Aspects of industrial purification of peptides using large-scale chromatography

By

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Introduction

Recent advances in peptide synthesis technology allow manufacturing of complex peptides on a very large scale [1]. Significant advances have been achieved in the fields of synthesis [2] and purification of peptides [3].

For purification of peptides, it is often difficult to use methods similar to those applied in the purification of other organic compounds, mainly due to their complexity. Purification of organic molecules often uses methods based on crystallization to isolate the desired molecule. As efficiency and high yields are of vital importance for optimal economy of any industrial manufacturing process, methods other than those based on crystallization have been explored for purification of peptides and peptide like molecules. These methods usually utilize various principles of chromatography such as ion exchange chromatography, gel permeation chromatography and medium- or high-pressure reversed phase chromatography. The examples mentioned are the most commonly used in peptide purification today but other methods have been used in the past, most noteworthy being counter current distribution [4] and partition chromatography [5].

In this communication we discuss various aspects of large-scale chromatographic purification of peptides. The discussion is based on PolyPeptide Laboratories´ experience from development work with new purification processes and the manufacture of a number of peptide-based drug substances.

Synthesis-related impurities

The ultimate goal for any purification process is to obtain a preparation that meets the quality requirements set for the compound to be purified. In the manufacture of active pharmaceutical ingredients, it is recommended that no single unknown impurity is more than 0.1% (e.g. as determined by high performance liquid chromatography, HPLC, and given as relative area percent) in the final substance [6]. To this end it is valuable if the nature of potential and actual impurities is known prior to the design and development of the purification procedure.

In peptide synthesis, the chemistry is well known and many different side reactions have been reported and described in the literature [7]. Examples of impurities that may be generated are diastereomers, hydrolysis products of labile amide bonds, deletion sequences formed predominantly in solid-phase peptide synthesis and insertion peptides and by-products formed during removal of protection groups in the final step of the synthesis. Polymeric forms of the

desired peptide are also known. These are often by-products associated with formation of cyclic peptides containing disulphide bonds.

The challenge faced when we are to develop a process employing chromatography as the purification principle is to isolate the desired peptide in the mother liquor from a complex mixture of related impurities such as those discussed above. In this context, it deserves mentioning that all of the impurities referred to here probably could not be removed by a single chromatographic method, but rather by a combination of methods.

Purification strategy

The purification process should be as simple as possible and contain a minimum of steps. We have found that a combination of at least two complementary methods operating via different chromatographic principles, such as ion exchange chromatography and/or gel permeation chromatography and reversed phase chromatography result in powerful purification processes.

In a typical purification procedure the crude synthetic peptide mixture is first subjected to a "capturing" step in which the bulk of the impurities is removed. This can be achieved, for example, by applying the crude peptide solution to an ion exchange column. A fraction highly enriched in the desired peptide is obtained after this initial purification removing for instance those by-products generated in the final deprotection of the peptide; impurities generated in this step are usually low molecular weight and uncharged structures.

If higher purity is needed, a "polishing" step is introduced by applying the peptide solution to a column packed with reversed phase resin. Additional purification by chromatography on other resins usually is not needed after this step. Other successful combinations of purification methods are exemplified by ion exchange chromatography followed by gel permeation chromatography and by reversed phase chromatography followed by an intermediary ion exchange step and final polishing on a column packed with reversed phase resin.

Chromatography media

Reversed phase chromatography (RPC). The most powerful method for peptide purification is without doubt reversed phase chromatography utilizing hydrophobic interactions as the main separation principle. It is characterized by the use of a stationary phase and an aqueous mobile phase containing an organic solvent such as acetonitrile or an alcohol. Various chromatography media have been used for large-scale purifications of peptides on reversed phase resins. Among the most popular are those based on C-4, C-8 and C-18 alkyl chains attached to a silica surface [8]. Phases based on synthetic polymers are also used and have recently received increased attention due to the chemical stability of these materials [9].

For industrial scale purifications on reversed phase resin columns, important considerations are shape and size of the particles of the stationary phase. Columns packed with spherical particles are preferred to those packed with irregularly shaped particles; the latter is highly likely to result in clogging of frits. Such a risk is imminent on extensive use of columns and when the column is operated under dynamic axial compression. Particle size is another important characteristic of bonded silica phases that strongly influences column efficiency.

For large-scale applications, a particle size of 10-16 microns normally yields satisfactory separations on reversed phase columns.

Ion exchange chromatography (IEC). In this technique separation is dependent on the ionic interaction between the support surface and charged groups of the peptide. Both cation and anion exchangers have been used with success for peptide purifications. For large-scale purifications, high flow-rates and efficiency are desirable characteristics. Such high performance ion exchangers of different chemical compositions are now commercially available. Examples of such materials are primarily based on agarose [10]. Synthetic polymers withstanding high concentrations of acid and base have also been reported [10, 11].

High mechanical strength is a desirable property of the ion exchange material as it allows large-scale purifications in columns under dynamic axial compression. Mechanical strength is often associated with a hydrophobic character of the stationary phase. This may lead to poor recoveries and may also have a negative impact on the separation efficiency. However, a possibility to circumvent this problem is to use an organic modifier in the mobile phase.

Gel permeation chromatography (GPC). This method separates molecules primarily on the basis of size exclusion. The technique is highly efficient for separation of polymeric forms of peptides and for desalting of peptide solutions. Sephadex ® is a well-known example of a commercially available gel permeation material and has been used successfully in purifications of various molecules [12]. Disadvantages with gel permeation chromatography are the low capacity and the relatively low flow-rates that can be applied for optimal separation on such columns.

Equipment

A system for large-scale chromatography of peptides may consist of the following subsystems and units.

- Column
- Detector
- Buffer preparation system
- Solvent delivery system
- Fractionation system
- Data collection system

As the column can be regarded as the heart of the chromatographic system, proper choice of column is of outmost importance for a successful large-scale purification. Aspects of column material (glass versus steel) and mode of compression (static versus dynamic) of the column are crucial factors worth considering in the design of a chromatography system for industrial-scale purification of peptides. Efficient column packing is another critical factor. Therefore it is necessary to use sensitive test methods for determination of theoretical plates and symmetry of the packed column.

As industrial purifications of active pharmaceutical ingredients are performed under strict regulations such as cGMP (current Good Manufacturing Practice), sanitary aspects must be given special attention in the design of the individual components of the purification system.

Careful documentation of the process is another requirement expected by the regulatory authorities. To this end, it is also desirable to collect data of chromatographic process parameters, such as flow rate, pressure, conductivity and pH during the purification and if possible measured directly in line. This is particularly desirable for the elution step. In ion exchange chromatography such data may be obtained directly by measuring pH and conductivity in line and in reversed phase chromatography by measuring the concentration of the organic modifier in the eluate by employing a near infrared detector (NIR).

The elution step

In reversed phase chromatography, elution is effected by an organic modifier, either applied isocratically or as a gradient, or a combination of both.

In large-scale reversed phase chromatography, the organic modifier should be selected on the basis of criteria other than those applied when considering small-scale chromatography. In addition to separation efficiency, aspects such as economy of the process and environmental and toxicological impacts of the modifier should be considered in the design of the chromatographic process. From this point of view, organic modifiers frequently used in small-scale reversed phase systems, such as acetonitrile, methanol, isopropanol and others, should be avoided for industrial-scale purifications. For large-scale reversed phase chromatography we have found that ethanol is an excellent substitute both with respect to the above discussion and as a proven efficient eluent.

Ion exchange chromatography utilizes both isocratic and gradient elution. The eluent is often a volatile salt, for example ammonium acetate, which can be removed by lyophilization or by subjecting the solution to reversed osmosis or solid phase extraction.

Solutions of aqueous acetic acid are the most frequently used eluents for large-scale applications of gel permeation chromatography.

Related to the above discussion is the introduction of the counter ion. This is normally introduced as an ion in the elution buffer, for example, acetate in solutions containing organic modifier and acetic acid. It can also be introduced via a separate desalting step on a reversed phase column. This is achieved by washing adsorbed peptide with a high concentration of the desired counter ion followed by actual elution of the peptide from the column.

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Isolation of the purified peptide

By far the most common method for isolation of peptides is lyophilization. Promising new alternatives, which probably are technologies more readily scaled-up than lyophilization, are precipitation and spray drying.

Prior to lyophilization there is often a need to concentrate the peptide solution from the last purification step. Normally, concentration of a solution containing the peptide is carried out

under reduced pressure. As big eluent volumes is a problem associated with large-scale processes, there is a need for high capacity alternatives to reduce collected volumes of solvent. Such an alternative is reversed osmosis (RO) since this technique provides both a mild and scalable method for removal of low molecular weight salts and organic solvents, and is a very rapid means for concentration of the peptide solutions. If reversed osmosis is used in combination with diafiltration, it is possible to produce peptide solutions with a predetermined concentration of the counter ion.

Using this strategy for isolation of the purified peptide, we have lyophilized peptide solutions containing up to 100 g of peptide per litre.

It should also be added that the isolation step described above not only provides the desired peptide in the form of a solid but also assists in the control of quality attributes such as content of water, counter ion and residual solvents.

Example

The following example will illustrate the strategy discussed above for large-scale purification of synthetic peptides. It will also demonstrate the advantage obtained by combining different chromatographic methods resulting in a powerful purification process. An overview of the purification procedure developed is shown in Scheme 1.

In the example, a crude peptide is purified in a two-step chromatographic procedure employing ion exchange chromatography and reversed phase chromatography. The crude peptide solution contains three critical impurities, depicted Impurity 1, Impurity 2 and Impurity 3 (Figure 1). Due to their similar migration through the analytical HPLC column, it is suggested that the three impurities have hydrophobicities similar to the mother peptide.

The content of desired peptide in the crude mixture is 0.5 g per litre and the purity about 74% (according to HPLC analysis). This solution is applied to an ion exchange column by loading 8 g of peptide per litre of stationary phase. After washing the column, adsorbed peptide is eluted by a combination of isocratic and gradient elution using ammonium acetate as the eluent. Figure 2 shows the pool obtained from the ion exchange purification step indicating that the level of the critical impurities has been reduced significantly. In the capturing step, the purity of the peptide increased to about 96%. The ion exchange step is performed on a 45-litre column (diameter 45 cm) and the total process time is 6 hours.

The pool from the ion exchange step is applied directly, without any additional treatment of the solution, to a column packed with silica based reversed phase chromatography media. The concentration of the desired peptide in the application solution is 1.5 g per litre and the load on the reversed phase column is 35 g of peptide per kg of the stationary phase. Elution of the adsorbed peptide is effected by a gradient of ethanol in the presence of acetic acid. Total process time is 5 hours and the chromatography step is performed on a 20 cm diameter reversed phase column packed with 8 kg of chromatography media. The HPLC purity of the peptide obtained after the polishing step on the reversed phase column is more than 99,5%. In this final purification step, the critical impurities have each been reduced to levels not exceeding 0.11% (Figure 3). All other impurities are present at levels below 0.05%.

The entire purification is summarized in Table 1. It shows that the overall molar yield after both chromatography steps is higher than 80%. It is worth pointing out that the chromatography is run without reprocessing of any side fractions. It should also be added that the successful purification of this peptide is the result of the combined approach using two different chromatography resins to obtain the desired purity of the peptide. It is highly unlikely that it would be possible to obtain the same efficient separation using purification on single columns only, neither on the ion exchange column alone nor on the reversed phase column alone. We believe that this is especially true for the separation of Impurity 2, which is structurally very similar to the mother peptide.

The peptide is finally isolated by lyophilization following concentration of the last peptide pool by reversed osmosis affording the active pharmaceutical ingredient as the acetate salt. The batch size is approximately 0.5 kg.

GMP considerations

The ultimate manufacturing goal for any peptide pharmaceutical is a process that combines good economy with efficiency and that meets the requirements of the regulatory authorities. The latter demand for example that chemical and analytical procedures are well documented and that test methods and specifications for raw materials, intermediates and finished drug substance are established in advance in order to guarantee that the manufacturing process is reproducible and under control.

As the purification and isolation of the drug substance are late process steps and as they remove impurities with an impact on the quality of the final substance, GMP requirements for the purification step are rigorous. For example, it is essential to identify critical steps and parameters of the purification and to determine limits for the parameters identified. The process is considered to be under control when it is validated indicating that it is reproducible within predetermined limits of critical process parameters. With reference to the purification step, the following parameters may be considered critical for the quality of the drug substance and for the yield of the manufacturing process.

- Column loading
- Flow rate
- Composition of elution buffer
- Column performance, i.e. plate height and asymmetry factor
- Column cleaning procedures
- In process storage time
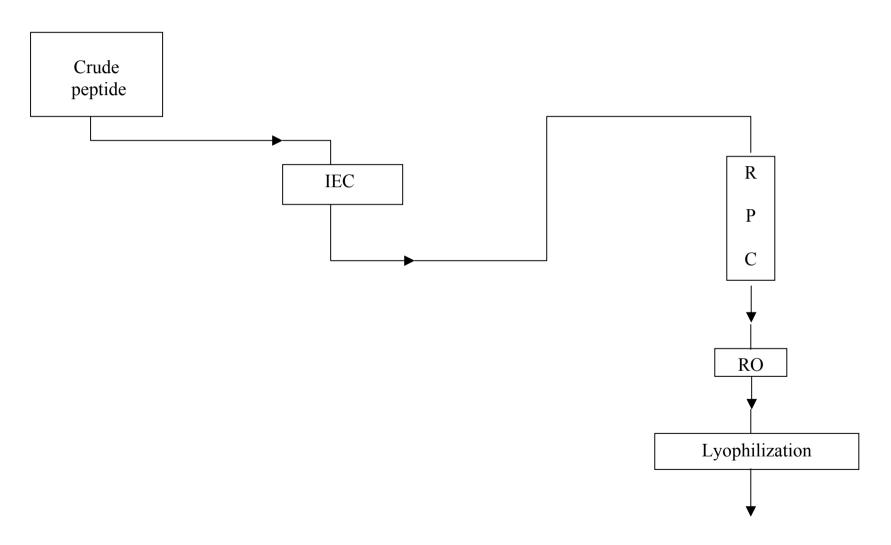- Pooling of fractions

Summary and outlook

Various methods based on chromatography for large-scale purification of peptides have been discussed. Special attention has been paid to ion exchange chromatography and reversed phase chromatography. It has been demonstrated that a highly efficient industrial purification process could be obtained by combining the two methods. Also, methods of isolation of the purified peptide such as prior concentration of the peptide solution by reversed osmosis and lyophilization to yield the desired substance as a solid have been discussed. In relation to the

manufacturing process, GMP requirements with special reference to the purification step have been mentioned. It should finally be added that due to recent advances in the area of drug delivery there has been an increased interest in peptides for therapeutic applications. This development will probably lead to an increased demand for industrial production of peptides in the future and is likely to provide considerable manufacturing challenges both in the area of synthesis and purification of peptides.

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Scheme 1. Overview of the purification process described in the Example using a combination of ion exchange chromatography and reversed phase chromatography

Crude peptide → IEC → RPC → RO → Lyophilization → Active pharmaceutical ingredient
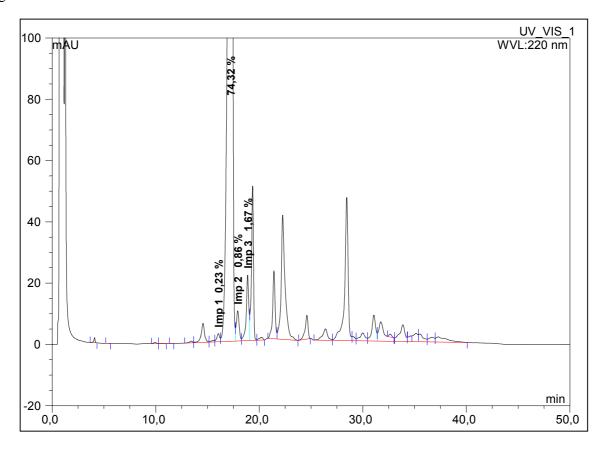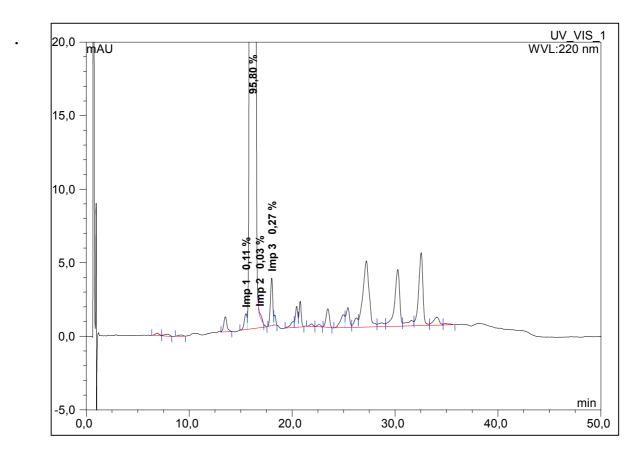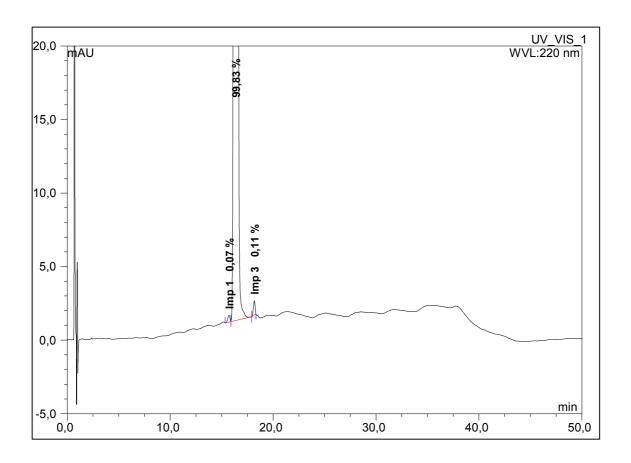
Figure 1

Figure 2

Figure 3

Legends

Figure 1: HPLC chromatogram of crude peptide solution.
Imp 1, Imp 2 and Imp 3 denote critical impurities. Details of the purification is given in the text under Example.

Figure 2: HPLC chromatogram of IEC pool. Further information as for Figure 1.

Figure 3: HPLC chromatogram of RPC pool. Further information as for Figure 1.

Table 1. Summary of data of the peptide purification discussed in the Example

Purification step	Yield (%)	HPLC purity (area %)	Impurity 1[1] (area %)[2]	Impurity 2[1] (area %)[2]	Impurity 3[1] (area %)[2]
Crude peptide	100	74	0.23	0.86	1.67
Ion exchange chromatography	~90	96	0.11	0.03	0.27
Reversed phase chromatography	~90	> 99.5	0.08	n.d[3]	0.11

[1] Denotes impurity in Figures 1-3
[2] According to HPLC analysis
[3] n.d. = not detectable