

Guide

for the elaboration of monographs
on **synthetic peptides** and
recombinant DNA proteins

European Pharmacopoeia

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1 **3. RECOMBINANT DNA PROTEINS**

2 **3.1. DEFINITION**

3 The Definition section states:

- 4 — monomer formula, except for glycoproteins;
- 5 — molecular mass (approximate figure for glycoproteins);
- 6 — physical form;
- 7 — amino-acid sequence of the protein chain and glycosylation sites, where appropriate;
- 8 — identity and biological activity of the substance and its naturally occurring analogue;
- 9 — specific biological activity (expressed in activity units per mass of protein).

10 **3.2. PRODUCTION**

11 The Production section states:

- 12 — appropriate details on the production method;
- 13 — specific biological activity if not covered by assay in the monograph;
- 14 — procedures designed to minimise or eliminate agents of infection;
- 15 — use of stabilisers and auxiliary substances;
- 16 — procedures for validation of the production process as described in the monograph
- 17 *Products of recombinant DNA technology (0784).*

18 **3.3. IDENTIFICATION**

19 **3.3.1. General considerations**

20 The identification tests should be specific for the product and should be based on unique
21 aspects of its molecular structure and/or other specific properties. More than 1 test (physico-
22 chemical, biological and/or immunochemical) is considered mandatory for the establishment
23 of identity; however, methods used in the determination of potency or purity may also be
24 employed or modified as appropriate to serve as identification criteria.

25 Whilst the precise range of tests that comprise the identification criteria depends on the nature
26 of the product and cannot be specified in advance, it can be considered that the Identification
27 section of a monograph generally comprises techniques that verify the size of the molecule, its
28 primary sequence, its isoelectric profile, its chromatographic properties and that the molecule
29 has adopted the correct functional configuration. Typically this battery of tests comprises:

- 30 — bioassay;
- 31 — peptide mapping;
- 32 — reversed-phase liquid chromatography (LC);

- 1 — size-exclusion LC;
- 2 — isoelectric focusing and/or capillary electrophoresis;
- 3 — for glycoproteins, glycan analysis.

4 Where peptide mapping provides appropriate replacement, *N*-terminal sequence analysis may
5 not be required.

6 **3.3.2. Bioassay**

7 Generally, for products of recombinant DNA technology, cross-reference to a biological assay
8 is an important identification criterion. It may be replaced entirely by physico-chemical tests
9 only in cases where:

- 10 — sufficient physico-chemical information about the drug, including higher-order
11 structure, can be thoroughly established by such physico-chemical methods; relevant
12 correlation to biological activity is established;
- 13 — biological activity of related proteins present in the product is known;
- 14 — there exists a well-documented manufacturing history.

15 **3.3.3. Peptide mapping**

16 Peptide mapping is considered to provide direct evidence of the sequence, and at present is
17 usually considered essential. There may be a move towards refinement of peptide mapping
18 technology by the use of LC/mass spectrometry.

19 **3.3.4. Liquid chromatography**

20 Cross-reference to 1 or more LC procedures (typically reversed-phase and size-exclusion LC)
21 is often used as an identification criterion. In instances where the nature of the protein renders
22 it not amenable to fractionation by these methods (e.g. for very large proteins, or for
23 heterogeneous glycoproteins), electrophoretic or ion-exchange chromatographic techniques
24 should be considered as replacements.

25 **3.3.5. Other tests**

26 Additional methods that may be employed as identification tests include:

- 27 — electrophoretic methods based on size or charge;
- 28 — antibody-binding methods, either alone or in conjunction with electrophoretic
29 methods (Western blotting);
- 30 — spectroscopic methods, including mass spectrometry;
- 31 — glycan analysis techniques, for glycosylated proteins.

1 3.4. TESTS

2 3.4.1. Purity tests

3 Analytical methods for proteins are usually developed on the basis of size, charge and
4 hydrophobicity (e.g. size-exclusion LC, ion-exchange LC, reversed-phase LC, SDS-PAGE,
5 isoelectric focusing and capillary electrophoresis). In elaboration of monographs it is usually
6 possible to remove one by demonstrating redundancy (e.g. ion-exchange LC can be covered
7 by reversed-phase LC or capillary electrophoresis). Additional tests such as sialic acid
8 determination or, where appropriate, other glycan analysis techniques are included if known
9 to be necessary for specific impurities, or if the combination of reversed-phase LC and size-
10 exclusion LC does not cover all relevant impurities. When SDS-PAGE is prescribed, the
11 conditions to be used should be those described in general chapter 2.2.31. *Electrophoresis*,
12 unless it is demonstrated that these conditions are not appropriate for the product to be tested.

13 Where specific impurities are known to have clinical consequences or to reflect good
14 manufacturing practice, methods for their detection should be transparent and appropriate
15 system validation reference materials or procedures provided to support the monograph.

16 Size-exclusion LC for the determination of dimers and higher-molecular-mass impurities
17 remains an important purity test as aggregated molecules generally exhibit immunogenicity.
18 Where possible, this test should be elaborated using non-denaturing conditions (neutral,
19 aqueous buffers), to avoid dissociation and consequent non-detection of non-covalent
20 aggregates.

21 3.4.2. Tests for bacterial endotoxins/pyrogens

22 The monograph contains the test for bacterial endotoxins (2.6.14) or a monocyte-activation
23 test (2.6.30).

24 3.5. ASSAY

25 Typically, the specific activity of the drug substance is stated in International Units per
26 milligram of protein. The Assay section of the monograph therefore comprises 2 procedures:
27 determination of protein content, usually by a comparative LC procedure against a defined
28 chemical reference substance; and a bioassay in terms of the International Standard. Bioassay
29 limits are calculated as specified in general chapter 5.3. *Statistical analysis of results of*
30 *biological assays and tests* and are typically expressed as an acceptable range for the
31 estimated potency (e.g. 80-125 per cent of the stated potency), and an acceptable range for the
32 confidence limits of the estimated potency (e.g. 64-156 per cent of the stated potency).

33 In exceptional circumstances, different strategies may be employed:

34 — where the battery of physico-chemical tests does not adequately characterise
35 structural aspects of the molecule known to affect the biological activity *in vivo*, for

1 example glycosylation, the monograph may include an *in vivo* bioassay; where an *in*
2 *vivo* assay is used to assess the extent of glycosylation, this should only be replaced
3 with an *in vitro* assay where it has been shown that the physico-chemical analysis
4 adequately addresses the glycosylation pattern;

- 5 — where the battery of physico-chemical tests has been shown to characterise
6 adequately the molecule, according to the criteria outlined in section 3.3.2 of this
7 guide, a physico-chemical assay alone may be employed; where a physico-chemical
8 assay procedure is used, the limits are typically asymmetric, the upper limit being
9 100 per cent + the permitted assay repeatability (typically up to 5.0 per cent for
10 reversed-phase LC), and the lower limit being 100 per cent – (the permitted assay
11 repeatability + the maximum permitted level of impurity).

12 **3.6. LABELLING AND STORAGE**

13 These sections give information on labelling requirements and storage conditions. Other
14 parameters are determined on a case-by-case basis.

15 **4. SYNTHETIC PEPTIDES**

16 **4.1. DEFINITION**

17 The Definition section states:

- 18 — elemental formula;
- 19 — molecular mass;
- 20 — physical form;
- 21 — structural formula;
- 22 — identity and biological activity of the substance and, where appropriate, its naturally
23 occurring analogue;
- 24 — assay specifications;
- 25 — route of production;
- 26 — salt form;
- 27 — any chemical modifications, such as esterification or amidation.

28 **4.2 CHARACTERS**

29 The appearance of the solid synthetic peptide should be described. The solubility, if
30 applicable, should be provided.

1 **4.3. IDENTIFICATION**

2 **4.3.1. General considerations**

3 Synthetic peptides may be considered to differ from products of recombinant DNA
4 technology in 2 aspects that affect the structure of the monograph:

- 5 — they are usually small, typically below 5000 Da;
- 6 — they may have chemical structures that do not occur naturally in proteins or peptides.

7 These features have 2 consequences in elaborating monographs:

- 8 — generally the battery of physico-chemical tests is sufficient to characterise the
9 product without the use of a bioassay;
- 10 — as stated in the monograph *Substances for pharmaceutical use (2034)* and in the
11 General Notices, alternative sets of tests may be used for identification; users may
12 therefore perform either amino-acid analysis or nuclear magnetic resonance (NMR)
13 spectrometry in addition to the other identification tests. Identification by NMR
14 spectrometry applies to peptides comprising up to approximately 15 amino acids.
15 Where appropriate, for instance where the peptide comprises unnatural amino acids,
16 or when prescribed in individual monographs, the use of NMR spectrometry may
17 also be required in addition to conventional methods based on analysis of the amino-
18 acid composition or sequence.

19 Typically, the Identification section of the monograph comprises:

- 20 — amino-acid analysis and/or NMR spectrometry;
- 21 — reversed-phase LC.

22 In many cases it may be appropriate to include more than one LC. The inclusion of multiple
23 chromatographic identification tests is particularly important if a spectroscopic method is not
24 included.

25 Where NMR spectrometry is prescribed for identification, a separate reference standard for
26 NMR identification is established from a bulk of the substance; no assigned content is
27 necessary.

28 **4.4. TESTS**

29 **4.4.1. Related peptides**

30 Typically, monographs for synthetic peptides contain a reversed-phase LC test for related
31 peptides. Such tests are validated for specified impurities known to be potential contaminants
32 and are transparent with respect to these impurities.

33 The provisions for synthetic peptides in the general monograph *Substances for*
34 *pharmaceutical use (2034)* apply to all synthetic peptides unless otherwise stated.
35 Monographs should include acceptance criteria for:

- 1 — each specified impurity;
- 2 — unspecified impurities, normally set at the identification threshold;
- 3 — total impurities.

4 Reference standards for specified impurities or, where appropriate, the reference peptide
5 spiked with the specified impurities are provided. Where necessary, specified impurities may
6 have to be separately quantified using independent methods. Where a monograph depends on
7 a single purity test, the capacity of the method to measure all relevant impurities should be
8 demonstrated.

9 **4.4.2. Optical rotation and absorbance**

10 These useful tests should be retained where appropriate. Chiral chromatography may offer
11 some potential to replace optical rotation.

12 **4.4.3. Acetic acid, loss on drying, water content**

13 Determination of acetic acid and water are general requirements for peptides. As a general
14 rule, the salt form of the peptide is not specified in the title of the monograph, and acetate
15 remains as a test. The method used for the determination of acetic acid is that described in
16 general chapter 2.5.34. *Acetic acid in synthetic peptides*, unless it has been demonstrated that
17 this method is not appropriate for the peptide to be tested.

18 Loss on drying is used less frequently because it requires large quantities of material. It is
19 often replaced by a water-content determination. The method for determining water content is
20 that described in general chapter 2.5.12. *Water: semi-micro determination* (Karl Fischer) or in
21 general chapter 2.5.32. *Water: micro determination* (coulometric titration).

22 **4.4.4. Tests for bacterial endotoxins/pyrogens**

23 The monograph contains the test for bacterial endotoxins (2.6.14) or a monocyte-activation
24 test (2.6.30).

25 **4.5. ASSAY**

26 Assays for synthetic peptides are generally comparative chromatographic procedures,
27 performed using a defined chemical reference substance as the standard. Results are normally
28 expressed in terms of the acetic acid-free, anhydrous substance. The permitted limits for such
29 tests are typically asymmetric, the upper limit being 100 per cent + the permitted assay
30 repeatability (usually ± 2.0 per cent), and the lower limit being 100 per cent – (the permitted
31 assay repeatability + the maximum permitted level of impurity).