Methodological guidelines for stability studies of hospital pharmaceutical preparations

Part 1: liquid preparations

Under the aegis of SFPC (French Society of Clinical Pharmacy) and GERPAC (Evaluation and Research Group on Protection in Controlled Atmospher)

1st Edition - October 2013

Methodological guidelines for stability studies of hospital pharmaceutical preparations

ISBN : 978-2-9526010-4-7
Printed by : Print Conseil

coordinator

Valérie SAUTOU
Hospital Pharmacist and Assistant Professor in Clinical Pharmacy
Faculty of Pharmacy, Auvergne University
University Hospital of Clermont-Ferrand

authors

Denis BROSSARD
Professor in Pharmaceutical Technology
Faculty of Pharmacy, Paris Descartes University

Valérie Chedru-Legros
Hospital Pharmacist
University Hospital of Caen

Sylvie Crauste-Manciet
Hospital Pharmacist and Assistant Professor in Pharmaceutical Technology
Faculty of Pharmacy, Paris Descartes University
Hospital of Poissy Saint-Germain-en-Laye

Sandrine Fleury-Souverain
Hospital Pharmacist
University Hospital of Genève

Frédéric Lagarce
Hospital Pharmacist and Professor in Biopharmacy and Pharmaceutical Technology
Faculty of Pharmacy, Angers University
University Hospital of Angers

Pascal Odou
Hospital Pharmacist and Professor in Biopharmacy and Pharmaceutical Technology
Faculty of Pharmacy, Lille 2 University
University Hospital of Lille

Sandrine Roy
Hospital Pharmacist
Antoine Beclère Hospital, Paris

Farshid Sadeghipour
Hospital Pharmacist
University Hospital of Lausanne

Valérie Sautou
Hospital Pharmacist and Assistant Professor in Clinical Pharmacy
Faculty of Pharmacy, Auvergne University
University Hospital of Clermont-Ferrand

sommaire

acknowledgments

The authors thank :

Jean Vigneron and Jean-Daniel Hecq for their participation in the first meetings of the study group and their contribution to the achievement of the chapter on validation of stability indicating methods.

Christine Barthélémy for her contribution to the writing of chapter 5.

Daniel Bourdeaux, Françoise Picq and Hélène Confolent for the conception of the sheets for internal qualification of equipment.

editorial

Drug compounding in hospital pharmacies allows the specific needs of hospitalised patients, needs that are not always satisfied by the pharmaceutical industry, to be met. . In recent years, the biggest development in this field has been a move towards a rationalisation of that activity, ensuring the clinical efficiency of these medicinal preparations whilst improving their quality. The rationalised development of that activity in hospital pharmacies should be further enhanced in the future by the implementation of risk analysis approaches designed to eliminate drug-related iatrogenia

In this context, the question of the stability of compounded medications remains a major preoccupation for pharmacists. The present work seeks to answer this concern by adopting an approach that is both didactic and pragmatic. This first opus is the fruit of an extensive collaboration in the form of a joint work group coordinated by Valérie Sautou and bringing together French and Swiss hospital and university hospital practitioners from two learned societies (SFPC and GERPAC), bringing a wealth of experience and skills to bear on the issue of the stability of sterile and non-sterile solutions. Basing their work on international benchmarks, the work group sought to adapt their methodology to focus specifically on the hospital environment, developing a genuinely useful tool for the management of stability studies. To this end, a complementary computer program has been created to offer users concrete assistance with the interpretation of their results. The other great advantage of this work is that it offers a new critical perspective on the existing literature dealing with stability studies.

This work will serve as a valuable tool for drug compounding in hospitals, contributing to the broader objective of optimising quality and making patient care ever safer.

We hope you will use this new preparation without moderation!

Marie-Claude Saux
President of the SFPC

Sylvie Crauste-Manciet
President of GERPAC

introduction

The use of pharmaceutical technologies in hospital environments has developed considerably in recent years, in response to new therapeutic, safety and economic demands. The importance of ensuring that the preparation of high-risk drugs is safe, while also rationalising costs, has seen the emergence of centralised preparation units for chemotherapies in oncology. This approach is currently being pursued further with the centralisation of other costly and/or toxic risk preparations and through the development of dose-banding. Hospital pharmacies are also required to respond to therapeutic needs not covered by the pharmaceutical industry, especially in the case of preparations intended for ophthalmology, paediatrics and allergology, or preparations required for use in biomedical research.

The production of such preparations is not limited to the study of clinical feasibility and the technical considerations inherent to their manufacturing. Pharmacists must ensure that the preparations they produce remain stable and thus retain all of their properties throughout storage time and right up to the point when they are administered to the patient.

For this to happen pharmacists require reliable stability data. They need to be sure that the information provided in the literature, where it exists, is based on firm scientific and methodological bases, and that it can be transposed to other applications without risk. Where the literature has no satisfactory data to offer, the pharmacist may, when availed of the necessary resources, implement a stability study. Currently, the stability studies conducted by industrialists on specialist pharmaceutical preparations rely on the recommendations of the ICH. These guidelines represent an essential methodological base for the development of medications, but they need to be adapted to reflect the realities of clinical practice, particularly in a hospital environment and in the context of outpatient care.
Ce guide constitue un soutien méthodologique pour la réalisation d'études de stabilité des préparations. Ces préparations peuvent être de simples reconstitutions et/ou dilutions de spécialités pharmaceutiques ou bien des préparations plus complexes formulées à partir de spécialités pharmaceutiques ou de matières premières.

The present guide provides further methodological support for the execution of stability studies on medicinal preparations. Such preparations can be simple reconstitutions and/or dilutions of specialised pharmaceutical products, or more complex preparations formulated from pharmaceutical specialities or raw materials.

In the first instance, this guide defines a methodological approach with a description of all the parameters to be taken into consideration within the scope of a stability study. It describes the methods for analysis suited to such studies and their implementation, and addresses how they are to be validated in accordance with specific criteria. As an educational resource, the guide provides tools to help conduct studies (details of operating procedures, validation software, qualification sheets for analysis apparati, etc..) and interpret the results. On the latter point, the decision-making assistance tool included here takes into account all the physico-chemical data obtained as well as clinical and toxicological data.

Beyond the methodological assistance this tool offers for pharmacists running their own stability studies, it also constitutes an aid for the interpretation of the data derived from the scientific literature. Using the methodological elements provided here, pharmacists will be in a position to evaluate the relevance of the stability data provided by scientific articles. They will then be capable firstly of judging as to whether the study's methodology allows the results to be applied in their domain, and secondly of determining whether the elements provided lend themselves to a transposition of the stability data presented to the preparation concerned.

Stability studies of preparations produced in strictly controlled conditions, respecting a pertinent methodology, help further enhance the safety of pharmaceutical production, ensuring that drugs retain all of their essential properties throughout the preparation process and right through to administration.

The stability of pharmaceutical preparations is a crucial aspect of their proper usage. The safety and effectiveness of a treatment can be affected by stability problems. A preparation is considered practically stable when over a given period of time its essential properties do not change, or change only in tolerable proportions.

Instability is primarily caused by chemical reactions occurring spontaneously or being delayed, inducing hydrolysis and oxidation-reduction phenomena. In liquid forms, instability can also result in physical phenomena such as the appearance of turbidity, precipitation, discoloration, viscosity, or - for dispersed systems - phase separation. If the integrity of the preparation's packaging is not maintained, instability of a microbiological nature may also occur.

In this chapter we describe the main instabilities to which preparations in a hospital environment may be subject, and the various parameters that can influence them.

CHAPTER 1

KEY INSTABILITIES: DESCRIPTION AND INFLUENCE FACTORS

1 CHEMICAL INSTABILITY

1.1 HYDROLYSIS

A substance is hydrolysed when it decomposes by the fixation of H+ and OH- ions from water dissociation. The acidity or basicity of a solution may cause significant degradation in the active ingredient. The pH may also influence oxidation, primarily because of the fact that the oxidation-reduction potential of many reactions is pH-dependent.

1.2 OXIDATION REDUCTION

The majority of active ingredient molecules come in reduced form. As a result, the presence of oxygen from the ambient air may cause instability problems. When this reaction is spontaneous, it is often referred to as auto-oxidation. Depending on the molecular structure of the active ingredient, this phenomenon can clearly be amplified by the pH of the medium. This problem is often avoided by replacing air with an inert gas (usually nitrogen) during filling, pH adjustment and stabilization with the use of a buffer, or by the addition of a chelating agent such as EDTA. Antioxidants, provided they do not create other degradation or toxicity problems, can also be a straightforward and practical solution.

1.3 PHOTOLYSIS

Daylight, or, more specifically, UV rays, can act as a catalyst to oxidation and hydrolysis reactions. Photolysis, depending on the molecule, is directly proportional to the intensity and wavelength of the light applied. The simplest solution for protecting a photolysis-risk preparation is to keep it away from light, either with the use of a suitable container, or by means of appropriate secondary packaging.

1.4 RACEMIZATION AND EPIMERIZATION

This phenomenon is limited, but when it does it can have therapeutic implications for chiral molecules, with much higher pharmacological activity for one of the enantiomers. Racemization or epimerization may cause a decrease in activity.

2 PHYSICAL INSTABILITIES AND INCOMPATIBILITIES

2.1 PRECIPITATION RISKS

In solutions, the precipitation of an active ingredient or destabilized excipient can occur at any time, and not just because of saturation problems in the vehicle used. Precipitation is not necessarily immediate, and its kinetic parameters depend on various factors. For example, molecules with poor water-solubility are often solubilized using co-solvents (such as ethanol, propylene glycol, or different polyethylene glycols). The dilution of what was an initially optimized solution may give rise to precipitation. In the case of weak acids or bases, the solubility of the AP is directly dependent on the pH of the solution. To stabilize the pH, the presence of buffers is often essential. Consequently, any changes in their dilution or overruns of their buffering capacity may directly cause precipitation. The formation of insoluble salts with components of another drug or additive during a mixture process may also prove to be problematic. The formation of complexes between high molecular weight anionic organic molecules and cations is another source of physical stability problems. A decrease in the solubility of an organic AP in non-ionic base form in the presence of electrolytes such as sodium, potassium, calcium or chlorides - depending on their concentration - or in the presence of pH changes, may cause precipitation.

2.2 SORPTION RISKS

Pharmaceutical preparations may be subject to contents/container interactions with the constituent materials of their packaging products, via sorption phenomena that may evolve over time during the storage period. These are essentially of 2 types: adsorption and absorption.
Adsorption is a surface phenomenon by which molecules in gas or liquid form may bind to the surface of the pharmaceutical products' primary packaging, filter, or any other part of the secondary material. When these molecules penetrate into the material it is referred to as absorption.
Adsorption is generally the result of functional group interactions between the active ingredient and binding or anchor sites on the surface of the material. Although treating the surface of glass containers may prevent ionic interactions by blocking silanol functions, it cannot eliminate interactions of a hydrophobic nature. Adsorption on polymeric, cellulose or plastic surfaces is also very common. Obviously, the initial concentration of the active ingredient has a crucial effect on the consequences of these sorption phenomena. Binding sites are limited, and consequently if the concentration of active ingredient is low, the relative impact on the available quantity will be more much more significant than if that initial concentration were higher.
Absorption is a phenomenon that evolves more slowly over time, making it much more difficult to control than adsorption. It is particularly common in molecules of a lipophilic nature when they come into contact with materials with a predominantly amorphous structure, such as polyvinyl chloride.

2.3 LEACHING RISKS

The packaging products used to store preparations, and the medical devices used in their administration, are made from materials containing additives that may migrate into the preparation. Some of these additives may present toxic risks to the patient, or may even cause the preparation to become unstable. It is therefore vital that particular care be taken in selecting the packaging used for a preparation. The primary examples are plasticizers leached from polyvinyl chloride (presence in infusion devices, droppers) and silicone oils, which may promote aggregation in active ingredients of proteinaceous origin.

2.4 OTHER INSTABILITIES AND PHYSICAL INCOMPATIBILITIES

Other phenomena are possible as well:
-The chelating and complexing of certain active ingredient molecules in the presence of polyvalent cations may also impede solubility. These complexes can also be formed with other excipient molecules, for example preservative agents normally present in some vehicles, such as simple syrup or even water.
-Color changes are also physical signs of instability, but are generally a result of chemical reactions or of the degradation of one of the components of the preparation.

3 MICROBIOLOGICAL INSTABILITY

Microbiological instability in a preparation implies that microbes have developed inside it during its shelf life. This may be the result of an undetected initial accidental contamination, or of contamination during storage, usually indicating that the integrity of the packaging has been compromised. Some parameters may impact the integrity of the preparation's packaging, rendering it permeable to microbes (temperature conditions, light, humidity, or the physicochemical instability of the preparation). It is therefore important that a physicochemical stability study be completed with a microbiological stability study for sterile preparations.

4 FACTORS INFLUENCING THE STABILITY OF A PREPARATION

4.1 PH

pH plays a significant role in the active ingredient's solubilization and thus in its bioavailability, but at extreme values it may also be responsible for significant degradation in the preparation. The rate of degradation is in fact much higher at extreme values. The optimum pH is often the same as the pH at which a given molecule is most soluble. Buffers are often included in pharmaceutical product formulations, and provide very good stability. However the formulation of preparations using these pharmaceutical products may change their pH and, crucially, their stability. Take for example fortified eye drop preparations incorporating antibiotics, where pH levels may be very low, thus potentially generating instability.

4.2 SURFACTANTS

Surfactants can be used to protect the active ingredient in hydrolytic groups such as hydroxyls, and limit their degradation. The different types of surfactants (anionic, cationic or non-ionic) may however form micelles in solution, thus trapping the active ingredient molecules and changing their bioavailability in solution.

4.3 TEMPERATURE

Temperature is one of the most important factors in drug stability. An increase of 10° C in storage temperature may lead to a 2 to 5-fold increase in the speed of degradation reactions. This is not, however, a generalized phenomenon, since in other cases it would be a drop in temperature that would be detrimental to the physical or chemical stability of the active ingredient. The precipitation of a saturated solution placed in a refrigerator is an example of this. For certain molecules, physicochemical stability is only optimal within a small temperature range, outside of which increased degradation is observed.
For most active ingredients, the kinetics of degradation reactions follows the Arrhenius law. Thus, when performing stability studies at elevated temperatures (at 40° C, for example), it is possible to determine the formulation's stability at ambient temperature. However, systematic extrapolation of expiration dates based on Arrhenius calculations would not be appropriate, and would in many cases give erroneous estimates. Stability studies in real storage conditions still need to be performed.
Depending on the molecule in question, temperature changes may have various effects. For example, peptides may be degraded at low temperatures by hydrolysis and oxidation, and become denatured at higher temperatures.
It is therefore very important that the results of predictive stability studies at different temperatures be interpreted with extreme caution, taking into account the attendant complexities of a variety of scenarios.

4.4 OXYGEN

The presence of oxygen in a preparation may cause instability via the oxidation of one of its components, as mentioned above in the paragraph on oxidation-reduction. Formulation (antioxidants) and manufacturing techniques (under nitrogen) need to be determined accordingly. Selecting an appropriate container and ensuring its integrity are also important elements to consider in order to prevent the infiltration of oxygen over time.

4.5 LIGHT

Light is a parameter that may cause chemical instability in photosensitive molecules. If preventive measures are implemented during manufacturing (selection of appropriate packaging material), it is important to check that they are maintained over time. Proper respect for packaging integrity as well as an appropriate selection of the administration device to be used to deliver the preparation must also be considered.

4.6 MATERIALS

Primary packaging products and their seals are crucial elements in the process, with an impact on the stability of the finished pharmaceutical product. The same applies for medical devices used for the administration of preparations, such as drip sets, risers, filters, etc. The constituent materials in these packaging products or medical devices may be the cause of different types of instabilities and incompatibilities.

- ***GLASS :*** though resistant to physical or chemical changes, its quality must be appropriate to its content. The choice of glass should be based on its hydrolytic resistance. For injectable preparations, type I is preferred.

- ***POLYMERS :*** With polymers and plastics in general, various issues with the substances contained in the materials migrating into the solution, or sorption issues (adsorption/absorption), must be taken into account. Furthermore, plastic's permeability to moisture (water evaporation through the package, and consequently the concentration of medications, with all the potential physicochemical consequences), oxygen (oxidative degradation), and carbon dioxide (decrease in pH and the possibility of physicochemical instability) is a significant concern. Ethylene Vinyl Acetate (EVA), which is commonly used for packaging parenteral nutrition mixtures made by hospital pharmacies, is completely oxygen-permeable.

- ***METALS:*** Several different metal alloys and aluminum are used in the composition of containers for semisolid forms (ointments, creams, etc.). These metals may react with active ingredients or even contaminate medications. It is highly recommended that containers with an internal lining made of a thin layer of polymer be used.

- ***RUBBER:*** the stoppers and seals used on injection vials are subject to the same concerns as containers made of polymers.

FOR MORE INFORMATION

To learn more about the various types of instabilities, the reader is encouraged to consult the paper published by LA Trissel (1990), and the book of JT Carstensen et al. (2000), referenced at the end of this guide.

When a stability study is being set up, a rigorous protocol must be established. In order to ensure that the results of the study will be reliable and replicable, it is important to define the following elements.

CHAPTER2
GENERAL METHODOLOGY

1 FORMULATING THE PREPARATION

As soon as a stability study is planned, the formulation of the preparation and the container selected, both need to be validated. When planning a stability study, it is assumed as a preliminary condition that the available literature has been consulted in order to establish that the formulation contains no known incompatibilities, and that there are no known content/container interactions involved. If no data is available, preliminary studies should be conducted.

1.1 DATA ON THE ACTIVE INGREDIENT

It is recommended that the first step should be to gather the following various elements regarding the active ingredient in the preparation, in particular its nature and origin

- The name of the raw material or pharmaceutical product
- The supplier
- The batch number
- If dealing with a proprietary medicinal product, obtain its exact composition and its pH. Indeed, some of the excipients in the pharmaceutical product may impact the stability of the preparation *(see examples in frame)*. The presence of co-solvents should draw attention to the risk of precipitation during the dilution of pharmaceutical products
- The nature of the active ingredient: It is important to know whether the product is used in base or salt form in order to express the results of the active ingredient assay in a manner consistent with the formulation of the preparation. If the product comes in several salified forms, you must make clear which salt was studied.

EXAMPLES

INFLUENCE OF PRESERVATIVES :

Influence of preservatives: Oldham et al. (1991) highlighted the instability of cefuroxime sodium (cloudy or precipitated) in the presence of 0.02% or more benzalkonium chloride (preservative)

INFLUENCE OF PH :

the buffer change in a 5-fluorouracil proprietary medicinal product (tris buffer replaced by sodium hydroxide) caused a change in pH and an increased risk of crystallization in solutions that were diluted and stored at 4°C (BARBERI-HEYOB M. et al., 1995)

All stability studies should be conducted with an active ingredient issued from the same batch.

1.2 ALL STABILITY STUDIES SHOULD BE CONDUCTED WITH AN ACTIVE INGREDIENT ISSUED FROM THE SAME BATCH.

Liquid preparations contain numerous excipients. We can include vehicles (reconstitution and dilution solvents), preservatives (antimicrobial agents), surfactants, viscosifiers, colorants and aromatic agents. For each of these components, it is necessary to have the exact name of the product, the supplier and the batch number. The stability study for each excipient will be conducted on the same batch of preparation.

1.3 PACKAGING ITEMS

The packaging has been chosen because it is suited to the practical conditions of use of this preparation, and because it has not given rise to any known content/container interaction.

The following elements should be taken into account and recorded:

- Type of packaging: vial, pouch, syringe, portable infusion devices, ampoules
- Capacity in ml
- Constituent Material(s): glass, polyethylene, polypropylene, polycarbonate, rubber, multilayer (record the nature of the materials comprising each layer).

  If glass, specify its quality (borosilicate, soda-lime, etc.) and its type (clear, amber)

In container descriptions do not forget to indicate the nature and composition of the sealing system: stoppers (butyl rubber, chlorobutyl, etc.) and caps for eye drop bottles, Luer-Lock closures, or pre-mounted needles for syringes, etc.

For eye drops, a dropper system may be fitted to the bottle: the stability of the preparation while in contact with the dropper must then be studied.

If outer packaging is used, specify its nature and quality.

2 CHOICE OF CONCENTRATIONS TESTED

The concentration of active ingredient in the preparation to be studied will always depend on the product's intended therapeutic use. When preparations are recognized as clinically effective only at a single concentration, the stability study will be conducted at that concentration.

In other cases the active ingredient is clinically effective over a specific range of concentrations. It is a situation that occurs frequently in the centralized manufacture of anti-cancer drugs

or anti-infectives: reconstitutions or dilutions will lead to different concentrations of the active ingredient.
Since the preparation's stability may vary as a function of this dilution, it is necessary to study the stability of the preparation at a minimum of two concentrations: one low and one high. Pharmacists must first identify the effective therapeutic range and then select the higher and upper concentrations. If the difference between the low and high concentrations is too significant (more than a factor of 10) an intermediate concentration stability study may be considered, depending on the clinical interest.

3 NUMBER OF TESTS

Stability studies should preferably be conducted on a single manufacturing batch, in order to avoid introducing variability derived from the fabrication process. Also, the test batch must include **at least 3 units**, so as to obtain a minimum of 3 independent measurements.

As far as possible units should be prepared in conditions which reflect the circumstances in which the product is intended to be used, at the same volumes, **and it is preferable to create one preparation unit per sampling time-point**.

If it is not possible to use different units for each sampling time-point (for a very expensive drug, for example), one unit may be used for all sampling time points, but care must be taken to limit the maximum volume taken in samples so as to not too greatly modify the surface of contact between content and container. It is also possible that multiple samplings may cause a seal failure in the container, and adversely impact the preparation's stability. Testing should then be repeated 3 times, on preparations from different batches.

4 STORAGE CONDITIONS

4.1 TEMPERATURE

Ambient temperature

So long as the active ingredient is not known to be heat-unstable, testing is performed at temperatures near 25°C. If the pharmacist has access to an environmental chamber, the units should be kept in the temperature conditions recommended by the ICH, namely 25°C ± 2°C.
Otherwise, the study may be performed at ambient temperature, while regularly recording it. The stability data for the preparation will then be shown for the temperature range measured during the study.

Refrigeration

If the data in the literature recommend refrigeration, or indicate that the principle active is known to be thermosensitive, or if analyses performed at 25°C show a rapid degradation of the molecule, a study at 5°C will be considered. If the pharmacist has a climate chamber with refrigeration, the study will be conducted under the conditions recommended by the ICH, namely at 5°C ± 3°C. Otherwise, stability will be evaluated in a refrigerator, with regular temperature measurements. The probe allowing temperature measurement will be inserted in close proximity to the preparation. There are indeed significant temperature variations within a refrigerator, which requires that care be taken about measuring temperatures.

Freezing-Thawing

If the data in the literature recommend freezing, or if the active ingredient degrades rapidly at ambient temperature and/or after refrigeration, a stability study on the frozen preparation will be programmed at approximately -20°C. Regular temperature recording must be performed in order, as far as possible, to maintain a temperature of -20°C ± 5°C.

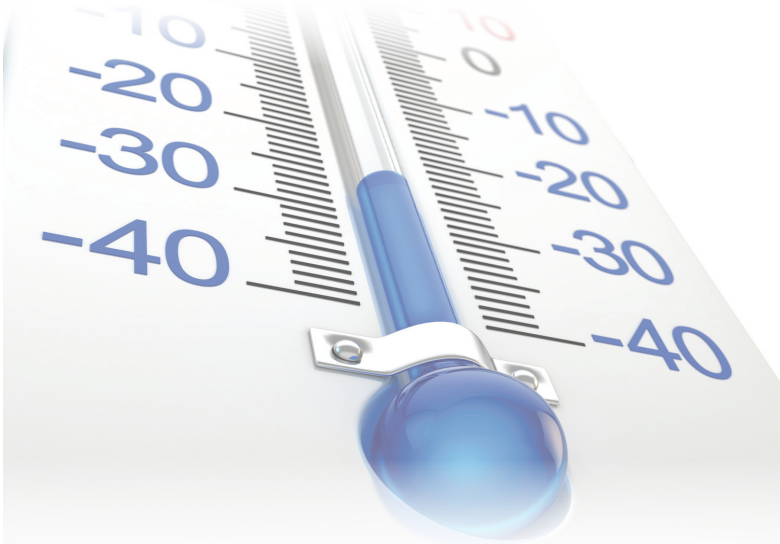
It is therefore important that conditions be defined for thawing the preparation and studying its stability after this thawing phase.

THE THAWING PHASE, AN INDISPENSABLE STEP IN A STABILITY STUDY

The thawing mode may influence the stability of the active ingredient
Cefuroxime is stable for 8 hours after thawing at 20-25°C, 4 hours at 33°C, and degrades immediately if thawing is performed in the microwave.
(C. Bontemps, 2010)

The case of portable infusors

Since portable infusors are intended to come into contact with the patient's skin, an additional stability study will be performed at 33°C.



4.2 RESIDUAL MOISTURE

If the pharmacist has a climate chamber, stability testing is performed at 25°C while maintaining the residual moisture at 60%± 5%, in order to achieve the conditions recommended by the ICH.
Otherwise, the study will be conducted under local residual moisture conditions, and it is recommended that measurements be taken regularly.

4.3 LIGHT

In the absence of data from the literature regarding the possible photosensitivity of the active ingredient, the use of day/night ambient light is recommended.

If the molecule is known to be photosensitive, tests will be carried while protecting the preparation with suitable packaging (amber bottle, opaque packaging, opaque outer packaging) and appropriate storage conditions, sheltered from the light.

Note: The stability of a preparation runs from its preparation phase right to its administration. Thus care must be taken regarding transport conditions, in order to avoid any deviation from the storage conditions validated in the stability study (chilled transport, frozen transport, pneumatic transport)

5 DURATION OF THE STUDY

Real-time studies are recommended, while limiting the storage period to 1 year in order to stay within acceptable limits with regard to normal hospital practices.

Upstream accelerated aging studies, may help to get an idea of the molecules' degradation pathways. In such case, pharmacists will work to the methodology dictated by the ICH.

6 SAMPLING TIME POINTS

Sampling at T0 is essential because it serves as a reference. It is performed immediately after fabrication.

Subsequent to this, sampling time points are calculated with reference to the maximum planned duration. We recommend establishing a minimum of 5 sampling time points between the initial time T0 and the maximum duration. We propose sampling frequencies corresponding to about 1/24th, 1/12th, ¼, ½ and ¾ of the maximum duration. These frequencies may be adjusted slightly to fit around a reasonable working schedule, staying within the laboratory's working hours, for example. However, for short delays (≤ 72 hours), it is recommended that the sampling schedule be followed as strictly as possible.
We propose the calendar below, based on durations of study ranging from 7 days to 1 year.

Start time (T0)	Intermediate sampling times							Total duration
0			6h	12h	24h	72h	5d	7d
0			24h	48h	7d	15d	22d	30d
0		24h	75h	7d	15d	30d	2m	3m
0		24h	7d	15d	30d	2m	3m	6m
0	24h	7d	30d	2m	3m	6m	9m	12m

Table1 : Sampling frequencies proposed as a function of stability study durations (d: days, h: hours, m: months)

For the specific case of post-thawing stability: sampling will be performed immediately after the preparation has thawed, and 24 hours after. The reference value remains the value obtained at T0, i.e., the value measured immediately after manufacture and before freezing.

This procedure is applicable to any study involving two successive storage modes (for example, refrigeration, then ambient temperature storage, or refrigeration after thawing)

If preparation instability is anticipated, sampling frequency should be increased at the start of the testing period.

If the study results show rapid alterations in the preparation, testing will be performed again with a shorter maximum duration, recalculating the sampling frequencies with reference to the table.

7 VOLUME SAMPLED

The volume taken for analysis will depend on the volume of the preparation, its usage (single dose or multi-dose) and the amount required for analysis.

If possible, it is preferable to prepare a separate preparation unit for each sampling time point, even for multiple doses.

8 ANALYSES TO BE PERFORMED

An assay of the active ingredient and monitoring of the appearance of degradation products are performed systematically.

The other analyses to be performed are determined according to the pharmaceutical dosage-form used for the stability study.

In the case of sterile preparations, the physicochemical stability study may be supplemented by a microbiological stability study.

These analyses, as well as their implementation and validation, are described in the following chapters of this methodological guide. A special chapter is dedicated to the interpretation of the results.

PROBLEMS WITH EXTRACTABLES

Some containers may release chemical compounds (such as plasticizers) during prolonged contact with a preparation. If pharmacists have access to the necessary analytical resources, they can monitor the migration of extractables from the container to the preparation. Otherwise it is recommended that care be taken in the choice of packaging: avoid PVC, which may leach plasticizers; check that no silicone has been used as a lubricant in the syringes, as this can result in the aggregation of protein based compounds; and check the composition of any stoppers that may leach toxic compounds.

All stability studies include an assay of the active ingredient and a study of the degradation products. In this chapter we will describe several techniques which can be used to perform an assay on a given active ingredient and/or their degradation products in view of performing a stability study. The analytical methods should be subject to prior validation, as described in chapter 4. Not all analytical methods are suitable for evaluating the stability of pharmaceutical compoundings.

CHAPTER3

Methods for the analysis of active ingredients and degradation products

1 CRITERIA FOR SELECTING ANALYTICAL TECHNIQUES FOR STABILITY STUDIES

In choosing testing techniques, preference should be given to separative methods that allow for differentiation of the various constituent ingredients of a mixture (for stability studies: separation of the active ingredient(s) from the degradation products and excipients contained in the pharmaceutical formulation) before assay using appropriate instrumental methods.

Liquid chromatography (LC) and capillary electrophoresis (CE) are the recommended methods. The majority of today's stability indicating methods use reversed-phase liquid chromatography, since most of the compounds being analyzed are hydrophobic. Moreover, a liquid phase chromatography unit is often one of the few instruments available in hospital pharmacies, if not the only one. This is why LC - and more specifically reversed phase LC - should be a primary method of choice when a stability study is required. Although technique selection is primarily dictated by what equipment is available, it is also strictly linked to the physicochemical properties of the compounds to be determined. Those properties will also guide the choice of the detector to be used for the assay.

In the interest of rounding out this chapter, however, it would be interesting to look at another technique, one particularly well suited for use in stability studies: capillary electrophoresis.

Two other separative techniques are briefly dealt with as well: high-performance thin layer chromatography and electrochromatography.

As part of a stability study, simple UV-Visible spectrometry - a non-separative technique - should not be used to perform active ingredient assays, though such techniques may be extensively used for the assay of active ingredients in a preparation in view of final batch validation and release.

2 LIQUID CHROMATOGRAPHY

2.1 PRINCIPLE

Among the various types of chromatography, the most widely used in the pharmaceutical domain is indisputably liquid phase chromatography (LC). This separative technique allows the components of a mixture to be isolated and quantified. The separation of compounds relies on the difference in the distribution of solutes between two immiscible phases: one stationary phase, which looks like a solid base (a column), and a mobile phase, consisting essentially of water and/or organic solvents. The efficiency of this technique derives from its high degree of selectivity. Indeed, the wide variety of commercially available stationary phases and the multitude of possible mobile phases allow for the analysis of a very broad range of compounds (non-volatile molecules, thermolabile molecules, molecules with high molecular weight or ionic molecules) using several separation mechanisms *(R. Rosset, 1991)*. A schematic diagram of LC instrumentation is shown in Figure 1.

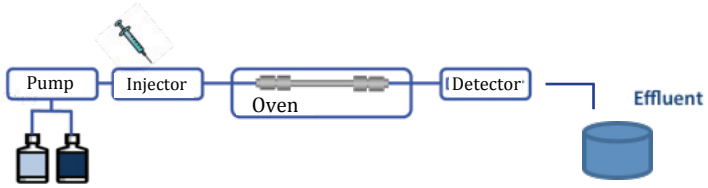


Figure 1: LC instrumentation scheme

2.2 EQUIPMENT

Chromatographic column

The column is a crucial component of any chromatography system, and the choice of column will be determined by the type of compound being analyzed. The LC column is usually a tube of between 5 and 30 cm in length, with an internal diameter of between 1 and 4 mm *(Figure 2)*. This tube can be filled with particles of 2 to 10 µm, held inside the column with the use of sintered seals placed at each end, or can be made in a high porosity one-piece form, such as monolithic columns.

Figure 2 : Standard chromatography column filled with particles

Injector

All modern LC systems come equipped with an automatic injector able to ensure constant injection volumes.

However, automatic injectors manufactured around fifteen years ago may present variability greater than 2% when used in "partial loop fill injection" mode, which may reduce the efficiency of this method. This is why it is recommended that the injection reproducibility of old automatic injectors be checked. If reproducibility is not demonstrated, use injections where the sample volume corresponds to the volume of the injection loop, or else use an internal standard. However, caution must be taken when using an internal standard with a method intended for use in a stability study. Not only must the reaction time of the internal standard be different from that of the active ingredient, but any possible degradation products must have different reaction times as well.

Pump

Given the low granulometry of the stationary phases, which causes significant pressure loss in the mobile phase, high-pressure pumps are indispensable to ensure consistent elution. These piston pumps generally operate at a constant rate of flow regardless of the pressure measured in the LC system. However, it should be pointed out that depending on the pump manufacturer and generation, the pumps may have highly different flow rates (from a few µL.min-1 to around ten mL.min-1). The rate applied is directly linked to the diameter of the chromatographic column used, and is determined according to the Van Deemter law, which allows us to calculate the optimum mobile phase flow for the column used, enhancing efficiency while preventing the pressure in the LC system from becoming too high.

Table 2 indicates the mobile phase flow rates generally applied, based on the characteristics of the chromatographic columns used.

Diameter of the chromatographic column	Mobile phase flow rate applied
4 - 4.6 mm	1 - 1.5 mL.min-1
3 mm	0.5 mL.min-1
2 mm	0.2 mL.min-1
1 mm	0.05 mL.min-1

Table 2 : Mobile phase flow rates applied as a function of chromatographic column diameters

Notice: be sure to follow column manufacturers' maximum pressure recommendations

There are two distinct types of pumps :

- An isocratic pump delivers mobile phase at a constant composition throughout the duration of the analysis. This is the simplest pumping system which allows for the separation of compounds in relatively simple mixtures (2-3 different compounds).
- A binary, tertiary, or quaternary pump allows 2, 3 or 4 types of mobile phase to be delivered simultaneously. This type of pump is capable of operating in gradient mode (change in phase composition as a function of time) or isocratic mode. Operation in gradient mode is preferable when complex mixtures are to be analyzed.

Oven

If the premises where the LC system is installed are subject to temperature variations (no air conditioning system), or if the analysis needs to be performed at temperatures other than the ambient temperature, it is recommended that a system be used to control the temperature of the column in order to ensure consistent measurement conditions. Ovens are the most commonly used option, though water baths and air flow systems may also be used.

Detector

A wide variety of detection systems can be coupled with liquid phase chromatography. The most current is certainly UV-Vis spectrophotometry. Indeed, approximately 80% of pharmaceutical substances have a chromophoric chemical entity, and can be detected by UV-Vis spectrophotometry. We can identify three types of UV-Vis detectors :

- Fixed wavelength detectors, which are able to work on only one wavelength (determined by the manufacturer); these are less and less common, and have almost become obsolete.
- Variable wavelength detectors, which can record absorbances at one or more wavelengths (set by the operator) between 190 and 700 nm; these are the most common nowadays.
- Diode array detectors, which are capable of recording absorbances over a whole range of wavelengths (set by the operator). The operator can thus obtain a UV-Vis spectrum for the selected wavelength range at any time during the analysis.

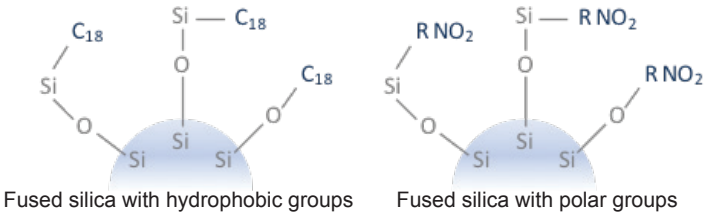
Other detection systems may also be used with liquid chromatography, such as fluorimetry, refractometry, electrochemistry, mass spectrometry, etc.

Generally speaking, the type of detection system used must above all be appropriate to the molecule in question. For stability indicating methods, it is not recommended to use a detection system requiring a derivatization phase. In most cases, UV-Vis spectrophotometry is perfectly suitable. A detection system capable of providing qualitative information (more selective than retention time) on the compounds to be analyzed and their degradation products is preferred, i.e., a UV-Vis, diode array, or mass spectrometry type detector. This type of detector is particularly useful for checking the separation between the active ingredient and the degradation products, and checking to ensure that the assay method is indicative of stability *(CHAPTER 4)*.

2.3 PRIMARY MODES OF LIQUID CHROMATOGRAPHY

There are different types of liquid phase chromatography. In this guide we will consider only partition chromatography, which is the most widely used. The separation mechanism is based on splitting a compound between a stationary phase and a mobile phase. Depending on the polarity of the stationary phase, this takes the form of either reversed-phase liquid partition chromatography (apolar stationary phases) or normal-phase liquid partition chromatography (polar stationary phases). In most cases, the stationary phases are silica-based, and have silanol groups on their

surface onto which apolar or polar units are fused, altering the initial characteristics of the silica (silanization reaction).



Fused silica with hydrophobic groups
-> reverse phase LC

Fused silica with polar groups
-> normal phase LC

Figure 3 : Fused silica

Reverse mode

Reverse mode is the most common mode for liquid partition chromatography. Indeed, it is particularly suited to drug molecules of a hydrophobic nature, because the fused units are generally octadecyl s(C18), octyls (C8), phenyls etc. Do not forget that during the silanization reaction, a certain proportion of the silanols remain free, and may be responsible for secondary interactions with compounds, reflected in exaggerated peaks. Various strategies have been deployed to address this problem, such as the use of patterns that reduce access to silanol compounds (steric protection, high density, long chains, polar group). A wide variety of silica-based media with low silanol activity are currently available commercially. This is why when positively charged basic compounds are being analyzed, and a modification of the pH of the mobile phase cannot be performed, it is recommended that these chromatographic media be used. Note that the silica-based columns are stable at pH values between 2 and 8. Although silica is still the base material for the chromatographic media, other materials based on alumina, titanium oxide, zirconium oxide, carbon or even organic polymers may be used. In reverse mode, the mobile phases consist of hydro-organic mixtures.

Normal mode

Normal mode, which is more rarely used, allows the separation of molecules of a polar nature because the fused units are diol, amino, nitro, nitrile etc. The mobile phases are comprised of mixtures of solvents: alkane paired with a more polar solvent such as tetrahydrofuran, chloroform, etc. As with reverse mode silica gels are widely used, with the same attendant pH restrictions, but other media are also available for liquid partition chromatography in normal mode.

Table 3 shows a few examples of reverse mode LC active ingredient assay methods. These methods have been verified by the authors as indicative of stability.

Analytes	Mode	Chromatographic conditions	References
Cefuroxime axetil	Isocratic	Stationary phase : Symmetry C18 (5 µm, 150 x 4,6 mm) Mobile phase : methanol / water (38/62 v/v) Detection : I = 280 nm	Ivanovic I. et al., 2006
Betamethasone sodium phosphate and betamethasone acetate	Linear gradient	Stationary phase: ACE3 C18 (3 µm, 150 x 4.6 mm) Mobile phase : - Aqueous solution: trifluoroacetic acid 0.5% v/v + Potassium citrate 31mM + potassium hexafluorophosphate 20 Mm - Phase A : dioxane/THF/aqueous solution : 4/4/92 v/v/v - Phase B : dioxane/THF/aqueous solution : 14/28/58 v/v/v - 60 min gradient from 100% phase A to 100% phase B Detection: I = 254 nm	Lu J. et al, 2010

Table 3: Examples of reverse mode LC assay methods

2.4 RECENT DEVELOPMENTS IN LIQUID PHASE CHROMATOGRAPHY.

As a general rule, the use of conventional liquid chromatography is ideal for the methods used in stability studies of medicinal preparations. In recent years, however, liquid chromatography has undergone numerous developments, and it is sometimes difficult to navigate the literature when looking to get up to date on a given stability indicating method. Among the various analytical developments, all aimed at reducing the testing time without diminishing the efficiency *(Guillarme D. et al, 2009)*, we will discuss the most common. We note, for informational purposes, that run times for conventional liquid chromatography usually fall between 20 and 60 minutes, while with these new approaches the times can be reduced to a few minutes (or even less than a minute).

→ Use of short chromatographic columns with ahigh mobile phase flow-rate

Columns of 3 to 5 cm in length, filled with particles of small size (about 3 µm in diameter) can be used at higher mobile phase flow rates, generating pressures compatible with conventional LC systems. However, the efficiency of this type of chromatography is impeded by the short length of the columns. This is why, when complex mixtures are to be analyzed, as is the case in stability studies, such short columns are not recommended unless a detection system with high selectivity (such as mass spectrometry) is used to counterbalance the weak chromatographic resolution resulting from the short columns. Analysis times below 5 minutes may be achieved.

→ Use of monolithic columns:

Monolithic columns, which have high porosity and also allow the pressure loss to be attenuated, can support mobile phase flow rates up to 10 times higher than the standard level while maintaining good efficiency and not generating high pressures. These columns are currently available on the market in diameters of 4, 3, 2 mm, or 100 µm, and the flow rates applied go from about a few µL.min-1 to 10 mL.min-1. Thus analysis can be performed very quickly, only taking about 1-5 minutes. As with short columns filled with small particles, the efficiencies obtained with short monolithic columns (5 cm in length) may prove insufficient for use in separating compounds out from a complex mixture, and so it is mandatory to have a highly selective detection system. Otherwise, it is preferable to use longer monolithic columns.

→ Ultra high pressure liquid chromatography

In recent years, ultra performance liquid chromatography, or UPLC, has been one of the biggest developments in liquid chromatography. UPLC uses short columns (about 5 cm) filled with very small particles (sub-2 µm) at pressures of over 400 or even 1000 bar. High efficiencies can thus be obtained for ultra-fast analysis times (less than a minute). This approach requires a system specially designed to work at high pressure, and allowing for the performance of ultrafast analyses (see the paragraph entitled “Caution!”). Today, manufacturers offer LC systems that can change between UPLC mode and conventional mode.

** CAUTION **

1. When unconventional flow rates need to be applied, as well as when using monolithic columns or short columns filled with small particles, it is important to ensure that the LC pump used is able to consistently deliver the desired flow rates.

2. When performing rapid tests, use a detection system calibrated to provide sufficiently rapid time constants and acquisition frequencies to record the elution of the compounds of interest. It is generally accepted that a peak is set correctly when it has a minimum of 20 points.

3. It is also possible to perform LC separations at temperatures above 60 °C so as to obtain rapid analyses, since the viscosity of the mobile phase decreases in such conditions. However, this mode of LC operation is strongly discouraged in stability studies, since the analytes may undergo changes at high temperatures. Moreover, many stationary phases are thermally unstable.

3 CAPILLARY ELECTROPHORESIS

3.1 PRINCIPLE

One alternative to chromatographic techniques is capillary electrophoresis (CE), which also allows for the separation and quantification of pharmaceutical substances.

Like LC, CE can also be a qualitative data source, thanks to its migration time, and potentially via the detection system used. The primary advantages of CE are its high efficiency, low organic solvent consumption, and the rapid development of the method. In addition, CE is a particularly selective technology at a lower price (the low price of capillaries compared to chromatographic columns), allowing for the analysis of a wide range of compounds, ranging from small inorganic ions to complex molecules such as proteins. The separation mechanism in electrophoresis is based on the differences in displacement speed among the compounds under a high electric field, which is directly dependent on their “charge-over-size” ratio.

3.2 EQUIPMENT

Electrophoretic capillaries

CE uses fused silica capillaries, with an internal diameter equal to or less than 100 µm. Each end of the capillary is immersed in electrolyte-rich independent solutions called «electrophoretic buffers,» immersed in which are two electrodes connected to a generator capable of delivering voltages up to 30 kV. A diagram of a typical capillary electrophoresis setup is illustrated in Figure 4. The samples are injected into the capillary either by pressure (hydrodynamic injection) or by electrical voltage (electromigration injection). The volume injected is generally between 1 and 10 nL. Because of the lack of reproducibility in capillary electrophoresis injections, it is indispensable that an internal standard be used for any quantitative analysis. For the development of stability indicating methods, separation must be obtained for the internal standard, the compound of interest and any possible degradation products as well.

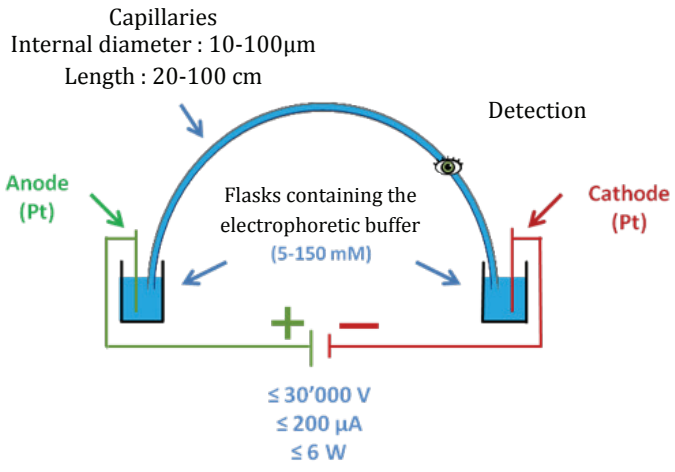


Figure 4 : Schematic diagram of a capillary electrophoresis instrumentation set-up

Detector

Various different detectors can be used in capillary electrophoresis. As in the case of LC, the most widely used system is still UV-Vis spectrophotometry. However, the short path travelled by the light (i.e., the internal diameter of the capillary), and the small amount of the sample injected, are responsible for the low sensitivity of CE. This is a real problem for the quantification of samples where the compound to be assayed is in a low concentration, such as in biological samples or in trace analysis; this is not the case for the analysis of pharmaceutical formulations, which in general have active ingredient concentrations on a scale of mg.mL-1. However, different strategies have been developed to improve the sensitivity of UV detection in CE, such as the use of “bubble” detection cells or “Z-cells.” These capillaries demonstrate diametric enlargement (a bubble) or form a “Z” on a small segment of the capillary at the detection site, thus allowing the optical path to be expanded. When the analyte does not fall into any chromophoric chemical group and/or when it is in a low concentration, other detection systems may be used, such as mass spectrometry, conductivity, laser-induced fluorescence or amperometry.

3.2 PRIMARY MODES OF CAPILLARY ELECTROPHORESIS

Depending on the nature of the electrolytes present in the electrophoretic buffer, various different modes of capillary electrophoresis may be applied, such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), or microemulsion electrokinetic chromatography (MEEKC). *(Suntornsuk L. et al, 2010)*

In all cases, the selectivity will depend on the type and pH of the electrophoretic buffer used, as well as on the possible presence of additives in the buffer.

Capillary zone electrophoresis (CZE)

CZE is the simplest and most widely used mode of CE. CZE is particularly well adapted to the separation of pharmaceutical compounds, which in most cases are ionic compounds (i.e., basic organic compounds, acids and/or inorganic ions). In general, the capillary is filled with a single aqueous buffer (the most commonly used: phosphate, acetate and borate buffers) and high voltage is applied. In positive mode, detection is performed at the cathode end of the capillary, and injection is performed at the other end of the capillary (anode). In such conditions, the order of migration is as follows: (1) positively charged compounds, (2) neutral compounds, and then (3) negatively charged compounds (Figure 5 A). In CZE, separation is governed by the pH of the buffer and the pKa of the compounds being studied.

In CZE mode, though the separation between the positively and negatively charged particles depends on their charge to size ratio, this is not the case for neutral particles, which migrate along with the electro-osmotic flow. If a separation of neutral particles from one another is desired, another CE mode will need to be used.

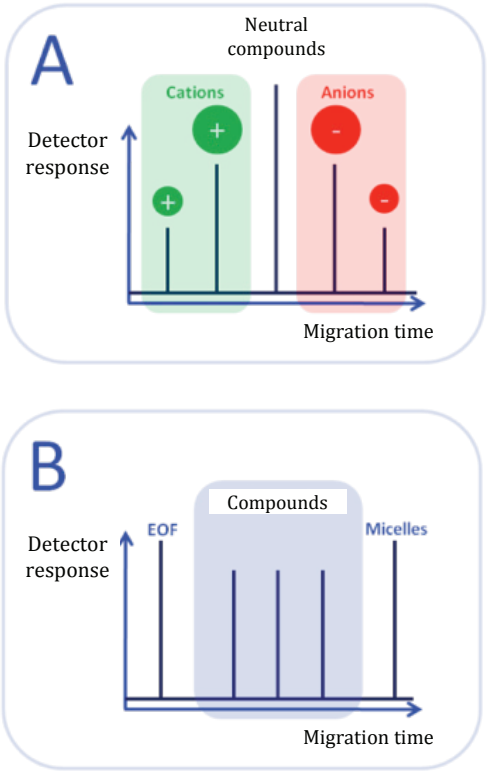


Figure 5 : Diagram illustrating the separation of compounds, A: CZE in positive mode B: in MEKC mode

(positive mode with negatively charged micelles)

Micellar electrokinetic chromatography (MEKC) and microemulsion electrokinetic chromatography (MEEKC)

MEKC *(Hiroyuki N.. et al., 1996)* and MEEKC *(Altria KD, 1999)* are used to separate neutral and ionic compounds. In the case of MEKC, surfactants such as sodium dodecyl sulfate (SDS) or cetyltrimethylammonium bromide are added to the electrophoretic buffer at concentrations above their critical micelle concentration, with the separation mechanism based on the partition differences of the compounds between the micelles and the solvent (Figure 5 B). In MEEKC, the separation of compounds is governed by their division between the buffer and the drop of oil; this allows for different selectivity than what can be obtained via MEKC. The microemulsion droplets are generally formed by the use of solvents such as heptane or octane in water. In order to stabilize the microemulsion and to obtain a homogeneous system, a surfactant to ionize the droplets and a co-surfactant to reduce the surface tension of the water-oil interface are added.

MEKC or MEEKC indicating methods can be highly effective, but sufficient expertise is required to implement such methods. Although these two modes of CE may prove suitable for the separation of neutral compounds, well documented limitations do exist, in particular the low sensitivity of these methods due to the use of ionic additives in the electrophoretic buffer, and the frequent recording of high currents.

Non-aqueous capillary electrophoresis (NACE)

It is possible to use organic solvents containing soluble salts that are formate-based (formic acid or ammonium formate) or acetate-based (acetic acid or ammonium acetate), instead of aqueous buffers, to perform electrophoretic separation in CE (Riekkola ML et al, 2000). NACE offers the following advantages compared to CZE in water:

- Different selectivities.
- Better solubility and stability of compounds in organic solvents.
- Rapid analysis times.

NACE is a sensible alternative for the development of stability indicating methods when testing in an aqueous medium is not possible. From an ecological and economic perspective, it is true that organic solvents are used in NACE, but in very low proportions compared to LC, as only a few microliters are needed to prepare the electrophoretic buffer. We emphasize that the primary drawback of NACE is the possible lack of sensitivity inherent in the UV absorption of certain organic solvents.

4 OTHER SEPARATION TECHNIQUES

4.1 HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Another chromatographic separation technique that can be used for the establishment of stability indicating methods is high performance thin layer chromatography *(J Sherman et al, 2010)*. The separation mechanisms involved in HPTLC are the same as those in liquid chromatography. In HPTLC, a thin layer (100µm) of stationary phase, composed of fine particles (about 5 µm) covers a rigid plate (of varying dimensions). As with LC, different types of stationary phase can be used for a separation in normal or reverse mode, depending on the nature of the analytes. The mobile phases are also the same as those used in LC, and allow elution of the compounds along the plate by capillary action. During elution, the sample previously deposited on the plate is conveyed and distributed between the mobile and stationary phases. Separation takes place over a distance of a few centimetres (3-5cm) in less than 15 min. Analyte detection occurs via densitometry (measuring the absorbance at the spot). Because of its low cost and its rapid development, HPTLC may serve as an alternative technique for either LC or CE. The ability to work in two-dimensional mode (two successive separations in perpendicular directions by changing the nature of the mobile phase) makes for high selectivity. It should also be pointed out that HPTLC is also used as a preliminary scan of the operating conditions, to prepare for a subsequent application of LC. One of its primary limitations is its low sensitivity and limited quantitative capability.

4.2 CAPILLARY ELECTROCHROMATOGRAPHY (CEC)

Capillary electrochromatography *(Dermaux A. et al, 1999),* as its name suggests, combines two separation techniques: liquid chromatography and capillary electrophoresis. The application of an electric field to the ends of a capillary filled with a solid media can elute the analytes into the capillary via electro-osmotic flow. The separation of the analytes is achieved not only thanks to their affinity for the media (stationary phase) and for the mobile phase, but also occurs as a function of their electrophoretic mobility. The stationary phases are identical to those used in liquid chromatography (fused silica, porous polymer monoliths). The mobile phase, in turn, generally consists of a hydro-organic solution. Although this technique combines the advantages of the CE and LC (high efficiency, high selectivity, low consumption of organic solvents, etc.), it has certain limitations that makes it more of an experimental technique than a routine practice. The low commercial availability of capillaries and their high cost are indeed major limitations to the use of CEC. For this reason many laboratories produce their own filled capillaries, which requires significant expertise in order to mitigate the non-reproducibility of manufactured media. The instruments used are those normally used for capillary electrophoresis and therefore are not always suitable for CEC. Indeed, capillary thermostatization is more difficult in CEC, because of the strong resistance of the filled columns (compared to hollow capillaries), and the use of an elution gradient which is difficult to manage. Furthermore, multiple parameters are involved in optimizing the separation (ionic strength, aqueous-organic mixture, applied voltage), which may be a negative aspect (complicated development) but a positive point as well (more adjustable selectivity).

Due to its complexity, CEC should only be considered as a potential technique for stability indicating testing when LC and CE cannot be used.

CONCLUSION

LC and CE are particularly useful techniques for developing stability indicating methods. Based on separation mechanisms different from those offered by partition LC, CE is a supplementary analysis technique that can thus resolve separations that are difficult to obtain with LC. If neither of these two techniques is available, pharmacists may turn to other separation techniques like HPTLC. As mentioned in Chapter 3, the choice of a technique to be used for the development of a stability indicating method is based first and foremost on the scientific literature. If operators can find no precedents in the literature, they should then refer to the physicochemical properties of the molecule of interest and the composition of the pharmaceutical formulation to guide their choice of what technology (and method) to use. Table 4 summarizes the primary question-answer pairs that operators should go through when developing a stability indicating method.

Questions	Responses
Properties of the molecule	
• What are the key properties of the molecule?	**Neutral :** • LC method or • appropriate CE method (MEKC, MEEKC, NACE) **Acid, Base :** • find the molecule's acid dissociation constant(s) (pKa); • LC: mobile phase with a pH appropriate for partitioning compound of interest/mobile phase/column or • CZE: acidic electrophoretic buffer for basic molecules (with pH ≤ pKa-2) and a basic electrophoretic buffer for acidic molecules (with pH ≥ pka +2)
• What is the nature of the molecule and its solubility?	**Hydrophilic :** • Normal phase or reverse phase LC • CZE with an electrophoretic buffer with the appropriate pH **Hydrophobic :** • Reverse phase LC • CZE with an electrophoretic buffer with the appropriate pH if the molecule is water-soluble, otherwise NACE
• Does the molecule have chromophoric groups?	**Yes**: LC or CE with UV/DAD detection **No:** Test other detection systems available or refer to the literature on the molecule or related substances
• Is the molecule present in sufficient concentration to permit its detection via the chosen system?	**Yes**: Proceed as with any other method development (by LC or CE) **No:** • Choose another detection system compatible with the analytical technique • Use capillaries equipped with «zeta» or «bubble» cells for UV-Vis CE • Pre-concentrate the sample by extraction (liquid-liquid or on solid media) prior to LC or CE analysis
• Is the molecule stable at ambient temperature?	**Yes**: Proceed as with any other method development (by LC or CE) **No**: • LC at an appropriate temperature (lower temperature for the column oven and for sample storage before LC injection) • Rapid LC. • avoid CE.
• Is the molecule stable in light?	**Yes**: use conventional laboratory glassware **No**: Use brown lab glassware, or protect conventional glassware with Aluminum paper.
Composition of the pharmaceutical formulation	
• Does the formulation contain any excipients interfering with the analysis?	**No**: Proceed as with any other method development (by LC or CE) **Yes** LC or CE, but the excipient must be clearly separated from the molecule to be assayed as well as from any of its degradation products.

Table 4: Question-response pairs for the development of a stability indicating method.

We observed in the previous section that the preferred analytical methods for stability studies were separation methods.

Implementation of the chosen assay method requires development and validation stages that will vary in length depending on the extent of existing data in the scientific literature.

The present section will address the essential criteria needed to appraise the quality of an analytical method used to perform a stability study, as well as providing practical advice enabling you to develop and validate a stability indicating method.

CHAPTER4

Validating the analytical method

1 BASIC CONCEPTS

An analytical method developed for the purposes of a stability study on a preparation must cater for the following :

- Identification and quantification of the active ingredient in order to monitor its evolution over time.
- Monitoring the appearance of any degradation products over time, with semi-quantitative analysis.

To answer to these objectives, the method must satisfy certain validation criteria:

For analysis of the active ingredient within the preparation:

- The method must be specific and thus allow the active ingredient to be clearly distinguished from other components in the preparation (excipients and possible degradation products). It is essential that the analytical method should provide an indication of stability
- To quantify the molecule of interest within the preparation, it is appropriate to define a dosing interval within which the method will present criteria of linearity (or possibly another calibration function), accuracy and reliability. To make any quantification, a calibration range will be necessary.

When seeking degradation products:

The method must permit the operators to observe degradation products and conduct a semi-quantitative analysis. The global surface of peaks will be determined and must not exceed a certain acceptable limit known as the exclusion limit.

Table 5 summarises the general parameters to be studied when assaying an active ingredient, and the semi-quantitative survey of degradation products per limit test.

Validation parameters	Active ingredient	Degradation products
Specificity /selectivity	+	+
Calibration response	+	-
Reliability: - repeatability - intermediate precision	 + +	 - -
Accuracy	+	-
Assay range	+	-
Limit of detection (LOD)	-	+
Limit of quantification (LOQ)	-	-

Table 5: validation parameters of an analytical method for use in stability studies

To obtain a precise definition of these analytical parameters, consult Appendix 1.

2 2. FROM DEVELOPMENT TO VALIDATION OF THE ANALYTICAL METHOD

Implementation of an assay method may require a development phase during which various operating conditions will be explored. Among them, the following can be mentioned:

- specificity: “stability indicating method” and “matrix” effect
- conditions of solvents and dilution,
- the dosing interval,
- standards,
- preparation of the sample.

If all the points mentioned above have to be documented, it may be wise to give priority to certain amongst them. Thus, the order of study proposed below may provide an example of a work plan to be followed :

- Seeking or establishing an analytical method having “stability indicating capability”. This stage is essential within the scope of a stability study and its path is described in the flowchart shown in figure 6.

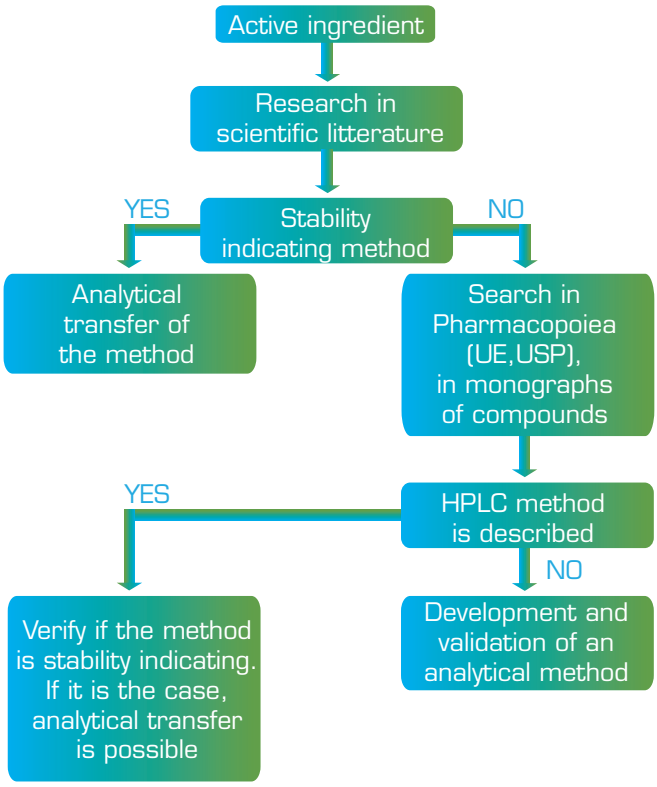


Figure 6: Determining an analytical method suitable for stability studies on an active ingredient

The first approach involves focusing on specific works or surveying the scientific literature. It is appropriate to conduct a critical analysis of the scientific literature on the topic as not all the methods published in reviews are indicative of stability. If the method is appropriate, it should provide the basis for the three subsequent stages.

WHERE TO FIND STABILITY INDICATING METHODS

Reference work: Xu QA, Trissel LA "Stability indicating HPLC methods for drug analysis", 2003, 2ndedition, APhA Publications, Houston.

Referenced methods in the Stabilis database: http://www.infostab.com/

Examples of reviews publishing stability studies conducted using stability indication methods: International Journal of Pharmaceutical Compounding; European Journal of Hospital Pharmacy; International Journal of Pharmaceutics; American Journal of Health System Pharmacy

If a stability indicating method is not available in the literature, it may be worth seeking out a monograph on the active ingredient in a Pharmacopoeia (European, American or other). Indeed, monographs on raw materials may provide a first approach. Such monographs list the main synthesis impurities. This most often concerns feedstock, sometimes the first degradation products. By way of example, the monograph for pilocarpine, which is an ester of pilocarpic acid, states that pilocarpic acid should be sought. The latter constitutes the first degradation product of pilocarpine due to the relative ease with which the ester bond splits. Nevertheless, it is important to bear in mind that the Pharmacopoeia fails to state whether the method of the monograph also provides an indication of stability. As a result, strictly speaking, the performance of forced degradation studies will prove to be indispensable.

If no analytical stability indicating method is to be found in the literature, the matter should be investigated as a matter of priority using the chromatographic method retained.

- Document the conditions of solvents, dilution and storage interval.
- Select calibration benchmarks.
- Establish the protocol for preparation of the sample whether for hospital pharmacy preparations or for standards.
- Study the matrix effect.

2.1 DEVELOPMENT OF A STABILITY INDICATING METHOD

Principle

A stability indicating method is an analytical procedure capable of distinguishing the drug to be analysed from its degradation products formed during the stability study in defined storage conditions. The method must be sensitive enough to detect degradation products in small quantities and sufficiently decisive to distinguish products with potentially similar structures.

To achieve this objective, there are two possible solutions:

- If the degradation products of the active ingredient to be analysed are available, you can check for the absence of interference between the various compounds using the chosen analytical method. Note that it is often difficult and even costly to obtain such compounds. Furthermore, hospital pharmacy preparations include excipients likely to be at the origin of other degradation products that may derive from a reaction with the active ingredient.
- If the degradation products are not accessible or if interaction of an excipient is suspected, forced degradation must be performed.

We advise conducting this forced degradation on a batch of the preparation used to validate the stability indicating capability of the analytical methods. The principle is described below together with some practical proposals for implementation.

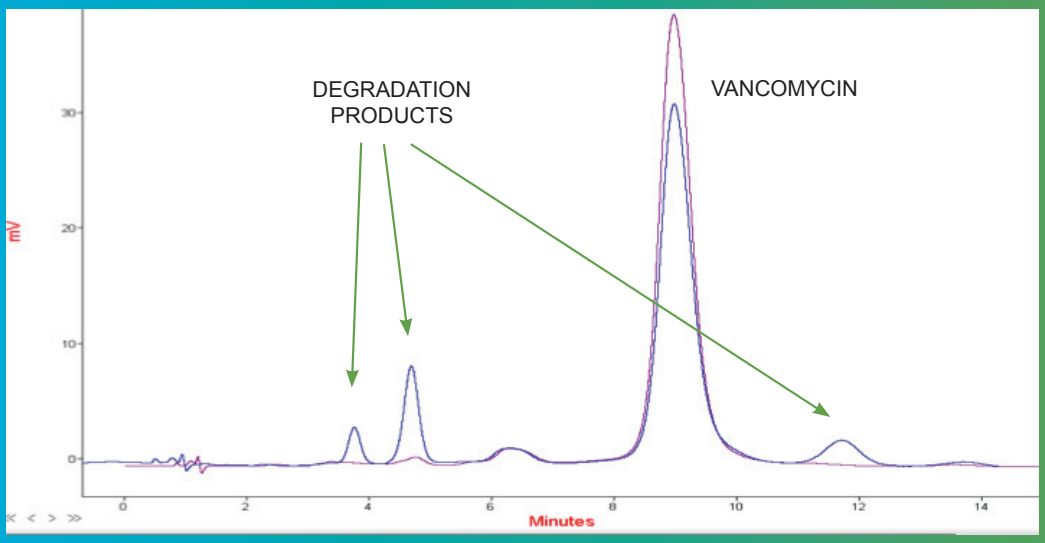
Forced degradation

The stability indicating capability is evaluated by a forced degradation that may also allow the product's degradation pathways to be determined. The nature of the forced degradation pathways will depend on the drug and its dosage form.
Forced degradation is generally performed on a batch and includes:

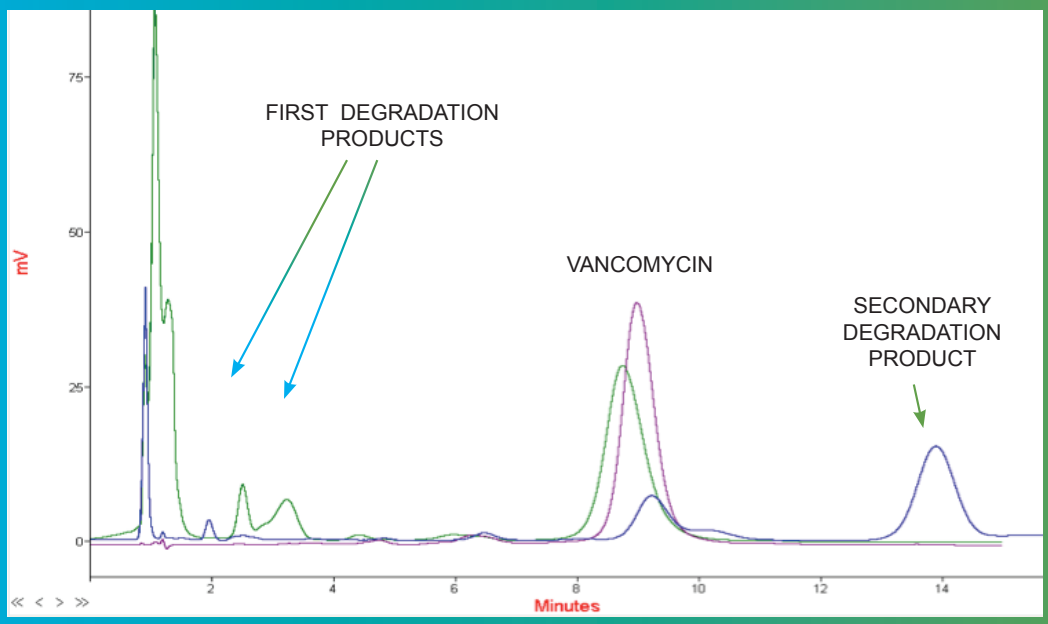
- significant variations in pH
- temperature variations
- an oxidation reaction
- a photolysis reaction

In practice, degradation will depend on the product and there is no standard method. The goal is not to destroy the entire molecule and obtain degradation products resulting from the first degradation products but to destroy about 20% of the molecule and highlight degradation products through their relative retention. Such degradation products must not be co-eluted with the molecule to be studied.

Intentional degradation - destroy just 20% of the molecule to visualise the first degradation products



Chromatographic plotting of a 125 µg/ml solution of vancomycin before degradation (violet curve) and after 4 hours at 80°C (blue curve): appearance of the first degradation products



Violet curve: 125 µg/ml vancomycin solution before degradation
Green curve: vancomycin after 1 hour in contact with a solution of NaOH: partial degradation of the molecule (about 20%) and appearance of the first degradation products
Blue curve: vancomycin after 24 hours in contact with a solution of NaOH: significant degradation of the molecule. Presence of secondary degradation products but no possible visualisation of the first degradation products. Excessively aggressive treatment – to be avoided.

(Charts taken from thesis by V. Maillot-Pyscszek, 2010)

The choice of an intentional degradation method may be influenced by the chemical structure of the active ingredient and its sensitivity to certain of these parameters. Some pointers are provided in the inset box.

EXPECTED DEGRADATION IN RELATION TO COMPOUNDS PRESENT

Hydrolysis: amides, esters, lactames, lactones
Oxidation: thiols, thioethers
Photolysis: olefins, acrylic halogenated derivatives, arylacetic acids, compounds with nitro-aromatic groupings, N-oxides

By way of an example, a method for a first approach to degrade a molecule is proposed *(method 1)*. This may be completed by methods 2 and/or 3 in relation to the known sensitivity of the product to oxidation and photolysis reactions.

Method 1

Several acid and base concentrations are used to degrade the molecule. According to the results obtained, the most adequate degradation condition is then modified to adjust the rate of degradation to about 20% of the solution's initial concentration.

A solution corresponding to 4 times the concentration of the middle of the range will be made, with this solution then subjected to variations in pH (HCl 0.1 N, HCl 0.5 N, HCL 1 N, NaOH 0.1 N, NaOH ,5 N, NaOH 1N) then neutralised with the corresponding acid or base.

Example: 1 ml of solution + 1 ml HCl 1N for 1 hour at 80°C then neutralised by 1 ml of NaOH 1N and diluted with 1 ml of mobile phase. The sample is thus diluted to a quarter and can be compared with a similar solution diluted to a quarter and not degraded *(see table 5)*.

To obtain the results mentioned above, the pH, temperature and duration conditions will be adjusted according to each molecule.

Preparation of solution								
Solution n°	1	2	3	4		5	6	7
Standard (mL)	1	1	1	0		1	1	1
HCl 0.1N (mL)	1				NaOH 0.1 N (mL)	1		
HCl 0.5N (mL)		1			NaOH 0.5 N (mL)		1	
HCl 1N (mL)			1	1	NaOH 1 N (mL)			1
Heat 1 hour at 80°C then neutralize by:								
NaOH 0.1 N (mL)	1				HCl 0.1N (mL)	1		
NaOH 0.5 N (mL)		1			HCl 0.5N (mL)		1	
NaOH 1 N (mL)			1	1	HCl 1N (mL)			1
Mobile phase (mL)	1	1	1	2		1	1	1

Table 5: Proposed method of forced degradation by acid, base and heat

These tests will be performed on standard solutions to assess the degradation specific to the active ingredient. Ideally, they will concern the active ingredient formulated in order to evaluate its behaviour in presence of excipients for the preparation.
«Micromethods» must sometimes be used due to the cost of the products (see box on bortezomib)

Degradation of bortezomib by micromethod according to Walker et al (2008)

The content of a vial of 3.5 mg of bortezomib is dissolved in 3.5 ml of distilled water to prepare a solution at 1 mg/ml. To 100µl of samples of this solution, 5µl of different concentrations of sodium hypochlorite are added (sodium hypochlorite at 1.00%, 0.80%, 0.70%, 0.50%,0.40%, 0.30%, 0.25%, 0.20%, 0.10% and 0.01%) prepared by dilution with distilled water). Each mix is shaken in the Vortex and immediately analyzed by chromatography.

Method 2

To study the oxidative degradation of a product within a preparation, the latter can be brought into contact with a solution of hydrogen peroxide between 3 and 30%.
For example, 1 ml of a solution containing 1 mg of product can be brought into contact with 10 ml of a solution of hydrogen peroxide at 6% and left in contact for 3 hours at 100°C. The percentage of hydrogen peroxide can then be increased if the product does not reach 20% of degradation, up to a maximum of 30%

Method 3

Forced photodegradation can be performed by exposure to light, using a combination of UV fluorescent, cold white lamps or metal halide or xenon lamps. The energy of the lamp will be at least 1.2 million lux.h-1 of fluorescent light (between 400 and 800 nm) and 200 Wh/m2 of UV light (between 320 and 400 nm). If decomposition is not observed, intensity can be increased by a factor of 5. Beyond this, the product will be considered to be photostable.
We recommend conducting at least one exposure of the preparation under a lamp generating UV A (320 to 400 nm): the preparation should be placed 15 cm from the lamp for a period of 6 hours.

The solutions obtained after degradation can then be analysed using the chosen analytical method and compared with the initial solutions before degradation.

The specificity of the HPLC method must be validated with respect to all the components. The method must allow a distinction to be made between degradation products of the active ingredient, excipients and additives to the preparation.

A detector giving access to the peak purity concept, meaning a diode array detector or a mass spectrometer has to be preferred.If this proves impossible, using a visible-UV detector will be acceptable on condition extreme care is taken when analysing the chromatogram. Any deformation of a chromatographic peak must lead us to suspect co-elution of the two products. This peak asymmetry may be studied in two different elution conditions: the operator may proceed with separation using a short gradient (30 minutes) or a long gradient (90 minutes) before proceeding to compare the profiles, with the long gradient allowing for more effective separation.

- Each degradation product will be identified by its relative retention time with respect to the retention time of the initial product (rather than the retention time, which will vary with the temperature if the column does not have a thermostat).

- The chromatogram of the initial solution before degradation will be compared with that of the solution after degradation to ensure identification and perform a semi-quantitative analysis of the degradation products

Mass balance

Analysis of the results obtained from this forced degradation should preferably be completed by evaluation of the mass balance.

In practice, the mass balance is obtained by comparing the peak surface of the non-degraded sample with the sum of the surface of peaks of the degraded sample and degradation products. The sum of all the peaks must be close to the surface of the non-degraded peak. If a significant difference exists, a number of hypotheses can be put forward:

- some degradation products are not highlighted by the chromatographic system and the method proves questionable,

- the response coefficient for the impurity is different to that of the active ingredient.

2.2 CONDITIONS OF SOLVENTS AND DILUTION

It is essential that the solvents used, especially when diluting standards or samples, allow the entire analyte to be rendered soluble when it is introduced in a known quantity and diluted in a known volume.

Various sources of information can provide a support, among which European Pharmacopoeia monographs that allow the degree of solubility of a molecule in a given solvent to be determined. In the case of a salified organic molecule, it is important to take into account the nature of the salt as differences in solubility can be observed in relation to the counter-ion.

Where data are not available or in the event of doubt, it remains possible to check rapidly whether the solubilisation conditions actually allow for complete dissolution of the analyte. To do so, two stock solutions with different concentrations are prepared by independent weighing. Each stock solution should then be diluted so as to obtain identical working concentrations. Each working concentration is injected pure and half diluted six times. Processing of the results involves comparing the following:

- The mean intensity of the signal obtained for each pure and diluted working concentration: these intensities must show a factor of 2. This means you can ensure that working concentrations do not lead to saturation of the detector.

- The mean intensity of each working concentration derived from different stock solutions. The signals must have intensities that are not significantly different. If the signals recorded for the working concentration derived from the most highly concentrated stock solution are lower than those obtained for the other working concentration, this may indicate a solubilisation defect.

2.3 ASSAY RANGE

The assay range depends on the molecule used. In the case of an assay method applied to an active ingredient in a dosage form, an assay range between 60 and 140% of the target value can be applied.

Expanding the range to add an additional point higher than the last point on the scale will allow you to check that the concentrations retained do not lead to a saturation of the detector response. The measurement range should be defined by independent weighing in order to ensure control over possible errors in preparation (weighing or dilution), or to prepare the range from a “stock” solution that diluted to match each point on the scale, using this range with reference to a quality control mechanism based on independent weighing.

2.4 CALIBRATION

All indirect dosing methods require implementation of calibration and thus a reference substance whose concentration/mass is perfectly well known and under control. This therefore involves knowing the purity and water content of the reference substance, and storing the substance in appropriate conditions.

A number of approaches can be envisaged:

- Using a reference from the European Pharmacopoeia,
- Using a suitable raw material and a batch other than that going into production,
- Using a chemical reagent that will be accompanied by an analysis report specifying the purity and water content.

The conditions for conservation of these samples will be directly dependent on the physico-chemical properties of the target molecule. The parameters to be set are the temperature for conservation, protection against light, humidity and oxidation.

Two remarks can be made concerning the choice of the standard substance:

- Many active ingredients are organic molecules that are administered in a salified form. It is possible to use another salt as a reference where it is easier to purchase or for reasons of stability. In this case, attention must be paid to conversions in relation to the molecular weights and respective solubility of the different salts.

Example: adrenalin is marketed in the form of a solution of epinephrine hydrochloride and adrenalin bitartrate can be used as a reference.

- In all events, it is absolutely not recommended to use a pharmaceutical speciality or a hospital pharmacy preparation as a standard as the drug dosage has to be determined accurately for each batch. Furthermore, the accuracy of the quantities given for packaged preparations is not sufficient for use in developing analytical methods.

Example: vancomycin content varies from 85 to 115%.

2.5 EVALUATING THE MATRIX EFFECT

In the case of interference caused by excipients, exploration must be conducted during the linearity study through preparing ranges with and without excipients, with the ratios of concentrations between the active ingredient and the excipients reproducing the composition of the finished product. The results for these concentrations can then be processed to ensure there are no significant differences between the slopes and the y-intercepts observed in each condition.

3 STATISTICAL VALIDATION OF THE METHOD

The assay method developed must be covered by statistical validation. To this end, it must answer to the various criteria defined below. The theoretical approach for validation of the method will be described and an experimental design proposed. By following the described experimental design, the results obtained can be entered into the computer program to ensure calculation of the parameters so as to qualify or invalidate the assay method.

3.1 THEORY

Response function

DEFINITION

This response function can take the form of a straight line (linear response) or any other function (non-linear response = quadratic response). The validation principles remain the same.

It should be noted that linearity is the response function model most frequently used. This is most often due to the equipment available in hospital pharmacies (UV spectrophotomers, HPLC coupled with a visible-UV detector or a fluorimeter). However, other regression models, as with a quadratic or logistic regression, can also be considered as alternatives where this corresponds to the response model of the detector used.

By definition, the linearity of an analytical procedure is its capacity within a given assay range to obtain results directly proportional to the quantity of analyte present in the sample.

DETERMINATION

The response function is determined by the successive passage of three independent ranges, each point of the range being prepared from an independent weighing.
In order to check whether a matrix effect exists, two types of range are produced:

- A range only containing the active ingredient
- A reconstituted range (with excipients)

Accuracy

DEFINITION

Accuracy expresses the closeness of the match between the mean value obtained from a series of test results and a theoretical value considered to be true. This expresses the systematic error of an assay method.

DETERMINATION

The accuracy will be studied from data obtained during study of the calibration function. For each point on the scale, the preparation is made from a weight value. As a result, the theoretical concentration (X) is readily known. The range obtained gives access to an equation (Y=aX+b). The intensity of the signals recorded for each point of the scale allows the

calculated concentrations (Z) to be computed on the basis of the inverse equation (=(Y-b)/a)The ratio between the calculated concentrations and the theoretical concentrations provides a rate of overlap (R%) defined by the following equation: R%=100.Z/X

Precision

DEFINITION

Precision expresses closeness of the match (coefficient of variation and degree of dispersion) between a series of measurements coming from the same uniform sample (independent test results) in determined conditions.

Reliability gives information only on the distribution of random errors and gives no information on the specified (or true) value. Reliability can be evaluated at different levels.
Repeatability concerns independent test results obtained within the same laboratory, with a determined analysis method, on identical test samples prepared by the same operator and analysed with the same equipment within a very short time slot.

Intermediate precision concerns independent test results obtained within the same laboratory, with a determined analysis method and identical test samples. The other operating conditions can be different: operators, equipment and longer time periods between tests (on different days).
Reproducibility concerns independent test results obtained with a determined analysis method and identical test samples. The other operating conditions (operators, equipment and laboratories) are different.

DETERMINATION

To study the **repeatability** of the method, 100% analysis of six points of the range is performed, each point being injected once and each point being prepared by an independent weighing
To document **intermediate precision** the process used to study repeatability can be reproduced twice.

Limits of quantification and detection

The limits of quantification and detection are not a priori necessary for analysis of an active ingredient within the scope of a stability study. For information, the definition and determination method will be outlined.

Limit of quantification

DEFINITION

The limit for quantification is the smallest quantity of analyte in a sample that can be quantified to a defined level of accuracy.

DETERMINATION

Determination of the limit of quantification can be performed through three approaches, the first two corresponding to analyses accompanied by graphic recording (case of chromatography for example) :

- Approach based on the signal/noise ratio: this approach can be considered if the analysis method used presents a background noise recording. In most cases, a signal/noise ratio at 10 is accepted as a limit of quantification. In practice, solutions with a known concentration are analysed. The solution will be diluted until the signal/noise ratio shows the expected value.

- Approach based on the maximum amplitude of the background noise: the maximum amplitude of the background noise is determined over a distance equal to 20 times the mid-height width of the peak for the dosed analyte. This maximum amplitude is noted “hmax“. The limit of quantification can be calculated by :

$$\text{Limit of quantification} = 10 \times h_{max} \times \frac{\text{Injected quantity}}{\text{recorded signal (height)}}$$

- Approach based on the response standard deviation

$$\text{Limit of quantification} = 10\ S_{blanc}$$

- This approach can be retained where test performance is not accompanied by a recording (case of potentiometry for example). This will thus involve measuring the response of a «blank” (matrix containing all components of the product analysed with the exception of the dosed analyte) injected at least 6 times and deducing from it the standard deviation (S_{blank}).

Limit of detection

DEFINITION

The detection limit corresponds to the smallest quantity of analyte in a sample that can be detected but not quantified.

DETERMINATION

Determination of the detection limit can be achieved using one of three approaches, with the first two corresponding to analyses accompanied by a graphic recording (case of chromatography for example):

- Approach based on the signal/noise ratio: this approach can be considered if the analysis method used presents a recording of the background noise. In most cases, a signal/noise ratio at 3.3 is accepted a detection limit. In practice, solutions with a known concentration are analysed. The solution will be diluted until the signal/noise ratio shows the expected value.

- Approach based on the maximum amplitude of the background noise: the maximum amplitude of the background noise is determined over a distance equal to 20 times the mid-height width of the peak of the analyte sought. This maximum amplitude is noted «hmax». The limit of quantification can be calculated using the formula :

$$\text{Limit of quantification} = 3{,}3 \times h_{max} \times \frac{\text{Injected quantity}}{\text{recorded signal (height)}}$$

→ Approach based on the standard deviation of the response

Limit of quantification = 3,3 S_{blanc}

This approach can be retained where test performance is not accompanied by a recording (when using potentiometry, for example). This will thus involve measuring the response of a «blank" (matrix containing all components of the product analysed with the exception of the dosed analyte) injected at least 6 times and deducing from it the standard deviation (Sblank).

3.2 EXPERIMENTAL DESIGN FOR VALIDATION

Day #1

→ Passage of a range without excipients at 5 points (between 60 and 140% of the target value), each point being prepared via independent weighing.

→ Passage of a range with excipients at 5 points (between 60 and 140% of the target value), each point being prepared from independent weighings.

→ Quality Control (QC) 100% of the theoretical value: preparation of 6 controls by independent weighings and injection of each solution once. The CQ is prepared as the 3rd point of the reconstituted range.

Day #2 & 3

→ Twice reproduce what was done on day 1.

All data will be entered into the Valid XXL program that will ensure statistical processing of the results. To determine the software's computation procedures, you may refer to Appendix 2.

3.3 INTERPRETATION OF THE VALIDATION RESULTS: TAKING A STEP FURTHER …

Validation of an analytical method does not represent a goal in itself. While it provides a response to legislative requirements, the results obtained must above all provide for knowledge as to performances of the analytical method, and accuracy and reliability representing respectively the systematic bias and random error.
Thus, *G. de Fontenay et al. (2011)* describe two approaches that can be used to evaluate the risk of false negatives (obtaining data outside specifications whereas the batch is compliant) that can be generated by a validated analytical method. They concern:

Capability: quality method described since 2007 in the USP that caters for the tolerance interval, bias and inaccuracy.
Operating Characteristic Curves (OCC) constitute graphic representations of curves for levels of risk relating to data being generated outside specifications although the batch remains compliant.

The authors of the present article recommend using these two approaches as from the development stages. So it will be possible to revisethe method if it does not present the adequate performances.

If plotting curves for OCCs requires a number of calculations to be made, the approach based on capability (Cp) is readily accessible. This is computed in accordance with the following formula: if mean accuracy includes the value 100%,

$$Cp = \frac{[\text{Difference between upper and lower limits of the confidence interval}]}{6\sigma}$$

If the mean accuracy is different to 100 %, the lower of the values obtained using the two following formulae will be retained

$$Cp = \frac{[\text{Upper limit of the confidence interval - mean accuracy}]}{3\sigma} \text{ or}$$

$$Cp = \frac{[\text{Mean accuracy - Lower limit of the confidence interval}]}{3\sigma}$$

According to the value obtained for Cp, the method's performances will be:

→ Unacceptable for a Cp value lower than 0.3: another method will have to be developed

→ Extremely poor for a Cp value between 0.3 and 0.6: improvements will have to be made to the method

→ Poor for a Cp value between 0.6 and 0.8

→ Average for a Cp value between 0.8 and 1.0

→ Good for a Cp value between 1.0 and 1.33. However transferring the method to another laboratory may prove difficult

→ Very good for a Cp value higher than 1.33: the method may then be used on a routine basis.

Within the scope of a stability study for a given preparation, analysis of the active ingredient and degradation products as described in section 3 must be supplemented by testing of the other parameters to ensure that the product retains its initial properties during storage. These parameters will depend on the pharmaceutical form covered by the stability study.

We have opted to address the different pharmaceutical forms individually, with a distinction between liquid forms and solid forms and, within liquid forms, treating solutions and dispersions (emulsions and suspensions) independently. To date, only the issue of solutions has been comprehensively covered. We then go on to focus on the analysis of dispersions, especially parenteral nutrition mixes, and then solid forms.

CHAPTER5

Other analyses depending on pharmaceutical forms

1 LIQUID PHARMACEUTICAL PREPARATIONS

Before considering the stability of liquid forms we should take a further look at the concept of solubility, an essential factor to be controlled. The physico-chemical characteristics of products to be solubilised will have a direct influence on formulation and bio-availability, but also on the stability of liquid preparations. Here, solubility depends on the chemical nature of the product to be dissolved and that of the solvent to which it is added. It should be remembered that solubility correlates directly with temperature. During stability studies, this parameter must be taken into account in relation to temperature.

1.1 SOLUTIONS

«From a pharmaceutical point of view, a solution is often formed by one or more liquids that are mutually miscible, known as solvents, in a majority proportion, and by one or more substances in the dissolved state known as solutes." (Wehrlé, 2012)

Solvents are generally aqueous, hydro-alcoholic or oily solutions. Depending on the solvents used, a number of properties can be verified according to the type of pharmaceutical application (oral, buccal, parenteral, ophthalmic, dermal, rectal or other routes). Refer to the relevant Ph. Eur. monographs.

In terms of stability, liquid preparations are more subject to physical and chemical instability (various degradations, hydrolyses, oxidation, etc.) or bacterial contaminations.

Various parameters can be tested to ensure that the initial properties of the solutions are maintained throughout their conservation lifetime.

The main parameters to be checked and the pharmaceutical techniques to be used are detailed hereunder.

1.1.1. CLARITY

Description

A solution is a monophase liquid preparation in which active substances and excipients are a priori totally dissolved. Three Ph. Eur. monographs address the matter of clarity:

→ «Clarity and degree of opalescence of liquids» (2.2.1.). this monograph describes a visual method allowing a liquid to be examined to be compared with extemporaneous control suspensions, and instrumental methods (nephelometry, turbidimetry, etc.) based on measurement of optical density after shaking. This is to be applied for example to solutions intended for oral, dermal, rectal and other routes.

→ While for certain solutions the number of particles remaining in suspension is of little significance, for others, and especially for parenteral solutions, such notions of clarity and absence of particles are compulsory. Indeed, in accordance with Ph. Eur., "Eye-drops that are solutions, examined under suitable conditions of visibility, are practically clear and practically free from particles." and "Eye-drops that are solutions, examined under suitable conditions of visibility, are practically clear and practically free from particles." Two types of particles are now listed under Ph. Eur.: visible particles (□ 50µm) and sub-visible particles as defined by 2 threshold sizes □ 10 µm and □ 25 µm.

Each type of particle is then covered by a dedicated monograph.

→ "Particulate contamination: visible particles» (2.9.20.).

→ "Particulate contamination: sub-visible particles» (2.9.19.).

Interest

The clarity test in stability studies is intended to check that a product really is fully dissolved and that it does not slowly precipitate after fabrication. Changes in appearance over time mean there has been a degradation of the preparation resulting in cloudiness or formation of a precipitate.

Apparatus

To determine the number of particles, as we have observed there are two methods described in Ph. Eur.

VISIBLE PARTICLES

Matt black panel
These installations, known as viewing stations, comprise white non-glare and matt black panels above which an adjustable lamp-holder (apparatus described in Ph. Eur. 2.9.20.) or a depolarising screen is positioned so that the particles in movement can be seen once the ampoules or vials have been slowly shaken. This visual method is the preferred one to monitor the evolution of visible particles.

Automatic particle counters
Various techniques are available on the market. The drawback with these systems is their inability to detect stationary particles. They will therefore not be retained to conduct monitoring of the appearance of visible particles within the scope of a stability study for a preparation.

SUB-VISIBLE PARTICLES

To determine particulate contamination, 2 processes are described by Ph. Eur. in monograph 2.9.19. (Particulate contamination: sub-visible particles). This monograph is applicable for injectable preparations and preparations for infusion, but it appears possible to extend its applicability within the scope of stability studies to other dosage forms in order to detect a possible instability by precipitation of very fine particles.

Optical microscope
The principle is described in method 2 of Ph. Eur.: microscopic particle count test. This involves filtering a solution over a dark membrane and, using a binocular optical microscope equipped with a micrometer, observing the presence of any particles. The number of particles found then has to be related to the volume filtered and compared with the threshold defined in the pharmacopoeia. Within the framework of a stability study, the result obtained after the conservation time will be compared with that determined immediately after production of the preparation.

Light opacity spectrophotometer
The principle is described in method 1 of Ph. Eur.: light obscuration particle count test. A laser beam traverses a sample of liquid in movement and the presence of those particles leads to a reduction in light proportional to their size. This means the size of the particles can be determined and thus counted. A dedicated Ph. Eur monograph explains how the method works, 2.9.31. “Particle size analysis by laser light diffraction.” 2.9.31. Analyse de la taille des particules par diffraction de la lumière laser

Table 6 summarises the limits in visible and sub-visible particles according to the dosing forms concerned.

	Visible particles	Sub-visible particles	
Injectable solution	0	≥ 10µm	≥ 25µm
<100 ml >100 ml	0	< 6000 particles / recipient* < 25 particules / ml	< 600 particles / recipient* < 3 particules / ml
Eyes drops in solution	0		

*The volume of the sample analysed must be at least 25 mL. If the volume of the preparation is <25 mL, bring together several recipients. Expression of the results must take into account the number of recipients.

Table 6: Limits in visible and sub-visible particles of preparations in the form of solutions.

1.1.2 PH

Description

Pharmaceutical solutions are often obtained by solubilisation of an active substance with a given pH. Indeed, the pH may allow an ionic form of an acid or base function to be obtained, thus facilitating solubilisation. The pH can also be set to allow for tolerance of the pharmaceutical form. Indeed, some pHs are incompatible with certain administration routes.

Interest

On conservation, a number of causes can lead to a change in pH, including a degradation of the active ingredient itself, degradation of an excipient, a container/content interaction or diffusion of ambient carbon dioxide through the packaging. Thus, any change in pH will reflect a change in the initial prepared solution.

Apparatus

There are two systems to measure pH: litmus paper and the pH-meter. However, within the scope of a stability study, the litmus paper technique is not sufficiently precise. Only the pH-meter will therefore be described in the present section. When the probe of a pH-meter is immersed in a solution, an electrical voltage (T) appears on the terminals of the electrodes due to an electrochemical cell phenomenon. When equilibrium is reached between the solution studied and the probe, this voltage becomes a decreasing linear function of the pH:

$$T=A-B.pH$$

where A and B are coefficients that depend on the nature of the electrodes, the solutions in which they are immersed, and the temperature. Due to this latter parameter, any calibration of a pH-meter must be performed coupled with a temperature sensor.

It should be recalled that the pH is a parameter which depends on the nature of the liquid medium. pH-meter probes specific to the solution must therefore be used. There is a difference between probes for aqueous media on the one hand and organic media on the other.

Calibration of the pH-meter allows for the adjustment of these 2 coefficients A and B thus permitting a precise determination of the pH. This technique is highly sensitive and perfectly suited to stability studies. The description of the apparatus, its operating method and the preparation of reference buffer solutions are referenced in Ph. Eur. monograph 2.2.3. “Potentiometric determination of pH”.

1.1.3 OSMOLARITY/OSMOLALITY

Description

L’osmolarité est la concentration de molécules osmotiquement actives par unité de volume. Elle se différencie de l’osmolalité qui est la quantité de molécules osmotiquement actives par unité de masse. On entend par molécule osmotiquement active, tout élément (ion, molécule) capable d’attirer les molécules d’eau au travers d’une membrane. Cette force d’attraction est appelée pression osmotique. En pratique, l’osmolalité est une façon globale de mesurer la contribution des différents solutés, présents dans une solution, à la pression osmotique de cette solution. Elle fait l’objet de la monographie 2.2.35. « Osmolalité » à la Ph. Eur.

Interest

In some situations, it can be advantageous to prepare an iso-osmolar solution in blood. The solution is then said to be isotonic. This property is sought for to ensure enhanced tolerance of eye drops, injectable solutions, etc. A solution that does not degrade keeps constant osmolarity. Within the scope of a stability study, measuring osmolarity can be considered to provide an additional parameter to help confirm chemical stability that should be highlighted by analysis of the active ingredient and monitoring of degradation products.

Apparatus

Within the scope or stability studies, the most appropriate apparatus is an osmometer. Osmometers currently marketed for hospital applications determine the osmolarity of solutions by measuring the freezing point depression, meaning the reduction in freezing point caused by chemical species in solution. The osmolarity of a solution can be calculated from the value of the freezing point depression (δc) using the following equation :

$$Cosm = -\frac{\delta c}{Kc} X\ 1000$$

Cosm : osmolarity en mOsm/kg
Kc: cryoscopic constant (Kc= 1.86 for an aqueous solution)
δc : freezing point depression in °C

For example, an isotonic solution of sodium chloride at 0.9% has a freezing point depression equal to – 0.52°C.

1.1.4 COLORATION

Description

A coloured pharmaceutical solution must not be subject to a change in its colouring at any time during its conservation. Colouring of a solution can be evaluated by the naked eye or by visible spectrophotometry.

In the monograph “Degree of coloration of liquids” (2.2.2.), Ph. Eur. describes two processes to assess the degree of coloration of liquids in the brown-yellow-red shades. This involves using the naked eye to assess shades in diffuse daylight as compared with control solutions produced in accordance with the protocol described in the monograph.

The other technique of absorption spectrophotometry in the visible is based on the Beer-Lambert law.

Supposing a monochromatic radiation with a fixed wavelength traversing a sample of thickness, the Beer-Lambert law shows that there is a proportional relationship between the signal measured (A) and the concentration of the solute (C):

that is : A=ε.l.C

With :
A : absorbance
ε : molar absorption coefficient
l : tank width in cm
C: molar concentration of the solution

Interest

A pharmaceutical preparation in solution form must, throughout its conservation, keep a constant composition, meaning constancy of all its physical properties, including coloration. Indeed, a change in colour means a change in composition. However, lack of change in colour does not mean that a change in composition has not occurred.

Apparatus

Visual inspections: testing scientists must maintain a reference colour palette in order to accurately establish the initial colour then, on each inspection, check that this colour is always the same. Generally, a control tube has to be produced with the newly created solution (extemporaneous preparation) and the coloration of this extemporaneous preparation has to be compared with the conserved preparation. There should be no visible difference. This method remains relatively insensitive.

Visible spectrophotometer: as soon as the substance is coloured it will show the property of absorbing in the visible, hence the use of a visible spectrophotometer (spectro-colorimeter) that, from a range, allows for an absorbance of the initial solution that should not change during conservation. This method is to be preferred to the previous one, as it is more sensitive.

This analytical technique is covered by a Ph. Eur. monograph “Absorption spectrophotometry, ultraviolet and visible” (2.2.25.). It should be stated that, in Ph. Eur., the coefficient known as specific absorbance of a dissolved substance (or specific extinction coefficient) is not expressed in moles, but represents the absorbance of a solution at 10 g/L (1%) having traversed a tank of 1 cm at a determined wavelength, whence:

$$A_{1\ cm}^{1\%} = \frac{10\ \varepsilon}{M_r}$$

où M_r : Masse moléculaire relative

1.1.5 ODOUR

Description

Odour can change during conservation. Ph. Eur. Specifies in the monograph “Odour” (2.3.4.) that the inspection must be performed on a watchglass, spreading 0.5 to 2 g of the substance to be examined. After 15 minutes, it is possible to define the odour or to ensure the absence of odour. Studies intended to evaluate the odour are only to be conducted in the presence of a non-toxic product.

1.1.6 VISCOSITY

Description

Viscosity can be defined as the friction forces that work to oppose the flow of layers of liquid in relation to each other. Each solution will have a more or less significant viscosity. Viscosity is directly related to temperature. The various ways to express this are defined in Ph. Eur. monograph 2.2.8. “Viscosity» where dynamic viscosity or the coefficient of viscosity η are defined as being the expression of the tangential force per surface unit (called shear stress τ and expressed in Pascals), needed to displace a layer of liquid in parallel to the slide plane by one square metre at a speed (v) of one metre per second in relation to a parallel layer located at a distance of one metre (x).

Interest

During conservation, viscosity can change either giving more fluid or, conversely, more viscous products. This property must not change over time.

Apparatus

A rotating viscometer is generally used. Its principle is based on measuring the shear forces that are exerted on a moving part when it is brought into rotation in a liquid at a constant rotation speed. Three pieces of apparatus are specified in monograph 2.2.10. “Viscosity – Rotating viscometer method»: concentric cylinder viscometers, cone-plate viscometers and pin viscometers. They can be used to measure the viscosity of liquids said to be Newtonian or non-Newtonian.

There is also another technique recorded in Ph. Eur. in monograph 2.2.9. “Viscosity – Capillary viscometer method» that involves calculating the time needed (to within 1/5th of a second) for the level of liquid held at 20°C to go from one mark to another in a precisely described calibrated tube (suspended ball level viscometer).

One further technique is detailed in Ph. Eur. in monograph 2.2.49.: ‘Falling ball viscometer method’, used in the case of Newtonian liquids (whose viscosity is independent of shearing). This involves determining the time needed for a ball in liquid held at 20°C to cover the distance defined between two marks, with the tube being positioned on a plane tilted by 10° in relation to vertical.

SUMMARY OF ANALYSES TO BE CONDUCTED

In stability studies, the order of priority will vary depending on the type of pharmaceutical preparation being tested. When conducting stability studies on solutions, Table 7 offers an indication of the order of priority of the tests to be performed, according to the dosage form of the preparation.

	Clarity	pH	Osmolarity	Colour[1]	Odour[2]	Viscosity
Syrup/potion	X	X		X	X	XXX
Dermal solution	X	XXX		XXX	X	
Injectable solution	XXX	XXX	XXX	XXX	X	
Eye drops in solution	XXX	XXX	XXX	XXX	X	X

[1] if the active ingredient is coloured in solution
[2] if the active ingredient is non-toxic

Table 7: Analyses recommended according to the dosage form of the solution (to be completed compulsorily by an analysis of the active ingredient and its degradation products)

Within the scope of a stability study, it is important to take into consideration all the parameters that can be used to check that the preparation has kept every one of its initial properties throughout its period of conservation. A simple definition of stability based on a variation in concentration of active ingredients within margin of ±10% is to be rejected. Such an approach would be over-simplistic as it would not take into consideration the therapeutic aspect (risk of under-dosing for certain active ingredients if there is a drop in concentration of less than 10%), the potential toxic risk of degradation products and possible instability of a preparation without loss of active ingredient.

CHAPTER6

INTERPRETATION OF RESULTS

As specified in the ASEAN guidelines (2005), the stability of an active ingredient or a pharmaceutical speciality is its ability to maintain its properties within specific limits throughout its lifetime. The chemical, physical, microbiological and biopharmaceutical aspects of the preparation must be taken into consideration. The problem lies in how specific limits are defined. Concerning the concentration in active ingredients and its change over time, most publications report an acceptable limit of 90% of the initial value. This limit is open to debate. Meanwhile, other parameters (degradation products, pH, turbidity, etc.) are not covered by defined acceptable limits.

The flowchart shown in figure 7 is offered as an aid to interpreting the results obtained during stability studies for preparations. This decision support tool designed on the basis of benchmarks and recommendations *(ICH Q1E 2003, ICH Q3A and Q3B 2006, Asean Guidelines 2005, Bardin et al 2011, European Pharmacopoeia 2013)* and hospital practice takes into consideration the results of all physicochemical analyses and replaces them in a clinical and toxicological context.

So as to allow for uniform interpretation of the results, the way the said results are expressed needs to be harmonised. We suggest that the recommendations described below be followed.

Expression of results

Active ingredient concentration: the active ingredient concentration is expressed as a percentage of the initial concentration obtained at T0, meaning immediately after manufacturing of the preparation. For each time, the mean percentage is calculated together with the 95% confidence interval around that mean *(see box).*

MEAN AND 95% CONFIDENCE INTERVAL

The 95% confidence interval (CI) corresponds to the interval of values that has a 95% likelihood of containing the true value of the estimated parameter. It allows the variability of analyses to be taken into consideration. Just taking into account the mean can falsify the results if their variability is too great.

Formulation of the confidence interval around an observed mean with a standard deviation observed σ (X) on a sample of size n.

$$\left[\bar{x} - 2\frac{\sigma(X)}{\sqrt{n}}; \bar{x} + 2\frac{\sigma(X)}{\sqrt{n}}\right]$$

The mean concentration obtained at T0 is estimated at 100%. The deviation as a percentage in relation to that value (deviation by more than ± 10% or between 0 and 10%) is followed as shown in figure 8.

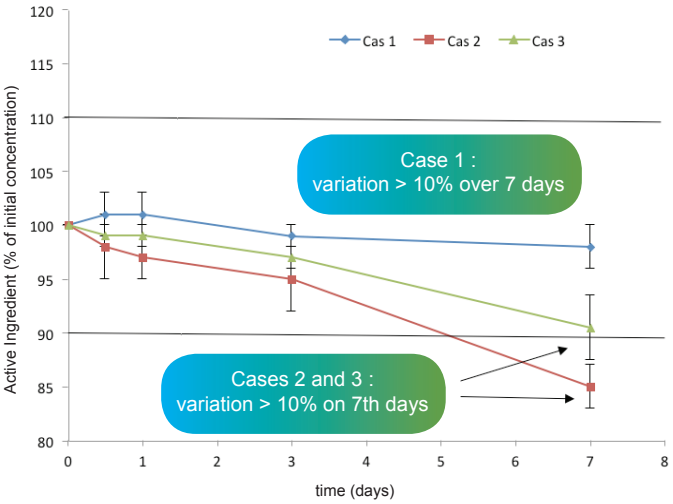


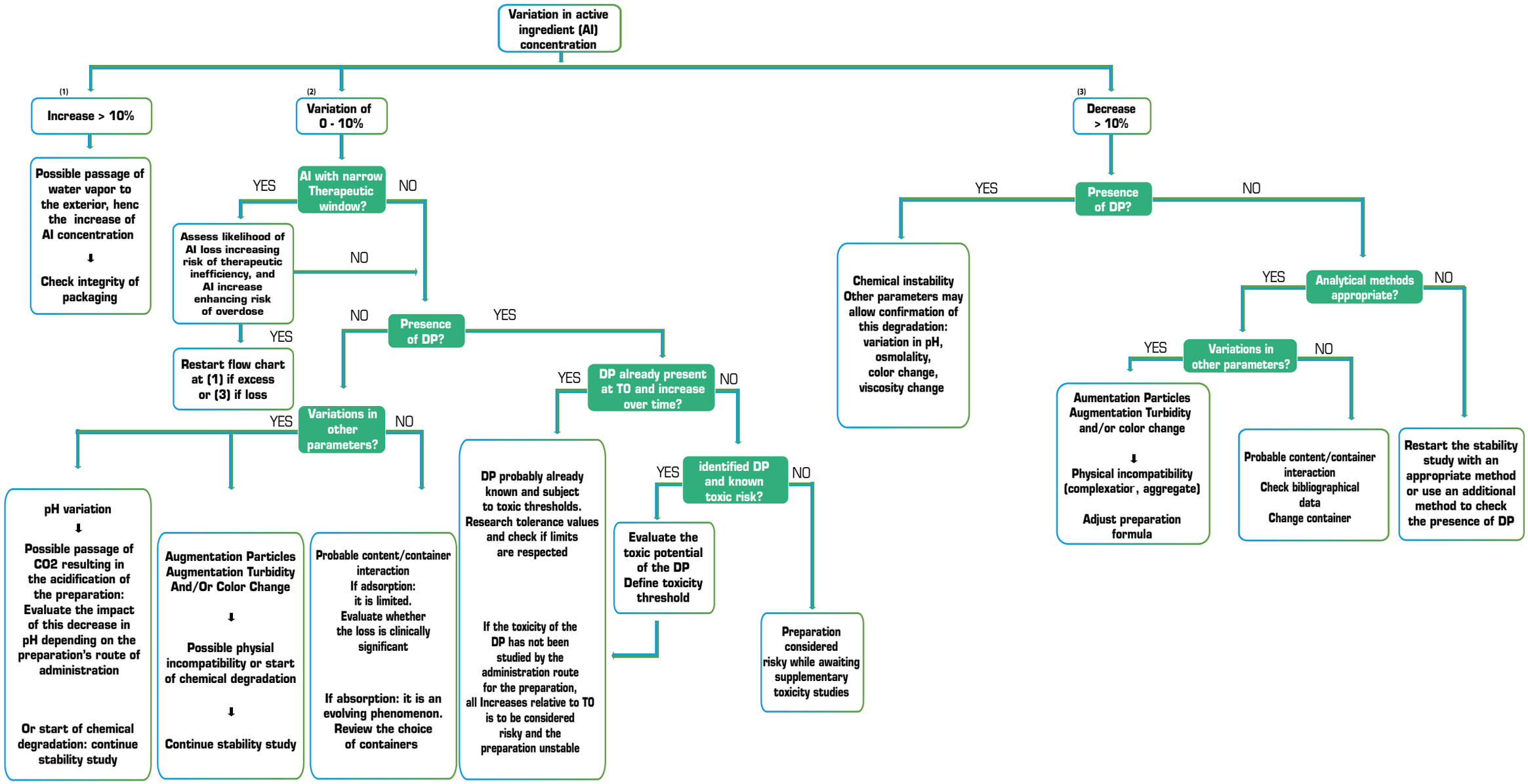
Figure 8: Evolution in concentration of active ingredient: expression of results

Degradation products: a quantitative analysis of degradation products is not required but there is a need for attentive monitoring of their evolution over time. During studies of intentional degradation *(see section 4)*, the degradation products are identified chromatographically by their relative retention time (tr) in relation to that of the active ingredient. Their presence in the studied preparation is sought thanks to tr and their change over time is estimated in relation to the increase in the surface of the corresponding peak(s).

Number of non-visible particles: the results are expressed in number of particles per recipient for preparations of less than 100 mL and in number of particles per mL for preparations of more than 100 mL in order to compare the results obtained with the values required by the European Pharmacopoeia (see section 5). An expression of the results as a percentage of the initial value is of no interest.

Values for **pH, turbidity and viscosity** are expressed in their respective units as specified in section 5. Monitoring of the mean value (with a CI at 95%) is performed over time. An expression as a percentage is not necessary as it contributes no useful information for data interpretation.

Colour change: evaluation of the change in colour is performed qualitatively in comparison with the colour of the preparation initially, immediately after manufacturing.



AI: active ingredient
DP: degradation products

Figure 7: Helps interpreting the results of a stability study on a preparation in solution form

Interpretation of results: Flowchart description (figure 7)

What is the variation in concentration of active ingredient after dosing?

1st case: the concentration of active ingredient varies by more than 10% in relation to the initial concentration.

This situation leads to the preparation being considered unstable. The physico-chemical instability expressed by this significant variation in concentration of active ingredient may be explained by analysis of the other parameters.

There are a number of possible situations:

The concentration of active ingredient increases over time (arm 1 of the flowchart): this situation of overconcentration of active ingredient suggests a reduction in the solvent in which the compound is diluted. The hypothesis of a diffusion of water vapour to the exterior of the container can be envisaged. It will therefore be important to check that a correct choice has been made with respect to the material(s) making up the packaging of the preparation to ensure it (they) are suited to the conservation conditions. If that is the case, the integrity of the packaging must be checked as it may have been subject to change during the preparation's conservation time.

The concentration of active ingredient diminishes over time (arm 3 of the flowchart). Another question then arises as to whether this loss of active ingredient within the preparation is isolated or related to a modification of other physico-chemical parameters, especially the presence of degradation products.

- The loss of active ingredient is not accompanied by degradation products. Firstly, there is a need to ensure that the dosing technique of the active ingredient is appropriate.

If the method does not indicate stability, the stability study is to be repeated using an appropriate technique.

If the method used does not allow the degradation products to be visualised (example of degradation products with absence of chromophore precluding detection under UV), an analysis of the samples using another technique may be considered (for example, by IR spectrometry).

If the analysis method proves to be appropriate, a loss of active ingredient without presence of degradation products or variation in the other physico-chemical parameters (pH, number de particles, turbidity and osmolarity) would suggest an interaction between the content and the container. It will then be necessary to check compatibility between the active ingredient and the materials making up the packaging. A change in the container should be proceeded with in the event of absorption type interaction. If the interaction is limited to an adsorption phenomenon (surface interaction whose evolution is limited over time) the sites may be saturated before packaging of the preparation.

A reduction in concentration of active ingredient without the presence of degradation products may also be observed in the case of physical instability as with a formation of aggregates or complexes. Particle counting and measurement of turbidity may provide additional information to interpret such instability.

The loss of active ingredient is associated with the presence of degradation products: the active ingredient is subjected to chemical degradation within the preparation. This may involve hydrolysis, photolysis or other phenomena as described in section 1. If other physico-chemical parameters (pH, osmolality, viscosity or colour change) are monitored this will allow such degradation to be confirmed and the mechanism to be interpreted. The origin will be pinpointed by the conservation conditions of the preparation concerned by that instability (e.g., light or temperature).

- Whatever the case, for any loss in active ingredient by 10% associated with the presence of degradation products, even in very small quantity, the preparation will be considered to be unstable in the studied conditions.

2nd case: the variation in concentration of the active ingredient does not exceed 10%.

In this case, the interpretation of results is more complex and must take into consideration other parameters but also clinical and toxicological concepts.

When the variation in concentration of active ingredient is significant and remains unexplained by analytical variations (the variation in concentration should be considered in relation to the accuracy of the analytical method), it will be appropriate first of all to determine whether it can have repercussions in terms of therapeutic efficacy and toxicity. For some active ingredients with a narrow therapeutic window, a slight variation in their concentration can be responsible for under- or over- dosing. For example, it is recommended for cytotoxics with a high haemato- or neuro- toxic potential to remain within the variability limit of 5%. For each preparation, the clinical impact of a variation in concentration must be evaluated taking into consideration the active ingredient's therapeutic window.

If the active ingredient has a narrow therapeutic window, a variation by more than 5% in its concentration in the preparation should not be considered acceptable. The interpretation of results will then be identical to the 1st case and will follow the process of the flowchart as shown along arm (1) in the case of an increase in concentration and arm (3) in the event of a reduction in concentration.

If the active ingredient does not have a narrow therapeutic window, any significant reduction in its concentration in relation to the method's analytical accuracy must be interpreted concomitantly with the data for the other physic-chemical parameters. In the first instance, the presence of degradation products will be sought.

Degradation products are not detected within the preparation. If the chosen technique gives pertinent results (indicating stability and allowing the degradation products to be visualised), a physical or chemical instability of the preparation can be considered. The other physico-chemical parameters will then help contribute to interpretation.

the other physico-chemical parameters (pH, osmolality, turbidity, particles, etc.) do not change over time *(see box)*: an interaction between the content and the container can be suspected. If this concerns a simple adsorption, the phenomenon will be rapid and limited. If the loss in active ingredient observed is considered to be clinically acceptable, the preparation can then be considered to be stable. If an absorption problem is involved (diffusion of the active ingredient in the material), the process may evolve over time and the loss of active ingredient may be accentuated. It will then be advisable to pursue the stability study to check out the process. In the case of absorption, a change in packaging will be required.

A variation in the physical parameters is observed, meaning an increase in the number of particles, in turbidity, a colour change and/or a modification to viscosity *(see box)*. A commencement of physical instability or chemical degradation must be considered. Here it will be advisable to pursue the stability study to check whether the loss of active ingredient changes in parallel with other physico-chemical parameters. Product degradation may also appear.

A variation in pH is observed. This may involve a start of chemical degradation that may be highlighted by pursuing the study. The observation of parameters like osmolality, colour or viscosity can sustain this hypothesis. A variation in pH may also be present with no change in the concentration of active ingredient or other physico-chemical parameters. Indeed, the container for the preparation may show a certain porosity to air. CO2 passing into the preparation is likely to acidify it and thus reduce the pH. The interpretation of this variation in pH must be conducted in light of the clinical use of the preparation and especially its administration route, bearing in mind that it is commonly admitted that a variation of at least one pH unit cannot be considered to be negligible *(see box)*

Degradation products are detected within the preparation. A quantification of such products would not appear to be required as it is difficult to define an acceptable limit. This will depend in particular on the toxicity of these products through the administration route concerned by the preparation. However, it is important to search for them on analysis of the active ingredient. On chromatographic analysis of the active ingredient, they will be characterised by their relative retention in relation to the peak of the active ingredient. Evolution of the surface of each peak is to be monitored throughout the stability study

In the event of presence of degradation products, a number of cases are possible:

• Degradation products were present at T0 (immediately after production of the preparation) and the surface of peaks continues to increase over the duration of the stability study. In this case, it can be assumed that the degradation products are already known and the toxic thresholds have been determined. For example, pharmacopoeia monographs refer to limit rates for impurities integrating degradation products and impurities relating to synthesis of the product. It is then advisable to determine whether limit rates exist for the active ingredient concerned. The administration route must also be taken into consideration. Indeed, for example, an acceptable threshold for degradation products by injection may no longer be applicable for administration by intracamerular injection. If the toxicity of degradation products has not been studied for the administration route of the preparation, any increase in these degradation products in relation to the initial value will be considered to be toxic and the preparation unstable as a precautionary measure.

• Degradation products were not present at T0. Here, a search through the literature is to be conducted to check whether they can be identified and if toxicity limits can be defined. If that is the case, those limits will be applied to the results of the study. If it is not the case, as a precautionary measure, the preparation will be considered to be unstable while awaiting more thorough toxicological studies.

WHAT ADDITIONAL DATA ON ACCEPTABLE VARIATIONS IN PHYSICO-CHEMICAL PARAMETERS?

Number of particles : an increase in the number of particles is an indicator to monitor a physical instability. This should remain within the limits defined by the European Pharmacopoeia for injectable solutions (see section 5).

Turbidity : there is no limit value for turbidity. However, in his publications, L.A. Trissel reckons that an increase in turbidity of more than 0.5 NTU (Nephelometric turbidity Units) has to be considered as a significant indicator of a solution's physical instability.

pH : there are no limits defined for variations in the pH of a solution. The pH must remain within the limits for acceptability related to its pharmaceutical form (for example between 4.5 and 11.5 for an eye drop according to Cadot et al, 2012), with the drop by one pH unit corresponding to an increase by a factor of 10 of the concentration of protons in the solution. A variation of more than one pH unit should not be considered to be negligible

Change in colour : special attention must be devoted to any change in colour of a solution. It should, however, be noted that some active ingredients are likely to show slight variations in colour before being prepared in solution. For example, the Pharmacopoeia mentions colourless to slightly yellowish powder. This tolerance will be considered in the interpretation of results.

Unlike physico-chemical stability studies, microbiological stability studies do not use metrological methods. From the microbiological perspective, this involves checking that the preparation retains its initial microbiological quality over time, right up to its being used.

It has already been observed that physico-chemical stability is studied from a preparation whose formulation has been validated. Clearly, microbiological stability too will be studied from preparations whose microbiological quality has been validated. For example, a sterile preparation must, immediately after its preparation, satisfy the requirements of the pharmacopoeia with respect to germs and endotoxins. Only conservation of this condition will be evaluated within the scope of a stability study.

CHAPTER7

Microbiological stability studies of preparations

Foreword

Microbiological stability will be understood differently according to the administration routes, in other words with regard to whether sterility of the finished product is required or not.

For a sterile preparation, it is expected that there be no germs, while for preparations that are not compulsorily sterile, there exist in the European Pharmacopoeia (7th edition, 2013) thresholds for tolerance in aerobic germs and moulds/yeasts as well as limits for specified germs.

The microbiological stability of forms that are not compulsorily sterile must thus show the conservation over time of these limit characteristics for contaminations as defined in the pharmacopoeia.

Concerning compulsorily sterile preparations intended for parenteral and ophthalmic routes, stability tests are expected to show conservation over time of sterility up to the moment of use.

The problem lies in the means used to measure this sterility over time, bearing in mind that the sterility test alone is inadequate to demonstrate the sterility of each unit of a batch.

Indeed, the test relies on analysis of a small sample of the batch; it thus suffers from a statistical limit and can only, as a result, detect cases of massive contamination. Furthermore, the sterility test will depend on the uniformity of the batch, conditions of manufacturing and the efficacy of the sampling plan, such that it will have to be shown that production is conducted uniformly so that the risk of contamination is the same for each of the manufactured units.

The Pharmacopoeia states in particular that using a terminal sterilisation method combined with physical measurements and automatic recording of the parameters will give a greater level of assurance of sterility than the result of a sterility test.

The problem of production in hospital pharmacies lies in the preponderant use of a manufacturing process by aseptic media fill of the injectable quality pharmaceutical speciality, as recommended moreover in French Good Manufacturing Practice for hospital and community pharmacies *(BPP, 2007).*

Here, use of the sterile pharmaceutical speciality as starting product is to be preferred to the use of the raw material. Thus, within the scope of aseptic preparation, definition of the batch is an essential issue, with uniformity being of the utmost importance.

Furthermore, it will be necessary to ensure control over the conservation conditions (packaging, temperatures and exposure to light) of the manufactured units reproducing the real conditions to be applied subsequently as a matter of routine.

Preliminary considerations

BATCH CONCEPT

It is necessary to define the perimeter of the batch on which the stability study is to be applied. The batch can, indeed, answer to quite different definitions.

For industrial production, the batch notion is pretty clear, with the batch comprising a homogeneous set of units manufactured in the same conditions using the same processes and in the same environments. Indeed, the stability test will have to be representative of that batch. Now, in hospital pharmacy, the batch notion can vary considerably and is closely related to the type of preparation. The batch can comprises a homogeneous number of units of the same composition intended for several patients or at the opposite extreme a single unit intended for a single patient, with the process being possibly either manual or automated.

The batch in the industrial meaning of the term comprising a homogeneous set of units manufactured in the same conditions using the same processes and in the same environments. It can be seen for example in hospital Central Intra Venous Additive Services (CIVAS) for preparation in series of injectable drugs. Two definitions for the batch were retained by the group of pharmacists of the Société Francophone de Nutrition Entérale et Parentérale (SFNEP) in order to determine the conditions for physico-chemical and microbiological inspection (Corriol O. et al. 2005). Thus for physico-chemical inspection, the batch is defined on the type of preparation while for microbiological inspection, the batch is defined on the mode of production:

The batch defined by the type of preparation that conditions physico-chemical inspection: the batch comprises several bags of the same composition, derived from the same initial admixture, or comprising a single bag for a single patient

The batch defined by the mode of production that conditions sterility inspection: in this case, the batch comprises several manufactured bags (extemporaneous and preparations in series can coexist) in uniform conditions: the process, conditions and staff for manufacturing are identical for all bags.

This latter definition of the batch is to be retained for the study of microbiological stability as it takes into consideration a set of elements likely to modify the quality of the finished product microbiologically, that is through the process, the operator and the environment.

When interpreting the results of the stability study it will be appropriate to take into account this batch notion, especially with respect to the value of representativeness of a sampling.

For microbiological stability, this notion of uniform conditions of manufacturing will be retained

NOTION OF MICROBIOLOGICAL RISK

Microbiological contamination of parenteral preparations produced in hospitals always constitutes a risk of nosocomial infection *(Macias et al 2010).* Respect for good aseptic preparation practices in accordance with Good Manufacturing Practices (BPP) must be implemented compulsorily to guarantee the microbiological quality of manufacturing. It is necessary to combine environmental conditions as defined in BPPs and appropriate training of operators in the aseptic technique as well as qualification of operators showing their ability to produce units aseptically.

The rate of microbiological contamination has recently been estimated by modelling of risks at 2.2 contaminated units per million units produced when the preparation is produced by staff qualified in aseptic preparation in an ISO 5 class environment *(Tidswell EC et al 2010).* The rate of contamination is directly related to the training of operators in aseptic techniques, as was shown by a comparative study before training/after training *(Isanhart CM, 2008)*. Finally, the more manual stages there are in the process, the greater the risk of contamination *(Stucki C. et al 2009).*

Benchmarks mention this risk during manufacturing, the angles of approach sometimes being different; thus in Good Manufacturing Practices, the microbiological risk is directly associated with the manufacturing process of the drug in closed system or open system in the controlled atmosphere zone.

Thus, microbiological risk is low when the preparation is made in a closed system and where the process answers to the following definition «Aseptic process allowing for a sterile product to be withdrawn and transferred to another sterile container in which the closing systems of the containers and the transfer equipment remain in place throughout the duration of the transfer process, ensured solely by a sterile needle, a sterile tubing or any other sterile transfer device. Transfer of the sterile product is performed such that it never comes into contact with the environment».

Microbiological risk becomes greater as soon as one of the stages in the process is in open system, for which at least one of the stages is conducted open incurring a risk of exposure of the solution to the environment. For the European recommendations derived from the inspectors' agreement (PIC/S guidelines) risk is also associated with environment quality control, and the process in closed system plays a role in minimising risk. The benchmark also stresses conditions at high risk of microbiological growth related to the nature of the product as fostering microbiological growth, the duration and the administration route.

Thus risk is associated with the intrinsic nature of the drug that can foster bacterial growth (for example, mix of parenteral nutrition, eye drops and irrigation solutions due to long infusion periods, portable infusion devices due to long periods and exposure to a temperature of more than 25°C) and the administration route (epidural and cardioplegic solutions).

Conversely, considering a priori that an active principle ingredient is not at risk of microbiological contamination is an error. This is especially the case for injectable cytotoxics for which the inhibiting power on the fertility of suspensions has not been systematically demonstrated, which confirms that prepared solutions can be contaminated just like other active principle ingredients *(Kramer I. 1998; Briceland LL et al 1990, Kasten A. et al 2007).*

Finally, some active principle ingredients can present a potency to inhibit microbiological growth, which is why it is essential beforehand to perform a sterility test to check for the absence of antimicrobial activity in accordance with the test from the Pharmacopoeia.

For the American pharmacopoeia, in its section specifically dedicated to hospital pharmacy preparation, USP <797>, microbiological risk is associated with the manufacturing process (closed or not), the number of active principle ingredients handled, the microbiological quality of the raw materials (sterile or not) and the manufacturing environment (controlled or not). Thus for USP, microbiological risk is low when the preparation is made in a closed system in a controlled environment and does not involve more than 3 products, dilution solvent included and two punctures maximum. The microbiological risk becomes average, when the preparation in closed system produced in a controlled atmosphere zone brings in more than 3 components (example of mixes in parenteral nutrition). Finally, microbiological risk becomes high when the raw material used is non sterile or when the sterile raw material is exposed to environmental conditions that are not controlled for more than an hour. This analysis of microbiological risk has led to proposing in USP <797>, and outside any sterility test, stability periods *(table 8)* in relation to the conservation temperatures (ambient, refrigerated or frozen). The author took care to specify that the physico-chemical stability conditions had to be verified.

Microbiological risk	Ambient temperature +20°C to +25°C	Cold +2 to +8°C	Freezing -10 to -25°C
LOW	48 hours	14 days	45 days
AVERAGE	30 hours	9 days	45 days
HIGH	24 hours	3 days	45 days

Table 8 : duration of microbiological stability for preparations produced in the hospital in accordance with USP <797> under reserve of physico-chemical stability data

This choice made by the US legislator is no doubt open to discussion but has the advantage of offering a pragmatic approach to the hospital pharmacist in the absence of available data and when implementation of the study is hard to proceed with.

General methodology

Microbiological stability studies will apply to preparations in series, in uniform batches **(hospital pharmacy preparations).** Heterogeneous production, as when manufacturing extemporaneous preparations, can hardly be covered by a stability study. While it remains possible to consider uniformity of production in the same conditions on preparations that are different and conduct a sterility test representative of a morning of production, it is hardly relevant to commit to long term stability of products prepared by the unit.
In the case of **extemporaneous preparations**, you are advised to stick to short conservation times and rely on the notions of microbiological risk found in the benchmarks (PIC/guidelines, USP <797>). Pharmacists may refer to the conservation lead-times proposed in table 8.

STUDY CONDITION :

The product batch must be the most uniform and representative of later production. It can only be produced by staff previously qualified for aseptic preparation, in a qualified, controlled environment and using a validated process.

The batch is to be kept in the conditions provided for later conservation, that is at ambient temperature, cold or frozen. Conservation at ambient temperature is the most unfavourable condition in conservation terms. The real packaging must be used in the test. This may consist of glass vials, plastic vials, flexible mini bags, syringes or portable devices that may be covered with a secondary packaging (possible overpack). For the stability study, the full final packaging (primary and secondary) must be used as it is provided for in real conditions. For example, when it comes to a portable infusion device, the stability test is made in the infusion device with its possible overpack as provided for in routine production.

TESTS PERFORMED ON THE BATCH:

It is expected that a sterility test be conducted at a periodicity defined over the duration of the study (that may be that proposed in section 2 and at least at D0 then at the end of the conservation period). The number of samples required will depend on the preparation studied and the volumes required to conduct the tests. The minimum quantities required to perform a sterility test in accordance with the recommendations of the Pharmacopoeia are specified in the following section describing the technique and its selection criteria. In parallel with the sterility test, it is recommended that the microbiological stability study be completed by checking that the integrity of the packaging for the preparation is maintained.

Monitoring endotoxins does not appear relevant within the scope of a microbiological stability study for a preparation. Indeed, it was clearly stated at the beginning of the section that stability studies were performed from preparations answering to the requirements demanded by the Pharmacopoeia. As a result, for any injectable preparation it will have been checked at T0 (immediately after preparation) that it is sterile and non-pyrogenic. Possible endotoxins that the raw material or a non-conforming item of packaging may contribute are therefore rejected. During the conservation period of the preparation (the object of the stability study), the only source that can introduce endotoxins will thus be that coming from microbial contamination. As a result, the sterility test performed during and on ending the period for conservation studied is a good indicator for microbiological stability of the preparation. Checking the integrity of the packaging may then provide proofs as to the origin of the contamination.

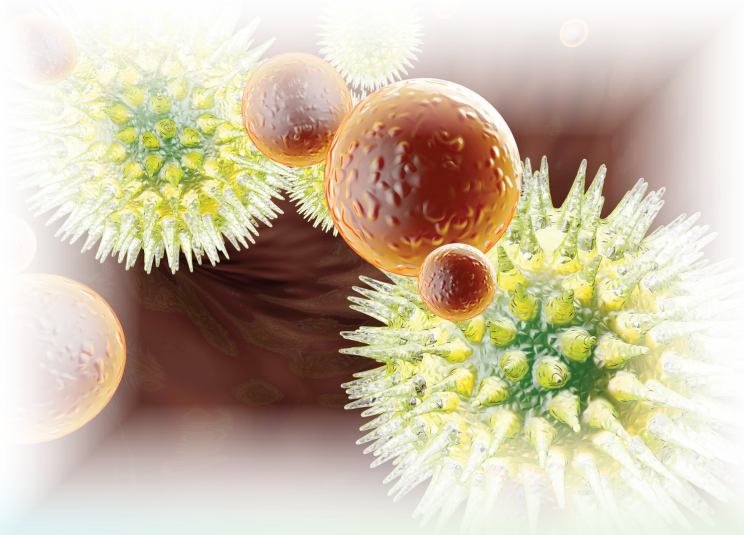
Analysis techniques

1 STERILITY TEST

For the sterility test, the European Pharmacopoeia should be referred to.

The sterility test must be associated with a prior validation test : validation of fertility of the culture media used for seeding and checking that the preparation does not in itself show an inhibitive effect on microbial growth. The stages in this validation test are described in **Appendix 3.**

Two plating methods are proposed in the Pharmacopoeia for the sterility test: the method by direct seeding and filtration on membrane. Filtration on membrane is generally preferred as it is more sensitive than direct seeding. However, alternative rapid microbiology methods may be highly advantageous in the hospital environment. Besides obtaining a rapid result for microbiological stability studies, the main advantage lies in allowing for a non-destructive test on part of the sample using closed system sampling allowing for re-analysis of the same sample during its



conservation over time.

1 - Membrane filtration method

Filtration on membrane requires more handling than the method by direct transfer and is thus more open to the risk of accidental contamination (false positive). The closed system must be given preference to the open system.

There are various completely closed systems to perform the sterility test, including Sterisart® *(Sartorius Stedim France)* and Steritest® *(Millipore)* thanks to which the risk of accidental contamination is very low.

These systems adapted to the various inspection zones (laminar flow hood, microbiological safety station or isolator) preclude handling stages as far as is possible: a peristaltic pump allows for transfer of fluids in a closed system. Micro-organisms are collected on the surface of a membrane with porosity of 0.45 microns that is in turn transferred into a culture medium. There are several versions available according to the type of container used (closed vials, open vials, pouches, ampoules, pre-filled syringes, dissolution kit for powder preparations, filtration surface, etc.).

CHOICE OF CULTURE MEDIA AND INCUBATION

The media generally used are liquid thioglycolate and the soybean casein medium. Thioglycolate is a liquid medium that allows growth of both aerobic and anaerobic microorganisms. Without agar, it does not interfere with the viscosity of a solution.

For adequate penetration of oxygen, the medium must be in tubes of dimensions defined to receive 15 ml of solution. The soybean casein medium allows for growth of a large number of aerobic bacteria and fungi (especially moulds).

The media must be incubated for at least 14 days at temperatures specified by the Pharmacopoeia and recorded.

EXAMPLE: Liquid thioglycolate (aerobic and anaerobic bacteria): incubation 30-35°C, 14 days; casein and soybean hydrolysate (aerobic bacteria and fungi): incubation 20-25°C, 14 days

The temperature and residual humidity of the oven are controlled and measured regularly.

CHOICE OF MEMBRANES

Filtration membranes must be chosen according to the physico-chemical properties of the filtered medicinal solution. This choice must take into account the risk of adsorption on the membrane that may lead to false negatives.

The main factors to be taken into account to limit adsorption are the chemical structure of the membrane, its thickness and its assembly on the support.

According to their polymeric structure, the molecules will adsorb differently on a membrane. The thickness of the membrane is another major issue to be taken into consideration. This varies between 100 and 300µm. During filtration, inhibitor residues can become trapped inside the membrane structure. As a result, the finer a membrane, the less will be the risk of adsorption.

Special attention must be given to assembly of the membrane on the filtration support in a closed system: a defective assembly can lead to false negatives. In some devices, the membrane is held in place by it being clipped between the edge of the filtration chamber and the base of the device. Such a design creates “pockets» containing product residues around the edge of the membrane that it is then difficult to flush away effectively. It is thus preferable for the membrane to be sealed to a support on the one side, and to the filtration/incubation chamber on the other.

Other factors allow adsorption of the anti-bacterial product on the membrane to be limited: pre-wetting of the membrane and rapid filtration of the diluted samples.

EXAMPLE : to test the sterility of antibiotic preparations, it is advisable to use a membrane that adsorbs a minimum quantity of product. It is possible to resort to a fine (100 µm) material with low adsorption (hydrophilic PVDF: polyvinylidene fluoride) so as not to trap inhibitor residues inside the membrane structure during filtration. A pore size of 0.45µm is appropriate as it retains enough micro-organisms while allowing for a high filtration flow rate in a negative pressure (USP, 31st)

SAMPLE VOLUME TO BE FILTERED, DILUTION LIQUID AND FLUSHING OF MEMBRANES

The filtration technique on membrane is used whenever the nature of the product so allows. Liquids are used to dissolve and dilute the products, or to flush membranes. The European Pharmacopoeia and USP indicate different types of fluids. The choice of fluid depends on the product to be tested.

In the case of viscous products for example, the appropriate fluid is used to dilute the product and make it filterable. Whenever possible, the total content of the recipient is used but taking care to respect the minimum quantities stated in the European Pharmacopoeia.

For parenteral liquid preparations, the minimum quantities are shown in table 9.

Preparation volume	Minimum preparation volume to be analysed for each medium
≤ 1 mL	Totality of recipient
1-40 mL	Half the content of each recipient but not less than 1 ml
> 40 et ≤ 100 mL	20ml
> 100 mL	10% of the volume but not less than 20 ml

Table 9: minimum volume sample to be analysed according to the European Pharmacopoeia

In the special case of antibiotic liquids, the volume prescribed in the pharmacopoeia is 1 ml whatever the preparation volume.
The number of units to be analysed depends on the batch size *(table 10)*

Number of units in the batch	Minimum number of units to be examined per medium
≤ 100	10% with a minimum of 4 units whatever the volume
> 200	10 units
> 500	2% with a maximum of 20 units (10 for big volume preparations)

Table 10: minimum number of manufactured units to be examined in accordance with the European Pharmacopoeia

In the special case of ophthalmic preparations, the number of units to be examined respects the indications of table 11.

Number of units in the batch	Minimum number of units to be examined per medium
≤ 200	5 per cent of recipients, with a minimum of 2
> 200	10 units
If the product is packaged in single-dose recipients, apply table 10 (parenteral preparations)	

Table 11: minimum number of manufactured units to be examined for ophthalmic preparations in accordance with the European Pharmacopoeia

If necessary, it is possible to top up to 100 ml with an appropriate sterile diluent (for example a neutral solution of meat or casein peptone at 1g/L). Except where justified as an exception, the total volume filtered through the same membrane must not exceed 1,000 ml (European Pharmacopoeia).

If the substance undergoing testing is a viscous liquid and is not suited to rapid filtration, it is possible to group together the content of different recipients in a single packaging and aseptically add a sufficient quantity of the dilution liquid before filtration in order to improve the flow (USP).

In the case of antibiotic based preparation, the fluids containing surfactants like polysorbate 80 are used to flush the membrane after filtration of the product to be tested; they improve flushing of the membrane and reduce the risk of inhibiting growth caused by residues adsorbed on the membrane. It is appropriate to note that the sterility test must be validated for any preparation: the absence of growth inhibiting effect of the preparation is then checked and the flushing solvent adapted (Appendix 3).

2 - Alternative method: rapid microbiology method

The conventional sterility test requires 14 days so as to allow for detection of micro-organisms with slow growth that can be in dormant state or subjected to a stress relating to extreme environmental conditions (e.g., cleaning agents and disinfectants, ultraviolet light or preservatives), while alternative rapid microbiology methods are especially useful to implement reiterated sterility tests on the same sample during its conservation.

Rapid microbiological methods based on indirect detection of growth can provide an alternative to the sterility test using membrane filtration.

Detection of CO2 using colorimetric methods or by change in pressure in a culture medium vial equipped with a C02 sensor of sensitivity adapted to the quantity produced by the micro-organisms (BACTEC® from Becton Dickinson and the BactT/Alert® system from Biomérieux). The samples of volumes defined for preparations or controls are seeded in the culture media of 50 ml (Bactec®) or 40 ml (BactT/Alert®). This system monitors microbiological growth in the vials every 10 minutes. An alarm is triggered in the event of microbiological growth. Identification of the germs can be conducted by PCR using the microSEQ ID system (Applied Biosystems, USA).

Detection of adenosine triphosphate (ATP) by bioluminescence (Rapid Milliflex® Detection System [RMDS]; Millipore). The samples of defined volumes of medicinal solution or controls are seeded with various concentrations of micro-organisms (0.1 to 100 UFC/ml). Each sample is aseptically filtered under laminar flow using a 0.45 ml membrane then flushed with 100 ml of peptone 0.1%. The membranes of each sample are incubated in culture media placed at 20-25°C and 30-35°C. A large number of samples are prepared with a view to evaluating microbiological growth every day, from the first to the fifth day of incubation: the membranes are separated from the solid culture media and then dried aseptically. A reagent highlighting the release of ATP or bioluminescence is sprayed onto each of the membranes: the ATP of viable micro-organisms reacts with the enzyme luciferase to produce light. The photons generated are captured by a camera with a coupled device and charged then the computerised image is analysed with a view to counting the number of units forming a colony. Detection of a CFU corresponds to a positive result. The micro-organisms are then replanted into new culture media to proceed with their identification: the DNA of the micro-organisms is amplified by PCR then the marked amplicon is placed in a capillary oven genetic analyser in which analysis of the sequences allows the germs to be identified by the microSEQ ID system (Applied Biosystems, USA).

Cytometry by laser detection in solid phase. the RDI scan allows the number of viable micro-organisms to be counted. This system is approved by the FDA to test the sterility of water.

The advantage of rapid detection using these methods can, however, be limited for slow growing germs. For example, the RMDS method only allows a single day to be gained as compared with conventional methods for micro-organisms with slow growth and low inoculum *(Parveen S. et al, 2009);* the detection period for P. acnes by BACTEC and BactT/Alert is equivalent to that for a conventional method *(Chollet R. et al, 2008; Khuu HM et al, 2004).*

There is a need to evaluate the risk of being able of contaminate the preparation with a slow growth germ in the manufacturing environment. For yeasts and moulds, there are specific media (BACTEC or BacT/Alert®) that can be used.

METHOD OF CHOICE

The use of media for haemoculture (BACTEC®, Beckon Dickinson / BacT/Alert®, Biomérieux) can provide a method of choice for the microbiological inspection of hospital pharmacy preparations. This method has the advantage of simplicity of use, the availability of the automated system in the hospital's clinical microbiology department, sampling in closed system that avoids destruction of the checked product, rapid response (24 to 48 hours) with automated read-off for detection of C02 production by the germs revealed by a coloured indicator's change in hue, and good sensitivity at 1 UFC/ml. *(Corriol O. et al, 2000).* It can be used to perform microbiological stability studies on preparations just like the sterility test described by the Pharmacopoeia.

The drawback is that the method is not quantitative and does not allow for a conclusion as to the presence or absence of germs. In the case of a positive result, it will be necessary to implement an identification of the germ. As for all methods, validation of the medium's use to check sterility of the preparation will have to be performed, especially to check for the absence of an inhibiting effect of the preparation on the medium, and fertility of media for the germs prescribed by the European Pharmacopoeia. Indeed, there may be risks of inhibition in the presence of certain preservatives or medications like antibiotics.

2 PHYSICAL TESTS ON THE INTEGRITY OF PACKAGING

It is relevant to check that the packaging of preparations is capable of maintaining the initial condition of sterility. Thus for each batch produced subsequently, the sterility test at D0, combined with tests showing that the packaging conserves its condition of initial quality should allow the microbiological stability of the product to be guaranteed. It is on these lines that a guide for the pharmaceutical industry has been developed by the FDA as an alternative utilisation to the sterility test.

For hospitals, using the test based on colorants can allow the weaknesses of a packaging and seals to be visualised. It is possible to use an ASTM F 1929-98 standard test method developed to detect leaks on porous packing material seals using a dye based on a solution of toluidine blue and Triton X-100 *(Standard ASTM F1929-98, 2004).*

REMEMBER…

Microbiological stability studies means you can check that the preparation preserves its sterility qualities throughout its period of conservation. The manufacturing process must not interfere in this stability study. The inspection conducted at D0, immediately after preparation, allows conformance to be checked. A microbiological stability study can only be performed if the manufacturing process has been validated and answers to Good Manufacturing Practices requirements. Thus the equipment, process and operators must be subject to validation as described in **Appendix 4**.

MICROBIOLOGICAL STABILITY OF NON-COMPULSORILY STERILE PREPARATIONS

For non-compulsorily sterile preparations, the microbial count test in accordance with the European Pharmacopoeia (2.6.12) and search for specified micro-organisms in accordance with section 2.6.13 applied to several conservation times can allow an answer to be provided to the question of microbiological stability of preparations that are not compulsorily sterile.

Microbiological quality acceptance criteria for non-sterile pharmaceutical products is based upon the total aerobic microbial count (TAMC) and the total combined yeasts/moulds count (TYMC) as defined in 5.1.4 of the European Pharmacopoeia in relation to the administration route. Thus the European Pharmacopoeia (5.1.4) defines the microbiological quality of non-sterile preparations with TAMC and TYMC in UFC per ml or per g and as also the absence of specified pathogenic micro-organisms.

For example, for an aqueous solution, where it is intended for oral administration, there must be no more than 102 CFU/ml TAMC and 101 CFU/ml TYMC and absence of Escherichia Coli (per ml); if it is intended for dermal route, it must respect the same limits in number of TAMC and TYMC, but the germs specified not to be present are different, these being Staphylococcus aureus and Pseudomonas aeruginosa.

The counting method used can be membrane filtration (seeding the filter on a nutrient agar) or counting on plate. The specified micro-organisms are sought.

The media are chosen according to the micro-organism sought.

The stages prior to counting in the preparation consist of validating the fertility of the media as also applicability of the counting method in presence of the product. For tests calling on specific microbiological competences, it is highly advisable to bring in the competences of microbiologists.

Numerous drugs originating in biotechnologies have proteinaceous active principles, for instance with monoclonal antibodies or enzymes. These products - whose use is growing - are expensive, and manufacturing in the open field is often sought. In such cases it is of interest to attain long-term stability. In addition it has been reported that the reconstitution process could alter these fragile macromolecules, especially if the protein undergoes mechanical stress (Lahlou et al, 2009). To ensure the quality of the prepared product, it is thus important for hospital pharmacists to be able to verify the stability of proteins over time or during a manufacturing process, from the moment they wish to deviate from the recommendations given in the Summary of Product Characteristics, which are often very restrictive.

CHAPTER8

Stability of preparations with a proteinaceous active principle

GENERAL MECHANISMS OF INSTABILITY IN PROTEINS AND PEPTIDES

Evaluating protein stability remains a complex matter. A distinction must be drawn between physicochemical stability and «organic" stability. In the latter the protein's biological activity needs to be maintained; this implies that the protein has remained chemically stable but also that its ternary and quaternary conformations have been maintained.

Physicochemical instability mechanisms that may potentially result in a loss of activity or in toxicity are of several kinds, and have recently been reviewed *(Manning et al, 2010).*

On the chemical side it is possible to observe deamidations involving the hydrolysis of the lateral amide functions of asparagine and glutamine residues. Aspartic acid can then cyclize, and then isomerize or undergo hydrolysis. Other reactions such as the Maillard reaction (formation of a Schiff base subsequent to a reaction between the carbonyl of a reducing sugar and the side chain, typically, of a lysine), oxidations on methionines or tryptophans, or the modification of disulfide bridges, are examples demonstrating the multitude of possible degradations and the great diversity of chemical conditions which may lead to this.

To these chemical instabilities must be added possible physical instabilities, such as denaturation by heat or cold, aggregation, precipitation, adsorption on container materials *(Hoehne et al., 2011)* or on medical devices (glass, polyethylene, PVC, etc.) or at air/water, water/ice interfaces, etc. There are many potential sources of protein instability, yet certain structures are more stable than others; peptides are generally considered more stable than high molecular weight proteins and specific structures such as antibodies may be relatively stable after dilution if stored properly *(Paul et al., 2012)*

METHODS FOR EVALUATING THE STABILITY OF PROTEINS AND PEPTIDES

To evaluate physicochemical stability, several analytical techniques need to be used in parallel. These techniques are complementary, and in order to increase the level of evidence, they should be combined.

Physical stability is either evaluated by turbidimetry at different wavelengths, between 340-360nm and 550nm, or by size measurements performed via light diffraction or size-exclusion chromatography (SEC); other techniques exist as well *(Mahler et al., 2009)*, including microscopy techniques. These techniques are used to evaluate aggregates due for example to mechanical stress, but also potential cleavages of dimers into monomers, or, inversely, dimerization phenomena. Qualitative analysis is also possible via gel chromatography, for example with SDS-PAGE type techniques.

Post-centrifugation UV spectroscopy was also proposed to assess protein aggregation; absorbance at 279nm allows access to the non-aggregated fraction, and the second derivative of the spectrum and the ratios of absorbance at 279 nm and 251nm were used *(Lahlou et al., 2009)*. This non-separative technique is however difficult to interpret, due to the excipients present in formulations of active ingredients of biological origin such as proteins.

To assess physical stability, a European consensus group *(Bardin et al., 2011)* recommends the combination of several techniques, including at minimum turbidimetry and SEC; still, most often at least four different techniques are combined.

To evaluate **chemical stability**, the same European consensus group recommends the combination of at least three SEC techniques, ion exchange chromatography (IEC), and peptide mapping (HPLC coupled with enzymatic treatments). It is also possible to use chromatography with detection via UV spectrometry, fluorescence or mass spectrometry.

Aside from the proteins' physicochemical stability, which allows the detection of changes that will cause loss of activity or side effects (immunological or renal intolerance, for example), the biological action of the protein of interest should be evaluated as well. An assessment of biological activity maintenance can be made by the recognition of structures exhibiting such activity via immunoenzymatic assay (ELISA); it should first be verified that the ELISA kit to be used will correctly recognize the domain of the protein exhibiting the activity. These analyzes, however, may be insufficient; the gold standard is to test the protein's biological activity in vitro, or, ideally, in vivo. These techniques, called «bioassays» are performed in specialized biology laboratories. It can be verified, for example, whether a monoclonal antibody properly inhibits its target, or at least attaches to it. The maintenance of biological activity is a necessary condition for a demonstration of stability, but alone is not sufficient. Indeed, there may still be residual activity when there are aggregates or denatured proteins present, which can potentially be toxic. Since measurements of biological activity are highly variable, it will on the other hand be difficult to conclusively establish the total maintenance of the integrity of the protein of interest in light of an activity similar to the control activity.

Like for conventional molecules, stability studies require protocols for forced degradation studies, which must mimic the physicochemical stress potentially encountered during storage conditions: high temperatures, accidental freezing/thawing, mechanical or oxidative stress, radiation, pH changes, etc. According to the ICH Q5C, these forced degradation studies can check whether accidental storage conditions can become sources of instability. They also allow us to highlight the primary degradation products, and thus to check the specificity of the analytical methods used. Standard and universally accepted forced degradation conditions still do not exist for proteins. A recent article *(Hawe et al., 2012)* gathers the primary sources of instability for proteins, and discusses the different options as regards protocols for forced conditions.

Thus, it seems that to carry out a stability study on a protein of therapeutic interest and conclusively establish the maintenance its activity on the basis of the absence of degradation products would be an adventure fraught with pitfalls and obstacles. A comprehensive and recent study *(Paul et al., 2012),* demonstrating - according to its authors - the stability of diluted rituximab over 6 months, combining

chemical and physical analytical techniques, and an assay on a cell in vitro, shows how broad the range of techniques required can be. To highlight the complexity of bioassays, we note that the direct cytotoxicity of rituximab observed in vitro in this study does not fully reflect its mechanism of action, which may be direct and thus measurable in vitro or mediated by the immune system, and would thus require an in vivo study *(Boross, et al., 2012).*

CONCLUSION

Due to the technical nature of the techniques to be used, it is clear that stability studies of proteinaceous active principles are beyond the capacity of hospital pharmacies, with the special exception of the leading specialized centers in the field. It is therefore necessary to subcontract these studies to expert laboratories or to rely on the available literature. Caution - regarding this last point: the stability of proteins is highly versatile and depends heavily for example on the excipients used, as is emphasized by the immunogenicity that was induced by the change in the formulation of EPO *(Schellekens et al., 2012; Casadevall et al., 2002)*. One should thus refrain from extrapolating from bibliographic sources when the preparation to be assessed is not strictly identical to that described in the reference publication. By “identical” we mean the same concentration, same pharmaceutical product, same container, and same vehicle.

APPENDIX

Appendix 1

DEFINITIONS OF THE VALIDATION PARAMETERS FOR TESTING METHODS

1.1 SPECIFICITY

The specificity of an analytic method is its capacity to unambiguously establish an analyte's existence in the presence of other compounds (other elements in the matrix such as excipients, degradation products). In the context of an assay method, not all methods are specific. So for terms of separation methods, specificity or selectivity will depend on the mode of detection used.

1.2 ASSAY RANGE

The assay range of an analytic procedure is the interval between lower level and upper level values, inclusively, for which it has been demonstrated that the procedure is appropriate in terms of its accuracy, reliability and calibration mode.

1.3 RESPONSE FUNCTION

This response function can be a straight line (linear response) or any other function (nonlinear response = quadratic response). The validation principles are the same.

It should be noted that linearity is the most frequently used response function model. This is most often due to the equipment available in the hospital pharmacy (UV spectrophotometers, HPLC coupled to a UV-Visible detector or fluorimeter). However other regression models, such as quadratic or logistic regression, are certainly possible if they correspond to the detector response model used.

The linearity of an analytic procedure is by definition its capacity, within a given assay interval, to obtain results directly proportional to the quantity of analyte present in the sample.

1.4 ACCURACY

Accuracy is a measurement of the closeness of agreement between the average value obtained from a series of test results and a theoretical value considered true. It reflects the systematic bias of a method of assay.

1.5 PRECISION

Precision expresses the closeness of agreement (coefficient of variation, degree of dispersion) between a series of measurements from a single homogeneous sample (independent test results) in a given set of conditions. Precision only indicates the distribution of random errors and does not provide information regarding specified (or true) values. Precision may be assessed at various levels.

Repeatability deals with the results of independent tests obtained in the same laboratory, with a fixed method of analysis and identical test samples, prepared by the same operator and analyzed with the same equipment within a very short period of time.

Intermediate precision deals with the results of independent tests obtained in the same laboratory, with a fixed method of analysis and identical test samples. Other operating conditions may be different: operator, equipment, the passage of a more significant period of time between tests (different days).
Reproducibility deals with the results of independent tests obtained with a fixed method of analysis and identical test samples. Other operating conditions (operator, equipment, laboratory) are different.

1.6. LIMIT OF DETECTION

The limit of detection (LOD) corresponds to the smallest quantity of an analyte in a sample which can be detected but not quantified.

1.7. LIMIT OF QUANTIFICATION

The limit of quantification (LOQ) is the smallest quantity of an analyte in a sample which can be quantified with a specific degree of accuracy.

Appendix 2

STATISTICAL PROCESSING OF RESULTS

It is considered in the present paragraph that the calibration curve is a linear regression. The following tests will be performed on ranges containing the active ingredient and on reconstituted ranges.

CALIBRATION MODEL

Uniformity of variances

In theory, linear regression can only be considered if the variances on each concentration are uniform (= similar). To check for this uniformity, the test most often used is Cochran's. The aim of the test is to determine the following ratio:

$$C = \frac{s^2_{max}}{\sum_{j=1}^{k} s_j^2}$$

With k, the number of times the test is performed (according to the model proposed k=3); N, the total number of tests (according to the model proposed N=15), s²max, the highest variance, and $\sum_{j=1}^{k} s_j^2$, the sum of variances for each group j (for each concentration). Variances will be considered to be homogeneous if the ratio C is less than the theoretical value $C_{(\alpha,k-1,N-k)}$ where α is the authorised risk. In this type of study, a risk of 5% is recommended.

Statistical verification of the regression

The adequacy of the model is to be checked in the following manner.
Let us assume a range of tests performed on i different concentrations (i=5) and j repetitions of each concentration (j=3). This means the following can be determined:

The mean values for responses can be :

The general mean : $\bar{Y} = \frac{\sum_i \sum_j Y_{i.j}}{i.j}$

The means of responses for a concentration i $\bar{Y} = \frac{\sum_j Y_{i.j}}{j}$

The method involves determining the following sum : $SCE_y = SCE_r + SCE_{nl} + SCE_{reg}$

Where

SCEy represents the total sum of squares of deviations in responses Y and is equal to $SCE_y = \sum_i \sum_j (Y_{ij} - \bar{Y})^2$

SCEr represents the sum of squares of deviations in responses Y due to residual error and is equal to: $SCE_r = \sum_i \sum_j j.(Y_{ij} - \hat{Y}_i)^2$ where Y is the value predicted by the regression for a concentration

SCE_nl represents the sum of squares of deviations in responses Y due to the inadequacy of the model (non-linear in this instance) and is equal to : $SCE_{nl} = \sum_i \sum_j j.(\hat{Y}_i - \bar{Y}_{ij})^2$

SCEreg represents the sum of squares of deviations in responses Y due to the regression used and is equal to :

$$SCE_{reg} = \sum_i \sum_j j.(Y_{ij} - \bar{Y})^2$$

The suitability test involves conducting a variance analysis that can be presented in the following manner:

Sources de variation	Sommes des carrées	degrés de liberté	Variances	F de Fisher expérimental	F de Fisher théorique
Régression	*SCEreg*	1	$s_{reg}^2 = \frac{SCE_{reg}}{1}$	$F_{reg}^{exp} = \frac{s_{reg}^2}{s_r^2}$	$F_{1,j.(i-1)}^{1\%}$
Non linéarité	*SCEnl*	i-2	$s_{nl}^2 = \frac{SCE_{nl}}{i-2}$	$F_{nl}^{exp} = \frac{s_{nl}^2}{s_r^2}$	$F_{i-2,j.(i-1)}^{1\%}$
Résiduelle	*SCEr*	j.(i-1)	$s_r^2 = \frac{SCE_r}{j.(i-1)}$		
Totale	*SCEy*	i.j-1			

Interpretation of this test is therefore performed as follows:

- The first hypothesis whereby the variation in Y correlates with the variation of X is verified if F_reg^exp>F_(1,j.(i-1))^(1%).

Stated otherwise, there exists a relation between the variation observed of Y and the variation produced of X.

- The second hypothesis, whereby the regression model used to explain the variation of Y as a function of X is adequate, is verified if F_nl^exp<F_(i-2,j.(i-1))^(1%)

In other words, there is no mismatch for the model explaining the variation of Y as a function of X.

(see Appendix 1 to see how to compute using Excel)

SPECIFIC FEATURES

In order to check that excipients in liquid form do not interfere in dosing, it must be proved that the regression line obtained with the active ingredient alone and that obtained in the presence of excipients are similar. The following stages must be followed to perform this test:

Compare slopes

To compare the slopes obtained with ranges of active ingredient alone (slope b1 with variance S²b1) and those obtained with reconstituted ranges (slope b2 with variance S²b2), a Student test is conducted :

$$t = \frac{|b_1 - b_2|}{\sqrt{S^2 b_1 + S^2 b_2}}$$

The slopes will not be significantly different to risk α if the value obtained "t" remains less than t (α ; N1+N2–4 ddl). For this type of study, it is recommended to have N1=N2=15, and a risk α at 5%
If the result obtained shows a significant difference, this can be said to express a matrix effect, leading to a review of the dosing procedure.

Compare intercepts

To compare intercepts obtained with active ingredient ranges alone (slope a1 with variance S²a1) and those obtained with reconstituted ranges (slope a2 with variance S²a2), a t-test is also to be performed :

$$t = \frac{|a_1 - a_2|}{\sqrt{S^2 a_1 + S^2 a_2}}$$

The intercepts will not be significantly different to the risk α, if the value obtained «t» is less than t(α; N1+N2–4 ddl). For this type of study it is recommended to have N1=N2=15, and a risk α at 5%

If the result obtained shows a significant difference, this can be taken to express a systematic error.

Compare each intercept with 0.

A further t-test is performed whose formula is : $t = \frac{a}{Sa} < t(\alpha, N - 2ddl)$

With «a», the intercept and «Sa», the standard deviation of the intercept. If the value of «t» obtained is less than that read in the Student t-table, the reference system used for dosing can be limited to point 100%. Otherwise, each dosing must be done using a set of calibration solutions.

ACCURACY

The overlap rates are calculated for each range point, considering the three ranges for the same series (for example the three «PA only" ranges if absence of matrix effect has been demonstrated). To do so, the ratio between the concentration observed for each range point and the theoretical concentration deduced from weighing having served to prepare the range point is calculated.

1 - Check the homogeneity of inter-group variances (Cochran test and variance analyses)

$$C = \frac{S^2_{max}}{\sum_{j=1}^{k} S^2_j} < C_{(\alpha, k\text{-}1, N\text{-}k)}$$

with k, the number of times the test is performed (according to the model proposed k=3), N, the total number of tests (according to the model proposed N=15), S^2max, the highest variance, and S^2j, the variance for each group j.

2 - Check the validity test for means that involves demonstrating that inter and intra-group variances are not significantly different (Fisher test).

$$F = \frac{S^2_C}{S^2_E} < F_{(\alpha, k\text{-}1, N\text{-}k)}$$

with S^2C inter-group variance and S^2E, intra-group variance.

3 - Calculate the mean overlap interval and the associated confidence interval: the value 100% must be included in that interval.

Intervalle de recouvrement moyen = moyenne des taux de recouvrement $\pm \frac{t_{(\alpha,N\text{-}1)} \times S_T}{\sqrt{N}}$

With ST, the standard deviation for the set of overlap intervals

RELIABILITY

Repeatability and reproducibility

We have three series of values with each series comprising six values. For each value, we know the theoretical concentration since each point has been constituted by an independent weighing. Each point has been analysed with a range and thus for each point and it is possible to deduce from this an observed value. This therefore gives access to a percentage for overlap by the ratio between the observed value and the theoretical value.

1 - Check homogeneity of variances (Cochran test, variance analyses)

2 - Calculate the relative standard deviation respectively for repeatability and reproducibility (see table below).

Number of groups	k	3
Sum of means	T_1	Sum of nj x mj (= sum of overlap rates)
Sum of mean squares	T_2	Sum of $n_j \times m_j^2$
Total number of measurements	T_3	18 (if the model retained was 6 QC taken at D1, D2 and D3)
Sum of measurement squares	T_4	Sum of n^2_j
Sum of variances	T_5	Sum $[(n_j-1)\ S^2_j]$ with S^2_j, the intra-group variance
Repeatability variance	S^2_r	$\frac{T_5}{T_3 - k}$
Inter-group variance	S^2_g	$\left[\frac{(T_2 \times T_3) - T_1^2}{T_3\ (k-1)} - S^2_r\right] \times \frac{T_3\ (k-1)}{T_3^2 - T_4}$
Intermediate reliability variance	S^2_R	$S^2_r + S^2_g$

LIMITS FOR QUANTIFICATION AND DETECTION

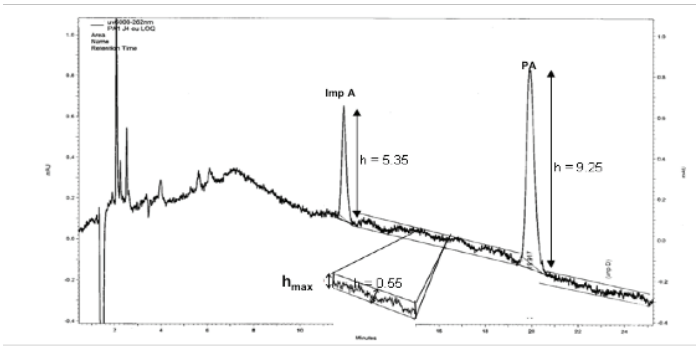
La figure ci-dessous illustre le calcul de la limite de quantification pour l'impureté A et le principe actif PA. Une solution contenant l'impureté A et le principe actif PA ont été diluées de façon à obtenir des signaux de l'ordre de 10 à 20 fois le bruit de fond. Les limites de quantification sont alors facilement accessible en appliquant la formule suivante :

$$\text{Limite de quantification} = 10 \times h_{max} \times \frac{\text{quantité injectée}}{\text{signal enregistré (en hauteur)}}$$

with : h_{max} = 0,55

- quantity injected: quantity injected respectively for each product knowing the concentration of the solution and the injection volume
- signal recorded (on height) = respectively 5.35 and 9.25 for impurity A and the active ingredient

The result will be expressed as quantity of material. It is to be noted that if the «quantity injected» is replaced by the concentration injected, the quantification limit result will then be expressed as a concentration.



An identical methodology will be applied to calculate the detection limit. The solution injected to proceed with the calculation should generate a signal not exceeding 5 to 10 times the background noise.

Appendix 3

VALIDATION OF THE STERILITY TEST

Any sterility test must be covered by a validation phase: a growth test validating the culture medium used and validation tests specific to the preparation concerned (validation of the technique and search for the microbial inhibition effect).

1. **Growth test** on each of the batches of culture media, whether these are prepared or purchased, in order to ensure that they will be capable of detecting the presence of micro-organisms (aerobic bacteria, anaerobic bacteria, yeasts and moulds). Other strains than those proposed by the Pharmacopoeia can be used on condition that they satisfy the growth test. The same strains must be used for growth tests and validation.

EXAMPLE : reference strains (e.g., Staphylococcus aureus ATCC 29213 and Pseudomonas aeruginosa ATCC 27853) are inoculated into a TS broth after seeding while they are undergoing an exponential phase of growth. After 24 hours at 37°C, bacterial growth should be observed. A count is made. An internal quality control is conducted (broth not seeded, placed open in the oven for 14 days at 30°C.

2 - **Test to validate the sterility test technique with respect to the preparation concerned.**
This allows you to ascertain that the technique will not hinder a clearly observable microbial growth visually comparable to that observed with the positive control when you transfer the medication into pre-defined culture media in the presence of a small number of viable microorganisms of 6 microbial strains as described in the Pharmacopeia (10-100 UFC). The evaluation also brings in analysis of a non-inoculated sample (negative control). A growth must be observed for the previously inoculated sample within 3-5 days maximum, according to the germ. This test must be conducted when the sterility test has to be applied to a new product or whenever a modification is made to the test experimental conditions.

3 - **Inhibition of antimicrobial agents contained in the preparation**. When conducting the sterility test on solutions containing antimicrobial agents (method by filtration) and in order to obtain the most favourable conditions for bacterial growth, the European Pharmacopoeia recommends flushing the membrane at least 3 times with a volume of injection water at least equivalent to the volume of the sample tested. The procedures for this test were not, however, provided for concentrated solutions (example of eye drops fortified with anti-infectives). A validation test on neutralisation of the antimicrobial agent must thus precede the sterility test so as to allow any trace of antimicrobial agent to be eliminated and thus obtain the most favourable conditions for bacterial growth (elimination of false negatives). This inhibition is performed by chemical neutralisation or by flushing with injection water or another solution if necessary (Letheen for example). A growth test is conducted in parallel (positive control). Microbial growth is observed, identical to that of the positive control if the antimicrobial activity of the 3 antibiotics is eliminated satisfactorily.

EXAMPLE : a sample of anti-infective eye drop solution is deposited on the filtration membrane, the latter then being flushed with a volume of injection water. A number of tests are conducted with decreasing volumes (e.g., 700ml then 600ml, then 500ml and so on). Each membrane is cultured in a trypticase-soy broth with an inoculum counted from 1ml of 10 to 100 UFC/ml. The bacterial species are chosen according to the antibiotic's spectrum of activity: Staphylococcus aureus for vancomycin, Pseudomonas aeruginosa for ceftazidime and Escherichia coli for amikacin. The results are read after 72 hours at 37°C. The control without antibiotic is a membrane inoculated with 1 ml of E. coli. Below validated flushing volumes, no visual turbidity is observed, meaning that the bacterial inoculum does not grow.

Appendix 4

MANUFACTURING PROCESS QUALIFICATION

VALIDATION OF EQUIPMENT AND QUALITY MONITORING

The equipment intended for the manufacturing of sterile medications must undergo a comprehensive qualification process (design, operational, installation and performance qualification) in accordance with French Good Manufacturing Practice for hospital and community pharmacies (BPP, 2007) or PIC/S guidelines. Besides qualification, such equipment must be regularly monitored and maintained in order to guarantee initial quality lasts over time. The results of microbiological inspections on controlled atmosphere zones must comply with good manufacturing practices and be attached to the batch file.

PROCESS MICROBIOLOGICAL VALIDATION

An aseptic media fill test is needed to demonstrate that the process is performed correctly and allows the sterility objective to be respected. In accordance with good manufacturing practices, the test is to be applied on performance qualification and must be renewed on a regular basis and whenever a major change in process or installation comes into effect (BPP, 2007; PIC/S guidelines, 2008).

Principle: the test principle is to reproduce the process exactly but replacing real products with culture media. As for the sterility test, there is a need to check the fertility of the culture media before the test is conducted.

Choice of culture media: the media used must allow for the growth of a broad spectrum of micro-organisms while keeping to a reduced quantity (≤ 100 UFC); in most instances triptycase soy broths are used.

Batch size: for batches of a size of less than 3,000 units produced (this being the general case in hospital pharmacies), the batch size must be equal to the maximum batch size.

Number of tests: in initial qualification, the test is reproduced 3 times. In requalification, a single test is required.

Test condition: in so far as is possible, the worst cases (where they have been identified) must be reproduced during the test. Operators must first have been qualified for aseptic preparation (cf. & microbiological validation for operators).

The process used must reproduce the process used in real production as faithfully as possible. In manual production, it will be necessary to introduce all production methods used in the test, as for example, sampling from an ampoule, solubilisation of powder, transfer of vials, and preparation of syringes and mini bags, while the number and type of actions must be as representative as possible of the activity.
In the production zone, it will be necessary to ensure there are no disinfection residues likely to inhibit the culture media. At the end of the test, all culture media residues that could foster microbiological growth in the environment will have to be eliminated.

Incubation: all the units are brought to incubate for 14 days at a temperature suited to micro-organisms of between 30 and 35°C (European Pharmacopoeia) and observed regularly in the interval to detect the appearance of possible disturbance indicating microbial growth. To detect a wide variety of bacteria and detect yeasts/moulds, 2 incubation temperatures can be recommended (Barillet L. et al., 2008), 20 to 25°C for 7 days immediately followed by 30-35°C for 7 days. If the result is positive, the micro-organisms as also their source (human or environmental) must be determined.

Media fertility test: there is a need to demonstrate that the culture media used is appropriated for microbiological growth of the strains prescribed by the European Pharmacopoeia. It is recommended to conduct the fertility test before using the media for validation of the processes but to apply it to units manufactured after incubation in order to ensure there is no influence of the process on performances of the culture medium (Barillet L. et al., 2008).

Expected results: no microbiological growth must be detected. In the event of growth, an inquiry must be proceeded with to determine the source of the process behind the contamination.

MICROBIOLOGICAL VALIDATION FOR OPERATORS

Validation of operators adopts the same media fill test principle developed above but applies to manufacturing by a single operator. The objective is to ensure that the operator has mastery over the aseptic process. This test must be performed before media fill for process validation.
An operator dedicated validation procedure for hospital pharmacies has been developed by the NHS UK Pharmaceutical Aseptic Services Committee (Sizer T. et al., 2001). Each test gives the operator the opportunity to make 29 transfers from vials, bags and ampoules and in the end make 10 preparations with the culture medium (5 syringes, 4 vials and one bag). The method can readily be reproduced by purchasing culture media marketed in bags and vials, but there are also ready to use kits (for example: Klerkit Universal Operator Broth Transfer Validation Kit, Shield Medicare).

This test's major limitation is the level of sensitivity for its application in a hospital pharmacy environment. Indeed, considering the ISO 5 manufacturing environment, the manufacturing process with its low risk of microbiological contamination (closed process in accordance with GMP) and the very small number of units produced, it is unlikely that microbiological contamination be highlighted. At least 3,000 units would have to be manufactured to detect a rate of contamination at 1 per 1,000 with a confidence interval of 95%, which is practically impossible. One way to improve the test's sensitivity to evaluate operators is to contaminate surfaces at risk on purpose with a challenge micro-organism, with manufacturing by operators being performed in simulated environment conditions (Sigward E. et al., 2012).

Appendix 5

SHEETS EQUIPMENT QUALIFICATION

Sheet 1 : Osmometer

Sheet 2 : Automatic pipette

Sheet 3 : Counter invisible particles

Sheet 4 : Chromatographic system (HPLC)

Sheet 1 : Osmometer

Operational qualification of an osmometer

Control date:
Operator :
Control number :

1. Osmometer calibration:

Calibration 50 mOsmol/kg ☐
Calibration 850 mOsmol/kg ☐

2. Osmometer control:

Frequency : Twice a year

290 mOsm/kg standard repeatability:

Sample	Value	
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
Mean		**Acceptability criteria:**
Standard deviation		**288-292 mOsm/kg** **2 mosm/kg**

3. Conclusion :

Osmometer in accordance with the specifications

☐ **Yes**

☐ **No**

Signature of the operator **Signature of manager**

Qualification of an automatic pipette (100µl)

Control date:
Operator :
Control number :

Single channel pipette with variable volume

RAININ EDP2/100
Serial number: 1693018

Gravimetric method : **Using the Mettler Toledo XS205DU**
N° CHU-05-090
Z (25°c) : 1,003774

1. Controlled volume : 10 µL

	measure 1	measure 2	measure 3	measure 4	measure 5
Weight (g)					
correction z (µl)	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**
Mean volume (µl)					
Standard deviation					

	Accuracy biais		Precision biais	
	µl	%	µl	%
Calculations				
Manufacturer tolerance	0,3	3	0,1	1
ISO 8655 norm	**0,8**		**0,3**	

2. Controlled volume : 100 µL

	measure 1	measure 2	measure 3	measure 4	measure 5
Weight (g)					
correction z (µl)	**0,00**	**0,00**	**0,00**	**0,00**	**0,00**
Mean volume (µl)					
Standard deviation					

	Accuracy biais		Precision biais	
	µl	%	µl	%
Calculations				
Manufacturer tolerance	0,80	0,80	0,20	0,20
Norme ISO 8655	**0,80**		**0,30**	

3. Conclusion :

In accordance with the manufacturer's tolerance ☐
In accordance with the ISO 8665 norm ☐

Signature of the operator **Signature of manager**

Sheet 3 : Counter invisible particles

Operational qualification of a subvisible particles counter

Control date:
Operator :
Control number :

Counter HIAC 9703 Hach Lange

1. Control of the counter 10 µL

Frequency : Twice a year

- Use of a standard Pharmtrol® (Thermo): polystyrene particles of 15 µm, 3800/ml ±15%, 25ml.

Counting (monograph 2.9.19 European Pharmacopoeia 7th edition) :

- 4 measures on a 5 ml volumefor particles >10µm and particles >25µm, mean on the 3 last measures.

	A : Number of > 10 µm particles /ml	B : Number of >25 µm particles /ml
Counting 1		
Counting 2		
Counting 3		
Counting 4		

Mean (A) : **Mean (B) :**

Acceptability criteria :

Mean (A) et mean (B) in the range 3230-4025 ☐
Relative difference between Mean A and Mean B <10% ☐

2. Conclusion :

Counter in accordance with the specifications :

☐ **oui**
☐ **non**

Signature of the operator **Signature of manager**

Sheet 4 : Chromatographic system (HPLC)

Qualification/Maintenance of a chromatographic system (HPLC)

Chaine HPLC Jasco 9000

Control date:
Operator :
Control number :

	Checking	Action	Reference pieces/parameters	Results
Pump	In-line filter	Clean or change	Ref 6688-H704B	
	Suction and discharge valves	Clean or change	Suction :6778-H109A Discharge: 6688-H455B	
	Piston seals	Change once/year	Ref 6688-H148A	
	Diaphragms Rinsing circuit	Change once/year	Ref 6688-H455B	
	Zero pressure test	Without column and pump working	Display : 0 bar	
	Leak test : maximal pressure	Seal front ; apply a maximum pressure of 500 bars, stop the pump.	No leaks if pressure remains unchanged	
	Flow check	With column connected	0.5 ml, 1 ml, 2 ml/min	
Autosampler	Rheodyne valve	rotor seal change / 2000 injections	Ref 7010-039	
	Needle	Change once/year	Ref 6705-H151A	
	Suction syringe	Change plunger tip once/year Change metal filter and seal once/year	Ref 2313-0045 Filter + seal ref 6705-H118A	
	Pressure test	Obture autosampler outlet; test a pressure of 300 bars, stop the pump.	No leaks if pressure remains unchanged	
Detector	UV lamp	Change if undervoltage (<1V) Voltage : shift 1 Number of hours : shift 6	Ref L-6300-51	
	Reset to zero the number of hours after change	Cf Guidebook		

Signature of the operator

Signature of manager

REFERENCES

CHAPTER 1 KEY INSTABILITIES: DESCRIPTION AND INFLUENCE FACTORS

THIS CHAPTER IS MAINLY BASED ON THE ARTICLE OF TRISSEL LA, 1990 AND THE REFERENCES THEREIN

Arnaud Y., Dauphin A., Mignot A. Interactions matières plastiques/médicaments . In Les matières plastiques à usage pharmaceutique (Coord E. Postaire). 2ème ed. 1991, Editions médicales internationales, Paris, France.

Carstensen J.T., Rhodes C. Drug Stability: Principles and Practices, 3rd Rev. 2000, Marcel Decker Inc, New York, USA.

Florence A.T., Attwood D. Drug stability. In physicochemical principles of pharmacy. 4th Ed. 2006, Pharmaceutical Press, London, UK.

Trissel L.A, Drug stability and compatibility issues in drug delivery, The Cancer Bulletin. 1990; 42:393-398.

CHAPTER 2 GENERAL METHODOLOGY

Bakshi M., Singh S. Development of stability-indicating assay methods: critical review. J. Pharm.Biomed. Anal. 2002; 28: 1011-40.

Barberi-Heyob M., Watelet M. Merlin J.L. Bleger C., Schroeder B. Stability of newly-formulated 5 fluorouracil solutions. Bull. Canc. 1995, 82(12): 1025-31

Bardin C., Astier A., Vulto A. et al. Guidelines for the practical stability studies of anticancer drugs: a european consensus conference. Ann. Pharm. Fr. 2011, 69: 221-231.

Bontemps C., Chataigner A, Sautou-Miranda V, Chopineau J. Étude de stabilité d'une solution de céfuroxime pour injection intracamérulaire. Congrès Hopipharm, Marseille 2009.

International Conference of Harmonization (ICH). Guidelines for stability Q1A à Q1F. http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html

Oldham G.B. Formulation and stability of cefuroxime eye-drops. Int. J. Pharm. Pract. 1991; 1: 19-22.

Singh D., Kumar V. Recent developments on long term stability test conditions. The pharma review. 2006: December: 61-68.

Vigneron J. Stability studies: ten pieces of advice. EJHP Sci. 2008; 14:2.

Williams L.A., Hastings MB. Identifying the criteria of a valid stability study. Int. J. Pharm. Comp. 2009; 13(1). 32-36.

CHAPTER 3 METHODS FOR THE ANALYSIS OF ACTIVE INGREDIENTS AND DEGRADATION PRODUCTS

Altria K. Application of microemulsion electrokinetic chromatography to the analysis of a wide range of pharmaceuticals and excipients. J. Chromatogr. A. 1999; 844 (1-2): 371-386.

Dermaux A., Sandra P. Applications of capillary electrochromatography. Electrophoresis. 1999 ; 20 (15-16): 3027-3065.

Guillarme D., Rudaz S., Veuthey JL. Nouveaux développements en chromatographie rapide dans le domaine pharmaceutique. Spectra Analysis. 2009; 267: 12-17.

Hiroyuki N., Terabe S. Micellar electrokinetic chromatography. Perspective in drug analysis, S. Terabe, J. Chromatogr. A. 1996; 735(1-2): 3-27.

Ivanovic I., Zivanivic L., Zecevic M. A stability indicating assay method for cefuroxime axetil and its application to analysis of tablets exposed to accelerated stability test conditions. J. Chromatogr. A. 2006; 1119(1-2): 209-15.

Lu J., Wei Y., Rustum AM. A stability-indicating reversed-phase high performance liquid chromatography method for simultaneous assay of two corticosteroids and estimation of their related compounds in a pharmaceutical injectable formulation. J. Chromatogr. A. 2010; 1217(1-2): 6932-41.

Riekkola M.L., Jussila M., Porras S.P. et al. Non-aqueous capillary electrophoresis. J. Chromatogr. A. 2000; 892 (1-2), 155-170.

Rosset R. Chromatographies en phases liquide et supercritique » 3ème éd., 1991, Editions Masson, Paris, France.

Sherma J. Review of HTPLC in drug analysis: 1996-2009. J AOAC Int. 2010; 93(3): 754-764.

Suntornsuk L. Recent advances of capillary electrophoresis in pharmaceutical analysis. Anal. Bioanal. Chem. 2010 ; 398(1) : 29-52.

CHAPTER 4 VALIDATING THE ANALYTICAL METHOD (+ APPENDIX 1 ET 2)

Carstensen J.T., Rhodes C. Drug Stability: Principles and Practices, 3rd ed. 2000, Marcel Decker Inc, New York, USA

De Fontenay G., Respaud J., Puig P. et al. Analyse des performances d'une Method analytique. Evaluation des risques lors d'une utilisation en routine. STP Pharma Prat, 2011, Vol 21(2), 123-33.

Gautier J.C., Nivet J.M., Algranti P. et al. Guide validation analytique. Rapport d'une commission SFSTP. I- Méthodologie. STP Pharma. Prat. 1992; 2(4): 205-26.

Hubert P., Nguyen-Huu J.J., Boulanger B. et al. Commission SFSTP. Validation des procédures analytiques quantitatives : harmonisation des démarches. STP Pharma.Prat. 2003 ; 13 (3): 101-134.

Hubert P., Nguyen-Huu J.J., Boulanger B. et al. Commission SFSTP. Validations des procédures analytiques quantitatives : harmonisation des démarches Partie II – Statistiques. STP Pharma. Prat. 2006 ; 16 (1) : 30-60.

Hubert P., Nguyen-Huu J.J., Boulanger B. et al. Commission SFSTP. Validation des procédures analytiques quantitatives : harmonisation des démarches Partie III - Exemples d'application. STP Pharma. Prat. 2006; 16 (2): 87-212.

ICH Q1A (R2). Stability testing of new drug substances and products. 2003 http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3A_R2/Step4/Q3A_R2__Guideline.pdf

ICH Q1B. Stability testing : photostability testing of new drug substances and products. 1996 http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1B/Step4/Q1B_Guideline.pdf

ICH Q1E. Evaluation of stability datas. 2003
http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1E/Step4/Q1E_Guideline.pdf

ICH Q2 (R1). Validation of analytical procedures : text and methodology. 1996
http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf

ICH Q3A. Impurities in new drug substances. 2006
http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3A_R2/Step4/Q3A_R2__Guideline.pdf

Trissel L.A. Stability indicating HPLC methods for drug analysis. 1st ed, 2008. Pharmaceutical Press. London, UK.

Walker S.E., Milliken D., Law S. Stability of bortezomib reconstituted with 0.9% sodium chloride at 4°C and room temperature (23°C). Can. J. Hosp. Pharm. 2008; 61(1): 14-20.

CHAPTER 5 OTHER ANALYSES DEPENDING ON PHARMACEUTICAL FORMS

Pharmacopée Européenne, 7ème édition 2013, Direction Européenne de la Qualité du médicament et des soins de santé, Conseil de l'Europe, Strasbourg. http://www.edqm.eu/

Wehrlé, P. Pharmacie Galénique : Formulation et technologie pharmaceutique. 2ème édition, 2012, Maloine, Paris, France

CHAPTER 6 INTERPRETATION OF RESULTS

Asean guidelines on stability study of drug product. Update review 22 Février 2005. 9th ACCSQ-PPWG Meeting, Philippines

Bardin C., Astier A., Vulto A. et al. Guidelines for the practical stability studies of anticancer drugs: a european consensus conference. Ann. Pharm. Fr. 2011, 69: 221-231.

ICH Q1E. Evaluation of stability datas. Février 2003
http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1E/Step4/Q1E_Guideline.pdf

ICH Q3A. Impurities in new drug substances.2006
http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3A_R2/Step4/Q3A_R2__Guideline.pdf

ICH Q3B. Impurities in new drug products. 2006
http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3B_R2/Step4/Q3B_R2__Guideline.pdf

Pharmacopée Européenne, 7ème édition 2013, Direction Européenne de la Qualité du médicament et des soins de santé, Conseil de l'Europe, Strasbourg. http://www.edqm.eu/

CHAPTER 7 MICROBIOLOGICAL STABILITY STUDIES OF PREPARATIONS

Bonnes Pratiques de Préparation, AFSSAPS, 3 Décembre 2007.
http://ansm.sante.fr/var/ansm_site/storage/original/application/a5d6ae4b3d5fdee013ca463462b7b296.pdf

Barillet L., Béchaud P., Chancel O. et al. De la pertinence des milieux de culture dans les simulations de procédés aseptiques. STP Pharma. Pratiques. 2008; 18(6): 503-510

Briceland L.L., Fudin J. Evaluation of microbial growth in selected inoculated antineoplastic solution. Hosp. Pharm. 1990; 1: 50338-340.

Chollet R., Kukucska M., Halter N. et al. Rapid detection and enumeration of contaminants by ATP bioluminescence using the Milliflex® Rapid Microbiology Detection and Enumeration system. J. Rapid. Methods. Autom. Microbiol. 2008; 16: 256-72.

Corriol O., Crauste-Manciet S., Arnaud P. et al. Recommandation pour la préparation des mélanges de nutrition parentérale. Nutr. Clin. Metabol. 2005; 19: 30-55.

Guide to good practices for the preparation of medicinal products in healthcare establishments PE 010-3. Pharmaceutical inspection co-operation scheme. PIC/S, October 2008. http://www.picscheme.org/pdf/23_pe0103-revisedgppguide.pdf

Isanhart C.M., McCall K.L., Kretschmer D. et al. Parenterals laboratory course to reduce microbial contamination rates in MFTs performed by pharmacy students. Am. J. Pharm. Educ. 2008; 72(2) article 27: 1-3.

Karstens A., Krämer I. Viabiability of micro-organisms in fluorouracil in novel anticancer solutions. Eur. J. Hosp. Pharm. Sci. 2007; 13: 27-32.

Khuu H.M., Stock F., McGann M. et al. Comparison of automated culture systems with a CFR/USP-compliant method for sterility testing of cell-therapy products. Cytotherapy. 2004; 6: 183-95.

Krämer I. Viability of microorganisms in novel antineoplastic and antiviral drug solutions. J. Oncol. Pharm. Pract. 1998, 4:32-7.

Macias A.J., Huertas M., Ponce de Leon S. et al. Contamination of intravenous fluids: a continuing cause of hospital bacteremia. Am. J. Infect. Control. 2010; 38: 217-21.

Parveen S., Simleen K., Selwyn A. et al. Evaluation of growth based rapid microbiological methods for sterility testing of vaccines and other biological products. Vaccine 2009; 29: 8012-8023.

Pharmacopée Européenne, 7ème édition 2013, Direction Européenne de la Qualité du médicament et des soins de santé, Conseil de l'Europe, Strasbourg. http://www.edqm.eu/

Sigward E., Fourgeaud M, Vazquez R. et al. Aseptic simulation test challenged with microorganisms for validation of pharmacy operators. Am. J. Health-Syst. Pharm. 2012; 69(15):1218-1224

Sizer T., Duncombe R., Needle R et al. Universal operator broth transfer, Version 9, UK Pharmaceutical Aseptic Services Committee (formerly UK National CIVAS Group) April 2001. update janv 2003, dec 2005. http://home.btclick.com/civas/universalbroth/Universal-Operator-Broth-Test-Version9-Dec2005.pdf

Standard ASTM F 1929-98 (Reapproved 2004). Standard test method for detecting seal leaks in porous medical packaging by dye penetration. Janvier 1998. ASTM International, West Conshohocken, USA

Stucki C., Sautter A.M., Favet J. et al. Microbial contamination of syringes during preparation: the direct influence of environmental cleanliness and risk manipulations on end-product quality. Am. J. Health-Syst. Pharm. 2009; 66: 2032-6.

Tidswell E.C., Rockwell J., Wright M.O. Reducing hospital-acquired infection by quantitative risk modeling of intravenous bag preparation. PDA J. Pharm. Sci. Technol. 2010; 64(2): 82-91.

The United States pharmacopeia, 36st rev., and the national formulary 31th ed. Rockville, MD: United States Pharmacopeial Convention; 2013.

CHAPTER 8 STABILITY OF PREPARATIONS WITH A PROTEINACEOUS ACTIVE PRINCIPLE

Bardin C., Astier A., Vulto A. et al. Guidelines for the practical stability studies of anticancer drugs: a European consensus conference. Ann. Pharm. Fr. 2011; 69(4): 221-31.

Boross P., Leusen J.H. Mechanisms of action of CD20 antibodies. Am. J. Cancer. Res. 2012; 2(6): 676-90.

Carvalho M., Paul M., Astier A. et al. Mechanically-induced aggregation of the monoclonal antibody cetuximab. Ann. Pharm. Fr. 2009; 67(5): 340-52.

Casadevall N., Nataf J., Viron B. et al. Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. N. Engl. J. Med. 2002; 14: 346(7): 469-75

Ebbers H.C., Crow S.A., Vulto A.G.. Interchangeability, immunogenicity and biosimilars. Nat. Biotechnol. 2012; 30(12): 1186-90.

Hawe A, Wiggenhorn M, van de Weert M. Forced degradation of therapeutic proteins. J. Pharm. Sci. 2012; 101(3): 895-913.

Hoehne M., Samuel F., Dong A. et al. Adsorption of monoclonal antibodies to glass microparticles. J. Pharm. Sci. 2011; 100(1): 123-32.

Mahler H.C., Friess W., Grauschopf U. et al. Protein aggregation: pathways, induction factors and analysis. J. Pharm. Sci. 2009; 98(9): 2909-34.

Manning M.C., Chou D.K., Murphy B.M. et al. Stability of protein pharmaceuticals: an update. Pharm.Res. 2010; 27(4): 544-75.

Paul M., Vieillard V., Jaccoulet E. et al. Long-term stability of diluted solutions of the monoclonal antibody rituximab. Int. J. Pharm. 2012; Oct 15; 436(1-2): 282-90.

Printing: www.printconseil.fr - Photos credits: fotolia