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Review

Toward greener analytical techniques for the absolute quantification of peptides in pharmaceutical and biological samples

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ABSTRACT

Peptide-based biopharmaceuticals represent one of the fastest growing classes of new drug molecules. New reaction types included in the synthesis strategies to reduce the rapid metabolism of peptides, along with the availability of new formulation and delivery technologies, resulted in an increased marketing of peptide drug products. In this regard, the development of analytical methods for quantification of peptides in pharmaceutical and biological samples is of utmost importance. From the sample preparation step to their analysis by means of chromatographic or electrophoretic methods, many difficulties should be tackled to analyze them. Recent developments in analytical techniques emphasize more and more on the use of green analytical techniques. This review will discuss the progresses in and challenges observed during green analytical method development for the quantification of peptides in pharmaceutical and biological samples.

Contents

Abbreviations: CE, capillary electrophoresis; HRMS, high resolution mass spectrometry; id, internal diameter; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RPLC, reversed phase liquid chromatography; SFC, supercritical fluid chromatography; UHPLC, ultra high performance liquid chromatography.

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1. Introduction

Peptides and peptide-based drug products are attracting increasing attention as diagnostics and therapeutics [1,2], due to the enormous diversity of functions of peptides in the human body and their involvement in almost every essential physiological process [3]. Today, approximately 100 therapeutic peptides are on the market in the three major regions, Europe, USA and Japan [4]. In 2012, six peptides have obtained marketing approval in the USA and five in the EU [5]. Moreover, the number of peptides entering clinical trials every year has increased till around 20 [4]. Peptides have higher potency, selectivity and specificity compared to small molecules, and they also show less off-target toxicity and drug–drug interactions [1,6,7]. The major drawback of peptides is their general poor oral bioavailability due their low permeability, in combination with a low metabolic stability. Recent advances to overcome these issues include for example modifications of the amino acid backbone and chemical conjugation with polymers [1,4,6,8]. Peptides are therefore one of the most promising areas for the development of new drugs with original mechanisms of action [4,9]. In this review, we follow the definition of peptides as proposed earlier [10,11], *i.e.* containing maximal 50 amino acid residues and having a molecular weight below 6 kDa.

The technique used for peptide synthesis depends largely on its size and chemical features [4,12]. Peptide manufacturing can be achieved entirely through chemical synthesis, recombinant DNA technology, cell-free expression systems, transgenic animals and plants or enzymatic synthesis [1,4]. Chemical synthesis is the most universal approach as it permits to include unnatural amino acids and pseudo-peptide bonds [1,4]. It remains the gold standard for the manufacturing of peptides between 5 and 50 amino acid residues [1,13,14]. Three major approaches are distinguished, namely solution-phase, solid phase and hybrid approaches [4,13].

Bioanalysis of peptides faces problems derived from the low concentration of these analytes in biological matrices, the large number of potential interferences and the limited sample volume [15]. Sample treatments usually involve isolation of the target analytes from the matrix in combination with their preconcentration, in this way improving sensitivity and selectivity of the assay [15,16] and reducing matrix effects [17,18]. Sample treatment has evolved in the last few years following three main trends, *i.e.* automation, miniaturization and simplification. This has led to the development of a number of microextraction techniques [15,19]. The characteristics of these microextraction techniques match with the requirements of bioanalysis. Indeed, automation results in improved throughput, allowing larger number of samples to be processed. Moreover, less sample volume is required due to the miniaturization. Finally, the reduction of number of steps in the sample preparation process, *i.e.* simplification, improves the precision of the method [15]. Sample pretreatment steps will not be further discussed, as they are outside the scope of this review.

Recently, the interest in green analytical chemistry has grown tremendously. An increased number of manuscripts on green analytical method development can be found in literature [20]. This term is used to describe analytical approaches that minimize the consumption of reagents and energy, as well as the reduction in the generation of hazardous waste. For analytical method development the focus is oriented toward reduction or elimination of toxic solvents and decreasing the analysis time [21]. The increasing importance of peptide therapeutics necessitates performance improvements in (bio)analytical techniques to support biopharmaceutical drug development [3]. Indeed, analytical methods are primordial to assess drug substance and drug product quality and to obtain reliable pharmacological and toxicological data [3,11,22]. The development of sensitive, selective, high-throughput methods for peptide analysis using greener analytical techniques is highly demanded [23]. Ligand-binding assays are still the current gold standard for peptide analysis, because they offer high sensitivity. Moreover, immunoassays are considered as green techniques as they provide rapid analysis, are mostly performed in aqueous solution, require simple sample pretreatment and low sample volumes [24,25]. However, the dynamic range is often limited and there can be large selectivity issues [3,26,27]. In addition, development of new antibodies can be time-consuming and costly [26]. Liquid chromatography (LC) coupled to mass spectrometry offers improved selectivity and reduced method development time, and can be used as alternative or as orthogonal assay [28]. LC remains today the method of choice for peptide analysis. However, both capillary electrophoresis (CE) and supercritical fluid chromatography (SFC) are complementary techniques which have also shown their usefulness for drug analysis [29,30]. This review will therefore focus on the progresses in and challenges observed during green analytical method development for the quantification of peptides in pharmaceutical and biological samples.

2. Challenges related to physicochemical properties of peptides

Independent of the used separation technique, there are several challenges that should be taken into account when quantifying peptides. These include the purity of the peptide standard, solubility of the peptide and related adsorption issues and also stability of these biopharmaceutical compounds. These issues will be discussed in more detail below.

2.1. Purity

One important and evident aspect necessary to perform quantitative analyses is the purity of the used peptide standard. Although the peptide manufacturers assure the peptide to be >95% pure, cases are reported where more than 50% of the peptide content comprised of impurities [12] or even a totally different peptide was present [31]. The following impurity classes can be distinguished: amino acid deletion or insertion, incomplete removal of protecting groups after synthesis, oxidation or reduction, diastereoisomerization, side- and end-chain impurities, dimers, peptide counter ions (usually trifluoroacetic acid), structurally unrelated contamination and miscellaneous impurities (for review, see [12]). The presence of any of these impurities has significant consequences on the biological function of the peptide and consequently on the outcome of the experiments. Thus, it is highly recommended to verify the quality of peptides before experimental use and to repeat the biological experiments with a second peptide batch, obtained from another source.

2.2. Solubility–nonspecific binding

Aspecific adsorption is a known property of peptides and is detrimental for sensitivity and carryover in all separation methods. Reduction of nonspecific binding should be investigated at the beginning of every method development phase. The extent of this nonspecific binding depends upon the characteristics of the peptide, such as charge of the peptide and hydrophobicity and should therefore be optimized for every peptide studied. Aspecific adsorption can be minimized by choosing the right dissolution solvent, dilution solvent for further standard preparation and injection solvent, but also the vial material, the temperature during standard preparation and during analytical separation should be carefully selected.

A widely applicable strategy to improve method sensitivity by intervening on all aspects of standard preparation, *i.e.* from