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Are low-priced peptides affordable?

INTRODUCTION

Towards the end of May 2006, a number of reports appeared in the press concerning the indictment of a biotechnology company and its owner on charges of allegedly shipping bogus research material to many corporate and academic researchers. According to these reports, the indictment alleged that employees of the company fraudulently misrepresented the purity of the peptide products they manufactured. Amongst other fraudulent activities, impurities in chromatograms were masked by cut-and-paste procedures. That this company has ceased activities should be no surprise to anyone. What is surprising, however, is that the company allegedly had to resort to "cutting and pasting" to fabricate HPLC chromatograms showing purities of presumably 95 percent or 97 percent. The "apparent" purity of the products could easily have been increased by simple manipulation of the HPLC gradient, in order to eliminate the separation between the product and closely-eluting impurities. One is tempted to speculate that the quality of the company's products must have been very low and there is ample justification now to question the results of research that used the peptides which the company sold. A high price to pay for low-priced peptides!
But before closing the door on what – however many institutions and projects were unfortunately affected – is almost certainly an isolated incidence of gross fraud, it is important to ask the question as to how important is the quality of peptides which are used for different stages of scientific investigations. The use of steep gradients and otherwise inappropriate and poorly resolving generic HPLC methods often fail to reveal the impurities in peptides. Expressed differently, are peptide users as a whole really aware of the purity of the products that they are buying, and what level of impurities can be tolerated in a peptide before it loses its pharmacological relevance?

THE RELEVANCE OF PEPTIDE PURITY

It seems almost needless to state that the purity and characterization of any molecule that is the object of a scientific study is important, but, in the context of investigations with peptides that are leading into preclinical and clinical studies, there are three pressing reasons for taking particular care in assessing these parameters:

1. regulatory issues;
2. the pharmacological profile of impurities; and
3. the peptide is often a new chemical entity (NCE).

Whilst regulatory concerns are only of importance for peptides that will be finally approved as drug substances, the consequences of the pharmacological profile of impurities and the NCE status are issues that apply to peptides in any stage of investigation.

Regulatory requirements: Regulatory requirements for peptide-based active pharmaceutical ingredients (APIs) are not well-defined. The only specific guidance on peptide APIs, the "Guidance for Industry for the Submission of Chemistry, Manufacturing, and Controls Information for Synthetic Peptide Substances (1994)" has recently been withdrawn. ICH Guidance for Industry Q3A, "Impurities in New Drug Substances" specifically excludes peptides. ICH Q6A, "Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances" states that the guidance is "not sufficient to adequately describe specifications of higher molecular weight peptides and polypeptides". These are indications that the ICH recognizes that impurity issues for complex peptides are special, but these guidance documents do not guarantee that less tight specifications than those for small molecules can be assumed to be acceptable.
Finding unexpected impurities in a peptide API late in the development process can put the project in jeopardy. From a regulatory standpoint, it pays to develop an optimized and robust manufacturing process, which can be shown to maintain impurities at an acceptable level, as soon as it becomes obvious that the process will be scrutinized by a regulatory agency. While it would be preferable to achieve this before starting clinical trials, this is often not practical, although the manufacturing process should be fixed at the latest before starting Phase III. A robust process can only be achieved if the accompanying analytical procedures can detect impurities derived from the manufacturing process, as well as degradation products. This, in turn, can only be achieved by the development of analytical methods which are specific for the peptide API. The development of a discriminatory analytical HPLC method may take up to several weeks for complex peptides. However, it should be an obligatory part of the pre-clinical development process.

Pharmacological profile of impurities: It is well-known that solid-phase peptide synthesis (SPPS), which is the default procedure for the initial synthesis of longer peptides, can generate crude peptides which contain a large number of impurities, such as deletion, insertion, truncated sequences, etc. Fortunately, most of these can be removed during purification, which is typically a one- or two-step procedure. However, those impurities that remain after the purification process may be difficult or impossible to resolve in a single HPLC method, and they may – by virtue of being closely-related analogs of the desired product – interact with the same receptors, thereby acting as antagonists or agonists. This

necessitates the use of high purity peptides (typically HPLC purity ≥97 per cent) for pharmacological studies (1). The danger of using peptides of lower purity is that the investigator will be no longer be studying the effects of a single substance, but a cocktail of potentially more than one pharmaceutically active species. There is no evidence whatsoever that impurities which are analogs of the desired product will have identical pharmacological profiles; in fact nearly all evidence argues against this.

New chemical entities (NCEs): The peptide under investigation is often an NCE which is being manufactured by the vendor for the first time or for the first time at significant scale. Purchasing an NCE is not the same as purchasing a commodity chemical, such as sodium chloride or a buffer salt. There is usually no history of analytical development and there is no reason to automatically assume that analytical methods have been evaluated in depth and/or can be treated with reliance.

ARE PEPTIDE USERS REALLY AWARE OF THE PURITY OF THE PRODUCTS THAT THEY ARE BUYING?

Analytical, reverse-phase HPLC is currently the default method for determining peptide purity. It is fast and it is flexible. There are a wide array of different supports and a virtually unlimited choice of mobile phases which can be used to develop analytical methods that resolve most peptides from their impurities. Most importantly from a manufacturing perspective, such optimized analytical methods can form the basis for the in-process control methods. The main weakness of any single HPLC method is that it only provides negative identification, i.e., it only shows impurities that resolve from the main peak, not impurities that co-elute. This deficit can be compensated by the use of one or more orthogonal HPLC methods that utilize different ion pairs or separation principles to reduce the risk of undetected impurities. This is demonstrated in Figure 1 for a sample of GLP-2 analyzed in mobile phases based on trifluoroacetic acid (TFA), triethylammonium phosphate (TEAP) and KPF_6. The impurities observed with the KPF_6 system could be reproduced with $NaClO_4$-containing mobile phases as well.

An additional weakness lies not in the HPLC method *per se*, but in the use of "soft" specifications. Although the use of terminology such as "the peptide should be greater than 97 per cent pure by HPLC", without giving details of the HPLC method (i.e., mobile and stationary phases, gradient, flow rate, column temperature, and wavelength), is common practice, the purity is not precisely defined. This is illustrated in Figure 2. When the same sample of PACAP (1-38) is analyzed in TFA/acetonitrile using a steep gradient at room temperature, and also in a shallow gradient at elevated temperature, a 10 per cent difference in peak purity can be demonstrated. It is, therefore, clearly possible, through relatively simple modifications of the HPLC method, to generate data which indicates that the product is of much higher purity than it actually is – sometimes even appearing to be 100 percent pure! The age of a column may also be a factor because performance will generally diminish over usage time.

Evaluation of the integration of peak areas is also open to many subjective factors. Depending on how baseline drift is compensated, the level at which noise is filtered out, and the method by which peaks are determined (e.g. baseline drop versus tangential skim), the peak areas of impurities can be interpreted differently. Even if due diligence is exercised by using a number of different, orthogonal mobile phases, enantiomeric impurities are exceptionally difficult to resolve and detect, as are β-aspartate transformations (2) because these impurities have very similar chemical and physical properties, as well as identical or near identical masses to the peptide product and, therefore, may not be detectable by LCMS. While agreement to tightly defined HPLC specifications, accepted by both vendor and customer, is common in an ongoing GMP scenario, it is not normally discussed in the context of preclinical studies, and not always during bidding processes for GMP peptides either. In these latter scenarios, the choice of HPLC method is typically left to the vendor, who usually has their standard methods or procedures for establishing them. The investment the vendor makes in developing suitable analytical methods will very much determine what the quality of the final peptides will be. The nominal purity on paper (proposal, certificate of analysis, chromatogram) must be assessed in the context of the resolving power of the HPLC methods used.

As a general rule, the closer a project draws to entering the decision stage for clinical trials, the more care needs to be exerted. Preclinical studies, in particular, are susceptible to failure due to late discovery of interfering chemical species. In the initial screening procedures, where many peptides have to be assessed for potency, such diligence is neither practical nor affordable. However, once the decision has been made to advance one or more lead substances into pre-clinical evaluation, it is important to know that the peptide has high purity. Cases of pharmacological actions due to false positive leads will be filtered out by default at this stage as the focus of the vendor moves from "reaching a high purity specification for non-GMP peptides" to "reaching a low impurity specification for GMP peptides". To be certain that any particular peptide has the purity required, the investigator should analyze the material by at least two orthogonal HPLC methods, if possible at raised temperature. If the peptide contains asparagine or aspartic acid residues (i.e., Asx), particularly in the combination Asx-Gly, Asx-Ala, Asx-Ser or Asx-Phe, an HPLC method capable of resolving isomerization products (i.e., those containing β-aspartyl residues) should be used

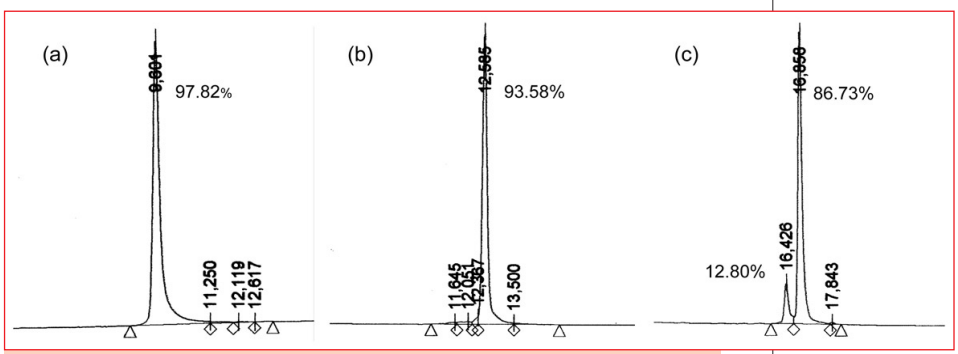

Figure 1. HPLC chromatograms of GLP-2 in: (a) Mobile Phase A = 0.1 percent TFA/H_20, Mobile Phase B = 0.1 percent TFA/Acetonitrile; Gradient = 35 percent - 60 percent B in 25 min.; Flow Rate = 1.5 mL/min.; T = room temperature; (b) Mobile Phase A = 0.1 M aqueous TEAP, pH 2.3, Mobile Phase B = Acetonitrile; Gradient = 35 percent - 60 percent B in25 min.; Flow Rate = 1.5 mL/min.; T = room temperature; (c) Mobile Phase A = 0.1 M aqueous KPF_6, Mobile Phase B = Acetonitrile; Gradient = 35 percent - 60 percent B in 25 min.; Flow Rate = 1.5 mL/min.; T = room temperature. A 4.6 x 250 mm Luna C18 column was used for (a) and (b), and a Purospher RP-18e column for (c). In all cases, the detection wavelength was 220 nm.

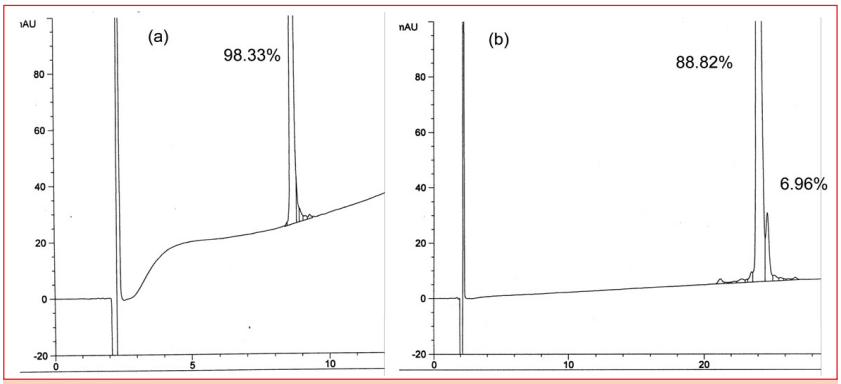

Figure 2. HPLC chromatograms of PACAP (1-38) on: (a) Discovery HS C18; Mobile Phase A = 0.1 percent TFA/H20, Mobile Phase B = 0.1 percent TFA/Acetonitrile; Gradient = 10 percent - 80 percent B in 20 min.; Flow Rate = 1.5 mL/min.; T = room temperature; (b) YMC ODS-AM; Mobile Phase A = 0.1 percent TFA/H20, Mobile Phase B = 0.1 percent TFA/Acetonitrile; Gradient = 19 percent - 27 percent B in 32 min.; Flow Rate = 1.5 mL/min.; T = 60°C. In all cases, the detection wavelength was 220 nm.

(3). The investigator should also be aware that chiral impurities may not be resolved from the main peak in longer peptides. In this case, an independent assessment of the chiral purity of the peptide should be carried out. If no in-house testing can be performed, the certificate of analysis should at least report purity using two orthogonal HPLC methods and copies of the HPLC chromatograms should be included, so that the investigator can independently assess the method of integration. In passing, it should, of course, be noted that, if the quality of peptides in any sort of screening procedure is not controlled, false negative leads are also lost, usually irrevocably. Who goes back to test a lead compound that was ineffective?

WHAT LEVEL OF IMPURITIES CAN BE TOLERATED IN A PEPTIDE BEFORE IT LOSES ITS PHARMACOLOGICAL RELEVANCE?

This question cannot be answered easily because it depends on the pharmacological properties of the impurities. However, it is known that minor changes to the sequence of a peptide may affect how it interacts with receptor systems or with proteases. Probably the best known examples are analogs of GnRH, where single amino acid substitutions can increase biological activity by an order of magnitude or more compared to the parent molecule, or even confer antagonist properties. The only sound advice is to invest in as pure a peptide as possible at any stage of development.
In this context, one should not lose sight of the fact that increasing the purity, say from 95 per cent to 97 per cent, is not just a 2 per cent increase in purity. It is also a 40 per cent reduction in potentially interfering impurities. Higher purity has implications for the cost of manufacturing. For small-scale (milligrams to grams) applications, the manufacturer will usually aim high on the crude peptide quantity and then just "cut" fractions with the required quantity and purity on a preparative column. In order to take a 90 per cent pure peptide to 97 per cent purity by simply "cutting" (no recycling of side-fractions), a substantial proportion of the peptide (possibly 25 per cent or more) must be discarded. So, clearly the manufacturer, who shows that the purity of a partially purified peptide is lower, will have to use more materials, solvents and time to fulfill an order for the same quantity of peptide as the manufacturer whose analytical methods are less discriminatory. Some of the best things in life may be free, but high quality peptides certainly do not fall into that category. You do, by and large, get what you pay for. However, it is important to note that the cost of the peptide, however expensive it may seem, is usually a very small proportion of the total investment that may be made in a project before it succeeds or fails. The extra cost of testing the peptide, or using an established manufacturer who provides analytical development, is usually insignificant.

OTHER PURITY ISSUES

Although the failure to effectively monitor HPLC purity is the error that is most likely to result in a misinterpretation of the pharmacological profile and in a deficient manufacturing process, it is certainly not the only quality issue that affects peptides (4). Cross-contamination with solvents, buffer salts, endotoxins and other agents is an uncommon, but serious issue; cross-contamination with even trace quantities of other peptides may lead to serious misinterpretation of data. In a GMP environment, where most peptides are supplied as acetate salts, TFA and HF (if Boc-synthesis is used) counterions are often not completely removed. Not all issues are related to the manufacturing process. Unintentional mishandling of peptides leads to many additional issues, of which the failure to anticipate the tendency of peptides to adsorb or lose moisture is the most common.

SUMMARY

A basic principle of scientific experimentation is that great care must be taken to minimize errors, particularly systematic errors, at the start of the project. Although seemingly small and irrelevant at the start, they will be propagated through each step of the experiment to have largely amplified consequences for the final results. The use of analytical methods, that fail to detect peptide impurities, is a systematic error. Applying sound scientific principles to the control of peptide purity can greatly reduce the risk of the failure of new therapeutic leads.

ACKNOWLEDGEMENTS

We would like to thank Dr. Rainer Gast for his contribution on the use of the KPF_6 buffer system, Dr. Aleksander Swietlow for his comments and contribution to the content of the text, and Yeeling Loh for her help with the chromatograms.

REFERENCES AND NOTES

1. The goal of obtaining an HPLC purity of 97 per cent on the first manufacturing run may not always be advisable or possible. For instance, multicyclic peptides and peptides containing free cysteines, can be particularly difficult to isolate in high purity. For toxicological studies, higher levels of impurities are also an advantage, as long as they can be removed at later stages of development.
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