

Technical Guide

for the Elaboration of Monographs
on Synthetic Peptides and
Recombinant DNA Proteins

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1 **TECHNICAL GUIDE FOR THE ELABORATION OF**
2 **MONOGRAPHS ON SYNTHETIC PEPTIDES AND RECOMBINANT DNA**
3 **PROTEINS**

4 **1. SCOPE**

5 This guide covers the elaboration of monographs on synthetic peptides and recombinant DNA
6 proteins. It does not cover the field of blood products or vaccines.

7 **2. ANALYTICAL METHODS: GENERAL PRINCIPLES**

8 **2.1. VALIDATION OF ANALYTICAL PROCEDURES**

9 Analytical methods included in monographs must have been validated according to the
10 principles set out in Chapter 3 of the Technical guide for the elaboration of monographs and
11 to those in ICH guidelines Q2A '*Validation of analytical procedures: definitions and*
12 *terminology*' and Q2B '*Validation of analytical procedures: methodology*', taking into
13 account specific issues concerning the unique tests used for analysing
14 biotechnological/biological products.

15 **2.2 REFERENCE STANDARDS**

16 Biological assays are calibrated against WHO international standards, against Ph. Eur.
17 standards or against in-house standards calibrated in terms of the primary WHO standard. If a
18 WHO or a Ph. Eur. standard has not been established, the manufacturer must have established
19 an appropriately characterised in-house biological reference material.

20 Physico-chemical analytical methods are calibrated against pharmacopoeial chemical
21 reference substances for the active drug substance and, where appropriate, for specified
22 impurities.

23 **Chemical reference substances: assignment of content**

24 The procedures for assigning the content of the chemical reference substance (milligrams of
25 peptide or protein per milligram of substance or per vial) vary significantly for the two
26 classes of product to be considered. In the case of reference substances for synthetic peptides,
27 the content is usually assigned on the basis of total material minus the combined value for
28 loss on drying (or water content), acetate (or any other ion) and related peptides.

29 For proteins, this approach is not applicable, and absolute determination must be undertaken.
30 Appropriate methods include amino-acid analysis and nitrogen determination.

1 **3. RECOMBINANT DNA PROTEINS**

2 **3.1 DEFINITION**

3 The definition section states:

4 — Monomer formula

5 — Molecular mass

6 — Physical form

7 — Amino acid sequence of the protein chain

8 — Identity and biological activity of the substance and its naturally occurring analogue

9 — Specific biological activity

10 — Route of production

11 **3.2 PRODUCTION**

12 The production section states:

13 — Appropriate details on the production method

14 — Specific biological activity if not covered by assay in the monograph

15 — Procedures designed to minimise or eliminate agents of infection

16 — Use of stabilisers and auxiliary substances

17 — Procedures for validation of the production process as described in the monograph on
18 *Products of recombinant DNA technology (0784)*.

19 **3.3 IDENTITY**

20 **3.3.1 General considerations**

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

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

32 — Bioassay

- 1 — Peptide mapping
- 2 — *N*-terminal sequence analysis
- 3 — *C*-terminal sequence analysis
- 4 — Reverse-phase liquid chromatography (LC)
- 5 — Size-exclusion LC
- 6 — Isoelectric focusing and/or capillary electrophoresis
- 7 — For glycoproteins, sugar side chain analysis

8 **3.3.2 Bioassay**

9 Generally, for products of recombinant DNA technology, cross-reference to a biological
10 assay is an important criterion of identity. The biological assay as a criterion of identity may
11 be replaced entirely by physico-chemical tests only in cases where:

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

17 **3.3.3 Peptide mapping**

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

21 **3.3.4 Liquid chromatography**

22 Cross-reference to one or more LC procedures (typically reverse-phase and size-exclusion
23 LC) is generally considered criteria of identity. In instances where the nature of the protein
24 renders it not amenable to fractionation by these methods (e.g. for very large proteins, or for
25 heterogeneous glycoproteins), electrophoretic or ion-exchange chromatographic techniques
26 should be considered as replacements.

27 **3.3.5 Other tests**

28 Additional methods that may be employed as tests of identity include:

- 29 — Electrophoretic methods based on size or charge
- 30 — Antibody-binding methods, either alone or in conjunction with electrophoretic methods
31 (Western blotting)
- 32 — Spectroscopic methods, including mass spectrometry

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, reverse-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 reverse-phase LC or capillary electrophoresis). Additional tests such as sialic acid
8 determination are included if known to be necessary for specific impurities, or if the
9 combination of reverse-phase LC and size-exclusion LC does not cover all relevant
10 impurities. When SDS-PAGE is prescribed, the conditions to be used should be those
11 described in chapter 2.2.31. *Electrophoresis* of the Ph. Eur., unless it is demonstrated that
12 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-weight 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 Typically, the monograph contains the test for bacterial endotoxins. In exceptional
23 circumstances, where the nature of the product renders the test for bacterial endotoxins
24 inapplicable or inappropriate, the rabbit pyrogen test may be used.

25 **3.5 ASSAY**

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

35 In exceptional circumstances, different strategies may be employed:

36 — Where the battery of physico-chemical tests does not adequately characterise structural

1 aspects of the molecule known to affect the biological activity *in vivo*, for example
2 glycosylation, the monograph may include an *in vivo* bioassay. Where an *in vivo* assay is
3 used to assess the extent of glycosylation, this should only be replaced with an *in vitro*
4 assay where it has been shown that the physicochemical analysis adequately addresses the
5 glycosylation pattern.

6 — Where the battery of physico-chemical tests has been shown to adequately characterise
7 the molecule, according to the criteria outlined in section 3.3.2, a physico-chemical assay
8 alone may be employed. Where labelling remains in units, the intrinsic specific activity of
9 the drug substance in units per milligram should be stated in the monograph.

10 — Where a physico-chemical assay procedure is used, the limits are typically asymmetric,
11 the upper limit being 100 % + the permitted assay repeatability (typically up to 5.0 % for
12 a reverse-phase method), and the lower limit being 100 % – (the permitted assay
13 repeatability + the maximum permitted level of impurity).

14 **3.6 LABELLING AND STORAGE**

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

17 **4. SYNTHETIC PEPTIDES**

18 **4.1 DEFINITION**

19 The definition section states:

20 — Elemental formula

21 — Molecular mass

22 — Physical form

23 — Structural formula

24 — Identity and biological activity of the substance and, where appropriate, its naturally
25 occurring analogue

26 — Assay specifications

27 — Route of production

28 — Salt form

29 — Any chemical modifications, such as esterification or amidation

30 **4.2 CHARACTERS**

31 The appearance of the solid synthetic peptide should be described. The solubility, if
32 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 two aspects that affect the structure of the monograph:

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

7 These features have two consequences in elaborating monographs:

- 8 – generally the battery of physico-chemical tests is sufficient to characterise the product
9 without the use of a bioassay,
- 10 – the use of spectroscopic methods such as nuclear magnetic resonance may be required in
11 addition to conventional methods based on analysis of the amino-acid composition or
12 sequence.

13 Typically, the identity section of the monograph comprises:

- 14 — Amino-acid analysis
- 15 — Reverse-phase LC
- 16 — Nuclear magnetic resonance spectrometry

17 In many cases it may be appropriate to include more than one LC or an additional
18 chromatographic procedure such as thin-layer chromatography. The inclusion of multiple
19 chromatographic identity tests is particularly important if a spectroscopic method is not
20 included.

21 **4.4 TESTS**

22 **4.4.1 Related peptides**

23 Typically, monographs for synthetic peptides contain a reverse-phase LC test for related
24 peptides. Such tests are validated for specified impurities known to be potential contaminants
25 and, where possible, are transparent with respect to these impurities. Where appropriate,
26 reference substances are provided for specified impurities. Where necessary, specified
27 impurities may have to be separately quantified using independent methods. Where a
28 monograph depends on a single purity test, the capacity of the method to measure all relevant
29 impurities should be demonstrated.

30 **4.4.2 Appearance, optical rotation and absorbance**

31 These useful tests should be retained where appropriate. Chiral-phase chromatography may
32 offer some potential to replace optical rotation.

1 **4.4.3 Acetic acid, loss on drying, water content**

2 Determination of acetic acid and water are general requirements for peptides. As a general
3 rule, the salt form of the peptide is not specified in the title of the monograph, and acetate
4 remains as a test. The method used for the determination of acetic acid is the one described in
5 chapter 2.5.34. *Acetic acid in synthetic peptides*, unless it has been demonstrated that this
6 method is not appropriate for the peptide to be tested. Loss on drying is used less and less
7 because it requires high amounts of material. It is often replaced by a water content
8 determination. The method for determining water content is that described in chapter
9 2.5.12. *Water: semi-micro determination* (Karl Fisher) or that described in chapter
10 2.5.32. *Water: micro determination* (coulometric titration).

11 **4.4.4 Tests for bacterial endotoxins/pyrogens**

12 Typically, the monograph contains the test for bacterial endotoxins. In exceptional
13 circumstances, where the nature of the product renders the test for bacterial endotoxins
14 inapplicable or inappropriate, the rabbit pyrogen test may be used.

15 **4.5 ASSAY**

16 Assays for synthetic peptides are generally comparative chromatographic procedures,
17 performed using a defined chemical reference substance as the standard. Results are normally
18 expressed in terms of the acetic-acid free, anhydrous substance. The permitted limits for such
19 tests are typically asymmetric, the upper limit being 100 % + the permitted assay repeatability
20 (usually ± 2.0 %), and the lower limit being 100 % – (the permitted assay repeatability + the
21 maximum permitted level of impurity).

22 **5. PRODUCTS FROM NATURAL SOURCES**

23 Products from natural sources are a heterogeneous group of substances and it is not possible
24 to apply a single set of criteria in this guideline. The issue of agents of infection does apply,
25 and monographs for these substances need to include appropriate statements.

26