaceutical agents, where peptide drugs accounted for 5% of the pharmaceuticals market in 2022,[1] especially in the antiviral, diabetics, and anti-infective therapeutics areas, requires cost-effective production on large scale. The current commercial production of peptides is *via* chemical synthesis in solid or liquid phase.[2,3] Following the synthesis of the peptides, the amino and carboxyl termini are free and are normally electrically charged. In order to remove this charge or for functional activity, the peptide might be N-acetylated at the amino terminus and/or C-amidated at the carboxyl terminus.

Currently, chemical methods, employing resins as a backbone on which the peptides are synthesized, are used for commercial production of pharmaceutical peptides. Chemical methods exist for the acetylation of the N-terminal group of the peptide while the amino acid side chains are protected.[4,5] The chemical solid phase synthesis of peptides as therapeutics however uses excess solvents and reagents which negatively impact the environment. Studies by Kekessie et al.[6] determined the process mass intensity (PMI) to produce these peptides. The researchers defined PMI as the total mass of materials used (raw materials, reactants, and solvents) to produce a specified mass of product. Synthetic peptides are particularly inefficient and the PMI ranges from 1684 to 34,585 (average 13,063) for peptides with amino acid units from 6 to 43 (average 19 AAs).[6] These figures indicate the necessity for greener methods to produce peptides.

The CSIR in South Africa in its Biomanufacturing Technologies Group has selected exenatide as a model to develop a heterologous production technology. This technology may address several disadvantages of chemical synthesis of peptides, such as cost and impact on the environment. A modified organism was used to produce the full-length peptide as a component of a larger fused protein, eliminating the large number of chemical steps as well as excess amounts of expensive reagents. This should reduce the overall cost and time of production dramatically. The heterologous production of the peptide produces a product which does not have the side-chains protected.

Following cleavage of the peptide from the fusion protein, some modifications, such as N-acetylation and C-amidation of the peptide may be necessary. Since the new technology involves production of the peptide *in vivo*, the side-chains of the amino acids are not protected and may take part in additional reactions. During chemical solid phase synthesis of peptides, the side chains of the amino acids are protected until capping of the end-points is complete to avoid unwanted reaction of these groups. To achieve the desired N-acetylation or C-amidation the reactions have to be chosen very carefully and will have to be very selective, to avoid unwanted side-chain reactions.

Many peptides, especially neuropeptide transmitters, require the presence of a carboxy-terminal α-amide group for biological activity. In the cell, this post-translational modification depends on the sequential action of two enzymes. The peptidylglycine α-hydroxylating monooxygenase or PHM and peptidyl-α-hydroxyglycine α-amidating lyase (PAL) are expressed in most eukaryotes as separate domains of a single protein (peptidylglycine α-amidating monooxygenase or PAM). The mechanism for carboxy-terminal amidation involves a glycine-extended intermediate and the two enzymes. PHM requires copper, O_2 and ascorbate, and is the rate-limiting step in amidation.[7] The PAM enzyme system can also be used *in vitro*.

Diabetes 2 and obesity have increased dramatically worldwide. The glucagon-like peptide-1 receptor agonists[8–13] such as exenatide, liraglutide,[14–17] and semaglutide are showing huge success in treating diabetic conditions and obesity,[16–18] especially in combination with a sensible diet and exercise[19] as reported by Tinsley et al.[20] Exenatide is a synthetic peptide used in the treatment of diabetes mellitus type 2 at a concentration of 2 mg once weekly[21] with an average molecular mass of 4186.6 Dalton (Da) and formula $C_{184}H_{282}N_{50}O_{60}S$. The original hormone is exendin-4 found in the saliva of the Gila monster and was isolated by Dr John Eng in 1992.[22] Exenatide is a 39 amino acid peptide and was approved in 2005 for the treatment of diabetes. The drug is considered to be an incretin mimetic. Incretins are a group of gastrointestinal hormones that cause an increase in the amount of insulin released from the beta cells of the islets of Langerhans after eating, even before blood glucose levels become elevated. The amino acid sequence is as follows:

His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2.

The analysis of amino acids and peptides has been widely reported, using various separation techniques and detectors. The most widely used technique is high performance liquid chromatography (HPLC) combined with a mass selective detector (MS). Recent developments have seen the move to ultra-performance liquid chromatography (UPLC) and the use of time-of-flight (TOF) or quadrupole time-of-flight (QTOF) mass spectrometers. The use of an UPLC-QTOF instrument to analyze peptides offers exceptional chromatographic resolution as well as accurate mass spectrometric data. Typical mass accuracies below 3 millidalton (mDa) would allow for the calculation of empirical formulae from accurate mass data. The problem of analyzing peptides that differ by 1 Da, the result of replacing a hydroxyl group with an amine, requires the selection of a suitable analytical column and carefully optimizing the chromatographic conditions.

Electrospray ionization (ESI) is a very useful technique for the detection of ionizable compounds, and the use of this process in positive ionization mode (ESI^+) is ideally suited for the detection of peptides. Peptides usually produce multiply-charged species in the gas phase, but software tools can predict the actual mass of the uncharged molecule.

Materials and methods

The natural exenatide standard was obtained from Prospectany (Israel), while uncapped and glycine-extended exenatide were

purchased from Peptide 2.0 Inc. Peptidyl α-amidating monooxygenase (PAM), derived from the skin of the frog *Xenopus laevis*, was purchased from Wako Chemicals. All chemicals for UPLC-MS work were of ultra-pure LC-MS grade and purchased from Fluka (Steinheim, Germany) or Honeywell (Burdick & Jackson, Muskegon, MI) and ultra-pure water was obtained from a Millipore Elix 5 RO system and Millipore Advantage Milli-Q system (Millipore SAS, Molsheim, France).

Production of the glycine-extended exenatide

Fernbach flasks with a volume of 2 L containing 1.2 L YPD medium were inoculated with single colonies and incubated on a rotary shaker at 180 rpm at 28 °C for 7 d. The flasks were harvested and protein measured using the Bio-Rad assay. The supernatant was then concentrated by ultrafiltration using a 10 kDa cutoff membrane. To remove all media components, the concentrated supernatant was further washed with 6 volumes of dH_2O (diafiltration) using the 10 kDa membrane. This material was then lyophilized to yield a final dry product.

Cleavage of the exenatide from the fused protein

For the cleavage of the Exenatide peptide from the fused protein, an initial 2 mg/1 mL was used and 7.5 µL of the EkMAx enzyme added in a 20 mM Tris buffer pH 7.4 containing 20 mM NaCl, 0.2 mM $CaCl_2$, and 5% glycerol at 28 °C for 6 hrs.

Amidation of the C-terminal

For the amidation of the glycine-extended exenatide, PAM was used.

Reactions with the PAM enzyme system were as follows:

Initially, glycine extended exenatide (0.5 mg) was used in the presence of either 50 mM sodium acetate pH 5.5 (conditions used by Wako) or 150 mM TES buffer pH 8.5. To each reaction was also added 10 µL of a 200 mM L-ascorbic acid solution (2 mM final concentration), 10 µL of a 1 mg/100 µL catalase solution (final concentration 100 µg/mL), 10 µL of a 400 µM $CuSO_4$ solution (4 µM final concentration) and 20 µL of the PAM enzyme (600 units). Reactions were done at 37 °C and samples (50 µL) were taken at 0, 30, 60, 120, and 240 min and at 24 hrs. The reactions were stopped by addition of 10 µL 50 mM EDTA and frozen until analysis on UPLC-MS.

Reactions were also done using uncapped exenatide. Reaction conditions were optimized using less PAM enzyme and testing 50 mM sodium phosphate buffer pH 8 instead of 150 mM TES buffer at pH 8.5.

Analytical methods

Instrumentation

A Waters UPLC coupled in tandem to a Waters SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. Chromatographic separation was done utilizing a Waters BEH130 C18 column (100 mm × 2.1 mm, 1.8 µm) thermostatted at 40 °C or a Waters CSH C18 column (150 × 2.1 mm, 1.7 µm) thermostatted at 60 °C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (pH of 2.3) and acetonitrile (containing 10 mM formic acid, Eluent B). The initial conditions for the BEH column were 95% A for 1 min followed by a linear gradient to 80% A at 1.1 min and kept constant for 0.9 min. The conditions were then changed to 62% A at 5 min and again kept constant for 2 min. The final ramp was to 5% A at 11 min. The column was allowed to wash for 1 min where after the system was re-equilibrated using the initial conditions. The runtime was 15 min and the injection volume was 10 µL. The initial conditions for the CSH column were 80% A for 1 min followed by a slow gradient (Curve 8) to 100% B at 22 min. The column was allowed to wash for 3 min whereafter the system was re-equilibrated using the initial conditions. The runtime was 30 min and the injection volume was 10 µL.

The SYNAPT G1 mass spectrometer was used in V-optics and operated in electrospray mode. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 3 mDa. The mass spectrometer was operated in positive mode with a capillary voltage of 3.5 kV, the sampling cone at 40 V, and the extraction cone at 5 V. The source temperature was 120 °C and the desolvation temperature was set at 400 ^{0}C. Nitrogen gas was used as the nebulization gas at a flow rate of 450 L/h. Data was collected over the mass range 100–5000 Dalton (Da) with a scan speed of 1 second per scan. The software used to control the hyphenated system and do all data manipulation was MassLynx version 4.1 (SCN 704) Milford, MA, USA. BioLynx software was used to transform the multiply charged TOF data. The analysis of Exenatide was done on the BEH column. The CSH column and its optimized conditions were used to distinguish between amidated and non-amidated peptides. Samples were supplied frozen and thawed directly before analysis and transferred to suitable low-volume insert vials. The Sample Manager and Sample Organizer were thermostatted at 4 °C.

Results and discussion

Y. lipolytica (MD3) was cultivated in shake flasks for 7 d and produced 0.12 g per liter of protein (as determined by Bio-Rad). The protein was purified using Ultra-filtration and freeze-dried before digestion with enterokinase to cleave the exenatide from the carrier protein.

An exenatide standard was obtained and exenatide fused to the lipase as a recombinant protein was produced *via* fermentation in-house. An experiment was done to determine if the peptide could be cleaved from the fused protein. In another experiment using the exenatide standard, enterokinase was added to determine whether there are other sequences in the exenatide which might be prone to the action of the enterokinase. From the results, it was found that intact exenatide were produced during the cleavage of the fused protein in less than 6 hours in the presence of the EkMax enterokinase.

Using the PAM enzyme, the glycine-extended exenatide peptide was fully amidated in 90 min at the lower pH at 5.5 (>98% based on % area) without any unwanted products being formed. To determine that it is the C-terminal amino acid which is amidated, a control experiment was done with normal uncapped exenatide without the glycine extension. No reaction took place confirming the specific amidation of the C-terminal. Using 125 units of PAM enzyme instead of 600 units also gave complete amidation. The peptide was fully intact.

For the analysis, an UPLC method to separate the non-amidated and amidated peptide was developed and tested with the standards. The initial analysis was done using a BEH C18 column, but no separation of the amidated and non-amidated exenatide could be achieved. Although the mass spectrometer could detect both compounds, no chromatographic separation could be obtained. Optimization parameters tested included temperature, pH, solvent composition, mobile phase flow rate, formic acid concentration (mobile phase modifier), column length and sample concentration. A new version of the BEH column chemistry became available that incorporated a charged-surface chemistry. This charged-surface hybrid (CSH) C18 column offers alternative selectivity to the BEH column but still have the inherent characteristics of the BEH hybrid column. The CSH column was tested using the same chromatographic conditions used for the BEH column, but no separation was observed. The strongest ionization of exenatide was obtained at an acidic pH, but pH changes between 2 and 5 did not assist with the separation. The best peak shape for exenatide was obtained with acetonitrile whereas methanol caused broadening of the peaks and significantly increase the back pressure. Typical acidic ESI analyses are done with a formic acid concentration of 0.1% (v/v). A systematic lowering of the formic acid concentration resulted in the separation of the amidated and non-amidated exenatide. The optimum formic acid concentration was 0.005% (v/v). Flow rate changes between 0.2 and 0.6 mL/min did not improve the separation, and it was found that 0.4 mL/min produced the best results. A 150 mm column length was required to get a good separation.

The Waters G1 mass spectrometer was acquired for the analysis of small molecules, and therefore equipped with a 4 kDa resolving quadrupole. This would not permit the direct detection of the intact and protonated exenatide and non-amidated exenatide, but monitoring of the multiply-charged ions made detection of the $[M+3H]^{3+}$ and $[M+4H]^{4+}$ species possible. Waters BioLynx software was used to calculate the average mass and accurate monoisotopic mass of each peptide sequence. This can be summarized as follows:

Exenatide uncapped (protonated)

Average mass = 4188.6227, Monoisotopic mass = 4186.0192
N-Terminus = 2H, C-Terminus = OH
Amino acid sequence: HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPS

Exenatide with GLY but not amidated

Average mass = 4245.6747, Monoisotopic mass = 4243.0406
N-Terminus = 2H, C-Terminus = OH
Amino acid sequence: HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPSG

Exenatide amidated (not protonated)

Average mass = 4186.6300, Monoisotopic mass = 4184.0273
N-Terminus = H, C-Terminus = NH2
Amino acid sequence: HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPS

Exenatide amidated (protonated)

Average mass = 4187.6380, Monoisotopic mass = 4185.0352
N-Terminus = 2H, C-Terminus = NH2
Amino acid sequence: HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPS

Waters TOF Transform software was used to transform the mass spectra of the multiply-charged species to predict the molecular weight of the intact compounds. This is done by de-isotoping the masses and realigning to a single charge state mass axis.

Figure 1 displays the separation between non-amidated and amidated exenatide obtained with the optimized chromatographic method on the CSH analytical column. The mass spectra of the non-amidated and amidated exenatide are represented in Figure 2. The non-amidated exenatide produced a multiply-charged mass spectrum with a base peak intensity (BPI) mass ion of 1047.7662 Da (red spectrum). By using the Tof Transform function included in MassLynx software, this mass spectrum could be transformed from a multiply-charged mass spectrum to a predicted single-charge-state mass spectrum (purple spectrum). The amidated exenatide produced a multiply-charged mass spectrum with a BPI mass ion of 1047.2719 Da (green spectrum). Similarly, this mass spectrum could be transformed from a multiply-charged mass spectrum to a predicted single-charge-state mass spectrum (black spectrum) (Figure 3).

The work done in this study showed that structurally similar compounds with a mass difference of 1 Dalton can be successfully separated chromatographically from each other resulting in good quality accurate mass MS data.

Although this method was developed for reaction monitoring purposes, the application as a semi-quantitative method was investigated. A functional calibration curve could be constructed from 0.01 to 0.1 µg/mL (3rd order; R^2 = 0.997) with a detection limit of 0.005 µg/mL. The stability of the method was also tested on different days after equilibration of the analytical column. The retention times did not differ (R_t 12.66 ± 0.0 min; n = 6; Figure 4) for the 30-min method. A short 10-min method was developed based on the same chromatographic conditions. It was found that the retention times differed nominally (R_t 4.09 ± 0.01 min; n = 10). The peak area response of ten repeat injections of the same concentration sample produced an average peak area of

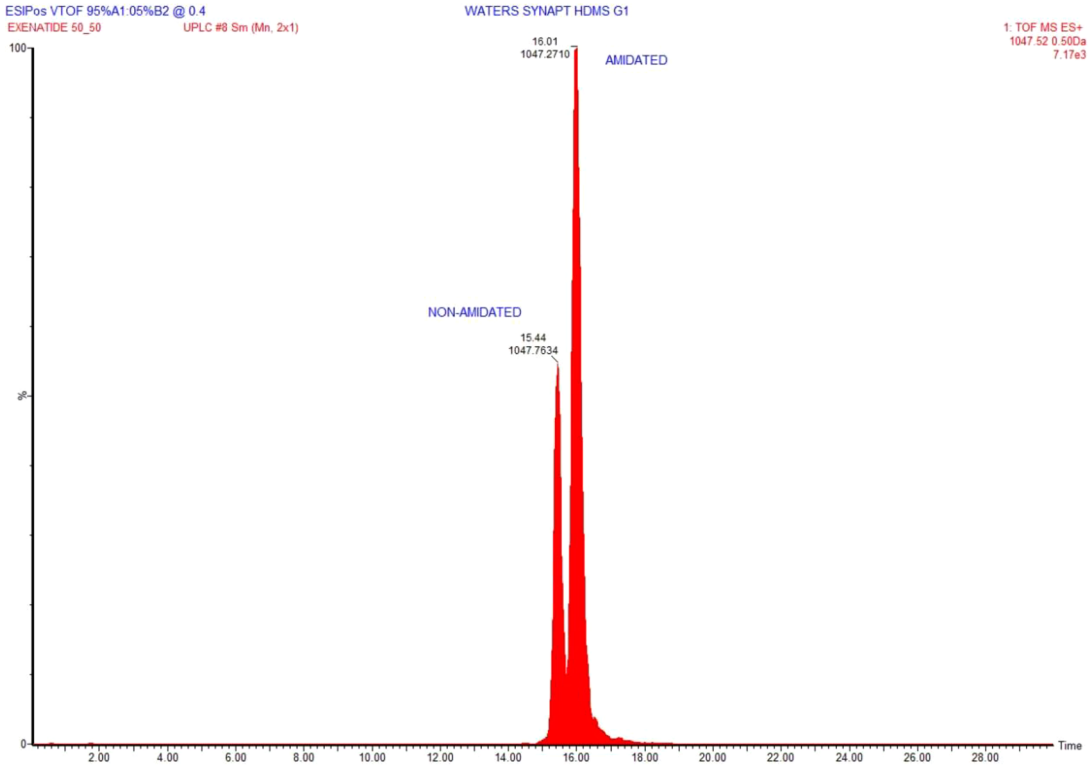


Figure 1. UPLC separation of non-amidated (R_t = 15.44 min) and amidated exenatide (R_t = 16.01 min) on the 150 mm CSH column using the optimized chromatographic conditions.

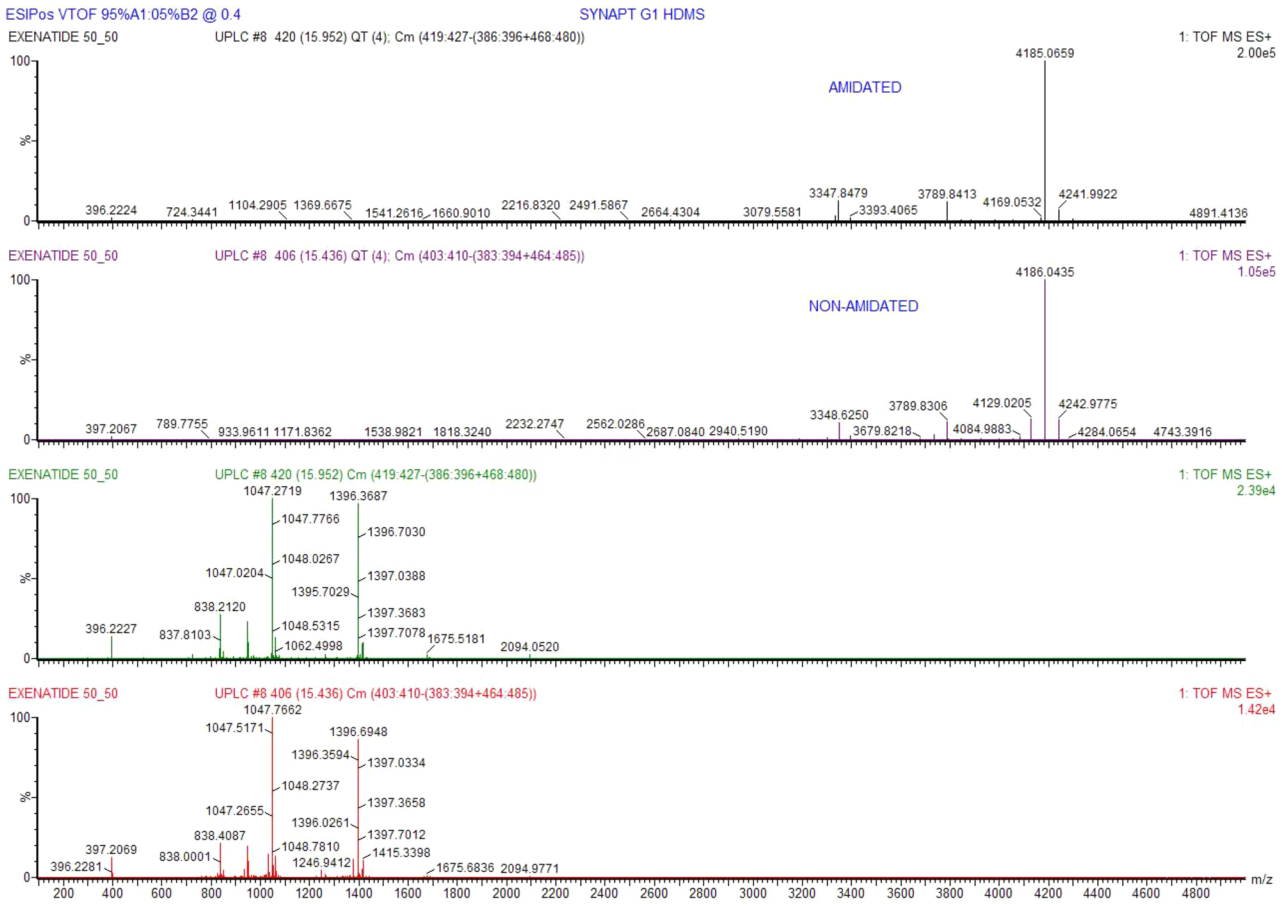


Figure 2. MS spectra of non-amidated (red spectrum) and amidated exenatide (green spectrum) as well as the predicted masses of the intact non-amidated (purple spectrum) and amidated (black spectrum) exenatide. The predicted masses were produced with the TOF Transform option in MassLynx.

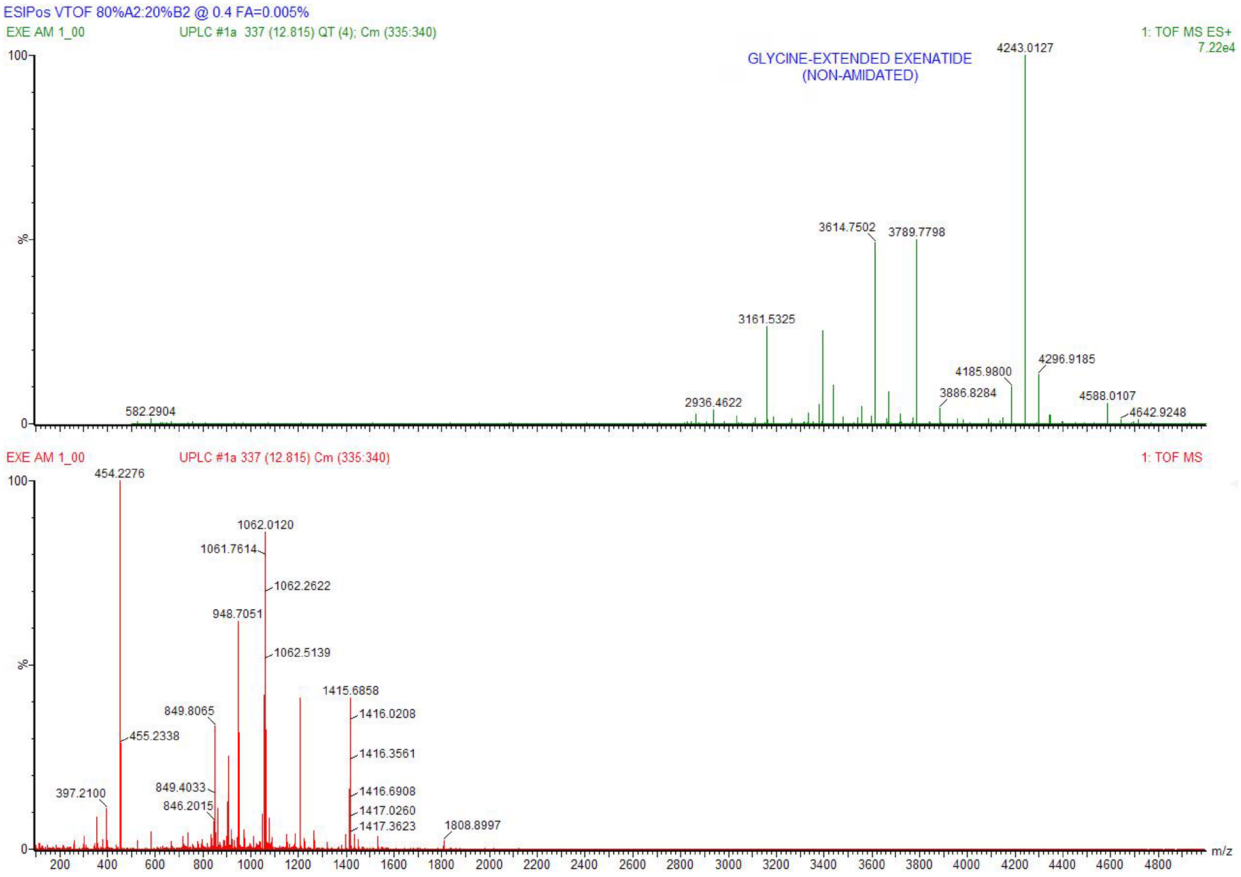


Figure 3. MS data to confirm the identity of the starting material (red spectrum) which was uncapped glycine extended exenatide with a mass of 4243 Da (green spectrum). The predicted mass was produced with the TOF Transform option in MassLynx.

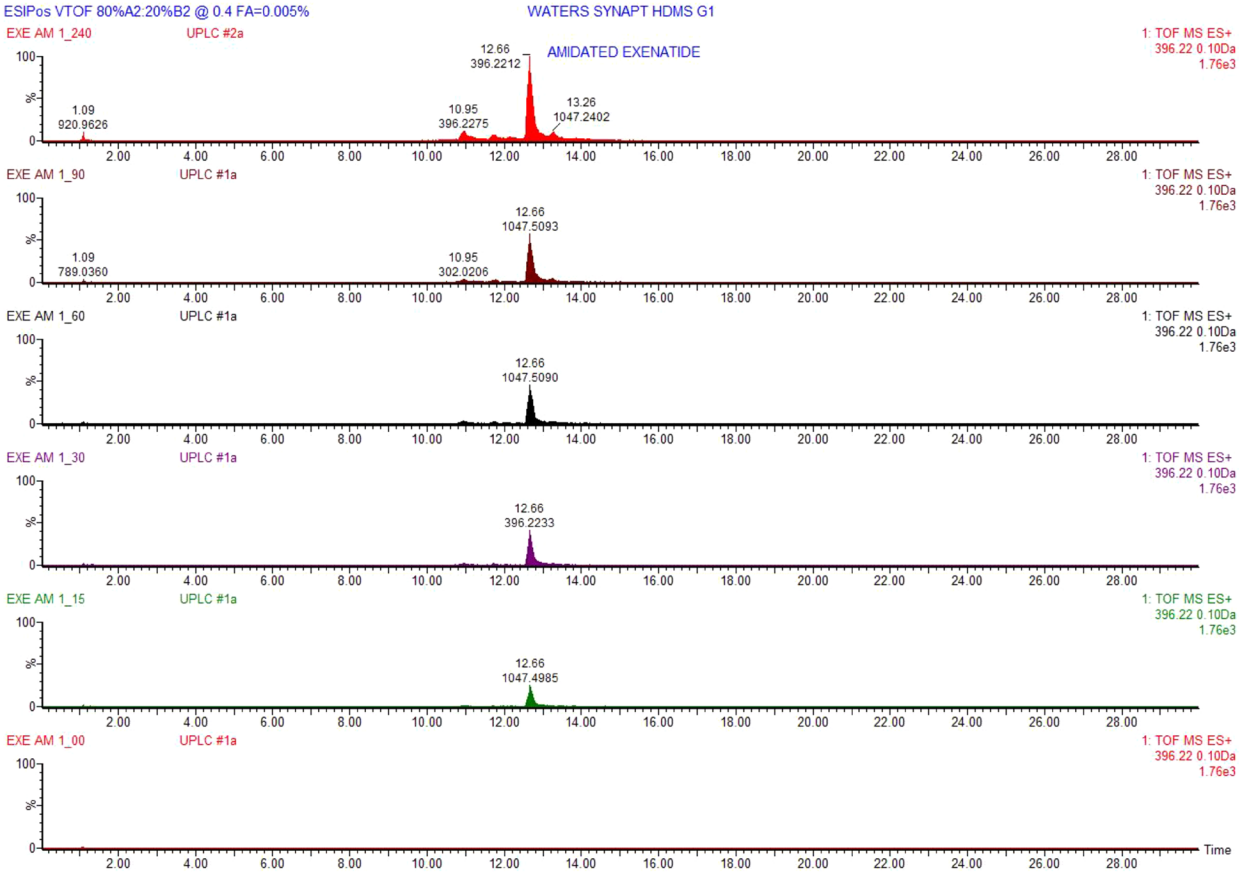


Figure 4. UPLC-MS data of the increase in C-amidated exenatide product (R_t = 12.66 min) with time (0–240 min). Annotation of the mass of each peak was done according to the base peak ion observed for each compound.

32,799 ± 816 (2.5%). The short method was tested on three consecutive days and produced similar results. The analytical columns were dedicated to exenatide development work for the duration of the study to minimize the possibility of contamination from alternative sources.

Conclusion

The increasing use of peptides in pharmaceutical applications has necessitated the development of technologies which will result in economically feasible methods to produce the compounds. Heterologous expression of a recombinant peptide is possible in prokaryotic and eukaryotic expression hosts and may result in more affordable peptide production. The peptides produced in this way may however still require modifications, such as N-acetylation and C-amidation for full activity and bioavailability. General methods which can be employed on almost any peptide for N-acetylation and C-amidation may be very valuable. The methods developed must be able to acetylate the N-terminal or amidate the C-terminal without requiring the side chains of the amino acids being blocked.

In order to follow all the reactions taking place, a very robust method of analysis is required to determine the success of the work. Using UPLC-QTOF-MS technology, chromatographic methods were developed and the multiply-charged reaction products detected with mass spectrometry. The initial chromatographic development was done on a Waters BEH C18 column, but no separation could be achieved between the amidated and non-amidated exenatide. The use of a 150 mm Waters CSH C18 column allowed for the separation of the co-eluting compounds after method optimization of the formic acid concentration.

Acknowledgments

The authors are grateful for financial support from CSIR Parliamentary grant funding, as well as the CSIR Genetic Engineering and Bioprocess teams for producing the exenatide in the genetically modified microorganism.

Disclosure statement

The authors declare no competing interests.

Data availability

The data supporting the study's findings are available upon reasonable request.

Funding

The research was from Parliamentary Grant funds from the CSIR.

ORCID

Lucia Steenkamp http://orcid.org/0000-0002-9240-4242
Paul Steenkamp http://orcid.org/0000-0002-4416-6796

References

[1] Rossino, G.; Marchese, E.; Galli, G.; Verde, F.; Finizio, M.; Serra, M.; Linciano, P.; Collina, S. Peptides as Therapeutic Agents: Challenges and Opportunities in the Green Transition Era. *Molecules* **2023**, *28*, 7165. DOI: 10.3390/molecules28207165.

[2] Merrifield, R. B. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154. DOI: 10.1021/ja00897a025.

[3] Jaradat, D. M. M. Thirteen Decades of Peptide Synthesis: key Developments in Solid Phase Peptide Synthesis and Amide Bond Formation Utilized in Peptide Ligation. *Amino Acids.* **2018**, *50*, 39–68. DOI: 10.1007/s00726-017-2516-0.

[4] Martos-Maldonado, M. C.; Hjuler, C. T.; Sørensen, K. K.; Thygesen, M. B.; Rasmussen, J. E.; Villadsen, K.; Midtgaard, S. R.; Kol, S.; Schoffelen, S.; Jensen, K. J. Selective N-Terminal Acylation of Peptides and Proteins with a Gly-His Tag Sequence. *Nat. Commun.* **2018**, *9*, 3307. DOI: 10.1038/s41467-018-05695-3.

[5] Lifetein. N-terminal Acetylation and C-terminal Amidation. https://www.lifetein.com/Peptide-Synthesis-Amidation-Acetylation.html?srsltid=AfmBOoomPAAn4CcZ6zon3XqSsT04oeF1ag6_C7xfNV9Wj4Hiu9cTagAF. (accessed Aug. 20, 2024)

[6] Kekessie, I.; Wegner, K.; Martinez, I.; Kopach, M. E.; White, T. D.; Tom, J. K.; Kenworthy, M. N.; Gallou, F.; Lopez, J.; Koenig, S. G.; et al. Process Mass Intensity (PMI): A Holistic Analysis of Current Peptide Manufacturing Processes Informs Sustainability in Peptide Synthesis. *J. Org. Chem.* **2024**, *89*, 4261–4282. DOI: 10.1021/acs.joc.3c01494.

[7] Mair, G. R.; Niciu, M. J.; Stewart, M. T.; Brennan, G.; Omar, H.; Halton, D. W.; Mains, R. E.; Eipper, B. A.; Maule, A. G.; Day, T. A. A Functionally Atypical Amidating Enzyme from the Human Parasite *Schistosoma mansoni*. *FASEB J.* **2004**, *18*, 114–121. DOI: 10.1096/fj.03-0429com.

[8] Holst, J. J. The Physiology of Glucagon-like Peptide 1. *Physiol. Rev.* **2007**, *87*, 1409–1439. DOI: 10.1152/physrev.00034.2006.

[9] Baggio, L. L.; Drucker, D. J. Biology of Incretins: GLP-1 and GIP. *Gastroenterology* **2007**, *132*, 2131–2157. DOI: 10.1053/j.gastro.2007.03.054.

[10] Lovshin, J. A.; Drucker, D. J. Incretin-Based Therapies for Type 2 Diabetes Mellitus. *Nat. Rev. Endocrinol.* **2009**, *5*, 262–269. DOI: 10.1038/nrendo.2009.48.

[11] Hayes, M. R.; Mietlicki-Baase, E. G.; Kanoski, S. E.; De Jonghe, B. C. Incretins and Amylin: neuroendocrine Communication between the Gut, Pancreas, and Brain in Control of Food Intake and Blood Glucose. *Annu. Rev. Nutr.* **2014**, *34*, 237–260. DOI: 10.1146/annurev-nutr-071812-16120.

[12] Hayes, M. R.; Schmidt, H. D. GLP-1 Influences Food and Drug Reward. *Curr. Opin. Behav. Sci.* **2016**, *9*, 66–70. DOI: 10.1016/j.cobeha.2016.02.005.

[13] Kanoski, S. E.; Hayes, M. R.; Skibicka, K. P. Glp-1 and Weight Loss: Unraveling the Diverse Neural Circuitry. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2016**, *310*, R885–R895. DOI: 10.1152/ajpregu.00520.2015.

[14] Knudsen, L. B.; Nielsen, P. F.; Huusfeldt, P. O.; Johansen, N. L.; Madsen, K.; Pedersen, F. Z.; Thøgersen, H.; Wilken, M.; Agersø, H. Potent Derivatives of Glucagon-like Peptide-1 with Pharmacokinetic Properties Suitable for Once Daily Administration. *J. Med. Chem.* **2000**, *43*, 1664–1669. DOI: 10.1021/jm9909645.

[15] Madsen, K.; Knudsen, L. B.; Agersoe, H.; Nielsen, P. F.; Thøgersen, H.; Wilken, M.; Johansen, N. L. Structure-Activity and Protraction Relationship of Long-Acting Glucagon-like Peptide-1 Derivative: importance of Fatty Acid Length, Polarity, and Bulkiness. *J. Med. Chem.* **2007**, *50*, 6126–6132. DOI: 10.1021/jm070861j.

[16] Knudsen, L. B.; Lau, J. The Discovery and Development of Liraglutide and Semaglutide. *Front. Endocrinol. (Lausanne)* **2019**, *10*, 155. DOI: 10.3389/fendo.2019.00155.

[17] O'Neil, P. M.; Birkenfeld, A. L.; McGowan, B.; Mosenzon, O.; Pedersen, S. D.; Wharton, S.; Carson, C. G.; Jepsen, C. H.; Kabisch, M.; Wilding, J. P. H. Efficacy and Safery of Semaglutide Compared with Liraglutide and Placebo for Weightloss in Patients

with Obesity: A Randomised, Double-Blind, Placebo and Active Controlled, Dose-Ranging, Phase 2 Trial. *Lancet* **2018**, *392*, 637–649. DOI: 10.1016/S0140-6736(18)31773-2.

[18] Lau, J.; Bloch, P.; Schäffer, L.; Pettersson, I.; Spetzler, J.; Kofoed, J.; Madsen, K.; Knudsen, L. B.; McGuire, J.; Steensgaard, D. B.; et al. Discovery of the Once-Weekly Glucagon-like Peptide-1 (GLP-1) Analog Semaglutide. *J. Med. Chem.* **2015**, *58*, 7370–7380. DOI: 10.1021/acs.jmedchem.5b00726.

[19] Bridges, A.; Bistas, K. G.; Jacobs, T. F. Exenatide. *StatPearls* May 29, **2023**. https://www.ncbi.nlm.nih.gov/books/NBK518981/.

[20] Tinsley, I. C.; Borner, T.; Swanson, M. L.; Chepurny, O. G.; Doebley, S. A.; Kamat, V.; Sweet, I. R.; Holz, G. G.; Hayes, M. R.; De Jonghe, B. C.; Doyle, R. P. Synthesis, Opimization and Biological Evaluation of Corrinated Conjugates of the GLP-1R Agonist Exendin 4. *J. Med. Chem.* **2021**, *64*, 3479–3492. DOI: 10.1021/acs.jmedchem.1c00185.

[21] Mayo Clinic. Exenatide. https://www.mayoclinic.org/drugs-supplements/exenatide-subcutaneous-route/description/drg-20068401. (Accessed 24 March) **2025**

[22] Göke, R.; Fehmann, H. C.; Linn, T.; Schmidt, H.; Krause, H.; Eng, J.; Göke, B. Exendin-4 is a High Potency Agonist and Truncated Exendin-(9–39)-Amide an Antagonist at the Glucagon-like Peptide 1-(7–36)-Amide Receptor of Insulin-Secreting Beta-Cells. *J. Biol. Chem.* **1993**, *268*, 19650–19655. [q5]