UNIVERSIDADE TÉCNICA DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

DEVELOPING AN ANALYTICAL CHEMISTRY KNOWLEDGE BASED ON PROCESS IMPURITIES DETECTION IN PHARMACEUTICAL FORMULATIONS

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Dissertação para obtenção do grau de Doutor em Química

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Março de 2011

Abstract

This thesis describes the application of modern methods of physico-chemical analysis fundamentally mass spectrometry, especially LC-MS-MS, on issues related to the quality of pharmaceutical products.

Were studied in detail the APIs and formulations prepared industrially. Methods have been developed that allow, with great sensitivity, identify and characterize the major impurities of the products studied.

In work undertaken for the aminoglycoside antibiotics under study, was possible to develop a method capable of quantifying an impurity derived from hydrolysis of API in an injectable formulation.

The anxiolytic, of benzodiazepines category, was a tablet formulation. After the method development of LC-MS-MS we proceeded to the synthesis of the main impurity and this was been properly characterized. After characterization of the impurity, studies have been conducted on several samples with the same and with different formulations to understand the evolution of the quantity of impurity over time. Some of the ingredients present in certain formulations inhibits increasing of impurity amount. In order to find a more convenient formulation was carried out a study of experimental design (DOE) to study the interaction between the API, excipients and factors such as external conditions.

The results demonstrate the importance of introducing modern physicochemical techniques such as LC-MS-MS for identification, monitoring and control of pharmaceuticals by increasing the quality of pharmaceuticals and improving performance in the industrial sector.

Keywords:

LC-MS-MS, pharmaceutical impurities, active pharmaceutical ingredients, formulations, method validation, impurity synthesis, pharmaceutical industry

Resumo

Esta dissertação descreve a aplicação de métodos modernos de análise fisico-quimica fundamentalmente espectrometria de massa, em particular LC-MS-MS, em problemas relacionados com a qualidade de produtos farmacêuticos.

Foram estudados detalhadamente API's e formulações preparadas industrialmente. Desenvolveram-se métodos que permitem, com uma grande sensibilidade, identificar e caracterizar as principais impurezas dos produtos estudados.

No trabalho desenvolvido para o antibiótico aminoglicósido em estudo foi possível desenvolver um método capaz de quantificar uma impureza resultante da hidrólise do API numa formulação injectável.

Para o ansiolítico, da categoria das benzodiazepinas, sob a forma de comprimidos foi desenvolvido método de LC-MS-MS, procedeu-se à síntese da principal impureza sendo obtido um padrão que foi devidamente caracterizado. Caracterizada a impureza, foram efectuados estudos em várias amostras com a mesma formulação e formulações diferentes para compreender a evolução da quantidade de impureza ao longo do tempo. Alguns excipientes presentes em certas formulações mostraram-se inibidores do aumento da quantidade de impureza. Para encontrar uma formulação mais conveniente efectuou-se um estudo de planeamento experimental (DOE) estudando a interacção entre o API e factores tais como excipientes e condições externas.

Os resultados obtidos demonstram a importância da introdução de técnicas físico-quimicas modernas, tais como LC-MS-MS, para a identificação, monitorização e controlo dos produtos farmacêuticos aumentando a qualidade dos produtos farmacêuticos e melhorando a performance no âmbito industrial.

Palavras-chave:

LC-MS-MS, impurezas farmacêuticas, princípios activos, formulações, validação de método, síntese de impurezas, indústria farmacêutica

Acknowledgements

Now that I am keeping this individual academic step but with various personal and institutional contributions, I have to express to them my thanks.

To Professor Doutor José Monteiro Cardoso de Menezes, my academic supervisor, I want to express special thanks for all the friendship, encouragement and guidance of this work released since the beginning of this whole project. Its unmatched enthusiasm and his lessons allowed me to broadcast overcome barriers that often judged not transferable. For his support once again my thanks.

To my academic co-supervisor, Professora Doutora Marta Pineiro, I wish to thank the full and always very close support since the beginning of this work. Her determination, clarity, scientific, constructive spirit coupled with the enormous friendship that has given me has been crucial to the development and completion of this work.

To Dr Pedro Alves Barata, my industrial supervisor since the beginning of the project, I appreciate the reception given me. Thank you for gave me interesting knowledge of the activity of the pharmaceutical industry.

To my industrial supervisor, Dr José Manuel Martins, I thank all the availability granted either in personal terms, and scientific resources needed to develop the project.

To Doutora Teresa Alves, I wish to thank the gracious proposal to hold a doctorate in Atral Laboratories. I want to make express my gratitude for the friendship you have given me over the years we have shared in various scientific problems.

To Senhor Comendador Sebastião Alves, I express my gratitude for the possibility of realization of this thesis in a company like Atral marked by his enterprising spirit, visible in its long history of activity.

I wish to thank Professor Elisa Serra to the friendly careful guidance of texts, particularly when they were in English.

To all colleagues that carried PhD in Atral-Cipan group, thank you for sharing health knowledge that helped me in various phases of this project. To my colleagues and friends Catarina Santos and Sílvia Gramacho I want to thank the support they always gave me during the working day.

To my family, especially my children Duarte and Diogo, my husband and my parents I want to thank all the patience, understanding and love that they always shown, even when my mood was not the best. Without your contribution none of this would be possible.

To my father, who over the years has always given me both scientific and personal incentive, and always have been decisive in the conclusion of my projects here I wish to express special thanks.

I want to thank Chymiotechnon the opportunity of performing all the work on equipment that is installed in the Chemistry Department, University of Coimbra and the chance to explore it giving me all the materials and conditions suitable for the project.

I appreciate the financial support provided by Laboratórios Atral SA and the Portuguese Foundation for Science and Technology (BDE/15593/2006) during the last four years.

Agradecimentos

Agora que cumpro esta etapa individual em termos académicos mas com vários contributos pessoais e institucionais, não posso deixar de os referenciar expressando em seguida os meus agradecimentos.

Ao Professor Doutor José Monteiro Cardoso de Menezes, meu orientador, quero deixar expresso um agradecimento especial por toda a amizade, incentivo e orientação deste trabalho dispensado desde o início de todo este projecto. A sua inigualável força de vontade e os ensinamentos que transmitiu permitiram-me ultrapassar barreiras que muitas vezes julgava intransponíveis. Por toda a sua disponibilidade mais uma vez o meu obrigado.

À Professora Doutora Marta Pineiro, minha co-orientadora, desejo agradecer o apoio incondicional e sempre muito próximo desde o início deste trabalho. A sua determinação, clareza científica, espírito empreendedor aliada à enorme amizade com que me tem presenteado foram decisivas para o desenvolvimento e conclusão deste trabalho.

Ao Dr Pedro Alves Barata, orientador da empresa desde o início do projecto, agradeço o acolhimento que me concedeu. Foram para mim uma mais-valia os ensinamentos que me transmitiu sobre a actividade da indústria farmacêutica.

Ao Dr José Manuel Martins, orientador da empresa, agradeço toda a disponibilidade concedida quer em termos pessoais, científicos e de recursos necessários ao desenvolvimento do projecto.

À Doutora Teresa Alves, desejo agradecer a amável proposta de realização de um doutoramento nos Laboratórios Atral. Gostaria de deixar expressa a minha gratidão pela amizade que me concedeu ao longo dos anos em que partilhámos vários problemas científicos.

Ao Senhor Comendador Sebastião Alves, expresso a minha gratidão pela possibilidade de realização deste doutoramento numa empresa como o Atral marcada pelo seu espírito empreendedor, bem visível na sua longa história de actividade.

À Professora Doutora Elisa Serra agradeço a orientação criteriosa e amiga da redacção dos textos particularmente quando em língua inglesa.

A todos os colegas que realizaram doutoramento enquadrados no Grupo Atral-Cipan, agradeço a partilha saudável de conhecimentos que me ajudaram em diversas fases deste projecto. Às

minhas colegas e amigas Catarina Santos e Sílvia Gramacho quero agradecer o apoio que sempre me concederam ao longo dos dias de trabalho.

À minha família, em especial aos meus filhos Duarte e Diogo, ao meu marido e aos meus pais quero agradecer toda a paciência, compreensão e carinho que sempre demonstraram, mesmo quando a minha disposição não era a melhor. Sem o vosso contributo nada disto seria possível.

Ao meu pai, que ao longo dos anos sempre me concedeu incentivos tanto a nível científico como pessoal, e que foram sempre decisivos na conclusão dos meus projectos desejo expressar aqui um agradecimento especial.

Quero agradecer à Chymiotechnon a oportunidade da realização de todo o trabalho no equipamento que tem instalado no Departamento de Química da Universidade de Coimbra e a possibilidade para o explorar concedendo-me todos os materiais e condições adequadas para a realização do projecto.

Agradeço o apoio financeiro concedido pelos Laboratórios Atral SA e pela Fundação para a Ciência e Tecnologia (BDE/15593/2006) durante os últimos 4 anos.

Table of Contents

CHAPTER 3

CHAPTER 4

CHAPTER 5

List of Figures

List of Tables

Abbreviations and Symbols

ACN - Acetonitrile

APCI - Atmospheric Pressure Chemical Ionization

API – Active pharmaceutical ingredient

B – Magnetic Sector

C18 – Octadecylsilane chromatographic phase

C8 – Octylsilane chromatographic phase

CB – Commercial Brand

CE – Capillary electrophoresis

CI – Chemical Ionization

Da – Atomic Mass Unit

DAD – Diode Array Detector

DC – Direct Current Potential

EDTA – Ethylenediamine tetraacetic acid

EG - 1-N-ethylgaramine

EI – Electron Impact Ionization

ESI – Electrospray Ionization

GC – Gas Chromatography

GC/FID/MS- Gas Chromatography with Flame ionization detector coupled with mass spectrometry detection

GC-MS – Gas Chromatography coupled with Mass Spectrometry

GRAS – Generally recognized as safe

FTIR – Fourier Transform Infrared Spectroscopy

FWHM - Full Width at Half Maximum

ICH – Internation Conference on Harmonization

ICR – Ion Cyclotron Ressonance

IR – Infrared Spectroscopy

IS – Internal standard

HPLC- High Performance Liquid Chromatography

HPLC-UV- High Performance Liquid Chromatography with ultraviolet detection

HR/MS – High Resolution Mass Spectrometry

LC – Liquid chromatography

LC-MS – Liquid Chromatography coupled with Mass Spectrometry

LC-MS-MS - Liquid-Chromatography-Tandem Mass Spectrometry

LIT – Linear Quadrupole Ion Trap

LOD – Limit of Detection

LOQ – Limit of Quantification

MALDI - Matrix-Assisted Laser Desorption Ionization

MDD – Maximum Daily Dose

MeOH - Methanol

MS – Mass Spectrometry

MS^n/MSn- Tandem Mass Spectrometry

m/z – Mass to Charge Ratio

NMR – Nuclear Magnetic Ressonance

Q – Linear Quadrupole

QIT – Quadrupole Ion Trap

RF – Radio Frequency Potential

RRT – Relative Retention Time

RSD or %RSD – Relative Standard Deviation

RX – X-Ray Diffraction

SIM – Selected Ion Monitoring

SFC – Supercritical Fluid Chromatography

S/N – signal to noise ratio

SPE – Solid-Phase Extraction

SRM – Selected Reaction Monitoring

TDI – Total Daily Intake

TFA – Trifluoracetic acid

TOF – Time of Flight

TLC – Thin Layer Chromatography

UV-Ultraviolet

UV-Vis - Ultraviolet-Visible

CHAPTER 1

THESIS OVERVIEW

1.1. Introduction

The work presented in this thesis was developed during my PhD, beginning in November 2006. This work was concluded in October 2010 followed by the presentation of this dissertation presented in order to obtain Degree of Doctor in Philosophy.

This project was financed with a grant provided in equal parts by the Portuguese Foundation for Science and Technology (BDE 15593/2006) and by Laboratorios ATRAL, S.A. This collaboration aims to promote advanced training in the business environment, in projects of interest to the Company, and its development will enable the student to obtain a doctoral degree awarded by the University.

Since the objective was the development of modern analytical methods to support the pharmaceutical industry, the project was totally developed in the laboratories of Chymiotechnon. With this partnership, it was possible to use techniques of mass spectrometry allowing a broad knowledge of some products existing in the company.

The aim of the PhD was the development of case studies that would result in advanced capabilities in R&D through physical-chemical methods, leading to the modern resolution of problematic issues typical of a company engaged in the production of drugs for placement in the most demanding and competitive markets and subject to global competition.

Liquid-chromatography coupled with mass spectrometry was the most frequently used technique and is described in great detail throughout this dissertation.

1.2. Motivation

Since obtaining my degree in Chemistry, the subjects of study have always focused on aspects directed mainly to chemical quality control. Upon completion of the Master's Degree, I began working in association with a research group belonging to the Coimbra Chemistry Centre, Department of Chemistry, University of Coimbra, collaborating in projects and providing community support. This was carried out through institutions linked to the Department aimed at

providing support to businesses and the community at large: Chymiotechnon - Institute of Science Technology and Innovation in Chemistry and Aemiteq - Association for Innovation and Quality. In order to more correctly accomplish these tasks, I attended courses to develop skills in using mass spectrometry instruments and use MS / MS. I had however, many contacts with the RIAIDT, in the University of Santiago de Compostela, for carrying out work using techniques of nuclear magnetic resonance, mainly uni-dimensional, for the characterization of active principles of pharmaceutical products (API's).

The studies requested by the pharmaceutical industry requires quality control of API's and finished products, patents and determination of impurities in order to identify production processes and obtain experimental results for the organization of technical dossiers with chemical information of API's.

The experience in scientific work and the existing links to quality centres with expertise in different areas and work out help solve business problems and also my university teaching activity originated the motivation for embarking on the implementation of advanced studies leading to a Doctorate degree, while at the same time doing work that is useful for the development of the candidate.

1.3. Objectives

The objectives of this work described in this dissertation must be in agreement with the interest of the project to the company. Atral is a company which is primarily dedicated to the production of medicines (finished products) using raw materials from many sources and active ingredients from different suppliers, including companies in the same group. Quality control and the guarantee of origin and production of the materials used in production of drugs are crucial to allow maximum quality of final products and to support the legitimate use of property rights, an issue that often arises in the drug market.

The main themes were to develop a study of the active ingredient and finished product, as well as the determination of purity and the nature of contaminations. We aimed to determine their content not only by the traditional chromatographic method of HPLC with UV detection, but also with modern methods of detection and mass spectrometry.

The objectives of the initial project can be summarized as follows:

I. Quantification and identification of impurities / degradation products by new methodologies in a product

II. Assembly and validation of analytical methods as stability indicators (tablets and injection), with the identification of potential degradation products, involving stages of work like forced degradation of the active substance under various conditions of stress, identification of major degradation products (using LC/MS and/or other techniques) and assembly of a selective HPLC method "indicator of stability".

The work developed was oriented towards a study which is focused on two themes, in particular, the development of the analytical method for the determination of an impurity in an injectable product and the study of a drug with respect to its impurities and stability.

1.4. Thesis Structure

The chapters of the submitted dissertation were organized as a result of studies conducted over the last four years.
The main purpose was the inclusion of new methodologies for the detection and control of trace impurities in pharmaceuticals, so there are of course issues that are included in multiple chapters. For this reason a graphical representation of the structure of the thesis is presented.

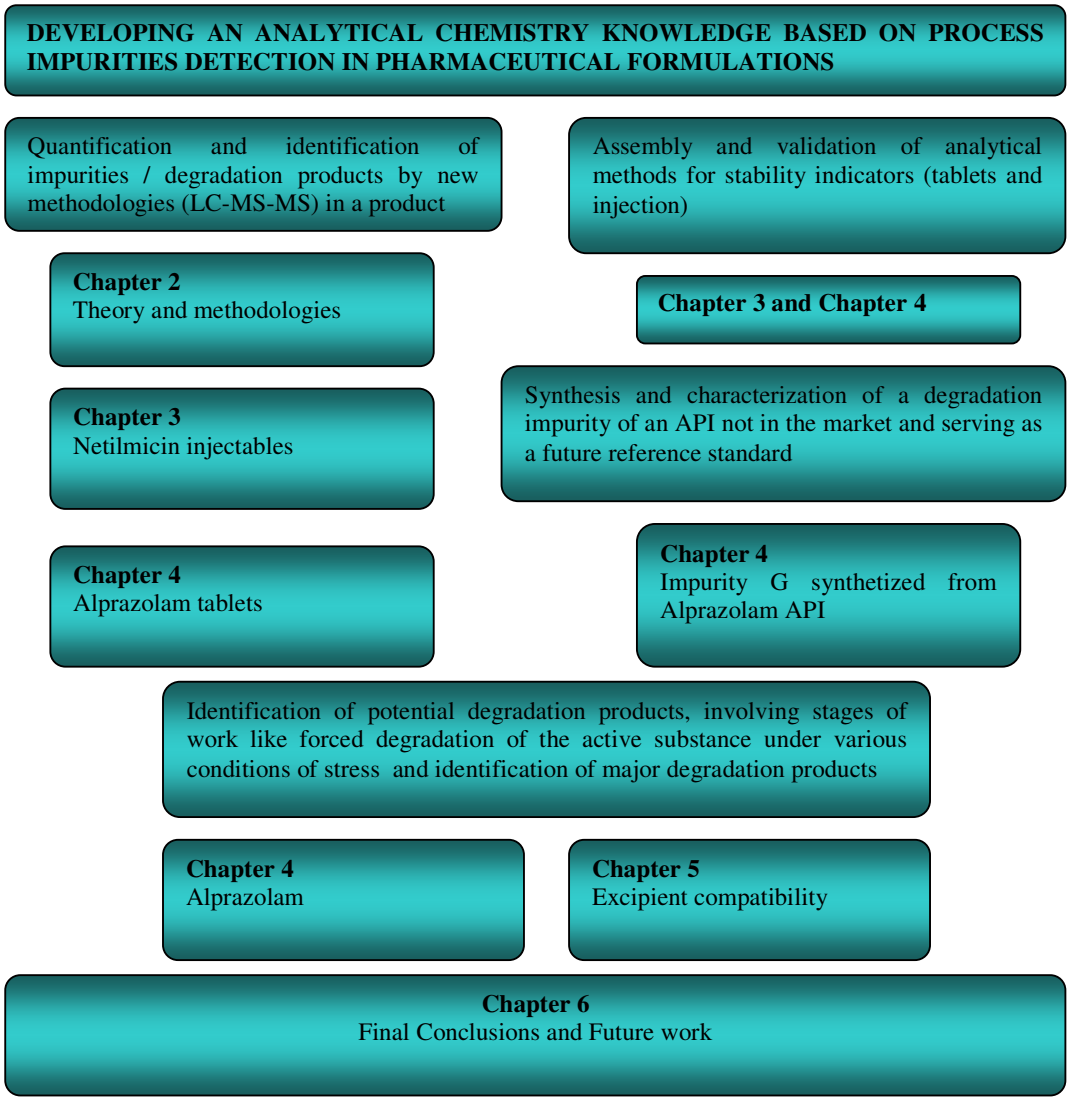


Figure 1.1 Graphical representation of the thesis.

CHAPTER 2

THEORY OVERVIEW

In this chapter a literature review is presented of the most relevant common techniques and themes used throughout this dissertation. The study of pharmaceutical impurities requires an adequate knowledge of the nature of all the materials involved, the origin and mechanisms of emergence of such impurities, the nature and capacities of the analytical methods to use, and also the knowledge of existing international regulation applicable.

The analytical approach has to take into account the need of detection and quantification usually of trace amounts and the complexity of the matrices were they are present requiring the establishment of adequate analytical strategies. Mass spectrometry is a particularly efficient and useful technique in this context therefore justifying a brief description of the technique and theory behind it, its historical development, instrumentation, advantages, disadvantages and development methods.

Other methods like NMR, FTIR, elemental analysis and UV-VIS spectroscopy are also important and were used in this work, mainly to confirm mass spectrometry based data. For this reason the description of these techniques is off the scope of this dissertation.

2.1. Pharmaceutical Impurities

An API (active pharmaceutical ingredient) is any substance or mixture of substances used in the manufacture of a drug product, which has pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease or to affect the structure and function of the body.

In the pharmaceutical industry, there is a strong need for qualitative and quantitative analysis of target analytes including drugs, impurities and degradation products in various mixtures including API's and finished products.

Impurities in pharmaceuticals are usually the unwanted chemicals that remain with the active pharmaceutical ingredients (API's) after its synthesis or extraction, or develop during formulation, or upon aging of both API and formulated API's to medicines [1]. Medicines are the formulated forms of API's. Impurities present in medicines before formulation with API are usually classified into the following categories: organic impurities (process and drug related), inorganic impurities, residual solvents, and may come from various sources [1, 2].

Organic impurities could be traces of starting materials or intermediates, by-products, degradation products and reagents, ligands or catalysts. In general, an individual API may contain all of these organic impurities.

Inorganic impurities originate from the manufacturing process and are usually known and include heavy metals, inorganic salts and other materials.

Residual solvents are normally organic volatile compounds used during the process, having known effects and toxicity and must be removed and controlled following appropriate guidelines [3, 4].

The impurities related with formulation or related with aging processes of both API and formulated API's can also results from various sources. The stability of the medicine depends not only on environmental factors but also factors related to the formulation. Environmental factors that may increase the level of impurities are exposure to adverse temperatures, light, moisture and gases such as oxygen and carbon dioxide. The main factors related to the formulation are the water content, particle size / surface area, pH,

solvents, the compatibility of cations and anions, the ionic strength, excipients, the patterns of distribution of the API and excipients and packaging [5].

The chemical degradation of the API has as immediate consequence a reduction of the levels of the active substance in the medicine and consequently the modification of the therapeutic dosage and in more serious cases lead to the formation of toxic impurities. It is of great importance a thorough knowledge of the type of chemical reactions more frequent in the degradation of the API's. More frequently and obviously related with the exposition to environmental factors are hydrolysis, oxidations and photodegradations. Since drug degradation is particularly relevant in this work, a more detailed brief description of these reactions follows. Other less common degradation reactions occur like hydration, racemization of chiral centers, decarboxylation, isomerisation, rearrangements, dimerization and polimerization, cyclization and deamidation [5]. It is important to note the possibility that some functional groups suffer more than one reaction of degradation. Such is the case of amines that will be discussed ahead in this dissertation (Maillard reactions).

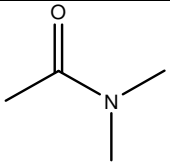
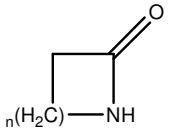
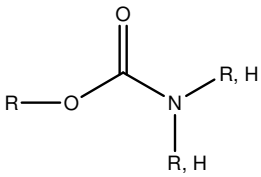
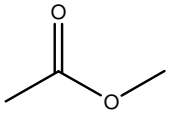
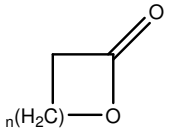
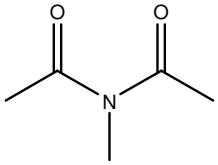
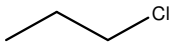
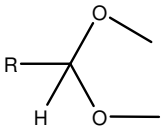
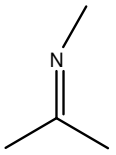
Hydrolysis

Hydrolysis is by far the most commonly occurring drug degradation process, either in solution but also in solid state,

$$RX + H_2O \rightarrow ROH + HX$$

Although the hydrolysis reactions can occur only in the presence of pure water they are usually catalysed by acids or bases [1, 5]. The chemistry of hydrolysis is largely determined by the presence of an electrophylic atom, often a carbonyl group carbon attached to a good leaving group. Some functional groups are particular sensitive to hydrolysis and some examples are summarized in Table 2.1. [6].

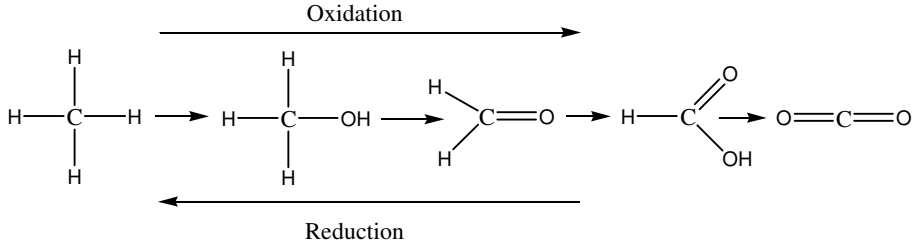
Table 2.1 Potentially hydrolysable functional groups in API and some example drugs ordered from most to least stable

Functional Group	General Structure	Reaction/Products	Examples
Amide		Carboxylic acid and amine	Chloramphenicol[7], indometacin[8], nicotinamide
Lactam		Ring opening with ends carboxylic acid and amine	Penicillins[9], cefalosporins[9], benzodiazepines[10]
Carbamic Ester		Nucleophilic attack of water on one or more of the tautomeric forms of the protonated molecule	Meprobamate[11], Neostigmine[12]
Ester		Carboxylic acid and alcohol	Atropine[13], aspirin, benzocaine[14]
Lactone		Carboxylic acid and alcohol	Warfarin[15]
Imide		Carboxylic acid and amide	Barbiturates
Alkyl Chloride		Substitution of halide for hydroxyl group	Chloramphenicol[16], clindamicin[17]
Acetal		Aldehyde or ketone	Erythromicine[18]
Imine		Carbonyl (ketone or aldehyde) and amine	Benzodiazepines[19, 20], rifampicin[21]

To stabilize the products with regard to hydrolysis is recommended keep the material under low humidity and low temperature conditions, control the pH and ionic strength, select the polarity of solvents.

Oxidation

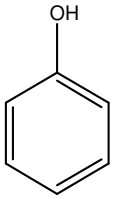
Oxidation processes correspond to an important degradation pathway, either in liquid and solid formulations [5, 22]. The presence of atmospheric oxygen can lead some API's to be converted in higher oxidation species generally illustrated by the following scheme for carbon compounds:



Some functional groups are particular sensitive to oxidation and some examples are summarized in Table 2.2.

The recommended procedures to prevent oxidation of the drugs are taken at the formulation and packaging. With regard to the formulation, must be taken into account the effects of dilution, control of impurities that contribute to oxidation such as metals and peroxides, control of pH and the possibility of adding antioxidants. In packaging is advisable to remove oxygen using inert atmosphere, and protection from light [23].

Table 2.2 Potentially oxidizable functional groups in API and some example drugs

Functional Group	General Structure	Reaction/Products	Examples
Amine	RNH_2 (primary)	Primary and secondary oxidize to hydroxylamines causing further imines or oximes	Fluoxetin[24], amphetamine[25]
	RNHR (secondary)		
	RNRR' (tertiary)	Tertiary amines origin N-oxides	
Alcohol	R-OH	Primary aldehydes or ketones and further carboxylic acids	Dexametasone[26]
Thiol	R-SH	Disulfide, sulfenic acid, sulfinic acid and sulfonic acid	Captopril[27]
Thioether	R_1-S-R_2	Sulfoxides and finally to sulfones	Fenotiazines[28]
Phenol		Quinones	Rifampicin[29]
		Dimerization	Morphine [30]

Photodegradation

Photodegradation, as the name implies, is a reaction of degradation by the action of light, i.e., is a form of electromagnetic radiation.

Exposure to ultraviolet (λ ~ 200-400nm) or visible light (λ ~ 400-800nm) can induce a series of chemical reactions as a result of the break of covalent bonds [5], because the energy of these photons is of comparable magnitude to the bonding energy of organic molecules [31]. No photochemical reaction can occur unless light is absorbed as established by Grotthus and Draper in 1918. The absorption spectrum of a compound is therefore an immediate way of determining the wavelength range to which the drug may be sensitive. Some drug substances and formulation excipients are colored, meaning that they

absorb light in the visible region. The color they display is complementary to the light they absorb, as an example a red powder is the result of absorption of blue light by the material. The great majority of therapeutic substances are white in appearance, meaning that they do not absorb light in the visible region. However, they may absorb in the UV region. The presence of aromatic residues and conjugated double bonds containing N, S, or O in the structure provides for the ability of the molecule to absorb light [31].

The photophysical process accompanying the absorption of UV-visible light can be described by an energy level diagram [5, 31] (Figure 2.1):

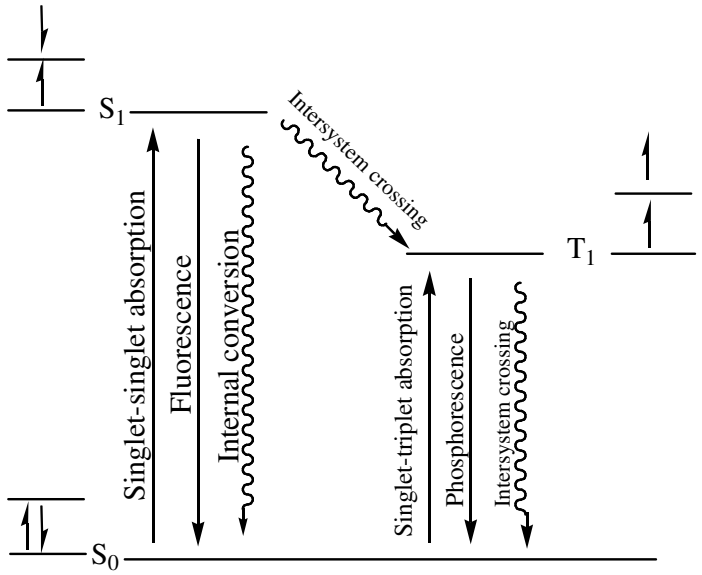


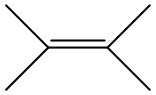
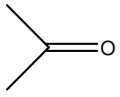
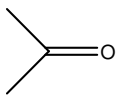
Figure 2.1 Energy-level diagrams for light absorption.

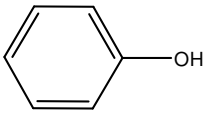
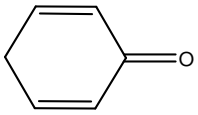
The photochemical processes are due to the absorption of light by a molecule experiencing a transformation to an electronic excited configuration. The electrons in the outermost shells of a molecule are the most susceptible ones. Spin state is a characteristic feature for electron excitation being usually the molecules in the ground state with paired electrons, singlet state (S_0) [5, 31]. When excitation occurs the spin can be maintained (S_1) or flipped (T_1). Inducing these electronic transitions, the absorbed radiation may promote the phenomena of fluorescence, phosphorescence, and radiant heat (photophysical processes), or the breaking of chemical bonds, leading to rearrangements or decomposition

(photochemical processes). The excited molecule can also behave as a free radical capable of initiating a reaction chain.

Some functional groups are particular sensitive to photodegradation as illustrated in examples of drugs and corresponding products shown in Table 2.3.

Table 2.3 Potentially photolabile functional groups in API and some example drugs

Functional Group	**General Structure**	**Reaction/Products**	**Examples**
Carbon-carbon double bonds		Isomerization	Tioxanthines, doxepin [32]
		Addition	Protriptyline [31]
Aromatic rings halogenated and heterocycles	Ar - X	Dehalogenation	Fluoroquinolones [33]
Sulfa drugs	$Ar - SO_2R$	Homolytic cleavage with SO_2 production	N-substituted sulfanylamide derivatives [32]
Nitro group	$R\text{-}NO_2$	Rearrangement to nitrito group with further fragmentation liberating NO Reduction to nitroso	Metronidazole[32]
Carbonyl group		Intermolecular hydrogen abstraction and fragmentation via α-cleavage (Norrish Type I)	Cortisone [31]
Carbonyl group		Intramolecular γ-hydrogen abstraction followed by C_α-C_β cleavage (Norrish Type II)	Dithranol [31, 32]

Phenols			Adrenaline [32]

The recommended procedures to prevent photodegradation of drugs involve either external or internal protection. External protection can be done avoiding light to reach the preparation, using an opaque container or, in the case of tablets and capsules, by means of an opaque coating. Internal protection can be done by incorporating in the preparation some additive that either absorbs the active light competitively with the API or quenches the photoreaction of the latter [32].

2.1.1. Regulatory aspects

From what has been previously described for impurities, knowledge of the chemical structure of an impurity is essential to assess its toxicological implications and to gain an understanding of its formation mechanism. Knowledge of the formation mechanism is critical to improve the synthetic chemical process and optimizing the drug formulation to reduce or eliminate impurity. Even after various purification processes and improvements to the synthesis process, it is impossible, in most cases, to completely eliminate impurities from various sources since no chemical reaction has 100% selectivity and as refered by Qiu and Norwood no chemical compound is "rock" stable [34]. Therefore, it is essential to carry out close monitoring and follow appropriate regulations.

Due to the importance and significance of the presence of impurities in pharmaceutical products, the current regulatory requirements on impurity identification are well defined in ICH Q3A (R2) – "Impurities in new drug substances" [2] and ICH Q3B (R2) – "Impurities in new drug products" [35]. The thresholds requirements for reporting, identification and qualification of organic impurities are based on maximum daily dose (MDD) and total daily intake (TDI). The first refers to the amount of API administered per day, while the TDI refers to the amount of impurities ingested. The thresholds, according to established requirements [2, 35] are presented in Tables 2.4, 2.5 and 2.6.

Table 2.4 Thresholds for impurities in new drug substances

MDD	Reporting Threshold	Identification Threshold	Qualification Threshold
≤ 2g/day	0.05%	0.10% or 1.0mg TDI (whichever is lower)	0.15% or 1.0mg TDI (whichever is lower)
> 2g/day	0.03%	0.05%	0.05%

The thresholds for substances and products are not the same in order to give more consideration to low dose drug products.

Table 2.5 Reporting thresholds for impurities in new drug products

MDD	Reporting Threshold
≤ 1g/day	0.1%
> 1g/day	0.05%

Table 2.6 Identification and qualification thresholds for impurities in new drug products

MDD	Identification Threshold	MDD	Qualification Threshold
< 1mg	1.0% or 5µg TDI (whichever is lower)	< 10mg	1.0% or 50µg TDI (whichever is lower)
1mg-10mg	0.5% or 20µg TDI (whichever is lower)	10mg-100mg	0.5% or 200µg TDI (whichever is lower)
>10mg-2g	0.2% or 2mg TDI (whichever is lower)	>100mg-2g	0.2% or 3mg TDI (whichever is lower)
>2g	0.10%	>2g	0.15%

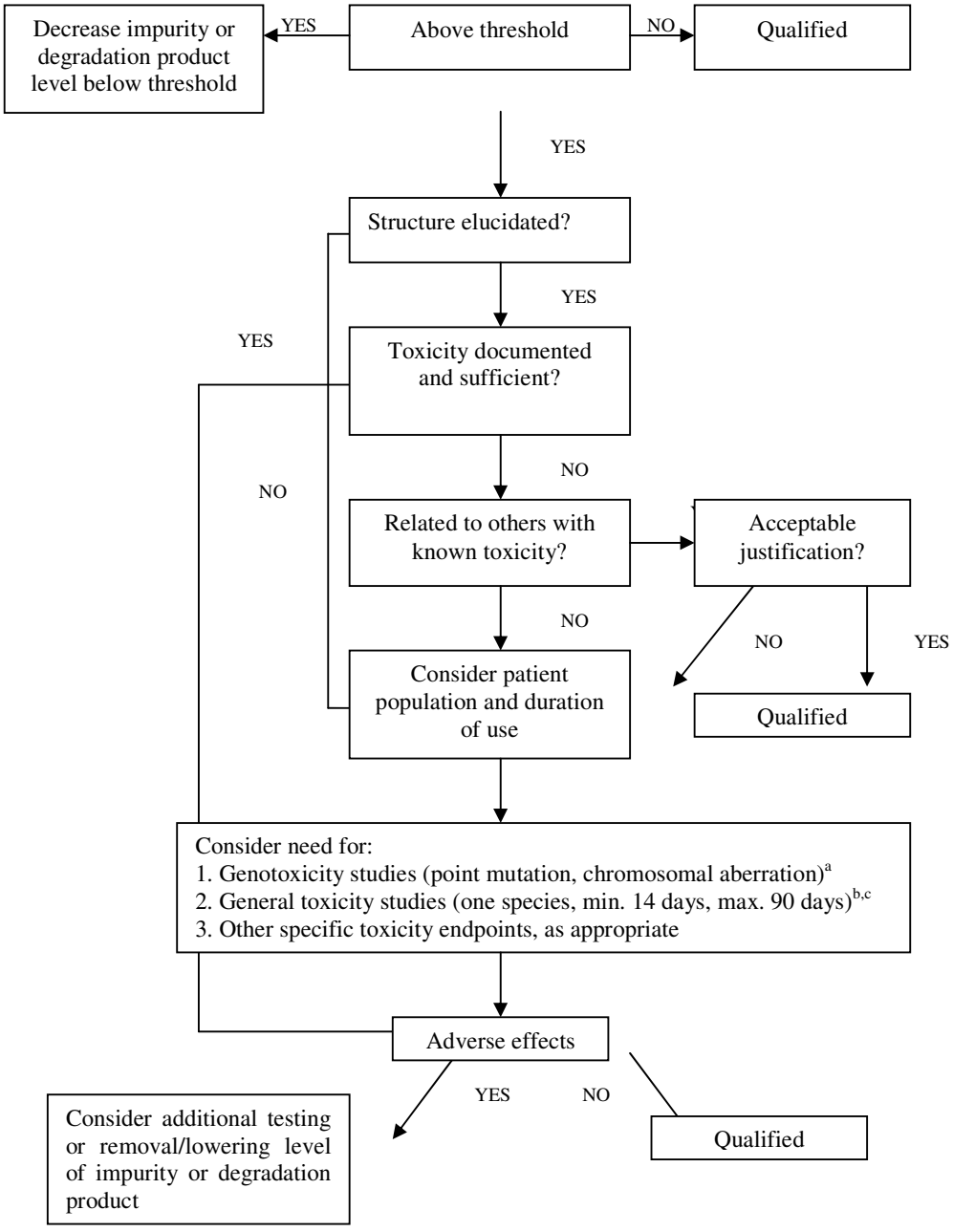
According to the regulations these thresholds may be higher if scientifically justified (an excipient, after all, is an impurity and can be present in extremely high amount) and smaller if the impurity is unusually toxic. Higher or lower threshold limits for qualification of impurities may be appropriate for some individual drugs based on scientific rationale and level of concern, including drug class effects and clinical experience.

Figure 2.2 presents the decision tree for safety studies for the qualification of impurities in a new drug substance or a new drug product when thresholds are exceeded. In some cases, decreasing the impurity below the threshold in the drug substance may be preferable to providing safety data. Decreasing the level

of impurity must be evaluated on a case-by-case basis, since such effort can add significantly to manufacturing costs. Reducing impurity levels in drug products has also to be considered, since these are defined as degradation products, but in this case, formulation, container/closure, or storage condition changes may be required. Adequate safety data may be available in the scientific literature to qualify an impurity. If data are not available, additional safety testing has to be considered, taking into account a number of factors, including patient population, daily dose, route and duration of drug administration, and

drug class [36]. The control of other impurities, as inorganic impurities and residual solvents, is well established in pharmacopoeia monographs and ICH Q3C (R3) [4], respectively.

Leachables is a class of impurities not covered by ICH guidance [34]. These impurities are not detailed in this dissertation, giving greater emphasis to organic impurities and their detection.



a. If considered desirable, a minimum screen for genotoxic potential should be conducted. A study to detect point mutations and one to detect chromosomal aberrations, both in vitro, are seen as acceptable minimum screen.

b. If general toxicity studies are desirable, study(ies) should be designed to allow comparison of unqualified to qualified material. The study duration should be based on available relevant information and performed in the species most likely to maximize the potential to detect the toxicity of an impurity. In general, a minimum duration of 14 days and a maximum duration of 90 days will be acceptable.

c. On a case by case basis, single dose studies may be acceptable, especially for single dose drugs, and when such studies are conducted using an isolated impurity. If repeat-dose studies are desirable, a maximum duration of 90 days would be acceptable.

Figure 2.2 Decision tree for safety studies [36].

2.1.2. Strategies for identification of pharmaceutical impurities

Isolation and characterization of impurities are required to establish biological safety and this is the basis for the need and scope of impurity profiling of drugs in pharmaceutical research. To isolate and quantify the impurities, various instrumental analytical techniques can and have to be used.

The impurity profile is variable due to changes in synthetic route, reaction conditions, source and quality of the starting materials, reagents and solvents used, the purification steps, crystallization conditions, drying, distillation and storage of materials. The same applies to drug formulation, too. As a consequence of these points the impurity profiles of bulk drugs and formulations made thereof have to be checked repeatedly not only during the research and development period but also in all cases if any changes take place in the above listed factors. For this reason the control of impurity profiles is a frequent task, especially in industrial research and quality control laboratories. Taking into account the time and labor consuming nature of these studies it is essential to find a strategy which enables the results to be achieved within the shortest possible time with the greatest possible certainty.

Depending on the nature and complexity of a particular impurity the detection can involve only a simple retention time in a matching exercise using an authentic standard or require a multidisciplinary effort using modern analytical techniques. The impurity profile can be divided into several steps: detection of impurities with simple identification of impurities by chromatographic retention matching with known potential impurities (standard available), application of chromatographic, spectroscopic and hyphenated techniques for structure elucidation and, in some cases, even requires synthetic preparation of the impurity. Published strategy schemes facilitating the work of identification and structural elucidation of impurities are known. Figure 2.3 is a schematic flowchart of the merged information from several references [34, 36, 37].

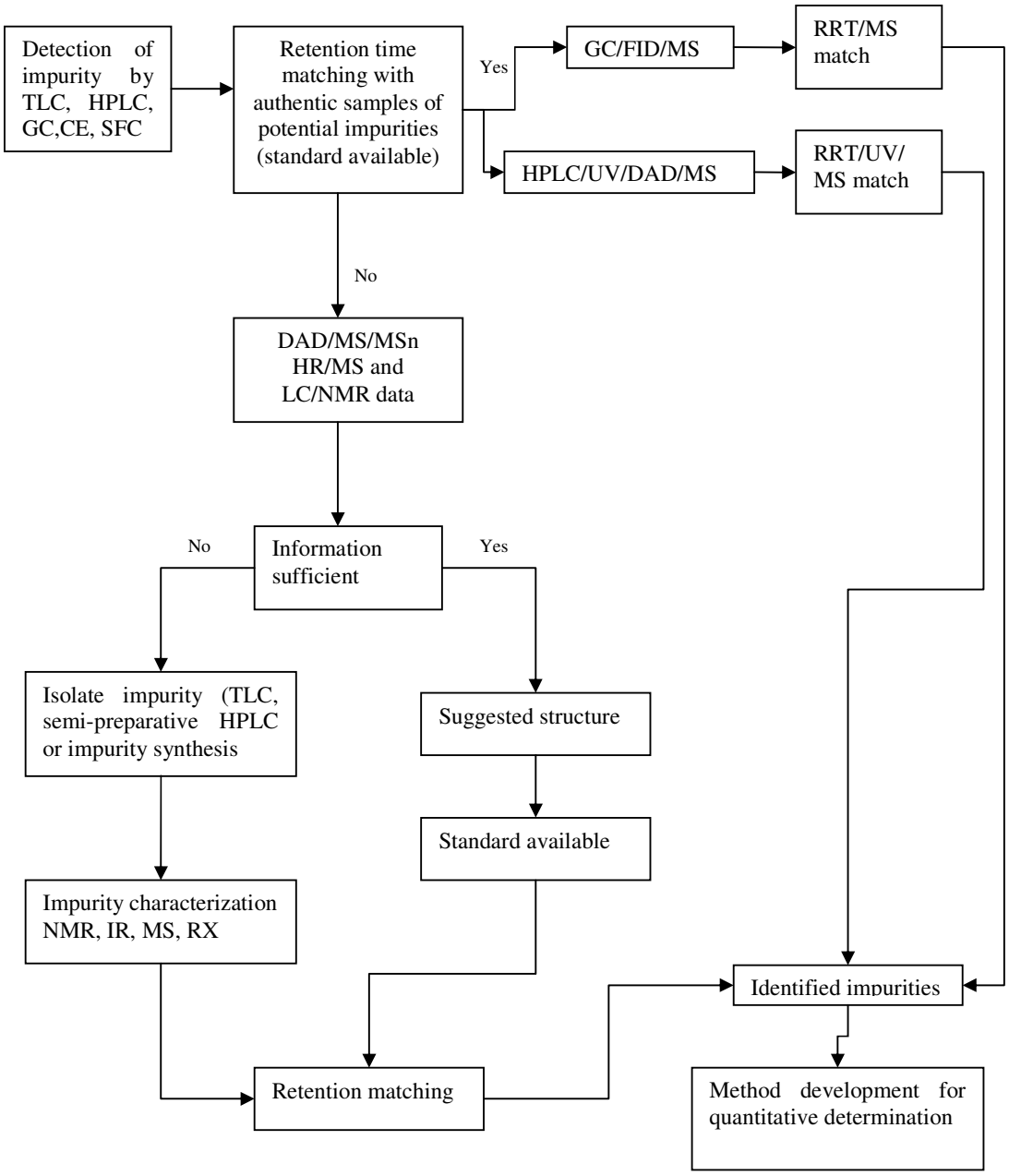


Figure 2.3 Flowchart for identification of unknown impurities in drugs. (CE – Capillary Electrophorese, DAD- Diode array, FID- Flame ionization detector, GC- Gas Chromatography, HPLC- High performance liquid chromatography, HR/MS- High resolution mass spectrometry, IR- Infrared, MS- Mass spectrometry, MSn- Tandem MS, NMR- nuclear magnetic resonance, RRT- Relative retention time, RX- X-ray diffraction, SFC- Supercritical fluid chromatography, TLC- Thin layer chromatography, UV- Ultraviolet detector)

2.2. Mass Spectrometry-based technology in pharmaceutical industry

2.2.1. Mass Spectrometry Historical Overview

The basic principle of mass spectrometry (MS) is to generate ions from either inorganic or organic compounds by any suitable method, to separate these ions by their mass-to-charge ratio (*m/z*) and to detect them qualitatively and quantitatively by their respective *m/z* and abundance [38]. In the next section the theoretical basis of the technique is discussed.

The first experiment which led to the technique that we now call mass spectrometry was carried out by J.J. Thomson, Nobel Prize in Physics in 1906 for the theoretical and experimental investigation on the conduction of electricity by gases while Thomson was investigating the properties of positive rays. In this experiment the ionization of neon atoms was produced and the isotopes ^{22}Ne and ^{20}Ne were separated [39, 40]. These were the first steps in developing a technique that was to become the extremely important analytical and structure elucidation tool that we presently know.

Electronic ionization and magnetic focussing was developed by Dempster (1918) and Aston (1919) measured the first atomic weights using MS [41]. In the first three decades of the 20th century Aston and other scientists redesigned the instruments to improve resolving power and began using them to separate and prove the existence of elemental isotopes. Until the 1940s, physicists dominated the MS field, using the technique mainly to resolve questions about the fundamental nature of atom. Only by the 1940s, mass spectrometers became commercially available, and MS was firmly established as a useful technique among physicists and industrial chemists [42].

Between 1946 and 1956, some major analyzers were developed such as time-of-flight, ion cyclotron resonance, magnetic double-focusing, quadrupole and high resolution MS. In the 60s there was a huge development of some ionization techniques such as chemical ionization, electrospray ionization, field desorption and plasma desorption [41].

By the 1980s, small organic molecules were routinely being analysed by MS. The great challenge was to analyse heavier molecules, such as proteins, nucleic acids and complex carbohydrates. To be able to do so as John Fenn, 2002 Nobel Prize in Chemistry for his

development of ESI, said it was necessary to "make molecular elephants fly". In 1983, Tanaka, Karas and Hillenkamp developed the technique of Matrix-Assisted Laser Desorption Ionization (MALDI). With the two techniques, ESI and MALDI it has become possible analyse large biomolecules [41, 42].

2.2.2. Mass Spectrometry Theory

Mass spectrometry allows determining the mass of a molecule by measuring the mass-to-charge ratio (*m/z*) of its ions. This means that to do the mass spectrometry experiment it is necessary to ionize molecules, separate those ions and finally detect them. All mass spectrometers have in common the simple basic setup: ion source, mass analyser and detector (Fig. 2.4).

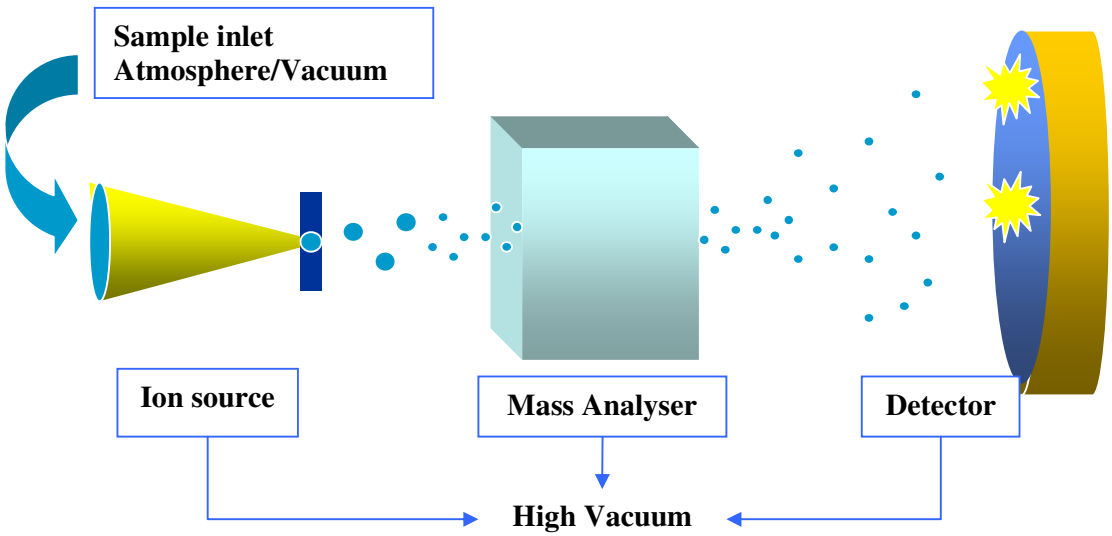


Figure 2.4 Basic setup of a mass spectrometer.

Some instruments combine the sample inlet and the ionization source, while others combine the mass analyzer and the detector. However, the sample molecules undergo the same process regardless of instrument configuration. The molecules of the sample have to be introduced into the instrument through some sort of sample inlet. Inside the instrument, these molecules are converted to ions in the ionization source, before being electrostatically propelled into the mass analyzer. Then, ions are separated according to their mass-to-charge ratio (*m/z*) within the mass analyzer. The detector converts the ion energy into electrical signals, which are then transmitted to a computer. The requirement of high vacuum is related to the need to provide a collision-free path to allow ions to reach the detector without undesirable collisions [39].

The mass spectrum obtained is a two-dimensional representation of signal intensity versus *m/z*. The intensity of a peak, as signals are usually called, directly reflects the abundance of ionic species of one *m/z* ratio which have been created from the analyte within the ion source. The mass-to-charge ratio, *m/z* is dimensionless by definition, because it calculates from the dimensionless mass number, m, of a given ion and the number of its elementary charges. The relative abundance is usually presented as a percentage, been assigned to the most abundant ion the 100% value [43].

2.2.2.1. MS Ionization Sources

As referred before, there are several available ionization sources, their selection and use depends on the sample polarity. The polarity of a substance is inversely proportional to its volatility. In general, for large relative molecular masses the number of functional groups also increases and therewith the thermal decomposition upon vaporization becomes more likely [40]. The wide differences in the characteristics and behaviour of molecules led to the development of several ionization devices being the most relevant examples, electron impact ionization (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption ionization (MALDI). The events that occur in the ionization sources listed are described in table 2.7 [38, 44]. Figure 2.5 outlines the applicability of the different ionization sources [45, 46].

Table 2.7 Summary of the general features of current ionization methods used in mass spectrometry.

Ionization source	Nature of analytes	Sample introduction	Mass Range	Event	Sensitivity
EI	Volatile; thermally stable	GC solid or liquid probe	<500Da	Collision of highly energetic electrons	picomole
CI	Volatile; thermally stable	GC solid or liquid probe	<500Da	Transfer charged species (an electron, proton or other) between the neutral analyte and ions from a reagent gas	picomole
APCI	Thermally stable	LC	<1200Da	Gas phase reaction between analyte molecules and solvent previously ionized by a corona discharge	High femtomole
ESI	Organic and inorganic compounds	Sample in solution	<70,000	Evaporation of charged droplets	High femtomole to low picomole
MALDI	Biomolecules (proteins, DNA, glyco-conjugates)	Sample is co crystallized with a matrix	up to 500,000Da	Photon absorption/Proton transfer	Low to high femtomole

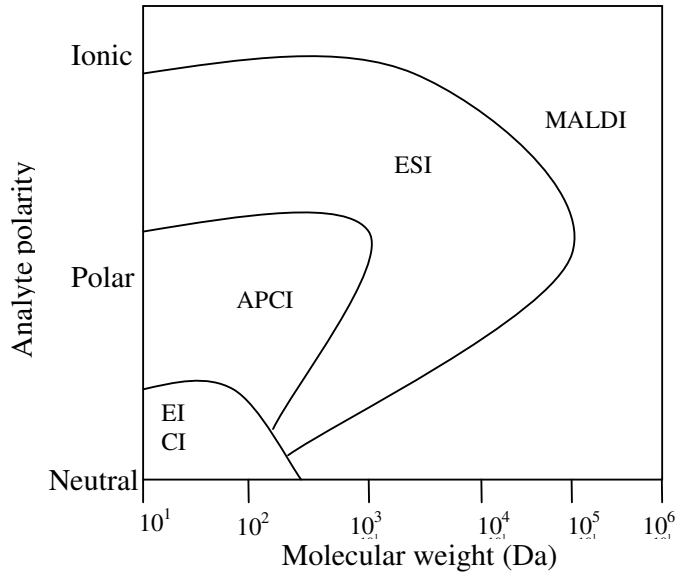


Figure 2.5 Use of ionization sources according to the molecular weight and analyte polarity.

Since the electrospray ionization (ESI) was the source most used in the development of studied methodologies included in this dissertation, a brief more detailed presentation of this ionization mechanism follows.

For several years there have been major problems of compatibility in coupling systems of LC with MS, such as the large differences in operating pressure between the two techniques and the problem raised by the need of disposal of most of the LC flow. The ESI source was one of the ionization types that could overcome in an extraordinary way the described problems.

ESI is a soft ionization technique that accomplishes the transfer of ions from solution to the gas phase. The solution is composed of a volatile solvent and the ionic analyte at very low concentration, typically 10^{-6}-10^{-3}M [38]. To the liquid passing through a capillary tube with a weak flux (1-10 µl min^{-1}) is applied a strong electric field (3-4kV), under atmospheric pressure, inducing a charge accumulation at the liquid surface located at the end of the capillary, which will break to form highly charged droplets [44, 46]. Figure 2.6 is a diagram of an ESI source [38, 39, 46]:

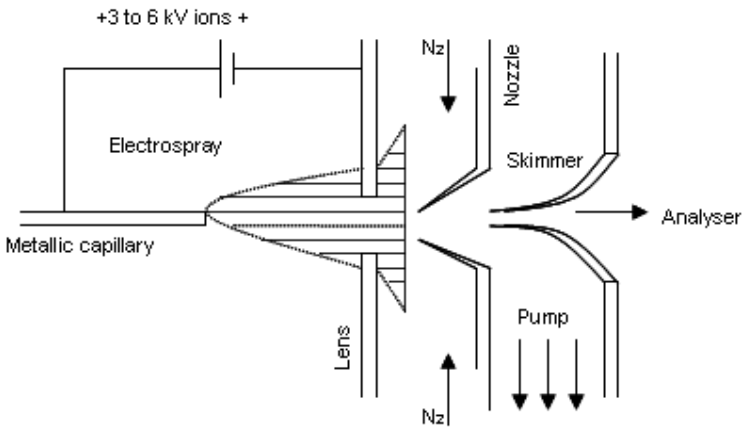


Figure 2.6 ESI source diagram.

The droplets are then driven electrically and are vaporized with the aid of a warm neutral gas (usually N_2). Under these conditions, the droplets break down and, while shifting inside the source, their size is continuously being reduced. Eventually, the repulsive forces, *coulombic forces*, among the ions on the surface of the shrinking droplets become very high. These forces will ultimately exceed the surface tension of the solvent, resulting in ions that desorbs into the gas phase. This theory of ESI ion formation is termed the ion evaporation method, and is believed to favour ions with relatively low *m/z* values. An alternative theory, which is supposed to be dominant in the case of ions with very high *m/z*, is the charge residue model, which involves continuous evaporation of the solvent accompanied by droplet fragmentation so that a single ion (probably multiply charged) is formed at the end of this process [44, 47]. These formation theories can be schematized as presented in Figure 2.7.

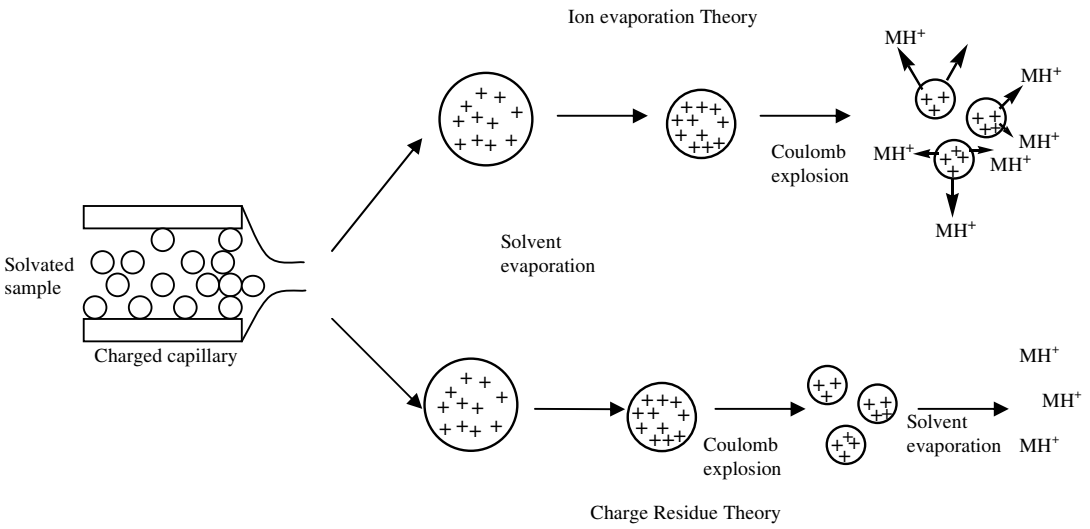


Figure 2.7 Electrospray ionization mechanisms.

As all sources of ionization, ESI has advantages and disadvantages that can be summarized in Table 2.8 [47].

Table 2.8 Advantages and disadvantages of ESI.

Advantages	**Disadvantages**
Practical mass range of up to 70,000 Da	The presence of salts and ion-pairing agents like TFA can reduce sensitivity
Good sensitivity with femtomole to typical low picomole sensitivity	Complex mixtures can reduce sensitivity
Softest ionization method, capable of generating noncovalent complexes in the gas phase	Simultaneous mixture analysis can be poor
Easily adaptable to liquid chromatography	Multiple charging can be confusing
Easily adaptable to tandem mass analyzers such as ion traps and triple quadrupole instruments	Sample purity is important
Multiple charging allows for analysis of high mass ions with a relatively low *m/z* range instrument	Carryover from sample to sample
No matrix interference	Some molecules tend to show multimer formation $[M+Adduct]^+$ where the adduct can be H, Na; NH_4 or otherwise

2.2.2.2. MS Analysers

The actual nuclear component of a mass spectrometer is the mass analyser, but not all analysers operate in the same way, while some separate ions in the space coordinate others separate them in the time coordinate. Therefore, the common characteristic of all mass analyzers is the place where the ions are separated based on their *m/z* values. Depending on the properties of the analytes to be analyzed, many different analysers with different principles of operation can be found. In table 2.9 we summarize some common mass analysers and their respective principle of operation.

Table 2.9 Mass analysers and their principle of operation [38, 39, 44, 46, 47].

Common mass analysers	Acronym	Principle
Time-of-flight	TOF	Time dispersion of a pulsed ion beam; separation by time-of-flight
Magnetic sector	B	Deflection of a continuous ion beam; separation by momentum in magnetic field due to Lorentz force
Linear quadrupole	Q	Continuous ion beam in linear radio frequency quadrupole field; separation due to stability of trajectories
Linear quadrupole ion trap	LIT	Continuous ion beam and trapped ions; storage and eventually separation in linear radio frequency quadrupole field due to stability of trajectories
Quadrupole ion trap	QIT	Trapped ions; separation in three-dimensional radio frequency quadrupole field due to stability of trajectories
Ion cyclotron resonance	ICR	Trapped ions; separation by cyclotron frequency (Lorentz force) in magnetic field

The three main characteristics of an analyser are the upper mass limit, the transmission and the resolution. The mass limit determines the highest value of the *m/z* ratio that can be measured. It is expressed in Da for an ion carrying an elementary charge, i.e. z = 1. The transmission is the ratio between the number of ions reaching the detector and that of the ions produced in the source. The resolving power is the ability to yield distinct signals for two ions with a small

mass difference [46]. Therefore, greater resolution corresponds directly to the increased ability to differentiate ions. The most common definition of resolution is given by the following equation:

$$\text{Resolution} = m/\Delta m$$

where m corresponds to *m/z* and Δm represents the full width at half maximum (FWHM). The analyzer's resolving power does, to some extent, determine the accuracy of a particular instrument.

The analyzer used in this work was essentially the ion trap, reason why a brief description of its characteristics and operation mode follows.

A quadrupole ion trap consists of a cylindrical ring electrode to which the quadrupole field is applied, and two end-cap electrodes, hyperbolic electrodes (Figure 2.8). One end-cap contains holes for the introduction of electrons or ions into the trap, while the other has holes for ions ejected out of the trap towards the electron multiplier. The end-caps are electrically connected and the DC (Direct current potential) and RF (Radiofrequency potential) potentials are applied between them and the ring electrode. The working principle of the QIT is based on creating stable trajectories for ions of a certain *m/z* or *m/z* range while removing unwanted ions by colliding them with the walls or by axial ejection from the trap due to their unstable trajectories [38].

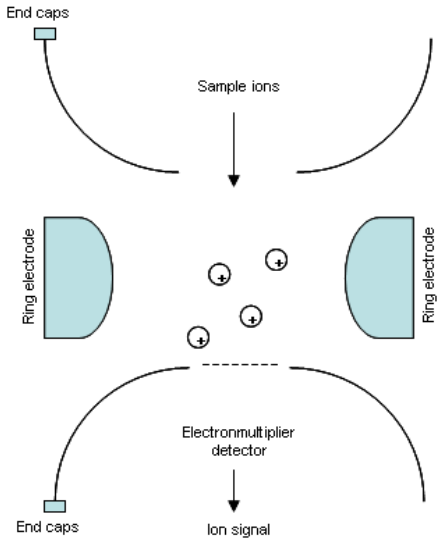


Figure 2.8 Ion trap mass analyser.

As the RF voltage applied increases ions of larger masses will be destabilized allowing different masses to be detected sequentially in time during the life of the pulse. The correlation between applied voltage and mass detected allows to obtain the mass spectrum of the sample [39].

There are four basic steps to all ion trap operation: trapping, isolation, excitation, and ejection. After collect, all ions, regardless of their mass, are allowed to enter the ion trap and are retained. Then the sequence of steps is processed until the ions are eventually ejected from the trap to the ion detection system. In the simplest case of full scan, all of the ions are collected and then ejected, resulting in a spectrum that displays the ion count of each mass initially entering the trap. In the SIM case, the ions are collected and during the time interval in which they are maintained trapped, voltages are altered to isolate a single ion, purging everything else. This isolated ion can be excited and fragmented to obtain the corresponding product ions that are sequentially scanned out [47].

Some of the ion trap analyzer, has advantages and disadvantages schematically described in table 2.10 [38, 39, 48, 49]:

Table 2.10 Advantages and disadvantages of ion trap mass analyser.

Advantages	Disadvantages
Small, easy to use and automate, fast	Low resolution
MS^n capability inherent (amount of structural information)	Interfering side reactions
Good sensitivity and specificity (greater efficiency than scanning systems)	Limited dynamic range (space charge effects diminishing the analyser performance)
Simultaneous store positive and negative ions at once for extended period of time	Do not support constant neutral loss and precursor ion scans
Ability to perform multiple stages of MS	Upper limit on the ratio between precursor m/z and the lowest trapped fragment is ~0.3

2.2.3. Mass spectrometry-chromatography coupling

In order to analyse mixtures, a gas or liquid chromatographic analyzer precedes the mass spectrometer in order to previously separate individual components. Chromatography is a physical separation method in which the components to be separated are selectively distributed between two immiscible phases: a mobile phase is flowing through a stationary phase bed. The chromatographic process occurs as a result of repeated retention/liberation steps during the movement of analytes along the stationary phase. The separation is due to

the differences in distribution coefficients of the individual analytes constituents of the sample. This separation occurs in the column, where is the stationary phase, due to the intra and intermolecular interactions between analyte molecules and mobile and stationary phases. The greater the forces between the solute molecules and those of the stationary phase, more the solute will be retained. Conversely, the stronger the interactions between the solute molecules and the mobile phase the more rapidly will the solute pass through the column. These interactions can be classified as polar, ionic and dispersive [50]. The nature of the compounds to be separated is determinant for the choice of the phase system.

The LC separations are based on four retention mechanisms, normal phase, reversed-phase, ion exchange and size exclusion. Reversed phase LC is the most widely used, probably for being the more versatile one, and also for the suitability to be applied to the system of LC-MS [36, 47]. In reversed-phase non-polar, chemically-modified silica, usually a result of the reaction of silica surface silanol groups with chloroalkyl- or alkoxyalkylsilanes, or other non-polar packing materials are used as stationary phases in combination with aqueous-organic solvent mixtures. The specific analyte-solvent interactions and solubility effects, are the factors most important for this type of separation, because the interaction of the analyte with the bonded-phase material is relatively weak of the type of non-specific Van der Walls interactions. The retention decreases with increasing polarity of analytes. Mixtures of water or aqueous buffers and an organic modifier, usually methanol and acetonitrile, are used as eluent. The percentage and type of organic modifier is adapted to the mixture under study [47].

The ideal case is the transfer of a method from a traditional analytical HPLC system, when a classical HPLC method is previously established for the sample under study, to an LC/MS system is a direct transfer without the need for any alterations to established conditions, leading to no doubts about the identity of minor impurities compared to the previously known [36]. Though, the coupling to the MS puts constraints to the buffers and organic modifiers commonly used in classical HPLC. In that case, proper chromatographic conditions have to be previously established selecting non-volatile buffer solutions which can be tolerated by the MS interface. Buffers serve to act in the chromatographic process to control and maintain the pH of the mobile phase in order to keep constant the ionization state of an analyte, and to adjust the pH of the mobile phase in such a way as to present the analytes to the MS in ionic form. Actually many of the complex mobile phases, non-volatile buffers and ion-pairing reagents, used in traditional HPLC result in clogging at the LC-MS

interface, build up of deposits in the ion source, and drastic reduction in ion production [50]. Phosphate buffers are traditionally applied in HPLC, since they span a wide pH range showing good buffer capacity, reducing protolysis of ionogenic analytes, which in ionic form show little retention [47]. To be compatible with LC/MS, alternative volatile buffers like ammonium formate, acetate or carbonate can be used. Buffer concentrations may also to be optimized in order to increase ESI sensitivity [52]. The factors that may effect generation of ions by API include: pH, pKa, temperature, mobile-phase additives, flow-rate, solvent composition and concentration of electrolytes and analytes.

For electrospray the response is greater when the organic concentration in the solvent system is higher [51], but there are not many options for organic solvents to be used in LC-MS. It is very important in reversed-phase LC the specific analyte-solvent interactions, solubility effects, because the interaction of the analyte with the bonded phase material is relatively weak. The retention decreases with increasing polarity of analytes. Mixtures of water or aqueous buffers and organic modifiers (acetonitrile and methanol) are used as eluents [47]. Because of the higher solvent strength and lower viscosity in mixtures with water, acetonitrile is often preferred to methanol [47, 52].

The sample preparation process has a direct impact on accuracy, precision, and quantitative limits and is often the rate determining step for many analytical methods. Aqueous samples usually must be processed in order to isolate and concentrate organic analytes from the sample matrix and provide a suitable sample extract for instrumental analysis. By using LC-MS, like in HPLC, it is often necessary to resort to extracting methods in order to remove impurities and to purify the sample. The classical isolation and purification techniques are filtration, recrystallization, sublimation, solvent extraction, distillation and chromatography [54]. The adoption of a particular isolation procedure will depend to a large extent upon the physical and chemical properties of the product. The extent of isolation, purification and concentration of the analyte is determined by [55]:

- the matrix itself (complexity, composition)
- the concentration of the analyte in the matrix
- the selectivity and sensitivity required in the subsequent analysis
- the analytical objectives (e.g., screening, quantitative or qualitative analysis).

A wide variety of sample pretreatment methods have been used in combination with LC-MS, like liquid-liquid extraction and solid-phase extraction [47]. The first one, liquid-liquid

extraction is based on the selective partitioning of analytes between two immiscible liquid phases. The process of extraction is concerned with the distribution law or partition law which states that if to a system of two liquid layers, made up of two immiscible or slightly miscible components, is added a quantity of a third substance soluble in both layers, then the substance distributes itself between the two layers so that the ratio of the concentration in one solvent to the concentration in the second solvent remains constant at constant temperature. Organic compounds are usually relatively more soluble in organic solvents than in water. This method is efficient on the removal of nonvolatiles being the analytes extracted by using solvents such as dichloromethane, ethylacetate, methyl-*t*-butyl ether or hexane and also provides an important cleanup [56]. Solid samples can also be extracted by using an adequate solvent like acetone, methanol or acetonitrile [47, 54]. The major disadvantages of this technique are the use of large amounts of solvents, the possible occurrence of emulsions and the need for evaporation of the solvent at the end of extraction [54, 56].

Solid-phase extraction (SPE) is a type of liquid column chromatography and is a more physical extraction process that brings a liquid in contact with a solid phase. The solid phase has a greater attraction for the analytes to be withheld, from now referred to as isolate, than the solvent in which the analyte is dissolved [55, 56, 57]. As the sample solution passes through the sorbent bed, the isolate concentrates on this surface, while the other sample components pass through the bed. Very selective extractions resulting in highly purified and concentrated isolates can be achieved by choosing sorbents with an attraction for the isolate but not for the other sample components [57]. The classical adsorbents are silica, magnesium silicate (Florisil), alumina, celite, ion-exchanging resins, and others [54, 55]. However, the introduction of silica bonded phases, similar to those used in liquid chromatography brings more extraction coverage. A silica bonded phase is a result of the reaction of silica surface silanol groups with chloroalkyl- or alkoxyalkylsilanes [55, 57]. A variety of different bonded silicas are commercially available, offering a wide range of selective properties of extraction. Available stationary phases have different mechanisms of interaction: non-polar (octadecyl-C18; octyl-C8; ethyl-C2; cyclohexyl-CH; phenyl-PH), polar (cyanopropyl-CN; diol-2OH; silica-SI; aminopropyl-NH_2; N-propylethylenediamine-PSA), ion exchange (benzenesulfonylpropyl-SCX; sulfonylpropyl-PRS; carboxymethyl-CBA; diethylaminopropyl-DEA; trimethylaminopropyl-SAX) [57]. As previously described for separation by LC, the mechanisms of interaction between non-polar phases

are the type of dispersion forces or Van der Waals forces, the polar phases rely on hydrogen bonds and dipole-dipole interactions, and the phases of ion exchange rely on electrostatic forces mechanisms [55, 57].The sorbent extraction interactions can be outlined as in Figure 2.9.

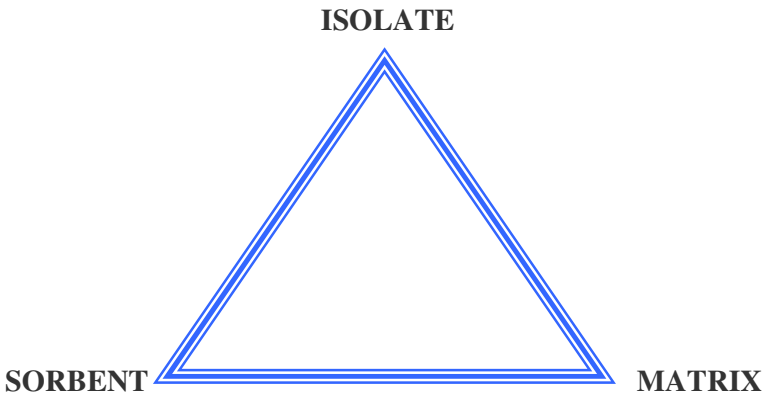


Figure 2.9 Sorbent extraction interactions.

During sorbent extraction, three simultaneous interactions must be considered. The first of these, sorbent/isolate interactions, results in isolate retention. Sorbent/matrix interactions are competitive, causing isolate elution. Matrix/isolate interactions also interfere with isolate retention by making the isolate unavailable to the sorbent.

The SPE process using silica bonded phases consists of five steps: wetting, conditioning, adsorption, washing and elution. Wetting serves to create an environment suitable to isolate retention since in the dry state the bonded alkyl chains are twisted and collapsed on the surface. Methanol is an effective solvating agent because it can interact with both the silanols on the silica and the carbon atoms of the bonded functional group. Conditioning is made with a solvent or buffer similar to the test solution. Adsorption step consists in the passage of the test solution at a controlled flow rate. Retention is the phenomenon where an attraction exists between the sorbent and isolate molecules, causing the isolate to be immobilized on the sorbent surface as the sample solution passes through the sorbent bed. Retention is a function of three factors: the characteristics of the isolate, the solvent, and the sorbent. The retention behavior of a given isolate can, therefore, be expected to change in the presence of different solvents and sorbents. To ensure a complete retention of the analyte(s) it may be necessary to change the test solution characteristics (by changing pH, polarity or viscosity). Washing step is performed by

passing a suitable solvent through the sorbent eliminating possible interfering matrix components, while the analyte(s) of interest remains adsorbed. Elution is the process by which an isolate is removed from a sorbent bed on which it has been retained. This is brought about by introducing a solvent to which the isolate is more strongly attracted than it is to the sorbent. The elution solvent chosen should elute the isolate from the sorbent bed in the smallest volume possible [55, 57].

There is an important distinction between the objectives of sorbent extraction and those of classical chromatography which also employs the terms retention and elution. In sorbent extraction, the goal is to retain an isolate on a sorbent strongly enough that the isolate does not move through the sorbent bed until the elution solvent is introduced [57].

The method development requires the selection of adequate solvents and sorbents according to analyte characteristics (pKa, solubility, molecular mass, polarity, molecular structure, presence of acid or basic functions), matrix characteristics (polarity, presence of co-extractable compounds) and the analytical method to be used subsequently [55]. There are already described some negative effects of SPE on the ionization in LC-MS, like ion suppression and/or enhancement due to extractables endogenous to the SPE hardware (polypropylene cartridges) that co-elute with analytes of interest during SPE methodology [58].

2.2.4. Developing new methodologies for quality control inside companies

The scope of drug analysis includes the analytical investigation of bulk-drug materials, the intermediates in their synthesis, products of drug research, drug formulations, impurities and degradation products of drugs, biological samples containing the drugs and their metabolites with the aim of obtaining data that can contribute to the maximal efficacy and maximal safety of drug therapy and the maximal economy of the production of drugs. The efficacy, safety and economy of drug therapy are extremely important issues not only from the point of view of public health, but also financial and political [59].

Mass spectrometry has an enormous impact on five major categories of the pharmaceutical industry: new chemical entity (NCE) characterization; biomacromolecule (BMM) characterization; bioanalytical quantification; metabolite identification; and impurity and degradation product identification [60]. However, the use of atmospheric-pressure ionisation

LC-MS for the quantitation of drug impurities and degradation products has been a poorly represented area of investigation in the literature of pharmaceutical analysis [61]. Several explanations are possible for this underutilization, including a perception that there is a little need for additional sensitivity and selectivity in much of the quantitative chemical analysis of drug substance and drug product development, a perception that the accepted precision requirements of drug-product-related substances (5-10%RSD) are tighter than those perceived achievable by atmospheric-pressure ionization LC-MS, and a conservative perspective by downstream organizations such as pharmaceutical quality-control groups or regulatory agencies that this type of approach would be inappropriate or scientifically invalid [61].

Impurity profiling hyphenated techniques, as LC-MS and LC-MS-MS, have proven to be better than classical methods [60, 62-66]. The reasons are varied such as the similarity of structural impurities with API, the frequent impossibility of isolating the impurities and the lack of authentic standards, among others. Significant added benefit of LC-MS-MS,

compared to UV diode array spectral matching, is that an MS/MS fingerprint may enable structures to be proposed for the non isolated impurities, avoiding time-consuming isolation [62]. The determination of high resolution mass spectrometry (HRMS) empirical formulas for unknown impurities could also be stated [60, 65]. The MS^n capabilities proved their efficiency in solving particularly challenging problems like mixture characterization [60, 62, 65, 67].

MS-MS detection is capable of detecting drug product impurities at or below the levels required for quantification. The additional modes of selectivity offered by MS, or more drastically MS-MS, analysis allow for more-rapid method development in that baseline chromatographic resolution of impurities and matrix components is nonessential [61]. Often, analysts perceive that MS detection does not provide the precision required for quantification of pharmaceutical drug products. This perception might be true in the case of determining API, which typically requires tighter precision (2%RSD); however, the determination of drug product impurities involves a more relaxed precision criterion of 10-15%RSD. Studies with tandem MS in the multiple-reaction monitoring mode can achieve these levels of precision with extremely low analyte concentrations [61].

The advantages of the use of hyphenated techniques such as LC-UV-MS / MS, in API's and formulations, are large and have been described: multiple dimensions of information for structure elucidation which includes relative retention time, UV area percent for quantitative

studies, UV-Vis spectra, molecular weight and substructural information via tandem mass spectrometry [67, 68].

In conclusion, the pharmaceutical industry has enormous advantages in developing multidisciplinary approaches [37, 69, 70] such as a strategy for drug impurity profiling based on different techniques whose results may be interpreted with process and product knowledge. In fact, should take special attention to modern hyphenated techniques, which allow multi-dimensional data, but it is often useful to study techniques with and without chromatographic separation, taking advantage of the separation techniques such as SPE [37] and TLC [70], and across important information from techniques as MS, NMR, IR, UV-Vis [37, 67-70].

It can be concluded that the requirements currently imposed on the quality of medicines led to routine chemical / physical analysis has become anything but routine with the need to develop methods much more efficient with greater regularity. Sample complexity has increased and more useful information for each sample at lower detection levels of detection is required, using more sensitive techniques and that until very recently were used only on high-end research applications. Now, this better analytical performance is now becoming a basic requirement for many routine applications. Since these new methods are much more demanding is essential to develop such projects allowing methods and workflows that simplify sample preparation and can isolate contaminants from the matrix in the most cost- and time-effective way.

CHAPTER 3

NETILMICIN

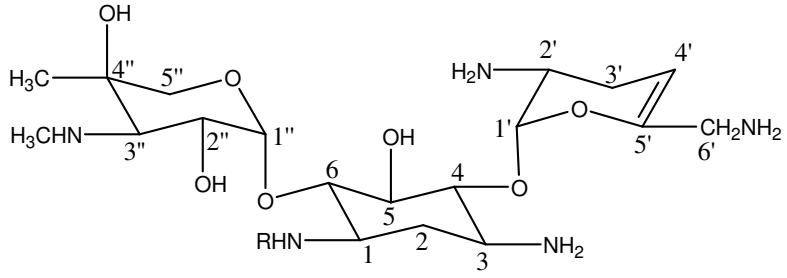
This chapter presents the study of determination and quantification of 1-*N*-ethylgaramine, an aminoglycoside antibiotic impurity, in injectables of netilmicin. A simple, sensitive, accurate and precise SPE-LC-MS method was developed and validated for the first time to quantify 1-*N*-ethylgaramine in intravenous formulations. Relevant aspects of the chemistry and reactivity of these compounds are presented. In order to develop an appropriate and sensitive method, a literature review of relevant methods for the analysis of aminoglycosides and related substances, is presented.

Methods of chromatographic separation of the analytes studied, methods of extraction from the matrix of injectables and also the adopted method for detection by mass spectrometry are described. The validation study of the overall method developed and its application in real samples is also presented.

3.1. Introduction

3.1.1. Chemistry

Netilmicin (Fig. 3.1), which is mainly used as the sulphate, 2-Deoxy-6-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl]-4-O-(2,6-diamino-2,3,4,6-tetradeoxy-α-D-glycero-hex-4-enopyranosyl)-1-Nethyl-D-streptamine sulphate, is a semi-synthetic water soluble aminoglycoside antibiotic [71].

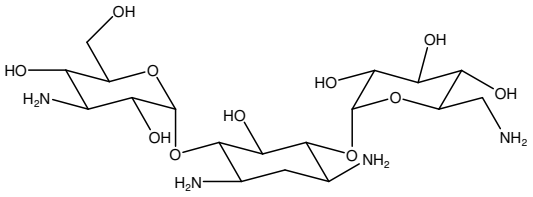


Netilmicin: R=CH_2CH_3

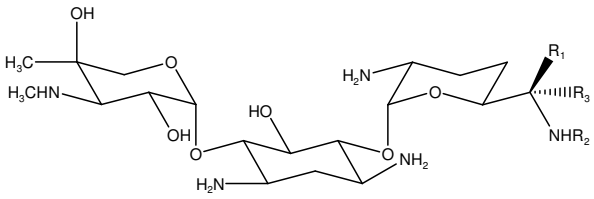
Sisomicin: R=H

Figure 3.1 Structure of netilmicin and its precursor sisomicin.

The aminoglycosides are a large class of antibiotics that contain two or more aminosugar groups linked by glycosidic bonds to an aminocyclitol. Aminoglycosides are basic compounds, extremely hydrophilic and thermolabile. Another important characteristic of such compounds is the absence of chromophores, since many structures have few or no double bonds, like gentamicin or kanamicin.



Kanamycin

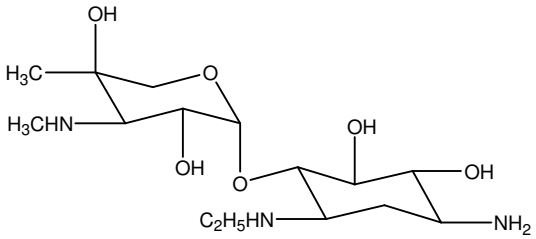


	R_1	R_2	R_3
Gentamicin C1	CH_3	CH_3	H
Gentamicin C2	CH_3	H	H
Gentamicin C1a	H	H	H
Gentamicin C2a	H	H	CH_3

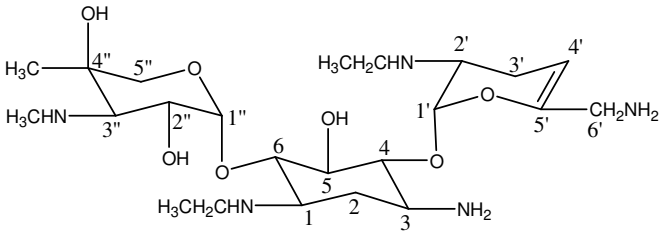
Figure 3.2 Structure of kanamycin and gentamicin.

The history of aminoglycosides began in 1944 with streptomycin and was thereafter marked by the successive introduction of a series of milestone compounds such as kanamycin, gentamicin, neomycin, tobramycin, which definitively established the usefulness of this class of antibiotics for the treatment of gram-negative bacillary infections [72, 73]. In the 1970s, the semisynthetic aminoglycosides dibekacin, amikacin and netilmicin demonstrated the possibility of obtaining compounds that where active against strains that had developed resistance mechanisms towards earlier aminoglycosides and displayed distinct toxicological profiles. These antibiotics are highly potent broad-spectrum antibiotics with many desirable properties for the treatment of life-threatening infections for people and animals [72].

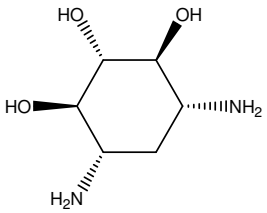
Netilmicin, the aminoglycoside under study, has a broad spectrum of activity against Gram-negative and against some Gram-positive bacteria, some of them resistant to other aminoglycosides, like sisomicin and gentamicin [74]. Netilmicin is prepared from sisomicin (Fig. 3.1) by the introduction of an ethyl group at the 1-*N*-position being reported various different synthesis [74, 75]. Therefore, sisomicin can be expected to be present in netilmicin samples, and also small amounts of some *N*-ethyl derivatives of sisomicin resulting from selective alkylation and acylation of the nitrogen functions of the initial component [74]. - The *N*-ethyl derivatives of sisomicin that can be formed, in small amounts are N^3, $N^{3"}$-, $N^{2'}$- and $N^{6'}$. The N^3- and $N^{3"}$- ethyl derivatives, which have very weak antibacterial activity, are normally removed by crystallization [74, 76]. Garamine is a known product of the hydrolysis of sisomicin. The corresponding *N*-ethyl derivatives such as 1-*N*-ethylgaramine (Fig 3.3) could be present in netimicin sulphate [71,74, 76]. In Figure 3.3 are presented some related structures that is expectable to be present in netilmicin samples [71, 74]:



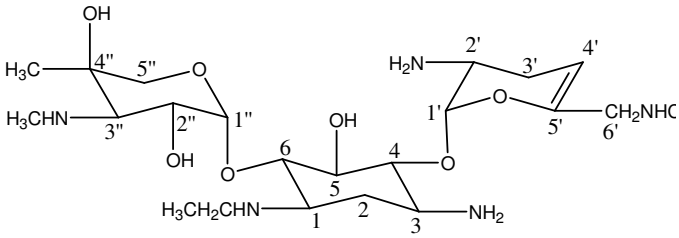
1-*N*-ethylgaramine



2'-*N*-ethylnetilmicin



2-deoxystreptamine



6'-*N*-ethylnetilmicin

Figure 3.3 Structure of related structures of netilmicin.

3.1.2. Literature Review

The nephro and ototoxicity of this class of compounds is a great concern and also their potential for acquired bacterial resistance [72-74]. They are widely use in veterinary medicine for the treatment of serious infections and in order to avoid exposure of humans to these compounds it must be respected the established residues limits. There are already some methods described to identify and quantify the most commonly used aminoglycosides in tissues of farm animals, milk and meat containing foods [72, 77-82].

Netilmicin exhibits less chronic nephro- and ototoxicity than others [74], but as other aminoglycosides, have a very narrow margin between their therapeutic and toxic dose (a too high plasma level of some aminoglycoside antibiotics may cause serious adverse toxicity) [74, 83]. Sensitive and accurate analytical methods for the analysis of aminoglycoside antibiotics in various drugs and body fluids are in great demand in medical practice for dosing guide, toxicity

preventing and efficacy ensuring [74, 83-87]. Some minor impurities containing in commercial bulk pharmaceuticals and their formulations also can exert harmful effects to human being's healthy, so quality control and quality assurance on aminoglycoside antibiotics' production also entail sensitive and reliable analytical techniques [74, 83, 88-90]. An example of the severity of the damage produce by a deficient control was the case reported in 2002 when 66 deaths in addition to hundreds of patients suffering from severe side effects after administration of gentamicin sulphate were to be caused by impurities related to faulty manufacture [90].

Quantifying aminoglycosides antibiotics with standard detection methods of refractive index and ultraviolet or visible light absorption is unsatisfactory because the extinction coefficient for these compounds is not optimum for accurate and sensitive detection [87]. Various methods to determine aminoglycosides and its impurities were described: pulsed electrochemical detection [76, 88, 89], HPLC-UV with aminoglycosides derivatization [77, 82, 84, 85], capillary electrophoresis with previous derivatization [86] and HPLC using amperometric detection [83].

The improvement on the methods to determine antibiotics and their related compounds was achieved using Liquid-Chromatography-Tandem Mass Spectrometry (LC-MS-MS) [72, 81]. LC-MS-MS methods for the analysis of gentamicin, neomycin and related compounds were previously reported [78-80, 90]. More recently Adams *et al* [74] described the characterization of impurities in sisomicin and netilmicin bulk samples by LC-MS-MS.

The study of netilmicin samples (API and formulations) and quantification of the major impurity, 1-*N*-ethylgaramine, is the mainly purpose of this study. With respect to regulations, the method described by the European Pharmacopoeia for related substances of netilmicin is the method that uses pulsed electrochemical detection, previously described by Adams et al [76]. The quantization of the major impurity of netilmicin is completely impossible with UV detection due to the lack of double bonds in its structure [74, 76]. The method previously described by Adams *et al* [74], using LC-MS-MS, was developed only to characterize related compounds of sisomicin and netilmicin, in bulk samples. In addition they prepare highly concentrated solutions to make such determinations.

Our aim was to develop and validate a more accurate method such as LC-MS-MS for quantification of 1-*N*-ethyl garamine in injectable formulations.

Netilmicin, as all aminoglycosides, is water-soluble and highly polar. Because of these qualities, they are not absorbed well from the gastrointestinal tract [73]. Therefore these drugs are usually administrated intravenous or intramuscularly. Another purpose of this study was to

determine the quantity of 1-N-ethylgaramine in injectable formulations. These products have various salts in its composition, a situation that led us to develop methodologies for effective extraction of the analytes under study. A validation method for API and final product was developed using neomycin as internal standard (Fig. 3.4).

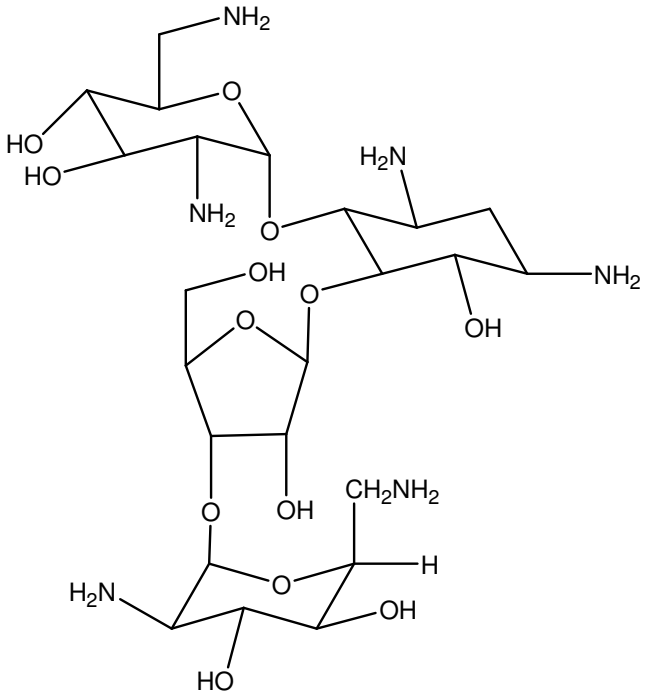


Figure 3.4 Structure of Neomycin, aminoglycoside used as internal standard.

3.2. Validation Procedure Description

3.2.1. Specificity

Specificity, according to ICH Q2(R1) [94], is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Tests were performed on 6 types of samples: 1) solvent used in the preparation of samples, 2) vehicle extract used in the preparation of injections without 1-*N*-ethylgaramine, 3) netilmicin sulphate, the raw material used in the preparation of injectables, 4) 1-*N*-ethylgaramine standard 5) ethyl garamine prepared in accordance with the protocol described in the analytical solution as standard for the determination of 1-*N*-ethylgaramine in injectable of netilmicin and 6) injectable extract Tilcin 150mg / 1.5 ml. All of these samples were prepared according to the analytical protocol described in the extraction methods. The chromatograms obtained for these samples are presented in Figures 3.5 to 3.10.

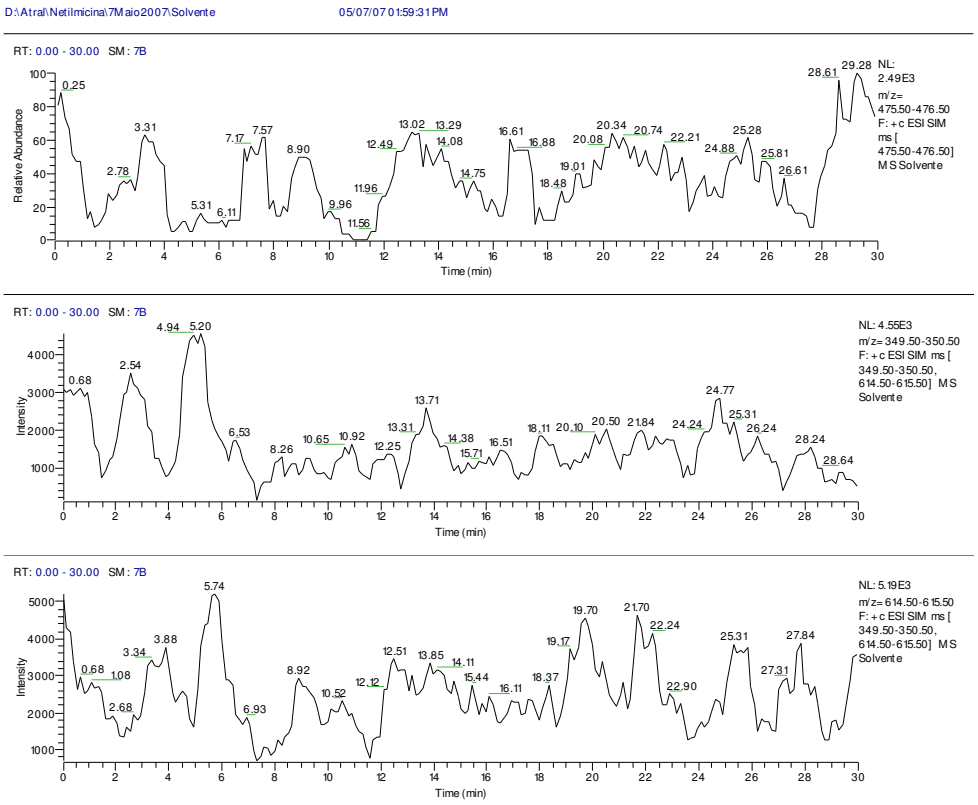


Figure 3.5 Solvent used in the preparation of samples

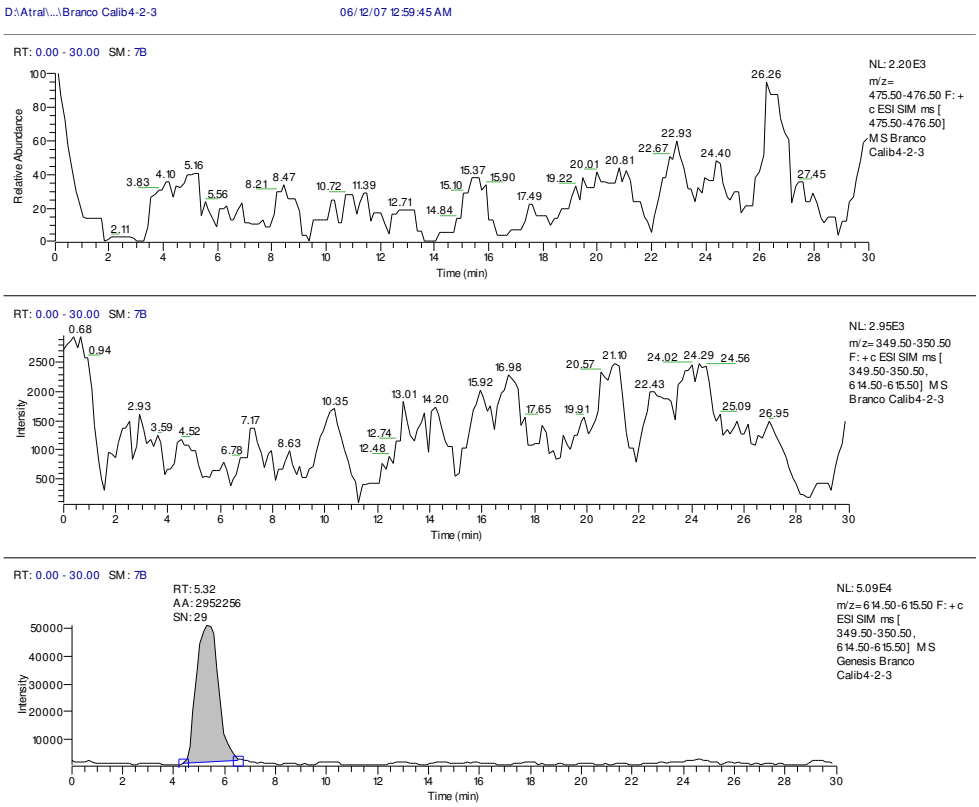


Figure 3.6 Vehicle extract used in the preparation of injections without 1-*N*-ethylgaramine.

(First cell – SIM $[M+1]^+$=476, second cell – SIM $[M+1]^+$=350, third cell – SIM $[M+1]^+$=350, 615)

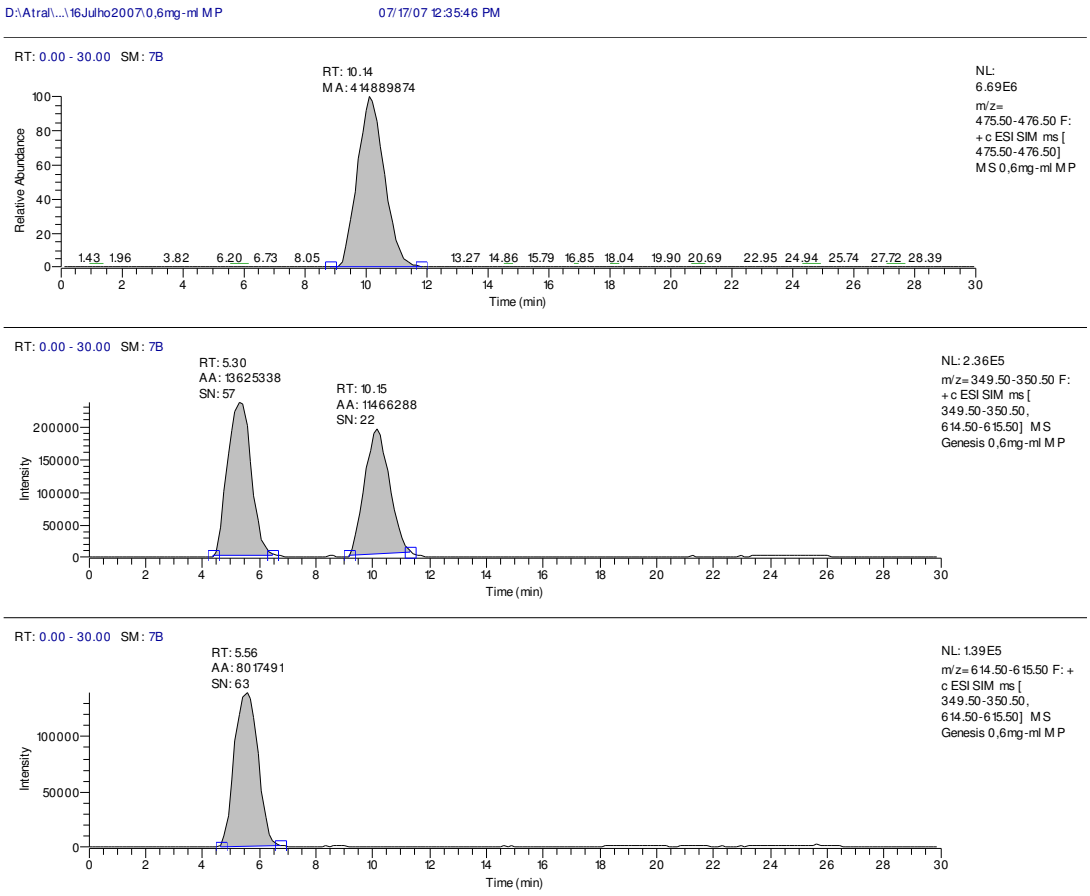


Figure 3.7 Netilmicin sulphate, the raw material used in the preparation of injections

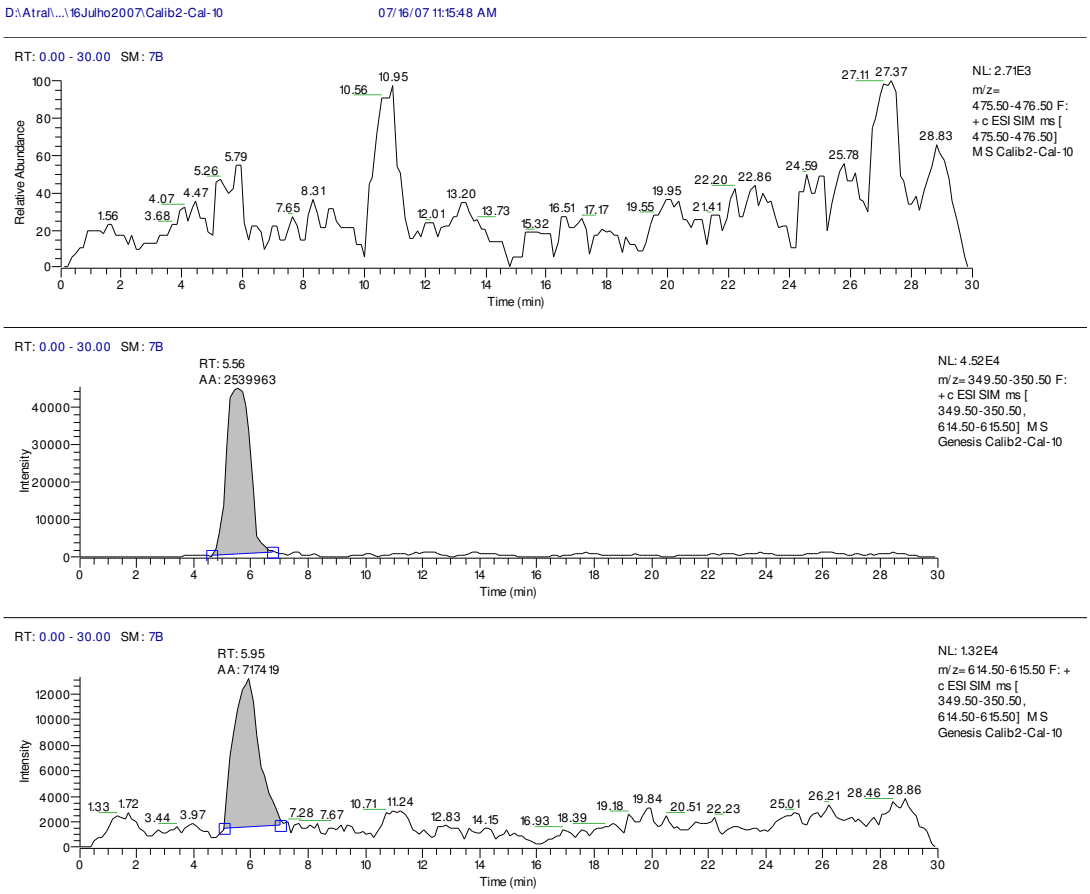


Figure 3.8 1-*N*-ethylgaramine standard.

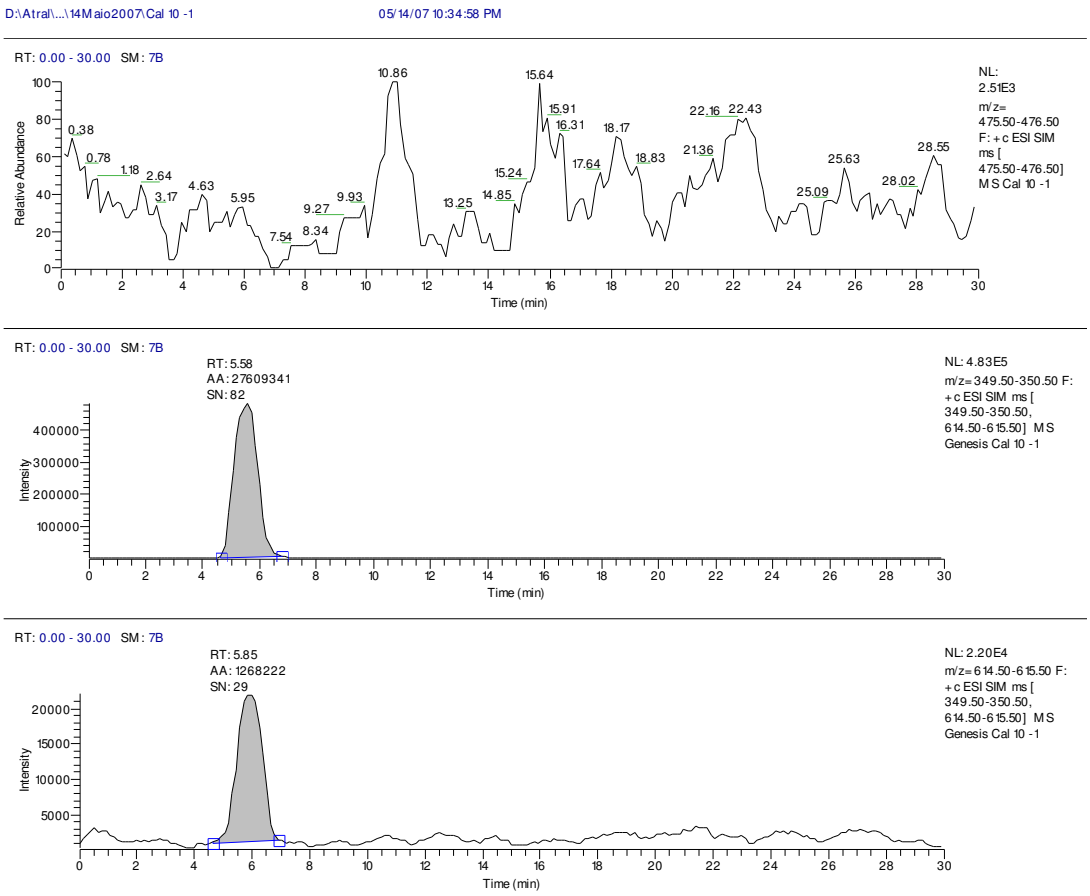


Figure 3.9 1-*N*-ethylgaramine prepared in accordance with the protocol described in the analytical solution as standard for the determination of impurity in netilmicin injectable.

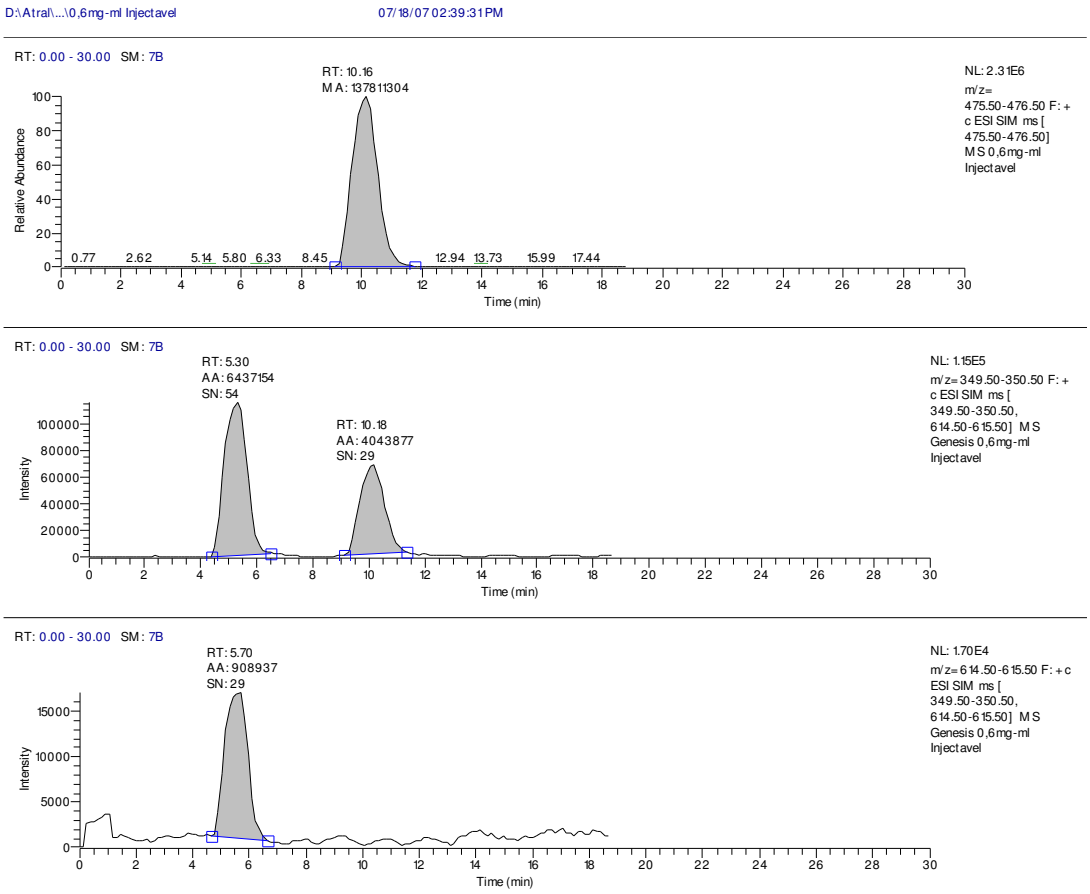


Figure 3.10 Injectable extract 150mg / 1.5 ml.

From the tests performed in these 6 different samples it can be detected unequivocally the various analytes in study. It were selected for the different analytes their respective $[M+1]^+$ mass: netilmicin, 476, 1-*N*-ethylgaramine, 350, and neomycin (IS), 615. The different retention times obtained (netilmicin, 10.14min, 1-*N*-ethylgaramine, 5.36min and neomycin, 5.6min) show that specificity is achieved.

From the results, it is clear that the method is selective for 1-*N*-ethylgaramine relation to other components present in netilmicin raw material, either in samples of injectable.

3.2.2. Precision

3.2.2.1. System Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained for multiple sampling of the same homogeneous sample under the prescribed conditions [94]. System precision, instrument precision or injection repeatability is the study of a minimum of 10 injections of one sample solution to test the performance of the chromatographic instrument [95].

It was prepared a standard solution of 1-*N*-ethylgaramine, according to the protocol described in the analytical to the raw material, with a concentration of 10mg/l and was injected 10 times, the areas were recorded and determined the residual standard deviation associated to the values obtained.
It was prepared a standard solution of 1-*N*-ethylgaramine, according to the analytical protocol described for injectables, with concentration of 10mg/l and injected 10 times, the areas were recorded and determined the standard deviation associated with residual values obtained.

For the determination of 1-*N*-ethylgaramine in raw material we obtained

Table 3.1 1-*N*-ethylgaramine in raw material system precision.

Cromatogram (29/05/2007)	**1-*N*-ethylgaramine (EG) area**	**IS area**	**EG area/IS area**
Cal 10 Prec 1	60651844	10771432	5.630806006
Cal 10 Prec 02	59891833	10240785	5.848363480
Cal 10 Prec 03	60967884	10661887	5.718301460
Cal 10 Prec 04	56398801	10186979	5.536361761
Cal 10 Prec 05	58745257	9956889	5.899961022
Cal 10 Prec 06	61779014	10939042	5.647570784
Cal 10 Prec 07	58359109	10851471	5.377990597
Cal 10 Prec 08	55681947	10240951	5.437185179
Cal 10 Prec 09	56739180	9478104	5.986342838
Cal 10 Prec 10	59556477	10340493	5.759539415
Average			5.684242254
SD			0.197894743
RSD (%)			3.481462156

For the determination of 1-*N*-ethylgaramine in injectable samples:

Table 3.2 1-*N*-ethylgaramine in injectable samples, system precision.

Cromatogram (14/05/2007)	**EG area**	**IS area**	**EG area/IS area**
Cal 10 Prec 1	19417542	678414	28.62196535
Cal 10 Prec 02	19889528	762458	19.48581838
Cal 10 Prec 03	18262694	878691	20.78397753
Cal 10 Prec 04	28544368	1205148	23.68536313
Cal 10 Prec 05	29951776	1200690	24.94546969
Cal 10 Prec 06	28764799	1533027	18.76340012
Cal 10 Prec 07	29150697	1232339	23.65477113
Cal 10 Prec 08	28854708	1161772	24.83680791
Cal 10 Prec 09	29212474	1436615	20.33423986
Cal 10 Prec 10	29182098	1497607	19.48581838
Average			22.45976315
SD			3.186950048
RSD(%)			14.1895978

The residual standard deviation obtained for the set of values of 1-*N*-ethylgaramine in the standard solution for determination of impurities in raw materials, RSD = 3.48% is less than the maximum value allowed for this parameter validation, 5%, concluding that the method has a precision appropriate to the aims of the method.

The residual standard deviation obtained for the set of values of 1-*N*-ethylgaramine in the standard solution for determination of impurity in injectables, RSD = 14.19%, is higher than the maximum allowed for this parameter validation, 5%. As described in the analytical protocol, all samples under study are first subjected to SPE, a procedure that necessarily leads this results dispersion.

3.2.2.2. Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time, being also termed as intra-assay precision [94].

Six determinations were made on 6 samples of raw material prepared in accordance with the analytical protocol with 0.7 mg of netilmicin. Six determinations were made on 6 samples of injectables prepared according to the analytical protocol with 0.6 mg of netilmicin. Analyses were performed on the same day, holding constant all variables of the experimental method, and was determined their % RSD values.

Table 3.3 Repeatability in EG validation method of raw material.

Cromatogram (30/05/2007)	**EG area**	**IS area**	**EG area/ IS area**	**EG (mg/l)**	**EG found in a raw material sample of netilmicin (in% on the amount of netilmicin)**
Cal 2 Repet.1	12405082	7954766	1.559452786	2.810327246	0.401475
Cal 2 Repet.1-2	11831498	7662575	1.544062929	2.78117979	0.397311
Cal 2 Repet.1-3	11662982	8431699	1.383230355	2.476572642	0.353796
Cal 2 Repet.1-4	12008510	7965725	1.507522542	2.711974511	0.387425
Cal 2 Repet.1-5	13164350	9077620	1.4501984	2.603406061	0.371915
Cal 2 Repet.1-6	14007112	8979251	1.559942138	2.811254049	0.401608
Cal 2 Repet.2	11314360	7244257	1.561838571	2.814845778	0.402121
Cal 2 Repet.2-2	10606342	7167712	1.479738862	2.659353906	0.379908
Cal 2 Repet.2-3	10600891	6983166	1.518063726	2.731938876	0.390277
Cal 2 Repet.2-4	10602748	7112067	1.49081104	2.680323939	0.382903
Cal 2 Repet.2-5	10803689	7708979	1.401442266	2.511064898	0.358724
Cal 2 Repet.2-6	11489821	7024055	1.635781753	2.954889683	0.422127
Cal 2 Repet.3	11768594	7654424	1.53748917	2.768729488	0.395533
Cal 2 Repet.3-2	11961693	7510628	1.592635529	2.873173351	0.410453
Cal 2 Repet.3-3	12346575	8445354	1.461936942	2.625638148	0.375091
Cal 2 Repet.3-4	8883960	6557315	1.35481672	2.422758939	0.346108
Cal 2 Repet.3-5	11842631	7409662	1.59826872	2.883842273	0.411977
Cal 2 Repet.3-6	13450714	7560048	1.779183677	3.226484237	0.460926

Cromatogram (30/05/2007)	EG area	IS area	EG area/ IS area	EG (mg/l)	EG found in a raw material sample of netilmicin (in% on the amount of netilmicin)
Cal 2 Repet.4-2	12992580	8108660	1.602309136	2.891494576	0.413071
Cal 2 Repet.4-3	11788233	6895216	1.709624905	3.094744139	0.442106
Cal 2 Repet.4-4	11166359	7728475	1.444833424	2.593245122	0.370464
Cal 2 Repet.4-5	13092360	7197184	1.819094802	3.302073488	0.471725
Cal 2 Repet.4-6	11861430	7104632	1.669534749	3.018815812	0.431259
Cal 2 Repet.5-2	12194970	7110070	1.715168768	3.105243878	0.443606
Cal 2 Repet.5-2	12223890	7203245	1.696997673	3.070828925	0.43869
Cal 2 Repet.5-3	11462090	6760696	1.695400888	3.067804712	0.438258
Cal 2 Repet.5-4	12305346	6461543	1.904397448	3.46363153	0.494805
Cal 2 Repet.5-5	10170007	6620672	1.536098904	2.766096408	0.395157
Cal 2 Repet.5-6	11688944	7125517	1.640434512	2.963701727	0.423386
Cal 2 Repet.6	12604857	7905275	1.594486846	2.876679632	0.410954
Cal 2 Repet.6-2	13136904	7661296	1.714710409	3.104375775	0.443482
Cal 2 Repet.6-3	13091423	7684233	1.703673353	3.083472259	0.440496
Cal 2 Repet.6-4	12155060	7457012	1.630017492	2.943972523	0.420568
Cal 2 Repet.6-5	12688333	7768258	1.633356281	2.950295988	0.421471
Cal 2 Repet.6-6	12147281	8290323	1.465236155	2.631886658	0.375984
Average			1.58955	2.867329325	0.409618
SD			0.123654	0.234192874	0.033456
RSD(%)			7.779173	8.167631	8.167631

Table 3.4 Repeatability in 1-*N*-ethylgaramine validation method of injectables.

Cromatogram (04/06/2007)	EG area	IS area	EG area/ IS area	EG (mg/l)	EG found in injectable sample of netilmicin (in% on the amount of netilmicin)
Cal 4-2 Repet1-1	2926528	669014	4.374389774	1.980928788	0.330155
Cal 4-2 Repet1-2	3830057	816616	4.690156695	2.121269642	0.353545
Cal 4-2 Repet1-3	2802720	851289	3.292324933	1.500011081	0.250002
Cal 4-2 Repet1-4	3559269	799421	4.452308608	2.015559381	0.335927
Cal 4-2 Repet1-5	3415430	919356	3.71502443	1.687877525	0.281313
Cal 4-2 Repet1-6	4206787	1197162	3.513966364	1.598518384	0.26642
Cal 4-2 Repet2-1	6413265	1471611	4.357989306	1.973639691	0.32894
Cal 4-2 Repet2-2	6644533	1723339	3.855615755	1.750362558	0.291727

Cal 4-2 Repet2-3	6138910	1650447	3.719543857	1.689886159	0.281648
Cal 4-2 Repet2-4	5995894	1682066	3.564600913	1.621022628	0.27017
Cal 4-2 Repet2-5	6246699	1764243	3.540724832	1.610411036	0.268402
Cal 4-2 Repet2-6	5390562	1440032	3.743362647	1.700472288	0.283412
Cal 4-2 Repet3-1	6750935	1418110	4.760515757	2.152540336	0.358757
Cal 4-2 Repet3-2	7151583	1542142	4.637434815	2.097837695	0.34964
Cal 4-2 Repet3-3	6989734	1534891	4.55389601	2.060709338	0.343452
Cal 4-2 Repet3-4	6825255	1426951	4.783103975	2.162579544	0.36043
Cal 4-2 Repet3-5	6832444	1353824	5.046774174	2.2797663	0.379961
Cal 4-2 Repet3-6	7416197	1417788	5.230822239	2.361565439	0.393594
Cal 4-2 Repet4-1	3336076	1040893	3.205013388	1.46120595	0.243534
Cal 4-2 Repet4-2	4678581	1015882	4.605437443	2.083616641	0.347269
Cal 4-2 Repet4-3	4548216	1038837	4.3781806	1.9826136	0.330436
Cal 4-2 Repet4-4	4002863	963700	4.153640137	1.882817839	0.313803
Cal 4-2 Repet4-5	3813619	870338	4.381767773	1.984207899	0.330701
Cal 4-2 Repet4-6	4630074	933124	4.961906456	2.242047314	0.373675
Cal 4-2 Repet5-1	7383268	1502605	4.913645303	2.220597912	0.3701
Cal 4-2 Repet5-2	6807671	1222694	5.56776348	2.511317102	0.418553
Cal 4-2 Repet5-3	4699464	1063535	4.418720587	2.000631372	0.333439
Cal 4-2 Repet5-4	6655532	1148060	5.797198753	2.613288335	0.435548
Cal 4-2 Repet5-5	6970754	1120179	6.222892948	2.802485755	0.467081
Cal 4-2 Repet5-6	7505208	1261081	5.951408355	2.681825936	0.446971
Cal 4-2 Repet6-1	3793752	713459	5.317407167	2.40004763	0.400008
Cal 4-2 Repet6-2	2986009	635909	4.695654567	2.123713141	0.353952
Cal 4-2 Repet6-3	4428906	863597	5.128440696	2.316062532	0.38601
Cal 4-2 Repet6-4	3650931	646696	5.645513502	2.545872668	0.424312
Cal 4-2 Repet6-5	2930555	777455	3.769420738	1.712053661	0.285342
Cal 4-2 Repet6-6	3869851	678834	5.700732432	2.570414414	0.428402
Average			4.602043	2.06943826	0.344906
SD			0.797892	0.351053697	0.058509
RSD(%)			17.33779	16.96372	16.96372

For the study of samples of raw material, the residual standard deviation obtained for the set of values of quantity of ethyl garamine as percentage of the amount of netilmicin,% RSD = 8.167631 is less than the maximum value allowed [95] for this parameter validation, 10% and can say that the method has adequate repeatability.

For the study of samples of injectables, the residual standard deviation obtained for the set of values of quantity of ethyl garamine as percentage of the amount of netilmicin, RSD = 16.96372%, is higher than the maximum allowed for this parameter validation 10%. Again, this deviation should be necessarily applied to the SPE procedure for extraction of the

complex matrix of injection. After an exhaustive study, it was not possible to reduce these deviations, which implies the use of this precision associated with this method.

3.2.2.3. Intermediate Precision

Intermediate precision expresses within-laboratories variations like different days, different analysts, different equipment, etc [94].

We made two sets of six real samples of homogeneous raw material (0.7 mg of netilmicin) and injectables (0.6 mg of netilmicin) in consecutive weeks, by the same analyst, holding constant the variables of the experimental method, determining their residual standard deviation. The results are presented in the following tables:

Table 3.5 Intermediate precision results of first weak in netilmicin raw material.

Chromatogram Weak 1	**EG Area**	**IS Area**	**EG Area/IS Area**	**EG (mg/l)**	**EG found in a sample of raw material netilmicin (In% on the amount of netilmicin)**
Cal 2 Repet.1	12554090	8500831	1.476807385	2.653801866	0.379114552
Cal 2 Repet.1-2	12582663	8750096	1.438002852	2.580308431	0.36861549
Cal 2 Repet.1-3	11101708	7505042	1.479233294	2.65839639	0.379770913
Cal 2 Repet.1-4	11907945	7304584	1.630201665	2.944321336	0.420617334
Cal 2 Repet.1-5	12493065	8454172	1.47773963	2.65556748	0.379366783
Cal 2 Repet.1-6	11620184	7306754	1.590334641	2.868815608	0.409830801
Cal 2 Repet.2	11384992	7135113	1.595628829	2.878842479	0.411263211
Chromatogram Weak 1	**EG Area**	**IS Area**	**EG Area/IS Area**	**EG (mg/l)**	**EG found in a sample of raw material netilmicin (In% on the amount of netilmicin)**
Cal 2 Repet.2-2	11085897	7334058	1.511563857	2.719628518	0.38851836
Cal 2 Repet.2-3	11377538	7308514	1.556751208	2.805210622	0.400744375
Cal 2 Repet.2-4	10575946	7098810	1.489819561	2.678446139	0.382635163
Cal 2 Repet.2-5	11140951	7298297	1.526513788	2.74794278	0.392563254
Cal 2 Repet.2-6	10850049	7322217	1.481798341	2.663254433	0.380464919

Cal 2 Repet.3	11398781	8058299	1.414539346	2.535869974	0.362267139
Cal 2 Repet.3-2	11421907	7904320	1.445020824	2.593600046	0.370514292
Cal 2 Repet.3-3	11707004	8458191	1.384102582	2.478224587	0.354032084
Cal 2 Repet.3-4	10730484	7927290	1.353613152	2.420479454	0.345782779
Cal 2 Repet.3-5	10418340	6519671	1.597985543	2.883305952	0.41190085
Cal 2 Repet.3-6	12271070	8099834	1.514977961	2.726094624	0.389442089
Cal 2 Repet.4-2	12332363	8644415	1.426627828	2.558764827	0.365537832
Cal 2 Repet.4-2	11409052	7534064	1.514329053	2.72486563	0.389266519
Cal 2 Repet.4-3	12561554	8089858	1.552753336	2.797638895	0.399662699
Cal 2 Repet.4-4	11569410	7835957	1.47645144	2.653127728	0.379018247
Cal 2 Repet.4-5	11945314	7801913	1.531075007	2.756581452	0.39379735
Cal 2 Repet.4-6	12375512	8124755	1.523185868	2.741639902	0.391662843
Cal 2 Repet.5-2	11098537	7366718	1.506578235	2.710186051	0.387169436
Cal 2 Repet.5-2	11094062	7911147	1.402332936	2.512751773	0.358964539
Cal 2 Repet.5-3	11067934	7496026	1.476506885	2.653232737	0.379033248
Cal 2 Repet.5-4	11471870	7518988	1.525719951	2.7464393	0.392348471
Cal 2 Repet.5-5	11383610	7369694	1.54465165	2.782294792	0.397470685
Cal 2 Repet.5-6	10572282	6965364	1.517836254	2.731508056	0.390215437
Cal 2 Repet.6	12518613	9365036	1.336739442	2.388521671	0.341217382
Cal 2 Repet.6-2	11794978	7103459	1.660455561	3.001620381	0.428802912
Cal 2 Repet.6-3	11088247	7507306	1.476994144	2.654155576	0.379165082
Cal 2 Repet.6-4	11675301	8292223	1.407982033	2.523450821	0.360492974
Cal 2 Repet.6-5	12358131	7932755	1.557861172	2.807312825	0.401044689
Cal 2 Repet.6-6	12841360	8273899	1.552032482	2.79627364	0.399467663

Table 3.6 Intermediate precision results of second weak in netilmicin raw material.

Cromatogram Weak 2	**EG Area**	**IS Area**	**EG Area/IS Area**	**EG (mg/l)**	**EG found in a sample of raw material netilmicin (In% on the amount of netilmicin)**
Cal 2 Repet.1	11109313	7597742	1.462186134	2.626110102	0.375158586
Cal 2 Repet.1-2	9911505	8238272	1.20310485	2.135425853	0.305060836
Cal 2 Repet.1-3	11389009	8684425	1.311429254	2.340585709	0.334369387
Cal 2 Repet.1-4	11232225	7861369	1.428787403	2.562854931	0.366122133

Cal 2 Repet.1-5	10675373	7977245	1.338228047	2.391340999	0.341620143
Cal 2 Repet.1-6	11002104	8043911	1.367755561	2.44726432	0.349609189
Cal 2 Repet.2	11092706	8794251	1.261358813	2.245755327	0.32082219
Cal 2 Repet.2-2	10427174	7802301	1.336422935	2.387922225	0.341131746
Cal 2 Repet.2-3	12628549	8624690	1.464232222	2.629985268	0.375712181
Cal 2 Repet.2-4	11848567	8387984	1.41256433	2.532129413	0.361732773
Cal 2 Repet.2-5	11269815	8131965	1.385866147	2.481564672	0.354509239
Cal 2 Repet.2-6	11944321	8248205	1.448111559	2.59945371	0.37135053
Cal 2 Repet.3	12299300	8688972	1.415506921	2.537702501	0.362528929
Cal 2 Repet.3-2	12517679	7935014	1.577524501	2.844553979	0.406364854
Cal 2 Repet.3-3	11761652	7668571	1.533747552	2.761643091	0.394520442
Cal 2 Repet.3-4	12624912	8227283	1.534517775	2.763101846	0.394728835
Cal 2 Repet.3-5	6694517	5248016	1.275628161	2.272780608	0.324682944
Cal 2 Repet.3-6	12558291	8265430	1.519375398	2.734423103	0.390631872
Cal 2 Repet.4-2	13244677	7934826	1.669183042	3.018149701	0.431164243
Cal 2 Repet.4-2	13265168	7789474	1.702960688	3.082122515	0.440303216
Cal 2 Repet.4-3	10540038	7047959	1.495473796	2.689154917	0.384164988
Cal 2 Repet.4-4	10362274	6884469	1.505166775	2.707512831	0.386787547
Cal 2 Repet.4-5	11906109	7988436	1.490418024	2.679579591	0.382797084
Cal 2 Repet.4-6	13186388	8495908	1.552086958	2.796376814	0.399482402
Cal 2 Repet.5-2	13301103	7817411	1.701471625	3.079302321	0.439900332
Cromatogram Weak 2	**EG Area**	**IS Area**	**EG Area/IS Area**	**EG (mg/l)**	**EG found in a sample of raw material netilmicin (In% on the amount of netilmicin)**
Cal 2 Repet.5-2	14025868	8472890	1.655381812	2.992011007	0.427430144
Cal 2 Repet.5-3	10942470	7492372	1.460481407	2.622881453	0.37469735
Cal 2 Repet.5-4	12945284	8067620	1.604597638	2.895828861	0.413689837
Cal 2 Repet.5-5	11283875	7491743	1.506174865	2.709422092	0.387060299
Cal 2 Repet.5-6	12467825	7149634	1.743841013	3.159547374	0.451363911
Cal 2 Repet.6	11439200	7646289	1.49604599	2.690238618	0.384319803
Cal 2 Repet.6-2	14141513	8273336	1.709287886	3.094105844	0.442015121
Cal 2 Repet.6-3	13564765	8098379	1.674997552	3.02916203	0.432737433
Cal 2 Repet.6-4	14101797	7912924	1.782122133	3.232049495	0.461721356

Cal 2 Repet.6-5	11409925	7926780	1.43941487	2.582982708	0.36899753
Cal 2 Repet.6-6	13632270	8203804	1.661701084	3.003979326	0.429139904
Average					0.385697
SD					0.030993
RSD(%)					8.035535

Table 3.7 Intermediate precision results of first weak in netilmicin injectables.

Chromatogram Weak 1	**EG Area**	**IS Area**	**EG Area/IS Area**	**EG (mg/l)**	**EG found in a sample of netilmicin injectable (In% on the amount of netilmicin)**
Cal 2 Repet.1	3329900	736059	4.523957998	2.047403555	0.341233926
Cal 2 Repet.1-2	3551850	677817	5.240131186	2.365702749	0.394283792
Cal 2 Repet.1-3	3826101	656248	5.830266911	2.627985294	0.437997549
Cal 2 Repet.1-4	3396242	779524	4.356815185	1.97311786	0.328852977
Cal 2 Repet.1-5	3299425	771333	4.27756235	1.937894378	0.322982396
Cal 2 Repet.1-6	3920980	672026	5.834565925	2.629895966	0.438315994
Chromatogram Weak 1	**EG Area**	**IS Area**	**EG Area/IS Area**	**EG (mg/l)**	**EG found in a sample of netilmicin injectable (In% on the amount of netilmicin)**
Cal 2 Repet.2	4943247	1232797	4.009781821	1.818880809	0.303146802
Cal 2 Repet.2-2	4808611	1297291	3.706655639	1.684158062	0.28069301
Cal 2 Repet.2-3	4861908	1259763	3.859383074	1.752036922	0.292006154
Cal 2 Repet.2-4	4689453	960137	4.884149866	2.207488829	0.367914805
Cal 2 Repet.2-5	4121754	1045629	3.941889523	1.788706455	0.298117742
Cal 2 Repet.2-6	5033374	1143001	4.403647941	1.993932418	0.33232207
Cal 2 Repet.3	5520869	994643	5.550603583	2.503690482	0.417281747
Cal 2 Repet.3-2	5810406	982096	5.916332008	2.666236448	0.444372741
Cal 2 Repet.3-3	5655202	936895	6.03611077	2.719471453	0.453245242
Cal 2 Repet.3-4	5138100	1027299	5.001562349	2.259672155	0.376612026
Cal 2 Repet.3-5	5839985	1063193	5.492873824	2.478032811	0.413005468
Cal 2 Repet.3-6	5724491	1038434	5.512619001	2.486808445	0.414468074
Cal 2 Repet.4-2	3798004	811241	4.681720968	2.11752043	0.352920072
Cal 2 Repet.4-2	3494387	860282	4.061908769	1.842048342	0.307008057

Cal 2 Repet.4-3	3550730	923574	3.844553874	1.745446166	0.290907694
Cal 2 Repet.4-4	3731973	691930	5.393570159	2.433897848	0.405649641
Cal 2 Repet.4-5	3411317	727274	4.690552667	2.12144563	0.353574272
Cal 2 Repet.4-6	3675233	763456	4.813942126	2.176285389	0.362714232
Cal 2 Repet.5-2	4169209	665141	6.26815818	2.822603636	0.470433939
Cal 2 Repet.5-2	5195443	771024	6.738367418	3.031585519	0.505264253
Cal 2 Repet.5-3	4750346	779467	6.094351653	2.74535629	0.457559382
Cal 2 Repet.5-4	4946389	833042	5.937742635	2.675752282	0.445958714
Cal 2 Repet.5-5	4961880	748201	6.631747351	2.984198823	0.49736647
Cal 2 Repet.5-6	4619449	791186	5.838638449	2.631705977	0.438617663
Cal 2 Repet.6	2543008	471343	5.395238712	2.434639427	0.405773238
Cal 2 Repet.6-2	2899421	462636	6.26717549	2.822166884	0.470361147
Cal 2 Repet.6-3	2670184	563189	4.741186351	2.143949489	0.357324915
Cal 2 Repet.6-4	2438394	580351	4.201584903	1.904126624	0.317354437
Cal 2 Repet.6-5	3008296	603491	4.984823303	2.252232579	0.375372097
Cal 2 Repet.6-6	2922961	612771	4.770070712	2.156786983	0.359464497

Table 3.8 Intermediate precision results of second weak in netilmicin injectables.

Chromatogram Weak 2	EG Area	IS Area	EG Area/IS Area	EG (mg/l)	EG found in a sample of netilmicin injectable (In% on the amount of netilmicin)
Cal 2 Repet.1	6665369	1291202	5.162142717	2.331041207	0.388506868
Cal 2 Repet.1-2	7020506	1535748	4.571391921	2.068485298	0.34474755
Cal 2 Repet.1-3	7003435	1105285	6.336315973	2.852895988	0.475482665
Cal 2 Repet.1-4	7192105	1342154	5.358628742	2.41836833	0.403061388
Cal 2 Repet.1-5	6955550	1350213	5.151446475	2.326287322	0.387714554
Cal 2 Repet.1-6	7803315	1418066	5.502786894	2.48243862	0.41373977
Cal 2 Repet.2	6738247	1273986	5.289106003	2.387469335	0.397911556
Cal 2 Repet.2-2	7077106	1340427	5.279739963	2.38330665	0.397217775
Cal 2 Repet.2-3	6615011	1115386	5.930692155	2.672618736	0.445436456
Cal 2 Repet.2-4	7345180	1164696	6.306521187	2.839653861	0.473275643
Cal 2 Repet.2-5	7115805	1392644	5.109564971	2.307673321	0.38461222
Cal 2 Repet.2-6	7001778	1252288	5.591188289	2.521728128	0.420288021
Cal 2 Repet.3	6712706	1238123	5.421679429	2.446390857	0.40773181
Cal 2 Repet.3-2	6912855	1246204	5.547129523	2.502146455	0.417024409
Cal 2 Repet.3-3	6589121	1281469	5.141849705	2.322022091	0.387003682
Cal 2 Repet.3-4	6469131	1380626	4.685650567	2.119266919	0.353211153
Cal 2 Repet.3-5	6347646	1357802	4.674942296	2.114507687	0.352417948
Cal 2 Repet.3-6	6499885	978401	6.643375262	2.989366783	0.498227797
Cal 2 Repet.4-2	6965404	1490632	4.672785771	2.113549232	0.352258205
Cal 2 Repet.4-2	6937973	1429687	4.852791555	2.193551802	0.365591967
Cal 2 Repet.4-3	3602873	712808	5.054478906	2.283190625	0.380531771
Cal 2 Repet.4-4	6896860	1418326	4.862676141	2.197944951	0.366324159
Cal 2 Repet.4-5	6540772	1264665	5.171940395	2.335395731	0.389232622
Cal 2 Repet.4-6	6681414	1346807	4.960929072	2.241612921	0.373602153
Cal 2 Repet.5-2	8443309	1303100	6.479402195	2.916489864	0.486081644
Chromatogram Weak 2	**EG Area**	**IS Area**	**EG Area/IS Area**	**EG (mg/l)**	**EG found in a sample of netilmicin injectable (In% on the amount of**

					netilmicin)
Cal 2 Repet.5-2	8061198	1014732	7.944164568	3.567495363	0.594582561
Cal 2 Repet.5-3	7875424	1055314	7.462635765	3.353482562	0.55891376
Cal 2 Repet.5-4	4255462	679036	6.266916629	2.822051835	0.470341973
Cal 2 Repet.5-5	7984909	1233216	6.474866528	2.914474012	0.485745669
Cal 2 Repet.5-6	8464290	1118431	7.568003748	3.400312777	0.566718796
Cal 2 Repet.6	7322822	1022901	7.158876568	3.218478475	0.536413079
Cal 2 Repet.6-2	7321112	1037579	7.055956221	3.172736098	0.52878935
Cal 2 Repet.6-3	4125154	727056	5.673777536	2.55843446	0.426405743
Cal 2 Repet.6-4	7706802	956835	8.054473342	3.616521485	0.602753581
Cal 2 Repet.6-5	7264884	835826	8.691861703	3.899805201	0.649967534
Cal 2 Repet.6-6	6883381	979227	7.029402784	3.160934571	0.526822428
Average					0.414432854
SD					0.078568
RSD(%)					18.95802

With regard to raw material, the residual standard deviation obtained for the set of values of relative percentage of ethyl-garamine in netilmicin is less than the value desirable for this parameter, 10% [95], indicating that the method has a average precision adequate for the purpose intended.

As to samples of injectables, the residual standard deviation values exceed the most desirable value. With this extraction methodology, SPE, which alone is effective in extracting the components from the matrix injection, it was not possible to obtain better precision.

3.2.3. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found, being also termed trueness [94].

In the initial study of accuracy have been added quantities of EG in three levels of concentration in the range of work established (0.2, 15 and 50mg / l) for the raw material and (1.5, 15 and 20mg / l) for injectables. The results of accuracy, the percentage of

recovery obtained were 90.0% for raw materials and 90.3% for injectables. It has been suggested to modify these concentrations.

For the study of accuracy in samples of raw materials, water solutions were prepared with 0.4% trifluoroacetic acid to which were added EG corresponding to about 60%, 100% and 140% of target concentration and proceeded then as described in the analytical protocol. It is understood that as a target concentration that was calculated in real samples and homogeneous through the calibration curve established. Tables 3.9 and 3.10 describe the results obtained for accuracy in raw material and injectables.

Table 3.9 Accuracy in netilmicin raw material.

Level	Chromatogram	EG added (mg)	EG Area/IS Area	EG recovered (mg)	Recovery (%)
60% (1,8mg/l)	Exac 60%-1	1.8	0.786834797	1.722986716	95.72148424
	Exac 60%-2	1.8	0.882248201	1.927298074	107.0721152
	Exac 60%-3	1.8	0.961576872	2.09716675	116.5092639
100% (3mg/l)	Exac 100%-1	3.0	1.358252945	2.946580181	98.21933937
	Exac 100%-2	3.0	1.29021512	2.800888909	93.36296362
	Exac 100%-3	3.0	1.444721054	3.131736731	104.3912244
Level	**Chromatogram**	**EG added (mg)**	**EG Area/IS Area**	**EG recovered (mg)**	**Recovery (%)**
140% (4,2mg/l)	Exac 140%-1	4.2	2.036561491	4.399061009	104.7395478
	Exac 140%-2	4.2	1.836825826	3.971361511	94.55622645
	Exac 140%-3	4.2	2.177597497	4.701065304	111.9301263
Average					102.944699
SD					8.091390384
%RSD					7.859938841

Table 3.10 Accuracy in netilmicin injectables.

Level	Chromatogram	EG added (mg)	EG Area/IS Area	EG recovered (mg)	Recovery (%)
60% (1,2mg/l)	Exac 60%-1	1.2	0.82887999	1.274683897	106.2236581
	Exac 60%-2	1.2	0.74973917	1.160975813	96.74798442
	Exac 60%-3	1.2	0.88332704	1.352912414	112.7427012
100% (2mg/l)	Exac 100%-1	2.0	1.58053862	2.354653188	117.7326594
	Exac 100%-2	2.0	1.38590753	2.075010824	103.7505412
	Exac 100%-3	2.0	1.53644926	2.291306411	114.5653206
140% (2,8mg/l)	Exac 140%-1	2.8	2.14673726	3.168156989	113.1484639
	Exac 140%-2	2.8	2.18893253	3.228782375	115.3136562
	Exac 140%-3	2.8	2.13690787	3.154034292	112.6440819
Average					110.3187852
SD					6.720447469
%RSD					6.091843249

The results for accuracy, combined with the residual standard deviation, are within the accepted parameters for the determination of impurities, are at the limit of the allowed range of 90-110% recovery for the raw material [95]. For injectables the value of recovery associated with residual standard deviation slightly exceeds these limits, which are the best possible results given the complexity of extracting the impurity matrix injection.

3.2.4. Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample [94].The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure [94].

Standard solutions were prepared as described in the analytical protocol for both the determination of EG in raw material, as in injectable. Tables 3-11 and 3.12 show the results for the analysis of prepared calibration standards. Internal standard concentration used was 9.92 mg/l.

Table 3.11 Calibration standards in netilmicin raw material.

Chromatogram 29/05/07	**EG Concentration (mg/l)**	**EG Area/ IS Area**
Cal 0 -1	0	0.021753554
Cal 0 -2		0.015021558
Cal 0 -3		0.011790806
Cal 0 -4		0.023987858
Cal 0 -5		0.017756748
Chromatogram 29/05/07		**EG Area/ IS Area**
Cal 0 -6		0.014809762
Cal 0,2 -1	0.307	0.175785615
Cal 0,2 -2		0.157955642
Cal 0,2 -3		0.166676346
Cal 0,2 -4		0.156452126
Cal 0,2 -5		0.168609941
Cal 0,2 -6		0.193303738
Cal 1,5 -1	1.533	0.89050871
Cal 1,5 -2		0.872618134
Cal 1,5 -3		0.912076428
Cal 1,5 -4		1.012920053
Cal 1,5 -5		0.988052825
Cal 1,5 -6		0.947349426
Cal 10 -1	9.808	5.483398016
Cal 10 -2		5.623500816
Cal 10 -3		5.67502742
Cal 10 -4		5.645857229
Cal 10 -5		5.699708742
Cal 10 -6		5.722738468
Cal 15 -1	15.019	8.243363673
Cal 15 -2		8.377242275
Cal 15 -3		8.83142768
Cal 15 -4		8.747047761
Cal 15 -5		8.470022451
Cal 15 -6		8.658118017
Cal 20 -1	19.923	11.57284628
Cal 20 -2		10.40775863
Cal 20 -3		9.983272065

Cal 20 -4		10.75285746
Cal 20 -5		10.12851341
Cal 20 -6		10.76469817
Cal 50 -1	50	22.68926325
Cal 50 -2		22.53228418
Cal 50 -3		22.84790694
Cal 50 -4		22.69455378
Cal 50 -5		23.91020999
Cal 50 -6		23.47708974

Table 3.12 Calibration standards in netilmicin injectables.

Cromatogram 14/05/2007	**EG Concentration (mg/l)**	**EGArea/ IS Area**
Cal 0 -1	0	0
Cal 0 -2		0
Cal 0 -3		0
Cal 0 -4		0
Cal 0 -5		0
Cal 0 -6		0
Cal 0.2 -1	0.1839	0.429219195
Cal 0.2 -2		0.305700159
Cal 0.2 -3		0.264146756
Cal 0.2 -4		0.387101764
Cal 0.2 -5		0.343495282
Cal 0.2 -6		0.280740663
Cal 1.5 -1	1.532	2.830588958
Cal 1.5 -2		3.277392254
Cal 1.5 -3		3.033042359
Cal 1.5 -4		3.099216391
Cal 1.5 -5		3.804288909
Cal 1.5 -6		3.09045974
Cal 10 -1	9.808	21.90177647
Cal 10 -2		23.26490926
Cal 10 -3		22.74682745
Cal 10 -4		21.44129586
Cal 10 -5		21.91661774
Cal 10 -6		22.07459107
Cal 15 -1	15.325	31.15994288
Cal 15 -2		31.75684702
Cal 15 -3		29.30031335

Cal 15 -4		35.21807175
Cal 15 -5		30.50687621
Cal 15 -6		33.85962195
Cal 20 -1		54.6749683
Cal 20 -2		53.65015387
Cal 20 -3		56.34000708
Cromatogram 14/05/2007	19.616	**EGArea/ IS Area**
Cal 20 -4		60.40007503
Cal 20 -5		50.84719076
Cal 20 -6		39.20228565

As for the results obtained for the raw material, was made a linear calibration curve and its correlation coefficient ($y = 0.462x + 0.618$, $r^2 = 0.992$). The linearity of the calibration function was tested using Fisher ratio revealing that the data exhibit non-uniform variance ($SN^2/S1^2 < F_{tabled}$ [95%, n-1]), needing to make a more appropriate statistical method, weighted regression, so as to give greater weight to points with higher precision [93].

The same treatment was made for the data obtained for injectables. The linear regression calibration curve was obtained and its correlation coefficient ($y = 1.97x + 2.36$, $r^2 = 0.977$). There was also a lack of homogeneity of variance and also weighted regression was used to treat the results.

This result took us to choose weighted least squares regression, by applying at each point weighting factors inversely proportional to the variance in that point. The individual weights were calculated with 3.1. The slope and intercept were obtained. The calibration curves for weighted regression obtained for the two methodologies are:

Raw material: $y = 0.528x + 0.0756$

Injection: $y = 2.25x - 0.0827$

In practice, the slope and intercept of the regression are similar to weight regression, the major difference lies in the calculation of confidence limits associated with readings of concentrations of real samples. This approach seems to us much safer to use, since the

methods developed for the analysis of injectables exceed the maximum limits for certain validation parameters.

3.2.5. LOD and LOQ

LOD or detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value [94]. LOQ or quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy [94]. The limits of detection and quantification were determined using the acceptance criteria based on signal to noise using S/N ratios of 3 and 10, respectively [94].

3.2.6. Summary of Validation Parameters

The results for the validation parameters studied in both matrices, API and injectables, are summarized in Table 3.13.

Table 3.13 Validation parameters obtained for the validation methods of quantification of EG in netimicin raw material and injectables.

Parameters		**API**			**Final Product**		
Accuracy		0.2mg/l	90.7%	**Average**	1.5mg/l	84.2%	**Average**
		15mg/l	95.3%	90.0%	15mg/l	89.9%	90.3%
		50mg/l	84.0%		20mg/l	96.7%	
Accuracy (using target concentration)		1.8mg/l	106.4%	**Average**	1.2mg/l	105.2%	**Average**
		3.0mg/l	98.7%	102.9%	2.0mg/l	112.0%	110.3%
		4.2mg/l	103.7%		2.8mg/l	113.7%	
System precision		%RSD=3.48			%RSD=14.2		
Intra-assay precision		%RSD=4.92			%RSD=16.8		
Intermediate Precision		%RSD=7.46			%RSD=17.6		
LOD		0.1mg/l			0.2mg/l		
LOQ		0.2mg/l (%RSD=6.12; 90.7%)			0.4mg/l (%RSD=7.64; 87.9%)		
Linearity	**Least Squares**	$y = 0.462x + 0.618$ $r^2 = 0.992$			$y = 1.97x + 2.36$ $r^2 = 0.977$		
	Weighted Least Squares	$y = 0.528x + 0.0756$			$y = 2.25x - 0.0827$		
Range		0.2-50mg/l			0.4-20mg/l		

3.3. Conclusion

A simple, sensitive, accurate and precise SPE-LC-MS method was developed and validated for the first time to quantify 1-*N*-ethyl-garamine in API. A simple and sensitive method, with a respectable accuracy and precision for injectables was developed. Simple solid phase extraction method was used to extract the analytes and an LC method was developed to analyze the samples. The sensitive detection was achieved using mass spectrometry detector and it was possible to quantify 1-*N*-ethyl-garamine in concentrations of 0.03%(*m/m*) in API samples and 0.06%(*m/m*) in intravenous formulations. The developed method was used to determine the 1-*N*-ethyl-garamine in various batches of commercial formulations.

The use of pharmaceuticals is always a balance between benefits and risks, but the same is not true for impurities in pharmaceuticals, impurities convey only risk. The challenge for regulatory agencies is to promulgate standards that assure that unavoidable drug impurities impart no or acceptable levels of risk. Our initial objective of developing a new methodology more sensitive in the determination of a known impurity was achieved. It was possible to validate a method, make the determination whether the content of impurity in the API or final product, according to the rules and thresholds for degradation product

CHAPTER 4

ALPRAZOLAM

Anxiolytic is a drug used for the treatment of anxiety disorders. These drugs are increasingly used as they help the treatment of various diseases of the modern world. As pharmaceutical drugs and having large use there is a greater concern in the study of their stability and impurities.

There are several anxiolytic drug classes. The current study focused on alprazolam, a drug belonging to the class of benzodiazepines. A literature review of the chemistry of alprazolam and related impurities and previously reported methods for their characterization is presented. This chapter deals with structural identification and characterization of impurities in alprazolam formulations, their study in various commercial brands (solid dosage forms of the API alprazolam) in long-run storage and forced degradation stability studies. Alprazolam degradation studies were performed in order to understand its predominant degradation mechanism. The synthesis and complete characterization of the major impurity is described.

4.1. Introduction

4.1.1. Alprazolam, a triazolobenzodiazepine

Benzodiazepines have the basic classical structure of 5-aryl-1,4-benzodiazepine, characterized by a benzene ring connected to a 1,4-diazepine ring in the fifth and ninth positions (Figure 4.1).

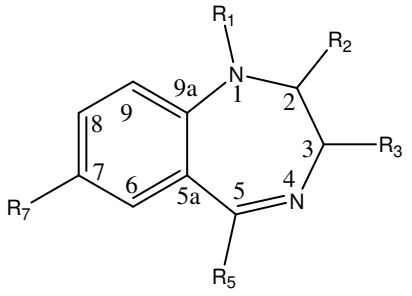


Figure 4.1 Scheme of 1,4-Benzodiazepines.

Since the discovery of diazepam and chlordiazepoxide more than three decades ago, new benzodiazepines have been introduced for the treatment of a broad spectrum of clinical disorders [98]. Benzodiazepines are prescribed as anxiolytics, sedatives, hypnotics, anticonvulsants, muscle relaxants, and some of them, are prescribed as antiepileptics [98-102]. Among the compounds of this family we find different durations of action. They are also prone to be abused and often used for suicidal and criminal purposes [98, 103]. Its popularity is due to the clinical margin of safety in use, the minimum side effects caused by them and the low potential for physical dependence [99]. More recently, a new generation of benzodiazepines belonging to the class of triazolobenzodiazepine (Figure 4.2) such as triazolam, estazolam and alprazolam, have set their importance due to their potent action at low doses [98].

Benzodiazepines are moderately polar compounds with low volatility, high boiling point, having the diazepine ring, an electron attractor group [102].

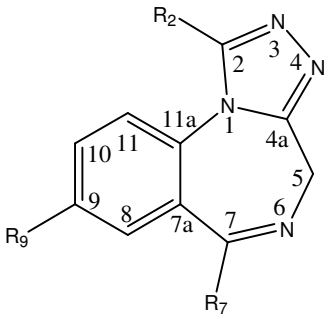


Figure 4.2 Scheme of Triazolobenzodiazepines.

The API 8-chloro-1-methyl-6-phenyl-*4H*-[1,2,4]triazole[4,3-a][1,4] benzodiazepine, commonly known as alprazolam (AL), is a compound of the benzodiazepines family with the following structure:

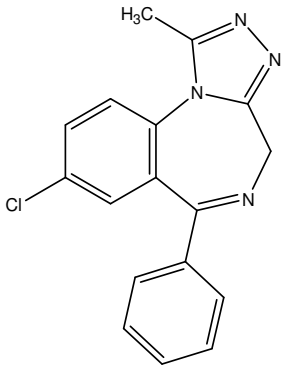
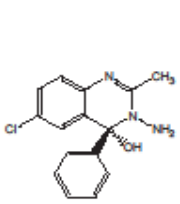


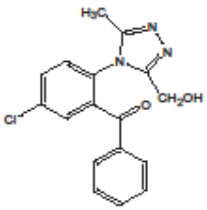
Figure 4.3 Scheme of Alprazolam.

The European Pharmacopoeia [104] describes various impurities associated with this compound, which are presented in Table 4.1.

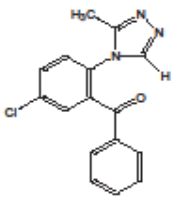
Table 4.1 Alprazolam related compounds structures.



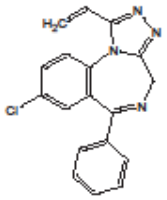
Impurity A
MW=287.74
(4RS)-3-amino-6-chloro-2-methyl-4-phenyl-3,4-dihydroquinazolin-4-ol



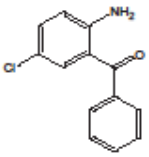
Impurity B
MW=327.76
[5-chloro-2-[3-(hydroxymethyl)-5-methyl-4H-1,2,4-triazol-4-yl]phenyl]phenylmethanone



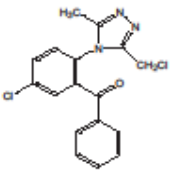
Impurity C (Imp C)
MW=297.73
[5-chloro-2-[3-methyl-4H-1,2,4-triazol-4-yl]phenyl]phenylmethanone



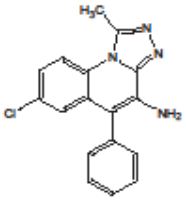
Impurity D
MW=320.77
8-chloro-1-ethenyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepine



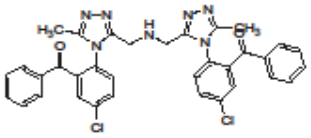
Impurity E
MW=231.67
(2-amino-5-chlorophenyl)phenylmethanone



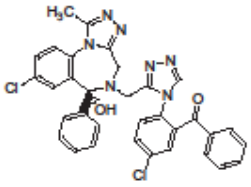
Impurity F
MW=346.21
[5-chloro-2-[3-(chloromethyl)-5-methyl-4H-1,2,4-triazol-4-yl]phenyl]phenylmethanone



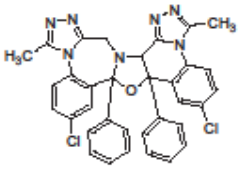
Impurity G (Imp G)
(also known by triazoaminoquinolaine)
MW=308.76
7-chloro-1-methyl-5-phenyl[1,2,4]triazolo[4,3-a]quinolin-4-amine



Impurity H
MW=636.52
bis[[4-(2-benzoyl-4-chlorophenyl)-5-methyl-4H-1,2,4-triazol-3-yl]methyl]amine



Impurity I
MW=622.50
[5-chloro-2-[3-[[(6RS)-8-chloro-6-hydroxy-1-methyl-6-phenyl-4H-[1,2,4]triazolo[4,3-a][1,4]benzodiazepin-5(6H)-yl]methyl]-5-methyl-4H-1,2,4-triazol-4-yl]phenyl]phenylmethanone



Impurity J
MW=618.51
2,17-dichloro-6,13-dimethyl-18b,19a-diphenyl-8b,19a-dihydro-10H,18bH-[1,2,4]triazolo[4'',3'':1'',2'']quinolo[3'',4'':4',5']oxazolo[3',2'-d]-1,2,4-triazolo[4,3-a][1,4]benzodiazepine

Alprazolam (AL) is extensively use for the control of panic attacks and in the management of anxiety disorders [105] being described their synthesis from the condensing of 1,3-dihydro-5-phenyl-*2H*-1,4-benzodiazepine-2-thione with an organic acid hydrazide [106]. The concern of the pharmaceutical industry with the quality of the product, in particular with the percentage of known and unknown impurities in the commercial formulation during the life time of the drug, stimulates the studies for the identification and quantification of the impurities of AL formulations. The stability of AL has been studied with respect to the photostability and excipient influence using LC-MS [107], spectrofluorimetric assay [108], capillary electrophoresis [109] and HPLC [110-112].

In 2002 Nudelman and Cabrera found that the drug is sensitive to both artificial light and to sunlight, the main degradation products being triazolaminoquinoleine (Impurity G), 5-chloro-[5-methyl-4H-1,2,4-triazol-4-yl]benzophenone and 1-methyl-6-phenyl-4H-s-triazolo[4,3-a]-1,4-benzodiazepinone (registered respectively as Imp G and Imp C in the Pharmacopeia [110].

In 2005, the same group reported studies of the hydrolysis and photodegradation of AL, emphasizing that Imp G is the result of a photodegradation. The authors suggested that the ring opening is a reversible reaction and they detected the presence of the opened-ring AL in the reaction mixture only by NMR analysis [113]. The different stability of AL against hydrolysis when it is in the presence of different excipients was studied concluding that Imp G is formed from the reaction of opened-ring AL with magnesium stearate and carboxymethyl cellulose (CMC) [111]. Barbas *et al.,* in 2007 [107], verified that Imp G, is formed rapidly in the presence of excipients under high temperature and humidity conditions, independently of the presence of light. An LC method for the determination of AL and Imp G was developed and validated but no fragmentation profile was achieved in MS [107]. Studying the influence of the different excipients, the authors conclude that AL degradation might be mediated by the Maillard reaction, including ring opening of Alprazolam and lactose leading to Imp G [114].

4.2. Characterization of the principal impurity of alprazolam

4.2.1. LC-MS-MS Studies

The impurity composition was analyzed by LC-MS-MS. The impurity produced a chromatographic peak with same retention time, the same spectrum and the same fragmentation profile of the impurity found in samples of tablets of alprazolam as seen in Figures 4.4 and 4.5.

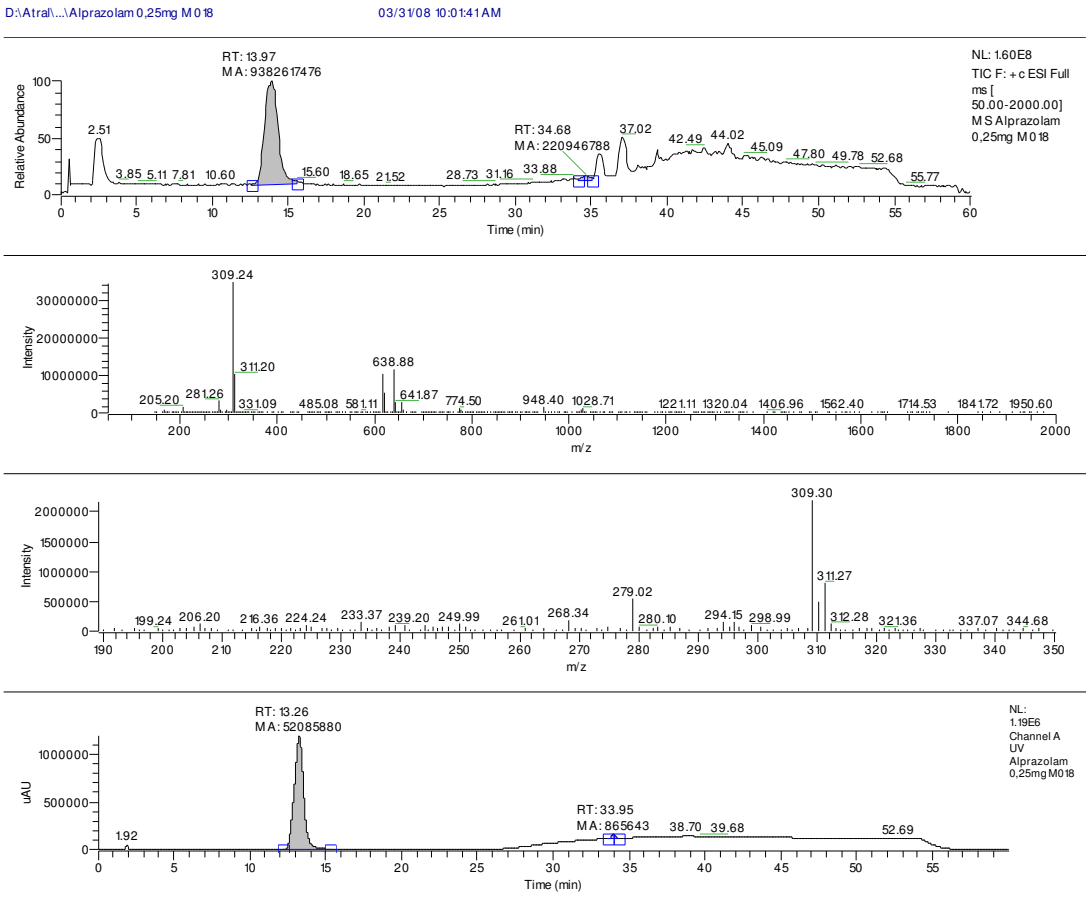


Figure 4.4 Analysis of Alprazolam tablet. (In the first cell we can see the full MS chromatogram; on the second and third cells we have the mass spectra peaks of 13.97 and 34.68 min, respectively. In the last cell we observe the chromatogram obtained by the detector photodiode array at λ=254 nm)

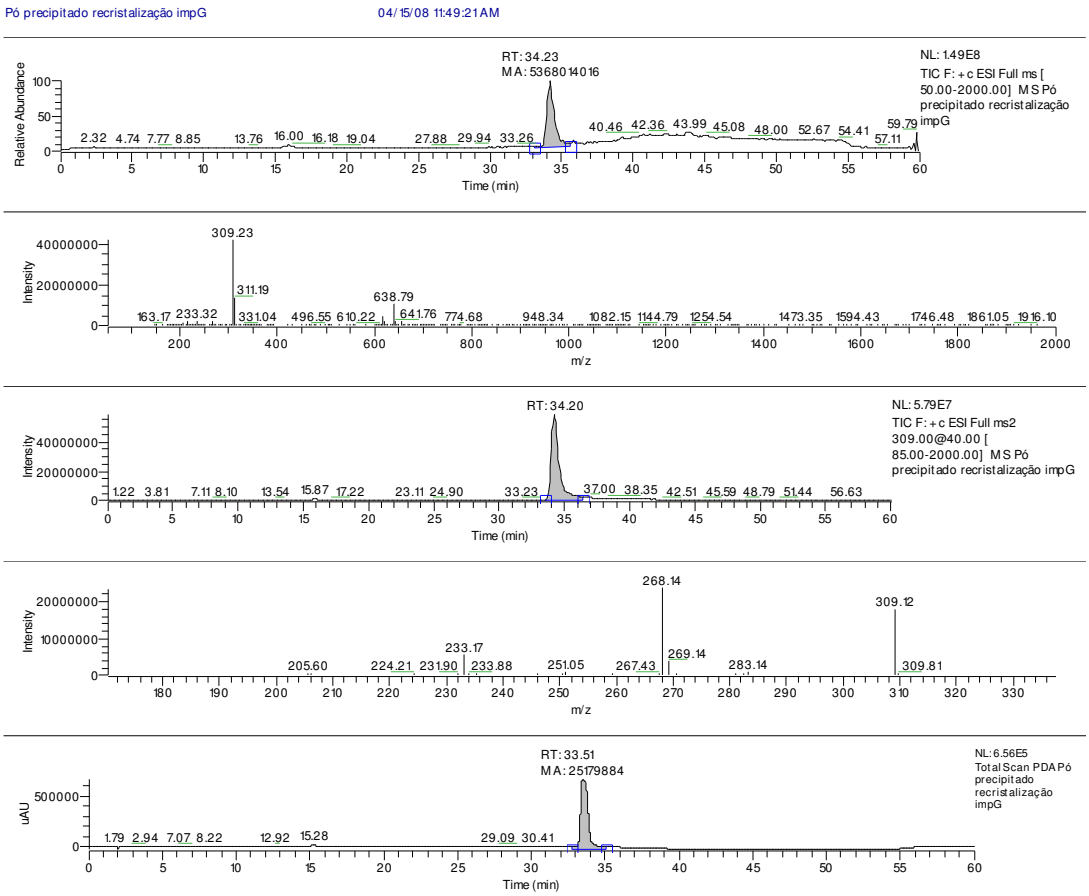


Figure 4.5 Analysis of the triazoaminoquinoleine (Imp G) synthesised. (In the first cell we can see the full MS chromatogram; on the second cell we have the mass spectra peak 34.23 min, on the third cell we have the SIM chromatogram of $[M+1]^+$=309, with the corresponding mass spectrum in the four cell. In the last cell we observe the chromatogram obtained by the detector photodiode array at λ=254 nm)

4.2.2. NMR Studies

Solutions of alprazolam API and synthesized impurity were analyzed by proton NMR, according to the procedure described in experimental section, and originated in deuterated methanol the spectra presented in Figures 4.6 and 4.7.

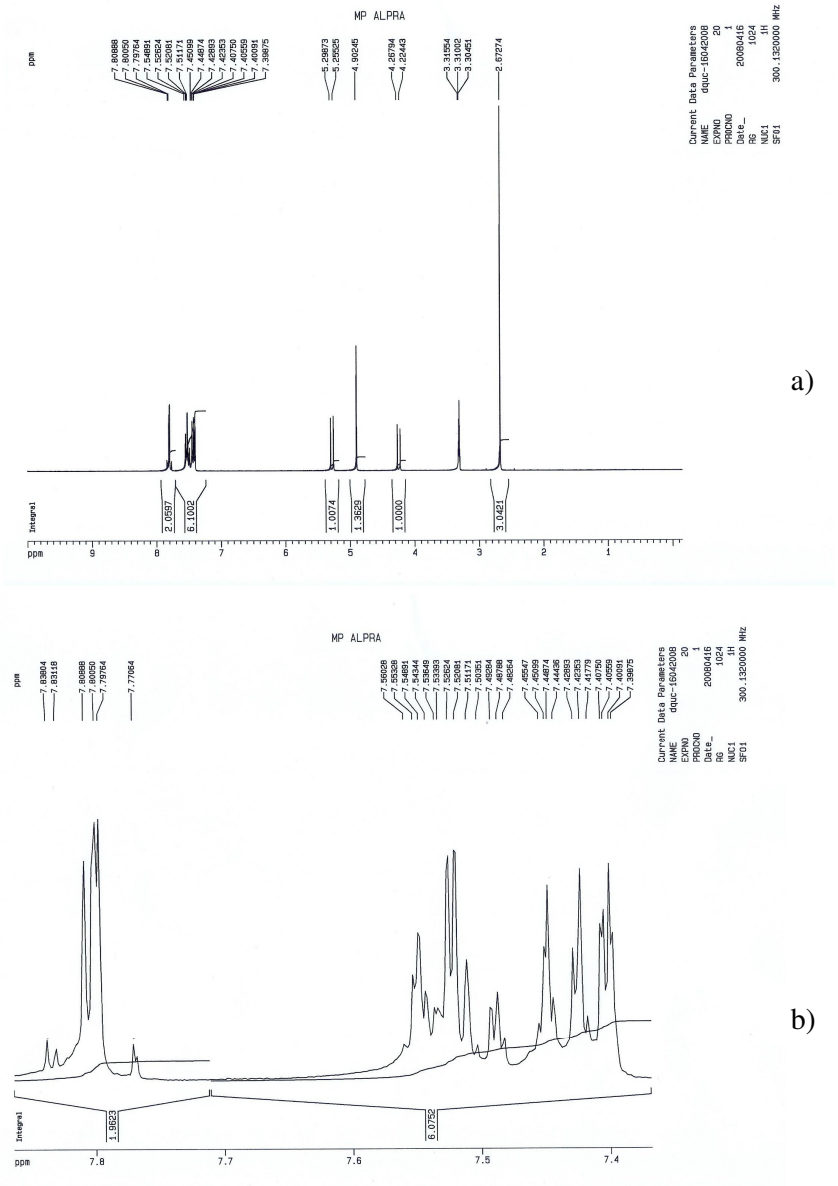


Figure 4.6 NMR analysis of API alprazolam. [a) Full spectra; b) expansion between 7.9 – 7.3 ppm]

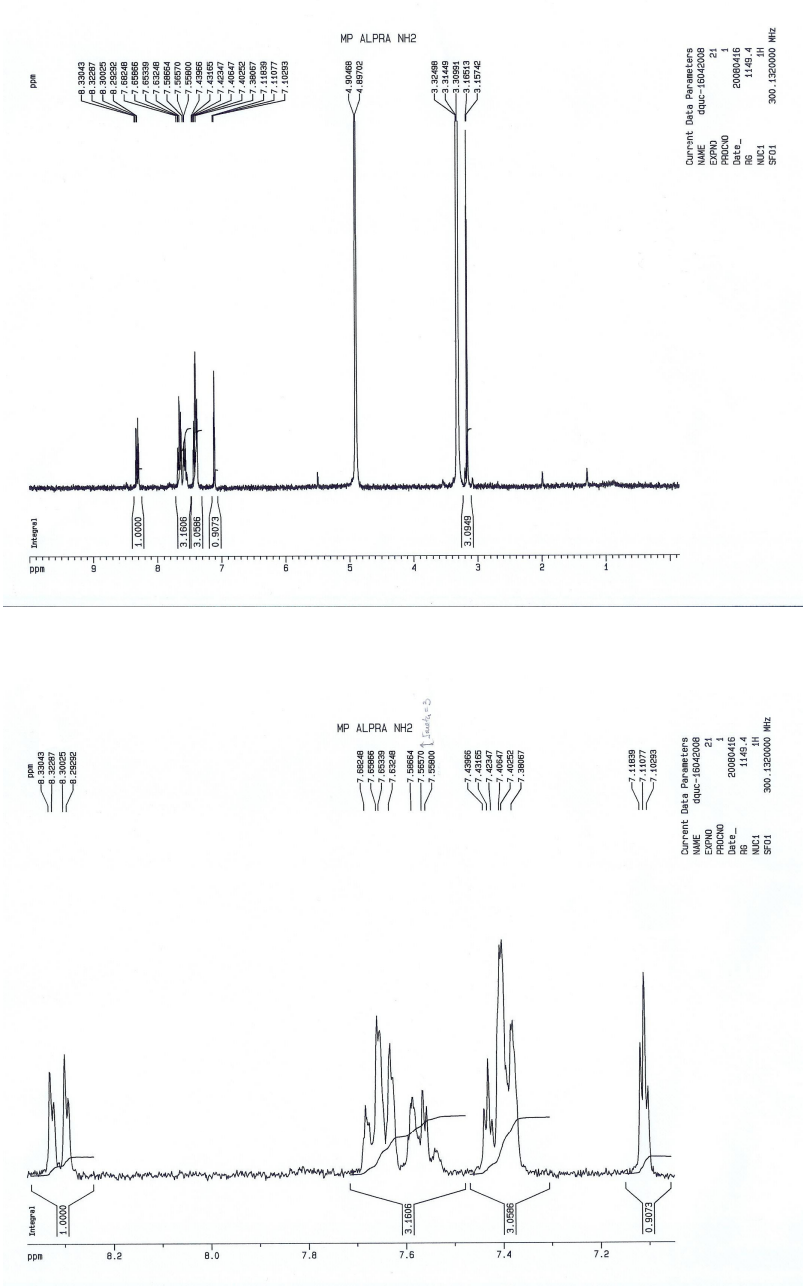


Figure 4.7 NMR analysis of triazoaminoquinoleine (Imp G) synthesised. [a) Full spectra; b) expansion between 8.4 – 7.0 ppm]

The spectra obtained allow us to make the assignments presented in Tables 4.2 and 4.3 for the AL and Imp G, respectively.

Table 4.2 Proton spectrum of AL (Peak at 4.9ppm correspond to MeOD; Ph-Phenyl group)

Chemical shift δ (ppm)	Assignment	Multiplicity
7.83 – 7.77	Protons H_7 and H_9	Multiplet
7.56 – 7,39	Protons Ph and H_{10}	Multiplet
4.22; 4.26 5.25; 5.29	Proton H_4	AX Sistem
2.67	Protons CH_3 (1)	Singlet

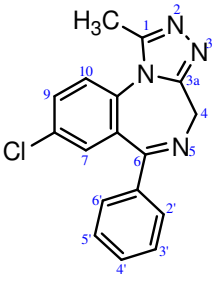
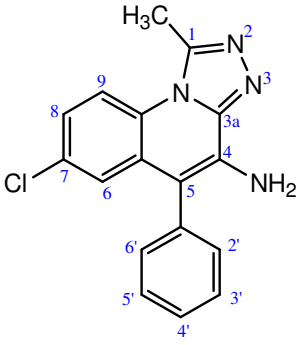


Table 4.3 Proton spectrum of Imp G (Peak at 4.9ppm correspond to MeOD; Ph-Phenyl group)

Chemical shift δ (ppm)	Assignment	Multiplicity
8.31	Proton H_8	Double Duplet
7.68 – 7.55	2 Protons Ph e H_6	Multiplet
7.44 – 7.80	2 Protons Ph e H_9	Multiplet
7,11	1 Proton Ph	Triplet
3.16	Protons CH_3 (1)	Singlet



Analyzing the results presented in the tables we can conclude that there are significant differences between the two compounds. As observed in the spectrum of alprazolam, the singlet methyl group with chemical shift is at 2.67 ppm and for the Imp G this singlet signal comes in lower fields (3.16ppm), suggesting an effect of deprotection probably related to the aromaticity of the rings. The typical sign of the methylene group (AX system) which is observed in the ^1H spectrum of alprazolam is not present in the spectrum of impurity G indicative of the disappearance of the diazepin group.

4.2.3. FTIR Studies

Since the amine was not observed in the NMR spectrum, because there is exchange with the solvent used to solubilize (-NH_2 + MeOD → MeOH+-NHD) were carried out FTIR spectra of impurity and alprazolam in order to identify unambiguously the amine.

The FTIR spectra were recorded with the sample dispersed in KBr pellets. The spectra obtained for AL and for Imp G are presented in Figures 4.8 and 4.9, respectivelly.

In the spectrum of the synthesized impurity we can check, as described in the literature for the existence of a set of peaks characteristic of primary amine, bands between 3160 – 3475 cm^{-1} corresponding to "Stretching", between 1600-1630 cm^{-1} to "bending" and between 700-800 cm^{-1} to "wag." As can be seen these peaks are not present in the FTIR spectrum of the sample of standard alprazolam.

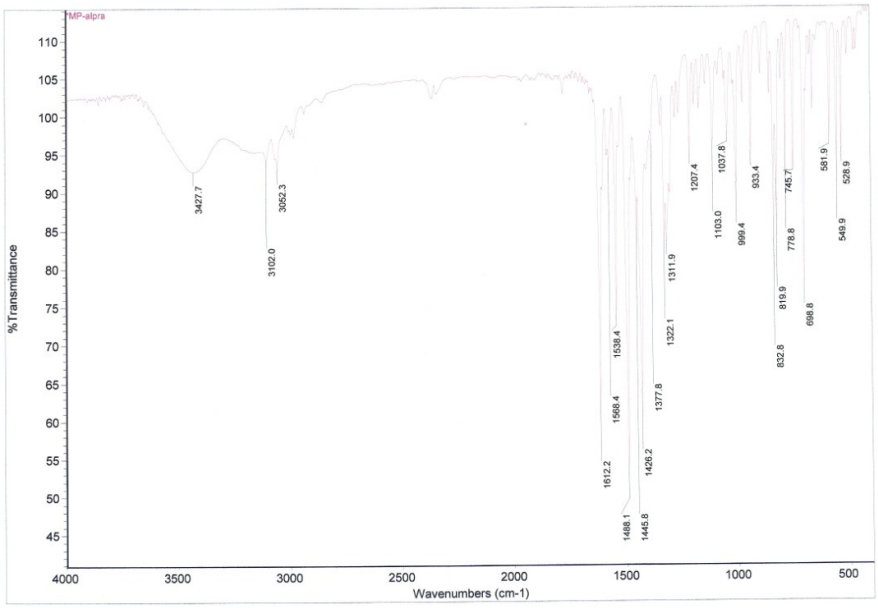


Figure 4.8 FTIR analysis of Alprazolam.

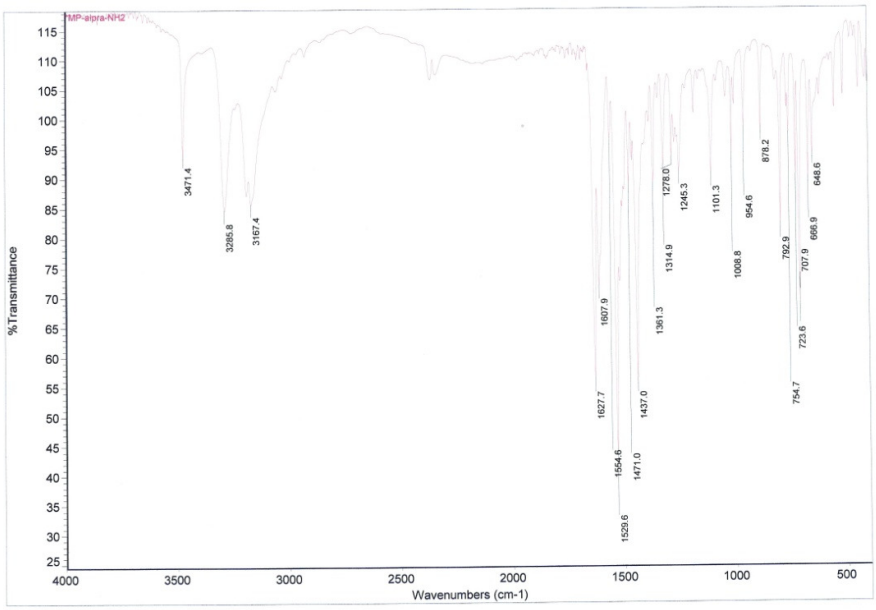


Figure 4.9 FTIR analysis of Triazoaminoquinoleine (Imp G).

4.2.4. Elemental Analysis Studies

Elemental analysis of the Alprazolam standard and synthesized triazoaminoquinoleine were performed on a Fisons Instrument EA 1108 CHNS-O and the results obtained are summarized in Table 4.4.

Table 4.4 Elemental Analysis of AL standard and Imp G synthesized.

Element	Theoretical Value (%) Molecular Formula $C_{17}H_{13}ClN_4$	Experimental Value for AL (%)	Error (%)	Experimental Value for Imp G (%)	Error (%)
N	18.15	18.26	0.11	18.12	0.03
C	66.13	65.61	0.52	65.44	0.69
H	4.24	3.94	0.3	4.03	0.21

Values obtained for elements N and H are within ± 0.3 %. Values obtained for carbon indicate a deviation which we reckon as likely due to impurity/solvent presence in our samples, including in AL USP standard.

As Imp G synthesized by us was extracted finally with methylene chloride it is possible a contamination with this solvent. Making an appropriate solvent CHN correction with methylene chloride we can obtain for AL and Imp G samples the results presented in Table 4.5. The corrections were carried out assuming that there are 0.035 mol and 0.05 mol of DCM as a contaminant in AL and Imp G, respectively.

Table 4.5 Elemental Analysis of AL standard and Imp G synthesized with solvent correction.

Element	Theoretical Value with solvent correction for AL (%)	Experimental Value for AL (%)	Error (%)	Theoretical Value with solvent correction for Imp G (%)	Experimental Value for Imp G (%)	Error (%)
N	17.97	18.26	0.29	17.90	18.12	0.22
C	65.63	65.61	0.02	65.42	65.44	0.02
H	4.23	3.94	0.29	4.22	4.03	0.19

In both cases the error obtained are within the acceptable error of ± 0.3 %.

4.2.5. UV Studies

Absorption spectra were recorded according to described in 4.2.1.5 and the curves obtained for AL and Imp G are presented in Figure 4.10.

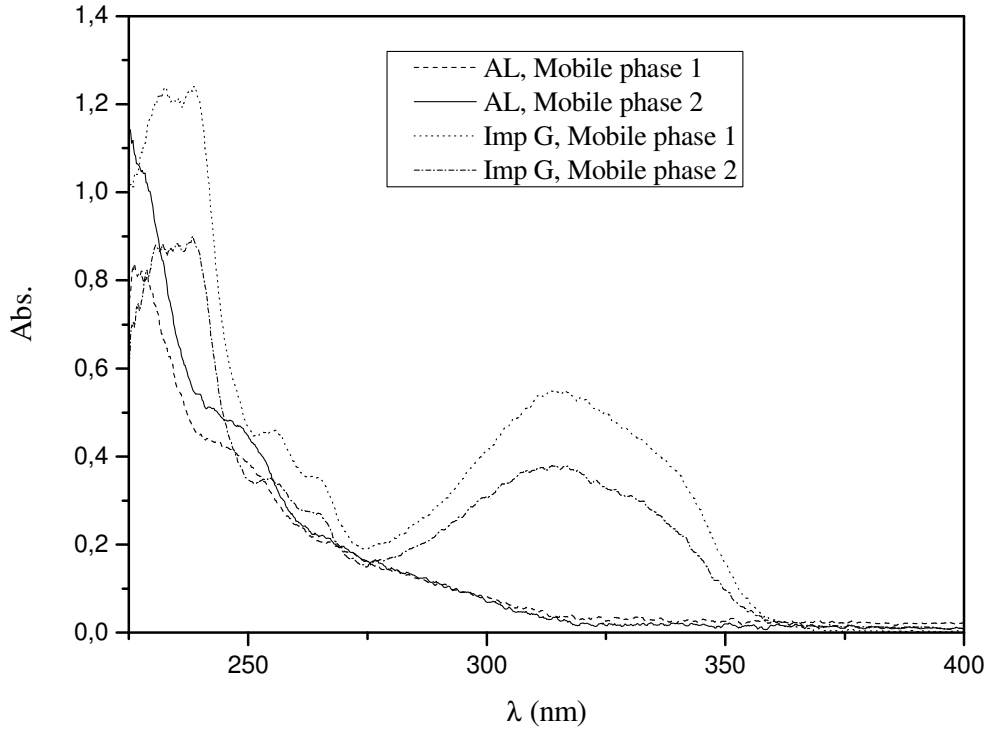


Figure 4.10 UV-VIS spectra of AL and Imp G in different mobile phases.

The absorption coefficient (ε) was determined in mobile phase A and B at 254 nm for AL and for Imp G the absorption coefficient at the maxima of the absorption bands was calculated in both solvents, Table 4.6. Considering the differences in the absorption coefficients in the two mobile phases used, the areas measured by LC do not represent the ratio between AL and Imp G; the concentration of Imp G corresponds to *ca.* half of the measured area.

Table 4.6 Maximum of absorption bands and absorption coefficients of Alprazolam in the two mobile phases used in the LC-MS-MS method.

Compound	λ (nm) ε $(mg/mL)^{-1}cm^{-1}$		
AL Mobile phase A	254		
	30.75		
AL Mobile phase B	254		
	39.82		
Imp G Mobile phase A	254	263	315
	34.36	26.66	38.48
Imp G Mobile phase B	254	263	315
	61.19	48.39	74.56

4.3. Solution degradation studies of Alprazolam

In order to expand the LC-MS-MS methodology developed, API was submitted to degradation under forced conditions to obtain samples with other impurities besides Imp G.

High humidity and temperature are known factors responsible for the increase in the rate of degradation of AL [107]. In order to probe the utility of the LC-MS-MS method to detect other known impurities, degradation of AL and commercial brands (CB) was accelerated [115]. We chose to carry out degradation in solution because solution interaction studies may have some predictive capability much faster [121].

Alprazolam API and tablets of 0.25 mg (expiration date 01/2011) were dissolved in equal parts of methanol/citrate buffer and kept in a water bath at 50 ºC in open vessels for 15 days. Aliquots were analysed during this period of time. In the LC chromatograms of AL and commercial tablets it was possible to identify, after 3 days, the Imp C at 9.89 min [104]. The MS spectrum shows a molecular peak at *m/z* 298 corresponding to $(M+1)^+$. The MS^2 spectrum shows only one peak at *m/z* 280. In order to obtain a fragmentation profile of this we performed a MS^3 spectrum, Figure 4.11. The main fragment is most likely to come from a water elimination originated from cyclization as proposed in Figure 4.12

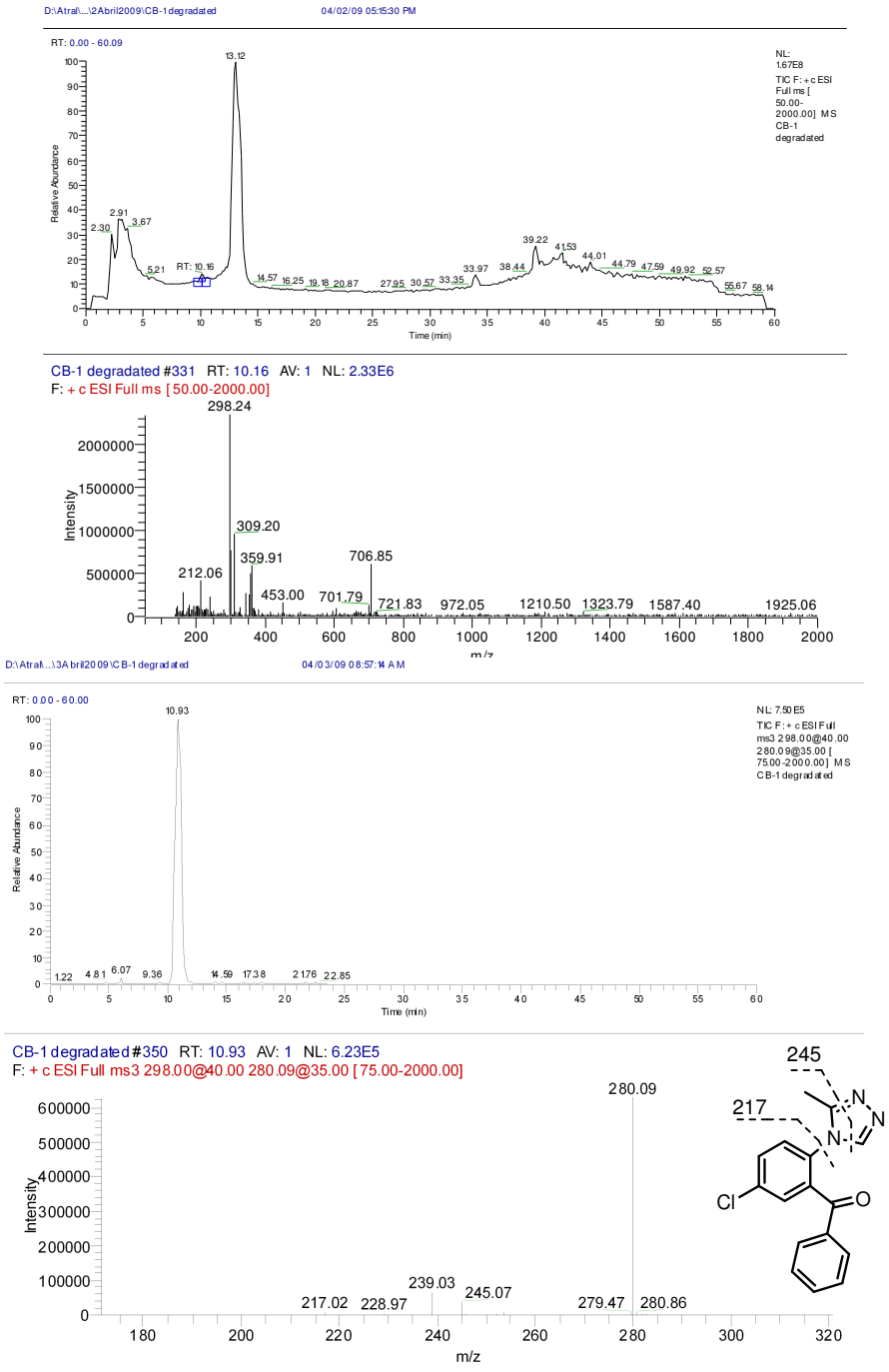


Figure 4.11 Identification and MS^3 spectra of Impurity C.

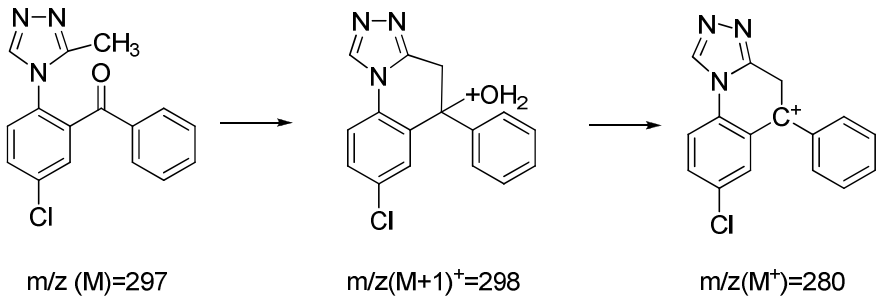


Figure 4.12 Proposal for the formation of the main fragment of impurity C.

Figure 4.13 shows the evolution of the relative percentage of Alprazolam, Imp C and Imp G in the forced degradation conditions. The degradation rate is higher in the CB than in the API solutions pointing out the influence of the excipients in this process. After 15 days the samples of AL present 7.54 % of Imp G and in the samples of commercial tablets the % of Imp G is 50.65, almost seven times higher than that obtained in the absence of excipients.

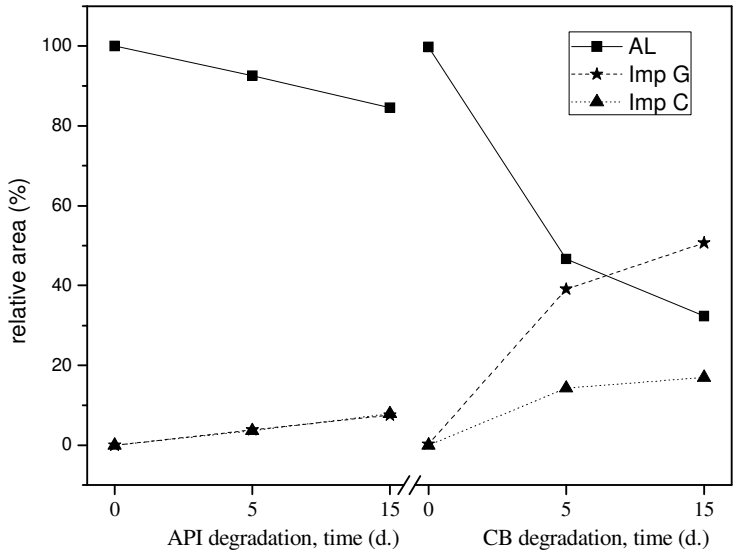


Figure 4.13 Evolution of the relative percentage of Alprazolam, Imp C and Imp G in the forced degradation conditions.

The opened-ring Alprazolam is the product of the hydrolytic reaction of Alprazolam. The hydrolytic breakdown leading to the benzophenone derivative was described for other benzodiazepines as triazolam [116] or loprazolam [117] as well as for the thienotriazolodiazepine known as brotizolam [118] as a room temperature reaction. The opened-ring Alprazolam is described for Barbas [114] and Nudelman and Cabrera [113] as the precursor of the degradation reactions, involving different excipients, leading to the formation of Imp G, still this compound only was possible to detect by NMR analysis.

In order to test our developed methodology we performed a forced degradation of AL with HCl 1M and MeOH:HCl 1M (1:1) [115]. The LC-MS-MS analysis of AL dissolved in HCl shows a small peak at 5.85 min. The same retention time but a more intense peak was obtained using the mixture MeOH:HCl, Figure 4.14.

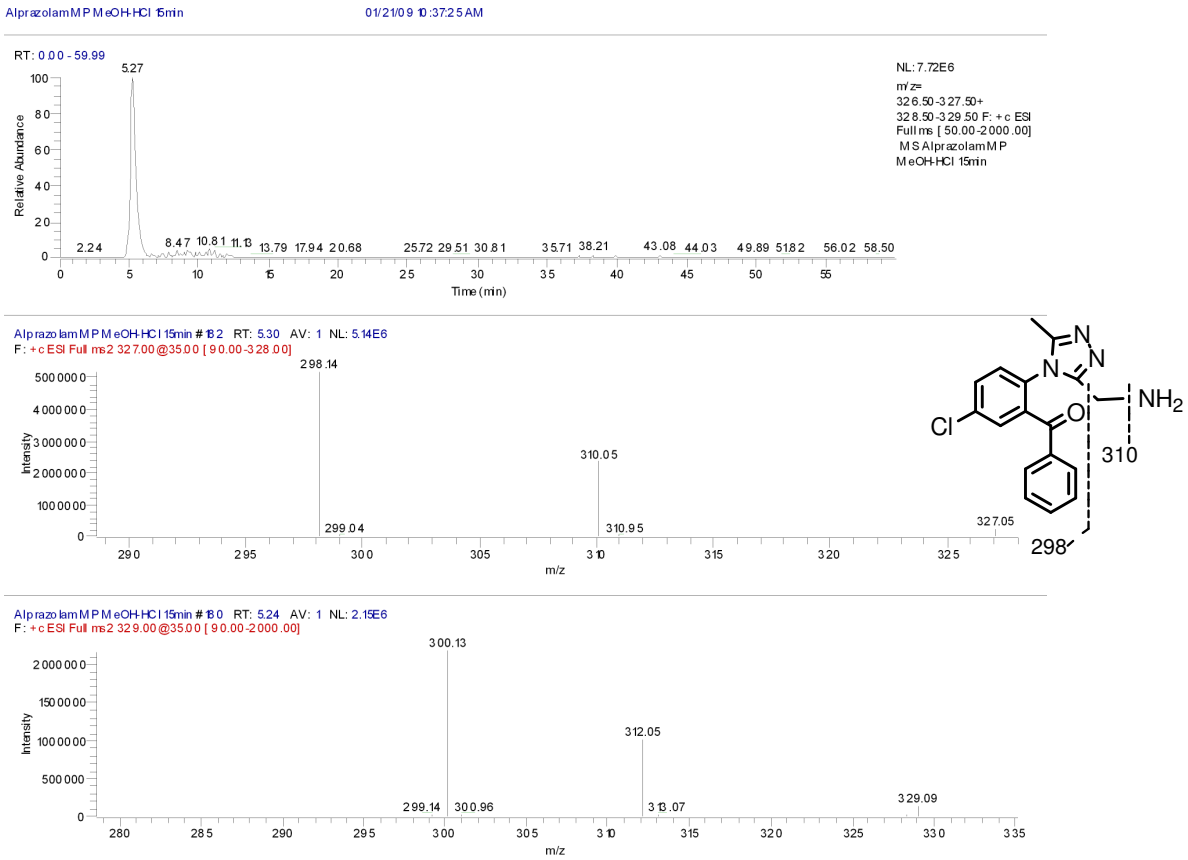


Figure 4.14 Alprazolam API subjected to degradation forced conditions.

Both solutions were refluxed overnight in MeOH:HCl mixture, observing an increase of peak area. The MS^2 analysis of that peak has two main fragments: 327(M+1), 310 and 298(100), Figure 4.14.

The fragmentation peak at *m/z* 310, could be interpreted as the loss of NH_3, the peak at *m/z* 298 is formed from the loss of CH_2NH_3. We confirmed by the isotopic analysis of ESI/MS-MS experiments that the isotope peak 329 gives a fragmentation pattern 329(M+1)→312→300(100). The spectrum of this compound has the M+1 of the opened-ring Alprazolam and the fragmentation profile is coherent with the structure.

Using the SIM mode it was possible to detect this compound, opened ring alprazolam, in the chromatograms of the tablets submit to the forced degradation conditions described above using MeOH:Citrate buffer solutions heated at 50 ºC during 15 days.

4.4. Conclusion

This chapter describes a study of impurities and the degradation products of alprazolam as API and in the form of finished product.

It was possible to identify the main impurities (Imp G, Imp C and opened-ring alprazolam), and make their characterization by various techniques.

As for the main impurity (Imp G) present in the finished product we synthesized a sample of this compound using the API as starting material and made an exhaustive characterization of this synthetic product through the analogy with Alprazolam.

Studies were performed (long run studies) using a commercial brand, demonstrating the increase of impurity G over time. The study was extended to various commercial brands, with different formulations, also in a perspective view of long run without forced degradation. The results were consistent with those previously described. With differing amounts Impurity G is common to all brands and increased over time.

In an attempt to understand the formation mechanism of the main impurity of alprazolam by degradation we have made some experiments with industrial API as well as the finished

formulated product. We observed the emergence of other impurities, impurity C and the precursor of impurity G. Both were characterized by MS. The precursor of Imp G, opened-ring alprazolam, had only been previously detected by NMR analysis [113], and it was now possible to detect it by single-run LC-MS-MS.

The LC-MS-MS methodology developed for the analysis of the Alprazolam API and its formulated product in commercial brands allows the separation and identification of Alprazolam and related impurities (impurity C and impurity G) as well as the hydrolysis product in acid media (the opened-ring Alprazolam), in only one chromatographic experiment. This involves the identification of impurity G through MS^2 analysis and detection of the opened-ring Alprazolam in SIM mode in one chromatographic assay

CHAPTER 5

CONCLUDING REMARKS

This final chapter aims to make an overall assessment of the work of this PhD project. The conclusions are related to the objectives proposed at the beginning of the project, to assess the importance of development achieved at the end of this work.

The results obtained using modern methods of mass spectrometry and that were addressed in this work clearly demonstrate that such work should be developed on other drugs and their formulations. As future work, was motivating the development of techniques for semi-preparative separation combined with accurate mass spectrometry.

5.1. Conclusion

As the main target of our study was the application of modern physicochemical methods to everyday problems of a pharmaceutical company, we conducted a selective literature review to allow a better fit the requirements that these structures are subject based on international regulatory guidance. It seemed to us equally important to describe in greater detail the method (LC-MS-MS) further studied in this paper.

As already mentioned in Chapter 1 the initial project objectives were divided into two key items I and II. We now have a detailed analysis of the findings obtained for each of these initial objectives.

I. Quantification and identification of impurities / degradation products by new methodologies in a product

The purpose of the use of new methodologies, in particular LC-MS-MS allowed the identification and quantification of impurities in two products on the market.

In the work developed to netilmicin, the development of this methodology allowed the determination of an impurity that is not detected by conventional methods (for example HPLC/UV) using prior isolation by SPE. Since this product is presented in the form of injectable formulations this study can act as a strict control of storage conditions on the product and monitor the conditions of formulation. It is worth noting the ease of hydrolysis of its API, so it is crucial to the strict control of several factors that may enhance this reaction. We developed an extensive work to validate the method for the determination of impurity in the API and for the determination of impurity in the finished product.

With respect to work done on alprazolam API and their formulations, the method of LC-MS-MS developed not only allowed the determination of some impurities described in the pharmacopoeia, but also the identification of a precursor of another compound impurity present in greater quantity allowing to draw conclusions about the mechanism of degradation. The developed method allows quantification of major impurity of alprazolam (Imp G).

II. Assembly and validation of analytical methods for stability indicators (tablets and injection), with the identification of potential degradation products, involving stages of work like forced degradation of the active substance in various conditions of stress, identification of major degradation products (using LC/MS and/or other techniques) and assembly of selective HPLC method "indicator of stability".

Although we have not conducted studies of degradation during this project in the developed work for the injection of netilmicin, in Chapter 3, we describe the conducted analysis of various samples degraded with various standard conditions used in industry, allowing us to conclude about the adequate selectivity obtained by the results of the method under study.

This second initial purpose of this project work on the alprazolam was more exploited. Once developed the method to determine the main impurities, it was necessary to carry out various studies of degradation in order to be able to determine the key enablers of that degradation. For this analysis was made with a view to long-run study, ie without resorting to forced degradation, several commercial brands and different dosages. It is possible to conclude that whatever the formulation is observable degradation and that is common to all brands to further deterioration in certain dosages.

After detailed analysis of several formulations were carried out degradation studies of API, in solid and solution. Demonstrated the high stability of the API, were made studies of degradation of the API with some of the ingredients present in several commercial formulations on the market. Like other studies in the literature, some excipients were more enhancers degradation. Other external factors such as temperature, light and humidity have also proved to be decisive in the process of degradation, which led us to develop a DOE study showing that there are factors, or combinations of factors that degrade to a greater extent the API as well as

others who can delay this same degradation. These results will undoubtedly be important for future optimization of formulations of alprazolam.

It was also possible, through appropriate synthesis methods, synthesize the major alprazolam impurity, thus allowing the detention of a purified impurity standard inexistent in the market. With this impurity synthesized by us, we have made further studies of degradation by the action of light, photolysis, which allowed us to conclude that in this particular case the extreme protection of tablets from light can not be a good methodology with regard to the amount of G imp that may be present.

5.2. Work assessment

The work developed and presented in this thesis clearly demonstrates the importance of applying new methods in industrial environments. From the perspective of sustainability and development of a company like Atral a global market and developing all the time was a strong imperative to prepare qualified professionals creating conditions to look for alternative solutions of guaranteed quality materials that acquires and produces. The work presented actually responded to these existing requirements.

Sustained development also meant to enter the area of production of some materials such as active ingredients and related impurities. With these developments in perspective, there was a growing need to have skills in quality control and validation of products that was actually achieved.

The requirements for quality control and validation of products subject to stringent certifications require the industry to prepare in order to impose their products with the most stringent quality standards. Knowledge about the potential of physical-chemical methods and their holding capacity with the utmost scientific rigor in solving each of the problems encountered was an essential requirement modern enterprise.

The requirements, competition and brand for a company to have visibility and impose himself in the modern market implies the ability to present its quality standards through original works that are real research projects where the desperate need for quality criteria for the second academic standards originality.

Analyzed the requirements for the pharmaceutical industry and currently doing a comprehensive analysis to the work presented, it seems clear that what was developed was a key lever in the quality control of pharmaceutical products, and in the long term may extend the developed methodologies to other problematic industrial cases.

5.3. Future work

The results show that there are several studies relevant to develop as future work.

The skills acquired subsequently allowed to develop, together with the company's unit galenic, formulations less susceptible to degradation thereby increasing the quality of products on the market that may even increase the life span of those same drugs, giving the company greater profitability.

The work of synthesis of impurities related to the various marketed API could be extended and allowing an impurities portfolio with properly characterization by various physicochemical methods like the work done to alprazolam.

Once we have these synthesized impurities can be perform detailed studies of the possibility of preventing its formation as well as studying the possibility of such damage. One example is the study that could be making with Imp G degradation in the presence of excipients, since we studied only their photodegradation.

The skills acquired in the area of physical-chemical methods can also be enormously helpful to a company with production facilities in the monitoring of conditions of operation from the perspective of working environment and occupational health and control of environmental impacts. With the skills acquired in the determination of compounds in trace amounts through methods such as LC-MS-MS, would be an asset to the adoption of these methodologies in the areas mentioned.

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Published papers within this thesis

Report prepared for the quality control of the company entitled:

"Report validation method for determining the impurity EG in netilmicin samples", November 2007

Report prepared for the Infarmed (Autoridade Nacional do Medicamento e Produtos de Saúde, IP) entitled:

"Identification and structural characterization of a related substance present in samples of tablets of alprazolam", July 2008

"Identification of Alprazolam and its degradation products using LC-MS-MS", Alexandra Rocha Gonsalves, Marta Pineiro, José Manuel Martins, Pedro A. Barata, José C. Menezes, Arkivoc 2010 (v) 128-141