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ORIGINAL ARTICLE

Quality evaluation of synthetic quorum sensing peptides used in R&D

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Abstract Peptides are becoming an important class of molecules in the pharmaceutical field. Closely related peptide-impurities in peptides are inherent to the synthesis approach and have demonstrated to potentially mask biomedical experimental results. Quorum sensing peptides are attracting high interest in R&D and therefore a representative set of quorum sensing peptides, with a requested purity of at least 95.0%, was evaluated for their purity and nature of related impurities. In-house quality control (QC) revealed a large discrepancy between the purity levels as stated on the supplier's certificate of analysis and our QC results. By using our QC analysis flowchart, we demonstrated that only 44.0% of the peptides met the required purity. The main compound of one sample was even found to have a different structure compared to the desired peptide. We also found that the majority of the related impurities were lacking amino acid(s) in the desired peptide sequence. Relying on the certificates of analysis as provided by the supplier might have serious consequences for peptide research, and peptide-researchers should implement and maintain a thorough in-house QC.

1. Introduction

Peptides are becoming an important class of molecules in the biomedical and pharmaceutical fields owing to their high affinity, strong selectivity for their targets and low toxicity [1]. Despite potential limitations such as overall low oral bio-availability, low metabolic resistance, potential immunogenicity, poor membrane permeability and financial aspects, several peptide drugs have entered the market [2,3]. The promising future of peptide therapeutics is further highlighted by the number of peptides in clinical and preclinical phases [4]. In 2012, approximately 200 peptides entered the clinical phase, while another 400 were at advanced preclinical stages [4–6].

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Quorum sensing peptides are a group of peptides currently attracting high interest. Quorum sensing, the process of cell-to-cell communication between bacteria, has been the subject of a great number of scientific research papers [7–9]. Three main groups of quorum sensing molecules can be distinguished: *N*-acylhomoserine lactone derivatives (AHL or auto-inducer-1), quorum sensing peptides and boron-furan derivatives (auto-inducer-2). The quorum sensing peptides, mainly found in Gram-positive bacteria, show a large structural diversity: short, linear fragments as well as cyclic (thiolacton) derivatives, with or without post-translational isoprenyl modifications, are observed [10]. These peptides bind to (i) bacterial membrane associated receptors, or (ii) cytoplasmatic receptors, after which the transcription of the target genes is activated. Interfering with this bacterial quorum sensing pathway might open interesting application perspectives.

Chemical peptide synthesis for medicinal purposes has become economically viable [11]. The possibility to produce small, medium (5–20 residues) to large (20–50 residues) peptides has evolved dramatically, hereby frequently outperforming the biotechnological approaches as they are known to date [6]. In 1965, Merrifield cleared the way for Solid-Phase Peptide Synthesis (SPPS), thus introducing and facilitating a totally new concept in peptide synthesis [12]: a peptidic chain, fixed to a solid support, is created by the consecutive addition of the appropriate amino acids. The reaction can be automated and possible solubilization-issues are avoided due to the fixation of the peptide to the solid matrix [13]. During SPPS, different side-reactions can occur, resulting in several types of peptide impurities [14]: *e.g.* (i) diketopiperazine structures [15,16], (ii) aspartimide residues [17–19], (iii) cysteine racemization [16], (iv) diastereoisomeric products [20,21], (v) dimers [22], (vi) acid precursors and protected sequences [20], (vii) oxidation and reduction of amino acids [19,23], (viii) amino acid deletions [15,23–26], (ix) amino acid insertions [23,24], (x) products of side chain reactivity and (xi) amino acid modifications during cleavage [16,19,23,24,26–30]. Thus, the crude peptides obtained from SPPS mostly will contain many by-products. These impurities, if present even after purification, have to be quantified and characterized to meet pharmaceutical regulatory requirements, but they need to be under control as well during the unregulated biomedical research and discovery phases [31,32]. Due to budgetary constraints, peptides used in research are often purchased at undefined or low purity levels, *e.g.* 70.0% [31]. Generally, the peptide purity used for biomedical research and discovery purposes ranges from as low as 50.0% to more than 95.0% [22,26]. However, closely related impurities of the target peptide may possess stronger binding affinities compared to the native peptide [26], thus potentially causing erroneous conclusions. Investigations by de Beukelaar et al. [33] indicated different immune responses of a protein-spanning peptide pool of 70.0% pure peptides due to the impurities. False-positive results in a HIV-vaccine trial were also ascribed to impurities in the peptide-mixtures [34]. Zhang et al. [35] were unable to reproduce their initial obestatin results in vitro, possibly due to impurities present in the initially examined peptide. Additionally, Verbeken et al. [31] observed different biofunctional responses in a set of tissue-organ bath experiments caused by impurities of the examined peptides. Impurities are thus able to potentially mask biomedical experimental outcomes and may cause false negative or positive results.

Quorum sensing peptides are currently being actively investigated, *i.e.* for their possible role in the crosstalk between the microbiome and its host [36,37]. Therefore, a thorough in-house QC analysis of a selected set of quorum sensing peptides will be conducted, followed by identification of the observed impurities. These data not only demonstrate the need for routine QC in peptide research, but also can help in building a global overview of expected related impurities in peptides.

2. Materials and methods

2.1. Chemicals and reagents

A set of 98 representative peptides was selected from the Quorumpeps database [38]. Linear peptide synthesis was conducted by an international supplier, while another supplier synthesized the cyclic peptides, both by means of Fmoc-SPPS. A minimal purity of 95.0% was requested at order. The sequences of the 98 peptides are provided in Table 1. Acetonitrile of HPLC–MS and UPLC-MS grades were purchased from Fisher Scientific (Aalst, Belgium). Formic acid (LC–MS grade) and DMSO (p.a. $\geq$ 99.9%) were obtained from Sigma-Aldrich (Diegem, Belgium). Trifluoroacetic acid (TFA), $NaH_2PO_4 \cdot H_20$ and Na_2HPO_4 were

Table 1 Peptides information.

Quorumpeps ID	Sequence	Molecular weight	Solvent	Column[a]	Purity (%)[b]
2	FNTIPSY	839.95	Water	C_{18}-FA	83.31
5	Ac-CGSLF, thiolacton linkage between C1 and F5	549.72	Water+50% DMSO	C_{18}-FA	88.30
7	FNTWPSY	913.00	Water	C_{18}-FA	98.46
10	ADLPFEF	837.93	Water	C_{18}-FA	99.20
11	AGTKPQGKPASNLVECVFSLFKKCN	2667.14	Water	C_{18}-FA	72.93
13	AIFILAS	733.91	Water+58% DMSO	C_{18}-FA	97.82
14	AITLIFI	790.01	Water+60% DMSO	C_{18}-FA	72.48
15	AKDEH	598.61	Water	C_{18}-TFA	69.26
16	AKTVQ	545.64	Water	C_{18}-TFA	96.20
17	ALILTLVS	829.05	Water+60% DMSO	C_{18}-FA	94.43
18	ARNQT	588.62	Water		92.33