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STUDY OF THE EFFECT OF DRY HEAT STERILIZATION ON ACTIVE PHARMACEUTICAL INGREDIENTS

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on Active Pharmaceutical Ingredients

Dedico este trabalho ao meu avô, Antônio Motta.

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"I am among those who think that science has great beauty."
(Marie Curie)

ABSTRACT

Sterile drug formulations must be subjected to a series of requirements to ensure the safety of health users. Sulfacetamide sodium and gentamicin sulfate are two bactericidal agents used in pharmaceuticals, however, terminal sterilization of these drugs is not possible due to possible degradation or instability. In these cases, all ingredients must be sterilized separately, and aseptic processing must be performed. The suitability of dry heat sterilization in a hot air oven was tested for both substances in different containers. Cycles ranging from 121°C for two hours to 180°C for 30 minutes were tested. The product integrity was investigated, where HPLC analysis had a critical role.

The analytical HPLC methods to determine the assay and related substances of sulfacetamide sodium required a revalidation due to an alteration in the solvent composition. The methods demonstrated to have a linear response within the working range, as well as robustness, precision, selectivity, and accuracy, appropriate limits of detection and quantification and a relative response factor of 1.9 of sulfanilamide.

Forced degradation tests performed on sulfacetamide sodium samples with acid, base, oxide, temperature, and UV light conditions, showed a decreased content of sulfacetamide sodium corresponding to an increased content of related compounds. The greatest degradation occurred when the samples were subject to oxidative conditions. Unknown impurities were formed therefore samples must be adequately monitored to prevent degradation during routine analysis.

For sulfacetamide sodium, dry heat sterilization in a sealed glass container resulted in the formation of a reddish-brown substance in cycles from 160°C to 180°C, with no color alteration for lower temperatures. The process demonstrated suitability for this substance in a moisture permeable sterilization pouch. Using this container, the ingredient maintained its physical properties for all cycles, and it showed no signs of degradation for a period of three months after a sterilization cycle of 160°C for 120 minutes.

The same process had a major impact on gentamicin sulfate, which went from a white to a yellow powder under all cycles and containers tested, showing a loss in content of, approximately, 10.0% after sterilizing it at 160°C for 120 minutes in a sealed nylon pouch with nitrogen atmosphere.

Keywords: Sterilization, Dry heat, Hot air oven, Validation of analytical methods, Sulfacetamide sodium, Gentamicin Sulfate.

RESUMO

Formulações estéreis devem ser submetidas a uma série de requisitos para garantir a segurança dos utentes. A sulfacetamida sódica e o sulfato de gentamicina são agentes bactericidas usados em medicamentos, porém, a esterilização terminal não é possível devido a possível degradação ou instabilidade. Nesses casos, todos os ingredientes devem ser esterilizados separadamente e implementado um processamento asséptico. A esterilização por calor seco em estufa de ar quente foi testada para ambas as substâncias em diferentes recipientes. Ciclos de 121ºC por duas horas a 180ºC por 30 minutos foram testados. A integridade do produto foi investigada, onde a análise por HPLC teve um papel fundamental.

Os métodos analíticos de HPLC para determinar o doseamento e substâncias aparentadas da sulfacetamida sódica exigiram uma revalidação devido a uma alteração na composição do solvente. Os métodos demonstraram ter uma resposta linear dentro da gama de trabalho, bem como robustez, precisão, seletividade e exatidão, limites de deteção e quantificação adequados e um fator de resposta relativo de 1,9 da sulfanilamida.

Os testes de degradação forçada foram realizados em amostras de sulfacetamida sódica com ácido, base, óxido, temperatura e luz UV, e os resultados mostram uma diminuição do teor de sulfacetamida sódica correspondente a um aumento do teor de impurezas. Com a maior degradação em condições oxidativas. Impurezas desconhecidas foram formadas, então as amostras devem ser bem monitoradas para evitar degradação durante análises de rotina.

Para a sulfacetamida sódica, a esterilização por calor seco em recipiente de vidro selados resultou na formação de substância castanho-avermelhada em ciclos de 160ºC a 180ºC, sem alteração da cor para temperaturas mais baixas. O processo demonstrou ser adequado para esta substância em uma bolsa de esterilização permeável à humidade. Com esse recipiente, o ingrediente manteve suas propriedades físicas em todos os ciclos, não apresentando sinais de degradação por um período de três meses após um ciclo de esterilização de 160ºC por 120 minutos.

O mesmo processo teve grande impacto no sulfato de gentamicina, que passou de um pó branco a amarelo em todos os ciclos e recipientes testados, apresentando uma perda de teor de, aproximadamente, 10,0% após esterilização a 160ºC por 120 minutos numa bolsa de nylon selado com atmosfera de azoto.

Palavras-chave: Esterilização, Calor seco, Estufa, Validação de métodos analíticos, Sulfacetamida sódica, Sulfato de gentamicina.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS AND ABBREVIATIONS

API – Active Pharmaceutical Ingredient

ATR - Attenuated total reflection

CAS - Chemical Abstracts Service Registry Number

CFU – Colony Forming Units

CRS – Chemical Reference Substance

CV – Coefficient of Variation

ELSD – Evaporative Light Scattering Detector

GAA – Glacial Acetic Acid

GMP - Good Manufacturing Practice

HPLC – High-Performance Liquid Chromatography

ICH - International Council for Harmonisation

IR – Infrared

LOD – Limit of Detection

LOQ – Limit of Quantification

LTB - Lauryl Tryptose Broth

NMR - Nuclear Magnetic Resonance

Ph. Eur. - European Pharmacopoeia

PVDF - Polyvinylidene Fluoride membrane

R^2 - Coefficient of Correlation squared

RF – Response Factor

R_F - Retardation Factor

RRF – Relative Response Factor

RSD - Relative Standard Deviation

SAL – Sterility Assurance Level

SD – Standard Deviation

TAMC - Total Aerobic Microbial Count

TSA – Tryptic Soy Agar

TSB - Tryptic Soy Broth

TYMC - Total Yeast and Mold Count

UV – Ultraviolet

VT – Test Value

1. INTRODUCTION

1.1. Context and motivation

The pharmaceutical industry produces many medicines that are directly applied to sensitive parts of the body, such as the ocular tissue. The manufacturers, then, must respect a Sterility Assurance Level in order to reduce the risks of infections associated with the growth of microorganisms.

According to the European Pharmacopoeia 5.1.1. (Methods of preparation of sterile products), terminal sterilization, where the final product is sterilized in its final container, is the preferred method due to its low possibility of recontamination. It is challenging to submit many pharmaceuticals to terminal sterilization, in some cases, because of the difference in each ingredient's resistance to different types of sterilization conditions.

Sulfacetamide sodium and gentamicin sulfate are two active ingredients used in eye drops and ophthalmic ointments produced by the company, and these substances are susceptible to suffer degradation under sterilizing conditions. The bioburden of both substances is particularly low, respecting the requirements, and all other substances included in these medicines are sterilized, followed by aseptic processing. Additionally, when it comes to anti-bacterial agents, the possibility of microbial growth is very reduced. However, this does not guarantee that the final product will remain sterile, therefore, sterilizing the active ingredients would add another level of safety to health users.

The Good Manufacturing Practice (GMP) expresses that, whenever possible, heat sterilization (moist or dry), should be the method of choice (EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use - Manufacture of Sterile Medicinal Products, 2008). Dry heat sterilization is a suitable method for many powder ingredients and is a promising process to eliminate all microbes present in the substances without causing degradation.

1.2. **Objectives**

With the previous context and motivation in mind, the goals of this work are the following:

- Performing different dry heat sterilization cycles in a hot air oven on both active ingredients;
- Verifying the efficacy of the cycles, verifying a Sterility Assurance Level (SAL) ≤ 10^{-6};
- Investigating the suitability of the dry heat sterilization process for both substances, through a complete analysis of their physical and chemical properties, including a Mass spectroscopy (LC-MS) analysis to detect any loss in content and formation of impurities;
- Monitoring the substances' sterility and stability after sterilization.
- Choosing the most suitable dry heat sterilization cycle for each API.

2. Literature review

2.1. Sterilization of Active Pharmaceutical Ingredients

Sterilization is achieved when an item is fully free from all microbes, which are, then, incapable of reproduction. It has a great importance when it comes to injectables, eye formulations and other pharmaceuticals for internal and external use. It differs from disinfection in the way that sterilization refers to a complete removal of all microorganisms, including bacterial spores and endotoxins, whilst disinfection concerns only the reduction of the number of microbes or microbial colonies (Rutala & Weber, 2013).

The goal of sterilization, from a pharmaceutical perspective, is mainly to avoid any chances of microbial growth, reducing possible risks of infection. In these cases, sterile products must be employed from production to administration. Under favorable conditions, such as humidity or a non-sealed package, bacteria, fungi, and viruses can grow and become viable again, meaning there is still a risk of non-sterility in sterile products. Therefore, to control the sterility of a drug, aspects such as manufacturing, processing, packaging, storage, as well as utilization are equally important. (Moondra, et al., 2018).

The methods of sterilization are divided into three categories (Moondra, et al., 2018).

- Physical removal – The physical absence of the organism by completely removing them.
- Physical alteration – The destruction, changing or altering the cellular or biochemical structure of the microorganism that affects the physiological function.
- Inactivation – The killing of microbes by complete and permanent disruption of the cellular or biochemical structure of microbes.

Drug delivery systems, as well as ophthalmic tools used in clinical practice, should be microbe free, due to the direct contact with the eye tissues, making sterilization required for both (Zielińska, et al., 2020).

The items that should be sterilized, from a pharmaceutical and medical perspective, are ophthalmic products, parenteral products containing drug substances, such as infusions, injections, vials and ampoules, formulated products, solutions, suspensions, and media, and products intended for use in sterile formulation packaging, such as containers, closures, and dropper bottles (Moondra, et al., 2018).

There are many aspects that may affect sterilization. According to Dağsuyu, et al. (2016) these include the population of microorganisms and their spatial arrangements, the microorganisms' intrinsic resistance, physical and chemical factors, and storage conditions. The population of microorganisms is the main element affecting sterilization, as the time required for the process is directly proportional to the number of microorganisms. Additionally, if the microorganisms are dispersed, the sterilization becomes easier than if they were clustered. The intrinsic resistance of a microbe modifies regarding the type of sterilization process, for that reason, the process used may vary with the object to be eliminated (Moondra, et al., 2018). Temperature and relative humidity are the main physical and chemical factors affecting sterilization. The process becomes more efficient when the temperature increases, however, excessive temperature may damage the product being sterilized. A humid atmosphere favors microbial growth, thus adversely affecting the sterilization process (Gerba, 2015). And finally, longer storage times may increase the microorganisms' resistance to sterilization (Moondra, et al., 2018).

According to Moriya & Módena (2008), the elements that should be observed when designing a sterilization process are the characteristics of the microorganisms, the resistance of the vegetative forms and of the spores producing bacteria, the number of microorganisms, and the characteristics of the sterilizing agent (Moriya & Módena, 2008).

From the Good Manufacturing Practice (GMP), the manufacture of sterile formulations should be performed in clean areas which should be kept to an appropriate cleanliness standard and supplied with air passed through filters of appropriate efficacy. Clean areas designated for high-risk operations, such as filling zone, open ampoules and vials, and making aseptic connections, are provided by a laminar air flow station. Clean rooms must be routinely monitored in operation, using methods such as settle plates, volumetric air, and surface sampling. A filtered air supply should maintain positive pressure and an air flow relative to lower grade areas. Temperature and relative humidity depend on the product and nature of the operations implemented (EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use - Manufacture of Sterile Medicinal Products, 2008).

The GMP refers that, in the course of all stages, including before sterilization, precautions to minimize contamination should be taken. Activities and movement of personnel should be kept to a minimum during aseptic operations. Microbiological contamination of starting

materials should be minimal, and the bioburden should be monitored for each batch prior to sterilization with specified limits on contamination before the process. Components, containers, and equipment should be handled after the cleaning process, as to not result in a recontamination (EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use - Manufacture of Sterile Medicinal Products, 2008).

The effectiveness of a sterilization process is assessed by a unit of measure called the Sterility Assurance Level (SAL). It represents a protection of the sterile product from contamination through the manufacturing process (Nguyen, et al., 2013). For this assessment, every log reduction (10^{-1}) represents chances of contamination of one in 1000. A sterilization process with a 6-log reduction (10^{-6}) is needed to attain, as it has a practically zero probability of microbial survival, representing one chance in a million that the product is not sterile (Shintani, 2016). It is possible to establish the SAL via testing the sterilization process on bacteriological endospores by using biological indicators, as their inactivation is a representation of the authenticity of the process (Moondra, et al., 2018). According to Archanjo, et al., (2012) the endospores are used on the considering they are the most resistant form against sterilizing agents. Therefore, a sterilization process can be represented by the destruction of all bacterial spores.

Mendes, et al., (2007) express that the choice of the sterilization method depends on the materials to be sterilized and on the impact that the method has on them. The chosen process may have negative effects on the products, and these can vary depending on the nature of the substances being sterilized. Therefore, choosing the correct method is crucial (Amadori, et al., 2015).

The sterilization process can be divided into terminal and nonterminal processes, based on the stage at which a substance undergoes sterilization. Aseptic processing, used for nonterminal processes, involves the use of techniques to process sterile products, avoiding additional contaminants. Sterile containers should be used for aseptically treated substances. A terminal process is where a finished product is sterilized in its primary container. Terminal sterilization is preferred to filtration and/or aseptic processing because a reliable SAL is possible to implement, which adds a safety margin. Inadequate technique during aseptic processing may cause accidental contamination, therefore, terminal sterilization provides the

highest assurance of sterility and should be used when possible (Guideline on the sterilisation of the medicinal product, active substance, excipient and primary container EMA/CHMP/CVMP/QWP/850374/2015, 2019).

Choosing the correct sterilization process is critical to ensure antimicrobial safety and the extended shelf life of the product, so well-optimized sterilization parameters are able to prevent the occurrence of degradation and limit the number of toxic residues released (Zielińska, et al., 2020).

The chemical and physical properties are influenced by the selected sterilization method. A suitable method must ensure sterility whilst maintaining stability and physico-chemical characteristics of pharmaceuticals, according to Archanjo, et al. (2012).

The methods available for sterilization are express in Figure 2.1.

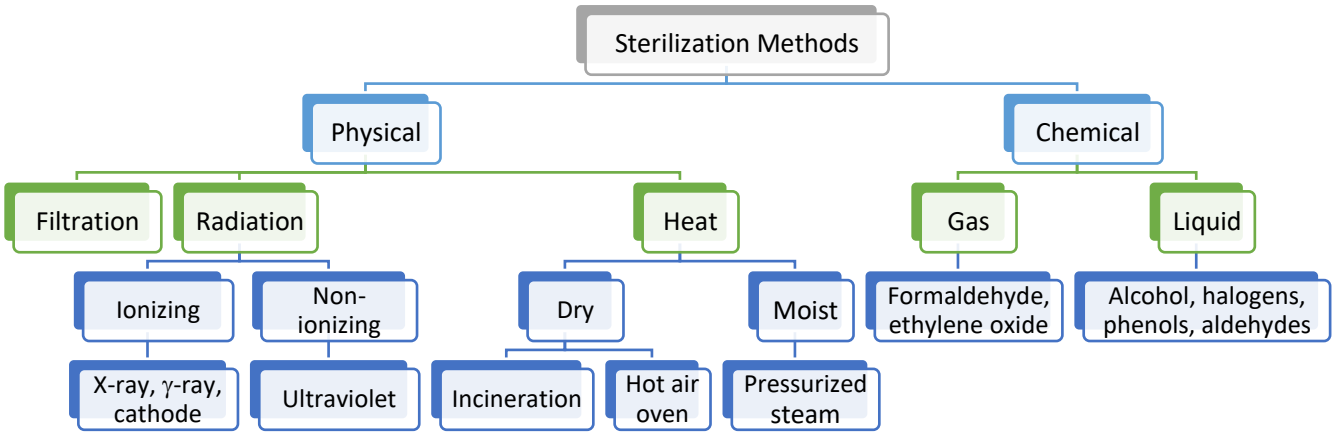


Figure 2.1-Methods of Sterilization. Adapted from (Moondra, et al., 2018)

Physical methods are divided in terms of temperature, exposure to radiation and filtration. Heat processes, both moist and dry, are not only the most used sterilization technique in the industry, but it is also the most economic and easy to control (Archanjo, et at., 2012). Regarding the physical methods, combining temperature and humidity results in most safe and effective processes. Chemical methods damage the structure of the microorganisms irreversibly (Mozachi, 2007). Although chemical agents deliver an inferior safety margin, they are an important alternative, given many materials are thermosensitive and cannot be sterilized by physical processes.

As stated in the European Pharmacopoeia (Methods of preparation of sterile products), finished products should be terminally sterilized whenever possible. When a terminal sterilization by heat is not possible, an alternative method of terminal sterilization, filtration and/or aseptic processing is considered. Terminal sterilization should not be dismissed only based on an increase in degradation products above the qualification thresholds or the impurity limits, and that should only happen if the degradation products are not qualified at the level at which they occur. The risk that a degradation generates should be balanced by the risk induced with aseptic processing (Guideline on the sterilisation of the medicinal product, active substance, excipient and primary container EMA/CHMP/CVMP/QWP/850374/2015, 2019).

According to the GMP, before a sterilization method is implemented, its product suitability and efficacy in achieving the desired sterilizing conditions should be demonstrated by physical measurements, or by biological indicators when appropriate. Where possible, heat sterilization (moist or dry), should be the method of choice (EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use - Manufacture of Sterile Medicinal Products, 2008). The selection of the sterilization process in case that the substance is a dry powder is presented by the decision tree in Figure 2.2.

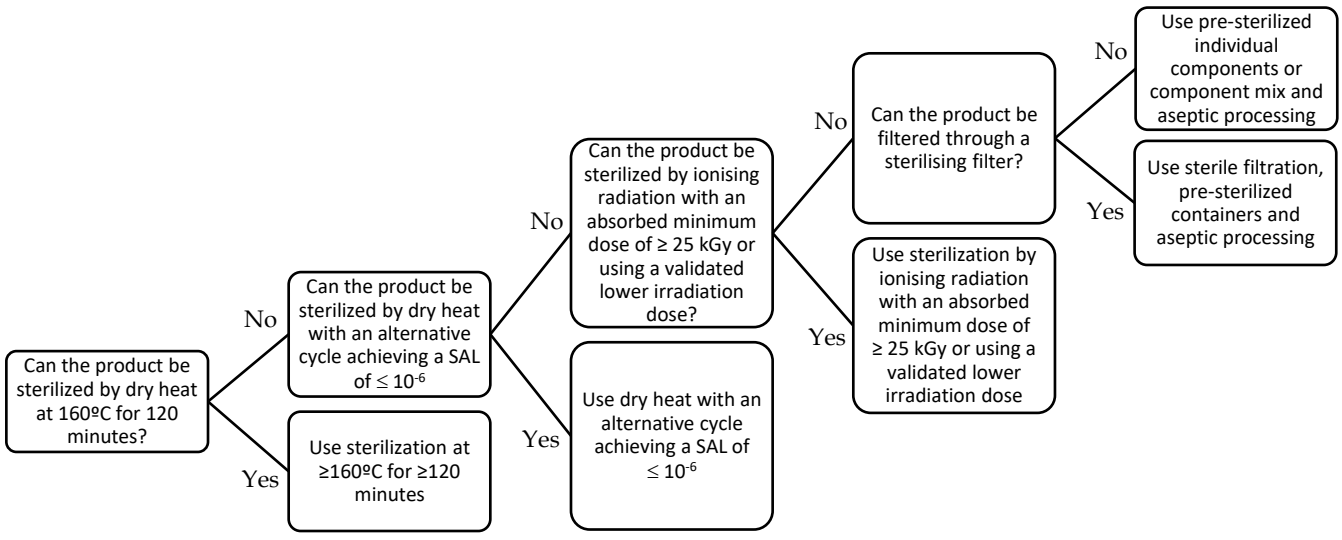


Figure 2.2-Decision tree for sterilization processes for dry powder products, non-aqueous liquids, or semi-solid products. Adapted from (Guideline on the sterilisation of the medicinal product, active substance, excipient and primary container EMA/CHMP/CVMP/QWP/850374/2015, 2019)

Sterilization is compulsory in the manufacturing of many medicines to reduce the risk of infection, however preserving the drug and material properties is also indispensable. An appropriate stability testing should be implemented to detect possible degradants during the shelf life of the substance (Zielińska, et al., 2020).

2.2. Sterilization by Heat

Heat sterilization (moist and dry) is the most utilized and consistent method of sterilization, where temperature is employed for the obliteration of microorganisms, as state Alfoqom Alazemi, et al., (2014). Sterilization by dry heat is the firstly thought method when it comes to dry powders. Dry heat works by oxidizing and dehydrating the chemical constituents of the microorganisms. High energy is required to break the peptide bonds of the proteins present in the microbes, due to the absence of water (Rachna, 2018). A microorganism's susceptibility to heat depends on many aspects, such as their individual resistance, spores forming capacity, amount of water and pH of the environment, and its composition (Moriya & Módena, 2008).

There are many benefits to using the dry heat method, given it is reliable and nontoxic, it has lower costs compared to others and it is easy to install, there are no chances of corrosion, since the instrument remains dry after sterilization, and it is not harmful to the environment (Rachna, 2018).

The thermal energy in the form of heat is capable of deactivating microorganisms and the degree of sterilization depends on the exposure time (Hirano, et al., 2014). According to Darmady, et al. (1961), it also depends upon sufficient heat penetration to the item, unlike the steam sterilization process, which needs the steam to come into direct contact with the surface of the object to kill the microbes. Powders are impenetrable by water or steam, therefore dry heat is highly suitable, given the heat penetrates all parts of the substance.

The most common apparatus used for dry heat sterilization is a hot air oven. It consists of a double-walled box, with hot air coming from an electrical resistance. The temperature inside is controlled by a thermostat and must remain constant throughout the sterilization time. The hot air oven is suitable for the sterilization of anhydrous materials, such as glass, syringes, needles, powders, cutting instruments, oils, Vaseline, etc. (Moriya & Módena, 2008).

Dry heat is easily diffused, and the process is rather simple, however, because of the air's relative difficulty as a heat transfer vehicle, dry heat cycles present longer times and higher temperatures than in the case of moist heat (Mozachi, 2007). Heat is absorbed from the

surrounding area of the equipment, moving to the next layers until the whole equipment reaches a set temperature and attains sterilization (Rachna, 2018). Larger volumes may influence the time of the process, given the heat may take longer to reach the central part of the load (Mozachi, 2007). Additionally, hot air ovens take close to one hour to reach sterilizing temperature. The apparatus must be mapped using sensors for the necessary temperatures prior to the sterilization, to ensure it does not show variations that may affect the process, which cannot exceed 5ºC (Darmady, et al., 1961).

The oven used in this study (Memmert™ UF55) was mapped for 160ºC and the assay report is shown in Table 2.1. The sensors were placed as it is demonstrated in Figure 2.3.

Table 2.1 - Mapping of the sterilization oven for 160ºC.

Sensor	Maximum (ºC)	Minimum (ºC)	Stability (ºC)	Average (ºC)
1	160.09	159.78	0.31	**159.93**
2	159.69	159.40	0.28	**159.55**
3	161.29	160.59	0.70	**160.96**
4	160.24	160.02	0.22	**160.13**
5	160.48	160.16	0.32	**160.30**
6	160.21	159.95	0.26	**160.08**
7	161.48	161.22	0.26	**161.34**
8	160.56	160.36	0.20	**160.47**
9	160.94	160.72	0.22	**160.83**
10	160.16	159.94	0.22	**160.06**

- The temperatures were acquired in intervals of 0m 10s ± 0.5s.
- The uncertainty associated to the maximum and minimum temperatures is ± 0.15ºC.
- The average presented was obtained by the average of all temperatures acquired after stabilization.
- The uniformity of the surroundings inside the chamber is 0.90ºC ± 0.24ºC, which is obtained by the difference between the maximum and minimum temperatures.
- The uncertainty of the stability is ±0.02ºC.

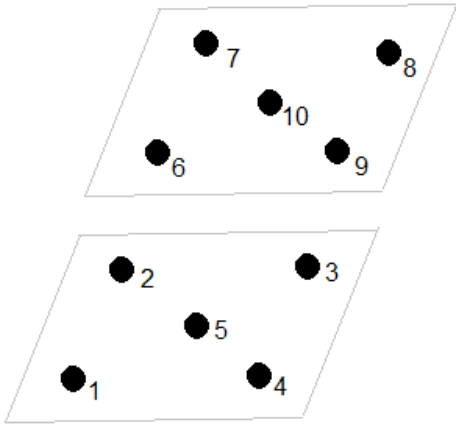


Figure 2.3-Placement of the sensors on both shelves of the sterilization oven, for the mapping of the apparatus at 160ºC.

- The bottom and top shelves are placed at 90 mm and 250 mm from the bottom of the oven, respectively.
- The side sensors are placed at 50 mm from the lateral walls.

According to the GMP, sufficient time should be allowed until the required temperature is reached before measuring the sterilizing time-period (EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use - Manufacture of Sterile Medicinal Products, 2008). Investigations showed that the temperature variations and the time to reach the necessary temperature may be reduced if the oven is allowed to heat up first and with the provision of a fan, forcing the circulation of air. Studies showed variations of 40ºC in an oven heated to 60ºC without a fan and not more than 5ºC in the same conditions with a fan (Darmady, et al., 1961). The GMP directs that the process should include air circulation within the chamber and a positive pressure should be kept, to avoid non-sterile air from entering and, after the cycle, precautions should be taken against contamination of the sterilized load during cooling (EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use - Manufacture of Sterile Medicinal Products, 2008).

The heat transfer properties and arrangement of the items inside the hot air oven are decisive in insuring effective sterilization. The efficiency of the sterilizer and reliability of mechanical components, as well as human error in operating the equipment are all factors to be considered (University of Missouri, 2020).

The reference conditions for dry heat sterilization, according to the Ph. Eur. 5.1.1. (Methods of preparation of sterile products) are a minimum of 160ºC for at least two hours.

For cycles with time and/or temperature lower than the reference conditions, physical and biological validation of the sterilization cycle should be provided to demonstrate a SAL $\leq 10^{-6}$, by demonstrating that the necessary parameters are achieved and by using biological indicators (Guideline on the sterilisation of the medicinal product, active substance, excipient and primary container EMA/CHMP/CVMP/QWP/850374/2015, 2019).

For active substances and finished products that are not used for parenteral administration, a maximum total bioburden limit of 10 CFU/g or 10 CFU/mL is acceptable (European Medicines Agency, 2019). According to the Eur. Ph. (Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use), the Total Aerobic Microbial Count (TAMC) acceptance criteria for microbiological quality of substances for pharmaceutical use is 200 CFU/mL and 2000 CFU/mL for the Total Yeast and Mold Count (TYMC).

The different possible temperatures and their correspondent times for dry heat sterilization cycles are demonstrated in Table 2.2.

Table 2.2-Combinations of time and temperature tested on dry heat sterilization cycles. Attained from https://casadobiotony.files.wordpress.com/2014/09/dfg.png (2014)

Temperature	Time
180ºC	30 minutes
170ºC	60 minutes
160ºC	**120 minutes**
150ºC	150 minutes
141ºC	180 minutes
121ºC	12 hours

The time and temperature combination is vital to attain sterilization using the dry heat method. It requires greater temperatures and lengthier times to reach microbial-killing effectiveness as compared with steam sterilization (Mohan & Gupta, 2016). Specific cycles must be determined for each type of material being sterilized (University of Missouri, 2020). The standard time according to the Ph. Eur. is 160ºC for 120 minutes. (European Medicines Agency, 2019) If that is the chosen cycle, validation is not necessary, however, if any of the other cycles are more suitable for the active ingredient, a validation of the cycle should be carried out.

2.3. **Sulfacetamide Sodium**

The first active ingredient to be analyzed was sulfacetamide sodium. This substance is included in the group of sulfonamides, which are high spectrum synthetic bactericidal agents (Purifarma, 2018). These have generally the same range of therapeutic action, inhibiting several gram-positive and some gram-negative organisms (Maggs, 2008). They work by blocking bacteria growth in the body through restricting the production of folic acid, which is required for them to multiply (Allergan, Inc., 2018). For this reason, medicines containing sulfonamides are prescribed as a treatment for a wide variety of bacteria caused infections, such as eye infections, bacterial meningitis, pneumonia, severe burns, and many others (Marks, 2015).

Sulfacetamide sodium is a monohydrate salt in the form of a whitish odorless crystalline powder with a bitter taste, highly soluble in water, that has low toxicity and great effectiveness as a drug. Consequently, it is the most prescribed sulfonamide for ophthalmic infections in the form of eye-drops and ointment, and it is mainly applied as 10-30% concentration solutions for topical application, according to Ahmad, et al. (1994). Sensitivity in therapeutic doses is greatly uncommon and sulfacetamide containing medicines are proved to be nonirritating to the ocular tissues even when it is at high concentrations, as express Ahmed, et al. (2017).

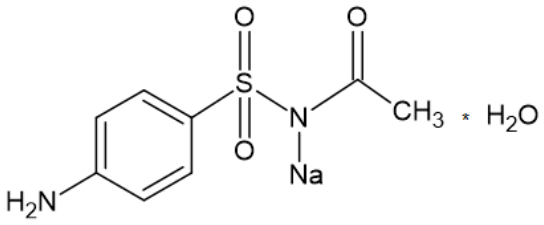


Figure 2.4-Chemical Structure of Sulfacetamide Sodium

Despite having anti-bacterial properties, it is still crucial that sulfacetamide sodium is sterilized, as well as all other APIs and excipients incorporated in any medicine that comes into direct contact with sensitive body tissues such are the eyes. Sulfacetamide sodium is the main active ingredient of ophthalmic ointments manufactured by the company. They are prescribed for the treatment of infectious and inflammatory ocular conditions, such as conjunctivitis. Considering these medicines contain more than one active ingredient, it is very challenging to perform a terminal sterilization, due to the different susceptivity to degradation of

each substance. Therefore, the logical route is for them to go through the process of sterilization separately, hence different conditions or even different methods may be applied.

For this study, it was necessary to understand whether sulfacetamide sodium was heat stable or if it would degrade under dry heat sterilization temperatures, forming its major degradation product, sulfanilamide, or other unknown impurities. This substance also has antibacterial properties; however, it is far less potent than sulfacetamide (Ahmed, et al., 2017). If sulfanilamide is formed above the limits, it will affect the efficacy of the medicines, thus it is essential that degradation factors are identified and avoided during sterilization and storage.

The thermal stability of this substance on aqueous solutions and ophthalmic preparations has been widely studied and discussed.

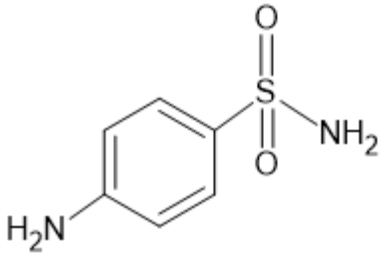


Figure 2.5-Chemical Structure of Sulfanilamide

According to the sulfacetamide sodium monograph in the European Pharmacopoeia, there are other impurities that may be detected in a sample of sulfacetamide sodium, although they are limited by a general criterion for unspecified impurities, which means it is not necessary to identify them. These impurities are N-(4-sulphamoylphenyl)acetamide, N-[[4-(acetylamino)phenyl]sulphonyl]-acetamide and dapsone.

Although there was a good deal of information about this substance's stability under moist heat sterilization conditions, the literature lacked data on studies performed at higher temperatures and the lack of moisture, which are representative of dry heat sterilization. With that in mind, there was an absence of knowledge of how sulfacetamide sodium would behave, since dry heat sterilization standard conditions are 160ºC for, at least, 2 hours. In addition, this active ingredient contains between 6 and 8% of water, which, upon evaporation, could create a moist environment, however not as much as in an autoclave, and this may possibly promote degradation (Ph. Eur.).

Furthermore, the studies completed by Ahmad et al., (1994) are related to aqueous solutions of sulfacetamide sodium, and those authors concluded that the solutions appeared darker with deposits of sulfanilamide after they were autoclaved in the presence of oxygen,

with no color change in an inert environment. Additionally, it has been found that they suffered a 1% loss when autoclaved at 115ºC for 30 minutes and at 120ºC for 20 minutes but when those were heated at 100ºC for 30 minutes, they were degraded by less than 0.5%.

However, for the posterior manufacturing process, the sterilization must be performed on the powder substance itself. For that reason, it becomes difficult to understand the thermal stability of this API, when the decomposition temperature is not determined, and knowing only that its melting point is around 260ºC (Fagron, 2015).

According to the authors (Ahmed, et al., 2017), the literature is missing information around sulfacetamide's degradation pathways and how the degradation products are formed, as well as their nature. Their argument is that the reactions of hydrolysis, photolysis and oxidation present some level of complexity and that is why they are not fully understood.

Based on photoinduced hydrolysis of sulfacetamide to form sulfanilamide and acetic acid according to (Ahmad, et al., 2016), the reaction in Figure 2.6. presents a suggestion for the hydrolysis of sulfacetamide sodium to form sulfanilamide and sodium acetate.

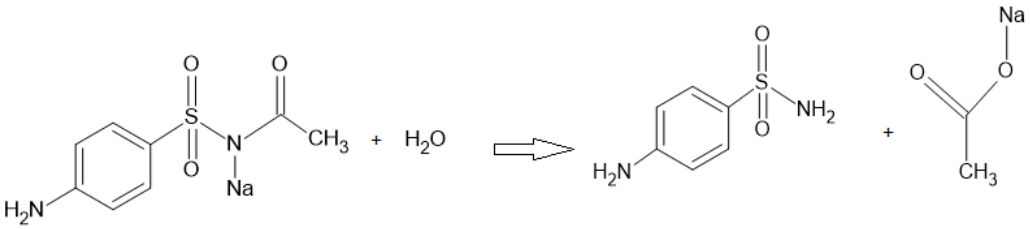


Figure 2.6-Hydrolysis of Sulfacetamide Sodium to form Sulfanilamide and Sodium Acetate. Adapted from (Ahmad, et al., 2016)

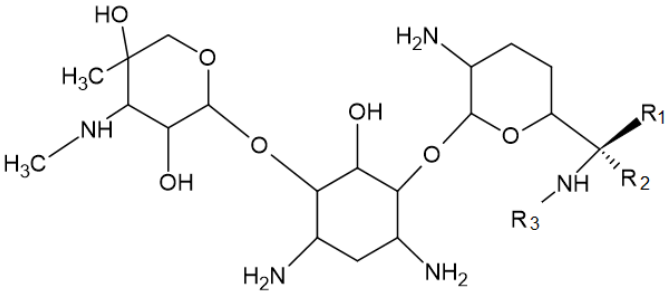
2.4. **Gentamicin Sulfate**

Gentamicin sulfate, the second active pharmaceutical ingredient to be analyzed, is also included in a few medicines manufactured by the company. It consists of an aminoglycoside antibiotic in the form of salt, which works by stopping bacterial protein biosynthesis, thus hindering the growth of many Gram-positive and Gram-negative microorganisms. It is a white, odorless powder, soluble in water and practically insoluble in alcohol and other organic solvents (Sigma-Aldrich, Inc.). This substance is characterized by a high spectrum and can be used to prevent bacterial infections in both animals and humans. It is isolated from microorganisms belonging to the *Micromonospora* species (Flurer & Wolnik, 1994).

Aminoglycoside antibiotics share pharmacokinetic features and present concentration-dependent killing, requiring parenteral administration due to their low oral absorption. They are effective against several aerobic Gram-negative bacilli, staphylococci, and a few mycobacteria and their uptake in the organism is promoted by inhibiting the synthesis of bacterial cell wall, although the mechanism through which that happens is still not comprehended. Although presenting a certain level of toxicity, and despite other alternatives for bactericidal agents that are far less toxic, aminoglycosides are still certainly useful in the treatment of many infections. Gentamicin remains the aminoglycoside of choice for infections caused by *Enterobacteriaceae* and *P. aeruginosa* (Edson & Terrell, 1999).

Gentamicin is not metabolized in the body, unlike almost every other drug, thus being eliminated unchanged. Additionally, it binds to multiple molecules, which results in it taking a long time to be excreted (Heslop-Harrison, 2012). Gentamicin comprises two pseudo-oligosaccharides, with identical polarities, C_1 and C_2, and they can be distinguished from other antibiotics belonging to the same family by its performance on paper chromatography (Weinstein, et al., 1963). Pseudo-oligosaccharides refer to microbial-derived metabolites with substantial biological activity that have been used in the treatment of human and plant infections, as state Alanzi, et al. (2018).

The three dominant components of Gentamicin are C_1, C_{1a} and C_2, however, Gentamicins C_{2a} and C_{2b} may also be found within the mixture (Flurer & Wolnik, 1994). Their chemical structures are illustrated subsequently.



Component	R1	R2	R3
C1	CH_3	H	CH_3
C1a	H	H	H
C2	CH_3	H	H
C2a	H	CH_3	H
C2b	H	H	CH_3

Figure 2.7-Chemical structure of Gentamicin components

Gentamicin sulfate is used by the company as an active ingredient in ocular antibiotics for the treatment of infections such as conjunctivitis and keratitis, as well as for eye burns and injuries by physical agents. Since they come in contact with the eyes, it is essential that all substances that are a part of this medicine are sterilized, or that a terminal sterilization is performed.

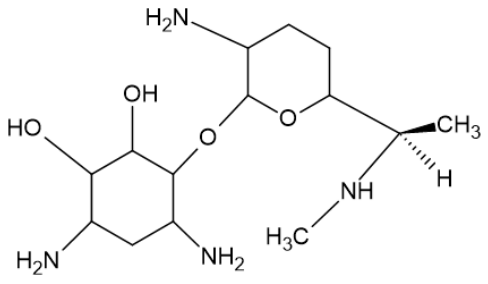


Figure 2.8-Chemical structure of Gentamine C1.

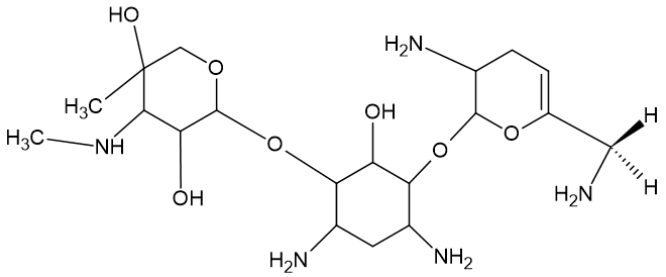


Figure 2.9-Chemical structure of Sisomicin.

According to the gentamicin sulfate monograph in the (Ph. Eur.), the impurities that might be detected, if present at a sufficient amount, are in Table 2.3. The unspecified impurities do not have to be identified, as they are limited by a general acceptance criterion.

Table 2.3-Gentamicin sulfate impurities (Ph. Eur.)

Designation	Type
Sisomicin (impurity A)	Specified
Garamine (impurity B)	Specified
Gentamicin B1 (impurity C)	Unspecified
L-streptamine (impurity D)	Unspecified
2-deoxystreptamine (impurity E)	Unspecified

Thamthaweechok, et al., (2018) and Traub & Birgit (1995) discovered that gentamicin sulfate solutions remained stable after moist heat sterilization at 121ºC for 15 minutes, which was assessed by the minimum inhibitory concentration against *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*. The authors Taplin (1965) and Casemore (1967) also investigated the bactericidal properties of this substance after autoclaving and concluded that those properties were not lost after the process.

On the other hand, the author Silveira (2017), stated that this active ingredient presents a yellowish coloration when exposed to an environment of moist and heat, adsorbing humidity. Investigations by Naveed, et al., (2014) were made, where a solution of gentamicin sulfate presented a 78% degradation, measured by spectrophotometry, after it was put in a water bath at 50ºC for 30 minutes. Additionally, the authors Ramos, et al. (2012) detected low

amounts of free radicals in samples of gentamicin sulfate sterilized by dry heat at 180ºC (30 minutes), 170ºC (60 minutes) and 160ºC (120 minutes).

Another study was made where gentamicin sulfate was put in a dextrose solution and then autoclaved with times ranging from five to 27 minutes, by Graham, et al. (1997). A comparison analysis was made between the heat-treated solutions and the ones left at ambient temperature. A yellow color was observed, going from colorless in the sample processed for five minutes to a deep yellow in the one processed for 27 minutes, indicating that gentamicin is affected by heat in dextrose solutions, with a considerable loss in potency. However, the same degradant peaks were detected in the samples that stayed at room temperature, as well as in the ones that were heat-treated, with a greater extent of degradation on the latter, and solutions maintained at room temperature remained colorless, therefore the formation of color was not considered related to the presence of degradants. Another solution of gentamicin was prepared in water and then heat-treated; however, no degradant peak was observed.

The last authors also concluded that the amino and hydroxyl group in this substance are susceptible to chemical oxidation, and Hanessian, et al. (1975) stated that the glycoside linkages of several aminoglycosides are known to be cleaved under oxidizing conditions.

In order to employ the substance in the pharmaceutical industry, it is important that it maintains all the same properties after the heat treatment, for instance, its appearance, pH, and so on.

3. Methods of analysis

3.1. High-Performance Liquid Chromatography

High-Performance Liquid Chromatography is the most important step when trying to ascertain the degradation of a substance. This analysis is adopted in the determination of the impurities and assay of either an active ingredient or a formulation.

The HPLC technique is one of the most used chromatographic separation tools. In this method, the mobile phase, containing the sample to be analyzed, is pumped with a specific pressure through a chromatographic column, which is filled with the indicated adsorbent material, and is maintained at a particular temperature in an oven. The analytes interact with the adsorbent, according to their individual characteristics, and the detector gives a response regarding each component's retention time, area, and height of the peak through a chromatogram (Patil, 2019). Therefore, an HPLC can separate the API from existing impurities and quantify them. Since impurities in pharmaceuticals are present at a low amount, a reliable analysis can only be accomplished after their isolation. Retention times can oscillate due to leaks, pump impairments, or alterations in the column temperature or in the mobile phase (Dong, 2005).

Figure 3.1-Schematic representation of a High-Performance Liquid Chromatography instrument

HPLC offers many assets, such as being highly precisive at identifying components in a sample and quantifying them, it allows gradient solvent systems, when necessary, it is greatly reproductible and provides high resolution, compared to other chromatographic techniques. At the same time, it can be relatively expensive, requiring regular maintenance, as well as

being challenging for beginner analysts (Patil, 2019). Additionally, the impurity profile methods do not take into consideration the potential of co-elution elution of impurities with product peaks (Ryan, 1998).

Impurity profiles are essential to understand the purity of substances. With the record of the impurities that may arise in a specific pharmaceutical, the method can exhibit chromatographic resolution between said impurities from the substance that is being analyzed. The impurities can be raw materials or process intermediates found in the pharmaceutical, process impurities that occur through a reaction, or degradants of the drug substances (Ryan, 1998).

Impurities related to pharmaceuticals can be assorted into three categories, according to ICH guidelines. These are organic impurities, elemental (inorganic) impurities, and residual solvents. Organic impurities are the ones that can occur in APIs or formulations during manufacture or storage. They may be known, unknown, volatile, or nonvolatile substances and can come from raw materials, intermediates unwanted byproducts or degradation products. These can be toxic or lead to undesired biological activity. Inorganic impurities can derive from APIs, raw materials, additives, excipients, catalysts, manufacturing processes and equipment used during the latter. Risk assessment is imperative to monitor these impurities in final medicines. Manufactures should further assess possible sources of contamination. Residual solvents are volatile organic chemicals that are used or generated at the time of production. Many of these are known to be toxic and environmentally hazardous (Agilent Technologies Inc., 2018).

Next, there are represented publications and guidelines for the control of pharmaceutical impurities (Agilent Technologies Inc., 2018).

- International Conference on Harmonization (ICH) Q3A (R2) Impurities in New Drug Substances, 25 October 2006.
- Guidance for Industry Q3A Impurities in New Drug Substances, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), June 2008.
- EMA/CPMP/ICH/2737/99 Note for guidance on impurities testing: impurities in new drug substances; ICH Topic Q3A (R2) Impurities in new Drug Substances, October 2006.
- ICH Q3B (R2) Impurities in New Drug Substances, 2 June 2006.

- Guidance for Industry Q3B(R2), Impurities in New Drug Products, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), July 2006.
- EMA/CPMP/ICH/2738/99 Note for guidance on impurities in new drug products; ICH Topic Q3B (R2) Impurities in New Drug Products, June 2006.

The HPLC technique is one of the most versatile methods for impurity profiling due to its high selectivity, particularly for routine analysis (Dong, 2005).

3.1.1. Evaporative Light Scattering Detection

The Gentamicin components are challenging to be analyzed by HPLC considering most of them are equipped with a UV detector. This happens because gentamicins do not carry UV absorbing chromophores, which is the region of the molecule responsible for its color. In order to overcome this obstacle, other methods are implemented to improve these molecules' chromatographic behavior, by adding a chromophore, thus enabling fluorescence or UV detection. These methods consist of derivatization of amino groups with o-phtaladehide, 2,4,6-trinitrobenzensulfonic acid and 1-fluoro-2,4-dinitrobenzene. The alternative is using electrochemical or evaporative light scattering detections (ELSD) (Grahek & Zupančič-Kralj, 2009).

The use of evaporative light scattering detectors has been growing over time due to its low cost, for being simple to operate and many other conveniences. This detector is considered pseudo universal. It does not require chromophores in the analytes, although substances more volatile than the mobile phase cannot be analyzed (Megoulas & Koupparis, 2005).

It can detect essentially all compounds, regardless of its physical and chemical properties, as long as it does not evaporate before passing through the beam of light. The detector is not affected by changes in the ambient temperature and is insensitive to changes in the mobile phase composition, resulting in a more stable baseline, making it easier to identify and integrate the peaks in the chromatograms, and offering better limits of detection, even for analytes with UV absorption. As for the setbacks of this type of analysis, the mobile phase must be completely volatile, otherwise it can leave residue in the heating chamber, blocking the optical cell. Additionally, samples with particularly low concentrations may not be detected (Urano, Rodrigues, & Berlinck, 2012). The ELSD is destructive, therefore, it should be last when used in series with other detectors (Megoulas & Koupparis, 2005).

The ELSD operates in three steps: nebulization, evaporation, and detection. The effluent that leaves the chromatographic column containing the sample is nebulized into small droplets with the assistance of a stream of an inert gas, usually nitrogen or helium, which may be heated, forming an aerosol. This aerosol is dragged through a heated tube by the gas, where the solvent is then evaporated. The remaining particles of the analyte pass through a beam of light, which scatter. The scattered light is then detected by a photodiode (Arndt & Brüll, 2013). The solvent used should be completely compatible with the analytes, so it does not change the nature of the original sample (Kreimer, Krull, & Rathore, 2012).

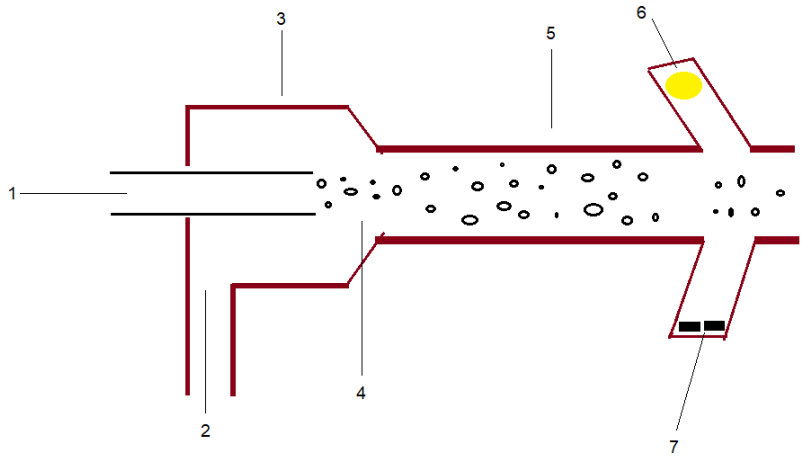


Figure 3.2-Scheme of an Evaporative Light Scattering Detector. Adapted from (J. H. Arndt & Brüll, 2013). 1 - Effluet; 2 - Inert Gas; 3 - Nebulizer; 4 - Liquid particles; 5 - Evaporator (Heated tube); 6 - Light source; 7 - Detector.

Light scattering depends on a series of aspects, including cell size, refractive index difference between the medium and the cell, refractive index differences within the cell, cell shape and cell orientation in the light beam. Additionally, it depends on the detector size and on its position regarding the light beam direction (John Wiley & Sons, Inc., 1999).

According to Urano, et al. (2012), the response of the ELSD depends on the size and shape of the particles. The amount of light scattered is only dependent on the quantity of the sample, and for this reason it does not require the analyte to contain a chromophore group. The tube temperature and the gas flow rate have the highest influence in the ELSD analysis; thus, they must be optimized. In the evaporation phase, choosing the right temperature is crucial, depending on the volatility of the analyte and the mobile phase. If the temperature or the gas flow rate is too low, the solvent might enter the detector in the liquid phase, resulting in excessive noise in the baseline. On the other hand, If the temperature is too high, it can

decrease the detector's sensitivity, if the substances are partially volatile. The use of a low flow rate improves the droplets size distribution, making it more homogeneous.

Evaporative light scattering detectors are, thus, able to facilitate HPLC analysis, by identifying substances that are not detectable under UV light, such as carbohydrates, sugar alcohol surfactants and natural synthetic molecules (Peak Scientific, 2017).

3.2. **Stability**

Determining the stability of pharmaceuticals is a crucial step to understand the efficacy of the whole sterilization process. A substance may present all parameters in accordance with the raw material requirements right after sterilization, however, the heat treatment may have an influence on some of its characteristics after a certain time. It is important to guarantee that the medicine remains stable throughout its shelf life.

The authors Bajaj, et al. (2012) define stability of pharmaceutical products as the duration to which substances preserve the properties and characteristics possessed at the time of its packaging, within the specific limits, throughout its period of storage and use.

Stability studies are able to secure product quality, safety and efficacy during a certain period, and they are considered a pre-requisite for the acceptance and approval of pharmaceuticals (Bajaj, et al., 2012). Stability tests on drug substances are specific in terms of temperature and relative humidity, with long term studies (12 months), intermediate studies (6 months) and accelerated studies (6 months) (European Medicines Agency, 2003), however, in this case, the active ingredients were stored for the short duration of the study (3 months) at a temperature of $20^{o}C \pm 5^{o}C$.

The potential adverse effects of instability are the following (Bajaj, et al., 2012).

- Loss of active ingredient – degradation of API resulting in less than 90% drug.
- Increase in concentration of active ingredient – solvent escape and evaporation can cause the product to show an increase in concentration.
- Alteration in bioavailability – changes in rate and extent of absorption on storage.
- Loss of content uniformity – loss of contents as a function of time.
- Decline of microbiological status – increase in number of viable microorganisms; contamination because of compromise package integrity.
- Formation of toxic degradation products – degradation of the drug component.
- Loss of package integrity – change in package integrity during storage.

- Reduction of label quality – deterioration of label with time, adversely affecting legibility.
- Modification of any factor of functional relevance.

3.3. **Validation of analytical methods**

Whenever a new analytical method is developed, analysts must validate it by following a series of previously defined parameters, or in the case of applying an alteration to an already developed and validated method, carrying out a revalidation is indispensable.

Revalidation of an analytical method should be considered whenever there are changes to the method, including changes to the mobile phase, column, temperature of the column, concentration or composition of the sample and standards, detector, etc. (World Health Organization, 2018). For this study, the HPLC method to determine the assay and impurities of sulfacetamide sodium required an alteration in the solvent as the acetic acid made it very difficult to dissolve the substance. Additionally, all HPLC analysis at the laboratory are completed with three injections for each sample. However, each impurities run can be very lengthy, so a revalidation using only two injections for each sample was suggested.

For a revalidation, it is acceptable to include only characteristics of relevance to the particular change and method (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018).

Although validation cannot prevent all possible issues, the process of developing and validating a method should address the most common ones (Green, 1996). Examples of common problems that can be avoided or minimized are the coelution of impurities with the analyte peak, a type of column that no longer achieves the separation necessary, the inability to achieve a certain detection limit, etc. Problems increase as additional people, laboratories and equipment are used to perform the method (Green, 1996).

Validating analytical methods are critical, considering analysts must make sure they are producing reliable results, providing confidence to the costumers. It is required that the values obtained are repeatable, that the method provides similar results when some experimental conditions are somewhat altered and that very low concentrations of the analyte can be quantified. Considering it is not possible to secure these requirements for every result, the international analytical institutions have developed a system that ensures the quality of the results if they have been obtained under certain circumstances (Boqué, et al., 2002).

Method validation represents the means of demonstrating that the method is fit-for-purpose, which, then, indicates that the reported results can be trusted (Boqué, et al., 2002). It is the process of verifying the suitability of methodology for providing useful analytical data (Taylor, 1983). The estimated values must relate to a level of uncertainty so as to ensure the compliance of that product with regulations. In addition, analysts must use a method whose limit of quantification for that parameter is below the legal limit, also, the rapidity with which the results are produced, and cost of the analysis should be taken into consideration (Boqué, et al., 2002).

When reference samples, analogous to the test samples, are available, the process of validation consists of analyzing a sufficient amount of reference samples and comparing the results to the expected values (Taylor, 1983). Reference materials with known and documented purity should be used in the analyses (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018). Validation requirements are constantly changing and vary depending on the substance being tested, the stage of drug development, and the regulatory group that will review the drug application (Green, 1996).

Results of analytical methods should be accurate, legible, contemporaneous, original, reliable, and reproducible. In pharmacopoeial methods, it must be proven that they are suitable for routine use. When they are used for determination of content or impurities, the methods should have been demonstrated to be specific to the considered substance. Acceptance criteria for all characteristics should be included in the protocols (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018).

Validating a method consists of obtaining experimental values for the selected performance criteria. These criteria are essential parameters that are used to investigate whether a method satisfies previously defined requirements. The basic parameters refer to the reliability of the method (Boqué, et al., 2002). Characteristics that should be considered during validation of analytical methods include specificity, linearity, range, accuracy, precision, limit of detection, limit of quantification, and robustness. Occasional exceptions should be dealt depending on the case (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018). These parameters provide a quantitative basis for judging performance capability and are useful for characterizing methodology and evaluating its suitability (Taylor, 1983).

3.3.1. Trueness

Trueness, or accuracy, can be described as the closeness of the test results obtained by the procedure to the true values (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018). It should be evaluated in terms of bias by analyzing reference samples (Boqué, et al., 2002). It can be evaluated by a comparison between a sample of known concentration and the value obtained in the analysis (Green, 1996). It is usually established on samples that have been prepared to quantitative accuracy and should be established across the specified range of the analytical procedure, for instance, three replicates of three different concentrations across the working range (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018).

3.3.2. Precision

Precision is defined by "the closeness of agreement between independent test results obtained under stipulated conditions" (ISO, 1993). The complete procedure should be performed repeatedly to separate identical samples from the same batch of material. It should be measured by the scatter of individual results from the mean and conveyed as the relative standard deviation (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018). These conditions depend on many aspects which include the laboratory, the operator the equipment, the calibration, and the day in which the results are acquired (Boqué, et al., 2002).

There are three types of precision that can be calculated: the repeatability, the intermediate precision, and the reproducibility (ISO, 1994). The reproducibility gives the largest expected precision, expressing the precision between different laboratories. Repeatability, on the other hand, gives the smallest value of precision, considering the results are obtained by the same operator, with the same equipment and within small periods of time (ISO, 1994). It should be measured using, at least, nine determinations over the range, for instance, three replicates of three concentrations, or a minimum of six determinations at 100% of the test concentration (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018). The intermediate precision is related to how the results may vary when one or more factors, such as time, equipment, and operator, are altered (ISO, 1994). It is a means of understanding which factors contribute to variability in the final result (Green, 1996). The intermediate precision is the most useful one, as it represents the type of variability that can be expected of the results, and because it is a part of the overall uncertainty of those

results. Furthermore, it is an estimate of the "internal reproducibility" of the laboratory (Boqué, et al.2002). If reproducibility is measured, the determination of intermediate precision is not necessary (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018).

3.3.3. Linearity and working range

In quantitative methods, the range of analyte concentrations for which they can be applied needs to be determined. The working range corresponds to the concentration interval in which acceptable accuracy, linearity, and precision are obtained (Green, 1996). The limits of detection and quantification correspond to the lower end of the working range, while many factors limit the range at the upper end (Boqué, et al., 2002).

Analysts must verify the linearity of the method within the working range, which means that there is a linear response in which the results are proportional to the concentration of analyte (Boqué, et al., 2002). For assay methods, this testing is commonly accomplished by analyzing standard solutions at different concentration levels, from 50 to 150% of the target concentration (Green, 1996). Samples should be prepared as to cross the claimed range of the procedure. If there is a linear relationship, the results should be evaluated by appropriate statistical methods, and a minimum of five concentrations should be used (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018).

To estimate these ranges, reference substances at different concentration levels may be used. The linearity of the calibration line can be determined by a high value of correlation coefficient and a good plot of the residual values, which must not show tendencies, must be randomly distributed and the number of negative and positive residual values must be roughly the same (Boqué, et al., 2002). In order to further check the linearity, a test for the lack of fit based on the analysis of variance may be implemented (Massart, et al., 1997).

3.3.4. Limit of Detection (LOD)

The limit of detection, or the minimum detectable net concentration, is defined as the minimum amount of substance that can be reliably detected by a given analytical method (Boqué, et al., 2002), producing a measurable response above the noise level of the system (Green, 1996). The LOD of a method must be defined before the measurements are made.

The detection limit is not necessarily determined (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018), and should be attained only for impurity methods in which chromatographic peaks near detection limit are observed (Green, 1996). Approaches may include visual evaluation, signal to noise ratio, standard deviation of the response and the slope, and calibration curve (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018).

3.3.5. Limit of Quantification (LOQ)

The limit of quantification serves as the ability of a method to properly quantify an analyte and it is interpreted as the lowest concentration of analyte that can be determined with an acceptable level of precision and accuracy (Eurachem, 2014). The limit of quantification can be fixed as the lowest standard concentration of the calibration range (Boqué, Maroto, Riu, & Rius, 2002). This limit is only required for impurity methods (Green, 1996). Approaches may include signal to noise ratio, standard deviation of the response and the slope, and calibration curve (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018).

3.3.6. Selectivity

Especially for HPLC methods, it is important to ensure that the signal produced is only due to the analyte of interest, and not to other interferences in the sample (Boqué, et al., 2002). Selectivity, or specificity, is the ability of a method to accurately determine the analyte of interest in the presence of other components in a sample, under the conditions of the test (Eurachem, 2014).

This analysis can be accomplished by using samples containing the analyte of interest and other suspected interferences. The samples are analyzed, and the analyte peak is evaluated for peak purity and resolution from the nearest eluting peak (Green, 1996). This way, it is possible to assess the ability of the method to analyze the analyte in the presence of other substances (Boqué, et al., 2002).

Other potential components and impurities are generated by exposing the analyte to stress conditions, such as heat, light, acid, base, and oxidant (Green, 1996).

3.3.7. Robustness

The robustness, or ruggedness, of an analytical method measures its ability to remain unaffected by small, but deliberate variations in certain parameters, thus, indicating its reliability during routine usage (ICH Harmonised Tripartite Guideline , 1994).

The robustness of the process is assessed by deliberately introducing small changes to a few factors and investigating their effects on the accuracy and precision of the results. This specification should be tested at an early stage during the optimization of the analytical method. Since many parameters may have a negligible effect, it is suggested to vary several of them at once. These parameters can be divided in continuous, such as extraction time, mobile phase composition, temperature, flow rate, etc., or non-continuous, such as the type of chromatographic column, brands, chemicals, etc. (Boqué, et al., 2002).

3.3.8. Stability of samples

In routine testing, it is important to investigate whether the solutions are sufficiently stable to allow for delays such as instrument breakdowns or overnight analysis, therefor the limits of stability should be tested. Samples and standards should be tested for over at least 48 hours, comparing them to freshly prepared samples. If the solutions do not exhibit stability, storage conditions or additives should be identified that can improve stability (Green, 1996).

A valid method alone is not sufficient for producing valid results. However, data obtained by a valid method used in a well-designed quality assurance program should be enough to accept the data's validity (Taylor, 1983). The quality of the results generated is directly connected to the quality of the process (Green, 1996).

The characteristics to consider during validation of analytical methods, according to the type of procedure, are present in Table 3.1.

Table 3.1-Characteristics to consider during validation of an analytical method.
Adapted from (Guidelines on Validation – Appendix 4. Analytical Method Validation CPMP/ICH/381/95, 2018)

Type of analytical procedure	**Testing for impurities**	**Assay**
Characteristics	**Quantitative tests**	**Content/potency**
Accuracy	+	+
Repeatability	+	+
Intermediate precision	+	+
Specificity	+	+
LOD	*	-
LOQ	+	-
Linearity	+	+
Range	+	+

- Not normally evaluated.

+ Should normally be evaluated.

* May be needed in some cases.

4. Materials and methods

In this chapter, the materials and operating procedures of all the analysis that were necessary to understand the effectiveness of the sterilization of both antibiotics are described. This includes sampling, validation, sterilization, chemical and microbiological analysis, and stability.

4.1. Sampling

The sampling of both APIs was done in similar ways, except for the pouches that each substance was put on, that were later used for the sterilization cycle. Tables 4.1. and 4.2. point out a few important aspects of the substances that were analyzed, as well as the characteristics of the sterilization containers.

Table 4.1-Characteristics of the active pharmaceutical ingredients

	Sulfacetamide sodium	**Gentamicin sulfate**
Manufacturer	Katwijk Chemie	Fujian Fukang
Purity	99,8%	100%
Internal batch number/ Manufacturer batch number	MP/19-0397/ 453339	MP/19-0340/ FG1901007
	MP/20-0382/ 454843	MP/20-0276/ FG1902047
CAS number	6209-17-2	1405-41-0

Table 4.2-Characteristics of the sterilization containers

	Sterilization recipient			
	Type	**Manufacturer**	**Material**	**Size**
1.	Erlenmeyer flask with screw cap	Normax	Borosilicate glass flask with polypropylene screw cap	100 mL
2.	Pouch	Steriking	PET/Polypropylene plastic laminate / Medical grade paper	75x200 mm
				100x300 mm
3.	Pouch	Duraline Systems	Nylon	100x250 mm

For the sampling, 15 g of each lot were collected into the containers, from different points, horizontally, of the original container. The points were chosen randomly. This was, approximately, the sufficient amount necessary to perform a complete analysis.

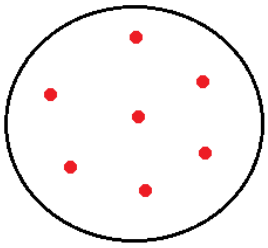


Figure 4.1-Example of points in the original container from where samples of the substances were randomly collected.

4.2. Bioburden

It is important to determine the bioburden of the drugs prior to the sterilization testing as a part of quality control, since it is a key step in pharmaceutical microbiology (Sandle, 2016). Considering biological indicators were used in the sterilization cycles, it is vital to prove that the substances have a bioburden well below the microbial population of the bioindicators, and that it complies with the bioburden limits of the raw materials.

The bioburden of sulfacetamide sodium was determined through the following method.

Materials

- 90 mL of letheen broth modified (LTB) 0.5% tween 80
- Ez-fit™ filtration unit with polyvinylidene fluoride (PVDF) membrane
- Milliflex™ vacuum pump

- Trypticase soy agar (TSA) plates
- 300 mL of rinse fluid D
- 10 g of each lot of sulfacetamide sodium
- 25 mL sterile pipette
- 10 mL sterile pipette
- Isopropyl alcohol 70%
- Safety cabinet class II - Baker™ SG503-HE
- Incubator (20ºC-25ºC) - Binder™ KD240
- Incubator (30ºC-35ºC) - Binder™ BD240

Operating procedure

In a vertical laminar flow cabinet, 10 g of each lot of the substance were added into 90 mL of LTB and the mixture was shaken vigorously until it the substance was completely dissolved. Then, the Ez-fit™ filtration unit with PVDF membrane was mounted into the Milliflex™ vacuum pump and the membrane was rinsed with 50 mL of fluid D, using a sterile pipette. 10 mL of the mixture containing the substance, with a sterile pipette, was filtered with the same membrane and the latter was cleansed with the remaining fluid D. This fluid is compatible with most antibiotics, and it is used for testing samples that contain lecithin or oil. It is also used to removed antimicrobial activity by membrane filtration (Sigma Aldrich). All materials were sprayed with isopropyl alcohol 70% before entering the cabinet. After that, the membrane was removed and put on the TSA plate, which was incubated for five days between 20-25ºC and then for two days between 30-35ºC.

The bioburden of gentamicin sulfate had been previously determined by the Laboratory.

4.3. **Sterilization cycle**

The sterilization cycles were chosen according to Table 2.2 on chapter 2.2. In an earliest stage, all cycles were tested for both APIs for color alterations. At this point, 1 g of each lot were tested at a time.

The smaller plastic/paper pouches (75x200mm) and the Erlenmeyer flasks were only tested in the earliest sterilization cycles, to investigate any color alteration that the heat could cause in the substances. In those cases, 15 g of each lot were collected into a plastic bag and then distributed into portions of 1 g to different pouches or flasks.

After choosing a cycle to carry on with a complete analysis, the larger pouches (nylon and plastic/paper) were used. The fan speed and the air flap position of the unit were both adjusted to 50%, considering those were the parameters used for the stability and uniformity assay.

The equipment used was the Universal oven UF55 by Memmert™. The unit comprises two shelves and can be used between +20 and +300ºC. Prior to each sterilization cycle, the desired temperature was set, and the containers were placed on a steel tray on the bottom shelf of the hot air oven once the set temperature was reached and stabilized, to reduce temperature fluctuations and to reduce the exposure of the substances to heat, thus minimizing degradation. Once the substance was in the oven, the timer was set as soon as the desired temperature was reached again. The containers were removed from the sterilizer immediately upon completion of the cycle.

Figure 4.2-Memmert™ Universal oven UF55

Biological indicator strips were used in each cycle to confirm the efficacy of the process and validate it. Therefore, it is possible to measure the direct lethality and secure a SAL ≤ 10^{-6}. The organism employed was the *bacillus atrophaeus* with a microbial population of $2.1x10^{6}$, as it is considered a highly resistant type of spore-forming bacteria. The strip should also be used in routine control of the sterilization processes (Sella, Vandenberghe, & Soccol, 2014).

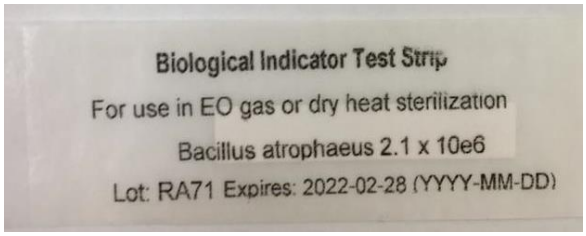


Figure 4.3-Biological indicator test strip - *Bacillus atrophaeus*

Figure 4.4. illustrates how the pouches were placed on the tray inside the oven. The load should not be place on the base or immediately under the top of the oven and it should not touch any of the walls. An inadequate loading could lead to exceeded temperatures or it could make it take longer to reach the desired temperature.

Figure 4.4-Placement of the sterilization pouches on the steel tray

4.4. **Inert packaging**

For gentamicin sulfate, the nylon pouches were used, and the substance was previously packed in inert atmosphere. The samples were put in the pouches and placed in a container. The nitrogen source was opened until the atmosphere was saturated. The pouches were sealed.

Figure 4.5-Nylon pouches with gentamicin sulfate in inert atmosphere

4.5. **Validation of the analytical method to determine the assay and impurities of sulfacetamide sodium**

A sulfacetamide sodium secondary standard with a content of 92.5% (lot MP/20-0382) was used in all the following tests. The analysis was accomplished by HPLC, in the conditions described in the Ph. Eur.

Column: Purospher™ RP-18 end-capped (5 µm). Size: 4x125 mm.

Mobile phase: Glacial acetic acid R (Fluka Honeywell), methanol R (PanReac Appli-Chem ITW Reagents), water R (1:10:89 V/V/V)

Solvent: Methanol R (PanReac AppliChem ITW Reagents), water (20:80 V/V)

Flow rate: 1.2 mL/min

Detection: spectrophotometer at 254 nm

Injection: 10 µL

Run time (assay): 12 minutes

Run time (impurities): 40 minutes

Column temperature: 25ºC

Autosampler temperature: 10ºC

The analysis was accomplished with a Hitachi LaChrom Elite™ HPLC system and the Empower™ software.

4.5.1. Linearity

Assay

For the linearity investigation of the assay method, five test samples at different concentrations were prepared, from 50 to 150% of the working concentration determined in the monograph. A stock solution was prepared by dissolving 250 mg of the substance in 25 mL of solvent (methanol 20%) and diluting 2 mL of this solution to 20 mL of solvent. The test samples were prepared from different dilutions of the stock solution.

Table 4.3-Preparation of samples for the linearity testing of the assay method of sulfacetamide sodium

Stock solution		1000	µg/mL
	Aliquot (mL)	**Dilution (mL)**	**Final concentration (µg/mL)**	
50%	2	20	100	
75%	3	20	150	
100%	4	20	200	Working concentration
125%	5	20	250	
150%	3	10	300	

Related substances

The linearity, in this case, was tested for unknown impurities with the sulfacetamide sodium secondary standard, and for impurity A (sulfanilamide) specifically. The range chosen went from the disregard limit to the defined limit for impurity A, which correspond to 0.05% to 0.2% of the test solution concentration (20 000 µg/mL). A stock solution was prepared

by dissolving 40 mg of the substance to 100 mL of solvent. Seven samples were prepared from this stock solution within the range mentioned.

Table 4.4-Preparation of samples for the linearity testing of the impurity method of sulfacetamide sodium

	Stock solution		400 µg/mL
	Aliquot (mL)	**Dilution (mL)**	**Final concentration (µg/mL)**
0,05%	2,5	100	10
0,08%	2	50	16
0,10%	2,5	50	20
0,12%	3	50	24
0,16%	4	50	32
0,18%	9	100	36
0,20%	10	100	40

In both cases, with the peak areas obtained from the HPLC analysis, the following parameters were determined.

- Coefficient of determination

$$R^2 = 1 - \frac{\sum_i e_i^2}{\sum_i (y_i - \bar{y})^2}$$

Where $\sum_i e_i^2$ correspond to the residual sum of squares and $\sum_i (y_i - \bar{y})^2$ to the total sum of squares.

- Homogeneity of variance

This parameter is determined through Levene's test for F (n-1, n-1, 99.0%). To evaluates if the working range is well defined, a PG value is calculated by the quotient of higher and lower standard variances ($\mathbf{S_1^2}$) and ($\mathbf{S_5^2}$) obtained by the signals of ten replicate injections of reference solutions at lower concentration (50% solution) and higher concentration (150% solution) of the working range. If PG is less that F, differences are not significant and working range is valid. If PG is higher than F, a narrower working range must be studied.

This test is only necessary for the assay method validation.

$$PG = \frac{{S_{10}}^2}{{S_1}^2} \; when \; {S_{10}}^2 > {S_1}^2$$

- Mandel's test

This test uses linear and polynomial adjustment to study which function can be adapted to experimental values. In this assay, a test value (VT) is compared with tabulated value (F). In fact, polynomial adjustment is always better to describe the function. However, if differences are not significant, a linear function can be used.

$$VT = \frac{DS^2}{S_{Y/X(2º)}{}^2}$$

$$DS^2 = (N-2)S_{Y/X}{}^2 - (N-3)S_{Y/X(2º)}{}^2$$

$$S_{Y/X} = \sqrt{\frac{\sum_i (y - y_i)^2}{N-2}}$$

$$S_{Y/X(2º)} = \sqrt{\frac{\sum_i (y - y_i)^2}{N-3}}$$

Where y and yi correspond to the average peak area and to the signal obtained with the adjustment, respectively.

- Residual analysis

  This shows the residuals of experimental points' distributions. In a linear function, residuals must be casually distributed.

- Rikilt

This is used for evaluation of the response factor (RF), to predict if it can be used in routine laboratorial work, instead of diary calibration curve.

$$\text{RF} = \frac{\text{concentration}}{\text{peak area}}$$

The quotient between the response factor related to each concentration and the average response factor must be 99.0-101.0%.

- LOD and LOQ

The limits of detection and quantification must be below the reporting threshold.

4.5.2. Selectivity

For the selectivity testing, different samples were analyzed to investigate any interference that might occur between the main analyte peak, which corresponds to sulfacetamide sodium, and other impurities, considering the resolution factor and the relative retention time.

The following samples were studied. For the reference solutions, a sulfacetamide sodium secondary standard was used, and for the test samples, a control sample (non-sterilized) was used.

- Solvent
- Mobile phase
- Impurity A 2 µg/mL
- Reference solution (a) (Impurity A 5 mg/mL + Sulfacetamide sodium 5 mg/mL)
- Reference solution (b) (Sulfacetamide sodium 20 µg/mL) – Impurities working standard
- Reference solution (Sulfacetamide sodium 200 µg/mL) – Assay working standard
- Impurities test sample (Sulfacetamide sodium 20 000 µg/mL)
- Assay test sample (Sulfacetamide sodium 200 µg/mL)

Stress testing

Seven different samples were prepared by dissolving 0.200 g of sulfacetamide sodium in 10 mL of solvent. 1 mL of hydrochloric acid 0.1 N and 1 mL of sodium hydroxide 0.1 N were added to two different samples, the third was left at 60ºC in a hot air oven, and the fourth was left exposed to ultraviolet lighting. Two other samples were tested with 1 mL of hydrogen peroxide at 3%. The last one was used as a control sample.

After four days, the first two samples were neutralized, and all the samples were diluted to assay samples with 1 mL to 100 mL of solvent. They were analyzed through HPLC to quantify the main peak and the impurities that were formed.

4.5.3. Precision

Assay

Six different samples at the working concentration (200 µg/mL) were prepared by dissolving, approximately, 0.200 g of the substance in 10 mL of solvent and diluting 1 mL of this solution to 100 mL of solvent. These samples were quantified against a working standard at the same concentration, that was prepared by dissolving 20 mg of sulfacetamide CRS in 100 mL of solvent.

The concentrations were obtained through the HPLC analysis, and the average of results should fall between 99.0% and 101.0%.

Repeatability

Repeatability was evaluated by the variability of the analytical response of a reference solution containing sulfacetamide sodium at 50%, 100%, and 150% of the test concentration. The variability obtained on each level should not surpass 2.0%.

Intermediate precision

This test was performed in the same manner, however on a different day, by a different analyst, chromatographic column and HPLC equipment of the same type and brand. In this case, the column used was the Inerstil™ ODS-3 (5 µm).

The variability of the results obtained by these two tests should not be greater than 2.0% for the assay method and 10.0% for the related substances method.

Related substances

Six impurities test samples were prepared by dissolving 0.200 g of sulfacetamide sodium in 10 mL of solvent. The amount of impurity A in the samples was calculated from the peaks obtained in the chromatograms.

The variability obtained on each level should not surpass 10.0%.

4.5.4. Trueness

Assay

Three samples at 50% of the working concentration (100 µg/mL), three samples at the working concentration (200 µg/mL) and three samples at 150% of the working concentration (300 µg/mL) were prepared by dissolving, approximately, 0.100 g, 0.200 g and 0.300g of the

substance, respectively, in 10 mL of solvent and diluting 1 mL of this solution to 100 mL of solvent. These samples were quantified against a working standard at the same concentration, that was prepared by dissolving 20 mg of sulfacetamide CRS in 100 mL of solvent.

Related substances

This testing was completed using sulfacetamide sodium and sulfanilamide. In the first case, three samples at 0.05%, three at 0.1% and three at 0.2% of the test solution concentration were prepared. For impurity A, three samples at the same concentrations were prepared by adding this impurity to test samples of sulfacetamide sodium. Considering samples of sulfacetamide sodium come with a small amount of sulfanilamide, this amount was previously calculated in the precision testing and withdrawn from the concentration obtained in the trueness testing, in order to calculate the exact amount recovered.

$$\text{Recovery } \% = \frac{\text{Experimental concentration}}{\text{Theoretical concentration}} \text{x} 100$$

The established acceptance criteria are 99.0 to 101.0 % for the assay method, and 90.0 to 110 % for related substances.

4.5.5. Robustness

Assay

Four parameters that could have an influence on the HPLC analysis were chosen. It was decided that, in this particular case, the following fields are more susceptible to variations in routine analysis: Time that samples are left in the ultrasonic bath., composition of the mobile phase – percentage of glacial acetic acid, column temperature, and flow rate.

Eight different analyses were completed where it was possible to evaluate the effect that each parameter variation and that the interaction between parameters had on the results.

Table 4.5-Parameters and their alterations for each analysis of the robustness test

	A1	A2	A3	A4	A5	A6	A7	A8
Time in ultrasonic bath (min)	10	10	10	10	**3**	**3**	**3**	**3**
GAA in mobile phase (%)	1	1	**0,95**	**0,95**	1	1	**0,95**	**0,95**
Column temperature (ºC)	25	**30**	25	**30**	25	**30**	25	**30**
Flow rate (mL/min)	1.2	1.2	**1.5**	1.2	**1.5**	1.2	**1.5**	**1.5**

Regular condition

Altered condition

For each parameter, the difference between the average of results obtained with a regular condition and the average of results obtained with an altered condition was calculated. The relative standard deviation should be below 1.0% for each parameter.

4.5.6. Stability of samples

For this testing, a reference solution and a test sample for assay and impurities were re-analyzed one week after they were prepared. The assay samples were stored in a refrigerator and the impurities samples were stored in a refrigerator and in the HPLC auto-sampler. They were quantified against a new working standard, prepared on the day of the analysis.

The stability of a reference solution and test samples was evaluated throughout the determination of the analyte concentration variation, expressed by:

$$\text{Variation (\%)} = \frac{|C_f - C_i|}{C_i} \times 100$$

C_i – Initial Assay (Day 0); C_f – Final Assay

The acceptance criteria for the variation are 2.0% for the assay method and 10.0% for the related substances method.

4.6. Complete analysis of sulfacetamide sodium

Appearance

The active ingredient was compared visually with a non-sterilized sample.

Solubility

The solubility of the substance in water and in anhydrous ethanol was investigated. 1 g of sulfacetamide sodium was added to 10 mL of water. 0.1 g of sulfacetamide sodium was added to 100 mL of anhydrous ethanol.

Identification B – Infrared absorption spectrophotometry

This test was accomplished by using a Thermo Scientific™ Nicolet™ FTIR Spectrometer and the OMNIC™ Software. The Attenuated total reflection (ATR) sampling technique was used in conjunction with the IR spectroscopy. With this technique, it is possible to analyze samples directly in the solid state without further preparation (Perkin Elmer Life and Analytical Sciences, 2005). The ATR accessory implemented was a monolithic diamond crystal. The blank sample was obtained by acquiring a spectrum before introducing the sample. The spectrum of a small amount of the sample powder, enough to cover the diamond accessory, was acquired and compared with the spectrum of a sample of sulfacetamide sodium CRS.

Solution S

1.25 g of sulfacetamide sodium were dissolved in 25 mL of water R. One solution S was prepared for each sterilized lot of the API, and one for a non-sterilized sample.

Identification F – Reactions of sodium

(a) 2 mL of a 150 g/L solution of potassium carbonate R were added to 2 mL of the solution S and. The mixture was heated to boiling. 4 mL of potassium pyroantimonate solution R were added, and the new mixture was once again heated to boiling. The glass beaker with the mixture was placed inside a container with iced water until cooled.

(b) 1.5 mL of methoxyphenyl acetic reagent R were added to 0.5 mL of the solution S and then, cooled in a container with iced water for 30 minutes. After that, the beaker was placed in water at 20°C, and the mixture was stirred with the help of a

glass rod for five minutes. 1 mL of dilute ammonia R1 was added. Lastly, 1 mL of ammonium carbonate solution R was added.

Appearance of solution S

A reference solution GY (greenish yellow) was prepared with 9.6 mL of yellow stock solution, 0.2 mL of red stock solution and 0.2 mL of blue stock solution. The reference solution GY4 was prepared with 0.5 mL of the refence solution GY and 9.5 mL of 1% hydrochloric acid solution. Solution S was then compared visually to the latter.

pH of solution S

The pH of solution S was measured with a Metrohm™ 827 pH lab meter, with integrated temperature sensor. The sensors were put directly into the beaker containing solution S, and the final pH and temperature values were registered once they stayed stable.

Sulfates

2.5 g of sulfacetamide sodium were dissolved in 25 mL of water R. 25 mL of acetic acid R were added, and the mixture was placed on a magnetic stirrer for 30 minutes and then filtered (S1).

3 mL of a 250 g/L solution of barium chloride R were added to 4.5 mL of a 10-ppm sulfate standard solution. The mixture was shaken and allowed to stand for one minute. In another test tube, 2.5 mL of this suspension were mixed with 15 mL of the solution S1, previously prepared, and 0.5 mL of acetic acid R were added (S2).

A standard was prepared in the same manner using 15 mL of a 10-ppm sulfate standard solution instead of solution S1.

After five minutes, the opalescence in the test solution was compared to that in the standard.

Water

The water content of sulfacetamide sodium was determined on three different occasions: The first was right after sterilization on the same day. The second determination happened on the day that the T0 assay and related substances testing was carried out, which was three days after sterilization, and again a week after the cycle, to check if there would be a rehydration after water loss during the sterilization process.

The water was measured resorting to a Karl Fischer titration, with a Metrohm™ 870 KF Titrino Plus, which performs an automatic titration. 75 mL of methanol was used as a solvent. 0.200 g of the substance was weighed into a glass weighing boat and then added to the solvent for titration. The result was the average value of two consecutive analysis.

Assay and related substances

The HPLC analysis conditions of the assay and related substances methods are described on chapter 4.5.

4.7. Complete analysis of gentamicin sulfate

Appearance

The active ingredient was compared visually with a non-sterilized sample.

Solubility

The solubility of the substance in water and in ethanol was investigated. 1 g of gentamicin sulfate was added to 10 mL of water. 0.01 g of gentamicin sulfate was added to 100 mL of ethanol.

Solution S

0.8 g of sulfacetamide sodium were dissolved in 20 mL of water R. One solution S was prepared for each sterilized lot of the API, and one for a non-sterilized sample.

Identification A – Thin-layer chromatography

Test solution: 25 mg of the substance to be examined were diluted to 5 mL of water R.

Reference solution: 25 mg of a gentamicin sulfate secondary standard were diluted in 5 mL of water R.

Plate: TLC silica gel plate R.

Mobile phase: the lower layer of a mixture of equal volumes of concentrated ammonia R, methanol R and methylene chloride R.

10 µL of each teste solution and standard, were applied next to each other on the lower end of the plate. The mobile phase was added to a chromatographic tank until the air was saturated. Then, the plate was placed inside the tank until the mobile phase had reached over

2/3 of the plate. After that, the plate was left to dry in air, and then sprayed with ninhydrin solution R1 and heated at 110ºC for five minutes.

Identification C – Reaction (a) of sulfates

1 mL of dilute hydrochloric acid R and 1 mL of barium chloride solution R1 were added to 5 mL of solution S.

Appearance of solution S

A reference solution Y (yellow) was prepared in a glass beaker with 2.4 mL of yellow stock solution, 0.6 mL of red stock solution and 7 mL of 1% hydrochloric acid solution. The reference solution Y6 was prepared with 0.5 mL of the refence solution Y and 9.5 mL of 1% hydrochloric acid solution. Solution S was then compared visually to the latter.

pH of solution S

The pH of solution S was measured with a Metrohm™ 827 pH lab meter, with integrated temperature sensor. The sensors were put directly into the beaker containing solution S, and the final pH and temperature values were registered once they stayed stable.

Composition

The components of gentamicin were assayed through a HPLC analysis.

Column: YMC-Triart™ 250x4.6 mm (5 µm)

ELSD temperature: 70ºC

ELSD gas pressure: 3.5 bar

ELSD gain: 11

ELSD filter: 10

Column temperature: 40ºC

Autosampler temperature: 15ºC

Injection volume: 18 µL

Flow rate: 0.5 mL/min

Mobile phase: Trifluoroacetic acid (Fluka Honeywell) 0.5%, Acetonitrile (Fluka Honeywell) (97:3 V/V)

Solvent: Water R

Run time: 30 minutes

Test samples: 25 mg of gentamicin sulfate to 50 mL of solvent

Working standard: 25 mg of a secondary standard of gentamicin sulfate to 50 mL of solvent. The control standard was prepared in the same manner.

The analysis was accomplished with a Hitachi Chromaster™ HPLC system with ELSD detector and the EZChrom Elite™ software.

Sulfate

Approximately 0.250 g of the substance to be examined were dissolved in 100 mL of water and the pH of the solution was adjusted to 11 with pH indicator test strips using concentrated ammonia R. 10 mL of 0.1 M barium chloride and about 0.5 mg of phthalein purple R were added to this solution. 50 mL of ethanol (96%) were added and a titration with 0.1 M sodium edetate was carried out until the violet-blue color disappeared completely.

The result was calculated, in anhydrous base, through the following equation.

$$\frac{(10 - V)x9.606}{m}x100$$

V – total volume of 0.1 M sodium edetate spent on titration, in mL.

m – real mass of substance weighed, in mg.

Water

The water content was determined on the day of the assay (composition) testing.

The water was measured resorting to a Karl Fischer titration, with a Metrohm™ 870 KF Titrino Plus, which performs an automatic titration. 75 mL of methanol was used as a solvent. 0.300 g of the substance was weighed into a glass weighing boat and then added to the solvent for titration. The result was the mean value of two consecutive analysis.

Sulfated Ash

A porcelain crucible was ignited at 600 ± 50ºC for 30 minutes and allowed to cool in a desiccator over silica gel. After that, approximately 0.50 g of the substance to be examined was weighed in the crucible. The substance was moistened with a small amount of sulfuric acid R and heated gently until the sample was thoroughly charred. After cooling, the residue was again moistened with a small amount of sulfuric acid R and heated gently until white fumes were no longer evolved. Then, the residue was ignited at 600 ± 50ºC until it was completely incinerated. The crucible was allowed to cool in a desiccator over silica gel and weighed.

The percentage of residue was calculated through the following equation.

$$\frac{W-T}{m}x100$$

W – final weigh of the crucible with the residue, in g.

T – tare weigh of the crucible, in g.

m – real mass of substance weighed, in g.

4.8. **Sterility**

The sterility testing was performed after the chosen sterilization cycle. The purpose of this test is to investigate the efficacy of the cycle, confirming that the drugs became sterile after the process was completed.

Sulfacetamide Sodium

Materials

- Steritest™ equinox pump
- Canisters with polyvinylidene fluoride (PVDF) membrane
- 100 mL of letheen broth modified (LTB) 0.5% tween 80
- 100 mL of rinse fluid A
- 300 mL of rinse fluid K
- 100 mL of thioglycollate broth
- 100 mL of tryptic soy broth (TSB)
- 10 g of sulfacetamide sodium
- 10 mL sterile pipette
- Isopropyl alcohol 70%
- Safety cabinet class II - ADS Laminaire™ Optimale 12
- Incubator (20ºC-25ºC) - Binder™ KD240
- Incubator (30ºC-35ºC) - Binder™ BD240

Operating procedure

In a vertical laminar flow cabinet, 10 g of sulfacetamide sodium was added to 100 mL of LTB and the mixture was shaken vigorously until the substance was completely dissolved. 10 mL of this solution was added to 100 mL of fluid A. After that, two canisters with a PVDF membranes were mounted into the Steritest™ equinox pump and the previous solution was filtered at a velocity of 70 rpm. The membrane was then rinsed with 300 mL of fluid K to

remove products that could inhibit microbial growth. The thioglycollate broth was added into the first canister and incubated at 30-35ºC to investigate the growth of aerobic and anaerobic bacteria. The TSB was added into the second canister and incubated at 20-25ºC to investigate the growth of aerobic bacteria, fungi, and yeast. All materials were sprayed with isopropanol before entering the cabinet. The results were obtained after 14 days.

Gentamicin Sulfate

Materials

- 10 mL of letheen broth modified (LTB) 0.5% tween 80
- 100 mL of tryptic soy broth (TSB)
- 100 mL thioglicollate broth
- 10 g of gentamicin sulfate
- 25 mL sterile pipettes
- Isopropyl alcohol 70%
- Safety cabinet class II - ADS Laminaire™ Optimale 12
- Incubator (20ºC-25ºC) - Binder™ KD240
- Incubator (30ºC-35ºC) - Binder™ BD240

Operating procedure

In a vertical laminar flow cabinet, 10 g of gentamicin sulfate was added into 90 mL of TSB. The mixture was shaken vigorously until the substance was completely dissolved. 5 mL of this solution was added into 100 mL of thioglicollate broth with a sterile pipette and incubated at 30-35ºC to assess the growth of aerobic and anaerobic bacteria. 5 mL of the solution was added into 100 mL of TSB with a sterile pipette and incubated at 20-25ºC to assess the growth of aerobic bacteria, fungi, and yeast. All materials were sprayed with isopropanol before entering the cabinet. The results were obtained after 14 days.

4.9. **Stability**

The stability testing of sulfacetamide sodium was accomplished by storing the substance in the sealed containers in which it was sterilized, at 20ºC ± 5ºC in a dry environment and protected from light. After one month (T1) and again after three months (T3) of the chosen

sterilization cycle, the complete analysis of the substance, as described in the European Pharmacopoeia, was repeated. The results were compared with the analysis that was carried out right after the chosen sterilization process (T0).

The stability of gentamicin sulfate was not obtained, considering this substance went through the chosen sterilization cycle at the end of the investigation period.

5.
Results and discussion

5.1. Validation of the analytical method

5.1.1. Selectivity

Assay

The results from the selectivity test are presented in Tables 5.1. and 5.2., for the assay and related substances method, respectively. The peaks that appeared on the chromatograms along with their relative retention times are reported. The resolution factors for the reference solution are in relation to the sulfacetamide sodium peak.

Table 5.1-Selectivity results for the assay method validation

Solution	Retention Time (min)	RRT	Resolution Factor
Solvent	NF	---	---
Mobile Phase	NF	---	---
Reference solution (Imp A 5mg/mL + sulfacetamide 5mg/mL)	2.466	0.46	5.80
	5.393	---	---
	9.988	1.85	8.63
Standard solution (200 µg/mL)	5.496	1.02	---
Sample solution (200 µg/mL)	5.496	1.02	---

NF – no peak was found above the reporting threshold.

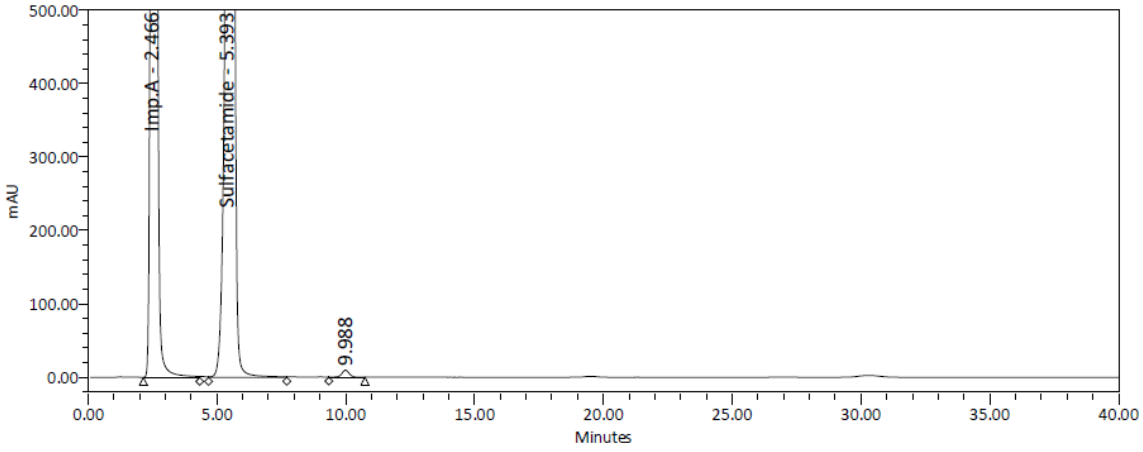


Figure 5.1-Chromatogram obtained for selectivity with the reference solution for the assay method validation

Related substances

Table 5.2-Selectivity results for the related substances method validation

Solution	Retention Time (min)	RRT	Resolution Factor
Solvent	NF	---	---
Mobile Phase	NF	---	---
Reference solution (Imp A 5mg/mL + sulfacetamide 5mg/mL)	NF	---	---
	2.492	0.44	5.19
	5.653	---	---
Imp A solution (2 µg/mL)	2.855	0.45	---
Standard solution (20 µg/mL)	5.699	1.01	---
Sample solution (20 000 µg/mL)	2.556	0.46	---
	5.579	0.99	4.25

NF – no peak was found above the reporting threshold.

The analytical methods for the determination of sulfacetamide sodium and its related substances in samples have demonstrated to be selective because the solvent, mobile phase and impurity A do not interfere with the analyte, and these are well separated from each other.

5.1.2. Linearity

The regression line of the analytical response as a function of analyte concentration was calculated by the method of least squares. The R^2, y-intercept, slope of the regression line, a plot of the data and an analysis of the deviations (D %) of the actual data points from the regression line are reported. The results from the Homogeneity of variance, Mandel's test, Rikilt's test, Residual analysis, and the limits of detection and quantification are also reported.

The results of the assay and related substances methods are presented subsequently.

Assay

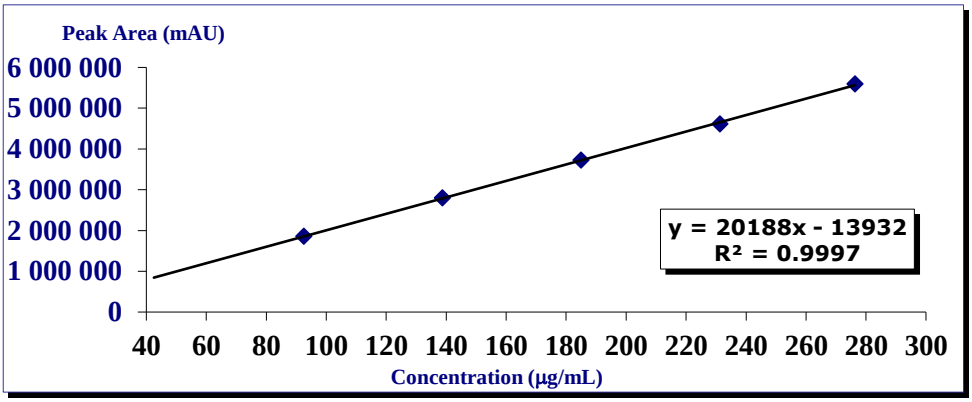


Figure 5.2-Linearity and coefficient of determination for the assay method validation

Table 5.3-Linearity parameters obtained for the assay method validation

Parameters	Results	Acceptance criteria
Range (µg/ml)	92.5 – 276.3	---
	Deviations	
Minimum/Maximum (%)	99.1 – 100.5	99.0 – 101.0 %
Average (%)	100.01	99.0 – 101.0 %
RSD (%)	0.56	≤ 2.0 %
R^2	0.9997	> 0.9950
	Regression line	
Slope	20188	---
Intercept	13932	

Homogeneity of variance

The parameters obtained for the homogeneity of variance from the linearity test are presented in Tables 5.4. and 5.5., for the assay and related substances method validation, respectively.

Table 5.4-Parameters obtained for the sample at 50% of the test sample on the Homogeneity of variance test for the assay method validation

y_1
Signal average for y_1 = 2112019
Standard deviation = 62784
Relative standard deviation = 2.97 %
Variance (S_1) = 3.94×10^9

Table 5.5-Parameters obtained for the sample at 150% of the test sample on the Homogeneity of variance test for the assay method validation

y_{10}
Signal average for y_{10} = 6226790
Standard deviation = 72030
Relative standard deviation = 1.16%
Variance (S_{10}) = 5.19×10^9

Where y_1 and y_{10} are related to the signals (peak areas) obtained for the samples at 50% and 150% of the test sample, respectively.

$$PG = 1.32$$

$$F_{(n-1,n-1,99,0\%)} = 5.35$$

$$PG < F$$

Mandel's test

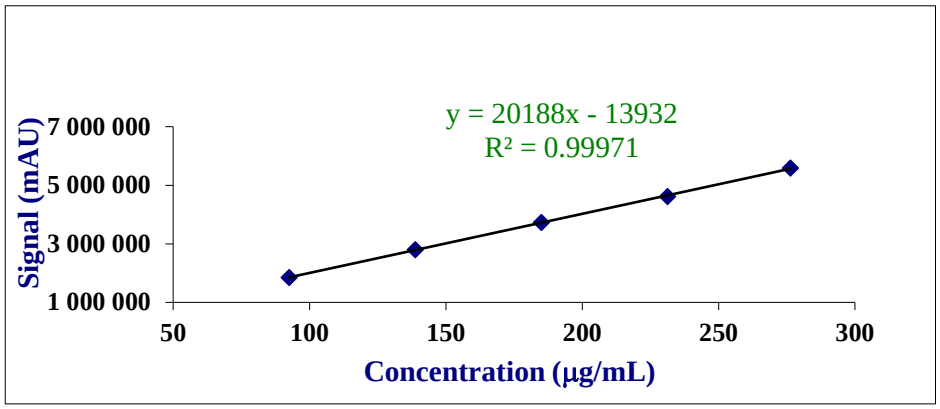


Figure 5.3-Linear adjustment from Mandel's test on the assay method validation

Table 5.6-Parameters obtained for the linear adjustment on Mandel's test for the assay method validation

Linear Adjustment	
(i)	**$(y-yi)^2$**
1	$3.3x10^6$
2	$1.8x10^8$
3	$1.2Ex10^7$
4	$1.7x10^9$
5	$6.7x10^8$
Sum =	**$2.5x10^9$**
N-2 =	**3**
$S_{y/x}$ =	**29116**

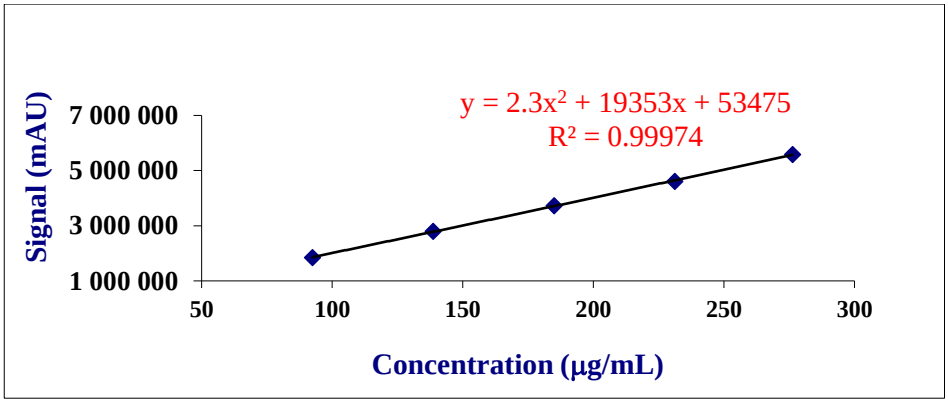


Figure 5.4-Polinomial adjustment of the assay method validation in Mandel's test

Table 5.7-Parameters obtained for the polinomial adjustment on Mandel's test for the assay method validation

Polinomial Adjustment	
(i)	**$(y-yi)^2$**
1	$1.3Ex10^8$
2	$3.4Ex10^8$
3	$1.7x10^8$
4	$1.3Ex10^9$
5	$2.6x10^8$
Sum =	**$2.2x10^9$**
N-3 =	**2**
$S_{(y/x)(2^o)}$ =	**33351**

$$DS^2 = 3.19x10^8$$

$$VT = 0.29$$

$$F_{(1,N-3,95.0\%)} = 18.51$$

$$VT < F$$

Residual analysis

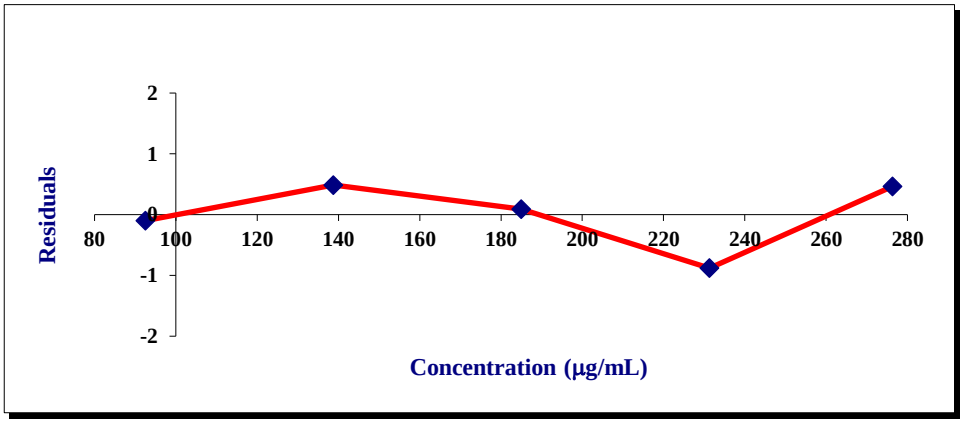


Figure 5.5-Residual analysis of the assay method validation

Rikilt

Table 5.8-Results of Rikilt's test for the assay method validation

	Response factor (yi/xi)	RFi / average RF	Acceptance criteria
Average	20103	100.0	99.0 - 101.0 %
Standard deviation	117	0.6	---
RSD (%)	0.6	0.6	≤ 2.0

LOD and LOQ

Table 5.9-Linear adjustment parameters obtained for the assay method validation

Slope (b)	20188
y-intercept (a)	-13932
Correlation coefficient (R)	0.9999
Coefficient of determination (R^2)	0.9997 (> 0.9950)
Sensitivity (e)	20188
Residual standard deviation ($S_{Y/X}$)	29116

$$Limit\ of\ detection\ (LOD) = 3.3\frac{S_{Y/X}}{b}$$

$$LOD = 4.8\ \mu g/mL$$

$$Limif\ of\ quantification\ (LOQ) = 10\frac{S_{Y/X}}{b}$$

$$LOQ = 14.4\ \mu g/mL$$

Related substances

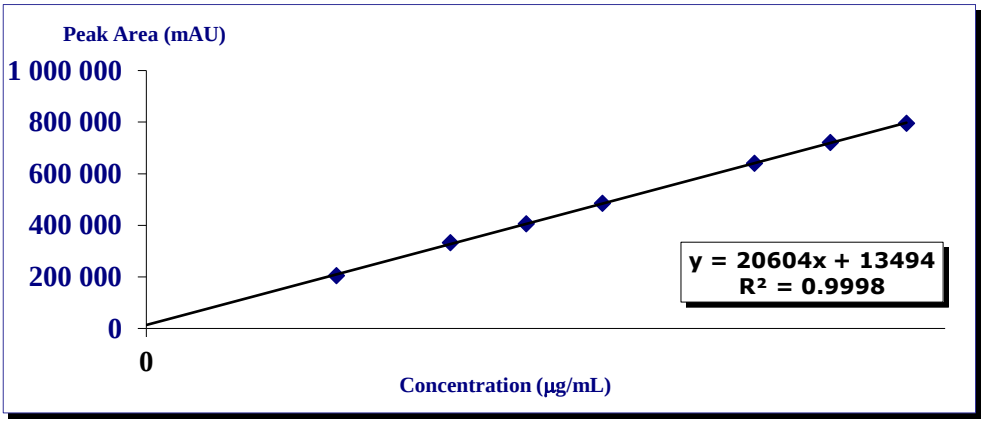


Figure 5.6-Linearity and coefficient of determination of the related substances method validation

Table 5.10-Linearity parameters obtained for the related substances method validation

Parameters	Results	Acceptance criteria
Range (µg/ml)	9.5 – 38.1	---
	Deviations	
Minimum/Maximum (%)	97.6 – 101.7	90.0 – 110.0 %
Average (%)	99.89	90.0 – 110.0 %
RSD (%)	1.23	≤ 5.0 %
R^2	0.9998	> 0.9950
	Regression line	
Slope	20604	---
Intercept	13494	

Mandel's test

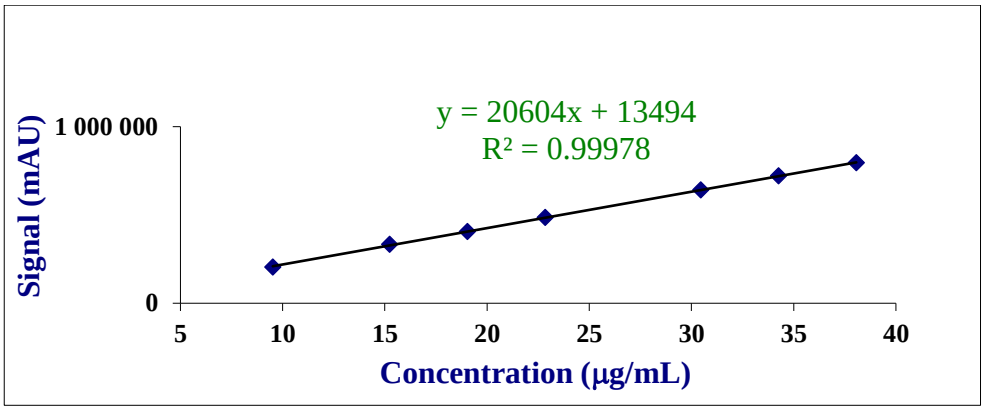


Figure 5.7-Linear adjustment of the related substances method validation in Mandel's test

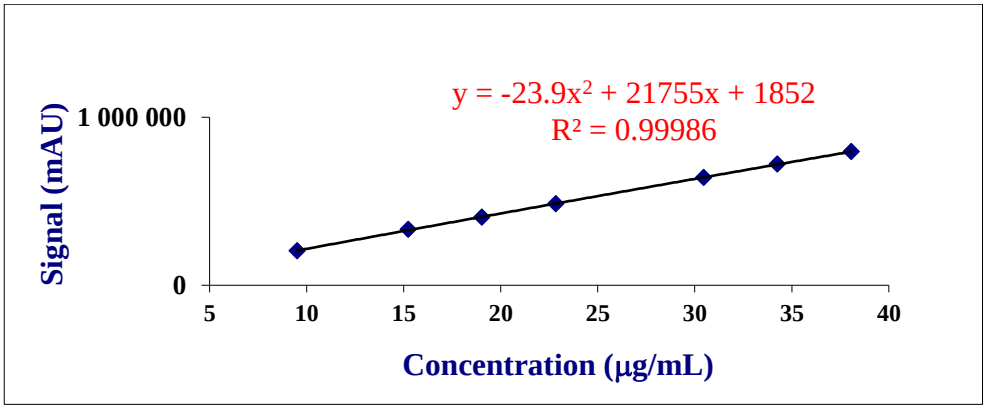


Figure 5.8-Polinomial adjustment of the related substances method validation in Mandel's test

$$VT = 2.35$$

$$F_{(1,N-3,95.0\%)} = 7.71$$

$$VT < F$$

Residual analysis

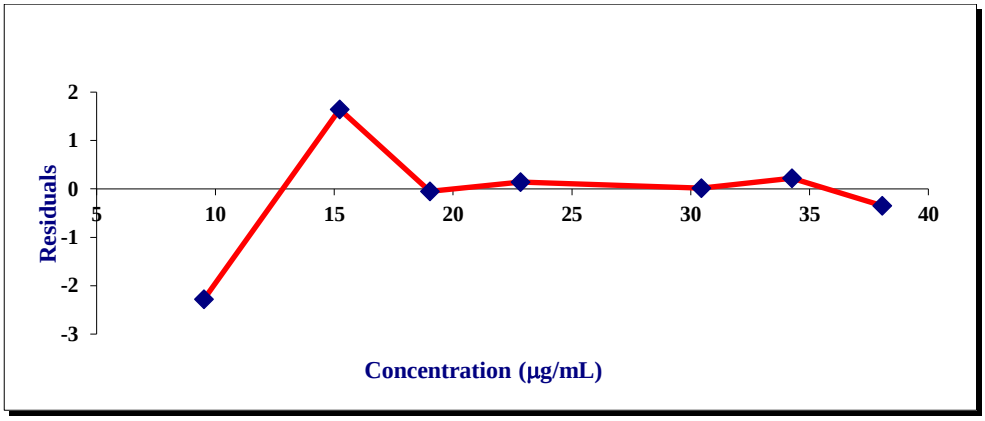


Figure 5.9-Residual analysis of the related substances method validation

Rikilt

Table 5.11-Results of Rikilt's test for the related substances method validation

	Response factor (yi/xi)	RFi / average RF	Acceptance criteria
Average	21268	100.0	98.0 – 102.0 %
Standard deviation	327	1.5	---
RSD (%)	1.5	1.5	≤ 2.0

LOD and LOQ

Table 5.12-Linear adjustment parameters obtained for the related substances method validation

Slope (b)	20604
y-intercept (a)	13494
Correlation coefficient (R)	0.9999
Coefficient of determination (R^2)	0.9998 (< 0.9950)
Sensitivity (e)	20604
Residual standard deviation ($S_{Y/X}$)	3536

$$LOD = 0.6\ \mu g/mL$$

$$LOQ = 1.7\ \mu g/mL$$

Related substances - Impurity A

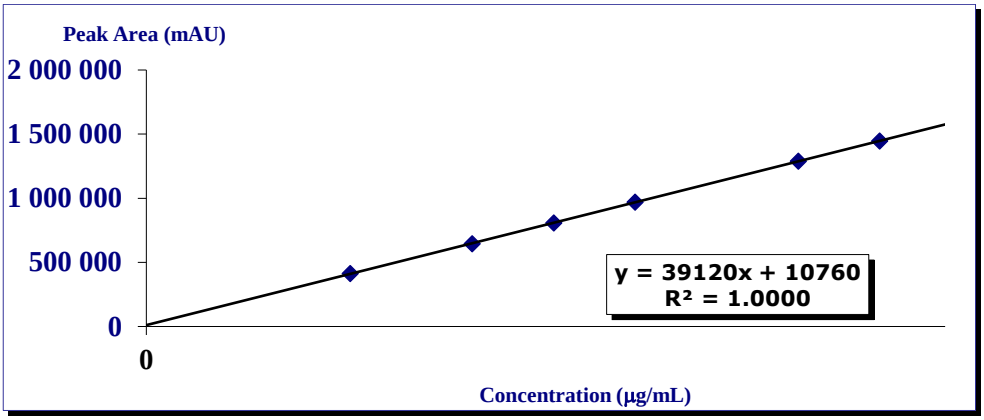


Figure 5.10-Linearity and coefficient of determination of the related substances method validation for sulfanilamide

Table 5.13-Linearity parameters obtained for the related substances method validation for sulfanilamide

Parameters	Results	Acceptance criteria
Range (µg/ml)	10.2 – 40.8	---
	Deviations	
Minimum/Maximum (%)	99.4 – 100.8	90.0 – 110.0 %
Average (%)	100.03	90.0 – 110.0 %
RSD (%)	0.42	≤ 5.0 %
R^2	1.0000	> 0.9950
	Regression line	
Slope	39120	---
Intercept	10760	

Mandel's test

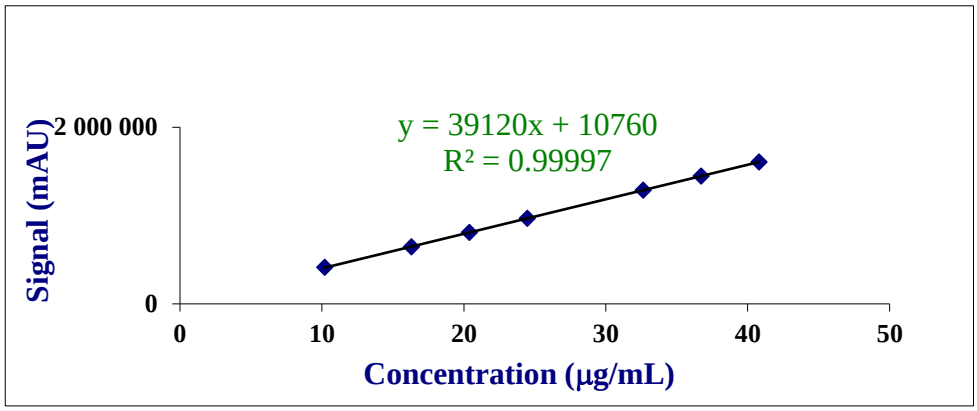


Figure 5.11-Linear adjustment of the related substances method validation in Mandel's test for sulfanilamide

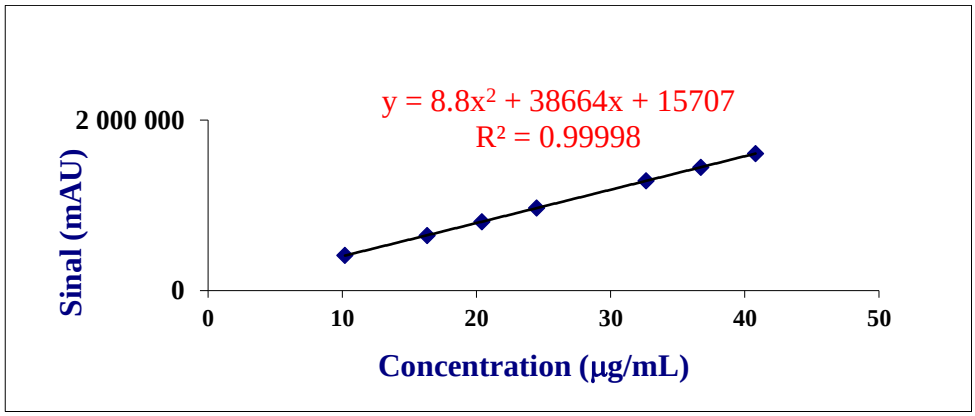


Figure 5.12-Polinomial adjustment of the related substances method validation in Mandel's test for sulfanilamide

$$VT = 0.61$$

$$F_{(1,N-3,95.0\%)} = 7.71$$

$$VT < F$$

Residual analysis

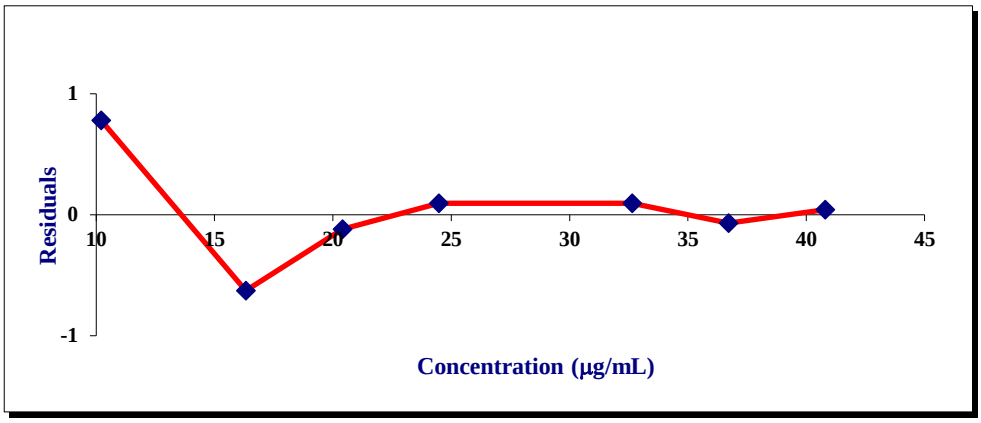


Figure 5.13-Residual analysis of the related substances method validation for sulfanilamide

Rikilt

Table 5.14-Results of Rikilt's test for the related substances method validation for sulfanilamide

	Response factor (yi/xi)	RFi / average RF	Acceptance criteria
Average	39642	100.0	98.0 – 102.0 %
Standard deviation	383	0.97	---
RSD (%)	0.97	0.97	≤ 2.0 %

LOD and LOQ

Table 5.15-Linear adjustment parameters obtained for the related substances method validation for sulfanilamide

Slope (b)	39120
y-intercept (a)	10760
Correlation coefficient (R)	1.0000
Coefficient of determination (R^2)	1.0000 (> 0.9950)
Sensitivity (e)	39120
Residual standard deviation ($S_{Y/X}$)	2512

$$LOD = 0.2\ \mu g/mL$$

$$LOQ = 0.6\ \mu g/mL$$

Relative response factor

Table 5.16-Relative response factor of impurity A (sulfanilamide) obtained from the related substances method validation

Compound	Average RF	CV (%)	RRF
Sulfacetamide sodium	21268	1.5	1.0
Sulfanilamide	39642	1.0	1.9

In all three cases, R^2 is higher than 0.9950, which is the established acceptance criteria,

The analysis of residuals shows that the analytical method has a random distribution of residuals in all three cases, therefore, the results are in compliance with the established acceptance criteria.

For Mandel's test, the tabulated F factor, for a confidence range of 95% and N-3 degrees of freedom, a TV < F was obtained, meaning the linear function is adequate for experimental representation.

The results obtained with Rikilt's test are consistent with the established acceptance criteria, indicating that the analytical method is linear in the working range. The response factor can also be used in the linear function, in routine work.

In the homogeneity of variance test from the assay method validation, the PG value obtained was less than F (5.35 for a confidence range of 99.0% and 9 degrees of freedom). This indicates that any differences between variances are not significant, and the working range is well defined.

The limits of detection and quantification obtained for the assay method were 4.8 and 14.4 μg/mL, respectively. This means that that method is able to detect and quantify the main analyte peak at a concentration much below the test sample concentration (200 μg/mL). The limits obtained for the impurity method using sulfacetamide sodium and sulfanilamide suggest that they can be individually quantified below the reporting threshold.

The relative response factor (RRF) of impurity A established in the related substances operating procedure is 2.0. The RRF obtained in this project was 1.9, which implies a good consistency with the original method of analysis.

5.1.3. Trueness

The trueness was evaluated by the assay variability of nine sample preparations of known concentration over three levels, 50%, 100% and 150% of the test concentration, covering the working range for the assay method. For the related substances method, the three concentration levels were 0.05%, 0.10% and 0.20% of the test concentration.

Three replicates of each concentration level were prepared, and the trueness was reported as a recovery percentage.

The results are presented in Tables 5.17 to 5.19.

Assay

Table 5.17-Recovery percentage for the three samples prepared at each level (50%, 100% and 150%) obtained on the Trueness test for the assay method validation

	Recovery (%)		
A1-50%	100.40	**Average (%)**	99.96
A2-50%	99.29	**SD**	0.59
A3-50%	100.19	**CV (%)**	0.59
A1-100%	100.05	**Average (%)**	100.35
A2-100%	100.55	**SD**	0.26
A3-100%	100.45	**CV (%)**	0.26
A1-150%	99.11	**Average (%)**	99.87
A2-150%	99.47	**SD**	1.02
A3-150%	101.04	**CV (%)**	1.02

Related substances

Table 5.18-Recovery percentage for the three samples prepared at each level (0.05%, 0.1% and 0.2%) obtained on the Trueness test for the related substances method validation

	Recovery (%)		
A1-0.05%	92.2	**Average (%)**	94.1
A2-0.05%	95.6	**SD**	1.7
A3-0.05%	94.6	**CV (%)**	1.8

A1-0.10%	94.4	**Average (%)**	94.5
A2-0.10%	94.5	**SD**	0.2
A3-0.10%	94.7	**CV (%)**	0.2

A1-0.20%	99.2	**Average (%)**	99.26
A2-0.20%	99.5	**SD**	0.27
A3-0.20%	99.0	**CV (%)**	0.27

Related substances – Impurity A

Table 5.19-Recovery percentage for the three samples prepared at each level (0.05%, 0.1% and 0.2%) obtained on the Trueness test for the related substances method validation for sulfanilamide

	Recovery (%)		
A1-0.05%	93.3	**Average (%)**	96.30
A2-0.05%	97.2	**SD**	2.71
A3-0.05%	98.5	**CV (%)**	2.81

A1-0.10%	94.9	**Average (%)**	96.2
A2-0.10%	97.4	**SD**	1.27
A3-0.10%	96.4	**CV (%)**	1.32

A1-0.20%	97.9	**Average (%)**	99.07
A2-0.20%	99.1	**SD**	1.14
A3-0.20%	100.2	**CV (%)**	1.15

The results are consistent with the established acceptance criteria in all three cases, which is 99.0 to 101.0 % of recovery for the assay method, and 90.0 to 110 % of recovery for the related substances method, showing that they are accurate in the established working ranges.

5.1.4. Robustness

Assay

The evaluation of robustness is used to determine the effect of variations in the method parameters. If variations in the conditions are likely to affect the measurements, these conditions should be properly controlled and reported in the procedure.

The evaluation of robustness was performed through the analysis of eight samples, which seven of them were submitted for different analytical conditions, corresponding to the combination of each variation in the analytical conditions. For each variation, the average of four samples results correponding to the nominal value of the parameter and the average of four samples results corresponding to the variation value of the parameter were obtained. The robustness was evalueted by comparison of each set of four samples average value. The results are presented in Tables 5.20. and 5.21.

Table 5.20-Average peak area results obtained from the Robustness test for the assay method validation

Factor	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**
Result (%)	101.02	100.73	100.65	101.73	101.78	102.82	100.81	99.87

Table 5.21-Comparison between the results obtained with the normal and altered parameters on the Robustness test for the assay method validation

		Average	Difference	RSD (%)
Time in ultrasonic bath	10min	101.03	0.29	0.20
	3min	101.32		
%Glacial acetic acid in mobile phase	1%	101.59	0.82	0.57
	0.95%	100.76		
Column temperature	25ºC	101.06	0.22	0.15
	30ºC	101.29		
Flow rate	1.2 mL/min	101.57	0.79	0.55
	1.5 mL/min	100.78		

The results for flow rate, time in ultrasonic bath, column temperature, and percentage of glacial acetic acid in the mobile phase parameters are in compliance with the established acceptance criteria, showing that the analytical method is robust in the working range for these parameters.

5.1.5. Precision

The precision was evaluated by the variability of the analysis of six replicates at 100% of the test solution. After that, two analysts prepared individually, on different days and using different equipment systems and different columns, 6 replicates of sample solution and a reference solution (Analysis Repeatability). The obtained results are presented in Tables 5.22. to 5.32.

Assay

Table 5.22-Concentration of the six samples prepared for the Analysis Precision test and the percentage results obtained for the assay method validation

	Concentration (µg/mL)	Concentration (%)
A1	199.95	100.05
A2	199.49	100.55
A3	199.77	100.45
A4	199.68	100.23
A5	199.49	100.57
A6	199.49	100.46

Table 5.23-Results from the Analysis Precision for the assay method validation

Average (%)	**100.39**
SD	**0.20**
CV (%)	**0.20**

Intermediate precision

Table 5.24-Concentration of the six samples prepared for the Intermediate Precision test and the percentage results obtained for the assay method validation

	Concentration (µg/mL)	Concentration (%)
A1	201.72	99.29
A2	201.81	99.06
A3	201.81	99.05
A4	201.81	100.75
A5	201.62	101.94
A6	201.53	100.54

Table 5.25-Results from the Intermediate Precision for the assay method validation

Average (%)	**100.19**
SD	**1.10**
CV (%)	**1.09**

Table 5.26-Comparison between the results obtained for different analysts, performed on different days with different equipment, from the Intermediate Precision test for the assay method validation

Intermediate precision			Acceptance criteria
Tests	**1**	**2**	---
Average (%)	100.3		99.0 – 101.0 %
RSD (%)	0.20	1.09	---
Variance	0.04	1.20	
Pooled average	100.3		
S^2	0.62		
S	0.79		
Pooled RSD (%)	0.79		≤ 2.0 %

Repeatability

The results obtained are presented in following table.

Table 5.27-Results obtained from the Repeatability test for the assay method validation

Precision of replicate injections response	50% (n=10)	100% (n=6)	150% (n=10)
RSD%	0.10%	0.81%	0.12%
			Acceptance criteria ≤ 2.0 %

Related substances

Table 5.28-Concentration of the six samples prepared for the Analysis Precision test and the percentage results obtained for the related substances method validation

	Concentration (µg/mL)	Concentration (%)
A1	18697.7	0.08
A2	19187.3	0.08
A3	18688.5	0.09
A4	18928.7	0.09
A5	18549.9	0.09
A6	19575.3	0.09

Table 5.29-Results from the Analysis Precision for the related substances method validation

Average (%)	0.09
SD	0.00
CV (%)	1.98

Intermediate precision

Table 5.30-Concentration of the six samples prepared for the Intermediate Precision test and the percentage
results obtained for the related substances method validation

	Concentration (µg/mL)	**Concentration (%)**
A1	18577.6	0.10
A2	18540.7	0.11
A3	187743.9	0.11
A4	18559.1	0.11
A5	18568.4	0.11
A6	18725.4	0.11

Table 5.31-Results from the Intermediate Precision for the related substances method validation

Average (%)	0.11
SD	0.01
CV (%)	1.79

Table 5.32-Comparison between the results obtained for different analysts, performed on different days with different equipment, from the Intermediate Precision test for the related substances method validation

Intermediate precision		
Tests	**1**	**2**
Average	0.10	
RSD (%)	1.98	1.79
Variance	0,00	0,00
Pooled average	0,10	
S^2	0,00	
S	0,00	
Pooled RSD (%)	1.88	
Acceptance criteria	For concentrations between 0.5% and 2.0%: ≤ 10.0 %	

The results comply with the established acceptance criteria, showing that the analytical method is precise in the working range.

5.1.6. Stability of samples

The variability results obtained between freshly prepared samples and the ones stored for the period of analysis are presented in Tables 5.33 to 5.35.

Assay

Table 5.33-Results from the Stability of samples test for the assay method validation

Working standard	
Concentration (%)	99.74
Concentration (µg/ml)	183.79

RSD (%)	**Conformity**
0.26	OK

Test sample	
Concentration (%)	100.34
Concentration (µg/ml)	200.73

RSD (%)	**Conformity**
0.04	OK

Acceptance criteria ≤ 2.0 %.

The results for the reference solution and for the sample solution are in compliance with the acceptance criteria, showing that solutions are stable during the analysis period at least for one week, stored protected from light in a volumetric flask in the refrigerator.

Related substances

Auto-sampler (10ºC)

Table 5.34-Results from the Stability of samples test for the related substances method validation, where the samples were stored in the HPLC auto-sampler

Working standard	
Concentration (%)	97.27
Concentration (µg/ml)	18.02

RSD (%)	**Conformity**
2.73	OK

Test sample	
Concentration (%)	0.12
Concentration (µg/ml)	21.41

RSD (%)	**Conformity**
40.39	NOT OK

Refrigerator (3ºC)

Table 5.35-Results from the Stability of samples test for the related substances method validation, where the samples were stored in a refrigerator

Working standard	
Concentration (%)	97.94
Concentration (µg/ml)	18.20

RSD (%)	**Conformity**
2.06	OK

Test sample	
Concentration (%)	0.10
Concentration (µg/ml)	19.40

RSD (%)	**Conformity**
21.50	NOT OK

Acceptance criteria ≤ 10.0 %.

The results for the reference solutions, in both cases, comply with the acceptance criteria, showing that they are stable during the analysis period at least one week, kept in a vial in the auto-sampler and in a volumetric flask in the refrigerator. The results for the sample solution do not comply with the acceptance criteria, which suggests that they are not stable during the analysis period, and that they should be prepared shortly before use.

5.1.7. Stress testing

The test samples were subjected to stress conditions and quantified against reference solutions. Figures 5.15 to 5.19 are examples of related substances chromatograms obtained for the samples subjected to stress conditions, with the HPLC analyses results presented in Tables 5.36 to 5.40. Figure 5.14 shows how the color of the related substances samples was altered after the time of this test.

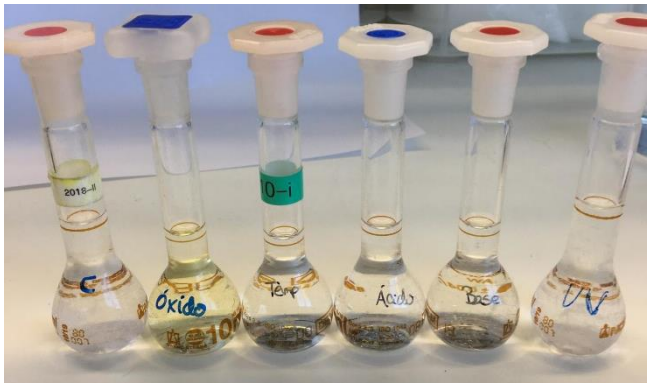


Figure 5.14-Comparison between the coloration of samples subjected to different stress conditions

Acid stress testing

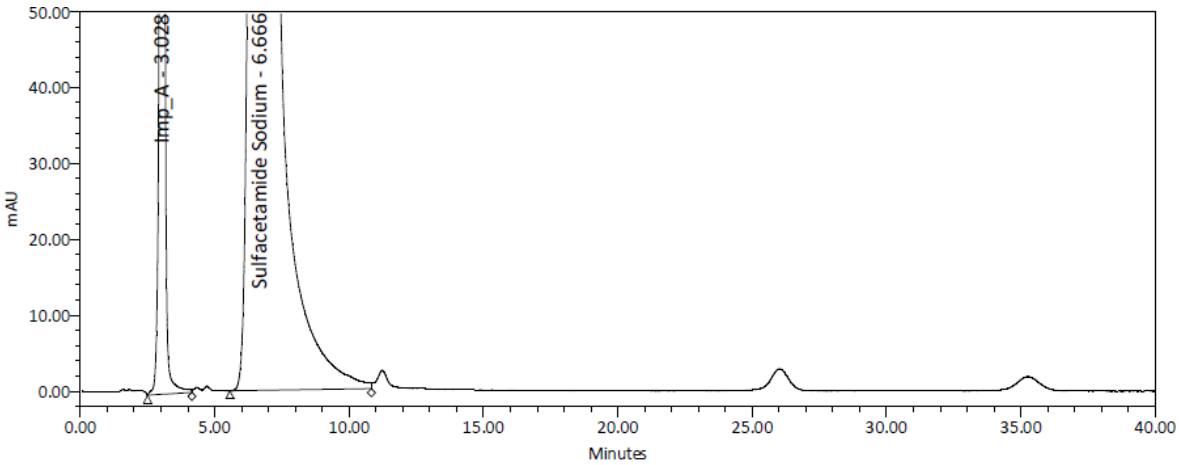


Figure 5.15-Chromatogram of a related substances testing run after acidic conditions

Table 5.36-Results obtained from the acid stress testing for the assay and related substances method validation

Assay (%)	91.71
H_2O (%)	7.16
Anhydrous base (%)	98.78
Impurities (%)	0.44
Mass balance (%)	99.22

Base stress testing

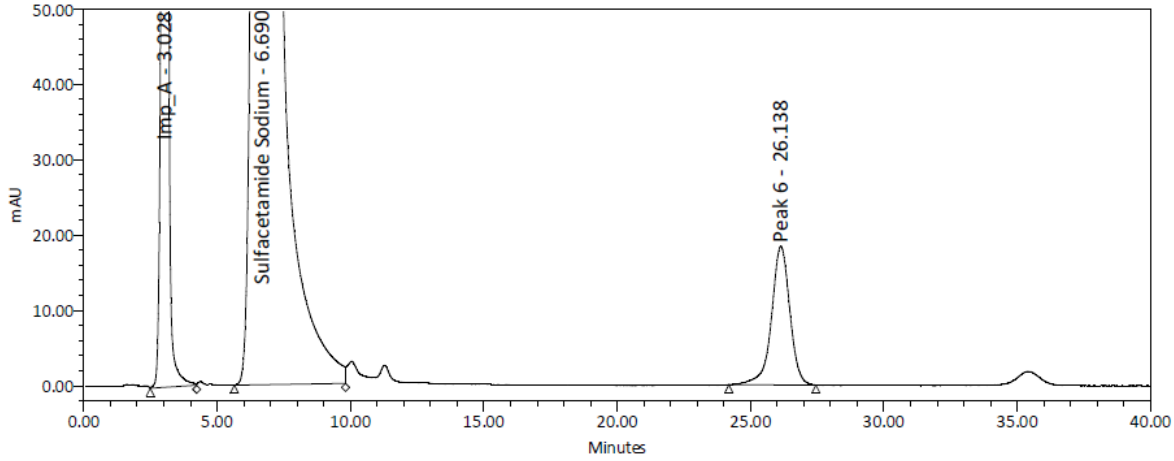


Figure 5.16-Chromatogram of a related substances testing run after basic conditions

Table 5.37-Results obtained from the base stress testing for the assay and related substances method validation

Assay (%)	90.33
H_2O (%)	7.16
Anhydrous base (%)	97.30
Impurities (%)	1.26
Mass balance (%)	98.56

UV light stress testing

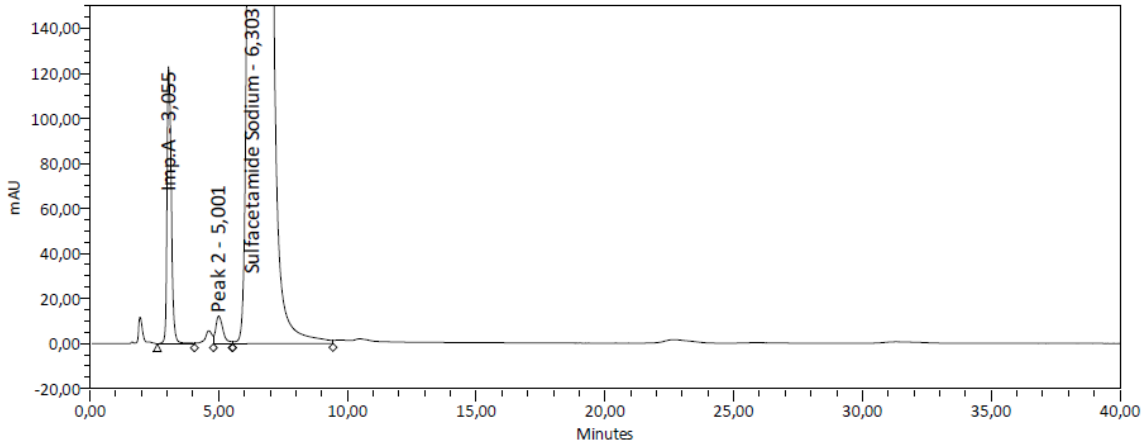


Figure 5.17-Chromatogram of a related substances testing run after exposure to UV light

Table 5.38-Results obtained from the UV light stress testing for the assay and related substances method validation

Assay (%)	92.66
H_2O (%)	7.16
Anhydrous base (%)	99.81
Impurities (%)	0.20
Mass balance (%)	100.01

Temperature stress testing

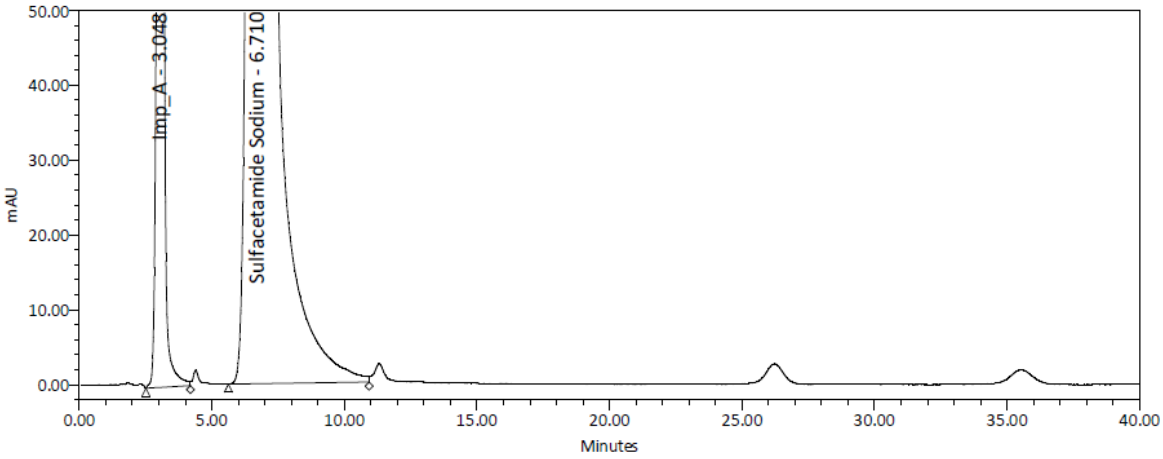


Figure 5.18-Chromatogram of a related substances testing run after exposure to high temperature

Table 5.39-Results obtained from the temperature stress testing for the assay and related substances method validation

Assay (%)	88.98
H_2O (%)	7.16
Anhydrous base (%)	95.84
Impurities (%)	2.48
Mass balance (%)	98.32

Oxide stress testing

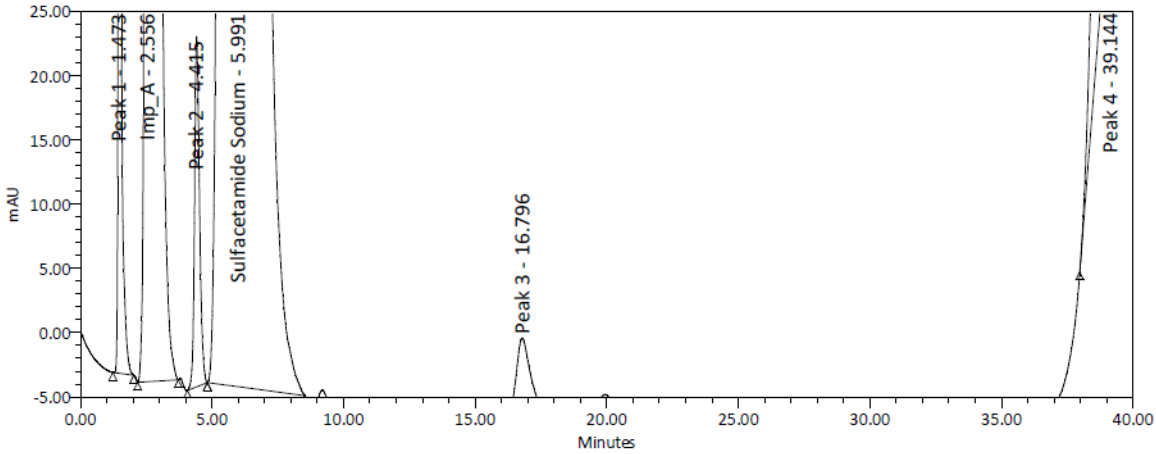


Figure 5.19-Chromatogram of a related substances testing run after exposure to an oxidizing agent

Table 5.40-Results obtained from the oxide stress testing for the assay and related substances method validation

Assay (%)	85.46
H_2O (%)	7.16
Anhydrous base (%)	92.05
Impurities (%)	5.15
Mass balance (%)	97.20

The results show a decreased content of sulfacetamide sodium that corresponds to an increased content of related compounds, mainly when the samples were subject to oxidative conditions.

According to Ahmad, et al. (1994), the thermolysis of sulfacetamide sodium solutions, as mentioned before, result in the formation of sulfanilamide. Additionally, the authors refer

that a brown color was formed within one hour of the addition of hydrogen peroxide to sulfacetamide sodium eye drops, with deposits of brown crystals. Tests with UV irradiation on sulfacetamide sodium solutions showed that they underwent hydrolysis and oxidation to form products identified as sulfanilamide, azobenzene-4,4'-disulfonamide,axoxybenzene-4-4'-disulfonamide, sulfanilic acid, azobenzene,4,4'-disulfonic acid and a blue product. The authors also describe that 20-30% ophthalmic solutions of sulfacetamide sodium show maximum stability at pH 7.3-8.5, which means that acidic and basic conditions may cause degradation of the ingredient in the solutions.

In this study, a few impurities were formed in the different tests, however, they were not identified, except for sulfanilamide. In some cases, the mass balance was not equal to 100%. This could happen due to analysis errors in sample preparation or equipment uncertainty, some samples could further degrade during the HPLC analysis, or some unknown impurities could have a response factor different than 1.0. Additionally, with the oxidizing agent, one peak appeared at the end of the test run and could not be entirely quantified, however, this peak does not interfere with the main analyte.

The samples must be adequately monitored to prevent any type of degradation during routine analysis.

5.2. Sulfacetamide sodium

5.2.1. Bioburden

As mentioned before, the bioburden determination represents a means of supporting the sterilization results. By, later, using biological indicators with a well-known microbial population, it is possible to understand if the sterilization process can be used for that specific substance. If it is demonstrated that the bioburden is much inferior to the biological indicator population, it gives the whole process a safety margin.

Table 5.41-Total Aerobic Microbial Count obtained for both lots of sulfacetamide sodium

MP/19-0397	TAMC < 1 CFU/100 mL
MP/20-0382	TAMC 1 CFU/100 mL

By analyzing the results, it is possible to see that the Total Aerobic Microbial Count (TAMC) is well below the microbial population of the biological indicator *(Bacillus atrophaeus* with a microbial population of $2.1x10^6$), which supports the efficacy of the sterilization process for sulfacetamide sodium. Additionally, according to the Ph. Eur., the TAMC acceptance criteria for microbiological quality of substances for pharmaceutical use is 200 CFU/mL, therefore the results obtained comply with the specified limits.

5.2.2. Sterilization cycles

For all combinations of time and temperature that were tested, a biological indicator was used, as a validation of the cycle.

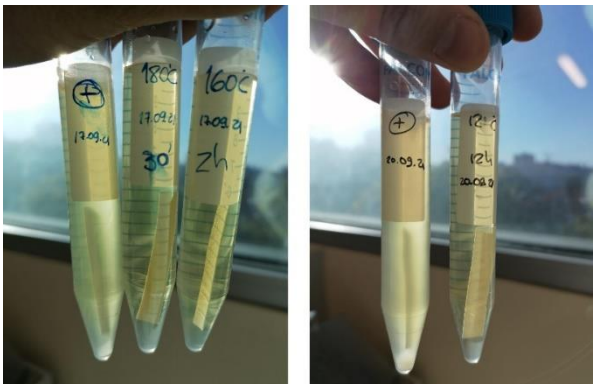


Figure 5.20-Results of the biological indicators for 180ºC-30min; 160ºC-120min; 121ºC-12h.

The turbidity in the positive culture media, which contained the bioindicator that was not subjected to sterilization, represents bacterial growth. The limpid media used for the bioindicators that went through the sterilization processes indicate that there was no bacterial growth, which assures a SAL $\leq 10^{-6}$ and validates the cycles, reaffirming their reliability.

The first sterilization tests were completed with a small amount, approximately 1 g, of sulfacetamide sodium, to investigate any visible effects that the heat exposure could cause. Firstly, a totally sealed glass flask was used, then a sterilization pouch made from plastic on one side and medical grade paper on the other was also tested. The pouch was sealed, but the paper side allows for the air and moisture to pass through.

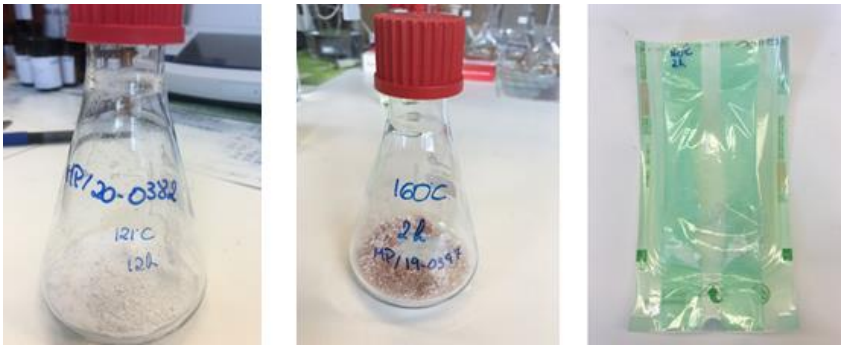


Figure 5.21-Sulfacetamide sodium samples after different cycles of dry heat sterilization in glass flasks and sterilization pouches

On the first image of Figure 5.21., it is possible to see that, after the sterilization process using the glass flasks, the substance remained white, with no color alterations. This happened for the following cycles.

- 121ºC, 12 hours.
- 140ºC, 180 minutes.
- 150ºC, 150 minutes.

This visual analysis is not, alone, an indication of lack of degradation, however, it is a suggestion that those cycles do not interfere with the physical characteristics of the substance and do not lead to the formation of colored impurities.

For the remaining cycles, from 160ºC and higher, a considerable part of the substance became brownish red, which points out to a major degradation of the substance into a colored impurity. The glass flasks were not ideal containers for sterilization, considering the increase in pressure caused by the higher temperatures that could cause the glass to crack, however, they were a good way to investigate the behavior of the substance in an environment with trapped moisture and high pressure.

When the same cycles were tested in the sterilization pouches, there was no color alterations or any darkening of the substance. This suggests that the moisture environment, along with the heat, was the key factor in the degradation of sulfacetamide sodium. This result is in agreement with the authors Ahmad, et al., (1994), who concluded that solutions appeared darker with deposits of sulfanilamide after they were autoclaved in the presence of oxygen although, in this project, the active ingredient was sterilized in the powder form and in a dry environment. Nevertheless, the evaporation of the water present in the substance could create

a surrounding with enough moisture to cause degradation in addition with high temperatures. At the cycles up until 150ºC and 150 minutes, the moisture and temperature are not sufficient to cause physical alterations on the substance. By using the sterilization pouches, when the moisture evaporates, it is able to escape the container, not staying in direct contact with the ingredient.

After these tests, another sterilization process was completed using larger sterilization pouches, on a greater amount of sulfacetamide sodium, approximately 15 g in each pouch, which was enough to perform a complete raw material analysis. Considering the cycle of 160ºC for 120 minutes on pouches did not result in visual modifications of the substance, this was the chosen cycle that was chosen to be repeated. This is the standard dry heat sterilization cycle, therefore, is does not require any type of validation in terms of microbial killing efficacy.

5.2.3. Raw material analysis

After the standard cycle was repeated in each lot of the substance, a complete raw material analysis was performed, following the operating procedure from the European Pharmacopoeia. This step was necessary to investigate any chemical effects that the heat sterilization could cause on the substance.

Appearance

The appearance of the powder substance was compared visually to a non-sterilized sample. The analytical method states that this active ingredient should appear a white or yellowish-white crystalline powder.

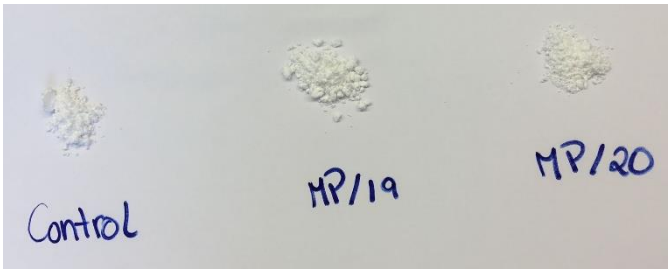


Figure 5.22-Comparison between sterilized and non-sterilized samples of sulfacetamide sodium after a cycle of 160ºC for 120 minutes

As it can be observed in Figure 5.22., both lots maintained the appearance of the control sample, which complies with the method parameters. The only difference is that the sterilized samples presented a drier appearance, suggesting a loss of their water content.

Solubility

The sterilized substances were tested for their solubility in water and in anhydrous ethanol. It was observed that they remained freely soluble in water and slightly soluble in anhydrous ethanol, which are the specifications in the operating procedure. This indicates that the heat did not provoke any changes that could increase or decrease the active ingredient's solubility.

Infrared absorption spectrophotometry

The infrared absorption spectrophotometry is an important active substance identification method. The spectrum obtained presents characteristic bands that correspond to the functional groups of the molecule, which provides information on the substance's structure. The sterilized samples were compared to a spectrum of sulfacetamide sodium CRS.

The infrared region between 1500 and 400 cm^{-1} is a very complex and informative part of the spectrum, which characterizes the analyzed molecule, known as the fingerprint region.

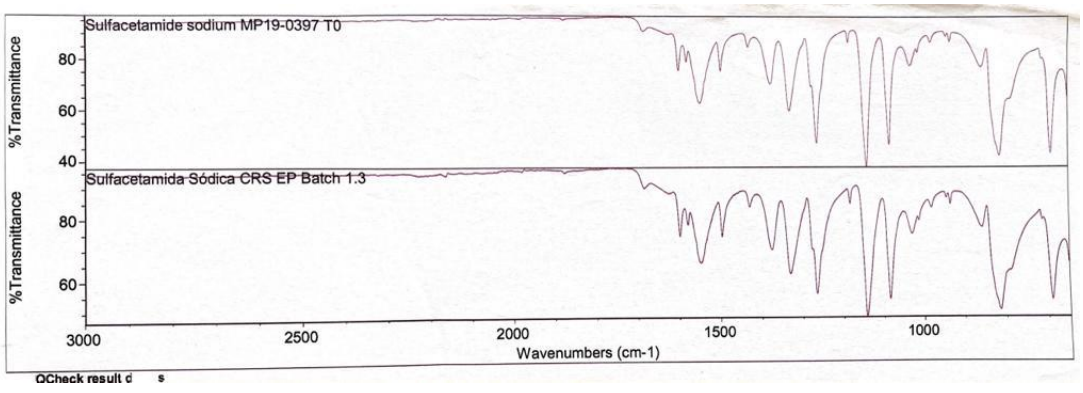


Figure 5.23-Comparison between the spectra of a sulfacetamide sodium sterilized sample from the MP/19-0397 lot and a sulfacetamide sodium CRS sample in IR absorption spectrophotometry

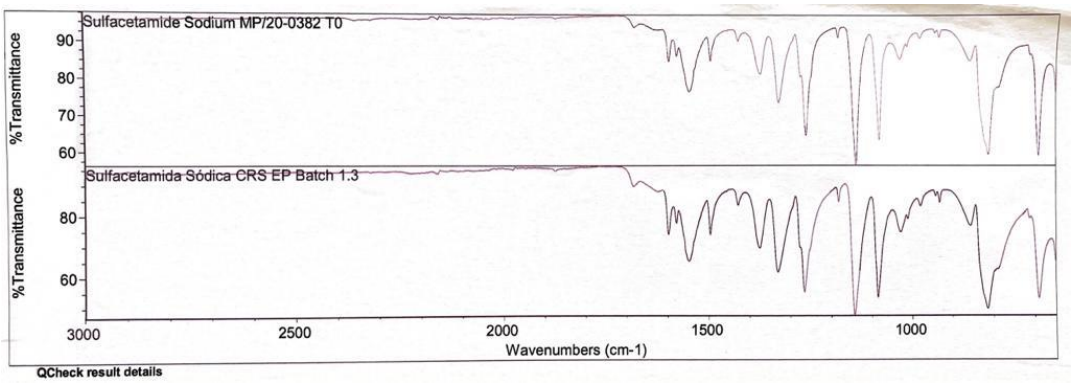


Figure 5.24-Comparison between the spectra of a sulfacetamide sodium sterilized sample from the MP/20-0382 lot and a sulfacetamide sodium CRS sample in IR absorption spectrophotometry

Table 5.42-Correlation obtained between the spectra of sterilized samples of sulfacetamide sodium and a sulfacetamide sodium CRS sample in IR absorption spectrophotometry

Correlation	
MP/19-0397	0.9688
MP/20-0382	0.9503

The sterilized substance shows the same bands in the spectrum as the reference sample, indicating that there were no modifications in the molecule's functional groups.

The correlation above 90% suggests a close enough proximity to the reference. The correlation numbers might not be higher simply due to noise, baseline shifts or atmospheric disturbances (Kondagula & Molt, 2010) or to an increase in impurities, especially sulfanilamide, which is already present in sulfacetamide sodium samples. This could also happen considering the reference sample is from a different lot and manufacturer as the sterilized substances.

Reactions of sodium

The reactions of sodium are the second identification analysis of sulfacetamide sodium, along with the infrared spectrophotometry. It is a process to identify the presence of the sodium functional group in the substance.

After the addition of the 150 g/L solution of potassium carbonate R and boiling, no precipitate was formed, as it appears on the first image of Figure 5.25. After the addition of the potassium pyroantimonate solution R and the new mixture was again heated to boiling and allowed to cool, a dense white precipitate was formed, as it can be observed on the second

image of Figure 5.25. This complies with the expected results, stated in the operating procedure.

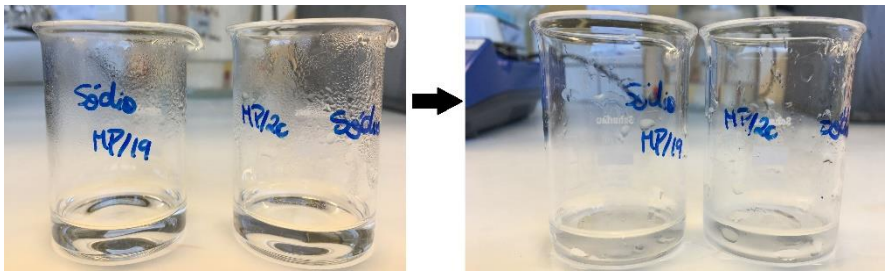


Figure 5.25-Results from the reaction (a) of sodium on sterilized samples of sulfacetamide sodium

In the reactions of sodium (b), a white, crystalline precipitate was formed after the addition of methoxyphenyl acetic reagent R and cooling in iced water for 30 minutes. Figure 5.26. shows how the precipitate completely disappears after the addition of dilute ammonia R1. This also complies with the expected results.

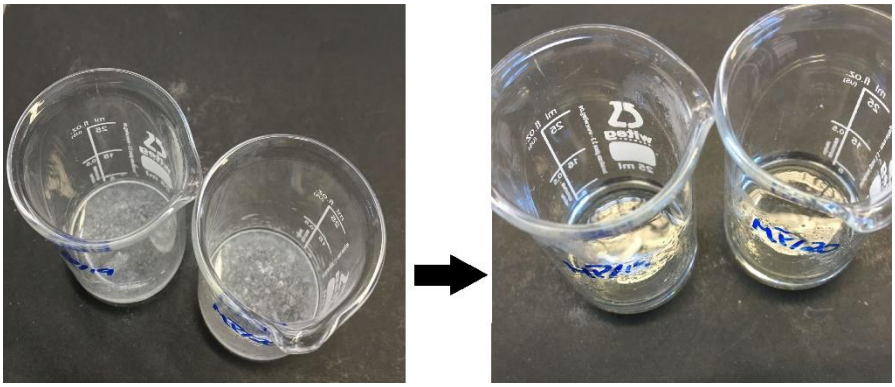


Figure 5.26-Results from the reaction (b) of sodium on sterilized samples of sulfacetamide sodium

Both these reactions demonstrate the presence of the sodium functional group in the active ingredient, indicating that sterilization had no impact at this level, which supports the results from the infrared absorption spectrophotometry.

Appearance of solution S

The solutions S of the sterilized samples were compared to the solution S of a control sample, which were all compared to reference solution GY4.

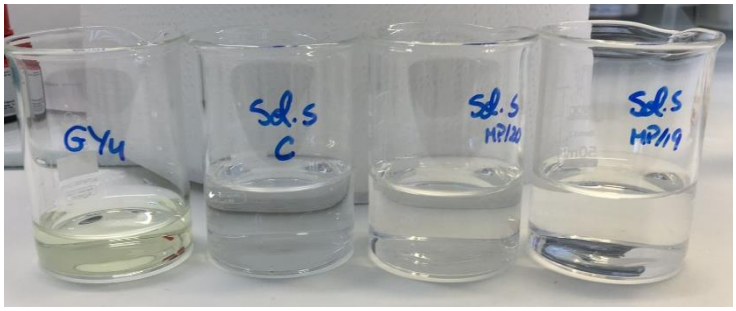


Figure 5.27-Comparison between the color intensity of solution S of sulfacetamide sodium sterilized samples to solution S of a control sample and a reference solution GY4

The specifications required the analyzed solutions to be clear and not more intensely colored than the reference solution, which, undoubtedly, can be observed in Figure 5.27. This result backs up the conviction that the heat did not alter the substances' physical characteristics, not producing any colored impurities.

pH

The pH, which is conventionally expressed as the hydrogen ion concentration of a solution, was determined from solution S. This value should be between 8.0 and 9.5.

Table 5.43-pH results obtained for both lots of sulfacetamide sodium

	pH	Temperature
MP/19-0397	8.12	25.0ºC
MP/20-0382	8.10	22.3ºC

By observing Table 5.43, the pH of both lots stayed within the required specifications, towards the lower end. The lower pH values are not a result of the water loss during sterilization since less water content would result in a higher pH. These values might vary with temperature, but they are very similar to the control samples, which is an indication that sterilization did not produce any more acidic substances.

Sulfates

This testing is a quantification of the sulfate group. The monograph specifies a maximum of 200 ppm and this limit can be verified by comparing the opalescence of the tested samples with a sample prepared with a sulfate standard solution.

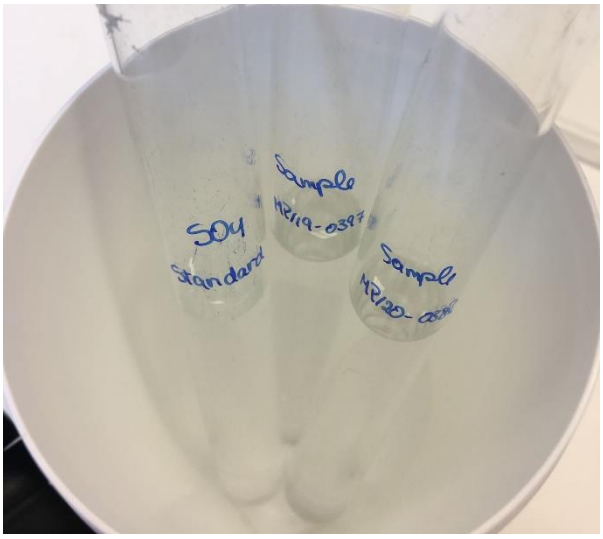


Figure 5.28-Comparison between sulfacetamide sodium sterilized samples after the Sulfates test to a Sulfate standard sample

As it can be observed from the meniscus in the tubes from Figure 5.28., the sulfacetamide sodium samples are clearer than the one in standard, which implies that the sulfates amount does not exceed the limit described. Therefore, the sterilization process did not increase availability of this particular functional group or, if it did, it was not enough to surpass the 200 ppm limit.

Water

After the sterilization cycle, a loss in the water content was expected. This could possibly make this parameter be out of specification which would demand adjustments in the manufacturing process of all the products that incorporate sulfacetamide sodium. However, this value was measured over a week period to confirm any changes over time.

Table 5.44-Water content results obtained right after sterilization for both lots of sulfacetamide sodium

Water content after sterilization	
Lot	**%H_2O**
MP/19-0397	0.21
MP/20-0382	0.21

Table 5.45-Water content results obtained three days after sterilization for both lots of sulfacetamide sodium

Water content three days after Sterilization	
Lot	**%H_2O**
MP/19-0397	3.04
MP/20-0382	4.47

Table 5.46-Water content results obtained one week after sterilization for both lots of sulfacetamide sodium

Water content one week after sterilization	
Lot	**%H_2O**
MP/19-0397	7.11
MP/20-0382	7.04

The water content requirement for this active ingredient is between 6.0 and 8.0%. as stated in the sulfacetamide sodium monograph (Ph. Eur.). It was noticed that right after sterilization, the water present in the substance was nearly zero, due to the evaporation during the process, which is considerably below specification. The value increases gradually over a few days, and, after a week, the substance presents the same amount of water as it had before the heat treatment. During this time, the sample was stored in the same pouches in which they were sterilized in, at ambient temperature and protected from light; similar conditions practiced at the company for this substance. This signifies that, even after losing water during sterilization, the substance will eventually rehydrate and regain the same characteristics, in terms of water content. The sterilization was performed on a rather small sample of sulfacetamide sodium (approximately 15 g), and the powder could be mixed before the water content determination, making the sample homogeneous in terms of contact with heat. These conditions are very different than what would have happened on a larger scale. When implementing this sterilization process on a large scale, it is best to wait at least one week for the rehydration of the substance, to minimize heterogeneity in terms of water content throughout the sample, to rule out any potential readjustments in the manufacturing of the pharmaceuticals, even if it already is a standard procedure at this company to measure the water of ingredients prior to the manufacturing.

Related substances

This section of the investigation, as previously discussed, had a major weight on the decision concerning the suitability of dry heat sterilization on sulfacetamide sodium. It is when any signs of degradation could be detected, and the impurities quantified.

The control of impurities in pharmaceuticals is essential to assure acceptable quality for users. If the heat tretament originates impurities above the stated limits, this should be enough to reject this type of sterilization for the substance.

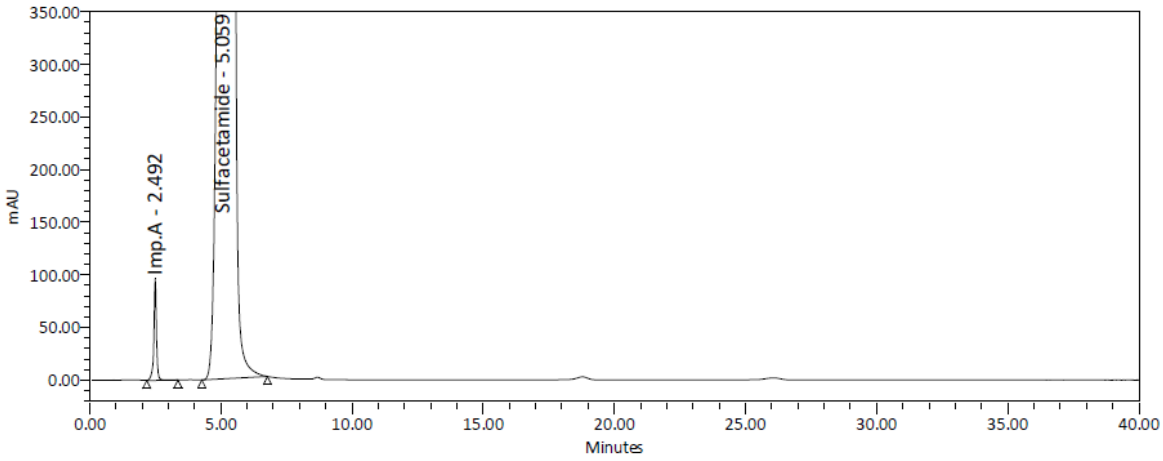


Figure 5.29-Chromatogram of a control sample in a related substances test run of sulfacetamide sodium

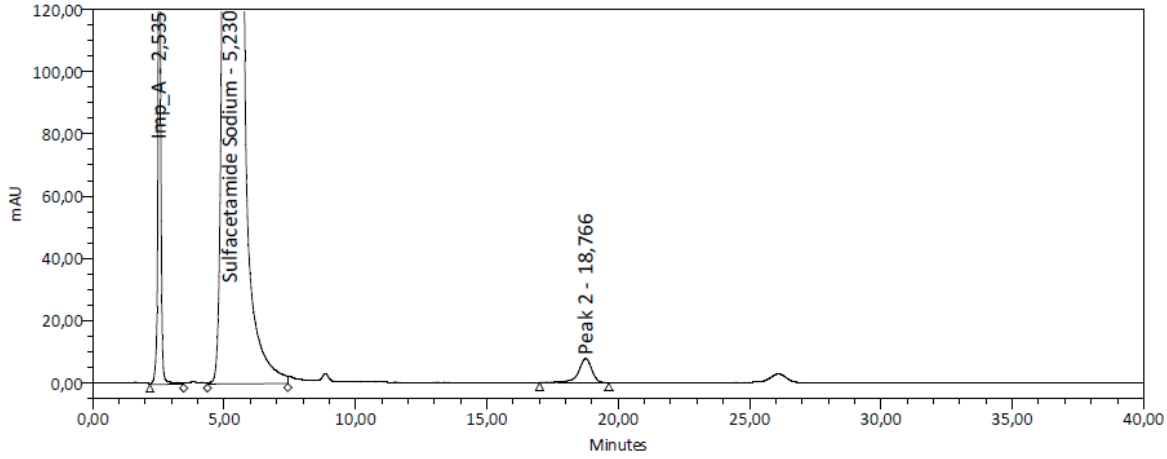


Figure 5.30-Chromatogram of a MP/19-0397 test sample in a related substances test run of sulfacetamide sodium

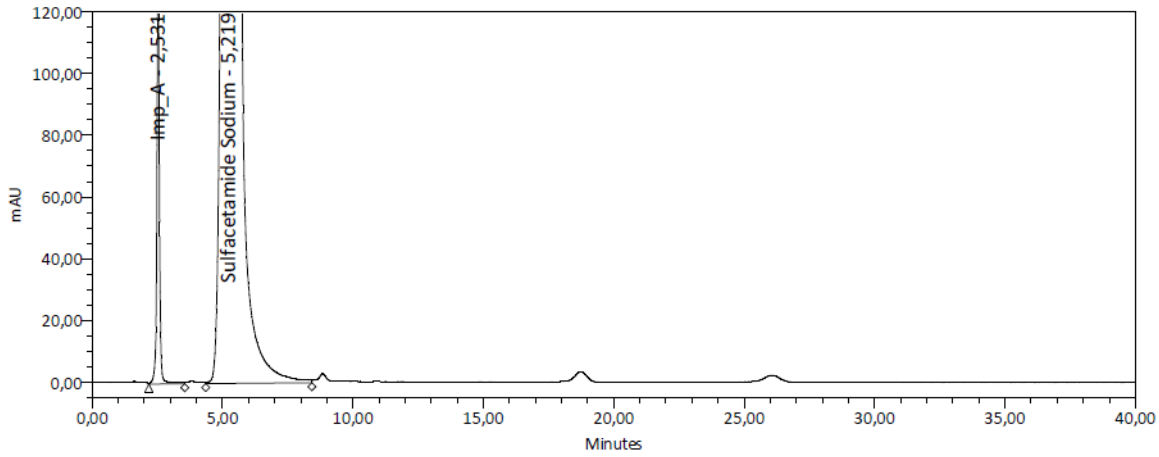


Figure 5.31-Chromatogram of a MP/20-0382 test sample in a related substances test run of sulfacetamide sodium

It can be observed in the chromatograms (Figures 5.29. to 5.31.) the major presence of impurity A, or sulfanilamide in the samples. Three other peaks of related substances appear in the non-sterilized sample as well as in the ones that were sterilized at 160ºC for 120 minutes.

This means that, after sterilization, no other impurities were produced. The monograph of sulfacetamide sodium declares three other detectable impurities, N-(4-sulphamoylphenyl)acetamide, N-[[4-(acetylamino)phenyl]sulphonyl]-acetamide and dapsone, which are potential impurities, not usually present above the identification threshold, and when that happens they do not need to be identified. (Ph. Eur.). The three unknown peaks, most likely, correspond to these, however their retention times are not specified.

Peak 2 in lot MP/19-0397 was quantified as it was just above the disregard limit, which is 0.05%. It is possible that this impurity was quite close to this limit in the other samples. This impurity might be present at a bigger quantity in this lot, considering it was purchased a year before the other, and the storage time and handling of the substance could have a slight impact on the amount of impurities.

Table 5.47-Results from the related substances analysis of sulfacetamide sodium for lot MP/19-0397

MP/19-0397		
	Control	**Sterilized**
Impurity A (%)	0.12	0.15
Peak 2 (%)	< 0.05	0.06
Other impurities (%)	< 0.05	< 0.05
Total impurities (%)	0.12	0.21

Table 5.48-Results from the related substances analysis of sulfacetamide sodium for lot MP/20-0382

MP/20-0382		
	Control	**Sterilized**
Impurity A (%)	0.08	0.10
Peak 2 (%)	< 0.05	< 0.05
Other impurities (%)	< 0.05	< 0.05
Total impurities (%)	0.08	0.10

Comparing the control to the sterilized samples present (Tables 5.47 and 5.48), a minor increase in sulfanilamide can be observed. From the investigations of Ahmad, et al., (1994), solutions of sulfacetamide sodium suffered a 1% loss when autoclaved at 115ºC for 30 minutes and at 120ºC for 20 minutes and a 0.5% degradation when heated at 100ºC for 30 minutes. They concluded that heating the solutions facilitates degradation and consequently, the prospect of sulfanilamide formation. The results obtained from the dry heat sterilization of the substance are in accordance with these authors' conclusions, however, in this case, the degradation is considerably less, believably due to the lack of water in the environment.

Nevertheless, with a limit of 0.20% for sulfanilamide, 0.10% for each specified impurity, and 0.50% total, the results comply with the requirements and still exhibit a good safety margin.

Assay

The assay is an important complementary analysis to rule out a significant degradation. Some impurities may not appear in the related substances analysis if they are not detectable

under the practiced chromatography conditions, or any unknown impurities that are quantified may have different response factors, leading to a misinterpretation of the results.

To confirm that the substance was not suffering a loss in content, the active ingredient was quantified in a more diluted sample against a standard, prepared in the same concentration.

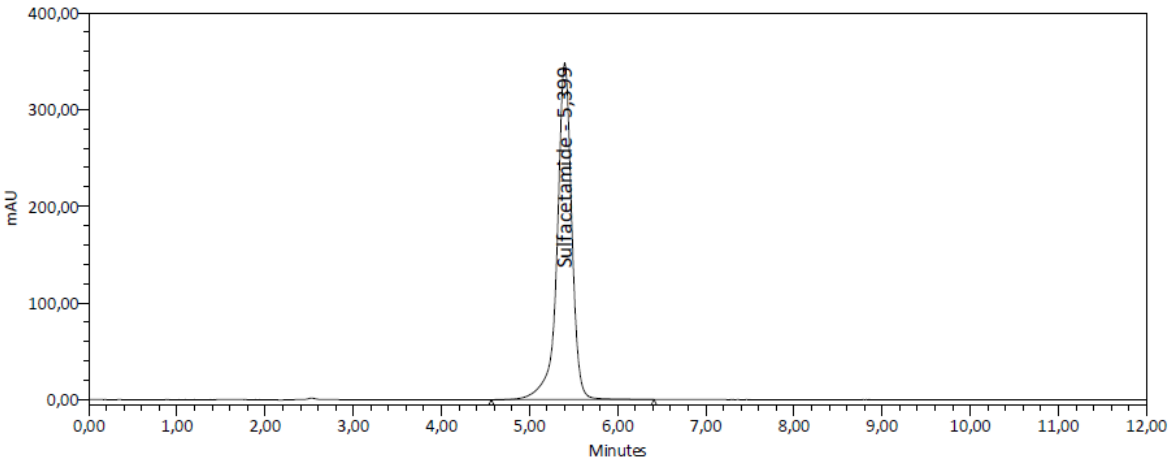


Figure 5.32-Chromatogram of a standard sample in an assay test run of sulfacetamide sodium

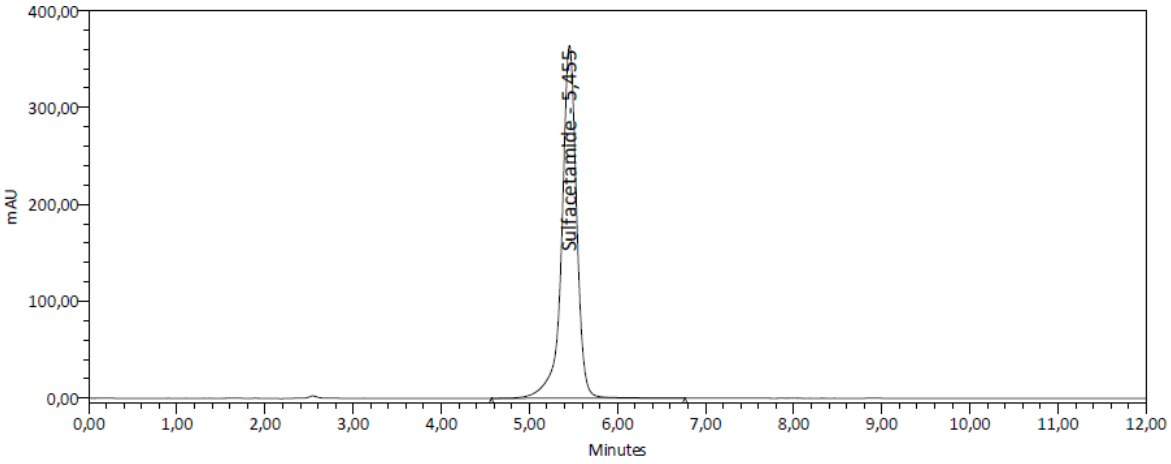


Figure 5.33-Chromatogram of a MP/19-0397 test sample in an assay test run of sulfacetamide sodium

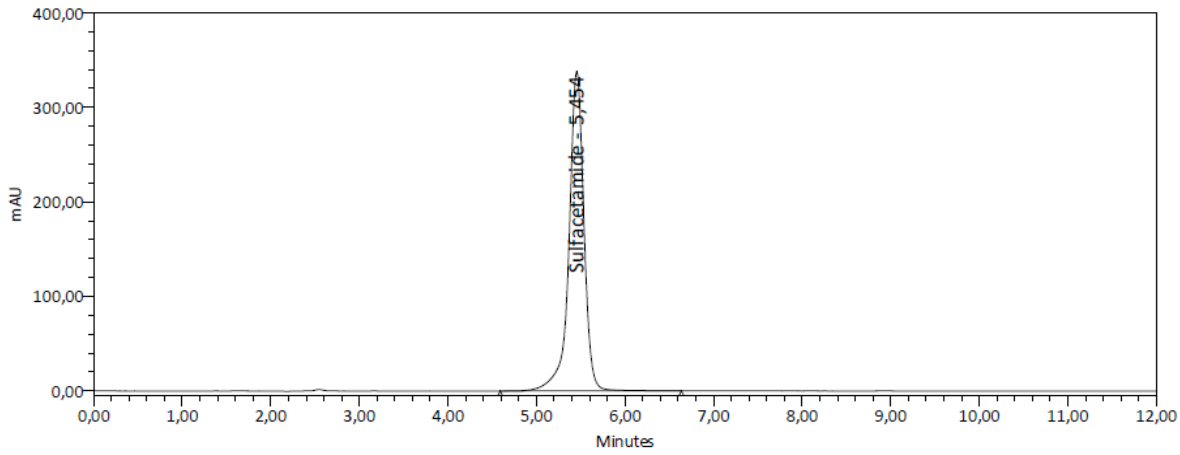


Figure 5.34-Chromatogram of a MP/20-0382 test sample in an assay test run of sulfacetamide sodium

Table 5.49-T0 results and mass balance from the assay and related substances analyses of sulfacetamide sodium for both lots

T0		
	MP/19-0397	**MP/20-0382**
Assay (%)	97.60 ± 2.30	95.95 ± 2.30
H_2O (%)	3.04	4.47
Anhydrous base (%)	100.66	100.44
Total impurities (%)	0.21 ± 0.01	0.10 ± 0.01
Mass balance (%)	100.87	100.54

The content of the anhydrous substance, as stated in the operating procedure, should fall between 99.0 and 101.0 %. The fact that the assay was above 100% could simply be related to small errors in sample preparation and in the HPLC analysis itself. The results certainly support the previous analysis, suggesting that sulfacetamide sodium is not suffering any significant degradation after heat sterilization.

5.2.4. Stability

With the goal of testing if the active ingredient remained stable over time after the sterilization process, all the tests in the operating procedure were repeated after one month, and again after three months, from the heat treatment. Free radicals could be formed during the heat process, causing unwanted interactions when used in a medicine.

All the qualitative tests, including solubility, reactions of sodium, sulfates and checking the appearance of solution S and of the powder itself, produced the same results. They were all in accordance with the expected limits and requirements, therefore, in those cases, the heat did not result in any instability of the substance. The results from the quantitative tests performed on sulfacetamide sodium are the following.

pH

The pH of both lots of sulfacetamide sodium was measured after a month (T1) and after three months from the sterilization cycle. The results are present in Table 5.50.

Table 5.50-T1 and T3 pH results obtained for both lots of sulfacetamide sodium

		pH	Temperature
T1	**MP/19-0340**	9.00	23.0ºC
	MP/20-0276	8.86	22.2ºC
T3	**MP/19-0340**	9.18	25.0ºC
	MP/20-0267	9.05	25.0ºC

Water

The results from the water determination of both lots of sulfacetamide sodium (T1 and T3) are presented in Table 5.51.

Table 5.51-T1 and T3 water content results obtained for both lots of sulfacetamide sodium

		%H_2O
T1	**MP/19-0340**	7.28
	MP/20-0276	7.19
T3	**MP/19-0340**	7.32
	MP/20-0267	7.25

Infrared absorption spectrophotometry

The T1 and T3 correlation obtained with the IR absorption spectrophotometry of both lots of sulfacetamide sodium are presented in Table 5.52.

Table 5.52-T1 and T3 IR absorption spectrophotometry results obtained for both lots of sulfacetamide sodium

Correlation		
T1	**MP/19-0397**	0.9645
	MP/20-0382	0.9665
T3	**MP/19-0397**	0.9623
	MP/20-0382	0.9573

Assay and related substances

After one month and three months, the assay and related substances determination by HPLC was repeated for both lots of the substance, and the results are presented in Table 5.53.

Table 5.53-T1 and T3 results, and mass balance obtained from the assay and related substances analyses for both lots of sulfacetamide sodium

	T1		**T3**	
	MP/19-0397	**MP/20-0382**	**MP/19-0397**	**MP/20-0382**
Assay (%)	92.31 ± 0.20	92.12 ± 0.81	93.11 ± 0.02	92.74 ± 0.13
H_2O (%)	7.26	7.19	7.32	7.25
Anhydrous base (%)	99.54	99.26	100.46	99.98
Impurities (%)	0.20 ± 0.01	0.08 ± 0.00	0.20 ± 0.00	0.08 ± 0.00
Mass balance (%)	99.74	99.34	100.66	100,06

The pH and water content increased over time but stayed within the limits required and are considered normal values for this substance. A slight further hydration was expected until the ingredient reached the content of the control sample, considering it was not stored protected from the moisture in the environment.

The infrared absorption correlation remained fairly stable over time, with no specific pattern of alteration, which indicates that the variations in the results could be caused solely by measurement uncertainty induced by the points mentioned before, which are noise, baseline shifts or atmospheric disturbances.

The results from the HPLC analysis confirm the stability of sulfacetamide sodium after three months from the sterilization process, where no other impurities were formed, there was no increase in the quantity of impurities that already existed, and the results stayed within the accepted limits, showing no particular pattern. The variations in the results could be induced by measurement uncertainty due to sample preparation and different HPLC equipment.

5.3. Gentamicin sulfate

5.3.1. Bioburden

The bioburden of gentamicin sulfate had been previously determined by the Laboratory and the results were also useful for the investigation of the sterilization cycle efficacy for this substance.

Table 5.54-TAMC and TYMC obtained for both lots of gentamicin sulfate

MP/19-0340	TAMC < 50 CFU/mL	TYMC = 50 CFU/mL
MP/20-0276	TAMC < 50 CFU/mL	TYMC < 50 CFU/mL

By observing the results (Table 5.54), it is possible to see that both Total Aerobic Microbial Count (TAMC) and Total Yeast and Mold Count (TYMC) are below the microbial population of the biological indicator *(Bacillus atrophaeus* with a microbial population of 2.1×10^6), which supports the efficacy of the sterilization process for gentamicin sulfate with a good safety margin. Additionally, even though the bioburden of this substance is much higher than the one of sulfacetamide sodium, the values are within the acceptance criteria, which are 200 CFU/mL for TAMC and 2000 CFU/mL for TYMC.

5.3.2. Sterilization cycles

All combinations of time and temperature previously determined for dry heat sterilization were tested for gentamicin sulfate.

At first, the sterilization tests were completed with a small amount, approximately 1 g, of substance, to investigate any visible effects that the heat exposure could cause. Just like for sulfacetamide sodium, the glass flasks and sterilization pouches that allow exchange of moisture and air were used.

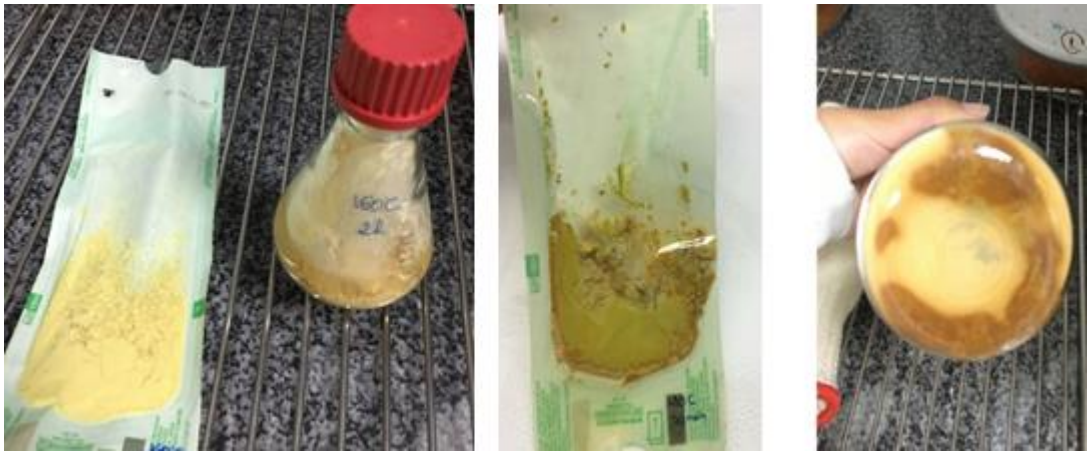


Figure 5.35-Gentamicin sulfate samples after different cycles of dry heat sterilization in glass flasks and sterilization pouches

Considering gentamicin sulfate is a white powder, it can be observed from Figure 5.35. how much the heat affected the appearance of the substance. The active ingredient became completely yellow after all sterilization cycles. Comparing the samples sterilized at 160ºC and at 180ºC, the samples were drastically darker at the higher temperature. It was noted that they got progressively darker from 121ºC to 180ºC. This suggests that the heat could be leading to the formation of impurities that grant coloration to the substance, even at a small quantity.

Figure 5.36-Comparison between samples of gentamicin sulfate sterilized by dry heat on a glass flask (right) and in a sterilization pouch (left)

Figure 5.36. is a comparison between two samples, after a sterilization cycle of 180ºC for 30 minutes. The first was sterilized in a sterilization pouch and the second in a glass flask. It is clear how much the flask sample is darker than the pouch one. This could happen for the same reason as sulfacetamide sodium, where the trapped moisture could be further boosting degradation, although the authors Thamthaweechok, et al., (2018) and Traub & Birgit (1995) discovered that gentamicin sulfate solutions remained stable after moist heat sterilization at 121ºC for 15 minutes, and the authors Taplin (1965) and Casemore (1967) concluded that those

properties were not lost after the process. This could mean that the moist heat cycle implemented is not sufficient to cause degradation, or that the degradation that occurs does not result in a significant loss in potency. Another option is that the impurities produce could also have similar bactericidal properties.

On the other hand, this result is consistent with the author Silveira (2017), who stated that this active ingredient presents a yellowish coloration when exposed to an environment of moist and heat, adsorbing humidity, and with the investigations by Naveed, et al., (2014) where a solution of gentamicin sulfate presented a 78% degradation after a water bath at 50ºC for 30 minutes.

The studies by Graham et al., (1997) which indicate that gentamicin is affected by heat in dextrose solutions, with a considerable loss in potency, also show the same degradant peaks were detected in the samples that stayed at room temperature, as well as in the ones that were heat-treated, with a greater extent of degradation on the latter, and solutions maintained at room temperature remained colorless, therefore the formation of color was not considered related to the presence of degradants. The solution of gentamicin that was prepared in water and heat-treated showed no degradant peak.

As mentioned before, the amino and hydroxyl group in this substance are susceptible to chemical oxidation, and Hanessian, et al. (1975) state that the glycoside linkages of several aminoglycosides are known to be cleaved under oxidizing conditions.

To investigate that, the same cycles were tested again, this time in totally sealed nylon sterilization pouches, in a nitrogen atmosphere. It was observed that the substance still became yellow, although not as dark as before, suggesting that the colored impurities are still being formed in inert atmosphere, even if at smaller quantities. One assumption is that the own hydroxyl groups of the gentamicin molecule could be responsible for oxidizing the amino groups of the substance.

The sample sterilized at the standard cycle of 160ºC for 120 minutes in inert atmosphere was chosen to proceed with the complete raw material analysis, as a way of investigating in what other ways the heat treatment could be affecting this active ingredient.

5.3.3. Raw material analysis

Appearance

After sterilization, the appearance of the powder was compared visually to a control sample. It is possible to observe, in Figure 5.37., the difference in color between the sterilized and the control sample. It can also be noticed the difference in color intensity between the substances sterilized at 121ºC and the ones sterilized at 160ºC. The monograph of this active ingredient requires the substance to appear as a white or almost white powder, which, undoubtedly, it does not. This color represents an impediment to make use of the substance in pharmaceuticals as it would possibly also have a major impact on the color of the final product.

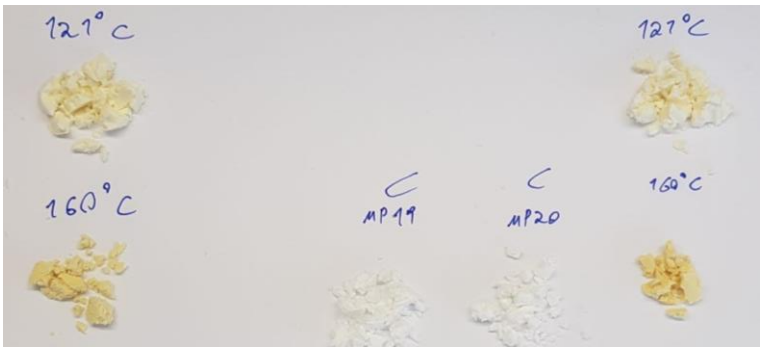


Figure 5.37-Comparison between sterilized and non-sterilized samples of gentamicin sulfate after a cycle of 160ºC for 120 minutes and 121ºC for 12 hours

Solubility

The sterilized substances were tested for their solubility in water and in anhydrous ethanol. It was observed that they remained freely soluble in water and practically insoluble in anhydrous ethanol, which are the specifications in the operating procedure. This indicates that the heat did not provoke any changes that could increase or decrease the active ingredient's solubility.

Thin-layer chromatography

The thin-layer chromatography is one of the identification tests of gentamicin sulfate, consisting of a separation technique based on adsorption, partition, ion-exchange or on com-

binations of these mechanisms. The spots in the chromatogram obtained with the test solutions are visually compared in terms of color, size, and retardation factor (R_F), to the corresponding spots obtained with the reference solution.

$$R_F = \frac{\text{distance from the point of application to the center of the spot}}{\text{distance travelled by the solvent from the point of application}}$$

The spots on the left correspond to the reference solution while the other ones correspond to both lots of gentamicin sulfate (Figure 5.38). As can be observed, the three spots in the test solutions are similar in position, color, and size to the three spots obtained with the reference solution, which are consistent with the operating procedure results stipulation. This implies that the same analytes are present in the sterilized samples as well as in the non-sterilized ones, therefore the heat process did not damage the substance as much as to fully degrade any of these components.

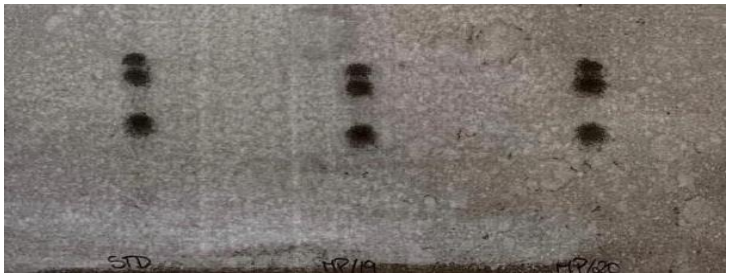


Figure 5.38-Chromatogram obtained in thin-layer chromatography for a reference sample, MP/19-0340 and MP/20-0276 (from left to right) of gentamicin sulfate.

Reaction (a) of sulfates

The reaction of sulfates serves of the second identification analysis of gentamicin sulfate. It is utterly a means to identify the presence of sulfates in the substance.

After the addition of dilute hydrochloric acid R and barium chloride solution R1, a white precipitate was formed, as it is seen in Figure 5.39. This result indicates the presence of sulfates in the sample, therefore, after sterilization, this substance remains identifiable through this method, which entails that this functional group was not significantly affected by heat.

Figure 5.39-Results from the reaction (a) of sulfates on sterilized samples of gentamicin sulfate

Appearance of solution S

The solutions S of the sterilized samples were visually compared to a reference solution Y6. Evidently, the color of the substance would greatly affect the color of solution S as well. The reference solution was much clear than the others, which confirms that this parameter is out of specification and the substance is unfit to employ in pharmaceuticals.

Figure 5.40-Comparison between the color intensity of solution S of gentamicin sulfate sterilized samples to a reference solution Y6

pH

The pH was determined from solution S and the value should be between 3.5 and 5.5.

By observing Table 5.55, the pH of both lots stayed within the required specifications, towards the lower end. These values might vary with temperature, but they are an indication that sterilization did not produce any more acidic substances.

Table 5.55-pH results obtained for both lots of gentamicin sulfate

	pH	Temperature
MP/19-0340	3.50	22.6ºC
MP/20-0276	3.54	22.6ºC

Water

After the sterilization cycle, a loss in the water content was expected. This analysis was done a week after the process and the water content was rather low, as can be observed in Table 5.56. The operating procedure only define a maximum of 15.0% for this parameter, so the results respect this requirement. However, this active ingredient is hygroscopic, so it is likely that it will absorb more moisture, but probably not exceeding the limit.

The difference between the water content of both lots might be due to storage arrangements, as one pouch may not have been as well closed as the other.

Table 5.56-Water content results for obtained both lots of gentamicin sulfate

	%H_2O
MP/19-0340	4.35
MP/20-0276	6.09

Sulfate

This test was important to determine the percentage of sulfate present in the sample. Through a titration, it was possible to measure this value, which should be between 32.0% and 35.0%, for the anhydrous substance.

Table 5.57-Weight of gentamicin sulfate in each sample and volume of 0.1 M sodium edetate spent on titration on the Sulfate test for both lots of gentamicin sulfate

		Weight (mg)	Volume (mL)
MP/19-0340	**A1**	264.6	1.15
	A2	252.0	1.30
MP/20-0276	**A1**	264.0	1.50
	A2	253.0	1.55

MP/19-0340

$$A1: \frac{(10 - 1.15)x9.606}{264.6} x100 = 32.13\%$$

$$A2: \frac{(10 - 1.30)x9.606}{252.0} x100 = 33.16\%$$

$$Water\ content = 4.34\%$$

Table 5.58-Results obtained for the Sulfate test of gentamicin sulfate MP/19-0340

% Sulfate (anhydrous base)	
A1	33.6
A2	34.7
	Average = 34.1 %

MP/20-0276

$$A1: \frac{(10 - 1.50)x9.606}{264.} x100 = 30.93\%$$

$$A2: \frac{(10 - 1.55)x9.606}{253.0} x100 = 32.08\%$$

$$Water\ content = 6.09\%$$

Table 5.59-Results obtained for the Sulfate test of gentamicin sulfate MP/20-0276

% Sulfate (anhydrous base)	
A1	32.9
A2	34.2
	Average = 33.5 %

The results from both lots are within the limits stated on the monograph, which supports the results obtained on the reaction (a) of sulfates. This means that the heat treatment did not increase availability or cause degradation on the sulfate group of gentamicin sulfate.

Sulfated ash

Through this method, it is possible to determine the amount of inorganic impurities present in a substance. It has a meaningful importance on a degradation investigation once

the determined limit could be exceeded if those types of impurities were somehow formed during the sterilization process.

Table 5.60-Values obtained along the Sulfated ash test for both lots of gentamicin sulfate

	W (g)	T (g)	m (g)
MP/19-0340	35.0080	35.0076	0.5026
MP/20-0276	27.4482	27.4475	0.5015

MP/19-0340

$$\frac{35.0080 - 35.0076}{0.5026} x100 = 0.08\% \approx 0.1\%$$

MP/20-0276

$$\frac{27.4482 - 27.4475}{0.5015} = 0.14\% \approx 0.1\%$$

As stated on the operating procedure, this parameter could not exceed 1.0%. The results suggest a low concentration of inorganic impurities, with a good safety margin, which gives no indication of their formation throughout the heat sterilization.

Composition

Considering gentamicin sulfate as a mixture of many gentamicin compounds, the HPLC analysis to determine the presence and assay of each component has a great importance for a degradation study. A validated method to determine the related substances was not available, therefore, any loss of the active ingredient components would be an indication of degradation. In this case, the limits specified correspond to each component or group of components.

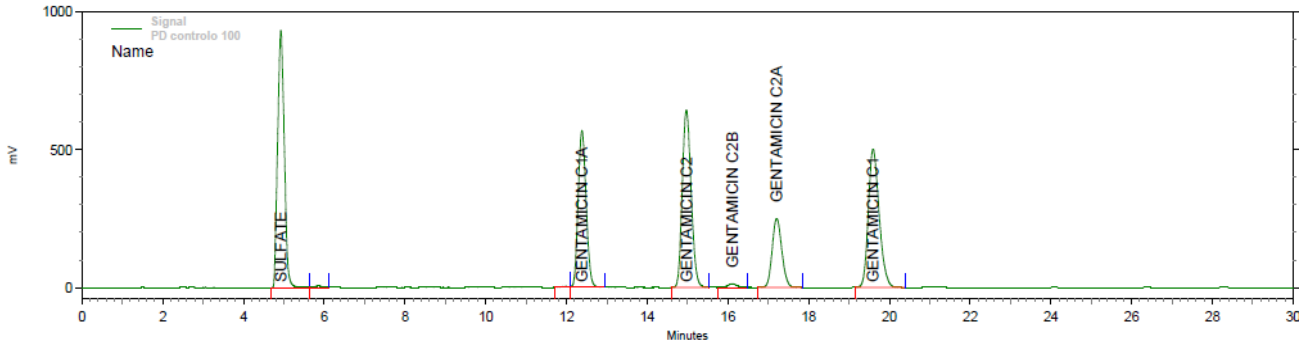


Figure 5.41-Chromatogram of a standard sample in a composition test run of gentamicin sulfate

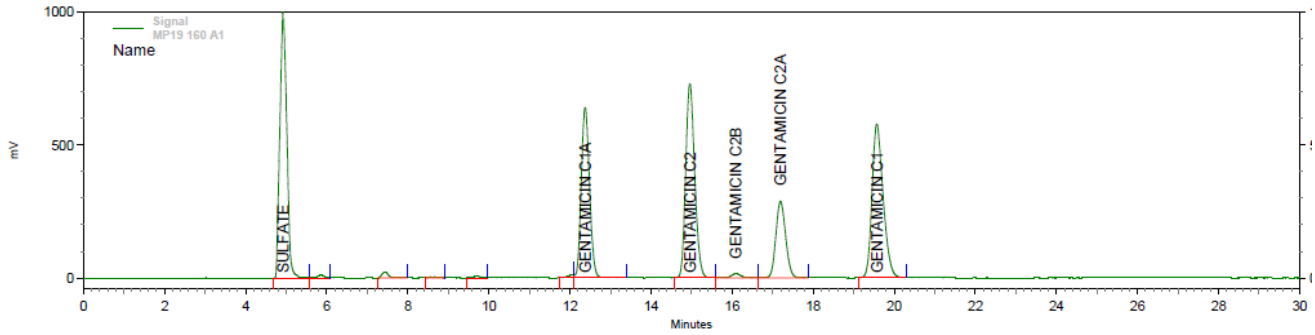


Figure 5.42-Chromatogram of a MP/19-0340 test sample in a composition test run of gentamicin sulfate

Table 5.61-Composition results for gentamicin sulfate MP/19-0340

	C1	C1a	C2+C2a+C2b
Limits	**25.0 - 45.0%**	**10.0 - 30.0%**	**35.0 - 55.0%**
Result (%)	27.61 ± 0.02	23.17 ± 0.05	40.97 ± 0.10
			Total = 91,8 %

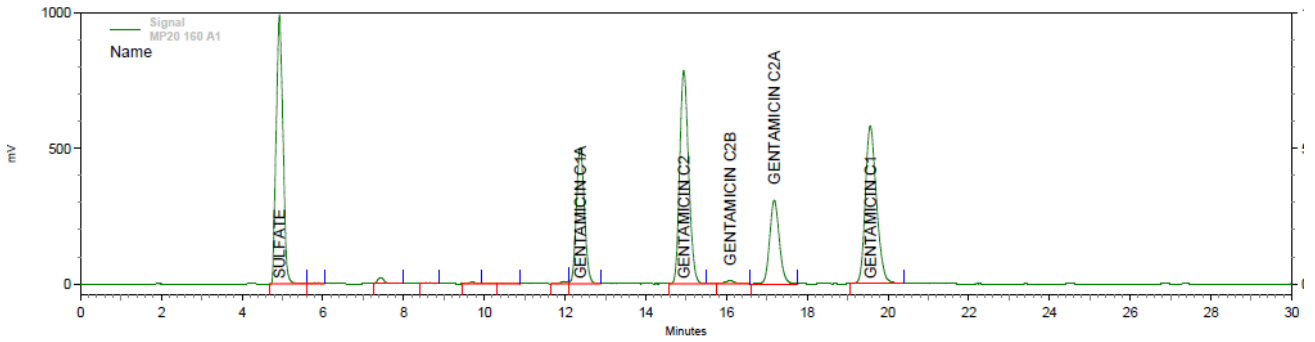


Figure 5.43-Chromatogram of a MP/20-0276 test sample in a composition test run of gentamicin sulfate

Table 5.62-Composition results for gentamicin sulfate MP/20-0276

	C1	C1a	C2+C2a+C2b
Limits	**25.0 - 45.0%**	**10.0 - 30.0%**	**35.0 - 55.0%**
Result (%)	27.26 ± 0.37	18.69 ± 0.45	42.21 ± 0.85
			Total = 88,2 %

By comparing the chromatogram obtained for the standard sample, the chromatograms of sterilized samples display other small peaks between the sulfate and gentamicin C1a peaks that were not quantified since they were under the limit of quantification, which indicate the

formation of small amounts of unknown impurities. This is consistent with the authors Ramos, et al. (2012), who detected low amounts of free radicals in samples of gentamicin sulfate sterilized by dry heat at 180ºC (30 minutes), 170ºC (60 minutes) and 160ºC (120 minutes). Nonetheless, for both lots, the assay of gentamicin components is within the established criteria, however the total loss of the active ingredient was approximately 10%, which is significant, even if the results may be accepted.

This means that, if the color of the substance was not out of specification, these results would be satisfactory to accept the method of dry heat sterilization for gentamicin sulfate.

5.4. **Sterility**

The sterility testing was accomplished to support the efficacy of the sterilization process and to reject any recontamination after the process, verifying the correct sealing of the sterilization pouches. The pouch containing the substance remained sealed until this test was started.

The sterility was determined for gentamicin sulfate right after the chosen sterilization cycle, and for sulfacetamide sodium right after sterilization (T0) and three months after (T3). For sulfacetamide sodium, it was possible to prepare two MP/19-0397 samples for T0 as a double verification of the results, however there was not enough left from the MP/20-0382 lot to do the same.

After 14 days of incubation, there was no cell growth in suspension in the culture media, as can be seen in Figure 5.44, which demonstrates the reliability of the heat process and of the sealing in the sterilization pouches in preventing recontamination.

Figure 5.44-Result of the sterility assay of sulfacetamide sodium and comparison to a control sample

6.
Conclusions and Perspectives

The main objective of this work was to investigate the suitability and choose the right conditions of the dry heat sterilization process for sulfacetamide sodium and gentamicin sulfate, two antibacterial substances that perform as the active ingredients of ophthalmic ointments and eye drops produced by the company.

The analytical methods to determine the assay and related substances of sulfacetamide sodium, which are considered the most relevant analysis in this investigation, required a re-validation, due to the difficulty in dissolving the substance with the solvent in the presence of the glacial acetic acid.

With the removal of this component, the methods showed accuracy within the working range, confirmed by the recovery percentage obtained. Based on results from the analysis precision, repeatability, and intermediate precision, it can be confirmed that analysis performed on different days, by different analysis and equipment do not have a significant influence on the results and that the methods are precise for the test sample concentrations. Furthermore, the assay analytical method is robust, being able to produce consistent results, even under slight alterations in the experimental conditions.

The assay method showed selectivity regarding the main analyte, with a suitable linear response, supported by a $R^2 = 0.9997$, and appropriate working range, 50% to 150% of the sample solution concentration (200 µg/mL). The limit of detection and quantification obtained were 4.8 µg/mL and 14.4 µg/mL, respectively. The method also showed random distribution of residuals, homogeneity of variances, and an average quotient between each response factor and the average response factor of 100.0 was obtained with Rikilt's test. Additionally, the suitability of the linear function for experimental representation was demonstrated through Mandel's test. Reference solutions and assay test samples were stable when stored in a refrigerator over the period of one week.

The related substances method also showed selectivity regarding the main analyte, with a suitable linear response, supported by a $R^2 = 0.9998$ within working range, 0.05% to 0.2% of the sample solution concentration (20 000 µg/mL). The limit of detection and quantification

obtained were 0.6 µg/mL and 1.7 µg/mL, respectively. The method also showed random distribution of residuals, homogeneity of variances, and an average quotient between each response factor and the average response factor of 100.0 was obtained with Rikilt's test. The suitability of the linear function for experimental representation was demonstrated through Mandel's test.

The related substances method, using a sulfanilamide reference substance also showed selectivity regarding the main analyte, with a suitable linear response, supported by a R^2 = 1.0000 within working range, 0.05% to 0.2% of the sample solution concentration (20 000 µg/mL). The limit of detection and quantification obtained were 0.2 µg/mL and 0.6 µg/mL, respectively. The method also showed random distribution of residuals, homogeneity of variances, and an average quotient between each response factor and the average response factor of 100.0 was obtained with Rikilt's test. The suitability of the linear function for experimental representation was demonstrated through Mandel's test.

Related substances test samples must be prepared before use, as they present instability after one week of preparation.

The relative response factor of Impurity A obtained was 1.9, compared with a RRF of 2.0 established in the operating procedure.

The results from the stress tests with base, acid, oxide, temperature, and UV light showed a decrease of content in sulfacetamide sodium samples, corresponding to an increased content of related compounds, mainly when the samples were subject to oxidative conditions, resulting in the formation of sulfanilamide and a few unknown impurities. Therefore, it is crucial to monitor samples to prevent any type of degradation.

For sulfacetamide sodium, reddish impurities were produced when the substance was sterilized in a glass flask from 160ºC (120 minutes) to 180ºC (30 minutes). When sterilized in paper/plastic sterilization pouches, the physical characteristics of the substance stayed intact.

Many tests were performed on the substance after a cycle of 160ºC for 120 minutes using sterilization pouches to verify its chemical integrity. All tests complied with the established requirements, and the water content increase progressively after sterilization, until it regained the water of the non-sterilized samples. It can be concluded that the trapped moisture in the glass flask, along with high temperatures, may encourage degradation. It was demonstrated that sulfacetamide sodium may be sterilized by the dry heat process, in containers that allow moisture outlet, as the substance maintained all its physical and chemical properties, with no signs of degradation, for, at least, three months after sterilization, supported by a mass balance

(assay + related substances) of 100.66% and 100.06% for MP/19-0397 MP/19-0340, respectively.

For gentamicin sulfate, the substance became completely yellow after the sterilization process in a glass flask as well as in the paper/plastic sterilization pouches, however with a darker color tone on the first, for all cycles tested. The same cycles were tested again using completely sealed nylon pouches in nitrogen atmosphere, and gentamicin sulfate still became completely yellow, but in a lighter color tone than in the first tests. This suggests a formation of yellow impurities that cause the substance to turn progressively darker with the increase of degradation, and that the hydroxyl group of the molecule may be oxidizing its own amino groups.

A complete analysis of this active ingredient was performed after a cycle of 160°C for 120 minutes using the nylon pouches to verify the heat effects on a chemical level. All tests showed compliance with the acceptance criteria, except for the appearance requirements. The composition analysis showed new peaks in the chromatograms, that may be related to degradants, and a loss in content of around 10.0%. The unknown peaks were not quantified. These results imply that dry heat sterilization is not suitable for gentamicin sulfate, as it leads to significant impacts in this active ingredient's characteristics.

Both active ingredients remained sterile until the pouches were opened, which demonstrates the efficacy of the sterilization process and of the sterilization pouches, as well as proper storage conditions.

These results are relevant for the company, as it indicated the dry heat process as a promising way to secure sterility sulfacetamide sodium, without impacts on the final pharmaceutical product. Additionally, the company is able to understand the impacts of dry heat sterilization on gentamicin sulfate and decide if it should be ruled out of the possibilities to sterilize this substance.

It is suggested that the following tests are performed as a complement of this work, and as to support these conclusions.

- Repetition of the complete analysis of sulfacetamide sodium after 6 months to one year after the sterilization process, to test its stability over a longer period.
- Identification of the colored impurity that was produced when sulfacetamide sodium was sterilized in a glass flask, through a Mass spectroscopy (LC-MS) analysis.
- Testing dry heat sterilization for sulfacetamide sodium on a larger scale, with the amount necessary for manufacturing, with bigger sterilization pouches.

- Identification and quantification of the impurities that appeared in the chromatograms obtained in the composition analysis of gentamicin sulfate, by using more concentrated samples on an HPLC analysis, with ELSD.
- Performing a nuclear magnetic resonance (NMR) spectroscopy of gentamicin sulfate to, possibly, identify the impurity that causes coloration of and researching how it is formed, in order to explore ways to prevent it.

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STUDY OF THE EFFECT OF DRY HEAT STERILIZATION ON ACTIVE PHARMACEUTICAL INGREDIENTS

NOVA SCHOOL OF SCIENCE & TECHNOLOGY