INVESTIGATION OF CHEMICAL DIFFERENCES IN MEDICATIONS OBTAINED FROM DIVERSE SOURCES USING NOVEL SPECTROSCOPIC AND STATISTIC APPROACHES

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Abstract

Generic medications are those medicines manufactured by a pharmaceutical company without a license from the company that has first invented and patented the same drug, when the related patent and other exclusivity rights have expired. Only studies of bioequivalence are requested as requirements to introduce a new generic medication in clinics, making easier and financially attractive for many pharmaceutical companies to participate in this typology of market.

Organised criminality is strongly attracted by this market both for the high profitability, and for the high similarity to the production and trafficking of illegal controlled substances associated to the extreme difficulties faced by the law enforcement authorities in effectively investigating the online market, because of its anonymity.

A significant paradigm continuously frequented in pharmacology is the confliction between views on generic medications that can be used interchangeably with the original medicines. Several clinical studies conducted in certain medical areas have shown as the generic medications present an overlapping therapeutic equivalence to the original ones. On the contrary, for certain other generics, both pharmacokinetics and pharmacodynamics issue have been reported. Also, in some cases issues on the stability of generics have been raised.

Despite the numerous research articles and reviews published on the matter of clinical equivalence among generic and original medications, no study to the scientific community has been presented on an analytical evaluation of the chemical composition of the different generic drugs that could shed some lights on the reason of the different clinical performances reported.

The main aim of this research was to develop a non-destructive quick qualitative analytical methodology to be able to discriminate differences in the chemical composition from generic medicines that have been reported not presenting similar therapeutic equivalence in clinical comparison studies, obtained from authorised pharmacies and non-authorised online sellers.

From the cardio-vascular area, digoxin (with the related cardiac glycosides digitoxin and digoxigenin) and amlodipine (in its different salts maleate, mesylate and besylate used in therapies). In the gastroenterology area, omeprazole both in its racemic and isomeric forms, have been selected as samples to be analysed. In the antihistamine area, cetirizine, in its racemic and isomeric forms, equally for the same reasons as before, have been considered. As starting analytical approaches, voltammetry, FT-IR, Raman spectroscopies and NMR have been considered and a statistical data analysis approach of the analytical data obtained based on

multivariate analysis such as principal component analysis, cross validation, correlation scatter plots and factor loadings has been implemented.

This work has matched the aims initially set, generating novel methods of analysis to investigate differences in the chemical composition within different groups of generic medications. This study has led to the creation and interpretation of new knowledge, through a systematic acquisition and understanding of a substantial body of scientific literature and through original research, and adjusting the project design in the light of unforeseen problems, conceptualizing, designing and implementing the research project for the generation of novel knowledge.

Preamble

Generic substitution has become common practise amongst healthcare professionals since generic medications can be used interchangeably with the original medicines. A significant paradigm continuously frequented in pharmacology is the confliction between views on generic medications. There is a substantial amount of clinical comparison studies that report how some generic medications show marked different therapeutic outcomes. There are concerns in the healthcare sector, as well as among patients, that not all only generic preparations are not equally clinically effective but also that their quality could be questioned, considering the risk of illegal introduction of fraudulent medications in the conventional chain of supply. Therefore, there is the need to develop a non-destructive quick qualitative analytical methodology to be able to discriminate differences in the chemical composition of similar medications from generic medicines, in order to adopt a flagging system able to give information on the nature of the medications tested.

In order to facilitate the reader an outline of the thesis is presented here below:

Chapter 1 gives the the outline of the thesis with introduction on the generic medication past and current situation, with a mention to the problematic of the counterfeiting criminal activities that revolve around the use of generic medicine.

Chapter 2 presents the aims and objectives of the research project.

Chapter 3 describes the analytical methods employed, giving an overview of their theoretical basis and of the medications analysed, chosen among the ones that have been reported presenting bioequivalence issues.

Chapters 4 to 7 present specific studies conducted using voltammetry, Raman and FT-IR spectroscopies on cardiac glycosides, calcium channel blocker, proton pump inhibitors, antipsychotic and anti-allergic medications aimed to find the optimal analytical conditions to set a flagging analytical system. It also introduces the statistical analysis of the data set obtained by these different analytical approaches.

Chapter 8 gives the conclusions of the research and presents the gains and limitations of the work.

Chapter 9 provides links to scope for future work.

1. INTRODUCTION

1.1. Generic and branded medications

1.1.1. Definition of generic medications

According to the definition given by the World Health Organisation (WHO), generic medications, or complementary equivalent medications, are those medicines manufactured by a pharmaceutical company without a license from the company that has first invented and patented the drug, when the related patent and other exclusivity rights have expired [1].

The assessment of the therapeutic equivalence between a generic medicine and the original one is based on the concept of bioequivalence. Two or more products are recognised as bioequivalent when, administrated at the same dose with the same formulation, reach the same site of action with the similar rate and extension [2].

Two pharmaceutical products are classified as therapeutically equivalent when they have the same clinical effect and safety profile [3].

Pharmaceutical equivalence is related to the same amount of the same Active Pharmaceutical Ingredient (API), in the same formulation, for the same route of administration, with comparable standard. Bioavailability is related to pharmacokinetics and pharmacodynamics characteristics of the comparing products. Pharmacokinetics parameters obtained from blood sample of volunteers involved in cross-over comparison studies are also considered when studying the bioequivalence. The pharmacokinetics is calculated as the area under the curve of the plasma drug concentration versus time (AUC) where peak concentration (C_{max}), time to peak (t_{max}), absorption lag time (t_{lag}) are the main parameters used to evaluate the bioavailability between different products. Particularly the peak concentration is used to assess the absorption rate, and the AUC is the feature used to evaluate the extend of drug absorption [4]

The foundation at the base of the model of bioequivalence is that if the different drugs are characterised as identical or similar, within a specified range, they will have same level of safety and therapeutic efficacy [5]. The FDA considers bioequivalent products

whose areas under the drug concentration is within 90 % confidence interval for the ratio sample versus reference. Another parameter taken into consideration is the maximum drug plasma concentrations which must fall between 80 and 125% [6].

When pharmacokinetic parameters are not obtainable because the medications specifically act on topic areas, for instance in case of comparison of the bioavailability of inhalers, pharmacodynamics features are compared [7].

Complementary equivalent medications are subjected to individual policies depending on the country of delivery and following different pharmaceutical regulations, even if produced by the same company. They are world wide labelled with a generic international non proprietary name (INN) adopted by the WHO [8]. The concept behind the introduction of a standard international name recognised worldwide is to guarantee recognisability and reducing prescribing errors. The INN for medication has been introduced by WHO in 1953.

Bioequivalence studies between innovator product and its generic alternative allows marginal chemical differences in the chemical structure of the drugs to be tested, for example the generic medications can be present as different salts or ester moieties [6].

Polymorphism is the aptitude of certain solid compounds to exist in more than one crystalline form. Although chemically identical, the polymorphs have dissimilar structural matrix which produce differences in their chemical-physical features such as hygroscopy, refractive index, melting point, spectral vibrational transitions, density. More than half of the active pharmaceutical ingredients are reported to present more than one polymorphic form [8,9].

These polymorphic forms of a drug differ in the physicochemical properties like dissolution and solubility, chemical and physical stability, flowability and hygroscopicity. These forms also differ in various important pharmaceutical outcomes such as drug efficacy, bioavailability, and even toxicity. Polymorphic studies are important as a particular polymorph can be responsible for a particular property which might not be exhibited by any other form.

The stereochemistry of the polymorphs is also a crucial parameter. The molecular dissymmetry and chirality associated is now being employed in the pharmaceutical drug development. Each optically active compound has different pharmacological activity

and it has been proved that chirality with dissymmetry, directly affects the pharmacological activity [10].

Consequently, it is critical to perform suitable polymorphic selection for each active pharmaceutical ingredient (API) and select the most stable polymorph for the product development. The most stable polymorph should be employed in the marketed formulation to prevent polymorphic alterations during manufacturing, delivery, or storage. In general, the selected polymorph should be thermodynamically stable during the drug development process and remain stable during the manufacturing process too [11]. In this context, various federal agencies require the appropriate description of the solid state of the API employed in the formulation [12].

Furthermore, on the regulatory aspect, currently there are concerns in reference to the clarity of the guidelines dealing with the polymorphic transitions of the API as well as the potential excipients [13].

Pharmaceutical companies introduce their products into the market under well-covered patents, in order guarantee the condition to generate income from the commercialization of their findings. Having the right of exclusivity on that medication allows the inventor company to avoid competitors to enter the same market with the same product for a certain period of time [14].

The cost for the introduction in the market of a new medication takes into consideration all the phases incurred in the drug development of the product, from the initial research and development, to the safety and efficiency studies, the manufacturing, regulatory approval and the patenting cost. In 2014, it was estimated that the average of such a cost was 2.6 billion American dollars [15]. Because of this level of financial commitment in the developing of new medications, the focus on recoup of the incurred costs and the expected profit is paramount.

During the period of validity of the patent, sometimes in the range of 20 years, the company benefits from a period of exclusivity. In this period the return and profitability is maximized covering even the costs incurred in the drug discovery and research of other drugs that have not been successful at any stage of the development path.

Generic medications when introduced into the market are sold at a considerably lower price than the original one. However, despite the lower price, the profit margins in the

complementary equivalent medications market are considerable, because of the fewer expenses incurred, not having had the burden of initial drug development.

1.1.2. History of generic medications

The use of generic medications is an old well-known alternative to the original ones, but it is only since the first decade of the 80s that have become known worldwide by the public. In fact, one of the first references of the use of generic medications, with the legal issues related to them, can be tracked down to the beginning of 20th Century when Bayer had to protect its most important drug, Aspirin, from the replicas produced intensively by other companies [16]. During the first period of co-existence in the market of both original and generic drugs, the regulatory affairs office of the FDA in America and the equivalent office worldwide were not particularly strict in applying the standards to accept generic medication for clinical use. The main safety test was essentially post-market, when the patients could report side and unexpected effects. The devastating drama of the Thalidomide overlooked usage forced a change in the regulation [13]. Thalidomide was introduced in the market in 1957 as over the counter available medication to treat the morning sickness in pregnant women. Shortly after the first years of use, around 10000 new births with malformation of limbs were reported all over the world [14].

As a result of such a scandal, in 1962, the FDA modified the related act for new admitted medications, strengthen the testing processes for authorization [15]. The safety and effectiveness control of new medications was then raised at a much higher standard, both for the original and for the complementary equivalent medications manufacturers. The pharmaceutical companies had to face lengthier and extremely expensive research and development studies before being able to introduce brand new drugs in the market. At this stage, because of the high cost, not many pharmaceutical company interested in producing generic medications were actually engaged in this market sector, being financially unrewarding.

In 1984 a new law, known as the Hatch-Waxman Act [15] changed some of the requirements requested to the generic medicines, making it financially convenient to produce complementary equivalent medications. Within the Hatch-Waxman Act

conditions, the pharmaceutical companies producing generic medications are no longer requested to repeat the the long lasting and costly pre-clinical and clinical tests, already done by the original company when initially produced the innovator branded medication. From 1984, within the Hatch-Waxman Act regulations, only studies of bioequivalence are requested [16]. Essentially, within these new regulations, to obtain the authorization to introduce a new complementary equivalent medication into the market, a generic manufacturer needs only to prove that its new generic product contains the same active pharmaceutical ingredients (API), have the same strength, use the same formulation and use the same route of administration [17] of the original branded used as reference. This change of the entry requirements attracted more pharmaceutical companies in this line of business, changing dramatically the therapeutic habits in the last decade.

1.1.3. Patent protection

A patent is the legal right of temporary exclusive exploitation of an invention hold by the inventor without having competitors to exert any commercial pressure by copying the invention itself [18]. The duration of legal protection varies among the different countries, being usually between 15 to 20 years. An invention to be able to be patented must meet specific requirements. Novelty is the main feature of a patent, being necessary that the invention is for the first time available to the public. Another main feature of an invention is that it must not be obvious for the technical expert of the sector. It must not be a simple straightforward implementation of the current technical knowledge but must retain a level of inventiveness, the so called ‘inventive step’, that place the invention is an adequate distance beyond or above the state of the art" [18].

Finally, the invention must have an industrial application, being able to be replicated in the manufacturing sites and having a market where to be sold [19].

Considering the high cost involved in pharmaceutical research and development, patenting is the preferred way to protect the investment and guarantee the expected financial return. When the protection period ends, the original inventor loses its exclusivity rights and other actors can be able to exploit the invention. Nevertheless, not

all the countries have patent laws enforced and in some states medications are not considered patentable.

In the pharmaceutical sector, the main types of patents cover the product, the process, and the formulation. The patent on the product, covers the active pharmaceutical ingredient as a new medication, and it is the most important but also the less frequent. The patent can cover the process to obtain the specific product or only the process to be used for the preparation of different products. The claim could be on the formulation of the the medication, i.e. on the end product not only on the API. This can include the excipients and the coating agents. Finally, the method of use can be also claimed in the patent, covering the new use of a drug to cure a medical condition. It is worth to remark that not all the categories of patents are recognised in all countries.

1.1.4. Financial implications in the switch from branded to generic medications

Switching from innovative brand to generic medications can halve the cost paid directly or indirectly by the patients. Particularly in developing countries with low and middle income the switch from innovative to generic medication generates great savings [20]. In China, it has been estimated that 370 million US dollars could be saved changing only four medications from originator to generic ones, with a potential saving of 65% [21]. In certain developing countries, for some medications, the gap in cost between branded and generic medication can even be more than ten-fold [22].

In the United Kingdom, the cost of prescribing in primary care has increased 4 times over the past 40 years, from two billion pounds in 1976 to 8 billion pounds in 2013 [23]. These figures are related to the increase in the volume of prescribed medication as a whole but it suggests a conversion in the prescription habits of the prescribers from originator to generic medications, with the relative changes in prices [24]. It has also been reported that the number of prescription of branded medications in the last 40 years has dropped by 33%. This change in habits on prescribing and dispensing complementary equivalent medications over branded ones has had an overwhelming effect on the productivity (measured as the ratio of the debit against the credit) of the prescribing budget in primary care [23]. Such increase is the result of the combination of the increased volume of generic medications prescribed and impact of new the

Pharmaceutical Price Regulation Scheme policies on costs for generic medicines [24]. On these basis, the NHS has saved approximatively 7 billion pounds in the last 40 years. Currently the ratio of generic medications prescribed is around 84% of the total of medications prescribed yearly. Certain medications, for different reasonable reasons, are still prescribed as brands, with an average cost 7 times higher than the equivalent generic drug [23, 24].

In 2007, the yearly prescription cost in the USA amounted to over 280 billion US dollars, raising the focus on the use of complementary equivalent medications in order to condense the financial impact on public funds [25,26]. In this country, generic medications represent approximatively 66% of the total medications prescribed annually, but account for less than 13% of the total cost [27]. It has been reported that a further exchanging of prescribing habits from original to generic drugs could lead to a continued reduction by 11% per year in the total USA financial budget for medication [28]. Particularly, in the cardiovascular clinical area, a reduction of 25% it is expected by the generic switching of the hypertension medications [29].

1.1.5. Perception of the use of generic medications from the patients

The increase of the volume of a multitude of generic medications manufactured by different companies for the same branded medicine causes perplexity within the general public [30].

It has been reported that spread feelings of uncertainty amongst both general public and health professionals on the equivalence in quality between branded and generic medications occur [31]. Specifically, the bioequivalence has been questioned in terms of generic substitution, in reference to the different industrialized processes and pharmaceutical technologies adopted. As a matter of fact, the efficacy of the method to test the bioequivalence and the concept of bioequivalence itself has been disputed. Furthermore, amid different countries, there are slight different approaches to test bioequivalence and this lack of international harmonization represents also an element to doubt about the results obtained [32].

Furthermore, in countries where the patent protection in the pharmaceutical business is not implemented, a reduced inclination to use generic medication prescribing and lack

of financial incentives for pharmacies to dispense generic drug have been observed. This is giving the impression to the general public that generic medications are considered as poor quality medications [33].

In a study conducted on adult individuals in Columbia (USA) to assess patients' awareness on generics, patients acknowledged that complementary equivalent medications are as safe as the reference branded and financially beneficial [34]. Nevertheless, despite the fact that 56% of the population interviewed agreed that more generic drugs should be adopted in therapeutic treatments, only 38% actually prefers to take complementary equivalent medications for themselves. In the USA, it has been reported that the usage of generic medications is inconsistent and that there are patterns of different use amongst different social groups. For instance, the perception of equivalence between generic and brand-named medications is lower in poorer communities, in younger generations and in patients who receive a regular care from specialists [35-37].

1.2. Clinical studies on therapeutic equivalence of generic medications

The idea that bioequivalence characteristics of two medicines are the ones to be taken into consideration to assess their therapeutic equivalence is generally accepted [38, 39]. For many years, the assessment of the bioequivalence has been used as official test to allow the introduction of new medications in clinical usage [39, 40]. However, in some circumstances when the formulation tested is not the same than the final formulation to be used clinically some more additional bioequivalence studied are necessary to demonstrate clinical equivalence [40].

The issue on the compliance with the bioequivalence requirements for a medication to be classified as a generic medicine relies on the fact that the tolerance accepted is not strict enough in case of medicines with a narrow therapeutic index or with an elevated intra subject and inter subject variability [38-41].

Based on a review that took into consideration three decades of literature referred to the effect of generic substitution on extended areas of therapy, a change in the modality to test the therapeutic equivalence of medications and a switch from measurements of average bioequivalence to individual and population bioequivalence has been proposed

[41]. The concept that bioequivalence is comparable to therapeutic equivalence has raised many criticisms. It has been reported that the tests for the approval of generics are not as stringent as the ones to allow the first introduction in therapy of new branded medications and that the level of the clinical equivalence tests among generics in comparison with the reference branded product is not acceptable [42].

Clinicians have identified a number of therapeutic areas where generic substitution ought to be applied carefully. In some particular areas of therapies, those concerns were so high that several comparison studies to check the interchangeability among generics and branded have been performed and published. Some of these studies have demonstrated the therapeutic equivalence amongst generic medications and original one, but some others have raised doubts about their similarity in clinical efficacies.

In the following paragraphs, it has reported an essential panorama of those medications where interchangeability has been proved and where strong concerns have been raised.

1.2.1. Clinical areas where therapeutic efficacy has been confirmed

1.2.1.1. Osteoporosis

Osteoporosis is a pathology characterised by a demineralization of of the bone structure leading to a reduction in density with a resulting decay of the mechanic features of the skeleton [43].

Osteoporosis is the first cause of bone fractures in the elderly [44, 45]. The demineralization occurs without any substantial symptoms and the breakage can consequently occur unexpected [46]. About 65-70% of population over 80 years old seems to be suffering of such condition [47-50]. Alendronic acid is a drug, belonging to the family of bisphosphonates, used in UK as first line of treatment [51] to inhibit the resorption of mineral from bone operated by the osteoclast cells.

Extensive studies on the efficacy of bisphosphonate have been produced [52-61]. Alendronic acid has shown to reduce significantly the breakage of bones [54-56] by improving bone mass and lower bone turnover [55-61].

The methods to assess the efficacy of on the mineralization of bone tissue is based on physical assessment of the mechanical properties of the bones such as bone mineral

density [62], X-Ray measurement [66], measurement of telopeptide in the cross linked structure of collagen [67]. In several studies [68-75] different branded and generic of alendronate medications have been compared in order to assess their efficacy and safety for patients. Different trials have been performed over significative period of time and different treatments on different test groups treated with the different manufactured alendronate tablets, at the standard dosage of 70mg once a week. Their bone mineral density was measured at different bone areas, more often on the lumbar spine, hip, and femoral neck. During and at the end of the test period of treatment, the bone mineral density for all patients was significantly higher than the values at the beginning. In this area of therapy, the generic medications have shown to be interchangeable.

1.2.1.2. Hypercholesterolaemia

Cholesterol is one of the main lipids that is used by cells to synthetize their membranes [76]. Cholesterol is insoluble in water and therefore needs to be transporter all over the body through the blood stream linked to lipoproteins. Despite the fact that this molecule is directly involved in the mechanism of production of cell membranes, a high concentration in the blood, hypercholesterolemia, is associated with pathology such as atherosclerosis, and particularly in the heart, coronary heart diseases [77-79]. Hypercholesterolemia is caused by two separate factors: genetic and life-style. A survey on the rate of population over 20 years old affected by hypercholesterolemia, shown as in 2010, around 13% presented blood level over the normal range [76]. When changes in life-style do not succeed in reducing the cholesterol level, a drug therapy is required. First line of treatment for anti-hypercholesterolemia is a group of medications called statins. They inhibit the HMG-CoA reductase, an enzyme involved in the biosynthesis of cholesterol. Different studies have been completed to ascertain the efficacy of such medications [77, 78]. Other studies have been performed to test the interchangeability of generic statins [79-90]. It has been highlighted the fact that replacement in therapy with generic statins represent a cost saving choice, being reassured on the efficacy, having the generic statins shown similar therapeutic efficacy to the referenced branded medications.

1.2.2. Clinical areas where therapeutic efficacy has not been confirmed

1.2.2.1. Glaucoma

Glaucoma is a group of ophthalmic conditions characterised by an increased intra ocular pressure (above 30mmHg) that stresses the optic nerve, damaging the connection between eyes and brain and leading to a loss of peripheral vision [91]. Open angle glaucoma is the most common, followed by the closed angle glaucoma and normal tension glaucoma [92]. In open angle glaucoma, the trabecular meshwork, is obstructed, allowing less fluid to leave the eye, increasing therefore the local pressure. This condition progresses gradually over time with no apparent symptomatology. It represents the 80-90-% of all the glaucoma conditions [91]. With closed-angle glaucoma, such a canal is still open but very narrowly, reducing the fluid drainage. This second type of glaucoma can develop slowly or rapidly. Normal angle glaucoma is less known, being characterised by a damaged optical nerve not associated to any high intra ocular pressure.

Globally, this pathology affects currently over 67 million people, with a predicted raise to 80 million in 2020, 74% of which will be open angle glaucoma [93]. In the USA, the overall population affected by this condition is about 2 million, being more common in older generations.

In glaucoma conditions, the main therapeutic goal is lowering the ocular hypertension. Topical treatment, with the use of eye drops, is the preferred therapy, being prostaglandin analogues the first line treatment recommended. Prostaglandin analogues, such as latanoprost, bimatoprost and travoprost, increase the outflow of the intra ocular liquid. Beta blockers, such as timolol, reduce the production of the intra ocular humor. Carbonic anhydrase inhibitors, such as dorzolamide, reduce the secretion of intra ocular liquid [94,95].

Studies on the interchangibility of latanoprost and combined dorzolamide-timolol generic medications in comparison with the relative branded equivalent have been performed [96-98]. Concern has been raised on the ability to obtain the same magnitude of results when switching from original medication to the equivalent generic ones.

The concentration of active ingredients and preservatives in 2 different generic and branded eye drops of dorzolamide and timolol and latanoprost, has also been monitored

using liquid chromatography in tandem with mass spectrometry (LC-MS). The bottles of eye drops have been tested and then stored at 25 °C for 30 days and then re-tested again. After this length of time, latanoprost in the branded product was still 94% of the original starting value while in both the generic eye drops the concentration dropped to less than 78%. Furthermore, the particulate counts of both dorzolamide-timolol and latanoprost generic had a significative increase in particulate material and in some cases long string-like contaminants were spotted.

1.2.2.2. Gastro Esophageal Reflux Disease

Gastro Esophageal Reflux Disease (GERD) is a condition in which an excess of gastric acid overtakes the stomach sack and reverses up into the esophagus, irritating its epithelium [99]. This is due to a not efficient closure of the lower esophageal sphincter that allows the passage of acid coming up from the underneath stomach. The esophageal epithelium is different than the gastric mucosa, being particularly sensitive to acid aggression. Factors that can trigger GERD are several [100, 101], the most recurrent ones are hiatal hernia, obesity, increased production of gastrin due for instance to a high level of calcium in the blood stream or to Zollinger-Ellison syndrome. Other factors are related to life-style and side effects of certain classes of medications.

GERD affects between 10 to 20% of western population [102], with daily episodes affecting 4-7% of the population. The incidence of the condition is higher in developed countries and lowering in African and Asian developing countries [103].

First line treatment for GERD addresses the use of Proton Pump Inhibitory (PPI) [104]. This class of medications inhibit specifically the proton-potassium ATPase pump in the stomach parietal cells, blocking the final step of acid production. The therapeutic action of omeprazole, the most common remedy, appears in an hour after administration and lasts up to three days [105]. Other medications used for GERD are H_2 antagonist, such as ranitidine, and antacids [106].

Several studies have assessed the therapeutic effect of PPI [107-109] highlighting the efficacy of the results with relatively non relevant contraindication.

High concerns have been raised by different clinical studies about the interchangeability of PPI among generic and branded medications, Comparison studies [110-112] have suggested that the acid suppressive action of generic PPIs are not necessarily

comparable, reflecting different clinical results, with the concept that bioequivalence not necessarily imply same therapeutic efficacy [113].

1.2.2.3. Bacterial infections

Antibiotics are a group of medications that are used to treat or prevent bacterial infections [114] by killing or preventing the growth of bacteria. Antibiotics are divided in different classes according to their mechanism of action and specific typology of infections they can treat. Nowadays, the majority of antibiotics are produced altering partially in manufacturer plants natural products [115].

Rifaximin branded and generic have been tested in a study of pharmacokinetics analyzing through HPLC-MS plasma and urine samples after set time from administration of the antibiotics. The results obtained showed remarkable differences in C_{max} and AUC between original and generic medications, highlighting a different bioavailability [116].

A review of articles related to the comparison of effectiveness of generic antibiotics suggests that there is a concern on the claims of therapeutic equivalence in this class of medications. Particularly for intravenous antibiotics, it has raised alarm on different level of impurity found. Also, pharmacokinetics and pharmacodynamics correlations have been reported being different in the comparison within same groups of generic antibiotics [117].

1.2.2.4. Cardio Vascular Diseases

Cardiovascular disease (CVD) is a large class of conditions that affect either the myocardial muscle and peripheral vessels or both. CVD represent the principal cause of death internationally [118]. Coronary artery disease, such as angina, myocardial infarction and stroke represent a cause of death for 80% of the male population and 75% in females [119]. Age is one of the main parameter to influence the epidemiology of these conditions. In USA, while cardiovascular disease affects 11% of the population aged between 20 and 40 years old, in the range of age between 60 and 80 years old the rate raises to 70 % [120]. In the western countries the average age of death from CVD is around 80 while in the developing countries it is around 68 [121]. CVD incidence is also sex related, occurring seven to ten years earlier in male population as compared to

women [122]. In a survey of nearly 50 articles, different classes of medications used in CVD have been taken into consideration to study the efficacy of complementary equivalent medications versus the reference brad-name medicines. Beta blockers, diuretics, calcium channel blockers, antiplatelet agents, statins, angiotensin converting enzyme inhibitors, alpha blocker have been compared. The results indicated a no evidence of superiority of branded medications over the generic ones but diverse therapeutic outcomes obtained with the different generics. More than half of articles analysed indicated a negative view on the interchangeability of medicines [123].

1.2.2.5. Allergic reactions

Antihistamine medications try to inhibit and suppress seasonal and perennial allergic reactions, such as allergic dermatitis, urticarial, rhinitis and ocular allergy without exerting a major effect on the autonomic central level [122]. Cetirizine and loratadine are relative recent antihistamines, defined as a second generation tricyclic antihistamine, with an effective long-acting selective peripheral H1-receptor antagonist activity, with a consequently reduced sedation activity. Tablets of different generics of loratadine [122] and desloratadine [123] were submitted to pharmacopoeial and non-pharmacopoeial tests, such as friability, disintegration, solubility and mechanical tests in order to evaluate and compare their response. Only two over six different generics proved to produce comparable results both for loratadine and for desloratadine probably to different polymorphs and formulation used.

1.3. Counterfeit medications

1.3.1. Introduction

Counterfeiting of medications is a serious and rapid growing danger for the patient's health and the pharmaceutical industry around the globe [124-126]. Fraudulent medications range from products without any active therapeutic ingredients to those with unsafe toxicological contents. They can be not only replicas of branded medicinal but also of generic medications or over-the-counter products as well as faked appliances or diagnostic tools [127-130].

According to the World Health Organization (WHO) designation [128] a counterfeit drug is a pharmaceutical item whose original descriptions have been purposely modified for deception intentions.

Furthermore, in May 2017, during the seventieth world health assembly, it has been agreed to adopt the definition of 'Substandard and Falsified medical products' as official term to be used by the member states [129]. Within this definition it has been incorporated the classification of 'substandard' and 'falsified'. Substandard defines those official medical products that lack of reaching the required standards of quality and/or to meet the necessary specifications. Falsified identifies those medical products that intentionally give false information on their nature, origin and structure.

The forgeries are extremely diverse and can encompass the adulteration of a product; the deceptive replication of an original product; the alteration of the related packaging [131-133].

To the class of Substandard and Falsified medical products belong also the items that:

-have an original composition in terms of active ingredient and excipients of the genuine pharmaceutical agent, appropriately boxed and stamped but illegitimately introduced in a country;

-items composed of the same constituents of the referenced medicine with original packaging but different amount of ingredients;

-products which do not contain any active pharmaceutical ingredient notwithstanding looking identical and with original look-like packaging;

-items visually similar to the genuine ones, with original boxing but containing unsafe ingredients in place of the genuine components;

-products with fraudulent boxing but original active ingredient;

-items with fake boxing but with dissimilar composition of active ingredients [134-136].

The forging of drugs could similarly be extended to:

-item being initially original but whose boxing was altered declaring a superior amount of active ingredient than the actual quantity in order to be marketed with a higher price.

-original products but expired can also be located inside boxes that show a posterior expire date [137-140].

Counterfeiting of medications is a quite obscured problem whose capacity is very hard to measure [141, 142], mostly when considering the adversities that the officials and

professional often face in discriminating a counterfeit drug from a genuine one. Based on a WHO report [142], counterfeit medicinal count internationally for nearly 10 per cent of the whole amount of medicines sold in the world. Nations like Australia, Canada, Japan, USA, New Zealand and those within the European Union are exposed only marginally to this phenomenon, reporting a very low share of counterfeit medicines of no more than 1% of the market value [143-146]. Nevertheless, the substantial volume of counterfeit medications incidents reported by developed states proves that this condition is extended to both industrialised [143-148] and less industrialised countries [149-157].

Predominantly in Africa [158-161], and marginally in Asia [162-164] and in Central and Southern America, forged medications sales range from 10% to more than 30% of the national legal market [145-165]. In the developing economies of many of the previous soviet block it is estimated an impact of the fraudulent medications of more than 20% of the national legal market [165-168]. FDA reported an 800 % surge in the incidence of counterfeit drugs in the interval 2000-2006 [169] in USA.

On December 2008, the Directorate General of Taxation and Customs Union of the European Commission (TAXUD) reported on the outcomes of the MEDI-FAKE action [127, 168, 170]. This was an operation conducted throughout the external area of the European Union and performed by the customs services of all the member states and coordinated by TAXUD. The operation accomplished a remarkable result, leading to a confiscation of 34 million illegitimate medications in two months' time. The fake medications ranged from antibiotics to anti-cancer, anti-malarial, anti-cholesterol medicines, painkillers, medications for erectile dysfunction [170].

In 2006, in the Russian federation, the federal health service stated that 10 % of all medications on the Russian market were fraudulent [167]. More recently, these share surged to 20 %, caused by a fast growing import of such products. In other countries of the area, such as Ukraine, this seems to be even worse with an estimated 40 % of the medications involved in the Ukrainian market being forged [166].

In 2005, in Peru, the local health authority, the general directorate of medicines, supplies and drugs (DIGEMID) of the department of health, confiscated 460000 fraudulent medicines and out of date genuine medications [171].

In the same year, 2005, in Dominican Republic, the local department of health stated

that 50 % of national pharmacies in the country functioned illegally, having found some medications had expired over 10 years before. Furthermore, the authorities reported 10 % of the drugs that were imported in the country were fraudulent [172].

The same year, in Kenya an unplanned inspection operated by the National Quality Laboratories and the Pharmacy and Poison Board disclosed that around 30 % of the medicines were fraudulent [173, 174]. Some of the solid counterfeit medications contained chalk powder and some of the liquid faked syrups bottles were only full of water, being marketed as genuine medications.

In 2004, in Nigeria, the ebony state task force on fraudulent and altered medications, stated that around 48 % of different items and medicines introduced into the country were substandard falsified goods [175-179]. Within this group of false medications, 19 % present a different composition with respect the original one; 16% have a dangerous or incorrect components such as chalk powder; 60 % lack of any active ingredients at all [172]. In India, as reported by the associated chamber of commerce and industry (Assocham) [183] 20 % of the drugs imported are fraudulent. that 38% of the medicines used in Indian national hospitals are counterfeit. Based on to two government investigations performed by the Cambodian ministry of health and the WHO [180, 181] the volume of purchased forged medicinal is in constant progressive increasing. In 2000, in Bangkok national drug-testing laboratories reported that 3.5% of the analysed antibiotics and analgesics specimens resulted to be forged, with a content inferior to 60% of the stated quantity of active principle ingredient reported on the packages. The same investigation was repeated in 2003 and the results shown an increased rate to 11% [172]. As a reaction to this results, in 2010, the inter ministerial committee (IMC), created with the purpose to contrast fraudulent and substandard medications import directed an operation that led 65% of illegitimate pharmacies to be shut down and five producers to stop from importing their altered goods in the country [172, 182].

All these are examples of a wide spread problem where countries are fighting a lonely war where health services and ministries are not collaborating internationally for fear to widespread panic in the population and lack of trust in the pharmaceutical systems.

Among the main medications introduced in developing countries, anti malarial medications have been heavily targeted by counterfeiters, exploiting the high incidence of these pathology in African and Asian regions [182-201]. Between 1999 and 2004, in

South East Asia the ratio of fraudulent over the counter antimalarial medications boosted from 38% to 53% [184].

Fake antibiotics [202-206] and anti viral medications of different typology [236-239] have are also entered dramatically the market of the same countries.

1.3.2. Manufacturing and distribution of medications

The manufacturing procedure and the production of medications is particularly intricate but it can be fractioned into two principal stages: the primary manufacturing stage and the secondary manufacturing stage [207]. The first stage basically is related to the manufacturing of the active pharmaceutical ingredient (API), which is the main substance with therapeutic characteristics. The second stage is related to the production of the end product, by incorporating the API in a mix of specific excipients that allow the API to be delivered to the wanted anatomical region and to act in a desired manner. When the end product is finalized the distribution stage is started. The distribution is itself divided in two stages: primary and secondary distribution. The primary distribution is the one relative to the delivering to the bigger warehouse directly from the manufacturers. The secondary distribution is the one operated from the bigger warehouse to intermediate distributors which in turns distribute to the final retailers, usually the pharmacies [207]. In certain circumstances, when there is an urgent need to supply a specific patient with a medication, the manufacturers sell straightforwardly to the pharmacies. In this case the medication is intended to be used by the specific patient and cannot re-enter the distribution chain [207, 208].

The manufactures may also decide to destine part of the medications produced for charity. The distributors usually do not deal with the full range of items of a single pharmaceutical company. They receive specific items of different manufacturers from different bigger distributors. The products can then be delivered to a final retailer or to another intermediate distributor, and restarting the process.

The business reason of all these intermediary distributions is the possibility to lower the final retail price of the item and being then beneficial to the final buyer. This is made possible, for instance, when intermediator obtain medications at lower prices when the product originates from surplus in production or storage [208].

The small dimension of the intermediate distributors allows them to be more flexible and ready to the changes in market demand. This is useful especially when there is a request of high volume of medications in a specific period of the year, for instance vaccines used in immunization campaigns. Thanks to this ability to respond quickly to the changes in the market demands, the intermediate distributors can counteract the larger distributors' shortages when a sudden and unforeseen surge in the demand of a particular medications occurs.

In different countries there could be different prices policies that make the cost of specific medication being different. This condition originates the phenomenon of the parallel import. Distributors buy the medication in the areas where the price is lower and sell it at increased price in other countries where the cost is higher [208].

This extreme intricacy of the distribution chain creates the conditions for the entry of fraudulent products [209].

Because of this complexity, the tracking of the drug journeys in the distribution chain from the initial manufacturer to the patients become very complicated.

Needless to say, the higher the number of intermediate distributors in the distribution chain the higher the complexity in chasing the journey of the medications and the possible infiltration in the official chain of illicit operations. Fraudulent medicinal might therefore be introduced into the distribution chain at virtually all levels in various modes [209]. For example, some of the elements that make the process weak, can be: the complication of the delivery process; the limited and infrequently implemented checking control during the distribution and re-packaging phases; shipping documentation that can be effortlessly altered [208, 209].

The effect of parallel import and the use of the internet as an international distribution network significantly facilitate the entry of forged medications in the official channels of distribution [209]. Furthermore, business operation processes put in place by some intermediaries, critically show how their conduct may enable the trade of fraudulent medications. Throughout the entire shipping activity, each intermediary distributor habitually masks the references of the previous suppliers on the shipping documentation to preclude their clients the possibility to bypass them on further transactions [210].

This practice is called 'neutralization' and it is used therefore by many distributors in order to defend their business interests and exclude as many competitors as possible

from the distribution chain [214, 228]. Furthermore, this procedure also hides all the previous shipping history of the product and, concealing any traces that can refer back to its origin, making in fact impossible to track back the drug.

Obviously, not knowing the origin of the medication within the distribution chain means not having elements to discriminate its quality. Notwithstanding the safety actions put in place to protect the legitimate supply chain, fraudulent medications might still find their way into the official distribution chain also because of intermediaries' behavior [214]. Some intermediaries may have a direct accountability as they deliberately disguise or merely disregard the actual source of the medication or medical device they market [234]. The 'One Touch' strips incident is a good example of how the role of unscrupulous intermediaries in the medicines distributor chain played an important key role [215]. In 2006, America imported from China fraudulent blood test strips, used to check glucose level in diabetic patients. They have been produced without applying the official production standards of manufacturing and they were then introduced through Canada to the USA. Such adulterated strips had also been found in significant volumes in 35 other countries worldwide particularly in Europe and Middle East. During the trial, the intermediary under investigation claimed that the company had only commercialised this product because they wanted to attain a more competitive lower prices and that they supposed the fraudulent strips were only lower priced parallel market products [215].

When legitimate medications travel in different countries and many distributors and retailers are involved, more complications to the identification of fraudulent pharmaceuticals arise. The repackaging procedure occur throughout the whole official distribution and shipment processes. They are necessary steps to guarantee that the container and instructions relative to a drug are understandable to the final patient. This process can be fulfilled directly by the importers, if specifically authorised, or by authorized companies [207]. The original container not only serves as an informative tool but also guarantees the originality of the medication if anti-counterfeiting features within the packages or labelling are applied [229]. After the opening of the product and the subsequent repackage these remedies might become inadequate. Furthermore, the code number used to identify a batch of drugs, important in case of a batch recall, every time that a repackage occurs, it is reprinted, creating the possibility for genuine mistakes

and illicit actions in the reprinting phase. Needless to say, this procedure offers huge chances of introducing counterfeit drugs into the official distribution channels. This recurrent change-hands procedure might be applied to conceal forged medications origin, making tracking nearly impossible. Additionally, there are further issues related to repackaging. In fact, the original packages should be disposed once they are switched with the new ones, otherwise they might be re-utilised by counterfeiters in order to be used as original packages for fake contents [236].

Other risks connected with repackaging may also be related to the possibility of alter the information on the original boxes. Two of the most common practices of falsification of packages are: the alteration of the quantity of active pharmaceutical ingredients (claiming more than was on the original information); the modification of the expiry date, allowing the distribution of out of date medicinal [231].

Another area exposed to risk of counterfeiting is the potential use of rejected hospital medications. This activity could be realised when the medication package does not present any anti counterfeiting protection system. In the case in which the unused hospital medication package still contains the medicinal, it could be remarketed while the original box can be used as a container for a forged medication [234].

1.3.3. Parallel import

Parallel import is related to a medication that after having previously been distributed through the different phases of the conventional distribution chain, it is bought by the major distributors and introduced into the parallel distribution chain with the intention of relocating the product into a new more convenient market using of parallel intermediate distributors [208, 209].

Parallel import, while is a lawful commercial practice in all the European Union, raises large criticisms related to its effective role on the infiltration of forged product in the official distribution chain [167, 217]. Parallel trade may simplify the entering of fraudulent medications entering in the official market in several different ways. Nevertheless, it is possible to group those ways in 3 main factors that characterise the parallel import distribution chain: the amount of commercial operators involved in the distribution of the goods; the volume of transfers of the same good in the delivery

system; the number of repackaging that the same good is exposed to [208-214]. All these three stages of the distribution chain represent weak points that can easily allow the access of fraudulent products when a definite regulatory system of control is lacking. Both parallel and intermediary distributors operate at the secondary distribution level. Parallel traders necessitate a license in order to operate in this area of business but it is not required any form of agreement with the manufacturer. This characteristic is similar for midway wholesalers who also do not have a predetermined contract with the manufactures. Midway wholesalers exploit fast changes in the market demands for a medication, subsidizing the increase of exchanges between the various stakeholders. As a result, before reaching the final user, the medication may be transferred several times [217-220]. Not having any sort of contract with the initial manufacturer of the medications, the intermediary distributors within the secondary distribution chain can have the possibility to buy the medications at a reduced price and resell it where the higher demand allows a growing profit. Such relocations, for the same product, can occur numerous times [220, 225-228]. On average, it is noted that a drug may be subjected to a multiple number of transactions and therefore such extension of level of transactions make a quality control approach on the originality of the goods extremely complicated. At the current time, it has been reported that there is no official method of validating the license of parallel distributors [244-247] Furthermore, the intermediary distributors have no obligation to keep a record of the batch number that identify the medications and allows tracking through all the transitional passages. In 2007, in the United Kingdom, 40000 packs of tablets were confiscated by the Medicines and Healthcare product Regulatory Agency (MHRA) [254, 255]. The fraudulent medications confiscated were allegedly anti-cancer, anti-psychotic and anticoagulant medications. These counterfeit medications had been boxed in France, manufactured in China, transported to Singapore, and subsequently to France, transferred to a parallel importer in Luxemburg and then finally sold them to other UK parallel importers. One of these British parallel distributor noticed some suspected alterations and contacted the manufacturer, which alerted the MHRA, leading to the confiscation [254, 255].

Based on a report from WHO [247, 254, 256], half of the medications traded on the internet sites are fakes. This peculiar market attracts people affected from different type of conditions, particularly those that are seen as taboos such as psychological and sexual

conditions. Pfizer conducted a study on a sample of 935 men, over 35 years of age, using regularly the internet market [216]. The outcomes showed that half of the examined people purchased online medications without a regular prescription, 67% for erectile dysfunction medications. 60% of the surveyed men also declared if they had known that the medications they were buying online were forged, they might not conclude the purchase.

The Food and Drug Administration released a study related to the effect of the internet market on the diffusion of the counterfeit medications. In this operation, the FDA studied a particular batch of counterfeit medicines available to buy on the internet [207, 213]. This product was advertised as produced in Canada but was actually coming from 27 different countries. The spam emails used to advertise the product were sent from an address licensed in Russia; the server for the website was located in China; the payee phone number was located in UK; the card payments processed in Australia and the product posted from US. Because of the single market regulation within the European Union, when a distributor obtains goods from an illegal internet source, can allow the spreading of the counterfeit product all over the European territory [208].

Counterfeiters usually penetrate the official distribution using as leverage the needs of the intermediary distributors to obtain goods at the lower possible price to maximize their profit. Once the products are introduced in the official distribution chain, they can therefore be marketed as all the other medications obtained from accredited manufacturer and it will be almost impossible to trace their origin [209-212].

1.3.4. The internet market

The anonymous nature of the online market makes the investigations performed by the law enforcement authorities significantly more difficult and consequently less effective [233], attracting the interest of criminals organizations to operate in this sector. In 2010, some unaware British higher education institutions were involved in a counterfeit medication scam involving the use of the ac.uk domains [235]. Counterfeiters exploited software flows in an extensively used technology named PHP utilized to make websites more interactive. Spammers injected a code associated with terms of the products they were trying to sell, such as Viagra, Cialis and other notorious names. Every time a

person looked for such medications online, British universities and colleges web addresses pop up, giving a sense of reassuring from such established institutions. Once the online buyer clicked on the links, they were immediately re-directed to the related online fake pharmacy. Those UK universities and colleges became unknowingly accomplices of such criminals.

1.3.5. Geographical distribution of medicine counterfeiting

According to the socio-economic conditions, in developed and less developed countries it is possible to identify diverse market choices operated by counterfeiters in respect of the distribution of different categories of fake medicines. This market variation is subjected to the different demands for specific medicines that exist in a given socio-economic context and highlight the professional level of organization of the groups behind this trade [151, 152].

In developed countries, such as West European countries, United States of America, Canada, the trading of counterfeit medication predominantly involves products that are lifestyle-related [188]. These so called lifestyle medications include pharmaceutical agents against erectile dysfunction, weight loss products, fake steroids, product that slow down the aging process. The trafficking of fake medication in less developed countries, normally is related to medications used to treat serious illnesses, such as different kind of antibiotics, vaccines anti-malaria and anti retro-viral for HIV. The diversity in the categories in counterfeit medications between these two general different areas reflect the specific marketing strategy implemented by traffickers [188-192]. Counterfeiters select only medications with the highest market share where the margin of profit are expected to be higher. In the developing countries the demand of certain medications is constantly high due to the constant exposure of the population to serious disease or epidemic catastrophes, making this market very appealing for the counterfeiters. The market area of developing countries is characterised by the high cost of the medications used to combat infections and other local typical diseases and at the same time by low supply of such products from the legitimate distribution chain [199-203].

The worldwide market of the counterfeit medications is remarkably very similar to its

legitimate counterpart. Thanks to the extensive networks, it permits a drug to be manufactured in a country that is very distant and not necessarily closely related to the ones of the final marketing [189-192].

1.3.6. Organised criminality involved in medicine counterfeiting

The reason behind the stringent interconnection between medicine counterfeiting and other forms of organized criminality can rely on the fact that there is an extensive similarity of between them [127, 128] For instance, organized crime involved in the production of narcotics, can use the same equipment to manufacture forged medicine. Different cases of cross-contamination have been reported, showing as the same clandestine laboratories used for the processing of narcotics have been used for the preparation of counterfeit medicines. For example, counterfeit antimalarial medications, seized in South East Asia have shown to contain traces of safrole [245]. This substance is a carcinogenic precursor to 3,4 Methylene Dioxy Meth Amphetamine (MDMA), better known as ecstasy, to substantiate the fact that the production of the forged medication has been performed in a plant used for manufacturing of ecstasy as well. A similar incident happened in a batch of counterfeit paracetamol elixir containing diethylene glycol derived from a contaminated manufacturing site that caused renal failure with fatal outcomes for the patient [246].

On this basis, it is clear that counterfeiting is a part of a more articulate organized criminality, which operates in an interconnect net of illegal operations. Unlawful distribution chains and trafficking itineraries already used traditionally for the movement of narcotics, as long as their manufacturing plants, can be used by counterfeiters to avoid investing in a new set of these infrastructures already established, and therefore maximize their profit [247-248].

One of the reason behind the success of the expansion of the illicit trafficking of counterfeit medications is indubitably the high demand of such products. Nevertheless, a distinction needs to be done. For an extensive range of forged medications, it is possible to consider valid the principle that wants the demand of such products being originated by the needs for medications that are not locally easily available through the traditional channels.

Another main reason that trigger the demand of forged medication is financial, because of the different cost of a medication available in pharmacies and the same obtainable from the alternative illegal ones.

However, in the case of fraudulent medications, the market requests that counterfeiters exploit is not only the one related to the illicit demands previously mentioned. When there is an increasing request of a medication, for various clinical reasons, in the legitimate distribution channels, counterfeiters operate intensively trying to enter in these official channels with their forged medications, being these the area of business that guarantee the highest level of profitability [248-250].

In 2010, in Peru, the commercialization of counterfeit medications generated 75 billion USD in the world, an increase of 92% with respect 2005 [171]. The Peru's association of pharmaceutical laboratories affirms that the value of sale of fraudulent medications in Peru has increased from 40 million US dollar in 2002 to a 66milion US dollar in 2006 [171].

The Kenyan association of pharmaceutical industry has reported that counterfeit drugs represent an estimated 130milion US dollar annually in sales in the country [174, 175].

The level of profitability of fraudulent medications commercialization has been projected being correspondent or even higher to that of the narco-trafficking [227]. For criminals involved in this area, the risks involved in trafficking in counterfeit medications is considerably lower, because worldwide the laws in this matter are either nonexistent or particularly severe [244]. Sentences in the majority of the countries are also less strict, only pecuniary, when compared with other serious crimes, which could involve long term detention. The lack of a strong and articulated reaction to this phenomenon from the law enforcement side is perhaps due to an initial impression that the occurrence of general counterfeiting was associated only to luxury products and for this reason did not create sufficient alarms to instigate an adequate reaction by law enforcement officials [244-247].

Medicine counterfeiting exploits the ability to produce a very low-cost product that, resembling the original one, can be sold with an incredibly higher final price counting on a well established organized criminal structure and a favorable environment. The participation of organized criminal groups in counterfeiting of fake medications has expanded the volume of medications copied, transforming the phenomenon into a large

trade illegal activity. According to WHO [248, 250], every year fraudulent anti-malarial and anti tuberculosis medications kill 700000 people and, from the same report, 200000 lives per year could be saved if malaria was treated without using counterfeit medications. In 1995, in Nigeria, almost 50000 people suffering from meningitis were treated with counterfeit vaccines, received as a gift by a country considered safe, and 2500 of them ultimately died. In 2001, China reported that 192000 dead occurred from the use of counterfeit medicines being administered instead those genuine ones [248, 250]. The ratio profit versus risks is particularly high and this represent the main reason at the basis of the commitment of the criminal organization in this line of illegal activity.

1.4. Literature review

The cost for the introduction of a new medication in the market, taking into consideration the overall phases incurred in the drug development of the product, was estimated averaged 2.6 billion American dollars in 2014. Generic medications are sold at a significantly lower price than the original one, but, nevertheless, not having incurred in the exceptional cost of the initial drug development phase, the lower price warrants considerable profit margins.

Within the Hatch-Waxman Act regulations produced in 1984, which regulates in the USA the entry requirements for the pharmaceutical companies to introduce a new generic medication, only studies of bioequivalence are requested, making easier and financially attractive for many pharmaceutical companies to participate in this typology of market. A consistent number of generic medications produced by a spread amount of manufacturers have permeated the therapeutic market world-wide.

Switching from the original brand to generic medications can considerably reducing the burden on the health assistance. It has been estimated that in the United Kingdom, the NHS has saved approximatively 7 billion Sterling in the last 40 years.

Health professionals and member of the public have expressed doubts on the real equivalence in quality and clinical efficacy between branded and generic medications. Spread feelings of uncertainty have arisen on these issues and the need for some more

robust bioequivalence studied to demonstrate clinical equivalence among generic and original correspondent medications has been expressed.

Over the last decades, a vast amount of literature has been produced on the matter of the therapeutic equivalence of generic medications to the relative brand-name ones.

Health professionals have recognized certain clinical areas where generic interchange needs to be applied carefully. In some particular therapeutic areas, the issue of generic substitutions raised so many concerns that numerous comparison studies to check the interchangeability among generics and branded have been generated. Several clinical studies conducted in certain medical areas have shown as the generic medications present an overlapping therapeutic equivalence to the original ones. For certain generics, both pharmacokinetics and pharmacodynamics issue have been reported. Also, in some cases issues on the stability of generics have been raised. In some of these areas a strong recommendation on the use of generic medications is encouraged, in light of the enormous financial advantages in using the generic against the original ones, helping to support the so much constrained budgets of the health sector in several countries worldwide.

Some of these studies have demonstrated the therapeutic equivalence amongst generic medications and original one, such as for osteoporosis and hypercholesterolaemia. Clinical areas where therapeutic efficacy has not been confirmed were ophtalmology, gastroenterology, antibiotics, cardio-vascular diseases, antiallergics.

Medicines counterfeit is an international grave and rapid growing danger for the patient's health and the pharmaceutical industry around the globe, both in industrialised and less industrialised countries.

Medications counterfeiting can embrace not only replicas of branded drugs but also of generic medications or over-the-counter products and also extended to faked appliances or diagnostic tools.

Primarily in Africa, and marginally in Asia and in Central and Southern America, counterfeit medications sales range from 10% to more than 30% of the national legal market. In many countries originated from the previous soviet bloc, the impact of the fraudulent medications is of more than 20% of the national legal market.

In the interval of time between 2000 and 2006, FDA reported an 800 % increase in the incidence of counterfeit drugs in the United States.

The effect of parallel import and the use of the internet market as a global distribution channel considerably facilitate the entry of fraudulent medicines in the official networks of distribution.

The law enforcement authorities face extreme difficulties in effectively investigating the organised crime perpetrated in the online market, because of its anonymity.

Organised criminality is strongly involved in this market because of the similarity of medicine counterfeiting and other more traditional forms of crime. For instance, organized crime involved in the production of narcotics, can use the same equipment to manufacture forged medicine but with a ratio profit versus risks extremely convenient, being the medicine counterfeiting in many countries not severely prosecuted.

Despite the numerous research articles and reviews published on the matter of clinical equivalence among generic and original medications, no study to the scientific community has been presented on an analytical evaluation of the chemical composition of the different generic drugs that could shed some lights on the reason of the different clinical performances reported.

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2. Aims and objectives of the study

From the considerations drawn in the literature review, it is clear that the different compositions in a formulation, the kind of medications being used (generic or branded) and the source of these are important from a clinical or therapeutically point of view given the impact on the patient response. This is particularly important in clinical areas where doubts on the therapeutic equivalence of generic medications have been raised.

The main aim of this research is to develop non-destructive quick qualitative analytical methodologies to be able to recognise differences in the chemical composition originated both from impurities in the formulation of the bulk agents and/or from instability of the active principal ingredient in generic medicines originated from the same brand-name medications and also obtained from different sources which could potentially provide counterfeit products. The scope of this study is to produce a flagging analytical tool able to quickly detect if a sample belongs to a class of approved generic medication or not.

This main aim can be broken down into the following objectives:

-To investigate generic medications that have been reported not presenting similar therapeutic equivalence in clinical comparison studies. From the results obtained through the bibliographic research, different drugs belonging to different therapeutic areas have been selected as specimens to be investigated. From the cardio-vascular area, digoxin (with the related cardiac glycosides digitoxin and digoxigenin) and amlodipine (in its different salts maleate, mesylate and besylate used in therapies) have been chosen because involved in investigations enquiring their clinical efficacies when used as generic medications. In the gastroenterology area, omeprazole both in its racemic and enantiomeric forms, have been selected as samples to be analysed since they have raised serious concerns about their generic medications' clinical efficacy. In the antihistamine area, cetirizine, in its racemic and isomeric forms, equally for the same reasons as before, have been considered.

-To select methods of analysis on these aforementioned generic drugs that can lead to significant and fast results. As starting analytical approaches, voltammetry, FT-IR, Raman and ^{1}H-NMR spectroscopies have been considered.

-To develop a statistical data analysis approach of the analytical data obtained based on multivariate analysis such as principal component analysis, cross validation, correlation scatter plots and factor loadings.

-To identify the analytical methods that present best responses among the previous taken into consideration on the basis of the results obtained.

-To expand the method of analysis to other generic medications: to apply the analytical and statistical method to other medicines and optimise the setting of such analytical approach.

-To apply the optimised method to medicines obtained from internet market to verify the authenticity of such medications.

3. GENERAL THEORETICAL PRINCIPLES OF THE ANALYTICAL TECHNIQUES ADOPTED IN THIS STUDY

3.1. Electrochemistry

Electrochemistry is the section of physical chemistry that investigates the correlation between identifiable chemical change and electricity. It is a measurable and quantitative occurrence with electricity considered an outcome of a particular chemical change. Voltammetry and Polarography are electrochemical methods based on the measurements of current i and potential V in chambers where electrolytic processes occur. Voltammetry measures the changes of current i as a function of the applied potential V and the diagrams that represent those variations are called voltammograms. The potential is modified continuously or at regular interval, being the independent variable, and the current is measured as the dependent variable. To perform a voltammetric investigation at least two electrodes are required. One electrode, called the working electrode, applies the desired potential in the desired conditions and interacts directly with the analyte. Furthermore, it assists the transfer of electric charge to and from the analyte. A second electrode is necessary to complete the electrolytic cell. This second electrode is characterised by a known potential, essential to correlate to the one of the of the working electrode. The second electrode needs to counterweight the electric charge that is added or removed by the working electrode during the execution of the experiment. This feature results to be particularly difficult to maintain, being particularly problematic for an electrode to provide a constant potential while passing current to counter redox reactions at the working electrode. To overcome this issue, the traditional original setting has been improved introducing a third electrode, the auxiliary electrode, dividing the supply of electrons and the provision of a reference potential executed by these two distinct electrodes, the reference electrode remains the half cell with a definite reduction potential and its only role remains to act as reference in measuring and controlling the potential of the working electrode. Thus, the reference electrode is not involved in passing any current during the electrolytic process. It is the auxiliary electrode that produces all the current necessary to balance the current registered at the working electrode. To realise this current, the auxiliary electrode fluctuates to extreme potentials at the extremes of the

solvent window, where it oxidizes or reduces the solvent or supporting electrolyte. These three electrodes, the working, reference, and auxiliary electrodes represent the set up of the current voltammetric apparatuses. In electrochemistry, the analytical signal is the current i, normally a faradaic current, which drifts through the cell during the electrolytic process of the analyte at the working electrode. The analyte may be a cation, an anion or a neutral molecule. The inventor of this technique, Jaroslav Heyrovsky, introduced as the working electrode a dropping mercury electrode. This type of electrode consists of a thick-walled glass capillary from which the mercury descents in drops into the sample solution, pushed by the pressure of a column of mercury. The recorded current-potential curves polarograms and introduced the term polarography. The term voltammetry derives from volt-am(pere)metry and it is different by the term voltametry (spelled with one m) which is indicated by IUPAC as a controlled current potentiometric measurement. The meanings of polarography and voltammetry are often used in the reverse sense or are used inaccurately. According to the IUPAC definitions, the term polarography should be used when the current-potential curves are registered by using a liquid working electrode whose surface can be renewed periodically or continuously (eg drops). This comprises the classic dropping mercury drop electrode (DME) and the successively improved static mercury drop electrode (SMDE) technique. Voltammetric analysis embraces all the techniques in which the current potential measurements are performed at a stationary and fixed working electrode, irrespective of its material structure. These comprise the thin mercury film electrode (TMFE), the hanging mercury drop electrode (HMDE), the glassy carbon electrodes (GCE) and carbon paste electrode (CPE). Working electrodes made of noble metals (such as platinum and gold) are used less recurrently. Different analytical approaches are addressed to the definitions of voltammetry and of polarography: these different methods vary in the way the measurement is performed and the procedure of electric potential in the determination activity.

In the classic case in Direct Current Polarography (DCP), the analysis is based on the measuring of the current that flows through the DME used as working electrode during a linear direct voltage modification. The counter electrode is routinely an electrode of the second category, such as an AgCl electrode or calomel, which contrarily to the more recent measuring settings, is at the same time the reference electrode. On closer observation it is possible to recognise that the current passing through the working

electrode is created by two elements. One is the Faradaic current iF, which is based on the reduction or oxidation of the analyte, according to the redox reaction occurring. The second element is the capacitive current iC, which is originated by the charging and discharging of the electrochemical double layer on the surface of the working electrode. For the majority of the polarographic analysis, while the faradaic current provides the analytical signal, the capacitive current represents the background noise interference.
In real terms analytical situation, the capacitive current, potential dependent, can increase up to 10-7 A and is then within the range of the Faradaic diffusion current iD of an analyte solution with 10-5 mol/L. When iC has the same value as iF (iF/iC=1), then the analytical signal can no longer be detached from the interference signal. The detection limit for direct current polarographic determinations is, therefore, restricted by this signal-noise ratio related to the connection between the measuring signal and interference signal. The maximum value for iF is the diffusion current iD and it is achieved when all the analyte conveyed to the surface of the mercury drop by diffusion have been reduced or oxidised. The correlation between the concentration of the analyte and the diffusion current is described by the Ilkovic equation:

$$\mathrm{iD} = 0.607 \times \frac{nD}{2} \times \frac{2M}{3} \times td \times \frac{Ca}{6}$$

where:
iD: diffusion current
n: number of electrons exchanged in the charge-transfer reaction
D: diffusion coefficient of the analyte
M: Hg flow rate
td: dropping time of the Hg drop
Ca: analyte's concentration

A higher sensitivity for a Polarographic measurements is only possible if the ratio iF/iC can be increased by other measuring techniques, weather by increasing iF or decreasing iC. Developments of this analytical technique aiming to reduce the capacitive current resulted in sampled DC polarography and to the pulse methods. Optimisations aiming to expand the faradaic current led to stripping voltammetry. In this last variant, the analyte is accumulated electrolytically at a stationary working electrode before being

voltammetrically determined. The adoption of the use of a static mercury drop electrode (SMDE) instead of the dropping mercury electrode DME and the introduction of digital instruments and has dramatically improved he performance of both voltammetric and polarografic methods.

Stripping voltammetry approaches are the highest performant electrochemical techniques for trace analysis and species analysis. The uncommon high selectivity and sensitivity are permitted by the accumulation of the analyte before its determination and that both accumulation and determination are electrochemical procedures whose advancement can be influenced. Determinations by stripping voltammetry are commonly more sensitive by a factor of 10^3 to 10^5 when compared to traditional polarographic analysis. The detection limits are usually between 10^{-9} to 10^{-11}mol/L, being in some instances even 10^{-12}mol/L. This values indicate that stripping methods are placed amongst the most sensitive analytic technique existing. In stripping methods, both the accumulation and the determination processes occur at the same electrode without the need to change vessels. This determines that the incidence of the systematic errors caused by contamination or evaporation can be maintained at a very low level. The term stripping is related to the fact that during the determination process, the accumulated analyte is stripped from the working electrode. The accumulation process always occurs at constant potential leading to the accumulation potential (E_{acc}). The accumulation process taking place at a stationary mercury drop, mercury film, graphite or noble metal electrode occurs for a controlled period, called the accumulation time (t_{acc}). The sample is accumulated electrolytically as a metal, or in alternative weather as a sparingly soluble mercury compound or adsorptively as a complex compound. The stripping of the deposed analyte species from the working electrode is the most important analytical step, and it is based on the oxidation or reduction process. In the typical instance where analyte is accumulated as an amalgam at the Hg drop or Hg film electrode, the determination occurs to be the reverse process to the accumulation, and this feature is where the term inverse voltammetry originates from. The term Anodic Stripping Voltammetry (ASV) is used where the determination of the accumulated product is not related to the oxidation process of the analyte but to its reduction. Anodic Stripping Voltammetry (ASV) is effective in determination of the metals which are able to dissolve in mercury with the formation of amalgams or which can be deposited electrolytically at noble metal or carbon electrodes. Cathodic stripping

Voltammetry (CSV) is adopted for the determination of organic and inorganic anions and varies from ASV both in the determination process and in the accumulation procedure. During the accumulation process, the sample is deposited anodically as a thinly soluble mercury salt or cathodically as an intermetallic compound at the electrode surface.

The combination of voltammetric accumulation and determination is defined as Adsorptive Stripping Voltammetry (AdSV) if the sample can be accumulated in an appropriate form by adsorption on the surface of the electrode and then voltammetrically determined by oxidation or reduction processes. Adsorptive accumulation is an important advantage to electrolysis because it makes stripping voltammetry applicable even for those analytes that cannot be accumulated or determined because of their lack of amalgam formation or irreversible electrode reactions at the mercury electrodes. Elements such as Aluminium, Iron, Cobalt, Nickel, Titanium, Chromium, etc. may therefore be traced by Adsorptive Stripping Voltammetry. Furthermore, Adsorptive Stripping Voltammetry is also applicable for the trace analysis of many organic compounds. It is worth to notice that while organic analytes with surface-active features are adsorbed directly at the electrode surface, traces of elements need firstly to be transformed into thinly adsorbable and soluble complexes. The determination procedures are related either to the reduction of the central atom, the reduction of ligands of the complex compound, or the catalytic hydrogen production. The determination limits of Adsorptive Stripping Voltammetry are in the range of nanograms per litre, making this technique's sensitivity even higher than anodic stripping voltammetry. The superior sensitivity of the Adsorptive Stripping Voltammetry is originated by the fact that the adsorbed analyte rests on the electrode surface while in anodic stripping voltammetry the accumulated metal diffuses into the Hg drop or Hg film. Consequently, the sample concentration available for the stripping process at the electrode surface, known as local accumulation factor, following adsorptive accumulation, is higher than the analyte concentration after electrolysis and amalgam synthesis.

3.2. Raman and Infra-Red Spectroscopies

An incident light beam interacting with a material can be absorbed, reflected, refracted, scattered or cannot interact at all, passing through it. When the level of energy of the

photon impacting with the material is correspondent to the to the difference between the ground state and the excited state of the molecules forming the impacted material, the absorbed photon causes a promotion to the higher electronic energy excited state of the molecules. Absorption spectroscopy measures this change in energetic level detecting the loss of energy of the out coming light after the impact with the sample material. Nevertheless, the light can also be impacting with the material and scatter from it. In this instance, the photon does not need to be at an energy level able to cover the difference of two energy level of the sample. This scattered light can be detected registering the scattered light at an angle to the direction of the incident light. When the incident beam photon has a energy level different from the electronic transition level of the sample material, not causing in this way any electronic transition, the efficiency rises at the fourth power of the energy of the incident light. Scattering is an analytical method widely used for the determination of different physical properties of materials, such as particle size and size distribution. Amongst the different scattering technique, Raman scattering is the principal analytical approach for molecular identification. One main way to characterise light is though its wavelength (λ), but, since spectroscopy is related to the interaction between incident beam light and energy states of the sample material, frequency (ν) or wavenumber are the principle feature then into consideration, being directly related to energy.

The condition in which light is utilised in Raman scattering and infrared absorption spectroscopies is quite different. In infrared absorption the analyte is exposed to a radiation of a broad range of wavelengths and when the specific incident light frequency matches the vibrational energy level of the analyte, the molecule is stimulated to a higher vibrational state. It is analysed the loss of energy of the incident radiation due to these absorptions. On the other hand, Raman scattering adopts one unique incident radiation at a fixed wavelength to interact with the sample material. The scattered light produced by the irradiated analyte is then monitored. Consequently, complementary to the infrared spectroscopy, Raman scattering does not demand an overlapped incident radiation to the energy change between the ground and excited level. In Raman scattering, the photons interact with the sample and alter (polarises) the electrons cloud to create a transitory state called a virtual state. The virtual state is unstable and the light is rapidly re-emitted. Since only an electrons cloud distortion occurs in scattering, the light is re-radiated with minor

frequency variations. This scattering re-radiation is called elastic scattering and is the principal process. At molecular level, this scattering process is named Rayleigh scattering. When nuclear motion is involved in the scattering process, energy is shifted from the incident light to the sample or from the molecule to the scattered light. In these instances, the process is deemed to be inelastic and the energy of the scattered light is different from the energy of the incident light by one vibrational energy unit. This is called Raman shift. The Raman scattering is a weak phenomenon, occurring in only one in every 10^6-10^8 photons which scattered. In the same time, other radiation processes can occur, such as fluorescence and sample degradation. The majority of the molecule exist at room temperature in the minor energy vibrational level. The virtual states are not natural states of the sample but are produced when the incident light interacts with the electrons cloud and causes polarization. The energy of these states is therefore determined by the frequency of the incident light source used. The elastic Rayleigh process is the principal intense process being the main process of scattering. Rayleigh process does not involve any permanent energy change in the electron cloud and consequently the light returns to the same energy state. On the contrary, the Raman scattering process involves an absorption of energy from the ground vibrational level to a higher excited vibrational state. This is called Stokes scattering. Nevertheless, some molecules may be present in an excited state due to thermal energy. Scattering from these excited states to the ground state is called anti-Stokes scattering. It involves transfer of energy to the scattered photon. The relative intensities of the two phenomenon depend on the population of the various states of the molecule. Anti-Stokes scattering will be weaker than the Stokes one and will reduce in intensity as the frequency of the vibration surges due to reduced population of the excited vibrational states. Furthermore, the rise of temperature induces an increase of the anti-Stokes scattering relatively to Stokes scattering. Generally, Raman scattering is recorded only on the low energy focussing on the Stokes scattering, but sometimes anti-Stokes scattering is desired. For instance, in the event of occurrence of fluorescence interference, this will appear at a lower energy than the excitation frequency and subsequently anti-Stokes scattering can be more beneficial to prevent or reduce interference. Furthermore, because of the temperature effect on this processes, the difference in intensities of Raman scattering in Stokes and anti-Stokes scattering can also be used to monitor temperature changes. Infrared absorption encompasses direct

excitation of the molecule exposed to an irradiant light holding exactly the energy differences between two state levels.

Raman scattering requires a much more intense radiation and measures the differences in energy between vibrational states as a difference between the energy of the scattered photon and that of the incident light. The strongest Raman scattering are produced from vibrations of the electronic cloud that create a variation in the polarizability of the electron cloud round the molecule. Normally symmetric vibrations produce the widest changes and create the greatest scattering. The dissimilarity with infrared absorption is remarkable. In infrared spectroscopy the most powerful absorption is produced by a variation in dipole and consequent more intense asymmetric vibrations. Among all the vibrations existing for a molecule, not all of them can produce both infrared and Raman processes and the two different spectroscopies usually give relatively distinctive intensity patterns. As a result, the two spectroscopies are frequently complementary and adopt together in chemical analysis to capture a better view of the vibrational structure of a molecule analysed. Both Raman scattering and infrared absorption are not active in centro-symmetrical molecule, because of the reflection of any part of the molecule through the centre will reach an identical point on the opposite part. This dissimilarity is remarkably valuable for small molecules where a comparison of the analysis obtained from infrared absorption and Raman scattering can be used to discriminate between cis and trans forms. The highest frequencies of vibrations will be given by light molecules and strong bonds while the lowest frequencies will be given by heavy molecules and weak bonds. During the assignment of the peaks in the spectroscopy diagram to the vibrations of the component of the molecule, it is important to bear in mind that two or more bonds of similar energies positioned close together in the same molecule can interact. In these cases, it is the vibration of the group of atoms linked by these bonds which is observed in the spectrum. For instance, methylene groups present both a symmetric and an anti-symmetric stretching rather than 2 separate stretches. Consequently, it possible to record different types of vibrations for the same chemical groups, because of the chemical surrounding and the geometry of the molecule. However, if the atoms forming a molecule are well separated, they can be treated separately and showing a large difference in energy between the vibrations in different bonds. In the assignment of the spectral peaks to the chemical bonds forming the structure of the molecule analised, it is possible to identify

energy ranges in which the characteristic frequencies of the most common groups occur, both in infrared absorption and Raman scattering. For instance, the interval between 4000 and 2500cm^{-1} is the region where single bonds absorb. The region between 2500 and 2000cm^{-1} is related to the multiple bond absorption. The 2000-1500cm^{-1} interval is where the double bonds occur. In the region below 1500cm^{-1} the majority of the molecules have intricate configurations of vibrations due to groups of atoms, usually in carbon chains, link by bonds of approximately the same energy level. This interval is referred to as the finger print region.

Surface Enhanced Raman Spectroscopy (SERS) is a surface-sensitive technique that enhances Raman scattering by molecules adsorbed on rough metal surfaces or by nanostructures such as plasmonic-magnetic silica nanotubes. The enhancement factor can be as much as 10^{10} to 10^{11} which means the technique may detect single molecules.

The exact mechanism of the enhancement effect of SERS is still a matter of debate with two principal theories accredited as the most probable. The electromagnetic theory suggests the excitation of localized surface plasmons, and can be applied regardless of the chemical composition of molecule being studied. This theory does not fully explain the magnitude of the enhancement observed in many systems.

The chemical mechanism proposes the formation of charge-transfer complexes for many molecules structured with functional groups with lone pair of electrons available that allow the formation of bond with the rough metal absorbed. In this theory the signal enhancement is of chemical nature, where the excitation wavelength is resonant with the metal-molecule charge transfer electronic states. The chemical theory cannot explain the observed signal enhancement in all cases but it is broadly accepted that the charge transfer between metallic nanostructures and molecular species is a key issue in the underlying chemical enhancement mechanism in SERS. The chemical mechanism only applies in specific cases and probably occurs in concert with the electromagnetic mechanism.

The Fourier Transform is a mathematical technique that convert any waveform into an alternate representation as the sum of sinusoidal functions. In Fourier-Transform Infra Red spectroscopy (FT-IR) the spectra are collected based on measurements of the coherence of a radiative source, using time-domain or space-domain measurements of the electromagnetic radiation. In the older technology, the original infrared instruments were of the dispersive type. These instruments separated the specific frequencies of energy

emitted from the infrared source by the use of a prism or grating. The detector measured the intensity of energy at each frequency which has passed through the analyte. This scanning process resulted to be rather slow. Fourier Transform Infrared (FT-IR) spectrometry was developed in order to overcome the time restriction met with the dispersive technology measuring all of the infrared frequencies simultaneously, rather than individually. A simple optical device, called interferometer, was developed, which produces a unique type of signal that embrace all of the infrared frequencies encoded into it. The unique signal can be measured quickly, usually on the order of seconds rather than several minutes as in the cases of dispersive type, and by Fourier transformation it can be decoded and converted into an interpretable traditional spectrum.

3.3. Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) is a physical phenomenon where nuclei exposed to electromagnetic radiations produced by a determined magnetic field, are able to resonate with them, absorbing and re-emitting specific resonance frequency. The resonance frequency varies according to the intensity of the magnetic field applied and to the magnetic features of the isotope studied. Particularly, the resonance frequency applied for a specific sample to analyse is directly proportional to the strength of the applied magnetic field. The resonance frequency is usually in the range of 60–1000MHz, the same of radio or television broadcasts. All elements' nuclei carry a charge. When nuclei are exposed to certain magnetic fields may interact with them and emitting electromagnetic radiations. When the spins of the protons and neutrons forming these nuclei are not paired, the resulting whole spin of the nucleus produces a magnetic dipole alongside the spin axis. The inherent scale of this dipole is a basic nuclear feature called the nuclear magnetic moment, μ. The internal structure of the nucleus determines the symmetry of the charge setting. In nuclei presenting a spherical charge distribution, the relative spin angular momentum number is ½. This is the case for instance for atoms like ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P etc. In nuclei where the charge distribution is non-spherical (analogous to e.g. a hydrogen 3d orbital) the spin numbers are higher (for example ^{10}B, ^{14}N, etc.). Nuclear Magnetic Resonance investigates quantum mechanical magnetic properties of the atomic nucleus, giving important information on the structural

architecture of crystal and non crystalline substances. NMR is also usually used in diagnostics, as an advanced medical imaging techniques, such as in magnetic resonance imaging (MRI). It is this characteristic that is used in imaging techniques; when a substance is located in a non-uniform magnetic field, the nuclei' resonance frequencies will vary according to the location of the magnetic fields. The resolution of the imaging technique is directly proportional to the intensity of the magnetic field gradient. Developments in increasing this feature have been sought, and the use of superconductors has led to a considerable improvement of the imaging resolution. Other developments to increase the efficiency of this analytical technique have addressed improving of hyperpolarization and multi-dimensional multi-frequency techniques.

The first approach to nuclear magnetic resonance spectrometry was based the application of continuous-wave spectroscopy (CW spectroscopy). This technique is based on the use of a determined fixed frequency and varying the current, and consequently the magnetic field, in an electromagnet in order to observe the resonant absorption signals. With the CW spectroscopy has been introduced the concept of "high field" and "low field" to indicate the low frequency and high frequency areas of investigation. CW NMR spectrometers often operating at 60MHz, are cheaper to maintain and operate, with correspondingly weaker (non-superconducting) electromagnets cooled with water rather than liquid helium. In these spectrometers, one radio coil runs constantly, sweeping through a variety of frequencies. A second perpendicular coil, devised not to absorb signals from the transmitter, received radiations from nuclei that reoriented in solution.
The downside of CW spectroscopy is that it investigates the NMR signals at individual frequencies in progression. Because of the intrinsic weakness of the NMR signal, the resulting spectrum is affected by a modest signal-to-noise ratio. This feature may be diminished by signal averaging, performing added repeated measurements. Whereas the NMR signal is constant between scans and so adds linearly, the background noise adds more gradually.

Fourier-transform NMR spectroscopy exposes the sample simultaneously to more than one frequency producing the NMR spectrum more efficiently than with the CW methods. The majority of NMR applications involve the full NMR spectra, where the intensity of the NMR radiation is function of the frequency applied. A significant improvement in NMR arose when short pulses of radio-frequency signals centered at the

middle of the NMR spectrum, started to be used. A short pulse of a given carrier frequency embodies a range of frequencies centered about the principal frequency, with the range of excitation being inversely proportional to the pulse duration. The Fourier transform of a short pulse contains contributions from all the frequencies in the proximities of the carrier frequency. Employing this short pulse to a set of nuclear spins concurrently excites all the single-quantum NMR transitions, orientating the magnetization vector away from its stable alignment. This alternating magnetization vector induces an electrical signal, known as the free induction decay (FID), oscillating at the NMR frequency. To acquire the frequency-domain NMR spectrum, as NMR absorption intensity versus NMR frequency, the time-domain signal, intensity versus time, must be Fourier transformed.

Multi-dimensional nuclear magnetic resonance spectroscopy is a modification of Fourier-transform NMR spectroscopy where there are two or more pulses applied with a methodic variation of the pulse sequence as the experiment is repeated. In multidimensional nuclear magnetic resonance there is a succession of pulses and one or more variable time period. For instance, in three dimensions, two time sequences vary and so on. These time intervals allow magnetization transfer between nuclei and, consequently, the identification of the relative nuclear-nuclear interactions.

Solid-state NMR spectroscopy is an analytical technique complements X-ray crystallography, performed on molecules in a solid phase. The development of this NMR spectroscopy has been extremely challenging because of the reduced signal related to the thermal motion in the solid state, where the majority of the molecules can only present limited vibrations and rotations at room temperature, each in a slightly different electronic environment, consequently showing a distinctive NMR absorption peak.

The sensitivity of the NMR technique depends on the intensity of the magnetic field and the relative magnitude of the nuclear magnetic resonance signals and over the decades, this technique has progressed also with the development of more powerful magnets. The sensitivity of nuclear magnetic resonance spectrometry is related to the abundance of nucleus able to magnetically resonate and, consequently, either on the natural concentration of such nuclides or on the possibility to artificially increase the experimental condition with such nuclides. In nature, the most abundant magnetically susceptible isotopes readily useful for nuclear magnetic resonance spectroscopy are

hydrogen and phosphorus. Contrarily, carbon and nitrogen have very low natural abundance magnetically susceptible isotopes.

In Nuclear Magnetic Resonance, the chemical shift is the frequency at which a nucleus under investigation resonate, with respect the resonant frequency of a reference substance exposed to the same magnetic environment. This feature is used as an analytical signal in NMR to characterise parts of the molecule under investigation. Within the molecular orbitals, the currents of electrons produce magnetic fields which can affect the total nuclear magnetic field. The electron distribution of the same type of nucleus investigated differs according to the electronegativity of the groups linked to the atom analysed. For example, in proton NMR, if the hydrogen is attached to a more electronegative group, the magnetic field necessary to produce nuclear resonance will be less intense than the one needed in a H-H bond. The reference substance is generally a molecule with a hardly distorted electron distribution that produce a strong peak of resonance, unambiguous to identify in the NMR spectrum. Tetramethylesilane (TMS) is usually selected as this standard because of its peculiar chemical structure, with four methyl groups linked to a single Si atom. The specific geometry of this molecule make the all twelve atoms of hydrogen equal when resonating with magnetic radiations. This leads to the production of a single and strong peak of resonance. Furthermore, in this molecule, the currents of electrons in the molecular orbitals of the C-H bonds are closer to the hydrogens than in the majority of the other organic compounds. This implies that these hydrogen nuclei are the most sheltered from the applied magnetic field. Consequently, the intensity of the magnetic field necessary to induce nuclear resonance has to be extremely higher than the one generally needed to induce resonance for the majority of the other organic molecules. These features allow to have a reference signal on the NMR diagram well distinguished and separated from the majority of the others.

3.4. Chemometric

Nowadays analytical chemistry employs equipment able to generate enormous amounts of data. An Infrared analysis may produce different thousands of data points as wavenumbers. A single analysis of Gas Chromatography coupled with Mass spectrometry usually generates 600K digital values that occupy around 2.5Mbytes. There

are two different ways to handle this huge quantity of information. The easiest way is to ignore the volume of the collected data. In the case of a spectroscopic analysis, for example, traditionally only the most representative peaks are taken into consideration for analytical purpose, disregarding the rest of the spectrum. In the case of Gas Chromatography coupled with Mass spectrometry the recording is set for a special unit of mass and not the full range of units of mass. Typically, quantitative analyses are univariate, identifying one variable, such as a peak current at one potential in an electrochemical measurement or the wavelength of maximum absorbance in a spectroscopic measurement correlated with a given sample concentration. This traditional approach to data analysis is incomplete and inefficient. Considering for example that a simple UV-Vis spectrum of an analyte containing 500 data points, with only one data point, such as absorbance at one wavelength, being used for quantitative purpose, after identification, 99.8% of the data will be discarded. On the basis of such consideration, it is not efficient therefore to collect data that will not be exploited. Moreover, a univariate approach is extremely exposed to interferents. It is almost impossible to differentiate a specific signal deriving from the analyte and from an interferent when both absorb at the one specific data point selected for the analysis. Without specific tools it has been impossible until recently to fully explore the total volume of data collected during an analysis, leaving unrevealed potentially important pieces of analytical information. On the other hand, the multivariate analytical approach involves the use of the multiple variables, such as the response at a range of potentials or wavelengths, or even over the entire range of data points gathered during analytical scans. With multivariate analysis it can be easier to identify and to remove noise when looking at the entire data points set, rather than focusing only on one point. Moreover, taking into account the full set of data points, the interferents profile will likely differ substantially from the analyte profile studied. Multivariate analysis increases the amount of possible information that can be obtained without loss. In the past, the application of multivariate models was hindered by the computational complexity of the data analysis. Nowadays these issues have been overcome with the development of dedicated software well supported by available personal computer. Developments in data processors computing over the past decades have transformed the analytical equipment in laboratories. Technological advances have made devices from one hand quicker, portable, and cheaper and on the other hand more

accurate, precise and available. Also data analysis approaches have also gained from innovations in technical computing, with accessible statistical software to be used with available powerful computers, allowing scientists to perform intricate statistical data analysis in relatively short period of time. Currently, the regular use of processors makes it possible to entirely elaborate vast data sets, with a extremely reduced loss of information. The range of principal goals of multivariate approach in analytical chemistry include data reduction, grouping, the classification of observations and the modelling of relationships that may exist between variables. By the intensive use of chemometric tools, it becomes possible to gain a deeper insight and a more complete interpretation of this data. The predictive feature is also an important aspect of some multivariate analysis, being relevant to predict whether a new investigation belongs to any pre-acquired qualitative groups or to evaluate some quantitative feature such as chemical concentration. Chemometric is defined as the the application of statistical and mathematical approach to the elaboration of analytical data obtained from analytical chemical investigation in order to gain maximum collection and extraction of useful information obtain increasing amounts of chemically relevant information from their data. Many commercial instruments are sold already with software containing algorithms that able to perform certain data analysis, such as Fourier transform analysis, data filtering, and peak recognition, automatically.

PCA is a visual illustrative method, not based on a probabilistic model of data but simply aims to provide geometric representation. Amongst the different multivariate analysis techniques, Principal Component Analysis (PCA) is the most frequently used, being a starting point in the process of data mining. PCA's main purpose is minimizing the dimensionality of the data. Normally, it is usual to deal with a vast amount of data where a number of *n* objects is described by a set of *p* variables. Those data are gathered in a matrix X, with n rows and p columns, with an element xij referring to an element of X at the i^{th} line and the j^{th} column. Usually, a row of matrix X matches with an "observation", that could be for instance a group of chemical dimensions or a diagram or, more commonly, an analytical curve generated from an investigation of an analyte, executed by an apparatus producing analytical curves as output data. A column in the matrix X is usually called a "variable". The generic average chemical analyses usually deal with multidimensional data n x p, with n and p of order of several hundreds or even

thousands. It is clear that with such an abundance of data, it is impossible to identify in this set any significant information without the support of a statistical technique such as PCA. Principal Component Analysis is used in diverse areas where data analysis is crucial, particularly in the food research laboratories and industries, where it is often used in combination with other multivariate procedures such as discriminant analysis.

Principal component analysis (PCA) is a mathematical approach to data analysis that rearrange a set of observations of possibly correlated variables into an orthogonal representation set of values of linearly uncorrelated variables called principal components. Therefore, PCA restructures high-dimensional data points set into a low-dimensional subspace component and a noise component. The main question in PCA is to how choose the number of principal components to be retained, that represent the dimension of the subspace. Different guidelines have been elaborated for evaluate the real dimensionality of the data points set. Some of these methods encompass the measurement of the percentage of total variation, the Kaiser's rule, and the screen test depending on a subjective options. On the contrary, other criteria, are methods based on statistical approaches that require distributional assumptions which are usually impractical. Another class of rules developed in chemometric, the cross validation (CV) methods, are fairly objective, not needing distributional assumptions. Notwithstanding traditional criteria, cross validation methods are not centred on the eigenvalues of the sample covariance matrix but on the predictive ability of different principal component models. The essential common idea of the cross validation models is the use of distinctive data sets for valuation and authentication of each principal component model. The training set is the data fraction used to estimate each principal component model. Considering a p-dimensional space where each individual dimension is linked with a variable. In such a space, the individual observation is depicted by its own coordinates related to the value of variables that define it. Because of the complexity of the original data, an interpretable representation of all the initial variables results to be inaccessible. Therefore, it is essential to condense the initial p-dimensional space into a reduced space than p, but retaining the greatest level of information. This amount of information is statistically expresses by the variances. PCA creates new variables by the mean of a linear combination of the original variables. This change of variables results geometrically in a change in orthogonal axes, called principal components. Each newly created axis, orthogonal each other, describes a direction that

defines a part of the overall information. The first principal component (PC1) is the first axis calculated in order to represent the main pieces of information, followed by the second principal component (PC2), orthogonal to the PC1, which expresses a lesser amount of information, and so on. In PCA therefore, the p original variables from the raw data points set are replaced by a group of new variables, the components, which are linear combinations of these original variables. The variances of components are classified in decreasing order. The whole set of components keeps all of the original variance in the processing of the PCA. This change of axis allows a better geometrical representation of the data, without altering the dimensions of the space.

Scores represent the coordinates of the observations on the Principal Components, and the relative diagrams are called score-plots. Loadings represent the influences of initial variables to the different components, and the relative diagrams are called loadings-plot. They can be thought as the projection of unit vectors characterizing the variables in the consecutive planes of the main components. While scores give a picture of the observations in the space produced by the new symmetric axes, the principal components, loadings express the variables in the space of principal components. Observations adjacent to each other in the space of principal components unavoidably present analogous features. This contiguity in the original space brings to a near neighbouring in the score-plots. Equally, the variables whose unit vectors are adjoining to each other are defined as positively correlated, indicating that their effect on the positioning of objects is comparable. These neighbouring are reproduced in the projections of variables on loadings-plot. On the contrary, variables positioned remotely from each other are described as being negatively correlated. Depending on the nature of the data, loadings can be differentiated in two different groups. When the data contains discontinuous variables, the loadings are represented as a factorial plan, such as PC1 vs. PC2, showing each variable in the PCs space. When the data contains continuous variables, for example in case of spectroscopic or chromatographic data, the loadings are represented differently. In this case, the values of the loadings of each principal component are represented in a diagram with the values of the loadings of component PC_i on the Y-axis and the scale corresponding to the experimental unit on the X-axis.

3.5. Validation

Standard deviation is a statistical feature that determine the dispersion of a set of data from its average value, also called the expected value. It is calculated as the square root of variance by determining the variation between each data point relative to the average. When the data points are sensibly scattered away from the average value, there is higher deviation within the data set and the correspondent standard deviation will be high. On the other hand, a low standard deviation indicates that the data points tend to be close to the mean of the data set, while a high standard deviation indicates that the data points are spread out over a wider range of values.

Relative Standard Deviation (RSD), often known as Coefficient of Variation (CV) is defined as the ratio of the standard deviation to the average and is expressed as a percentage. When RSD is high, the data set are spread out over a wide range of values, while when RSD is low, the data collections are tightly clustered around the mean.

The Limit of Detection (LOD) and Limit of Quantification (LOQ) are two relevant features used in method validation processes. They test the performance of a describe analytical procedure giving information on the smallest concentration of an analyte that can be detected. According to the IUPAC definition, Limit of Detection is the lowest amount of analyte in a sample which can be be reliably distinguished from zero, but not necessarily quantitated as an exact value. The LOQ gives more practical information, being defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of test. These two parameters can be determined in different ways, taking into account different related statistical features. One typical way, is two measure the two limits from the value of the standard deviation (SD) and slope of the calibration curve.

LOD is calculated as $3.3\times\frac{SD}{s}$, where ‘s’ is the slope of calibration curve, i.e. the coefficient of the concentration in the equation that describe the calibration curve. The SD is obtained from the blank response. LOQ is calculated as $10\times\frac{SD}{s}$, giving a higher value in concentration. In case of a linear calibration curve, it is assumed that the instrument response y is linearly related to the measured standard concentration x, for a limited range of concentration, and it can be express as a linear function y=ax+b. This model is used to compute the sensitivity a and the LOD and LOQ. This approach is

particularly applicable when the analytical method to validate is not affected by a relevant background noise.

Linear regression analysis produces a mathematical model that best explains the collection data obtained with the minimum total residual error. In a linear representation of the data, like in the typical case of a calibration curve, the coefficient of determination r^2 produces a measure of the degree to which the values of x and y are linearly correlated. The higher the R^2 the more linearly correlated are the concentrations and the correspondent analytical signals of the analytical method studied.

3.6. List of excipients present in the tablets analysed

Excipients are chemical components of a medicinal formulation, in addition to the active principle ingredient, that provide the requested chemical and physical features to the final medicine. The functions of excipients are mainly to contribute to reach the expected pharmacokinetic features of the finale dosage form, such as enhancing solubility, facilitating drug absorption, and to facilitate the manufacturing process, for instance improving the powder flowability or reducing adherence forces between particles and machineries.

-Anti-adherent excipients are used during the preparation of solid formulation to reduce the adhesion between the tablet and the press of the compression machine and also used to help protect tablets from sticking each other. The most commonly used is magnesium stearate.

-Binders are used in the preparation of solid forms with the purpose to hold all the ingredients together conferring to the final product the required mechanical strength. Typical substances used as binders are polysaccharides and their derivatives, proteins, synthetic polymers.

-Coatings protect tablet formulations from external air humidity and mask unlikable taste of some tablets. Typical substances used as coatings are cellulose ethers, such as hydroxypropyl methylcellulose (HPMC).

-Colour is important to contribute to an easier identification of a medication and to improve the look and feel of medications. Typical substance used as colour agent is titanium oxide

-Disintegrant excipients facilitate the tablet dissolution in the digestive system. absorbing water with consequent expansion and dissolution of the solid formulation. Typical substances used as disintegrants are crosslinked polymers, modified starch sodium starch glycolate.

-Glidants are employed to enhance powder flow by reducing inter-particles friction and cohesion. Typical substances used as glidants are fumed silica, talc, and magnesium carbonate.

-Lubricants are excipients employed to improve manufacturing features of the solid formulation reducing the adherence between the tablets and the machineries. Typical substances used as lubricants are talc, silica, magnesium stearate or stearic acid.

Pharmaceutical manufacturers tend not to provide information about the quantitative composition of the excipients used in the preparation of their medicinal formulations, being this considered as an industrial secret used to maintain a competitive advantage against the competitors. They have to reveal the qualitative list of excipients adopted in the preparation of a final medicines, listing them in quantitative decreasing order, citing the most abundant first.

Following, the qualitative list of the excipients present in the composition of the tablets analysed, according to the patient information leaflets provided by manufacturers,

Tab.1. List of excipients obtained from the patient's information leaflet provided.

Medication	dose	Cellulose	Lactose	Na laurilsulphate	Starch	Macrogol	Na stearyl fumarate	glycerol	Hypromellose	calcium hydrogen	Methacrylate	iron oxide	Talc	copovidone	sucrose	Silica	Silicon Dioxide	magnesium stearate	Titanium dioxide
Carbamazepine Tegretol (brand reference)	200mg	x									x						x	x	
Carbamazepine carbagem (generic)	200mg	x		x							x		x			x		x	
Levocetirizine Mylan (brand reference)	5mg	x	x			x			x									x	x
Cetirizine Bristol (generic)	10mg		x		x				x				x					x	x
Amlodipine Besilate (brand reference)	10mg	x			x					x								x	
Amlodipine Mesilate (generic)	10mg	x			x					x								x	
Amlodipine Maleate (generic)	10mg	x			x											x		x	
Omeprazole Dexcel (brand reference)	20mg		x		x		x		x			x							x
Omeprazole Sandoz (generic)	20mg	x			x								x	x	x	x		x	x
Omeprazole GPO (generic, internet supply)	20mg																		
Omeprazole Hycid (generic, internet supply)	20mg																		
Omeprazole Olzep (generic, internet supply)	20mg																		
Esomeprazole Actavis (brand reference)	10mg								x		x		x					x	
Esomeprazole AstraZeneca (generic)	10mg							x	x		x	x							
Digoxin Lanoxin (brand reference)	250μg		x															x	
Digoxin Accord (generic)	250μg		x															x	

4. Determination of Cardiac Glycosides by Voltammetry assays using Hanging Mercury Voltammetry Solid Electrodes and PCA analysis

4.1. Introduction

Cardiac glycosides (CG) are a family of bioactive molecules that exert an action on the sodium potassium ATPase pump influencing the cell metabolism. Their action is focused on the myocardial muscle, increasing the output force in the heart pulsing and reducing the rate of contractions. Cardiac glycosides are therefore particularly used in the treatment of cardiovascular condition such as congestive heart failure where an increase of the output force is required and in cardiac arrhythmias, where there is the need of a decreasing of the contraction rate. Nevertheless, these beneficial clinical effects are counteracted by a very narrow therapeutic index. The generic chemical structure of a cardiac glycoside is based on a central steroid glycoside molecule linked to a sugar and an organic group. The steroid glycoside consists of four rings linked to other functional groups which are co-responsible for the biological activity. The solubility and pharmacokinetics profile of the cardiac glycosides are influenced by the specific sugar group attached. The organic groups attached to the other moiety of the molecule confer different structural properties and are used to classify the cardiac glycoside in two sub-categories, cardenolide or bufadienolide.

Cardiac glycoside are normally obtained from Lanatoside C, a matrix glycoside present mainly in the leaves and seeds of *Digitalis purpurea* (commonly known as Foxglove) [1]. Certain cardiac glycosides are used for the treatment of congestive heart failure, a condition in which the heart is unable to pump blood efficiently around the body [2]. This, in turn, can cause fluid to collect in the lungs and body tissues and lead to congestion.

Digoxigenin, digitoxin and digoxin (Fig.1, 2 and 3) are amongst the most representative cardiac glycosides used in clinical therapy and toxicology.

Digoxigenin (Fig.1) is considered a hapten, with high immunogenicity used in many biochemistry applications. Haptens are small compounds that exert an immune response only when conjugated to a large molecule; the molecule can be one that also does not produce an immune response by itself, such as a protein.

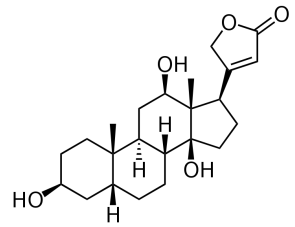

Fig.1. Digoxigenin, chemical structure

Basically, antibodies generated against haptens have higher affinities for their targets than other antibodies, so haptens are conjugated to other biological molecules to deliver immunological studies. Digoxigenin can be linked to a single species of RNA nucleotide triphosphate (usually uridine), and subsequently integrated into RNA as it is produced by the cell metabolism. When incorporated into the RNA chain, the digoxigenin markers can be followed with anti-digoxigenin antibodies. In a very analogous approach, digoxigenin may be linked to specific sugars to investigate cellular glycosylation mechanisms. In terms of chemical structure, digoxigenin is the aglicone of the digoxin, i.e. the the same structure of digoxin but with the lack of the sugar.

Digitoxin (Fig.2) present a comparable biochemical activity to digoxin, with a longer half-life. Contrarily to digoxin, digitoxin is only subjected partially to renal excretion, being partially exposed to hepatic catabolism.

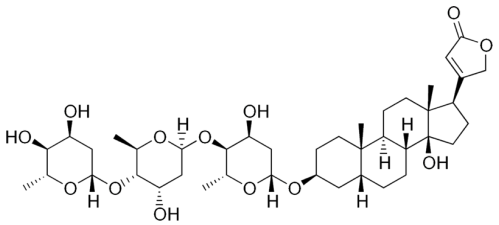

Fig.2. Digitoxin, chemical structure

It is therefore indicated more than digoxin for the treatment of cardiovascular conditions in patients with impaired kidney functions. Nowadays, it is not commonly used in clinical practice, preferring digoxin, characterised by an improved therapeutic and toxicological profile.

Amongst the different cardiac glycosides, digoxin (Fig.3) represents the most important for its use in current clinical practice. Cardiac glycosides act as stimulants on

the cardiac muscles as well as on un-striated and skeletal muscles, renal tubules and stray nerve centres.

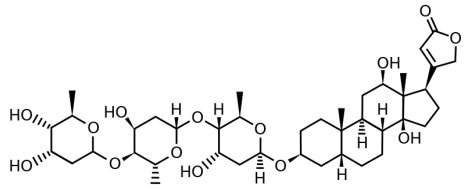

Fig.3. Digoxin, chemical structure

Digoxin is utilised to solve this as it is believed to inhibit Naþ–Kþ ATPase in addition to activating contractile elements of muscle fibres [2]. It increases the cellular calcium uptake in cardiac cells and enhances Naþ–Ca2þ exchange. Determination of the concentration of digoxin in blood, the heart and the kidney is, therefore, vital in order to confirm poisoning from ingestion of this compound. Digoxin has a double interest as a clinical molecule but also as a potential poison that could lead to heart failure [3]. The therapeutic action of digoxin starts around two hours after oral administration and 15–30 min after intravenous administration. The half-life period of digoxin via these methods is 1.5–2 days. Digoxin concentration in blood should be tested in order to find the appropriate dose for the given individual. However, digoxin has only a small therapeutic range of concentrations (0.5–2.0ng/mL) [1] and because of this index, digoxin therapy requires strict monitoring of blood levels in order to minimize toxicity [2-5]. Digoxin concentrations in vitro or in blood and urine samples have been determined previously using many techniques such as SEM [1, 6]; HPLC (with and without pulsed amperometric detection) [7-13]; LC-MS [14-18]; voltammetry [19,20], chemi-luminescence immunoassay [21] and reverse-phase thin-layer chromatography [22]. Digoxin tablets have been analysed using laser-induced fluorescence polarization [23]. Digoxin has been extensively studied using adsorptive stripping voltammetry by Wang et al. [19], where mercury electrodes were analysed by differential-pulse voltammetric analysis. The use of this technique allows a sensitivity of nanomole order. However, the hanging mercury drop electrode can present some shortcomings due to the inner mechanisms required to produce the drop. When using the hanging mercury drop technique the size of the drops has to be reproducible and remain unchanged to guarantee accurate and reproducible results. In order to achieve this, a mercury flow

from the Hg reservoir to the tip of the electrode is stopped at selected time intervals to produce a static as opposed to a growing drop. There are many factors that can lead to an unstable drop size. Firstly, during the filling of the mercury reservoirs, small bubbles of air can become trapped. Even a small bubble of air can cause serious interferences with the stability of the drop. Secondly, regulation of the pressure applied to the drop at the end of each analysis has to be accurately set up. It is important that the pressure is high enough to continuously replace the drop but not too high that the drop becomes stressed during the scan and its size changes.

Environmental issues, due to the toxicity of Hg, must be taken into account as well. Although mercury can be regenerated and reused, disposal of this element poses danger to the operator as well as large costs for disposal treatments.

Digitoxin and digoxigenin result to be by-products of the degradation of digoxin, which is currently used in cardio-vascular therapy [15].

The aim of this study, therefore, was to investigate the use of carbon glass, gold and silver electrodes to analyse digoxin tablets, generic and as branded, and the relative digitoxin and digoxigenin released by the degradation of the original digoxin, and compare the results with those obtained from Hg electrode.

In order to perform this study on the final pharmaceutical formulations, a preliminary voltammetric study was focused on the standards of the three cardiac glycosides, assessing the possibility to develop an analytical method able to investigate quantitatively the three cardiac glycosides pure standards in four different buffered acidic environment, resulting in pH5, 7.4, 10 and 11, using the traditional voltammetry based on mercury electrode and then comparing the results obtained with the outcomes resulting from voltammetric analyses of the same glycosides pure standard using solid electrodes, such as carbon glass, gold and silver electrodes.

4.2. Materials and Methods

4.2.1. Materials

-Digoxigenin (CAS No.: 129273-26-3, IUPAC name (3β, 5β, 12β) -3, 12, 14-Trihydroxycard-20 (22) -enolid;

-Digitoxin (Cas No.: 71-63-6, IUPAC name (3β, 5β) -3- {[2, 6- Dideoxy-β-D- ribo-hexopyranosyl- (1->4)-2, 6- dideoxy-β-D- ribo-hexopyranosyl- (1->4)- 2,6- dideoxy-β-D- ribo- hexopyranosyl] oxy} -14-hydroxycard- 20 (22)- enolide;

-Digoxin (CAS No.: 20830-75-5, IUPAC name 3- [(3S, 5R, 8R, 9S, 10S, 12R, 13S, 14S, 17R) -3- [(2R, 4S, 5S, 6R) -5- [(2S, 4S, 5S, 6R) -5- [(2S, 4S, 5S, 6R) -4,5-dihydroxy-6-methyloxan -2- yl] oxy-4-hydroxy-6-methyloxan-2-yl]oxy-4-hydroxy-6-methyloxan-2-yl]oxy-12,14-dihydroxy-10,13-dimethyl-1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 15, 16, 17- tetradecahydrocyclopenta [a] phenanthren -17- yl] -2H-f uran -5 - one;

-Alumina (Aluminum oxide Al_2O_3, CAS No.: 1344-28-1, 150 mesh);

- Silica (Silicon dioxide amorphous CAS No.: 112945-52-5, fumed, particle size 0.007μm)

were all obtained from Sigma Aldrich, UK.

Methanol, (CH_3OH, CAS No.: 67-56-1) was obtained from Fischer Scientific, UK. Sodium Acetate Trihydrate ($CH_3COONa \cdot 3H_2O$, CAS No.: 6131-90-4), Sodium Hydroxide (NaOH, CAS No.: 1310-73-2), Sodium phosphate dibasic dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, CAS No.:10039-32-4), Sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$, CAS No.: 13472-35-0), Sodium Carbonate (Na_2CO_3, CAS No.: 497-19-8) and Sodium hydrogen carbonate ($NaHCO_3$, CAS No.: 144-55-8) were obtained from Fischer International, UK. Glacial Acetic Acid (CH_3COOH, CAS No.: 64-19-7) was obtained from BDH Acids HP, USA. Gold and silver electrodes were obtained from Metrohm, UK.

Digoxigenin, Digitoxin and Digoxin stock solutions were prepared by dissolving the relative cardiac glycoside in methanol at a range of concentrations around 100μg/mL. These stock solutions were stored in the dark at 4°C.

The pH5 buffer was prepared using glacial acetic acid (0.2M) and $CH_3COONa \cdot 3H_2O$ (0.2M) at a ratio of 30:70 v/v.

The pH=7.4 buffer was prepared using $Na_2HPO_4 \cdot 12H_2O$ (0.2M) and $NaH_2PO_4 \cdot 2H_2O$ (0.2M) at a ratio of 81:19 v/v.
The pH=10 buffer was prepared using Na_2CO_3 (0.1M) and $NaHCO_3$ (0.1M) at a ratio of 60:40 v/v.
The pH=11 buffer was prepared using NaOH (0.005M) solution (Fischer International, UK).

4.2.2. Methods

Voltammograms were obtained using a 757 VA Computrace Metrohm connected to a Compaq deskpro personal computer. The reference electrode was Ag/AgCl (KCl 3M). The working electrode was a valve-controlled hanging mercury drop electrode (HMDE). Platinum electrodes were used as auxiliary electrode. To perform voltammetry analyses using HMDE electrode, 10mL of a selected buffer solution was poured into the measuring cell. Consequently, using the method of standard addition to build the correspondent calibration curve, it was added with different aliquots of 200µL of stock solution. The resulting solution, each time before the following addition, were deoxygenated by a flow of nitrogen for 8 minutes. In the cyclic voltammetric analyses, an accumulation potential of -0.9V was applied. 3 cyclic voltammetry scans were performed after each addition , with a RedOx potential range of +2V to -2V. The sweep rate was kept at 0.05V/s and the voltage step at 0.0059V. All data were obtained at 25°C. In the voltammetric analyses using solid electrodes, to perform cyclic voltammetry analyses, 10mL of a selected buffer solution was poured into the measuring cell with an aliquot of cardiac glycoside stock solutions to reach varying concentrations from 3.9- 14.8µg/mL. The resulting solutions were deoxygenated by a flow of nitrogen for 5 minutes. No accumulation potential was applied. 3 cyclic voltammetry scans were performed on each buffer solution, with a RedOx potential range of +2V to -2V. The sweep rate was kept at 0.05V/s and the voltage step at 0.0059V. All data was obtained at 25°C.The working solid carbon glass, gold and silver working electrodes also acted as stirrer. Platinum electrodes were used as auxiliary electrode. Before each new analysis and after the last of the set, each electrode was cleaned in order to remove traces of any electro-active substances that may be present.

First, the electrode was removed from the apparatus and washed under a flow of deionised water. Next, a single drop of deionised water and a spatula tip sized amount of alumina was then mixed in a petri dish to make a paste. The tip of the electrode was then cleaned in this paste by making 10 circular rotations in each direction, three times, as well as a ten figure of 8 movements in each direction, three times. The top of the electrode was then washed under a flow of deionised water before finally being sonicated in deionised water for 3 minutes.

4.3. Results and Discussion

The selected three cardiac glycosides have been analysed by hanging mercury voltammetry in order to evaluate the possibility to use this technique for investigative purposes. In order to identify the effect of different environmental grade of acidity on the response of these analytes on the analytical technique, 4 different pHs (pH5, 7.4, 10 and 11) have been chosen to test their voltammetric behaviour.

4.3.1. Investigation of the effect of different pHs on hanging mercury voltammetry of digoxin

At pH5, in the first attempt to build the calibration curve, the initial results gave information about the range of concentrations to adopt. At the first addition (with 1.5µg in 10.2mL of initial buffer) no peak was observed. At the second addition (with 3µg in 10.4mL) a peak is observed. At the third addition (with 4.5µg in 10.6mL) an overlapped peak to the one already obtained is observed. It seemed, therefore, that the first addition (with a concentration of 1.5µg in 10.2mL equivalent to 0.147µg/mL) was under the detectable limit of our system, while at the third addition (with a concentration of 4.5µg in 10.6mL equivalent to 0.424µg/mL) there was a saturation condition. In Fig.4 is reported a typical voltammogram obtained during these analyses. We had, therefore, to operate within these parameters for the next attempt to build a calibration curve.

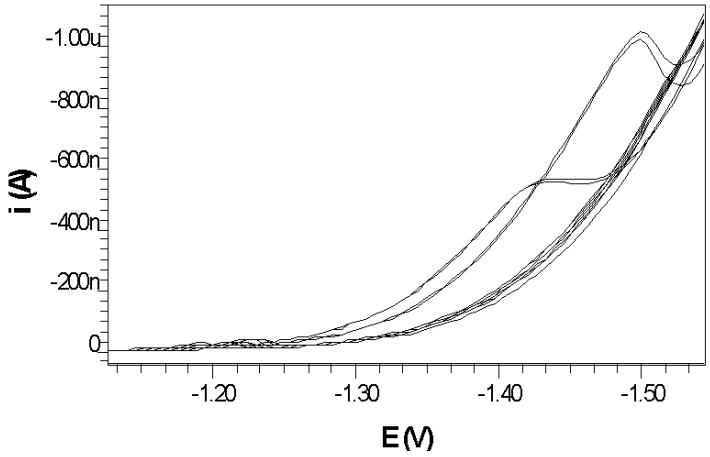

Fig.4. Typical mercury voltammogram of digoxin, showing a saturation condition registered at the third addition of aliquot of standard solution, with 4.5µg in 10.6mL

The second calibration curve attempt was performed starting from 10mL buffer spiked with 200µL of a solution of 7.5µg/mL and then making the following addition with 200µL of a less concentrated solution (1µg/mL, equivalent to 0.2µg) in order to avoid to reach saturation conditions.

After the first peak (conc 1.7µg in 10.2mL equivalent to 0.167µg/mL), the following addition seemed to lead to an immediate saturation. It was then decided to restrict again the range, this time between 0.15-0.167µg/mL.

After different setting and trial-error approach it was realised that this acidic condition was not analytical significant to perform voltammetric study on this molecule.

As performed for the calibration at pH5 shown before, the study of the calibration curve at pH7.4 was obtained starting with 10mL buffer adding aliquots of 200µL of 1µg/mL digoxin stock solution and making addition of 200µg each time. Six additions were performed and each addition was tested twice. The voltage of the peak used to follow the voltammetric response at the diverse concentrations was -1.390V.

Following the previous procedure, to perform the analyses at pH10, 10mL of the carbonate-bicarbonate buffer (60mL Na_2CO_3 0.1M + 40mL $NaHCO_3$) was added with serial aliquots of 200µL of the 1µg/mL digoxin stock solution. Six additions were performed and each addition was tested twice.

With the increase of the concentration of the solution under examination, a shift of the peak potential towards slight lower voltage was observed. It was speculated that it could have been caused by a chemical transformation.

In order to select the best representative peak value at which refer for the calibration curve, two peaks have been selected, at -1.403V and -1.409V, chosen by visual analysis of the voltammograms and their correspondent current values utilise for the construction of the calibration curve. The calibration curve relative to the peak at -1.403V resulted to present a slight improved linear regression (r^2) of 0.8766 with respect the linear regression of 0.8653 obtained at -0.1403V.

As in the previous pH conditions, 10mL of pH11 buffer were spiked with aliquots of 200µL of the 1µg/mL digoxin stock solution. Nine additions were performed and each addition was tested twice. Two peak values were preliminarily identified for the construction of the calibration curve, at -1.4796V and -1.4856V.

The calibration curves built considering the two different potential peaks appeared to be nearly identical with same slope for the independent variable x. The curve originated from the peak at -1.4796V showed a slight better linear regression of 0.9684 against the one obtained from the peaks at -1.4856V with a r^2 of 0.9625 and therefore was chosen as the analytical value to register the voltammetric signal.

4.3.2. Investigation of the effect of time on hanging mercury voltammetry of digoxin

During the execution of the voltammetric analyses at pH5, the solution after the scan was transferred in a vial and checked at set interval of time in order to investigated the action of the specific pH on the stability of the glycosides. It was noticed how, during the time, the peak obtained at the first scan (time=0) (-1.18V) tended to disappear, while a new peak (at -1.21V) was appearing and tending to increase with the time (Fig.5). It has been considered that a chemical process was happening, maybe a hydrolysis triggered by the slight acidic condition. In acidic condition, in the digoxin chemical structure, the bond that links the digitoxose sugar moiety to the digoxigenin part has been reported being separated by hydrolysis reaction [16]. This could lead to the release of a fraction of the original molecule with electrochemical behaviour.

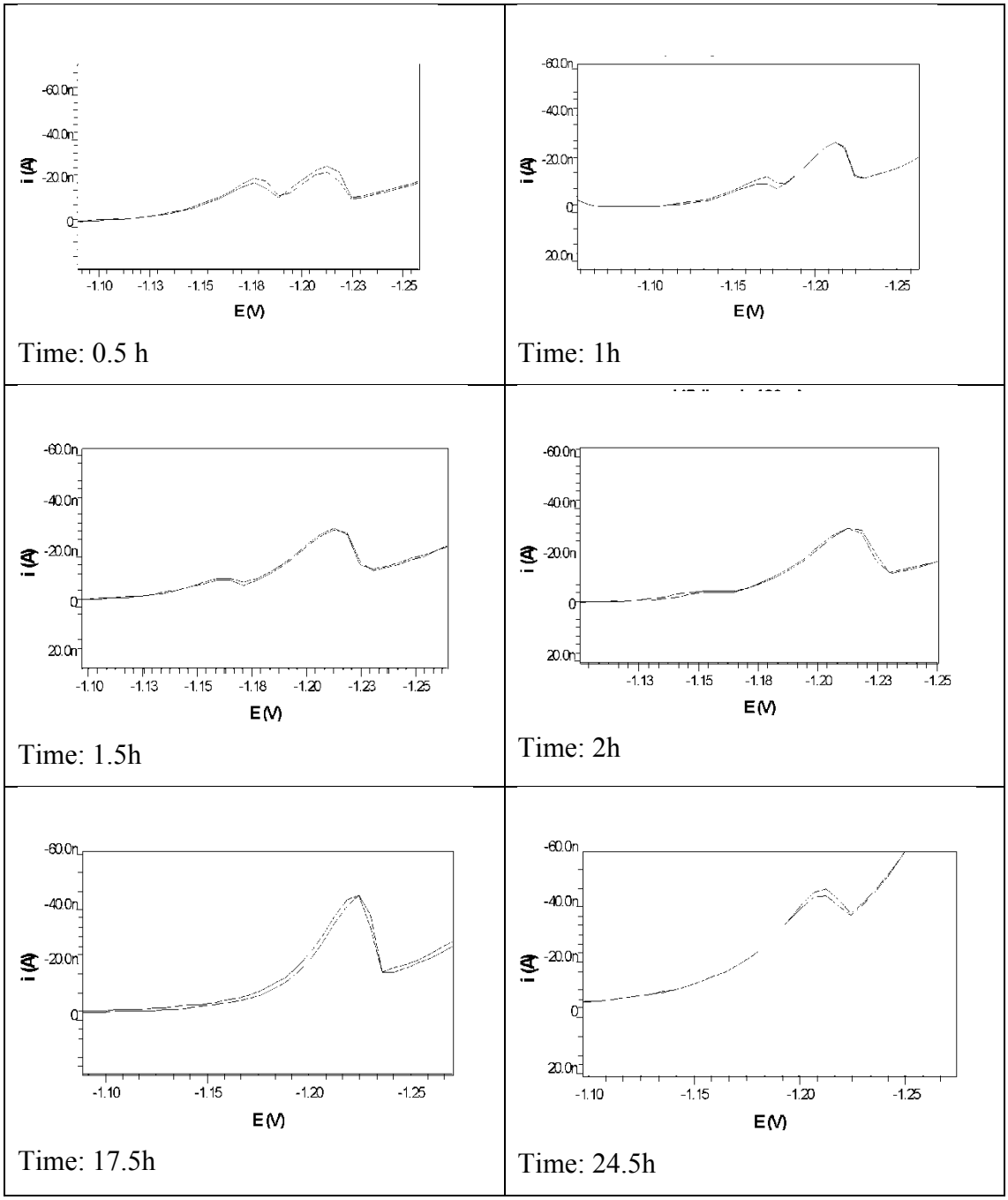

Fig.5. Variation of of digoxin mercury voltammograms obtained at pH5 tested at different interval of time

The same investigations conducted at the other pHs (7, 10, 11) did not produced any changing in the voltammograms obtained, indicating that no chemical transformations were happening at those pHs.

4.3.3. Investigation of the effect of different pHs on hanging mercury voltammetry of digitoxin

Digitoxin is characterised by an even lower solubility with respect digoxin, missing one hydroxyl group and being consequently less hydrophilic than digoxin. Because of this issue, the stock solution with higher concentration that it was possible to achieve was of 1.001µg/mL, in methanol as solvent. As in the previous setting of experiments performed on digoxin, in order to replicate and validate the analytical approach, 10mL of the same four different buffers were spiked with aliquots of 200µL of the digitoxin stock solution and analysed voltammetrically. The analyses conducted at pH5, 7.4, 10 did not show any variation from the baseline obtained from the voltammetric scan of the buffer on its own when the aliquots were added. Only the experiments performed at pH11 showed some electrolytic activity during the standard addition of the digitoxin aliquots. Eleven additions were performed and each addition was tested twice. In order to identify the lowest concentration traceable with this analytical approach, different concentrations obtained from the dilution of the original stock solution were tested and the lowest concentration that presented a relevant voltammetric response were of 0.1001µg/mL As presented before for the digoxin analysis, two different potentials were identified as representative of the calibration curve (at -1.4737V and -1.4796V) and the two relative calibration curves were built accordingly. The two different values of the peak led to similar equation of the curve, with similar slope for the x and similar linear regression. Based on the slight better r^2 of 0.9785 at -1.4796V, this value was chosen as analytical value to be used.

In order to investigate the effect of the exposure to the different pH on the chemical structure of digitoxin, voltammetric scansions were performed at different interval of time. The voltammograms obtained indicating that no chemical transformations were happening at those pHs.

4.3.4. Investigation of the effect of different pHs on hanging mercury voltammetry of digoxigenin

Digoxigenin presents as the digitoxin a reduced solubility, because of its lower polarity due to the lack of the hydrophilic sugar moiety. The maximum concentration that it was possible to reach in methanol was of 1.00µg/mL before to reach saturation. Exposing this digoxigenin stock solution to the same voltammetric approach defined previously for the other cardiac glycosides, as in the case of digitoxin, only pH11 was responsive to the test. Eleven additions were performed and each addition was tested twice. It was noticed that per each standard addition, the first scan gave always a peak higher than the following second scan, possibly due to a saturation process due to the alkaline condition of the buffer that reduce the polarity of the digoxigenin.

Three peak values were preliminarily identified for the construction of the calibration curve, at E(V): a)-1.4120; b)-1.1472; c)-1.1531. The linear regressions of the calibration curves obtained from these signals resulted to be not particular significative. The calibration generated from the potential at -1.4120V was the best of the three when considering the whole set of measurements (with first and second scan per aliquot) but not analytically relevant, with a linear regression of just around 0.4. When considering only the results obtained from the first scan, the signals recorded at -1.1472V were the ones that produced the better calibration curve, but still with a low r^2 of 0.8222. The second scan gave better results at -1.1531V, where the calibration curve resulted with a linear regression of 0.8637.

4.3.5. Investigation of the effect of different pHs on the voltammetric characteristics of digoxin, digitoxin and digoxigenin using solid electrodes

Examples of the use of electrochemical methods using solid electrodes for the determination of drugs in clinical [29, 30] or forensic applications [31-35] is not novel. Due to the selectivity and sensitivity achieved by the electrochemical techniques it is becoming more and more popular for their applications in sensors or sensing elements. Solid electrodes presents a range of advantages with respect the traditional mercury electrode. Solid metal electrodes, contrarily to mercury electrode, have no environmental implications and provide an accurate polishing phase at the end of each

analysis, which guarantees a constant analytical surface and in turn ensures reproducibility of results. Furthermore, despite the excellent cathodic potential range of mercury, its anodic range is severely limited with respect to main solid metal electrodes due to its ability to oxidise easily. Solid electrodes also have the potential to be used in portable analytical devices.

Solid electrodes, such as glassy carbon, silver and gold, have not been previously reported being used in any voltammetric studies to analyse digoxin, digitoxin or digoxigenin, representing a novelty in this area of research.

The aim of this study, therefore, is to investigate the use of carbon glass, gold and silver electrodes to analyse these cardiac glycosides and compare the results with those obtained from Hg electrode.

Investigation of the effect of different pHs on the voltammetric characteristics of digitoxin, and digoxigenin using carbon glass, gold and silver electrode did not produce any significative analytical results.

4.3.6. Investigation of the effect of different pHs on the voltammetric characteristics of digoxin, using carbon glass electrode

The electrochemical properties of the three cardiac glycosides were studied using solid electrodes replicating the same conditions adopted for the study using mercury electrode, with the same buffer solutions, of pH5, 7.4, 10 and 11.

In general, the results have not been as reproducible as when using HDME and this electrode has shown to be not indicated for the analysis of those cardiac glycosides. The vast majority of all the voltammograms obtained resulted showing no trace of signals at all. It was considered the use of KCl as enhancer to increase conductivity in the electrolytic cell, but without any analytical benefit. Only digoxin has shown a slight responsive behavior when tested at pH5 (Fig.6).

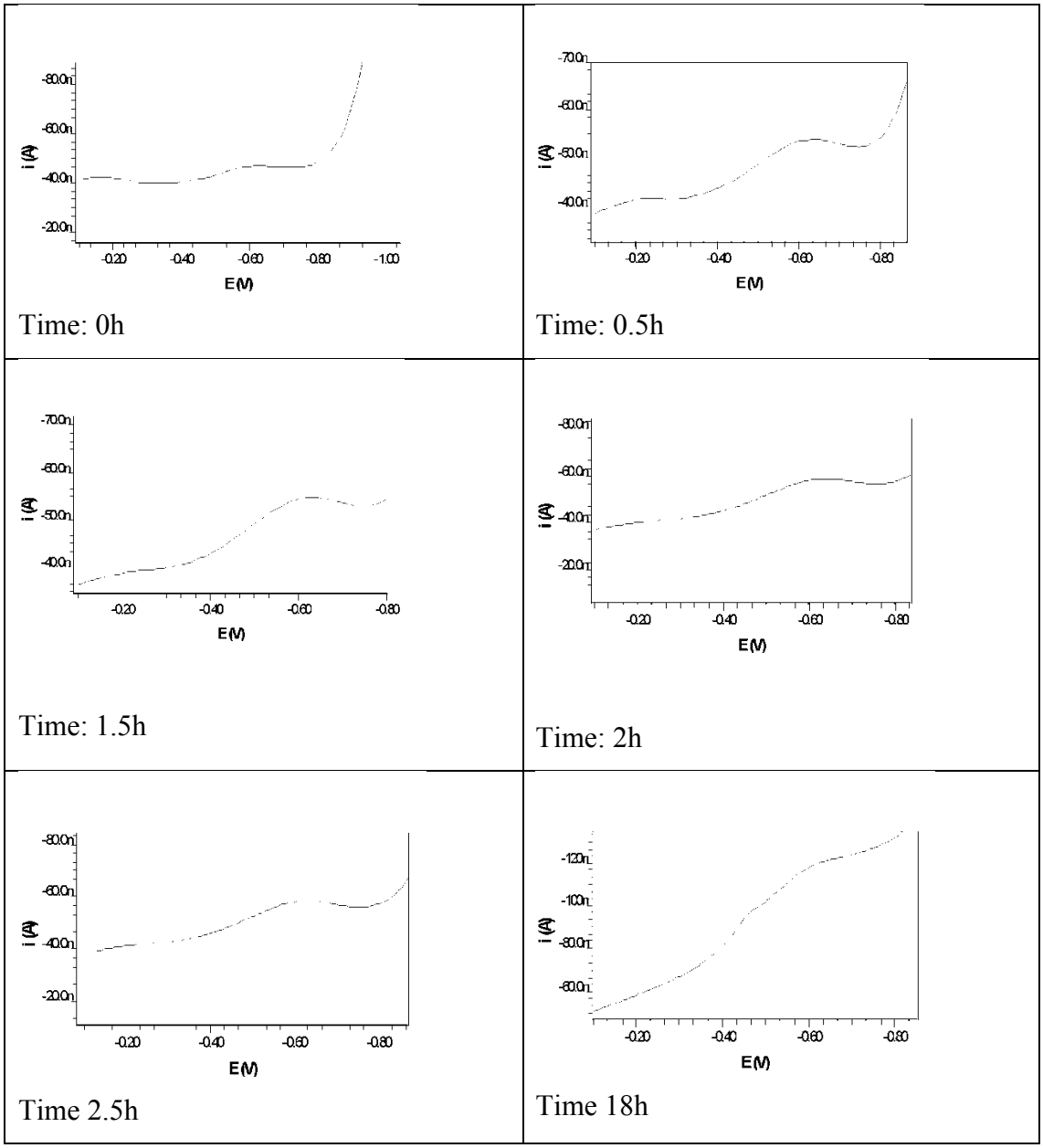

Fig.6. Voltammograms of digoxin tested with carbon glass electrode at pH5, at different times.

As in the identical case of the experiment conducted at this pH with mercury electrode, it has been noticed that when left the solution and re tested during the time a decomposition process seemed to occur, with a new peak gradually appearing and the initial one decreasing.

The kinetic of the disappearing peak was $y = 2\times10^{-11}x - 4\times10^{-8}$ while the kinetic of the appearing peak was $y = -8\times10^{-11}x - 5\times10^{-8}$. The rate of variation of the intensity of the peaks detected by carbon glass voltammetry was lower than the

correspondent rate noticed by mercury voltammetry. In the decomposition investigated by mercury electrode voltammetry, the disappearing trend line of the first peak and the formation of the second peak trend line had a coefficient of the time of the order of 10^{-10}. In the case of the results obtained by carbon glass, the same coefficients were of the order of 10^{-11}, where at the same changing of the variable time (x) the effect on the correspondent intensity of the peak (y) was lower.

4.3.7. Investigation of the effect of different pHs on the voltammetric characteristics of digoxin, using gold electrode

At pH5 and pH7.4, gold electrode shows an analytical peak during the reduction process at -1.2138 and -1.2733V, leading to a linear regression of the calibration curve of 0.9568 and 0.9072 respectively. At pH10, gold electrode shows good analytical signal both during the cycle RedOx in the reduction phase at 0.2322V, giving a linear regression of 0.976, and during the cycle OxRed, still during the reduction process at 0.2019, with r^2 of 0.9204.

This electrode showed the best linearity of data (r^2=0.9948) at pH11. At this pH, gold electrode showed to be able to analyse digoxin both in the RedOx cycle and in the OxRed cycle. In the RedOx cycle, actually, it was possible to use two different points of the RedOx curve at 0.601V in the anodic sweep and at 0.041V in the cathodic sweep respectively to build the calibration curve (with r^2=0.9267 and 0.9948 respectively). In the OxRed cycle the analytical peak was at 0.5887 in oxidation phase, with r^2=0.9792. The linear regression of the calibration curve obtained in these condition resulted to be of the order of 0.9, with a slope in order of 10^{-6}.

Figure 7 shows the oxidation of gold at different pHs from oxidation to reduction sweep. First, the gold electrode in a buffer solution (digoxin-free) was investigated in an oxidation-reduction scan cycle to explore the extent of its ability to oxidise and then return to its original state via a reduction process.

In an acidic condition, such as that of pH=5, the gold electrode does not present a visible peak in the oxidation direction and presents a slight peak in the reduction direction. Pasta, Mantia and Cui [24] suggest a probable reaction for the oxidation of gold to be:

$2Au+3H_2O \leftrightarrow Au_2O_3 + 6H^+ + 6e^-$

Such reaction is driven by alkaline pHs where the H^+ may link to the OH^- of the solutions, driving the equilibrium of the reaction towards the formation of oxidised Au.

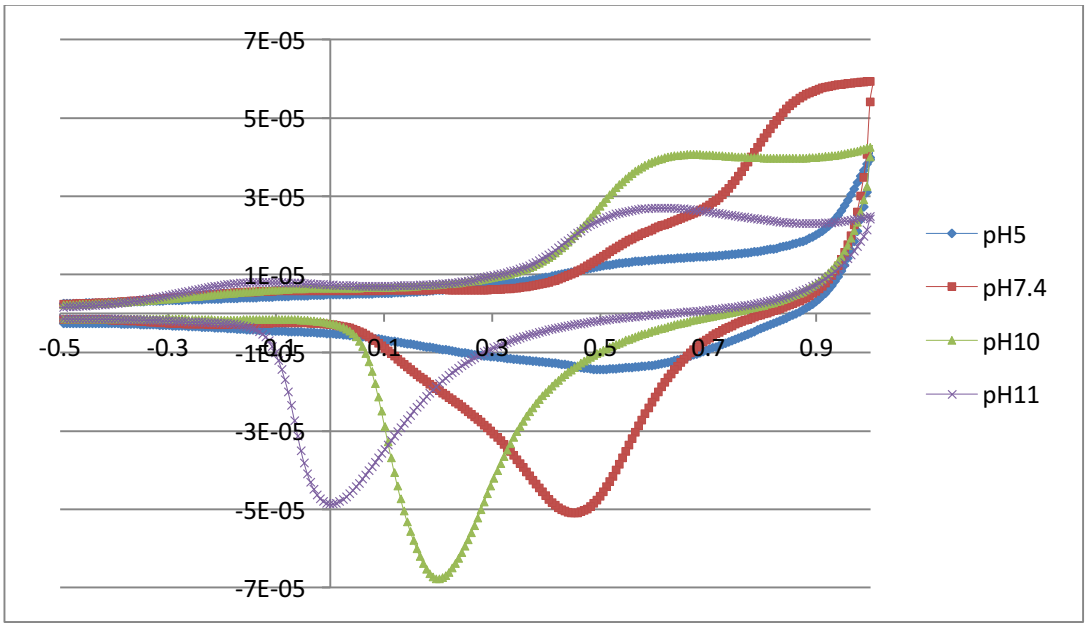

Fig.7. Voltammogram obtained using a gold electrode as a working electrode and different buffer solutions at different pHs of 5, 7.4, 10 and 11. Scan direction from -0.5V to +1V. Sweep rate = 0.05V/s; Voltage step = 0.0059V; T= 25°C.

A reaction such as this would be driven by alkaline pHs because the H^+ and OH^- ions may come together in solution, in turn driving the equilibrium of the reaction towards the formation of oxidised Au. It can be noted that as the pH of the buffer solution moves from acidic to alkaline the reduction peak shifts towards the negative potential, whereas the oxidation peak remains more or less constant at a potential of between +0.6V. The cathodic shift from right to left as the pH becomes increasingly alkaline can be explained by the consideration that the basic pH seems to favour the stability of the oxidised gold, which explains the higher voltage gap for the reduction current peak at the higher pH's [24]. This is in line with Kirk, Foulkes and Graydon [25] which reported the electrochemical formation of monolayers of Au(I) hydroxide in basic conditions close to pH=11.

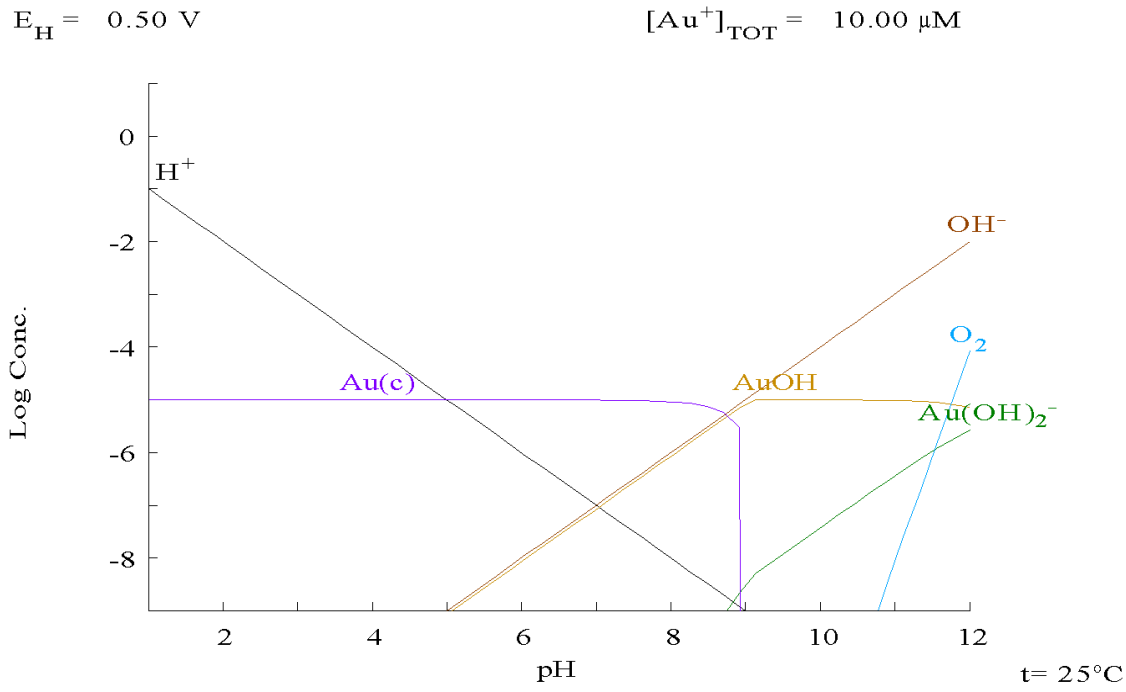

Fig.8. Theoretical oxidation-reduction equilibria for Au at 25°C and 10µM Au at different pHs.

Figure 8, obtained from Medusa software, shows the theoretical concentration of the different forms of oxidised Au as a function of pH at a fixed potential of 0.5V and a metal concentration of 10µM to simulate an equilibrium process using thermodynamic criteria.

It is noticeable at pH9 a sudden conversion of Au in $Au(OH)^-_2$; at pH5 oxidation of Au from metallic state to oxidised AuOH. This is in line with the results obtained (Fig.7) and with the Pourbaix diagram (Fig.9) that reports the different oxidised species of Au at different potentials function of pH.

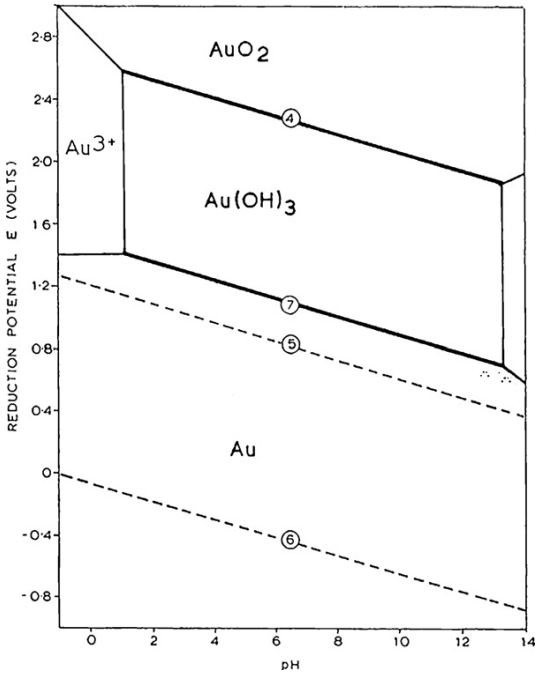

Fig.9. Pourbaix diagram of Au (extracted from Harris et al [24])

As the pH increases up to pH=9, a constant amount of gold is precipitating out of the solution and the concentration of H^+ ions is steadily decreasing. From pH5, the concentration of OH^- ions steadily increases.

Across pH=7, the level of H^+ and OH^- ions are at equilibrium, which is to be expected as at a neutral pH the concentration of H^+ and OH^- ions should be consistent. It can be noted that at pH9, the concentration of gold decreases dramatically, demonstrating the sudden conversion of gold to $Au(OH)^-_2$ ions. It is possible to identify (Fig.7) the formation of broad peaks in both reductive and oxidative scans, due to the chemisorption of hydroxide ions to the gold surface, in line with the report from Kirk et al [25]. At pH=5, Figure 8 also shows oxidation of gold from its metallic state to AuOH. This supports the results obtained from the cyclic voltammetry (Fig.7) for which an increase on surface oxides were obtained with an increase in pH. Similar results were reported both by Pasta, Mantia and Cui [24] who visualised the different oxidised species of Au at different potentials as a function of pH in the form of a Pourbaix diagram and by Nicol [36] who shown as gold at pH=7-12 is oxidized to Au(III) hydroxides, confirming the shift of the potentials shows in Figure 7. The same Figure shows the oxidation of the gold at different pHs in the positive sweep, and the following reduction in the negative sweep. It is noticeable an appearance of oxidative current

peaks in the in the range of 0.5-0.6V, increasing the alkalinity of the buffer solution in the oxidation phase.

This is in accordance with literature [24,25]. Y. Cui et al [24] speculate that in that condition the gold surface is covered by a layer of gold oxide subjected to the following reversible reaction:

$$Au_nO_{m-1} \leftrightarrow Au_nO_m+H_2O+2e^-$$

In the reduction sweep it is the signal of reductions on the gold electrode surface with peaks at 0.49V for pH7.4, 0.22V for pH10 and nearly 0V for pH11. The alkaline pH favours the stability of the oxidised Au, this explains the higher voltage gap for the reduction current peak at higher pHs. In the cycle starting from reduction and ending with oxidation, Red-Ox (Fig.7) gold electrode shows the same features, giving reduction peaks pH dependent of the oxidised Au with peak voltages decreasing with the increase of the alkalinity, 0.5V at pH7.4, 0.27V at pH10 and 0V at pH11.

The oxidation reactions appear at the same range of voltage applied in the cycle Ox-Red, showing that the reactions occurring at the electrode surface are fully reversible.

For the gold electrode the limit of detection (LOD) ranged from 55µg/mL at pH5 to 1.7µg/mL at pH11 (data reported in Appendix 1). This could be explained with the better response of the conversion of the equilibrium of oxidised Au in alkaline medium as discussed earlier. This assertion is corroborated by the values of the standard deviations (data reported in Appendix 1) showing the lower dispersions at this pH both in Red-Ox and in Ox-Red sweeps. The standard deviations and the coefficients of variation show that the results obtained are quite repeatable, as confirmed also by principal component analysis (PCA) as shown in Fig.10 and 11. PCA of the results shows how the data obtained are well grouped in isolated clusters, especially the ones obtained at pH11.

Furthermore, the gold electrode was investigated in a reduction-oxidation scan cycle to discover the ability of the gold electrode to reduce and then revert back to its original state via oxidation. Similarly to the results observed in the oxidation-reduction scan, reduction peaks dependent on the pH of the oxidised gold are observed. The peak voltages decrease from 0.5V to 0V as the pH increases towards the alkaline region from pH=7.4 to pH=11. The oxidation reaction on the reduction sweep appears at the same range of voltages as those uncovered in the oxidation-reduction cycle. All of these

factors demonstrated that the reactions on the surface of a gold electrode are fully reversible as expected.

By observing the chemical structure of digoxin, successful reduction and oxidation of the compound would be expected due to the presence of electroactive groups. As suggested by Ivanovskaya [20], the double bond conjugated to the carbonyl group of the molecule can be prone to reduction from an alkene to an alkane group, as well as oxidation as a result of the lactone ring being opened.

When using a gold electrode, at pH=5 and pH=7.4, an analytical peak during the reduction process at -1.21 and -1.27V, can be respectively seen, producing linear calibration curves with correlation coefficients of r^2=0.9568 and 0.9072, respectively.

At pH=10, the gold electrode also showed a good analytical signal in the reduction phase at 0.23V, producing a linear regression of r^2=0.976. This electrode showed the best linearity of data (r^2=0.9948) at pH=11. At this pH, the gold electrode showed to be able to analyse digoxin in a reliable manner. It was possible to use two different points of the curve, at 0.601V in the anodic sweep and at 0.041V in the cathodic sweep respectively to build the calibration curve (with r^2=0.9948).

For the gold electrode the limit of detection (LoD) ranged from 55µg/mL at pH=5 to 1.7µg/mL at pH=11. The optimum pH for analysis was chosen to be pH=11.

This could be explained with the better response of the conversion of the equilibrium of the oxidised Au in alkaline medium as discussed earlier. This is also corroborated by lower values of the standard deviations at pH=11.

The average Coefficient of Variation also followed this trend showing a higher precision of 2.07% and 4.13% for both the anodic and cathodic peaks respectively at pH=11.

Principal Component Analyses of the different digoxin solutions were also performed, as shown in Figures 10 and 11 . This PCA analysis of the results showed how the data obtained are well grouped in isolated clusters, especially the ones obtained at pH=11 where the points are closer to each other. The different clusters were helpful to discriminate the best pH conditions under any concentration, even blanks, to show any trends or surface behaviour and potential interactions. It is clear from the results achieved that pH is a key variable to determine the chemical reactions occurring on the surface of the electrode. Even the absence (blanks) or presence of digoxin in solution at

different concentrations seems to be a secondary factor in the clustering as demonstrated by the fact that all voltammograms clustered together. Different pHs will affect the position of the reduction and oxidation peaks and, as seen before, the reproducibility of these processes. Hence, to select the best possible analytical conditions for the analysis of digoxin on a gold electrode this is a factor that needs to be considered. The presence of surface oxides will greatly condition the electrochemical response. However, this is not a factor that prevents the analytical use of this electrode in the determination of digoxin as demonstrated by the calibration curves. The variability achieved in the analysis of each of the PCA plots was given by the percentage of variability for the different PCs. In this way for Fig.10 and Fig.11 this was PC1 (53.79%) and PC2 (26.86%) and PC1 (51.30%) and PC2 (29.94%), respectively.

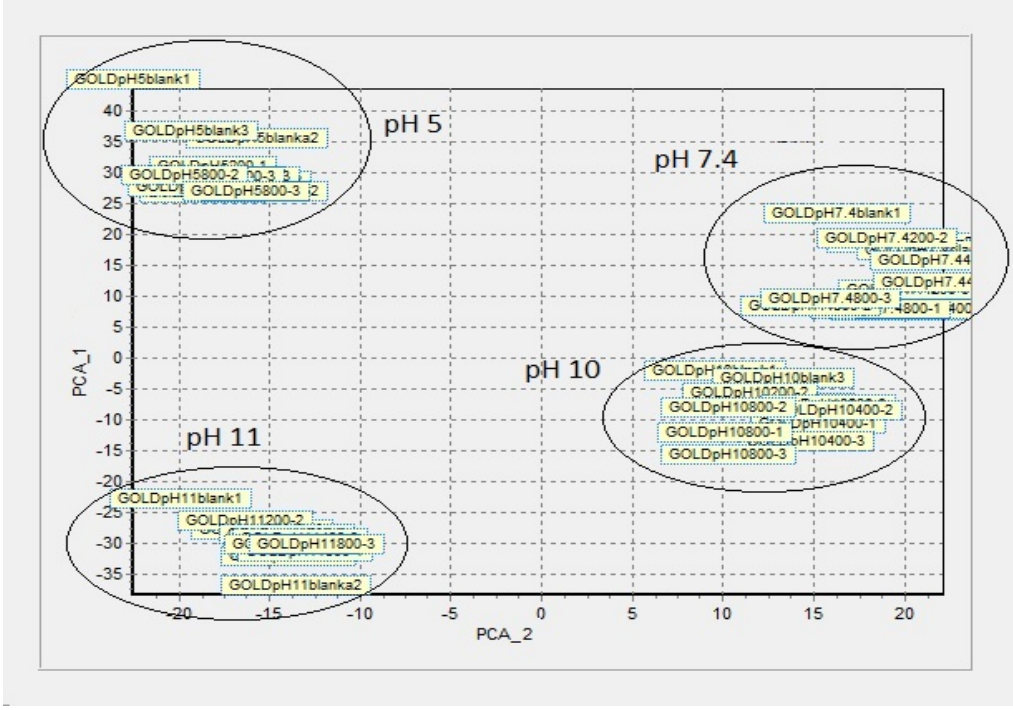

Fig.10. PCA scatterplot of the electrochemistry RedOx of a gold electrode at different pHs of 5, 7.4, 10, 11. Percentage of variability: PC1=53.79%; PC2=26.86%.

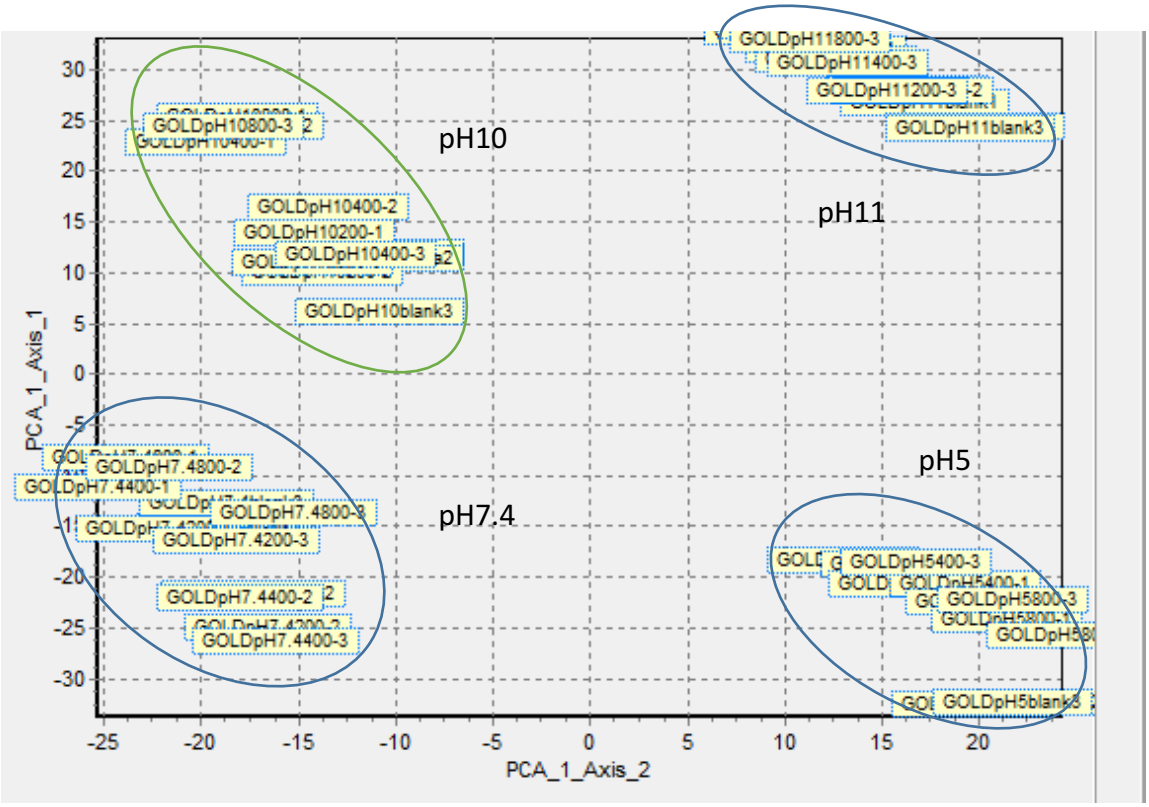

Fig.11. PCA scatterplot of the electrochemistry OxRed of a gold electrode at different pHs of 5, 7.4, 10, 11. Percentage of variability: PC1=51.30%; PC2=29.94%.

4.3.8. Investigation of the effect of different pHs on the voltammetric characteristics of digoxin, using silver electrode

At pH5, silver electrode showed the possibility to analyse digoxin both in OxRed and RedOx cycles at 0.0055V in the reduction phase and 0.0121V in the reduction phase respectively, with calibration curves r^2 correspondently of 0.9269 and 0.9874.

pH7.4 was a non-ideal environment for silver electrode that was not be able to detect digoxin in neither the cycles.

At pH10, silver electrode could analyse digoxin in in RedOx cycle at -0.0594V in the reduction phase (r^2=0.8561) and in OxRed cycle at -0.1195V during the reduction phase, achieving a calibration curve with a r^2=0.9104.

At pH11, this solid electrode was able to detect digoxin in both cycle, obtaining calibration curves with r^2=0.9111 in RedOx cycle at -0.2736V in the reduction phase and r^2=0.9923 in OxRed cycle at -0.2921V in the reduction phase.

Silver electrode, in the OxRed cycle (Fig.12), shows not particular evident signal of oxidation during the positive sweep at the acid environment of pH5, and at pH11, while at pH7.4 and 10 an anodic peak is shown at 0.505 and 0.535V respectively.

Reversing the potential scan, in the negative sweep at pH11 a weak signal is noticeable at -0.239V, while at the other pHs strong cathodic peaks are registered, with peaks at 0.02V for pH5, 0.03V for pH7.4 and -0.10V for pH10.

In the RedOx cycle (Fig.13), in the reduction sweep two peaks are visible for both pH7.4, at 0.012 and -0.149V, and pH10, at -0.053 and -0.357V. A single peak is noticeable at pH5, at 0.02V, and at pH11, at-0.405V. In the positive sweep, still two peaks are visible for pH7.4, at 0.560 and 0.815V, and for pH10, a weak one in the region around 0.350V and a stronger one at 0.590V.

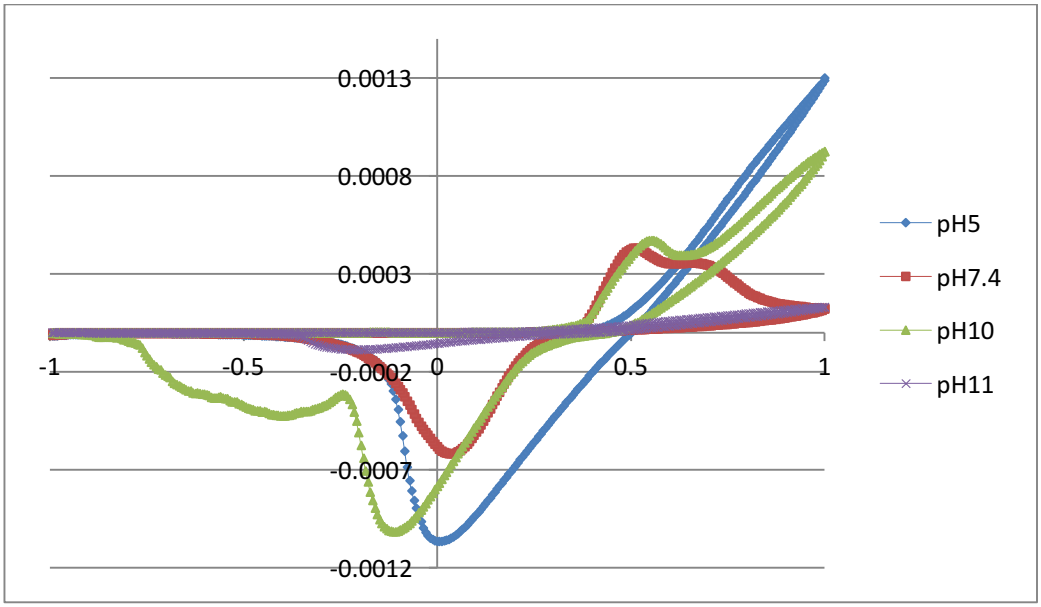

Fig.12. Voltammogram obtained using a silver electrode as a working electrode and different buffer solutions at different pHs of 5, 7.4, 10 and 11. Scan from -1V to +1V. Sweep rate = 0.05V/s; Voltage step = 0.0059V; T= 25°C.

Similarly to the investigation carried out for the gold electrode, the silver electrode was studied in an oxidation-reduction scan cycle first to discover the extent of its ability to oxidise and then reduce (Fig.12).

In the oxidation-reduction cycle where the silver electrode was employed, there is no evidence of an oxidation signal during the positive sweep in the acidic environment of pH=5. At the remaining pH levels of 7.4, 10 and 11, however, anodic peaks can be observed, in agreement with the results shown by Hassan et al [26]. When the scan is then reversed to reduce the products of the initial oxidation, only a weak signal was detected (-0.239V) in the alkaline conditions of pH11. The pH regions of 7.4 and 10 registered strong cathodic peaks.

Following this, the silver electrode was then studied by a reduction-oxidation scan cycle (Fig.13). In the reduction sweep of the silver electrode two peaks are visible at pH7.4 (0.012V and -0.149V) and pH10 (-0.3905V and -0.04745V) and only one at pH5 (0V). When the scan then moves into the oxidation phase, sweeping towards the more positive potential, two peaks are present at pH=7.4, however at pH=10 only one oxidation peak is observed. No oxidation peaks were detected at pHs 5 and 11.

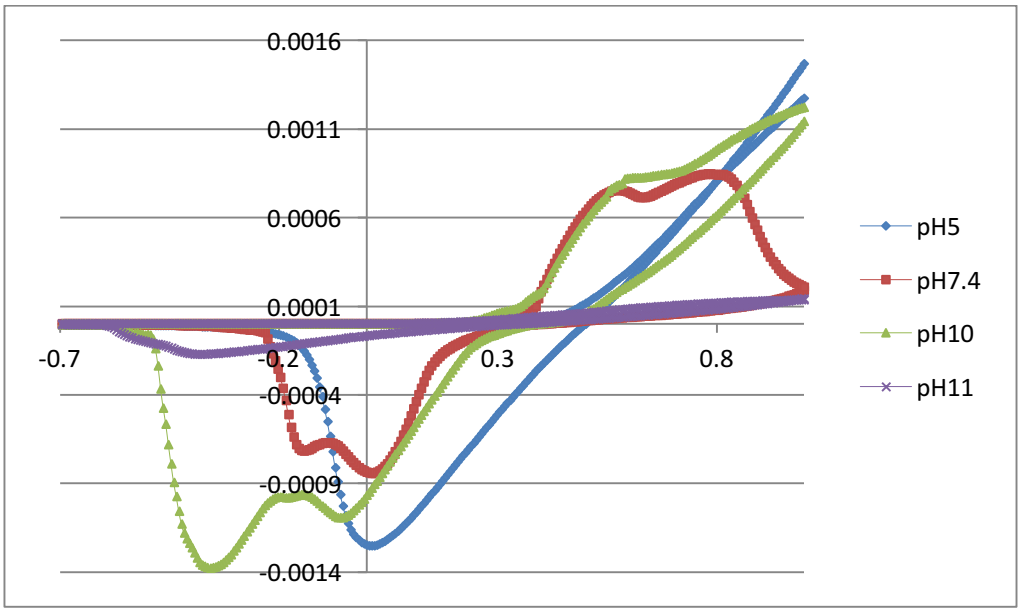

Fig.13. Silver electrode voltammogram in a reduction-oxidation scan cycle at different pHs of 5, 7.4, 10 and 11. Scan from +2V to -2V. Sweep rate = 0.05V/s; Voltage step = 0.0059V; T= 25°C.

Figure 14 shows the concentration of the different forms of oxidised silver as a function of the pH at a fixed potential of 0.5V and silver concentration of 10μM. As the pH increases up to pH10, a constant amount of silver is precipitating out of solution and the concentration of H^+ ions steadily decreases (expected as the pH is moving towards the alkaline condition).

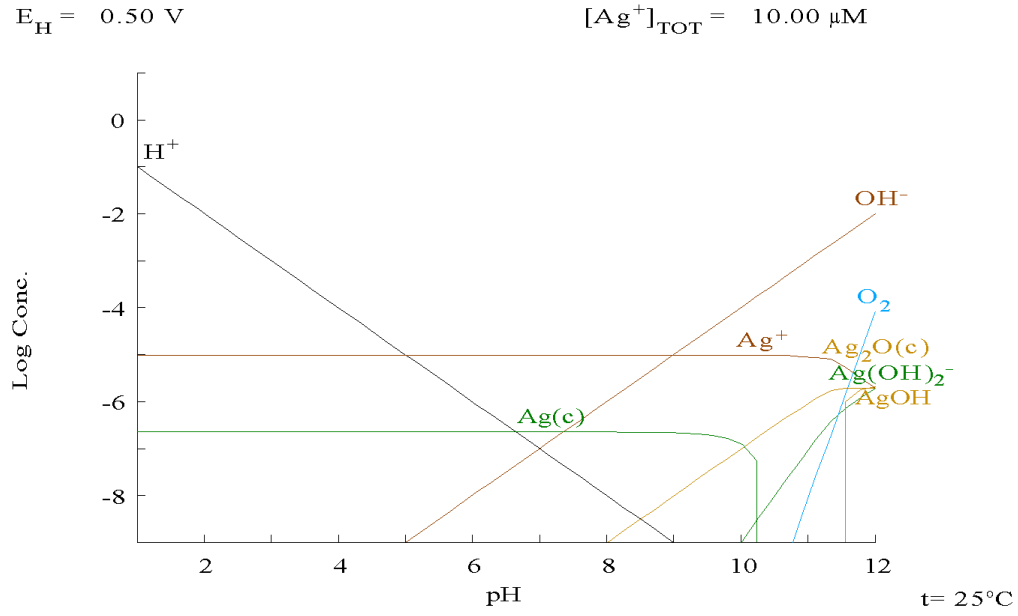

Fig.14. Theoretical oxidation-reduction equilibria for Ag at 25°C and 10µM Au at different pHs.

At the time that this is happening, up to pH11, a constant amount of Ag^+ ions are forming. It can be noted that at pH8 Ag_2O also starts to precipitate out of solution.

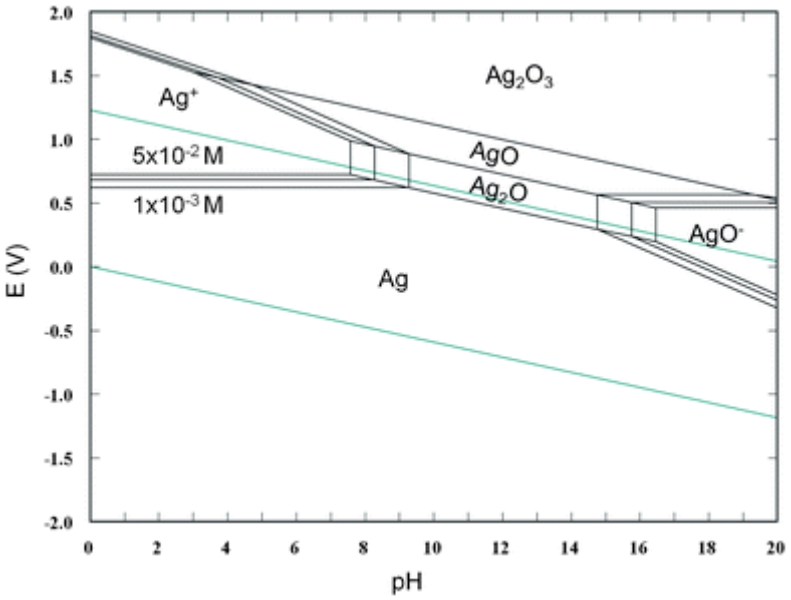

Fig.15. Pourbaix diagram of Silver (extracted from Hassan et al [26])

Silver electrode at pH5 showed the possibility to analyse digoxin both in OX-RED (Fig.12) and RED-OX (Fig.13) cycles at 0.0055V in the reduction phase and 0.0121V in the reduction phase respectively, with calibration curves r2 correspondently of 0.9269 and 0.9874. pH7.4 was a non-ideal environment for silver electrode that was not be able to detect digoxin in neither the cycles. At pH10, silver electrode could analyse digoxin in OxRed cycle at -0.1195V during the reduction phase, achieving a calibration curve with a r2=0.9104, and in RED-OX cycle at -0.0594V in the reduction phase (r^2=0.8561).

At pH11, this solid electrode was able to detect digoxin in both cycle, obtaining calibration curves with r^2=0.9923 in OxRed cycle at -0.2921V in the reduction phase and r^2=0.9111 in RED-OX cycle at -0.2736V in the reduction phase. For the silver electrode the LoD ranged from 16µg/mL at pH10 to 0.9µg/mL at pH5 and pH11.

The concentration of both these ions begins to plateau at around pH11. From pH10 a sudden increase of $Ag(OH)_2^-$ ions can be seen, followed by an appearance of AgOH ions between pH=11 and 12 (Fig.15). The presence of all of these ions at the differing pHs visually demonstrates the cyclic voltammetry results and the presence of surface oxides on the electrode, as well as supporting those results presented by Pasta, Mantia and Cui in the form of a Pourbaix diagram (Fig.15) [24] and [26]. It is clear, from the evidence supplied here, that an important factor to consider when digoxin is to be added to a solution and analised by an electrode system is the presence of metal oxides. Digoxin will have to compete with the electrode in both the oxidation and reduction processes occurring during cyclic voltammetry. This leads to a reduction in signal as the concentration of digoxin increases. There is a definite evidence of a linear dependence between the signal obtained and digoxin concentration.

Digoxin solutions at pH=5 showed the possibility to be also analysed using silver electrodes yielding peaks at 0.0055V and 0.0121V (in the reduction phase), showing calibration curves with r^2= 0.9269 and 0.9874, respectively. Solutions at pH=7.4 showed a non-ideal environment for the analytical use of silver electrodes as they were not able to detect digoxin in neither the cycles possibly due to the lack of interaction with surface oxides. At pH=10, silver electrodes could analyse digoxin at -0.12V during the reduction phase, achieving a calibration curve with a r^2=0.9104.

At pH=11, silver electrodes were able to detect digoxin obtaining calibration curves with r^2=0.9923 at -0.29V. For the silver electrode the LoD ranged from 16µg/mL at pH=10 to 0.9 µg/mL at pH=5 and pH=11, respectively. The best percentage of recovery obtained using gold electrode in OxRed was at pH10 with 115% and in RedOx it was at pH10 with 75% and at pH11 with 110%. An appreciative percentage of recovery resulting from the use of silver electrode was obtained only in OxRed condition at pH10 and it was of 120%.

The Standard deviation and the average Coefficient of Variation also followed the trend observed for gold electrodes showing a higher precision of -3.90% and -1.61% at pH=5 and 11, respectively.

Principal Component Analysis of the different digoxin solutions were also performed for silver electrodes, as shown in Figure 16 and 17.

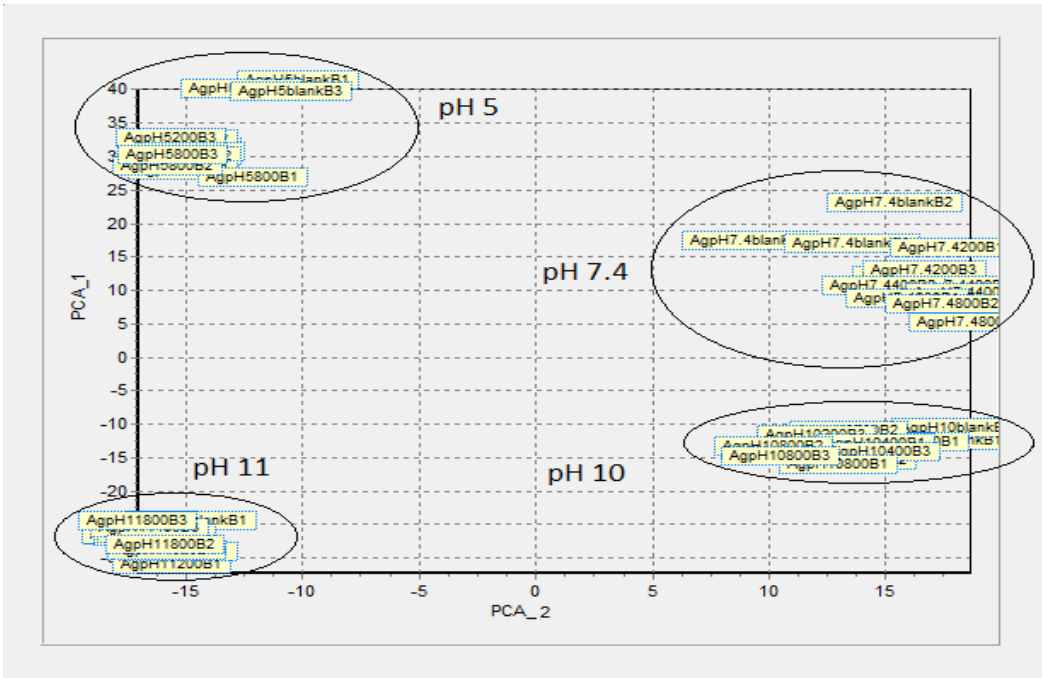

Fig.16. PCA scatterplot of the electrochemistry RedOx of a silver electrode at different pHs of 5, 7.4, 10, 11. Percentage of variability: PC1=51.30%; PC2=29.94%.

The PCA analyses showed a similar behaviour to that observed in gold at pH=11 and showed a better clustering than other pHs. Hence, the best possible analytical conditions for the analysis of digoxin on a silver electrode should be conducted at pH=11. Also,

the variability achieved in the analysis of the PCA plot for Fig.16 was PC1 (51.30%) and PC2 (29.94%) and for Fig.17 was PC1 (54.02%) and PC2 (20.61%).

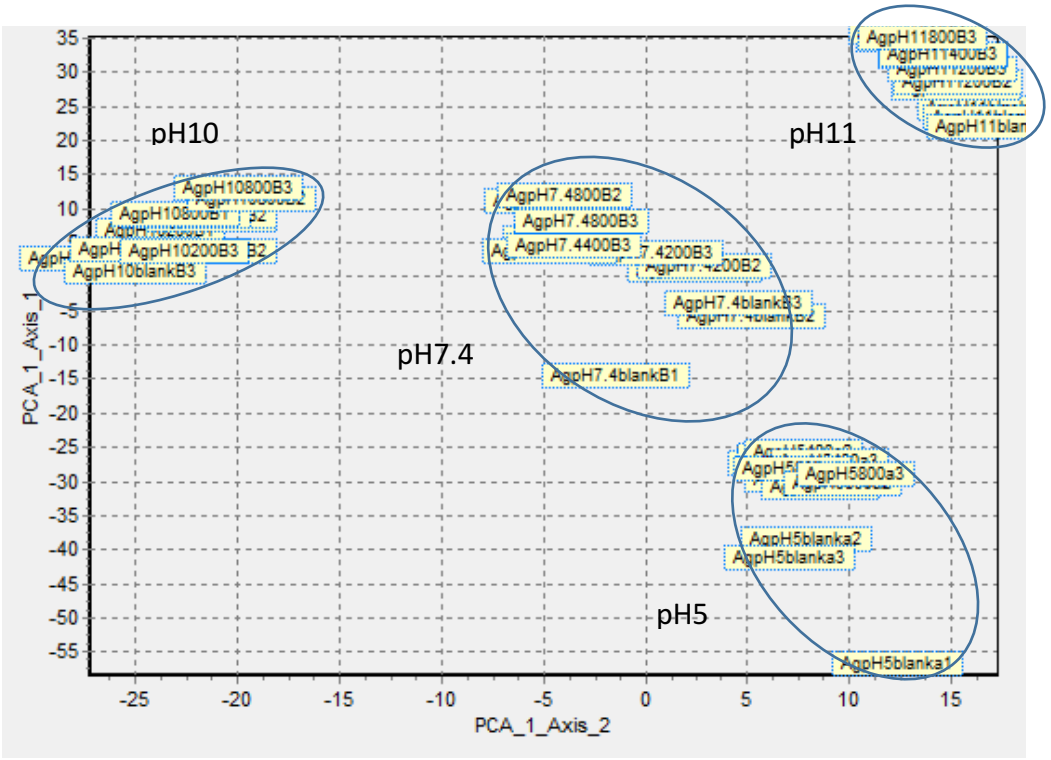

Fig.17. PCA scatterplot of the electrochemistry OxRed of a silver electrode at different pHs of 5, 7.4, 10, 11. Percentage of variability: PC1=54.02%; PC2=20.61%.

For the gold electrode in RedOx sweeps, the voltage potential peaks selected as analytical signals to follow in quantitative determinations were: at pH 5, -1.2138V; at pH7.4, -1.2733V; at pH10, 0.2322V; at pH11, 0.0418V in the reduction phase and 0.6012V in the oxidation phase. In OxRed sweeps, at pH5 and pH7.4 no signal was analytically significative; at pH10, 0.2018V; at pH11, 0.5886V.

For the silver electrode in RedOx sweeps, the voltage potential peaks selected as analytical signals to follow in quantitative determinations were: at pH5, 0.0120V; at pH7.4 no signal was analytically significative; at pH10, -0.0593V; at pH11, -0.2735V
In OxRed sweeps, at pH5, 0.0055V; at pH7.4 no signal was analytically significative; at pH10, -0.1194V; at pH11, -0.2920V.

4.4. Investigation of digoxin tablets by voltammetry

After having assessed the suitability of this analytical technique to investigate the cardiac glycosides in their standard pure form, the investigation was extended to the real pharmaceutical samples. Digoxin tablets from one generic and one branded version were submitted to voltammetric investigations, reiterating the same procedure applied previously for the standards. The tablet of digoxin contained 250μg of active pharmaceutical ingredient and as excipient lactose and magnesium stearate. The information found on the excipients were only qualitative, no information on the quantity were disclosed, being protected by industrial patent. The weight of the tablets averaged around 212mg, both for the generic and for the branded ones, leading to an extremely low ratio API to excipients of 0.1%w/w. The low ratio represented a strong limitation in the analysis of the API, because of the potential strong shielding effect of the signals generated from the bulk of excipients, covering the specific response of the analyte, as previously reported [24]. This could have been the reason of the poor results obtained in the voltammetric analyses of the digoxin in tablet form. In fact, when digoxin tablets have been submitted to the same analytical procedure of the correspondent standards, they showed a scarce response, of no analytical significance, to the voltammetric investigations both with mercury and with solid electrodes.

4.5. Raman characterization of digoxin, digitoxin and digoxigenin pure standards

In the light of the unsatisfactory results obtained from the voltammetry of the pharmaceutical formulations, in order to adjust the project design of the research, the possibility to use a different analytical technique to analyse the same previous sample was investigated.

Raman spectroscopy was chosen as a potential more appropriate analytical technique. Raman is a non destructive analytical method used in the last decades in the pharmaceutical sector with advantages to test in-situ solid pharmaceutical forms, often not even requiring to extract the tablets or capsules off from their original containing blisters and this has made Raman spectroscopy a valid candidate analytical method for quality control purposes [25-27].

4.5.1. Examination of the cardiac glycosides by Raman spectroscopy

The three pure standards of cardiac glycosides (digoxin, digitoxin and digoxigenin) investigated by Raman spectroscopy exhibited a good response to this analytical technique. Particularly, digoxigenin showed a quick significative response to Raman spectroscopy (Fig.18), while digoxin and digitoxin when exposed to conventional Raman analysis did not produced analytical results,

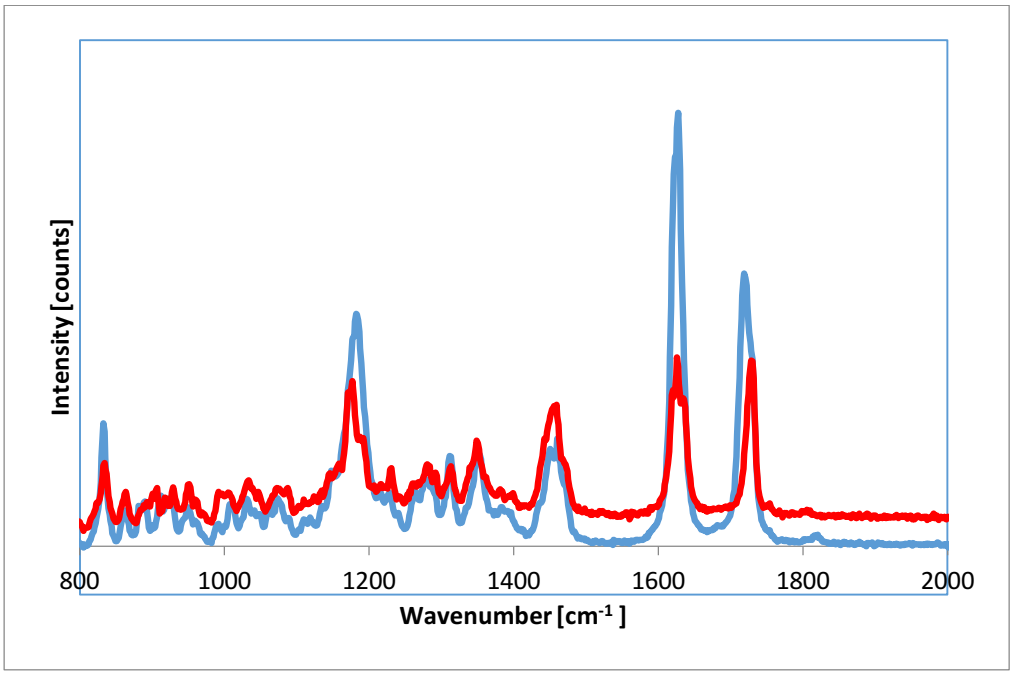

Fig.18. Comparison of SERS (blue line) and conventional Raman analysis (red line) of digoxigenin (spectra are shown offset in order to distinguish each other)

In order to amplify the Raman signals of the two non-responsive molecules, SERS analysis was performed on the standards of digoxin and digitoxin. When expose to SERS, the spectra obtained from the two compounds resulted changed, characterised by characteristic peaks (Fig.19, 20). Both digoxin and digitoxin showed a dramatic increase of the response to this analytical technique.

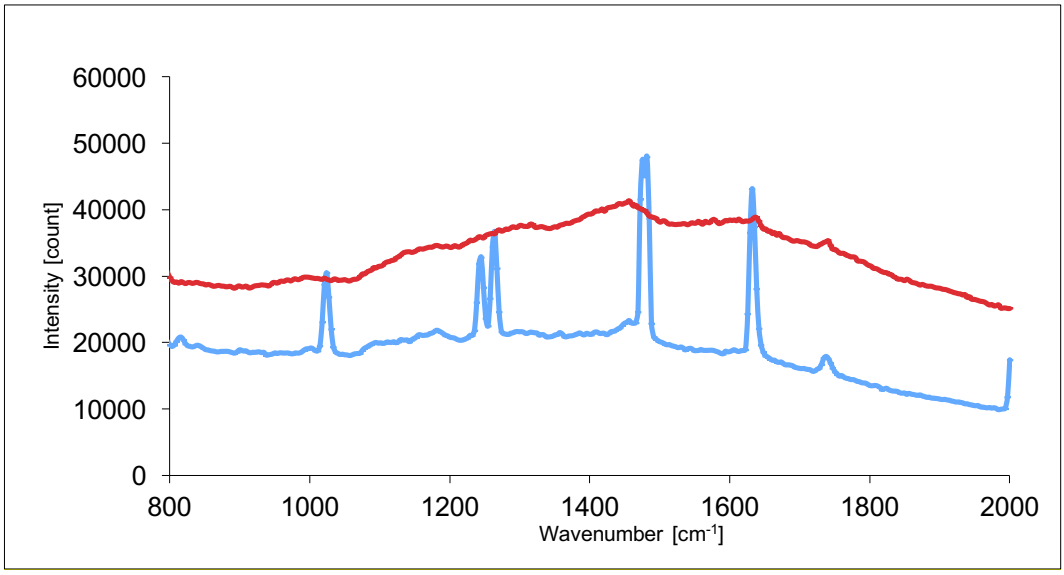

Fig.19. Comparison of SERS (blue line) and conventional Raman analysis (red line) of digoxin

On the contrary, when digoxigenin was submitted to SERS analysis the correspondent spectrum resulted to be very similar to the one obtained with conventional Raman spectroscopy.

SERS amplifies the signals produced by nucleophilic functional groups which have electron-rich atoms able to donate a pair of electrons to the silver colloid to form a new covalent bond. It is possible that the sugar moiety covers the Raman scattering of the steroid glycoside in digoxin and digitoxin, which benefit from a dramatic enhancement with the SERS, that shows a increase of the signals obtained from functional groups present in the steroid glycoside moiety. In Fig.20 it is possible to notice the sharp peak at 1470cm^{-1}possibly due to the the stretching of the double bonds C=C conjugated to the carboxylic group. At 1630cm^{-1} a strong signals probably attributable to the carboxylic group.

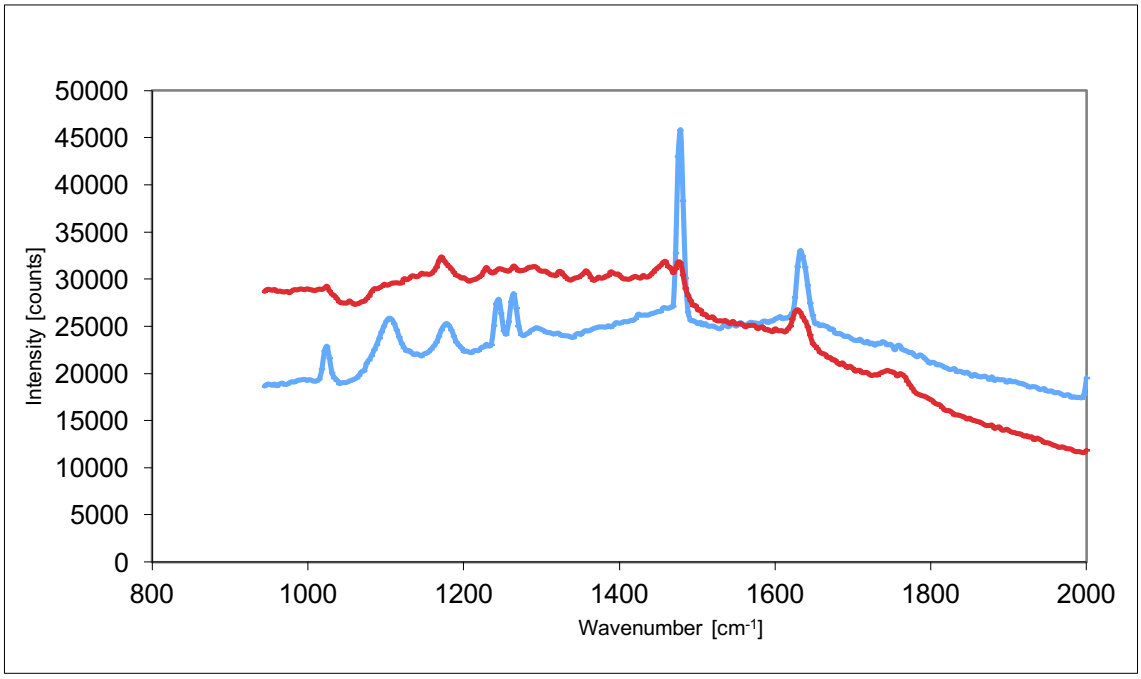

Fig.20. Comparison of SERS (blue line) and conventional Raman analysis (red line) of digitoxin.

In Fig.21 are plotted the spectrograms obtained for the three pure standards of cardiac glycosides investigated. In the region from 800 to 1000cm^{-1}, only digoxigenin seemed to show a significative Raman activity. The peak at 850cm^{-1} could be assigned to the stretching of the C-O-C in the cyclobutenolide ring.

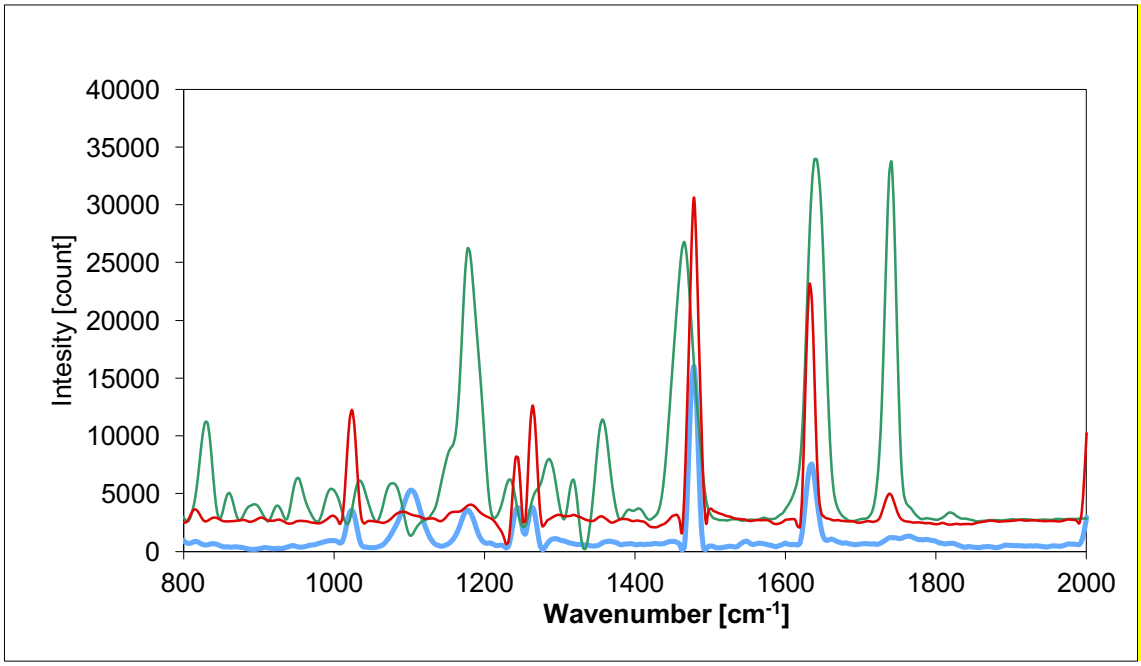

Fig.21. SERS Comparison of pure standards of Digoxin (red line), Digitoxin (blue line), and Digoxigenin (green line). (Spectra reported with baseline correction). At 1020cm^{-1} peaks due to asymmetric stretching C-O-C; at 1180cm^{-1} peaks due to CH_2 twisting; at 1470cm^{-1} stretching of the conjugated double bond C=C; at 1660cm^{-1} stretching of the carboxylic group; at 1750cm^{-1} the alicyclic ester stretching

The peaks in the range from 850 to 970cm^{-1} can be referred to the stretching of the diverse C-C alicyclic bonds (Fig.20). At 1020cm^{-1}, both digoxin and digitoxin, showed a sharp signal characteristic of asymmetric stretching C-O-C. Slightly shifted to 1040cm^{-1} digoxigenin presented a peak that can be associated to the same stretching. At 1100cm^{-1} digitoxin only presented a peak attributable to the OH rocking. At 1180cm^{-1} digoxigenin and digitoxin showed sharp signals, referable to CH_2 twisting, while digitoxin seemed not to present the same spectroscopic behavior. In the region from 1220 to 1250cm^{-1} all the three glycosides showed two sharp peaks, probably due to the OH scissoring of the hydroxyl groups both on the aglicone core and on the glycoside moieties. Digoxigenin showed a peak at 1320cm^{-1} and a prominent peak at 1360cm^{-1} due to bending of methyl groups. In the region between 1470 and 1490cm^{-1} all the three cardiac glycosides produced a sharp peak, possibly due to the stretching of the double bond C=C. In the region between 1640 and 1660cm^{-1} the three compounds emitted very strong signals attributable to the the stretching of the carboxylic group. At 1750cm^{-1} the alicyclic ester stretching is shown for digoxigenin and digoxin.

Raman spectroscopy resulted to be a valid technique to analyse the pure standards of cardiac glycosides. The extension of signals obtained were sufficient to identify and

differentiate the three samples. Particularly, digoxigenin seemed to respond directly to normal Raman investigation, without the aide of the metal colloids used to perform SERS investigation. However, SERS analysis was instead necessary for the digoxin and digitoxin, non responding to normal Raman analysis. Being the presence of the sugar moiety the main difference between the chemical structure of the digoxigenin and digoxin and digitoxin, it may be speculated that the presence of the hydroxyl groups in the sugar moiety seemed to interfere with the response of the steroid core alone, attenuating the intensity of the signals. Nevertheless, this attenuation has been resolved efficiently by the use of SERS for the analyses of the three cardiac glycosides, enhancing the signals of the nucleophilic functional groups, showing that this technique can be effectively adopted to investigate such products.

4.5.2. Raman characterization of digoxin tablets

Eight tablets of digoxin, generic and branded, were analysed with conventional Raman and SERS in order to explore the possibility to use this analytical technique to investigate this analyte in its pharmaceutical formulations. The signals obtained submitting the two different kind of digoxin tablets to both normal Raman and SERS analysis resulted analytically significative (Fig.22).

Lactose is the main excipient used in the manufacturing of the digoxin tablets tested. It presents a structure that can resemble the one of the polysaccharides side of the digoxin interfering with the analysis. The SERS spectrum of the main excipient lactose has been compared with the digoxin-tablet. The digoxin tablets and the lactose show different Raman spectra features, with some common peaks, and some peculiar ones.

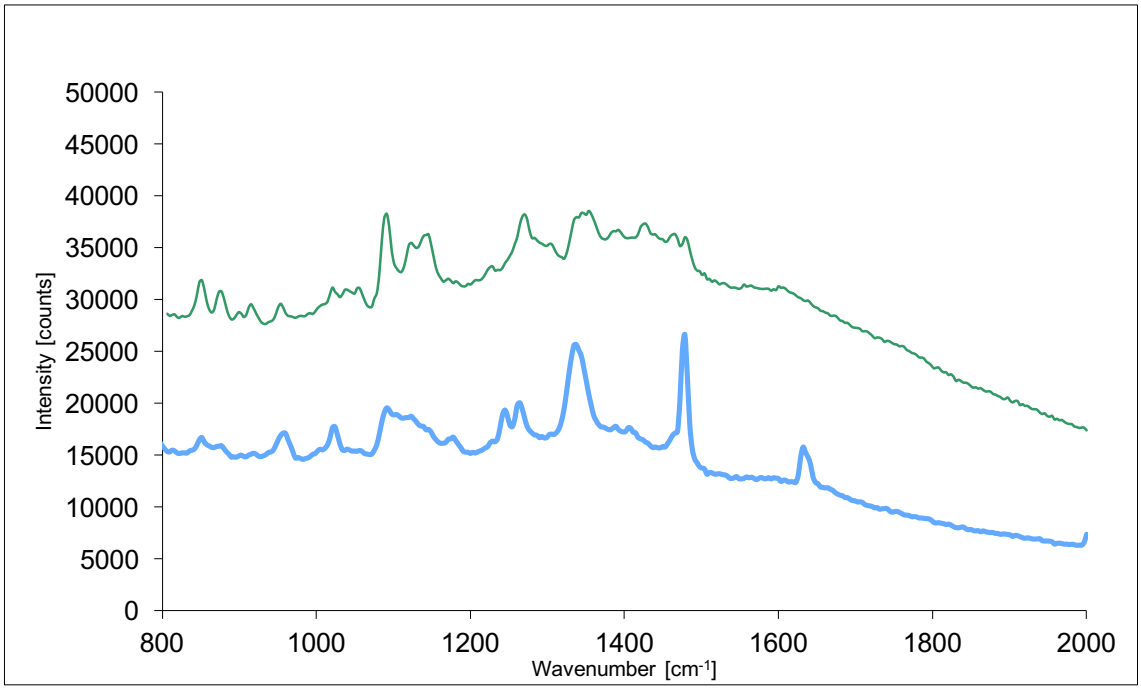

Fig.22. SERS spectroscopy of digoxin tablet (blue line) and lactose (green line).

The peaks in the range from 850 to 970cm^{-1} can be referred to the stretching of the diverse C-C alicyclic bonds present both int structure of the digoxin and in the lactose. At 1080cm^{-1} a sharp signal probably characteristic of asymmetric stretching C-O-C of the cyclobutenolide ring in digoxin is evident. At 1140cm^{-1} lactose showed a peak referable to CH_2 twisting, while digoxin seemed not to present the same spectroscopic behavior. In the region from 1220 to 1250cm^{-1} digoxin showed two sharp peaks, probably due to the OH scissoring of the hydroxyl groups both on the aglicone core and on the glycoside moieties. Digoxin showed a peak at 1330cm^{-1} due probably to bending of methyl groups. In the region around 1470cm^{-1} digoxin produced a sharp peak, possibly due to the stretching of the conjugated double bond C=C. At around 1630cm^{-1} digoxin produced a strong signals attributable to the the stretching of the carboxylic group.

4.6. Conclusions

Digoxin, digitoxin and digoxigenin were used in a comparison study of the electrochemical interactions of carbon glass, gold and silver solid metal electrodes in different pH buffer solutions of pH5, 7.4, 10 and 11, using cyclic voltammetry.

A good comprehension of the features of the different electrodes under different pH conditions was key to understand the electrochemical behaviour of these cardiac glycosides and their interactions with the different solid electrode surfaces. The results obtained shown that only digoxin could be investigated with a significant analytical response using this voltammetric approach.

The results obtained studying pure standards of these cardiac glycosides with solid electrodes carbon glass, gold and silver showed that only digoxin results offer sufficient value to be analytically investigated by voltammetry analysis using solid electrodes, particularly in gold and silver electrodes. In fact, carbon glass electrode resulted not able to register signals with analytical significance.

Nevertheless, the results obtained from the analysis of digoxin standard with Ag and Au electrodes showed that both electrodes were suitable for this investigation. Furthermore, in most of the cases, digoxin could be investigated, for the same pH, both in RedOx and in OxRed conditions. pH7.4 has shown to be a critical condition for both electrodes: the silver electrode has shown not to be sensitive at all, while the gold one has shown a response only in RedOx cycle.

When digoxin standard is added to the solution, competition with the electrode in the oxidations and reductions processes occurs the electro-activity of the electrode goes in competition with the electro-activity of digoxin, leading to a reduction of the signal with the increasing of the concentration of digoxin. This current peaks intensities, both at anodic and cathodic potentials, are highly correlated to a linear dependence on digoxin concentration.

Studying the chemical structure of digoxin, the double bond conjugate to the carbonyl group of the molecule seems to be prone to reduction from alkene to alkane group and to oxidation with opening of the lactone ring.

In terms of sensitivity, the better conditions have been for the Ag-electrode at pH10.

The very narrow solubility of digitoxin and digoxigenin it has been considered the major limiting factor to perform an adequate investigation in liquid condition of these molecules.

The results obtained on digoxin with the carbon glass electrode were overall inconclusive. Only digoxin has shown a slight responsive behavior when tested at pH5, as in the identical case of the experiments conducted at this pH with mercury electrode.

On the other hand, the results obtained by studying digoxin with Ag and Au electrodes in solution at different pHs showed that both electrodes were suitable for the analytical determination of digoxin in solution in the range of concentrations from 3.92 to 14.81μg/mL.

Using a gold electrode, the best analytical conditions, given by the LoD and the linearity of the calibration curve, showed that the alkaline environment at pH11 is the ideal one.

For this the LoD was 1.7μg/mL, with a corresponding average coefficient of variation of 2.09% and 4.13% at pH11 for both RedOx and OxRed sweeps, respectively.

For the gold electrode, the best percentage of recovery obtained in OxRed was at pH10 with 115% while in RedOx it was at pH10 with 75% and at pH11 with 110%.

Silver electrodes showed the best LoD (0.9μg/mL) at pH5 and at pH11. The results obtained characterised by an average coefficient of variation of -3.90% and -1.61% at pH5 and 11, respectively. For silver electrode, a significative rate of recovery was obtained only in OxRed condition at pH10 and it was of 120%.

The metal electrodes, gold and silver, studied seem to be good candidates in the analysis of digoxin via cyclic voltammetry replacing the mercury one and being able to quantify trace of this alkaloid in the range in optimum alkaline solutions at pH11. It is also important to emphasize the role that the surface oxides may play in digoxin redox behaviour and the catalytic influence in the electrochemical behaviour of this molecule in solid electrodes.

Micromole sensitivity has been achieved for both of the electrodes, showing that the use of these sensors could represent an alternative choice, more practical and less environmental impacting than using mercury electrodes.

Having confirmed that voltammetry analysis was indicated for the digoxin pure standard, the same investigation was extended to the tablet formulation of digoxin. Poor

results, not analytically representative, were obtained in the voltammetric analysis of the digoxin tablets.

The low solubility of the digoxin represented a strong limitation in the analysis of the digoxin tablets. The bulk of excipients is not electroactive, consequently the strong shielding effect of the signals showed in the spectroscopic analysis should not represent a limitation in voltammetry. When digoxin tablets were submitted to the same analytical procedure of the correspondent standards, they showed a scarce electrochemical response, of no analytical significance, to the voltammetric investigations both with mercury and with solid electrodes.

In view of the inadequate results obtained from the voltammetry analysis of the pharmaceutical formulations, in order to adjust the project design of the research, the possibility to use a different analytical technique to analyse the same previous sample was investigated.

Since cardiac glycosides have not been particularly studied using Raman spectroscopy, it seemed there were elements of novelty to undertake this study. The three pure standards of cardiac glycosides investigated by Raman spectroscopy exhibited a good response to this analytical technique. Particularly, digoxigenin showed a quick significative response to conventional Raman spectroscopy, while digoxin and digitoxin required SERS investigation to produce a significant analytical response. The response obtained submitting the two different kind of digoxin tablets to both normal Raman and SERS analysis resulted analytically significative. Even though the structure of the excipients used in the manufacturing of the digoxin tablets resembles the structure of the sugar moiety of digoxin, interfering with the analysis, some specific peaks attributable to the digoxin were registered.

4.7. References

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5. Differences Observed among Amlodipine Salts used in Medicine through Raman and FT-IR Spectroscopy with Chemometric Analysis

5.1 Introduction

A significant paradigm continuously frequented in pharmacology is the confliction between views on generic medications. As introduced earlier, "generics" are drugs comparable to their patented predecessors in terms of dosage, form, route of administration, quality and performance characteristics, and intended use [1, 2]. For a generic drug to be accepted by the appropriate authorities, the manufacturer must demonstrate that the replicate meets bioequivalence expectations whilst containing the same quantity of active pharmaceutical ingredient (API) [3]. Generic drugs should be used interchangeably with the innovators drug; however, generic substitution has become common practise amongst healthcare professionals [1]. Despite this commonplace normality there are concerns in the healthcare sector as well as amid patients that not all generic preparations are equally clinically effective as their brand siblings, and that brand-name drugs may be clinically superior [5-7]. In order to introduce into the market a generic medication, pharmaceutical companies have previously produced different salts of an approved patented medication [4]. The different salt forms of a molecule can change its pharmacokinetic and stability features, which leads to needs for more exhaustive regulatory considerations [8, 9].

Kesselheim *et al.* raised the issue of generic drug substitution in the cardiovascular area [10]. They reported how 23 of 43 publications expressed negative views of generic drug substitution within cardiovascular science, and advised against the interchangeability of generic medications. Dong *et al.*, however, studied the effect of different generics of Levothyroxine on a hypothyroidism condition, and concluded that there was not a considerable difference in the therapeutic properties [11]. In the area of epileptic medications, Burkhardt *et al.* suggest that phenytoin-branded drugs and their generics do not produce equivalent therapeutic effects in some patients and therefore substitution was not advised [12]. Borgheini and Guberman *et al.*, also within the epileptic discipline, reported a general level of discomfort among neurologists with regards to their views towards generic medications [13, 14].

Amlodipine provides an interesting example of a drug that pharmaceutical companies have introduced in different salt forms of the same active pharmaceutical ingredient moiety in order to attempt replication of an approved patented medication. Currently in therapy, amlodipine is available in the maleate, mesylate and besylate salt forms (Fig.1) [15,16].

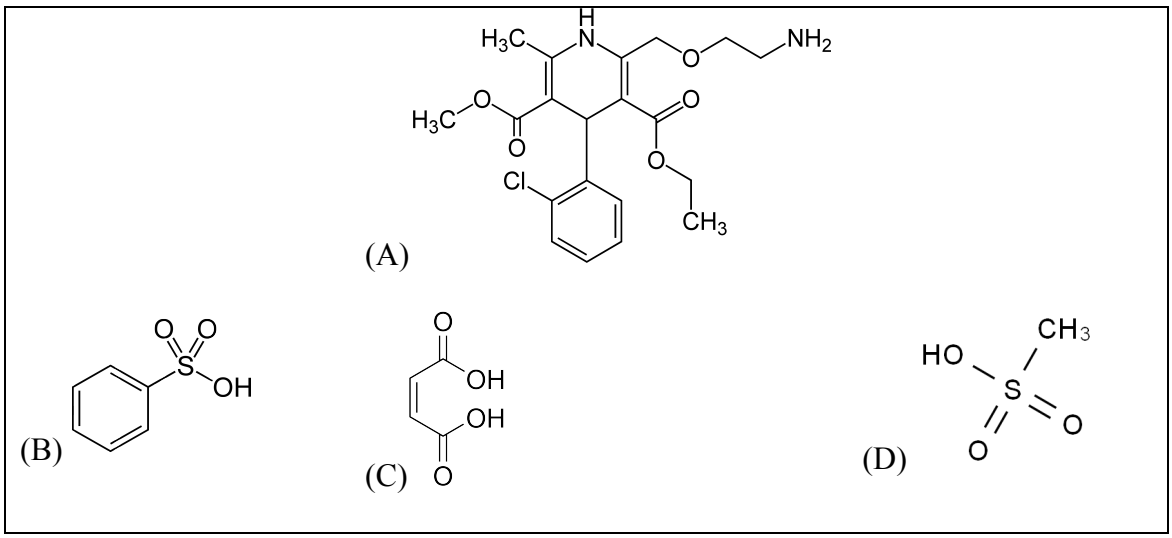


Fig.1. Chemical structure of (A) Amlodipine with relevant salt moieties; (B) Besylate, (C) Maleate and (D) Mesylate.

Amlodipine is a calcium channel blocker that acts on the cardiac muscle and its peripheral vessels. Acting on the myocardial cells, which are responsible for the conduction of the electric stimuli of the heart, amlodipine causes a decrease of the myocardial contractility [1]. Amlodipine causes dilation of coronary and peripheral arteries which is an important treatment of hypertension, as well as increasing blood flow to the cardiac muscle which aids angina [2].

The Food and Drug Administration has already raised concern that the maleate salt of amlodipine, unlike the besylate and mesylate salts, suffer from intrinsic chemical instability resulting in the formation of a biologically active degradation product [17]. Some studies, including that by Suh *et al.*, report how one of the by-products obtained by the degradation of the maleate salt moiety is a derivate of aspartic acid [18-20]. A citizen's petition was organised in 2002 to revoke acceptance of amlodipine maleate as a clinically equivalent option to amlodipine besylate, due to the high biological activity of the impurities obtained from the degradation of the maleate moiety [21]. This was

acknowledged by the United States Court of Appeals for the Federal Circuit which temporarily prevented the generic maleate version from entering the market while it underwent further investigation [22].

The amlodipine salts are available to purchase in the solid-state form of tablet. The majority of medications currently developed and prescribed are in the form of tablets or capsules due to their ease of consumption by the patient. The identification and characterisation of tablets and capsules is a vital process within the drug development timeline, as the pharmaceutical form of a substance can impact significantly its stability and bioavailability [23]. Taylor & Langkildl explain how useful it could be to be able to identify these solid-state medications *in situ*, particularly where different salts are present. Furthermore, tablets often contain low doses of API, which can present analytical challenges. [24]. The design of the pharmaceutical industry has demonstrated considerable challenges in the need for new analytical systems that can discriminate between salt forms of a single drug moiety for quality control purposes, especially when doubts on the nature of the source of manufacturing and distribution are raised.

Since the interchangeability of amlodipine medications has been questioned, here we explore differentiation of three salts of amlodipine, namely besylate, maleate and mesylate, in tablet formulation. Raman spectroscopy was chosen as one technique for this investigation as it has previously been selected to evaluate substances of abuse in combination with statistic aids. O'Connell et al reported how Raman spectroscopy, coupled with chemometric methods, can be used for on site analysis, both qualitative and quantitative, of narcotics deriving from illegitimate distribution chains [25]. In this study, O'Connell et al demonstrated that the first derivative of the Raman spectra from the fingerprint region yielded the best classifications and using cross-validation applied only on the fingerprint region between 750–1900cm^{-1} a correct classification rates of better than ~90% was achieved. They showed that even with very high degrees of sample variance it was possible to gain accurate identification. In a different study, Ryder explained how using PCA and Raman spectroscopy it is possible to discriminate between cocaine, heroin, and MDMA mixtures even with very similar and complex Raman spectra [26]. Moreover, restricting the variables for principal component analysis to the most intense peaks in the Raman spectrum of the pure narcotic resulted in a rapid discrimination method for classifying samples according to the narcotic type.

At the present time no similar published study has been extended towards PPIs medications. FT-IR was chosen as the second technique for this investigation, as it is usually coupled to Raman spectroscopy given complementary correlated results, but, similarly as for the Raman spectroscopy, its use on the three salts of amlodipine medications result to be innovative. Furthermore, Raman spectroscopy was chosen as one technique for this investigation as it has previously been selected to evaluate the co-action of amlodipine on cardio-vascular conditions, as well as for amlodipine tablet dissolution testing [27, 28] but not yet used to investigate the three therapeutic salts. FT-Raman has been previously used for the characterisation of various solid-state forms, including salts [29] but not for these amlodipine salts. FT-IR was chosen as the second technique for this investigation, as it is an instrument frequently deemed suitable for the analysis of tablets [30]. FTIR has previously been used to analyse crystal forms of amlodipine besylate as well as amlodipine inclusion complexes [31, 32] but not extended to a comparison study of the three salts. The application of these two analytical techniques in the examination of the three different salts of amlodipine with a subsequent chemometric evaluation presents an element of novelty in pharmaceutical analysis.

5.2. Materials and Methods

5.2.1. Materials

-Amlodipine maleate tablets ((Z)-but -2- enedioic acid; 3-O-ethyl 5-O-methyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate) were purchased from Discovery Pharmaceuticals;

-Amlodipine mesylate tablets (3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl] -4-(2-chlorophenyl) -6-methyl-1,4-dihydropyridine-3,5-dicarboxylate; methanesulfonic acid) were purchased from Actavis;

-Amlodipine besylate tablets (benzenesulfonic acid;3-O-ethyl 5-O-methyl 2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate) were purchased from Sandoz. Sixteen tablets of each Amlodipine salts were analysed internally and externally by Raman and FT-IR.

5.2.2. Methods

Spectroscopic analysis was carried out by Raman spectroscopy, surface-enhanced Raman spectroscopy (SERS) and FT-IR. Raman analysis was performed on a FORAM 685-2 instrument with a laser operating at 532nm, to ensure high levels of sensitivity. The Raman instrument was also equipped with an integral video microscope. Analyses were performed between the wavenumbers 800 and 2000cm^{-1}.

A Perkin-Elmer Spectrum 100 was used for the FT-IR analysis. This was supported by a Motorola DSP56303 Digital Signal Processor and a near infrared detector. The FT-IR was equipped with a Perkin-Elmer Autoimage microscope, with a IR performance of 9000:1 p/p signal to noise ratio and a resolution greater than 10μm. The range of wavenumbers investigated was between 4000 and 500cm^{-1}.

Sample Preparation: eight tablets were taken for Raman analysis. To test the external side of the tablets using Raman spectroscopy the tablets were exposed to the laser at five points for each tablet, to achieve 40 repeated representative analyses for each amlodipine salt. To test the internal side, the tablets were broken in half and again exposed to the laser five times for each tablet.

SERS enhances the sensitivity of standard Raman scattering by depositing a metal colloid on the surface of the sample being analysed. For this part of the investigation, silver colloids were prepared as follows: silver nitrate was reduced using sodium citrate in water, and concentrated by centrifuging at 5000rpm. The eight tablets for SERS analysis were covered with 2μL of the prepared colloid solution and 2μL of NaCl 1M after analysis by normal Raman, and analysed immediately. To test the external side of the tablets using Raman spectroscopy the tablets were coated in the silver colloid preparation and exposed to the laser. This was again repeated five times for each tablet, with the laser directed at a variation of external sites, to achieve the 40 representative analyses for each amlodipine salt. To test the internal side, the tablets were broken in half prior to the addition of the silver colloid, and again exposed to the laser five times for each tablet.

Eight tablets were also taken for FT-IR analysis. To study the external side of the tablets by FT-IR the samples were prepared by scratching the surface of the tablets onto the ATR plate with a small spatula. This was done in order to attain adequate contact of the sample on the ATR crystal, so a successful spectrum could be produced.

Due to the shape of the tablets this contact would not have been achieved if the tablet was kept intact, and would have resulted in poor transmittance. To study the internal side, the tablets were ground to ensure homogenisation and subjected to the aforementioned procedure. Each tablet was analysed individually using a scan cycle of 40.

Principal Component Analysis (PCA), Factor Loadings, Cross Validation, Leave-one-out were performed using Tanagra™ data mining software (University of Lyon, France). Analytical data were exported from the correspondent analytical apparatus to an excel spreadsheet and subsequently uploaded onto Tanagra.

5.3. Results

5.3.1. Raman Spectroscopy of Amlodipine Salts

The conventional Raman analysis of the three salts of amlodipine examined, both internally and externally, resulted to produce spectra with broad peaks, not analytically significant (Fig.2, 3).

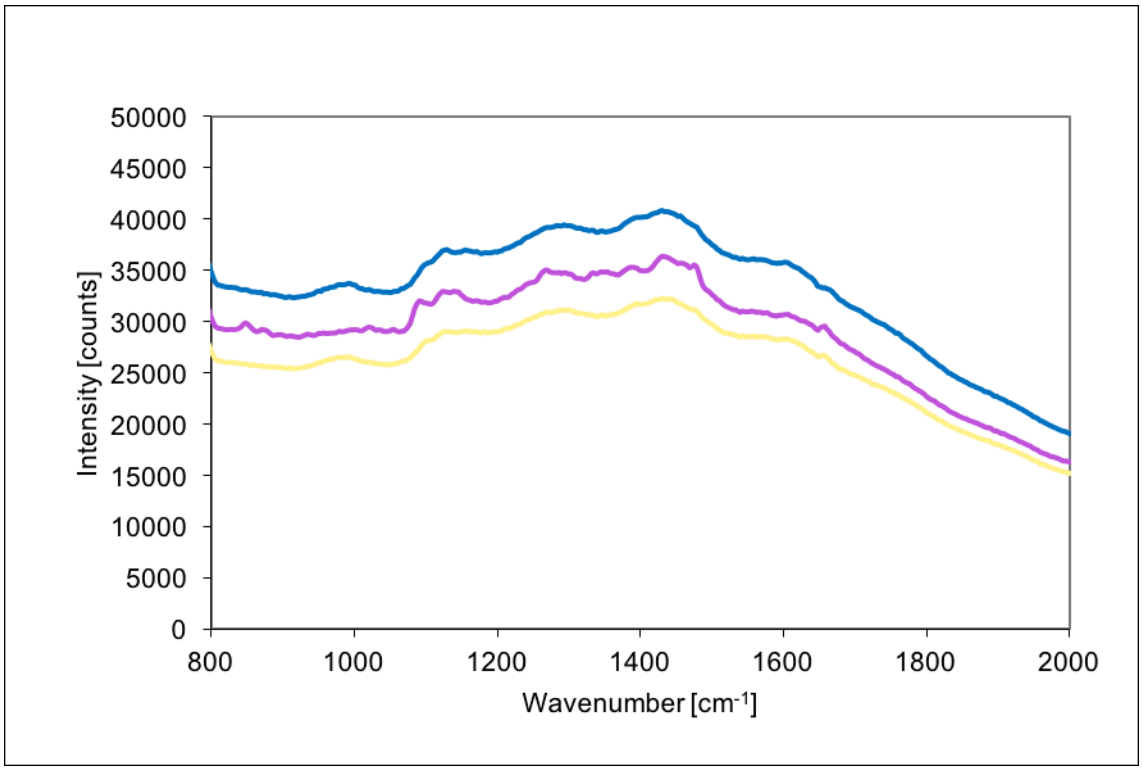


Fig.2. Raman analysis of tablets of amlodipine besylate (blue line), amlodipine maleate (purple line), amlodipine mesylate (yellow line), external sides

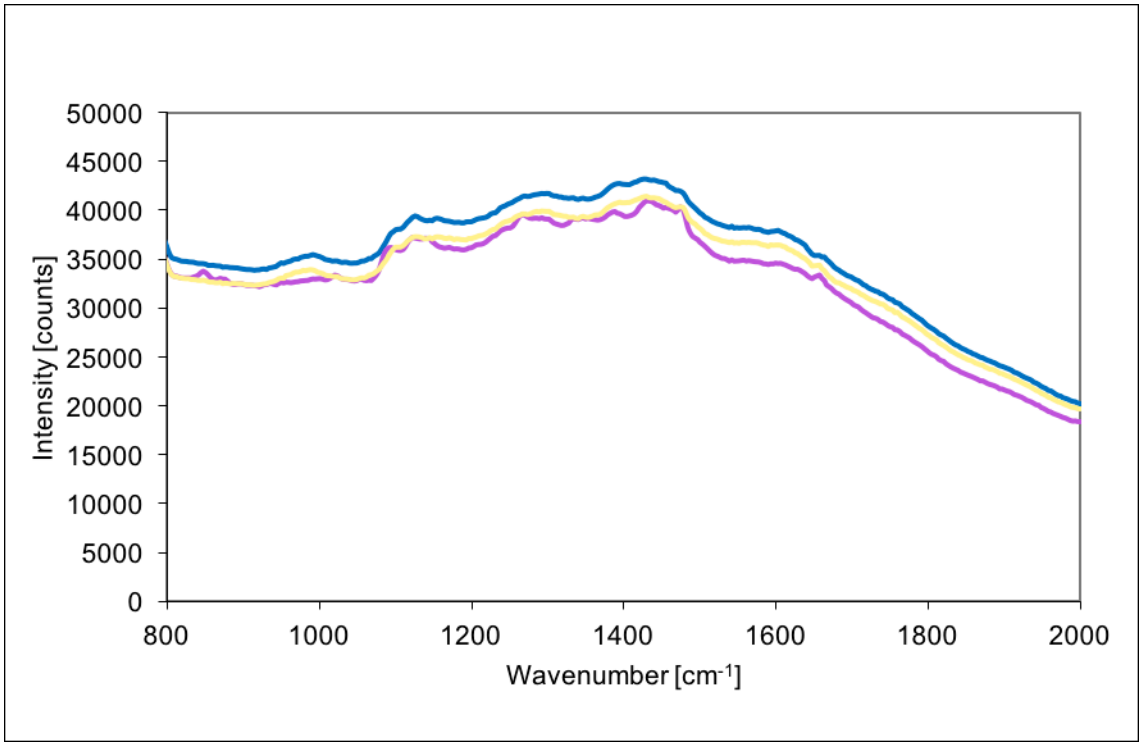


Fig.3. Raman analysis of tablets of amlodipine besylate (blue line), amlodipine maleate (purple line), amlodipine mesylate (yellow line), internal sides

Baseline correction is a significant pre-processing technique of the obtained spectrum, aiming to separate true spectroscopic signals from several type of interferences. Baseline correction attempts to remove mainly the spectral contributions originated by fluorescence effects associated to the Raman scattering, and the laser fluctuations contribution, which will be reflected in the process data as an overall intensity shift. In fact, Raman intensity of the peaks obtained can often vary sensibly amongst replicates (Fig.4) and this fluctuation can have a profound influence on the associated chemometric model by causing extra factors to appear. On the contrary, adopting preprocessing techniques, such as baseline correction, allows to improve the performance and robustness of the statistical models adopted.

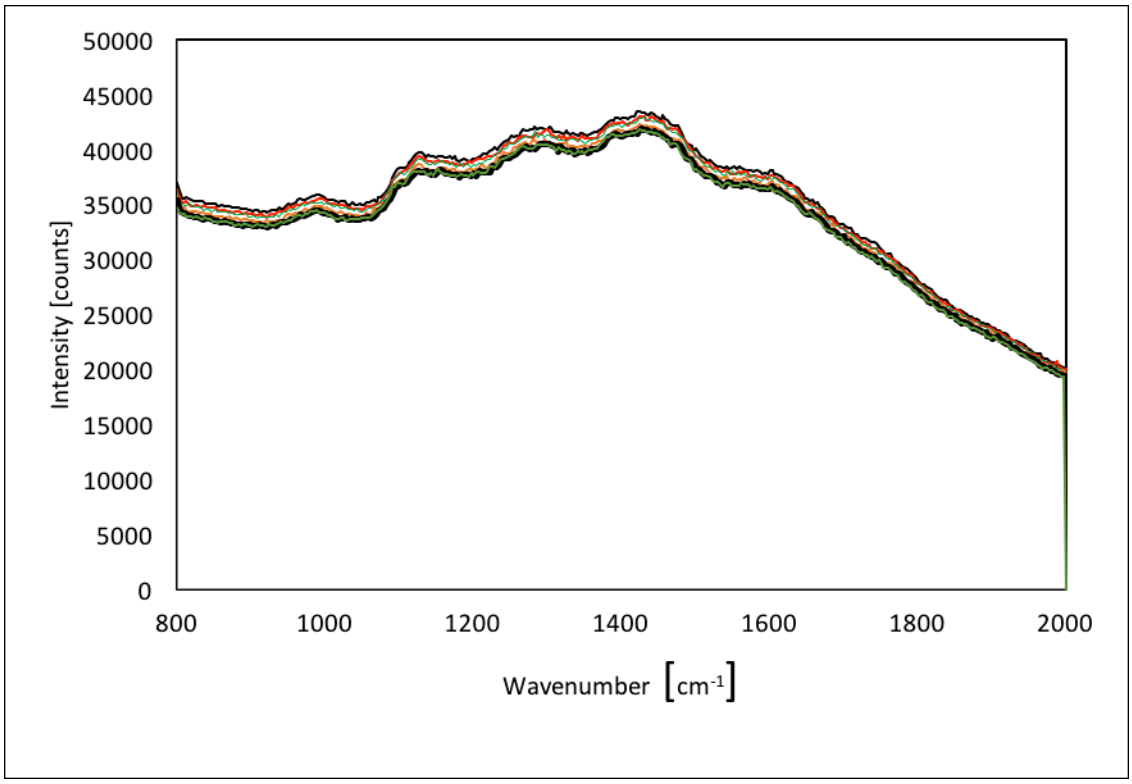


Fig.4. Raman analysis of tablets of six replicates of amlodipine besylate), internal sides, showed as example to highlight the variation of intensities of the generated spectra.

Opposite to the results obtained by conventional Raman, SERS analyses generated remarkably distinctive spectra thanks to the presence of several nucleophilic functional groups in the chemical structure of both the anionic and the cationic moieties of the amlodipine salts. The SERS spectra obtained from the analysis of the surfaces and of the internal areas of the samples, after their breakage, of the three salts investigated

have been displayed together in Fig.5 and 6. The spectra clearly show the similarity between forms with structure specific peaks obtained for each of the amlodipine salt.

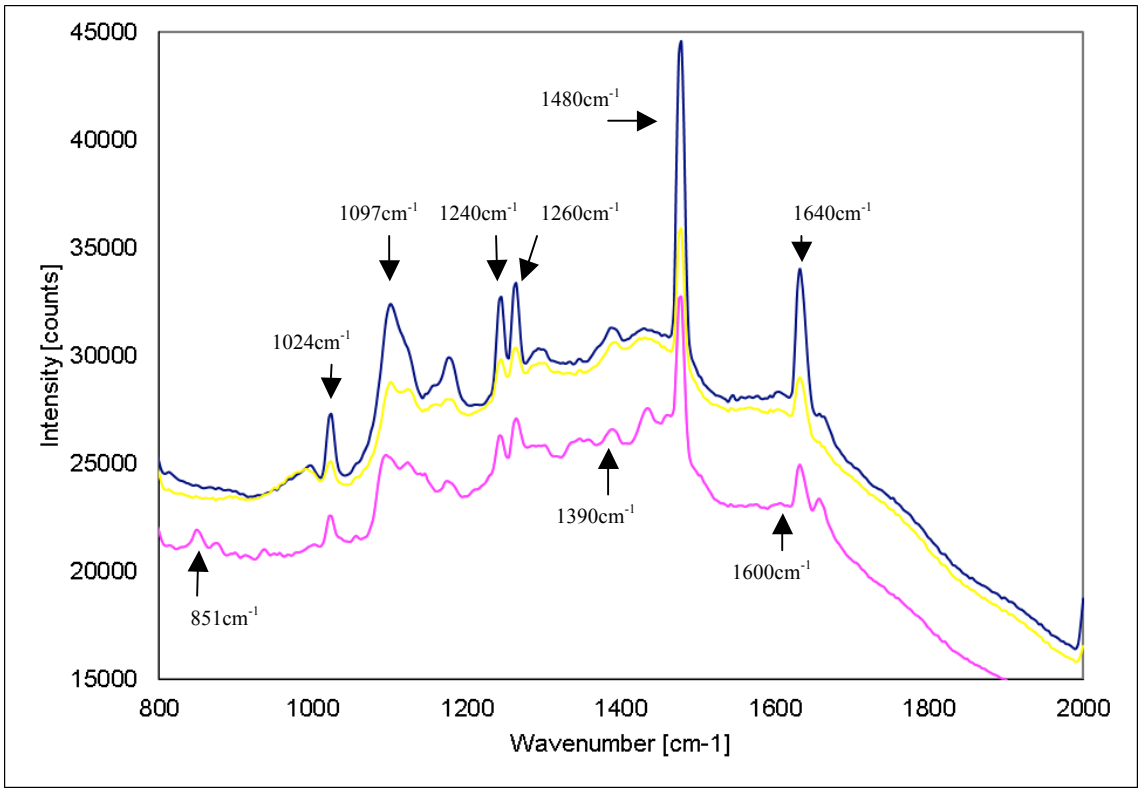


Fig.5. SERS analysis of tablets of amlodipine besylate (blue line), amlodipine maleate (pink line), amlodipine mesylate (yellow line), external sides. Peak at 851cm^{-1} due to stretching of the carboxylic group of the maleate; at 1024cm^{-1} peak due to the asymmetric stretching of the ether group; at 1097cm^{-1} the stretching vibration of the C-C aliphatic chain is shown; at 1240cm^{-1} peak due to the stretching of the C-C aliphatic chain; at 1260cm^{-1} peak due to the presence of multiple C-N stretching vibrations; at 1390cm^{-1} peak due to the cationic aromatic ring moiety which emits C-C vibrations; at 1480cm^{-1} peaks are related to the aromatic C=C stretching; at around 1600cm^{-1} it is observed that a small peak associated to asymmetric in plane bending for a NH_2 group; at 1640cm^{-1} sharp peaks related to the C=O stretching.

From Fig.1 it is clear that the cationic moiety is linked by its primary amine to the acid group of each of the anionic counterpart, respectively. In Tab.1 the tentative band assignment of the peaks obtained from SERS analysis is reported.

Starting at 1024cm^{-1}, all salts present a peak which correlates to the asymmetric stretching of the ether group in the cationic moiety. In the spectra of all three salts, the stretching vibration of the C-C aliphatic chain is demonstrated at 1097cm^{-1}. In the region of 1200-1250cm^{-1} there are two peaks present. The first at 1240cm^{-1} is also as a result of the stretching of the C-C aliphatic chain, and the second at 1260cm^{-1} is due to

the presence of multiple C-N stretching vibrations. All amlodipine salts present a peak at 1390cm^{-1} due to the C-C vibrations of the cationic aromatic ring moiety.

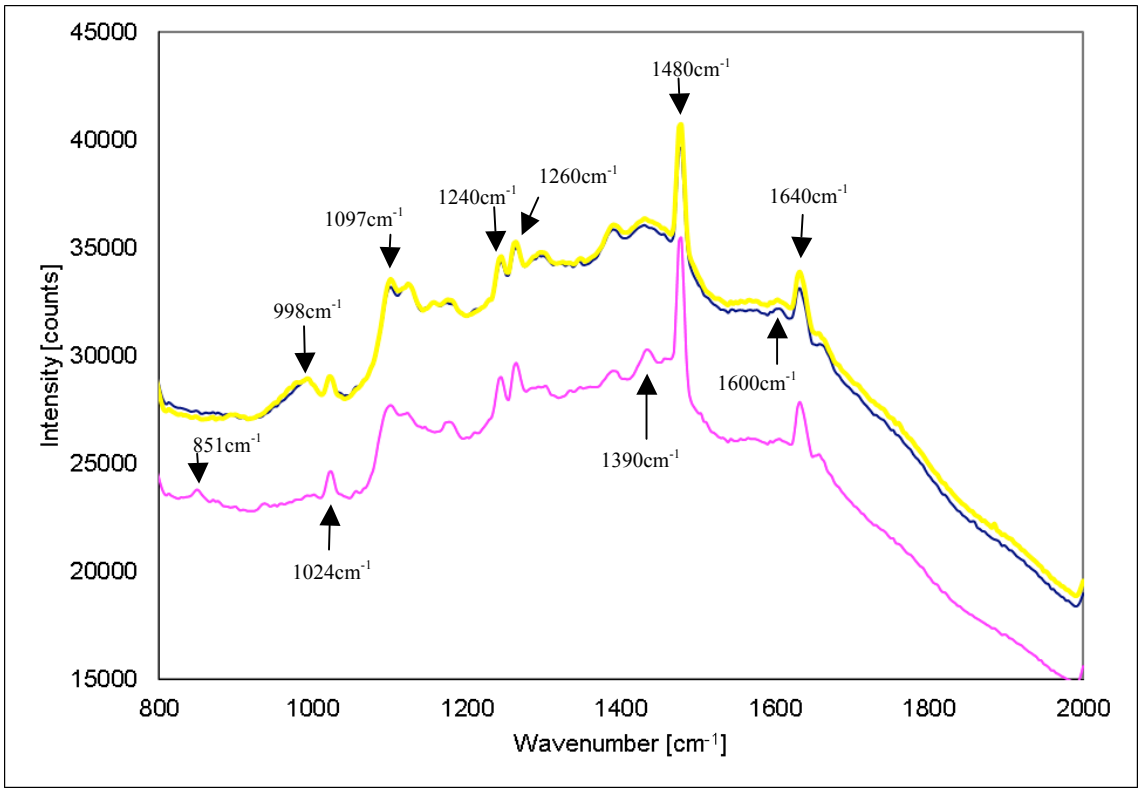


Fig.6. SERS analysis of tablets of amlodipine besylate (blue line), amlodipine maleate (pink line), amlodipine mesylate (yellow line), internal sides. Peak at 851cm^{-1} due to stretching of the carboxylic group of the maleate; peaks at 998cm^{-1} are related to sulfonic group stretching; at 1024cm^{-1} peak due to the asymmetric stretching of the ether group; at 1097cm^{-1} the stretching vibration of the C-C aliphatic chain is shown; at 1240cm^{-1} peak due to the stretching of the C-C aliphatic chain; at 1260cm^{-1} peak due to the presence of multiple C-N stretching vibrations; at 1390cm^{-1} peak due to the cationic aromatic ring moiety which emits C-C vibrations; peaks at 1480cm^{-1} are related to the aromatic C=C stretching; at around 1600cm^{-1} it is observed that a small peak associated to asymmetric in plane bending for a NH_2 group peaks at 1640cm^{-1} are related to the characteristic carboxylic group stretching.

The characteristic peak at 1480cm^{-1} is relative to the C=C stretching resultant from sections of the aromatic ring. The C-N group within the amlodipine molecule is accounted for by the broad C-N stretching aromatic peak at 1570cm^{-1} and 1610cm^{-1}. Around 1600cm^{-1} it is observed that a small peak is present for all salts, which has previously been associated to asymmetric in plane bending for a NH_2 group [28]. At 1640cm^{-1} all spectra produce a sharp signal, which is justified by the presence of the C=O unit in the carboxylic and ester groups. This has been selected in some studies as a reference peak for validation models [35]. The peaks mentioned here can be associated

to amlodipine itself, as the salt part of the molecule and any excipients between the different tablet types are different.

In addition to these peaks, there are spectral regions where differences between the salt forms are pronounced. In the amlodipine maleate spectrum, there is a prominent peak at 851cm^{-1}, in both external and internal analyses, that is not present in the mesylate and besylate spectra. This is due to the additional stretching of the carboxylic group of the maleate salts structure (Fig 1), not present in the besylate and mesylate structures. Furthermore, in both internal and external set of experiments (Fig.5, 6) the mesylate and besylate spectra show a peak evident at 998cm^{-1} which is not as strong in the maleate spectra. This wavenumber is often related to stretching of the aromatic C=C but also to the the stretching of the sulfonic group, presented only in the besylate and mesylate but not on the maleate moiety.

Tab.1. Tentative band assignment of SERS spectra of different salts of amlodipine tablets

Wavenumber [cm^{-1}]	Functional group
851	C=O maleate stretch
998	S=O besylate/mesylate
1024	COC asym stretch
1097, 1240	C-C aliphatic chain stretch
1260	CN stretch
1390, 1480	CC aromatic ring stretch
1570	CN aromatic stretch
1600	NH2 bending
1640	CO stretch

To aid the interpretation of the vast amount of wavenumbers generated by the spectroscopic techniques, both SERS and conventional Raman, PCA was used. PCA of the SERS data obtained by analysis of the external side of the amlodipine tablets shows identifiable distribution profiles for each salt (Fig.7).

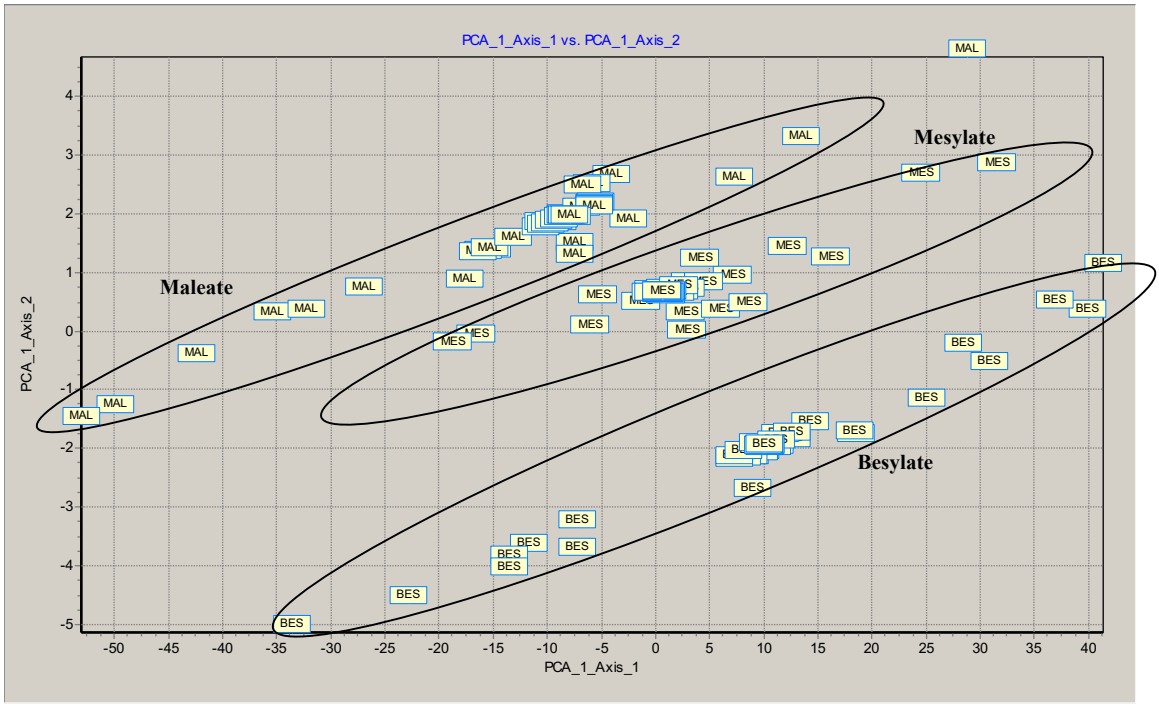


Fig.7. Principal Component Analysis of the results obtained by SERS analysis of the external side of amlodipine besylate, amlodipine maleate, amlodipine mesylate tablets. PCA1=97.91%, PCA1+2=99.27%

Principal component PC1 accounted for 97.91% of variation, with PC2 and PC3 being responsible for 1.36% and 0.65% respectively. The Kaiser-Guttman criteria advises to only use PCs greater than 1, for which PC3 has a score of 1.66, however as PC3 is accountable for less than 1% variation it was dismissed. PC1 and PC2 are therefore responsible for an accumulative 99.27% of the variation. The salts were separated due to their individual association with the two main components. All three salts gave a positive correlation across the two PC axis' but can be seen to separate over the value of PC2. Amlodipine besylate clusters at a lower PC2 value, the maleate salt at a high PC2 value whilst the mesylate salt clusters centrally around 0 for both components. Predictive accuracy of the PCA model obtained was validated via cross validation and leave-one-out approach (Tab.2, 3).

Tab.2 Cross validation of the PCA shown in Fig.7 on SERS spectroscopy obtained from the surface analyses of the amlodipine salts

Error rate				0.043			
Values prediction				Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0.01	0.03	BES	39	0	1	40
MAL	0.03	0.05	MAL	0	38	2	40
MES	0.03	0.05	MES	0	2	38	40
			Sum	39	40	41	120

The total error rate of the predictive model evaluated via cross validation accounted for around 4%. From the confusion matrix, it is possible to notice that out of 40 specimens per each salts analysed, besylate gave 39 samples correctly classified, correspondent to 97.5% of the total; maleate and mesylate salts gave 38 samples correctly classified, equivalent to 95%. These data reflect the visual representation on the PCA (Fig.7) were the three classes of samples are separated in three distinctive clusters, even if spread within each group.

Tab.3. Leave-one-out validation of the PCA shown in Fig.7 on SERS spectroscopy obtained from the surface analyses of the amlodipine salts

Error rate				0.041			
Values prediction				Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0.12	0.026	BES	39	0	1	40
MAL	0.14	0.051	MAL	0	38	2	40
MES	0.07	0.05	MES	0	2	38	40
			Sum	39	40	41	120

Furthermore, the predictive accuracy of the model was tested via leave-one-out validation. The cumulative error rate accounted for around 4%, with similar results in terms of predictions than the ones obtained from correspondent the cross validation. From the confusion matrix, it is also possible to observe that the correct classification for all the specimens analysed is comparable to the cross validation data.

PCA of the SERS data obtained by analysis of the internal side of the tablets (Fig.8) shows distribution profiles for each salt, but without the same clarification.

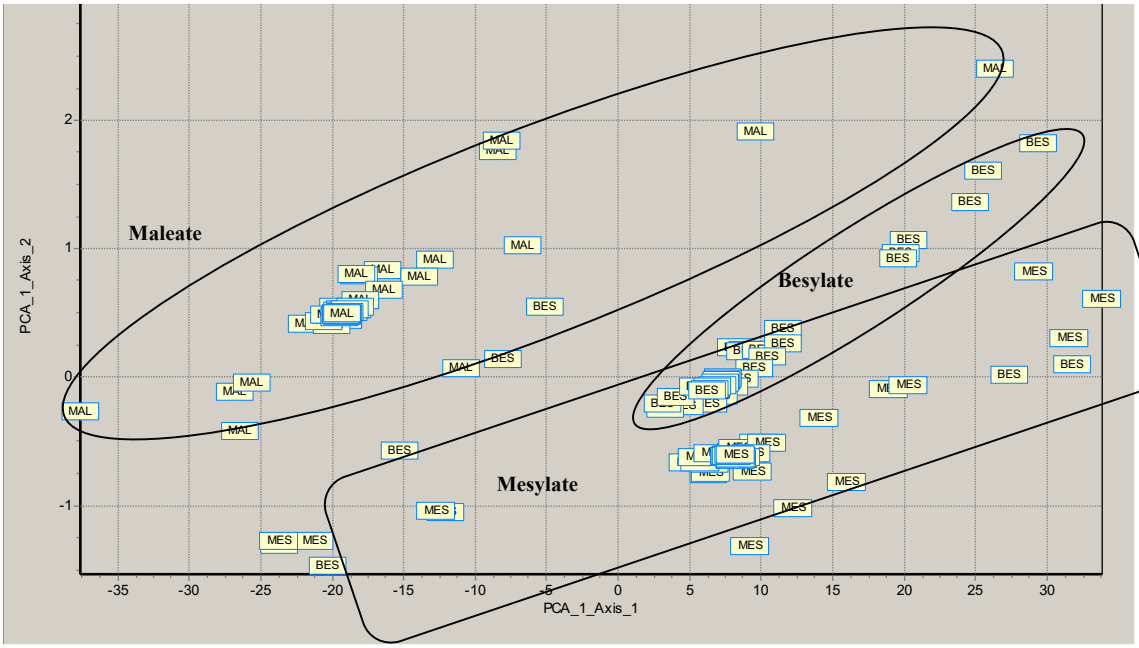


Fig.8. Principal Component Analysis of the results obtained by SERS analysis of the internal side of amlodipine besylate, amlodipine maleate, amlodipine mesylate tablets. PCA1=99.68%, PCA1+2=99.92%.

For this analysis, PC1 determines 99.68% of variability and PC2 0.24%. Here, amlodipine mesylate and besylate seem to have changed positions, with mesylate appearing to cluster at a lower PC2 value. As before, the model prediction of the principal component analysis model was validated by cross validation leave-one-out processing (Tab.4, 5).

Tab.4 Cross validation of the PCA from Fig.8 on SERS spectroscopy obtained from the internal analyses of the amlodipine salts

Error rate			0.5221			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0.375	0.4	BES	15	4	21	40
MAL	0.35	0.3944	MAL	8	22	10	40
MES	0.35	0.3615	MES	19	9	12	40
			Sum	42	35	43	120

The PCA model for the internal analyses performed by SERS gave a total error rate of 52%, with the error rates similar for the individual class of sample. From the confusion matrix it possible to observe that 22 of the 40 samples of maleate salts were correctly classified. This is accordance with the PCA (Fig.8) that shows the cluster for the maleate well separated from the others, even if with the individual maleate analyses resulted to be spread within the group. Besylate and mesylate data resulted to be less well classified, confirming the overlapping of their two clusters visible on the PCA scatterplot

Tab.5. Leave-one-out validation of the PCA from Fig.8 on SERS spectroscopy obtained from the internal analyses of the amlodipine salts

Error rate			0.5117			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0.375	0.455	BES	14	9	17	40
MAL	0.25	0.29	MAL	8	23	9	40
MES	0.38	0.4116	MES	6	20	14	40
			Sum	28	52	40	120

The total error rate of the predictive model estimated by leave-one-out validation accounted for 51%. Besylate and mesylate salts presented again the highest error rate, (Tab.5) in line with the correspondent cross validation and the visual representation on

the PCA scatterplot. From the confusion matrix, it is possible to notice that for all the classes of samples, the classification reflects the one obtained by cross validation.
The similarity in chemical structure of the amlodipine salts and the bulk agents used to manufacture the tablets (see list of excipients in chapter 2) could justify the results obtained, finding, on average, half of the samples correctly classified.

PCA of the conventional Raman data obtained by analysis of the external side of the tablets show clear distribution profiles (Fig.9). Principal component PC1 accounted for 99.37% of variation, with PC2 being responsible for 0.59%.

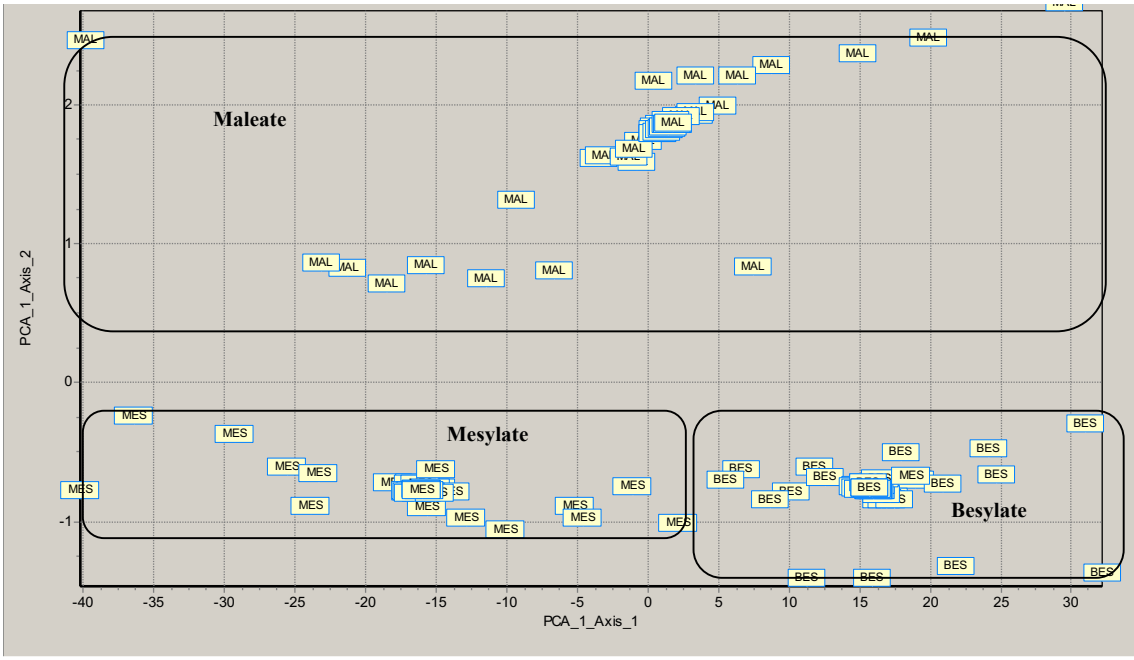


Fig.9. Principal Component Analysis of the results obtained by Raman analysis of the external side of amlodipine besylate, amlodipine maleate, amlodipine mesylate tablets. PCA1 explains 99.37% of the variability and the difference between PCA1 and PCA2 is 99.96%.

As before, the model prediction of the principal component analysis model was validated by cross validation leave-one-out processing. In Tab.6 and 7 the two prediction model validation are illustrated.

Tab.6. Cross validation of the PCA from Fig.9 on conventional Raman spectroscopy obtained from the surface analyses of the amlodipine salts

Error rate			0.3082			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0.175	0.249	BES	33	0	7	40
MAL	0.21	0.3112	MAL	2	34	4	40
MES	0.18	0.3011	MES	7	1	32	40
			Sum	42	35	43	120

The cross validation reported in Tab.5 shown a total error rate of the predictive model evaluated accounting for around 31%. The three salts presented a similar error, data which is supported visually by the PCA (Fig.9) that shows the three classes of samples grouped in separated but spread clusters. From the confusion matrix, it is visible that out of 40 specimens per each salts analysed, besylate gave 33 samples correctly classified, maleate and mesylate salts gave 34 and 32 samples correctly classified, respectively. Apart from the comparable chemical composition already mentioned, the similarity in classification obtained by the cross validation for this dataset could be explained taking into consideration that conventional Raman analysis was maybe not powerful enough, or less powerful with respect the correspondent SERS analysis, in its ability to identify minor details for the differentiation of the three salts.

Tab.7. Leave-one-out validation of the PCA from Fig.9 on conventional Raman spectroscopy obtained from the surface analyses of the amlodipine salts

Error rate			0.2945			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0.16	0.238	BES	32	0	8	40
MAL	0.24	0.3056	MAL	1	33	6	40
MES	0.19	0.32977	MES	8	1	31	40
			Sum	41	34	45	120

The predictive accuracy of the model tested via leave-one-out validation gave a cumulative error rate accounted around 29% with results in agreement with the ones obtained from the correspondent cross validation (Tab.7). The validations of this predictive model supported the visual representation data in the spreadsheet, with good rate of classification per class of samples analysed.

PCA results generated by normal Raman analysis for the internal side of the tablets also shows distribution profiles for each salt (Fig.10). With this investigation, the majority of the data points have clustered together more tightly in separate salt groupings with respected the previous analyses.

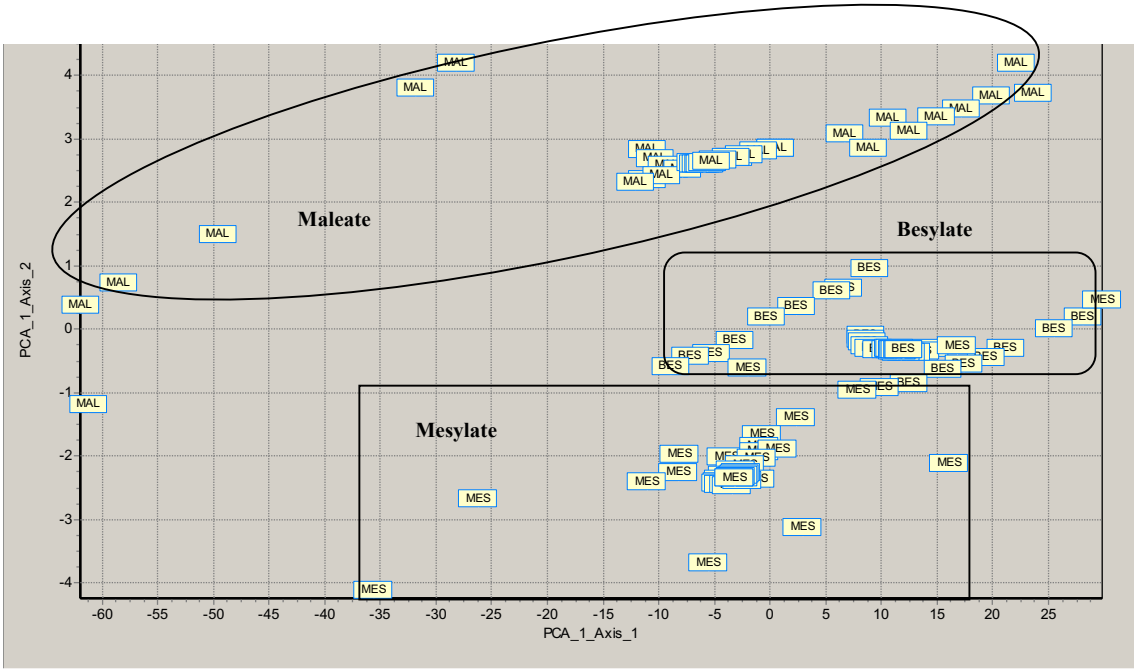


Fig.10. Principal Component Analysis of the results obtained by Raman analysis of the internal side of amlodipine besylate, amlodipine maleate, amlodipine mesylate tablets. PCA1 explains 98.04% of the variability and the difference between PCA1 and PCA2 is 99.82%

For this analysis, PC1 determined 98.04% of variability and PC2 1.78%. Amlodipine maleate still appears to favour a higher PC2 value, however this salt has a main cluster with several outlying points. The besylate salt has formed the tightest cluster of the three, and sits around a central 0 value for both components, with mesylate generating negative PC1 and PC2 scores as in the previous PCA.

Validation of this predictive model is shown in Tab.8 and 9, as seen before.

Tab.8. Cross validation of the PCA from Fig.10 on normal Raman spectroscopy obtained from the internal analyses of the amlodipine salts

Error rate			0.46872			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0.175	0.2711	BES	27	5	8	40
MAL	0.325	0.3292	MAL	11	19	10	40
MES	0.125	0.2744	MES	7	9	24	40
			Sum	45	33	42	120

In Tab.8 the relative cross validation is reported. The predictive model is associated to an amount of error of around 47%. Besylate and mesylate salts present a similar individual error rate, with 27 and 24 out of 40 besylate samples correctly classified, respectively. This data reflects the PCA correspondent, with visual grouping of the three class of samples. Maleate salts resulted to be more spread around the belonging cluster, this justifies the 19 maleate samples classified correctly shown in the confusion matrix.

Tab.9. Leave-one-out validation of the PCA from Fig.10 on normal Raman spectroscopy obtained from the internal analyses of the amlodipine salts

Error rate			0.4312			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0.18	0.14	BES	29	6	5	40
MAL	0.35	0.3771	MAL	11	16	13	40
MES	0.21	0.201	MES	8	7	25	40
			Sum	48	29	43	120

The predictive accuracy of the model tested via leave-one-out validation (Tab.9) gave an error rate around 43%, with the smallest error rate for the besylate group. This reflects the visual representation on the PCA scatterplot where besylate cluster formed

the tightest group. Furthermore, besylate cluster presents 29 of the 40 samples correctly classified. Maleate samples were more spread out in their own group, this is consistent with the confusion matrix data where only 16 samples result to be correctly classified. Mesylate samples present as well a consistent match between the validation data, with 25 samples out of 40 correctly classified, and the visual representation on the PCA scatterplot.

Factor loading was also employed, and demonstrated a very similar picture to that of the PCA. Each wavenumber gave a factor value of 0.80 or above, with most giving a value superseding 0.95. This suggests that each wavelength, and therefore each PC, is accountable for the majority of variability in the data.

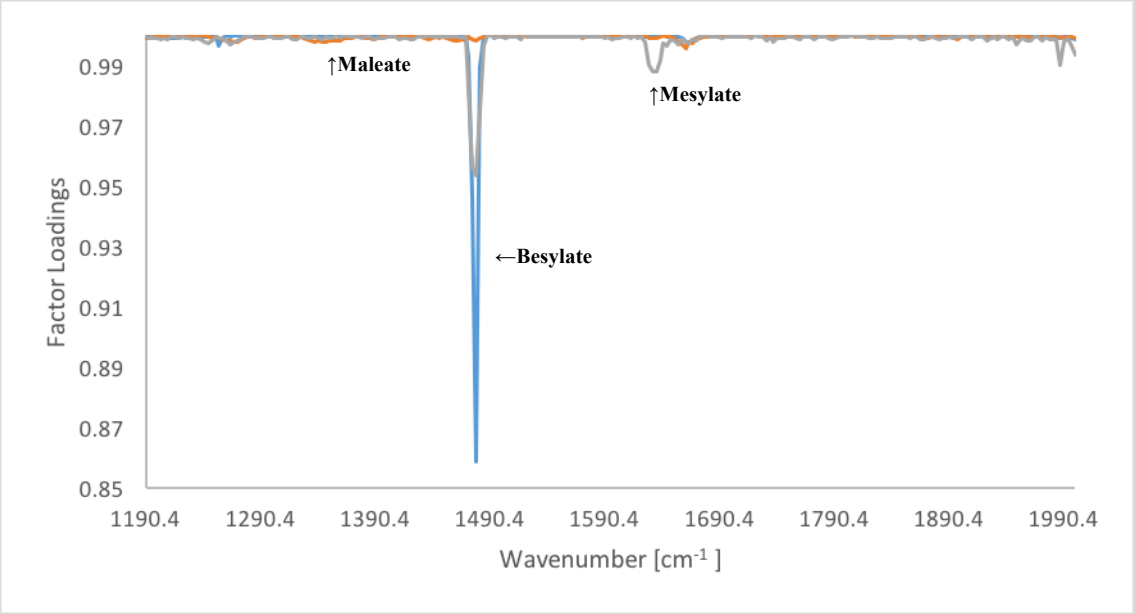


Fig.11. Factor Loadings of the three amlodipine salts versus attributed wavenumbers in Raman analysis of external area of samples. Amlodipine besylate (blue line), maleate (orange line), mesylate (grey line)

The factor loadings calculated against PC1 were then plotted against the correspondent wavenumbers in an attempt to identify a significant signal or signals that could be utilised for distinct characterisation between the amlodipine salts All the wavenumbers resulted to present a factor loading close to one with the exception of sharp peaks at 1480cm^{-1} for the external and internal and in the region around 1890cm^{-1} for the internal (Fig.11, 12).

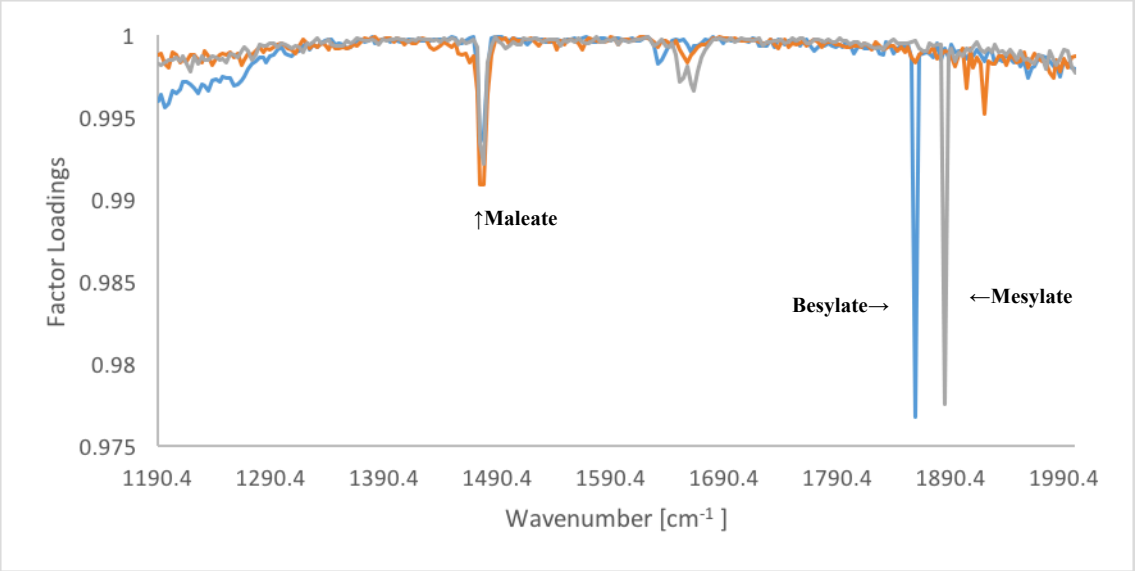


Fig.12. Factor Loadings of the three amlodipine salts versus attributed wavenumbers in Raman analysis of internal area of samples. Amlodipine besylate (blue line), maleate (orange line), mesylate (grey line)

Two peaks identify sources of potential separation power. Wavenumbers between 1465-1488cm^{-1} overlap for the three salts, however, and were discredited as possibilities. Amlodipine besylate shows a peak around wavenumber 1635cm^{-1} which could be used to separate it from the other two salts.

5.3.2. FT-IR Spectroscopy of Amlodipine Salts

The FT-IR spectra of the three Amlodipine salts have been overlaid in Fig.13 for the analysis of the external part of the samples and Fig.14 for the analysis of the internal sides. The spectra have been represented off-set on the figures in order to facilitate the band identifications.

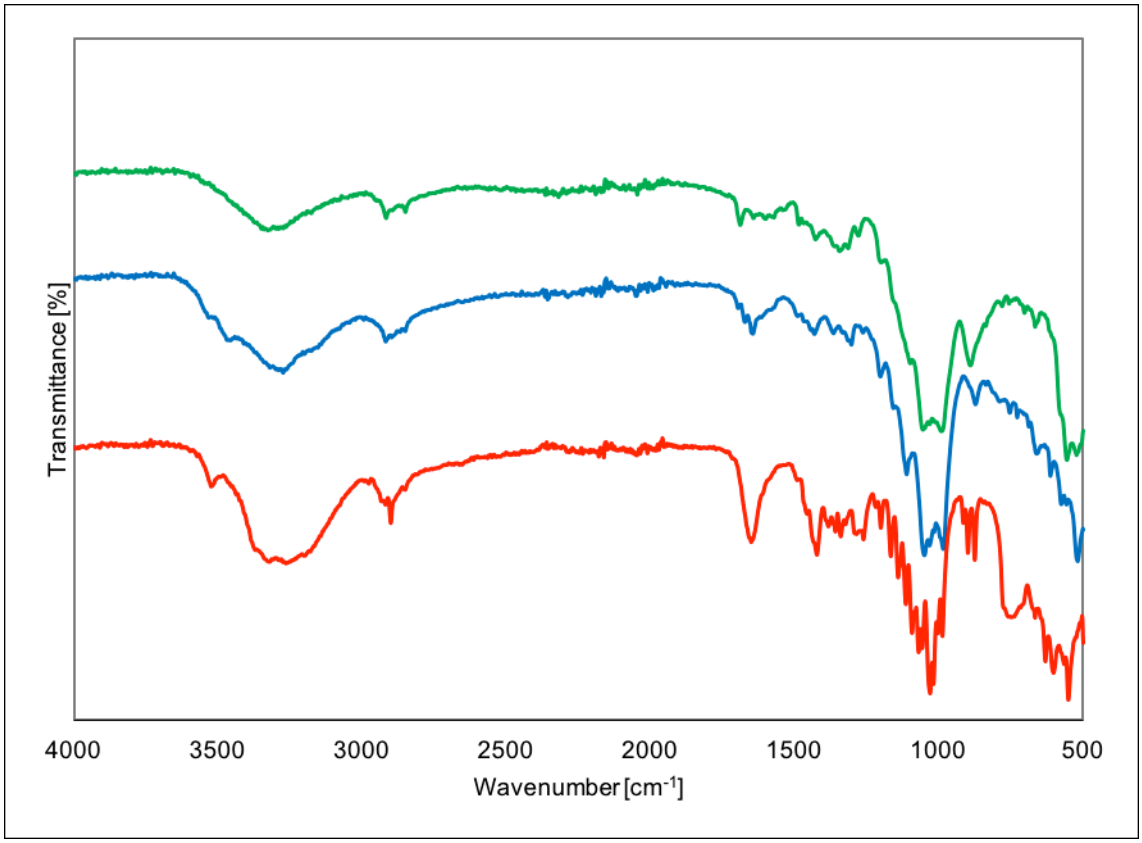


Fig.13. FT-IR analysis of tablets of amlodipine besylate (blue line), amlodipine maleate (red line), amlodipine mesylate (green line), external sides (spectra are shown offset in order to visualise the differences). At 3300cm^{-1} broad peaks due to the symmetric stretching of N-H and OH groups; at 2900cm^{-1} peak due to C-H stretching; at 1650cm^{-1} peaks due to carboxyl group stretching; at 1450cm^{-1} peak due asymmetrical in plane bending of CH_2 groups; at 1380cm^{-1} there is a peak descriptive of methyl CH_3 bending; at 900-1100cm^{-1} symmetrical and asymmetrical stretching of the ethyl group

Each spectrum contains structurally characteristic peaks which can be associated to certain parts of the Amlodipine molecule. The FT-IR analysis of both part of the specimens seemed to provide same peak location.

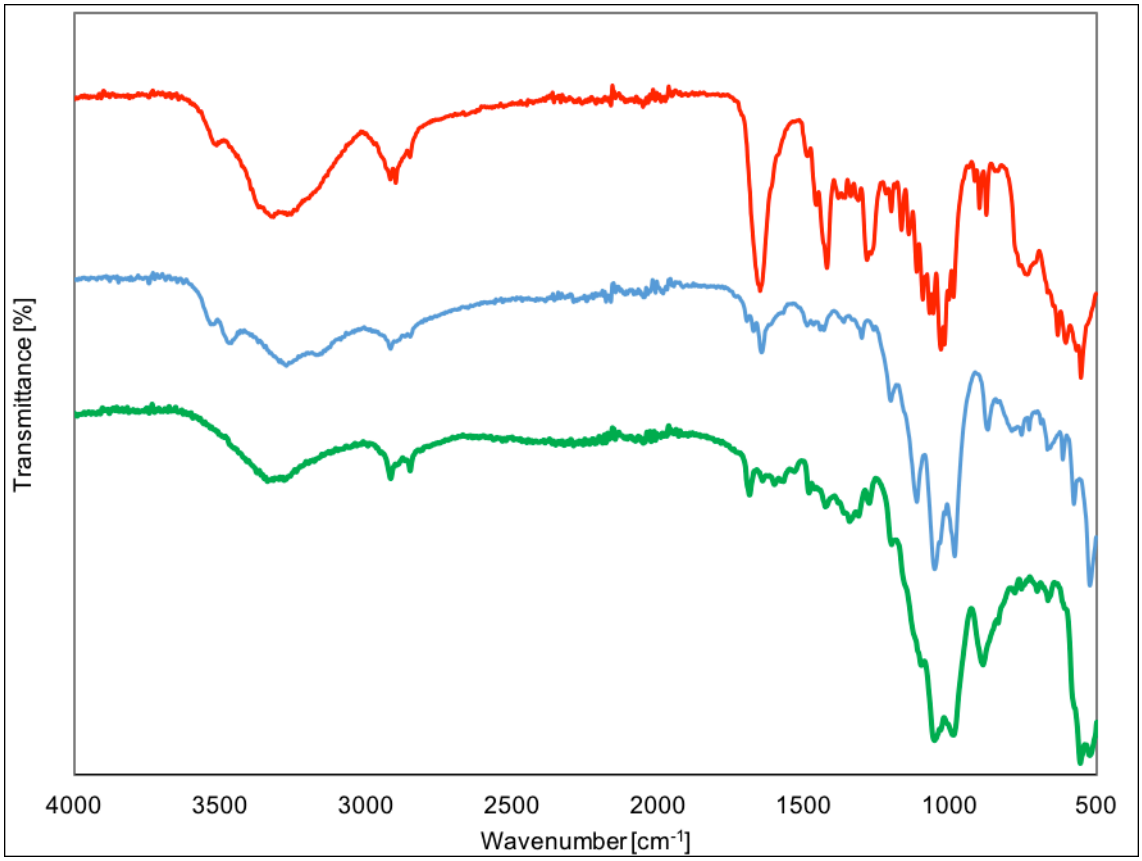
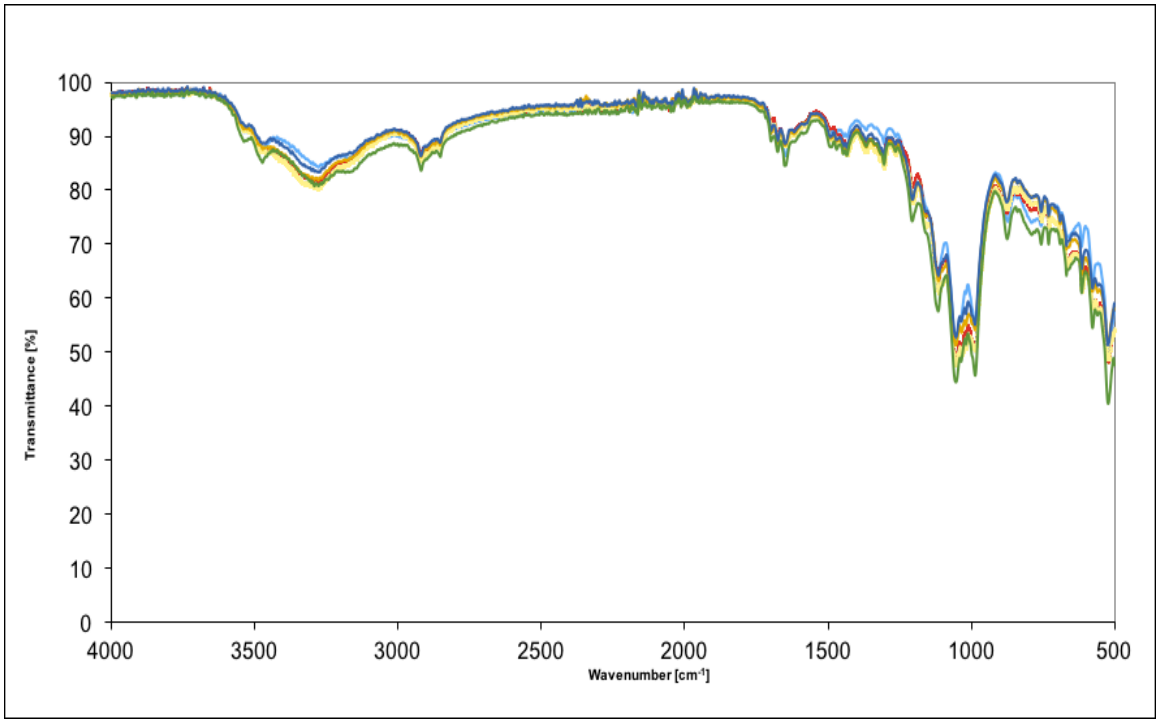


Fig.14. FT-IR analysis of tablets of amlodipine besylate (blue line), amlodipine maleate (purple line), amlodipine mesylate (yellow line), internal sides (spectra are shown offset in order to visualise the differences). At 3300cm^{-1} broad peaks due to the symmetric stretching of N-H and OH groups; at 2900cm^{-1} peak due to C-H stretching; at 1650cm^{-1} peaks due to carboxyl group stretching; at 1450cm^{-1} peak due asymmetrical in plane bending of CH_2 groups; at 1380cm^{-1} there is a peak descriptive of methyl CH_3 bending; between 1300cm^{-1} a strong peak for the ether group attached to the primary amine chain; at 900-1100cm^{-1} symmetrical and asymmetrical stretching of the ethyl group



In Tab.10 the tentative band assignment of the peaks obtained from FT-IR analysis is reported.

Tab.10. Tentative band assignment of FT-IR spectra of different types of amlodipine tablets

Wavenumber [cm^{-1}]	Functional group
3520	OH stretching
3300	NH asym stretching
3100	CH asym stretching
2900	CH stretching
1740	Ester CO stretching
1650	C=C aromatic stretching
1450	CH_2 bending
1380	CH_3 bending
1300	C-C alicyclic stretchng
1300-1000	COC stretchng
1100	Ethyl asym stretching
900	Ethyl stretching
558	CCl stretching

Starting at 3520cm^{-1}, peaks are observed for all three salts as a result of the hydroxyl O-H stretching. At 3300cm^{-1} broad peaks are generated due to the asymmetric stretching of N-H groups, as seen by Szabó *et al* [36]. Between 2900 and 3100cm^{-1} a small but sharp dual set of peaks are seen for each salt. The first at 3100cm^{-1} is accounted for by asymmetric C-H stretching and the latter at 2900cm^{-1} by standard C-H stretching. Within the recognised 'fingerprint region' of the spectra, at 1740cm^{-1} a small but sharp peak is recognised as a C=O stretch of an ester group. At 1650cm^{-1} a peak characteristic for aromatic group stretching is noted. In the region of 1450cm^{-1} peaks associated with asymmetrical in plane bending of CH_2 groups are present. At 1380cm^{-1} there is a peak descriptive of methyl CH_3 bending. At 1300cm^{-1} the stretching vibration of C-C alicyclic and aliphatic chains are generated. Between 1300-1000cm^{-1} there is a strong peak for the ether group attached to the primary amine chain. The symmetrical and asymmetrical stretching of the ethyl group is found at 900-1100cm^{-1} [37]. The C-Cl in the cationic moiety is responsible for the peak at 558cm^{-1}. The similarities in peaks mentioned here can be associated to the amlodipine core. Variances in salt type and excipients amid all three tablet types would therefore be expected to generate differences.

In addition to these FT-IR peaks, there are points of interest where differences between the salt forms are noted. In the amlodipine maleate spectrum, it is noted that the transmittance for the peak at 3540cm^{-1} is higher, due to the presence of extra O-H moieties as part of the carboxylic group in its structure. Again, at the peak at 1650cm^{-1}, the maleate salt has a greater transmittance than the other salts, and the peak itself is sharper due to the extra carbonyl groups attached to its form. Finally, the asymmetric C-H stretching of the benzene sulphonate ring of the besylate salt is slightly visible at around 3100cm^{-1}.

PCA of the FT-IR data obtained by analysis of the external side of the tablets show clear distribution profiles (Fig.13). Principal component (PC)1 accounted for 97.73% of variation, with PC2 being responsible for an additional 1.28%.

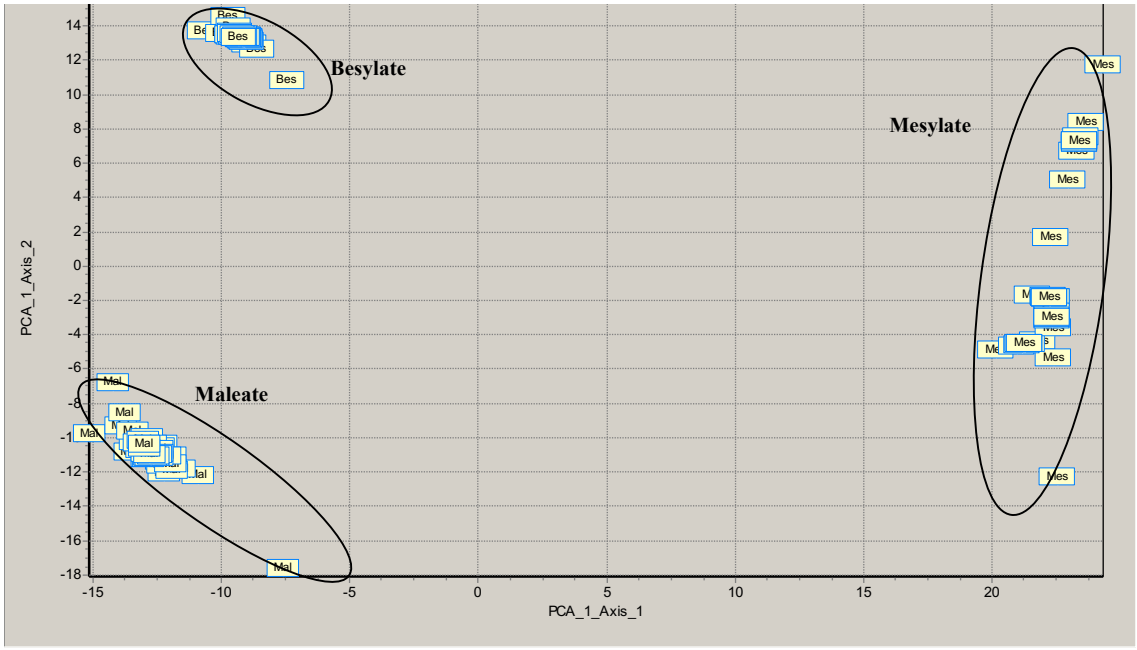


Fig.15. Principal Component Analysis of the results obtained by FT-IR analysis of the external side of amlodipine besylate, amlodipine maleate, amlodipine mesylate tablets. PCA1= 97.73%, PCA2=99.01%

The visual interpretation of the PCA scatterplot, shows how the three classes of samples resulted to be separated each others and tightly clustered, especially the besylate and maleate groups. Validation of this predictive model is shown in Tab.11 and 12, as seen before with cross validation and leave-one-out, respectively.

The leave-one-out validation (Tab.12) was in accordance with the results produced by the cross validation, showing the same trend confirming the good level of separation of the dataset.

Tab.11. Cross validation of the PCA from Fig.13 on FT-IR spectroscopy obtained from the surface analyses of the amlodipine salts

Error rate			0			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0	0	BES	40	0	0	40
MAL	0.01	0	MAL	0	40	0	40
MES	0.02	0	MES	0	0	40	40
			Sum	40	40	40	120

In Tab.11. the relative cross validation of the PCA scatterplot of Fig.13 is reported. The predictive model is associated to an amount of error of 0% with all the three data set points correctly classified.

Tab.12. Leave-one-out validation of the PCA from Fig.13 on FT-IR spectroscopy obtained from the surface analyses of the amlodipine salts

Error rate			0			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0	0	BES	40	0	0	40
MAL	0	0	MAL	0	40	0	40
MES	0.03	0	MES	0	0	40	40
			Sum	40	40	40	120

The leave-one-out validation (Tab.12) was in accordance with the results produced by the cross validation, showing the same trend confirming the good level of separation of the dataset.

PCA results for the internal side of the tablets by FT-IR analysis also shows distribution profiles for each salt, although without the high level of clarity (Fig.16).

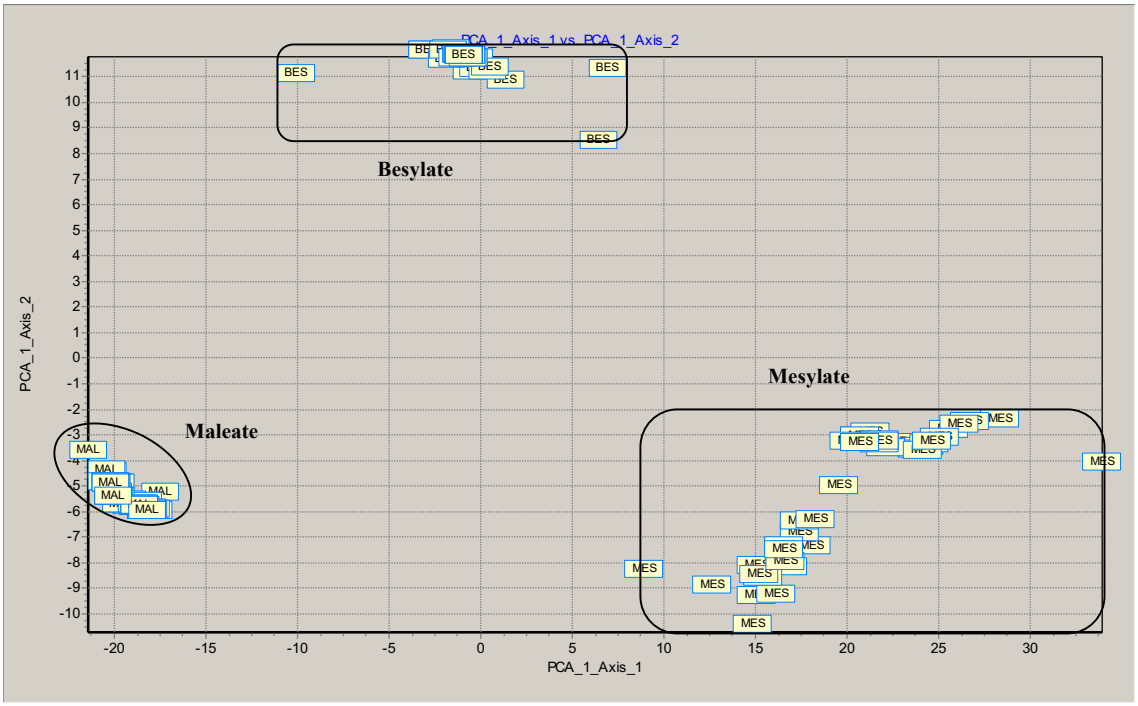


Fig.16. Principal Component Analysis of the results obtained by FT-IR analysis of the internal side of amlodipine besylate, amlodipine maleate, amlodipine mesylate tablets. PCA1= 97.11%, PCA2=98.77%

For this analysis, PC1 determined 97.11% of variability and PC2 a further 1.66%. The three different classes of samples resulted to be well separated each other and clustered together, better for the maleate specimens and less for the mesylate.

Tab.13. Cross validation of the PCA from Fig.16 on FT-IR spectroscopy obtained from the internal analyses of the amlodipine salts

Error rate			0			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0	0	BES	40	0	0	40
MAL	0	0	MAL	0	40	0	40
MES	0.02	0	MES	0	0	40	40
			Sum	40	40	40	120

The cross validation reported in Tab.13 shown a total error rate of the predictive model evaluated accounting for 0%, with all the dataset points correctly separated.

Tab.14. Leave-one-out validation of the PCA from Fig 16 on FT-IR spectroscopy obtained from the internal analyses of the amlodipine salts

Error rate			0			
Values prediction			Confusion matrix			
Value	Recall	1-Precision		BES	MAL	MES	Sum
BES	0	0	BES	40	0	0	40
MAL	0	0	MAL	0	40	0	40
MES	0	0	MES	0	0	40	40
			Sum	40	40	40	120

The leave-one-out validation (Tab.14) of the predictive model, presents similar results obtained from the correspondent cross validation, confirming the good percentage of data classification.

Factor loading was utilised for FT-IR statistical interpretation and, similarly to the Raman data, demonstrated a similar result to the PCA (Fig.17, 18).

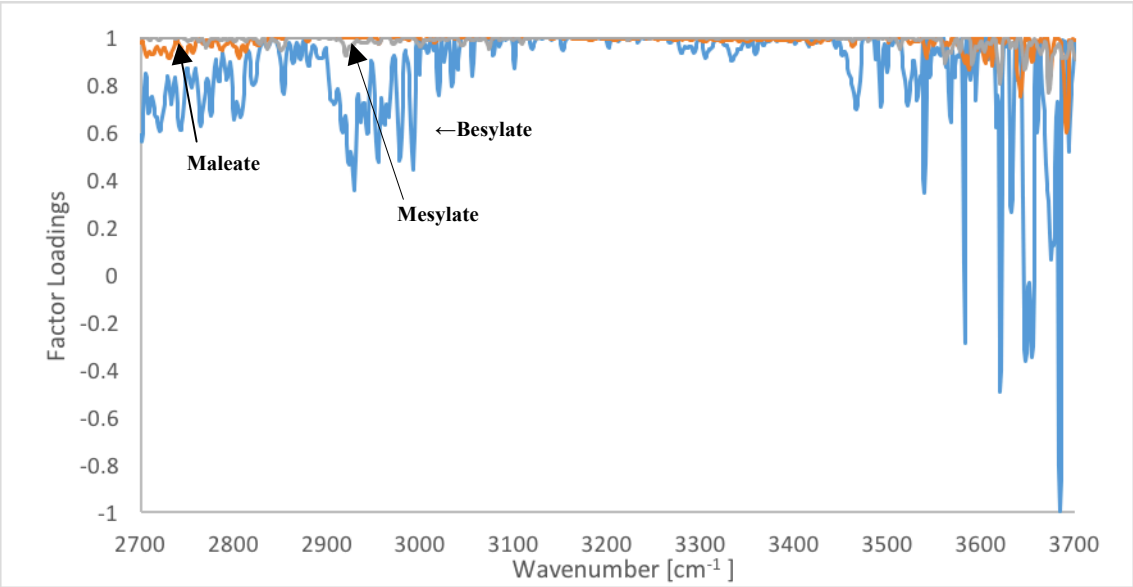


Fig.17. Amlodipine Salts Factor Loading vs 3700-2700cm^{-1}. Amlodipine besylate (blue line), maleate (brown line), mesylate (grey line).

A large portion of the wavenumbers studied gave a factor value of 0.80 or above. The factor loadings were again plotted against the correspondent wavenumbers in an attempt to identify a significant signal or signals that could be utilised for distinct characterisation between the amlodipine salts The large amount of data are reported in two different Figures (Fig.17, 18) for clarity.

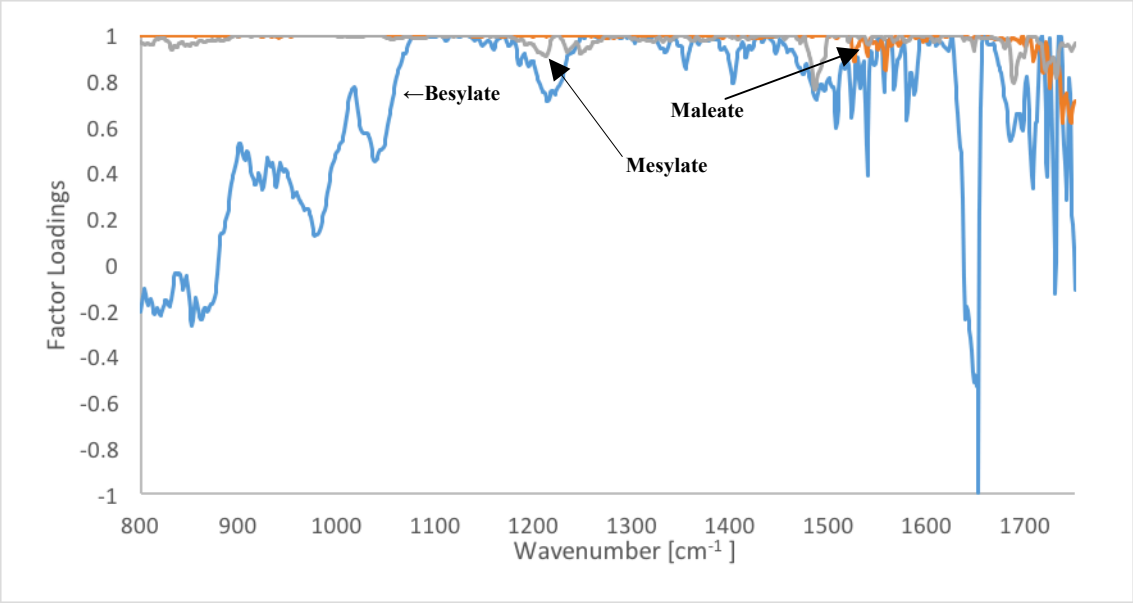


Fig.18. Amlodipine Salts Factor Loading vs 1750-800cm^{-1}. Amlodipine besylate (blue line), maleate (brown line), mesylate (grey line).

FT-IR plots generate surplus peaks which could be used to potentially separate the three salts. These wavenumbers, however, are all within areas of a spectrum where there are broad peaks, resulting in any small differences between the salts spectra being insignificant. Subsequently, separation of the three Amlodipine salts would be entirely using PCA clustering.

5.4. Discussion

5.4.1. Raman spectroscopy of Amlodipine.

The analysis of the three different amlodipine salts does not give a remarkably distinctive spectrum when studied via conventional Raman spectroscopy, both internally and externally. Using SERS, however, it was possible to observe characteristic peaks of the main amlodipine molecule.

Amlodipine anionic moiety presents an abundance of electronic clouds from nucleophilic groups such as primary amine, two different ester groups, ether group and aromatic structure able to link with the silver colloid and enhance the correspondent signal on the spectrum. Equally, the cationic counterparts of the amlodipine salts are characterised by electron donating groups.

Although an amlodipine standard was not used in this study, previously reported spectra compared to those achieved here demonstrate that there are variances that can be attributed to the excipients added to the tablets and the differences in the salt forms [28]. Results from the internal and external analyses are very similar although some differences in quality and resolution could be due to a possible coating effect on the tablets. Han & Faulkner noted that, using spectroscopic techniques, the external surface of the tablets appeared to accrue the signal from the API in the tablet core, and discussed what implications this effect could have on the measurement of API content between batches of tablets [38]. SERS analyses of the external side seem to show the same peaks but more pronounced than the internal area. This could be due to how the tablets are compressed in manufacture, effectively giving a higher concentration at the external surface compared to that of the internal examination [38].

Although the main amlodipine core gives a representative spectrum, the three salts do not appear at first glance to produce any evident differences in their spectra. This was a hypothesised result as it was realised that the chosen techniques needed to be powerful and sensitive enough to detect differences in salt forms despite the low dose of API in the tablet, the high excipient content, the near complete similarities in salt forms and possibility of a non-homogenous solid-state medication. The table reported in chapter 2, with the list of excipients present in the formulation of the tablets, shows as for all the three different classes of amlodipine investigated, cellulose and starch represent the main ingredients.

An explanation for the lack in differences could be provided from the additional chemical structure of the three salts to the main molecule. The differences in the structures of the three salts themselves are not eminent enough to separate, for example; the besylate and the mesylate salts both contain SO_3 groups. In the besylate form, the sulfonate is linked to an aromatic ring, while in the mesylate it is linked to a methyl group. The Raman signal of these sulfonic groups are not distinguished enough to separate the two different salts [36]. Furthermore, the maleate salt presents conjugated carboxyl groups which absorb in the same region of the aromatic ring, making difficult its traceability [37].

Despite the great similarities between the spectra of the three salts the application of chemometric methods, namely PCA and factor loading analysis, allowed

for the minor differences to be exploited. The PCA algorithm used within the Tanagra software generated score plots which meant visual clustering of the different amlodipine forms could be observed. Throughout PCA analysis of both Raman and SERS data, amlodipine besylate separated and clustered more tightly than the other salts. In addition to this, it was seen that the best separation power occurred from the data collected by analysis of the external side of the tablets.

There are many advantages of SERS over normal Raman, not least being its enhanced sensitivity and therefore selectivity, which would lead one to hypothesise that SERS data would allow for better discriminatory abilities [41, 42]. Interestingly, however, the PCA demonstrates that using data from standard Raman analysis provides this superior separation. One reason for this could be the intensity of background signal and noise peaks from contamination with regards to the bulking agents and excipients present in the tablets or to different polymorphs presenting different analytical response. Some studies have reported that compared to normal Raman, SERS actually considerably increases background noise compared with the transmittance from the analyte signal peaks [43]. If this is the case here, the explanation for this aforementioned phenomenon could be that the SERS analysis is actually too sensitive for this analysis and Raman is a more suited technique. This would, however, need to be investigated in much more depth.

Predictive accuracy of the model validated with both cross validation and leave-one-out gave a positive report on the accuracy of the prediction models with acceptable amount of error. The total error rates for all the PCAs performed on the Raman spectroscopies performed were around or below 50%, both from cross validation and from leave-one-out evaluation. The prediction model that was associated with the best validation was the one obtained from SERS investigation of the external parts of the tablets, with error rate ranging around 25%.

5.4.2. FT-IR spectroscopy of Amlodipine.

Analysis of the Amlodipine salts by FT-IR, similarly to Raman and SERS, does not produce three vastly unique spectra. The backbone of amlodipine generates several main peaks of interest which can be linked back to its structure, but there are limited

regions that can be associated with the individual salt forms. This could be because the core of the molecules is the same, or that the excipients within the tablets are covering any of the more noticeable differences, or perhaps the addition of the salt moiety simply adds to a stronger transmittance of already existing peaks. The high concentration of hydroxyl functional groups presents in these two sugar could, in principle, create a covering effect of the signals originated from the amlodipine molecules. However, despite the excess of signals originated from the excipients, the amlodipine salts shown to be detectable from FT-IR technique, revealing the proper distinctive peaks.

Prior to statistical interpretation, each spectrum was processed to attempt unambiguous identification of the salts by removing the main peaks concomitant with the Amlodipine backbone. These residual peaks would, in theory, match those of the salt molecule but this was unsuccessful as so similar. Lactose is one of the main bulking agents in all of the purchased tablets, which could be masking components of the API due to its broad sugar associated peaks. This is evidenced by the stretching C-O-C and C-OH peaks at 1100cm^{-1} from the sugar ring of the lactose [39]. Additionally, there have been reports that mention drug-excipient interactions which could suggest an alternative to the masking effect [40].

The differences that can be established include the stretching of the carboxylic groups at 1650cm^{-1} in the maleate spectra, which can be associated to the additional carbonyl groups attached to the structure of the salt. Also in the spectra of the maleate salt, there are three peaks in the internal analysis at 1660cm^{-1}, 1430cm^{-1} and 1290cm^{-1} which are more prominent than in the mesylate and besylate salts. These can be attributed to stretching of C=O and C=C bonding. In the 1500cm^{-1} region of the besylate salt there is evidence of N-H stretching, as previously reported by Koradia *et al* [44]. Assumptions may be made that these discrepancies between the spectra, which allow for differentiation, will all be located in the molecular fingerprint region [45]. Although there are these small differences in this area, in this case we are also presented with some minor variances in the left side of the spectra. In data for all three salts there is a major peak at 3520cm^{-1} due to the stretching of O-H bonding. Amlodipine maleate, however, has noticeably better transmittance for this peak due to the presence of an extra O-H moiety within the carboxylic group in its structure. In addition, there is a

visible presence of asymmetric C-H stretching at 3100cm^{-1} due to the benzene sulphonate ring of the besylate moiety.

Supporting the results of the Raman and SERS chemometric analysis, PCA of the FT-IR data also shows how the three salts can be successfully separated. The multivariate analysis here presented good discrimination power, although less than Raman and SERS, but some loss as error can be associated to the physical similarities in the salt compounds, the low dosage of API and the high proportion of excipients.

Some will argue that Raman is better and a more useful technique than FT-IR in this situation as the spectra here are so similar. Raman peaks are mostly sharper than those seen in FT-IR analysis and therefore makes it easier to spot spectral differences [26]. It has been reported that in pharmaceutical analysis, in many occasions it is not feasible to identify and characterise entire formulations using a single spectroscopic technique [46]. Clarke *et al*. combined Raman and IR spectroscopies in thoroughly determining the heterogeneous make up of solid-state dosage forms [47]. While Raman directly targets each part of the molecular structure of a given compound, IR presents the analyst with a subtler approach to the existing combinations of molecular functional groups and bonding patterns. Taylor & Langkilde support this notion by explaining that vibrations producing strong IR absorptions typically produce faint Raman signals, and vice versa [26]. The correlations that present themselves between the salt spectra and the calculated factor loadings are more difficult to establish in the FT-IR data due to the broad bands present in the spectra. As a result of the peaks encompassing a wider range of wavenumbers, it leads the factor loading algorithm to believe that more wavelengths are of necessity than in the Raman analysis. There is no clear spectroscopic evidence in the factor loadings for any salt. This is not surprising given the broad peaks, which make it problematic to detect a loading that contains a signature for one of the salt forms without spectral interferences from the excess material within the tablets. Previously single intensities and integrated areas from SERS data sets have been plotted against analyte or API concentrations to produce a univariate calibration [48, 49].

The validations of the chemometric model gave error rate around 20% for the analyses conducted on the external area of the specimens, and around 30% for the ones conducted on the internal areas of the tablets, with consequently good separation

amongst the different classes of samples, with besylate samples presented the best rate of correct classification.

5.5. Conclusions

It is inferred from the analyses that there are enough spectral differences, when coupled with chemometric methods, to successfully separate the three salts. Despite the minimal samples used and similarity in chemical structure, separation of the Amlodipine salts has been statistically proven successful. The proposed method gives a level of sensitivity and selectivity never before achieved with alternative techniques, as well as being fast and requiring minimal sample preparation. This study presents a method that could have large applications in the pharmaceutical industry, distinguishing between three salt forms of a pharmacologically active compound, for which some argue can produce unrequited side effects.

The combination of the two analytical techniques employed were successful in separating the besylate, maleate and besylate salts. Each technique provided peaks indicative of the Amlodipine backbone, as well as minor differences for each of the salt forms. These were extrapolated further with the use of PCA which allowed separation to be visually presented by clustering groups. Raman generated the best statistical separation of the salts with PC1 and PC2 accounting for an accumulative 99.96% of all variability within the data set. Cross validation and leave-one-out validation technique proved that the predictive models produced were reliable, allowing separation amongst the classes of samples studied.

The non-destructive nature of the technique, plus the minimal sample preparation and speed of analysis presents this as a very attractive choice for this type of investigation. Despite this, the FT-IR clusters visually appear to separate with the most clarity.

This is the first report to successfully identify and characterise amlodipine besylate, maleate and mesylate by Raman, SERS and FT-IR spectroscopy.

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An Investigation of different Esomeprazole and Omeprazole generic medications obtained from traditional and internet market using FT-IR, Raman, NMR spectroscopies and Chemometric Analysis

6.1 Introduction

Proton pump inhibitors (PPIs) are a family of medications that block or diminish the production of gastric acid acting on the proton pump present in the parietal cells of the stomach [1]. Omeprazole (Fig.1) and esomeprazole belong to such a family, being the first a racemic mixture and the second the related levo-enantiomer [2]. In the intracellular canaliculi of the parietal cell, particularly acidic, omeprazole is concentrated and converted to the active form, being a weak base, and interacts with H^+-K^+-ATPase, the proton pump, reversibly reducing or blocking its activity [3]. This influences the final step of the gastric acid secretion process and act irrespectively both in the basal acid secretion and in the one instigated by stimulus. PPIs are quickly active and with a unique daily dose can maintain control of the inhibition of the gastric acid secretion [2].

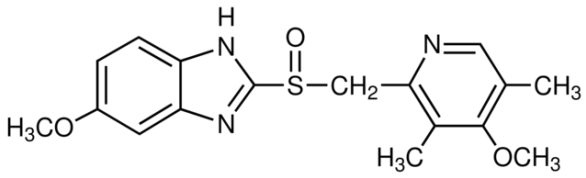

Fig.1 Omeprazole, chemical structure.

Because the reflux of the acidic gastric content into the esophagus plays a major role in the pathogenesis of symptoms of GERD (gastro-esophageal reflux disease) and lesions of erosive esophagitis, acid suppression with a PPI is currently a mainstay of anti-reflux therapy [3,4]. There is a strong correlation between the degree of acid suppression provided by a given drug and its efficacy. The superiority of PPIs over other drugs (antacids, prokinetics and H_2-receptor antagonists) has now been established beyond doubt, both for short- and long-term treatment [5]. Nevertheless, patient with erosive esophagitis are more reactive to PPIs than those with non-erosive reflux. Sometimes the action of PPIs in patients with atypical gastric condition is reduced to a reduction of the

symptomatology of heartburn. Different studies conducted on the safety of use of PPIs both in short and long periods have provided reassuring conclusions on their safety [6-9]. PPIs have shown improved healing yield in case of severe erosive esophagitis, with quicker relief of the associated symptomatology [9]. The successful results obtained with the use of PPIs for the treatment of erosive esophagitis is having an important reflection on the prescribing habits of clinicians of primary and secondary care organizations in the United Kingdom [10]. The vast therapeutic success of PPIs has incredibly increased their use both in primary and in secondary care. With the attempt to reduce their financial impact on the health system, generic medications, obtained both from tradition distribution chain and from parallel import, have represented the main type of PPIs used in clinics recently [11, 12]. This is one of the clinical areas previously cited, where there are concerns among health-care professionals and patients that not all generic and brand preparations can be equally clinically effective [6, 7]. Different clinical trials have been performed comparing brand medications versus generics, in order to evaluate any therapeutic differences. In [8, 9] Shimatania et al compared, in prospective, randomized, open-label, crossover studies, the acid-suppressive effect of generic omeprazole [8] and lansoprazole [9] with that of the original brand, measuring the intragastric pH at the regular interval of time, drawing the conclusion that acid-suppressive effects of some brands of generic omeprazole and lansoprazole are not the same as the original ones. Besides these clinical studies, no information has been found in the literature concerning the chemical characterization of PPIs generics, aimed to investigate if different clinical features are associated with specific chemical characteristics. In fact, generic drugs are actually chemically equivalent to their brand-name counterparts or among them just in terms of active ingredients, but they may differ in peripheral features, such as different polymorph forms of the same API, inert binders and fillers, shape, color and the specific manufacturing process, which could lead to diverse clinical responses [13-15]. This scenario highlights the need for a development of an analytical approach capable of discriminating amongst the different PPIs generic medications.

Since the interchangeability of omeprazole medications has been questioned, in this study, it was used Raman and FT-IR spectroscopies to characterize different omeprazole and esomeprazole generic formulation produced by different manufactures and obtained

from traditional pharmacies and from the internet market in order to establish what kind of differences could be found to substantiate any different clinical performances.

6.2 Materials and Methods

6.2.1 Materials

Omeprazole, (5-methyl-2-{[(4-methoxy-3,5-dimethyl-2-pyridinyl) methyl] sulfonyl}-1H-benzimidazole)

In order to characterise omeprazole and esomeprazole generics, seven different PPIs medications have been tested: five of these were 20mg omeprazole (Dexcel and Losec obtained from traditional pharmacies, and GPO, Hycid and Olzep obtained from internet websites) and two were 10mg esomeprazole Rambaxy and Esopral, obtained from traditional pharmacies.

Sixteen tablets of each PPIs were analysed internally and externally by Raman, FT-IR and NMR and the results submitted to chemometric evaluations.

6.2.2. Methods

Spectroscopic analysis was carried out by Raman spectroscopy, surface-enhanced Raman spectroscopy (SERS) and FT-IR. Raman analysis was performed on a FORAM 685-2 instrument with a laser operating at 532nm, to ensure high levels of sensitivity. The Raman instrument was also equipped with an integral video microscope. Analyses were performed between the wavenumbers 800 and 2000cm^{-1}.

A Perkin-Elmer Spectrum 100 was used for the FT-IR analysis. This was supported by a Motorola DSP56303 Digital Signal Processor and a near infrared detector. The FT-IR was equipped with a Perkin-Elmer Autoimage microscope, with a IR performance of 9000:1 p/p signal to noise ratio and a resolution greater than 10μm. The range of wavenumbers investigated was between 4000 and 500cm^{-1}.

Sample Preparation: eight tablets were taken for Raman analysis. To test the external side of the tablets using Raman spectroscopy the tablets were exposed to the laser at five points for each tablet, to achieve 40 repeated representative analyses for each

amlodipine salt. To test the internal side, the tablets were broken in half and again exposed to the laser five times for each tablet.

SERS enhances the sensitivity of standard Raman scattering by depositing a metal colloid on the surface of the sample being analysed. For this part of the investigation, silver colloids were prepared as follows: silver nitrate was reduced using sodium citrate in water, and concentrated by centrifuging at 5000rpm. The eight tablets for SERS analysis were covered with 2µL of the prepared colloid solution and 2µL of NaCl 1M after analysis by normal Raman, and analysed immediately. To test the external side of the tablets using Raman spectroscopy the tablets were coated in the silver colloid preparation and exposed to the laser. This was again repeated five times for each tablet, with the laser directed at a variation of external sites, to achieve the 40 representative analyses for each amlodipine salt. To test the internal side, the tablets were broken in half prior to the addition of the silver colloid, and again exposed to the laser five times for each tablet. Eight tablets were also taken for FT-IR analysis. To study the external side of the tablets by FT-IR the samples were prepared by scratching the surface of the tablets onto the ATR plate with a small spatula. This was done in order to attain adequate contact of the sample on the ATR crystal, so a successful spectrum could be produced. Due to the shape of the tablets this contact would not have been achieved if the tablet was kept intact, and would have resulted in poor transmittance. To study the internal side, the tablets were ground to ensure homogenisation and subjected to the aforementioned procedure. Each tablet was analysed individually using a scan cycle of 40.

^{1}H NMR spectra were obtained from a Bruker Avance 500 at 500.1MHz using TMS as internal standard. Chemical shifts for proton resonances were given inppm (δ). Signal multiplicity was characterized by s (singlet), d (doublet) and dd (double doublet).

NMR spectra were recorded using 128k complex points and a recovery time of 4 seconds 16 transients were generally sufficient to achieve good signal-to-noise.

Principal Component Analysis (PCA), Factor Loadings, Cross Validation, Leave-one-out were performed using Tanagra™ data mining software (University of Lyon, France). Analytical data were exported from the correspondent analytical apparatus to an excel spreadsheet and subsequently uploaded onto Tanagra.

6.3. Results

6.3.1. Raman spectroscopy of omeprazole generic medications

The SERS spectra related to the analysis of the surfaces of the omeprazole tablets investigated are reported in Fig.2. Tablets from all the seven different formulations have been submitted to the preparation for SERS analysis as introduced earlier. The spectra obtained from the SERS analyses of the surfaces showed both similar features and few distinctions for all the classes of samples. Hycid, indicated with the red line and GPO in purple, resulted to be overlapped.

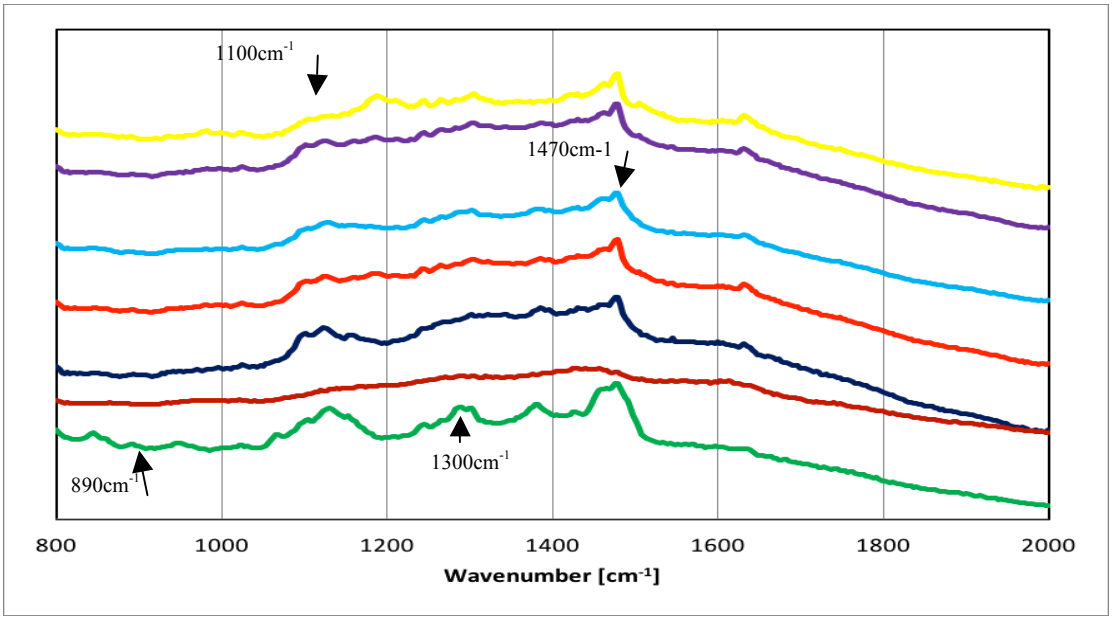

Fig.2. SERS analysis of omeprazole generic medications, external sides. Spectra have been off-set in order to avoid overlapping and facilitate observation. Rambaxy (black), Losec (yellow), Olsep (light blue), Esopral (green), Dexcel (brown). Hycid (red) and GPO (purple). At 890cm^{-1} and 1100cm^{-1} stretching of the ether groups present in the two aromatic rings; 1300cm^{-1} symmetric stretching of the chain vibrations of the two aromatic rings; at 1470cm^{-1} methyl asymmetric bending.

At 890cm^{-1} Esopral presents a more defined peak than the others, possibly related to the stretching of the ether groups present in the two aromatic rings of the omeprazole molecule. In the region around 950cm^{-1} Esopral and Losec produce smooth peaks, while the others do not present any peak, possibly attributable to the stretching of the C-C. In the region around 1100cm^{-1} all formulations produce signals with different intensity which might be assigned to the stretching of the ether groups. Hycid and GPO spectra

looked overall overlapped. In the region around 1260cm^{-1} all samples produce peaks which may be referred to the stretching of the thiocarbonyl group. In the region of 1300cm^{-1} all samples show peaks, possibly attributable to the symmetric stretching of the chain vibrations of the two aromatic rings. At 1380cm^{-1} strong peak for Esopral, small for the others and non for Dexcel possibly due to the symmetric bending of methyl groups. The asymmetric bending of the same groups laid in the region between 1440 and 1500cm^{-1} can explain the signals recorded in the region 1460-1475cm^{-1} for all formulations. At 1500cm^{-1}, Losec presents a peak probably to be referred to the asymmetric bending of the CH_3. At 1632cm^{-1} all samples present peaks with different intensity possibly related to the stretching of the aromatic rings.

To perform the investigation of the internal parts of the samples, the tablets of the seven different formulations have been broken in half manually and then submitted to the preparation for SERS analysis as indicated previously. The spectrum of GPO, shown with purple line, and Hycid, in red line, resulted to be overlapped, as also seen previously in the SERS analyses of the surfaces. Starting at 860cm^{-1}, all formulations present signals (Fig.3), more prominent for Dexcel, GPO and Hycid, which might be referred to the stretching of a ether groups present in the aromatic rings of the omeprazole molecule. At 880cm^{-1} Dexcel, GPO and Hycid present marked signals while the others show all smooth peaks. These signals may be attributable to the stretching of the different C-O-C linked to the other aromatic ring.

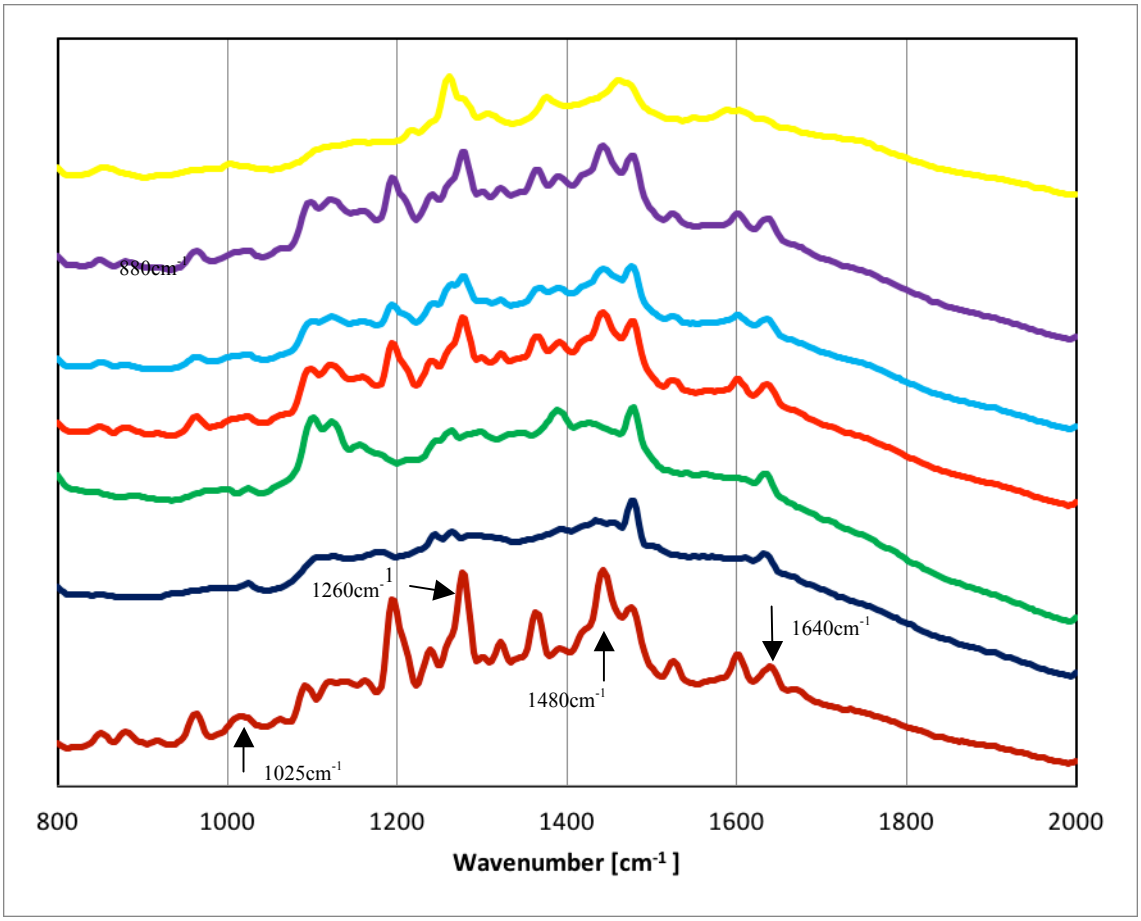

Fig.3. SERS analysis of omeprazole generic medications, internal sides. Spectra have been off-set in order to avoid overlapping and facilitate observation. Rambaxy (black), Losec (yellow), Olsep (light blue), Esopral (green), Dexcel (brown). Hycid (red) and GPO (purple). At 880cm^{-1} peak due to ether groups stretching; at 1025cm^{-1} peaks due to the stretching of the thiocarbonyl group C=S; at 1260cm^{-1} peak due to the stretching of the thiocarbonyl group; at 1480cm^{-1} peaks due to bending asymmetric of the methyl groups; at 1640cm^{-1} due to CN aromatic stretching.

At 970cm^{-1} Dexcel and Hycid present sharp peaks while Olsep at the same wavenumber shown a smaller peak, possibly related to the stretching of the C-C. At 1000cm^{-1} all formulations present a small peak followed by another at 1025cm^{-1}, which may be referred to the stretching of the thiocarbonyl group C=S. The stretching signals from the two aromatic rings are accounted for by the peaks in the region around 1100cm^{-1}. At 1160cm^{-1} all formulations present a broad signal that could be related to the asymmetric stretching of the ether group, C-O-C. The C=S stretching is demonstrated at 1260cm^{-1} with peaks for all the formulations with the exclusion of GPO and Hycid. At 1280cm^{-1} all formulations produce a signal even if with different intensity, possible to relate to the CC stretching of the aromatic chains. At 1395cm^{-1} it was noticed the presence of peaks for all formulations, with exclusion of Losec, due to bending symmetric of the methyl

groups. At 1480cm^{-1} all samples present sharp peaks, due to bending asymmetric of the methyl groups. At 1530cm^{-1} peak for Dexcel, Olsep, Hycid, GPO; at 1610cm^{-1} peak for Hycid, Olsep, GPO, Dexcel, with Losec presenting two peaks at 1590 and 1610cm^{-1} possibly to be referred to the stretching of the aromatic rings. At 1640cm^{-1} all formulations produce signals with different intensity, due to stretching of CN in the aromatic rings.

The conventional Raman spectrograms of the analyses of the surfaces of the omeprazole tablets under investigation are reported in Fig.4. As for the analyses of the internal sides shown earlier in SERS investigation, the spectra obtained from the surface analyses show common tracts and dissimilarities within the different tablets of omeprazole studied.

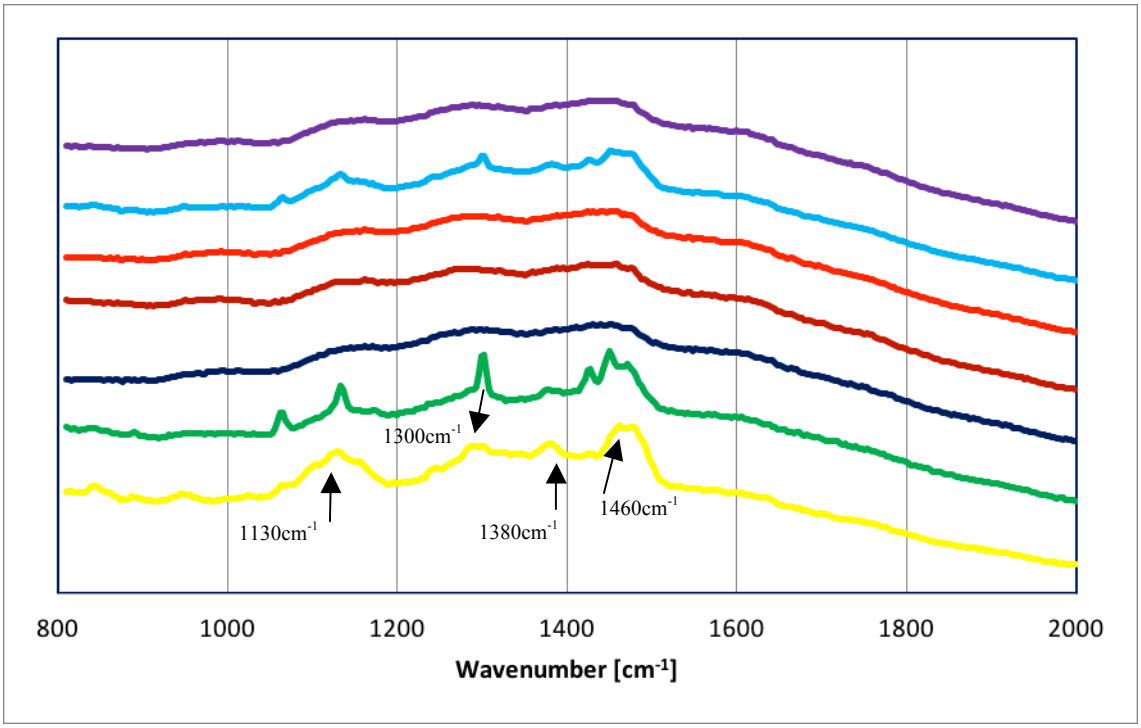

Fig.4. Raman analysis of omeprazole generic medications, external sides. Spectra have been off-set in order to avoid overlapping and facilitate observation. Rambaxy (black), Losec (yellow), Olsep (light blue), Esopral (green), Dexcel (brown). Hycid (red) and GPO (purple). At 1130cm^{-1} peaks due to the asymmetric stretching of C-O-C in the benzimidazole and in the pyridinylic rings; at 1300cm^{-1} symmetric stretching of the aromatic ring chain vibrations; at 1380cm^{-1} peaks due to symmetric bending of the methyl groups; at 1460cm^{-1} peaks due to the asymmetric bending of the CH_3.

At 840 and 890cm^{-1} sharp peaks are noticeable for Losec, and less prominent for the others, possibly due to the stretching of C-O-C in both the pyridinylic and benzimidazole rings. At 950cm^{-1} a sharp peak for Losec and a broad one for Dexcel tablets are noticeable, possibly due to the stretching of the C-C. The stretching of CS is accounted for by the peak at 1025cm^{-1} in Losec's spectrum. At 1065cm^{-1} Esopral and Olsep present sharp peaks possibly due to the symmetric stretching of the aromatic rings. At 1130cm^{-1} sharp peaks for Esopral, Olsep, and Losec possibly to be attributable to the asymmetric stretching of C-O-C in the benzimidazole and in the pyridinylic rings. At 1175cm^{-1} a small peak might be assigned to the stretching of CS. At 1245cm^{-1} small peak for Losec and at 1300cm^{-1} shifted peaks for all formulations, sharp for Esopral, possibly related to symmetric stretching of the aromatic ring chain vibrations. The symmetric bending of the methyl groups is demonstrated at 1380cm^{-1} with a sharp peak for Losec and medium for Esopral, Olsep. At 1430cm^{-1} small peak for Losec, and sharp for Olsep, Esop, possibly due to symmetric bending of CH_3. At 1450-1470cm^{-1} sharp

peak for Esopral, Olsep. Losec presents a sharp peak slightly shifted at 1460cm^{-1}, possibly related to the asymmetric bending of the CH_3.

The Raman spectra of the analysis of the internal side of the tablets are reported in Fig.5. The spectra show the similarities and differences amongst the different formulations of omeprazole under investigation. Structure specific peaks are clearly observed in the spectra obtained for each omeprazole formulation.

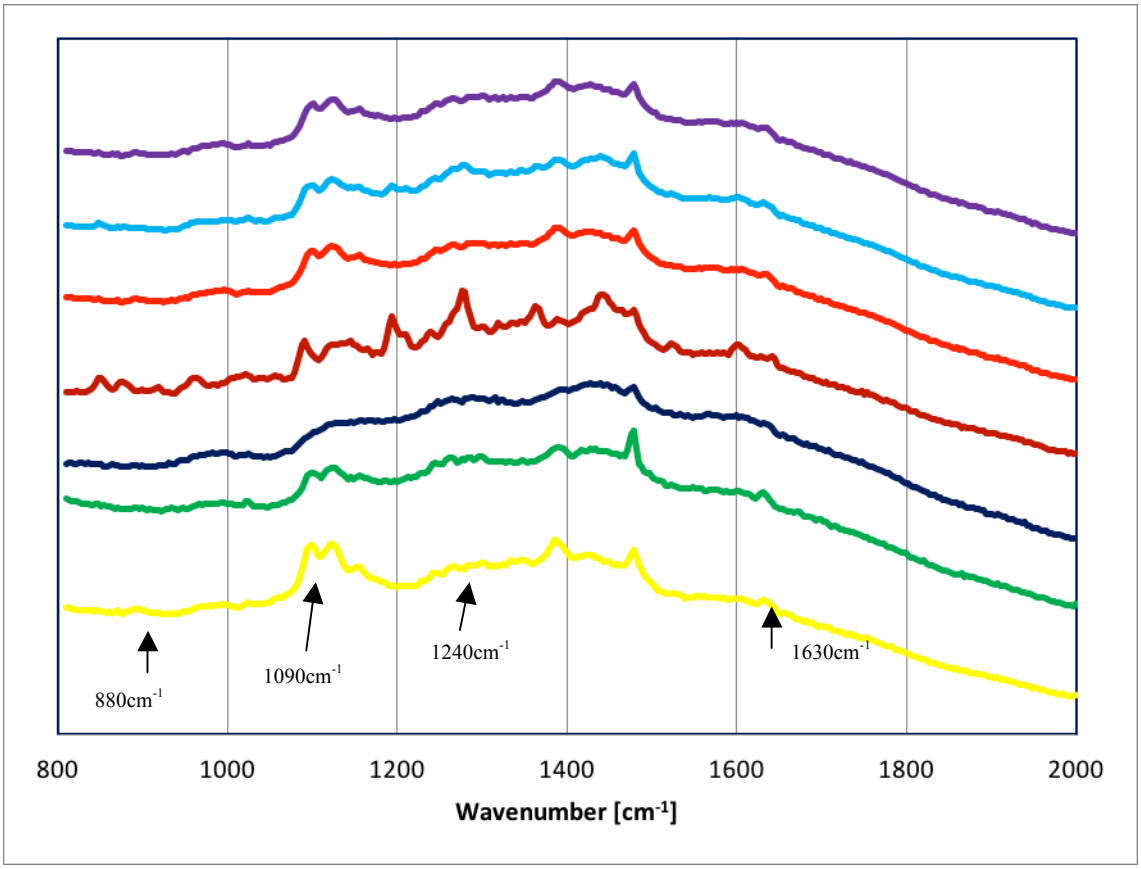

Fig.5. Raman analysis of omeprazole generic medications, internal sides. Spectra have been off-set in order to avoid overlapping and facilitate observation. Rambaxy (black), Losec (yellow), Olsep (light blue), Esopral (green), Dexcel (brown). Hycid (red) and GPO (purple). At 880cm^{-1} peak due to the symmetric stretching of C-O-C of the ether groups in the aromatic chains; at 1090cm^{-1}of the stretching of C=S of the sulphur conjugated to the benzimidazole ring; at 1240cm^{-1} peak due to the stretching of the aromatic ring chains; at 1630cm^{-1} peak due to the stretching of CN in the benzimidazole

Starting at the spectral region around 800cm^{-1}, Dexcel presents a strong peak at 850cm^{-1} and a second one at 880cm^{-1}. These peaks may be attributed to the symmetric stretching of C-O-C of the ether groups in the aromatic chains [18]. At 880cm^{-1}, Hycid presents a small peak possibly due to the same previous group but shifted. In this interval, the other formulations present minor peaks at around 850cm^{-1} which correlates possibly to the stretching of O-O from the lactose used as excipient. At 920 and 965cm^{-1}, two strong peaks for Dexcel are shown, possibly due to C-O-C asymmetric stretching shifted at different value than GPO and Hycid. The other formulations present a broad peak at 990cm^{-1} possibly related to the C-C stretching. At 1020cm^{-1} all present a peak, more remarkable for Dexcel and less for Rambaxy. The stretching C-C of the aromatic rings is demonstrated at at 1060cm^{-1}. In this area of the spectra, Dexcel presents a sharp peak, while all the other formulations only a minor one. At 1090cm^{-1} Dexcel presents a

peak, the others show similar peak at higher wavenumbers, with the exclusion of Rambaxy, as a result of the stretching of C=S of the sulphur conjugated to the benzimidazole ring. At 1125cm^{-1} all present substantial peaks, with the exclusion of Rambaxy and weak peak for Dexcel, possibly related to the asymmetric stretching of the ether groups present in the pyridinylic ring. At 1150cm^{-1} Dexcel present a weak peak that may be referred to the previous group shifted at higher wavenumbers. At 1155cm^{-1} all formulations, with the exclusion of Rambaxy and Dexcel, produced a peak, possibly due to the asymmetric stretching of C-O-C in both the aromatic rings of the omeprazole molecule. At 1190cm^{-1} it was present a strong peak for Dexcel, small for GPO, Hycid, Olsep, Esopral, absent for Rambaxy and Losec, possibly to be assigned to the stretching of the CS. At 1240cm^{-1} a peak present for all, with the exclusion of Rambaxy, due possibly to the stretching of the aromatic ring chains. At 1270cm^{-1} Dexcel presents a sharp peak, possibly due to a shifted signal from the aromatic rings. At 1365cm^{-1} and 1380cm^{-1} peaks possibly due to the symmetric bending of methyl groups, while the asymmetric bending of the same groups laid in the region between 1440 and 1500cm^{-1}. The CN in the pyridinylic ring is accounted for by stretching peaks at 1530 and 1600cm^{-1}. At 1630cm^{-1} a peak probably due to the stretching of CN in the benzimidazole ring [18] can be also observed. This has been selected in some studies as a reference peak for validation models based on the consideration that excipients generally do not interfere in this region of the spectrum not containing functional groups that emit at these wavenumbers [19].

To aid the interpretation of the vast amount of wavenumbers generated by the spectroscopic techniques, both SERS and conventional Raman, PCA was used. PCA of the SERS data obtained by analysis of the external side of the omeprazole tablets shows a partial identifiable distribution profiles for some of the classes of samples investigated (Fig.6).

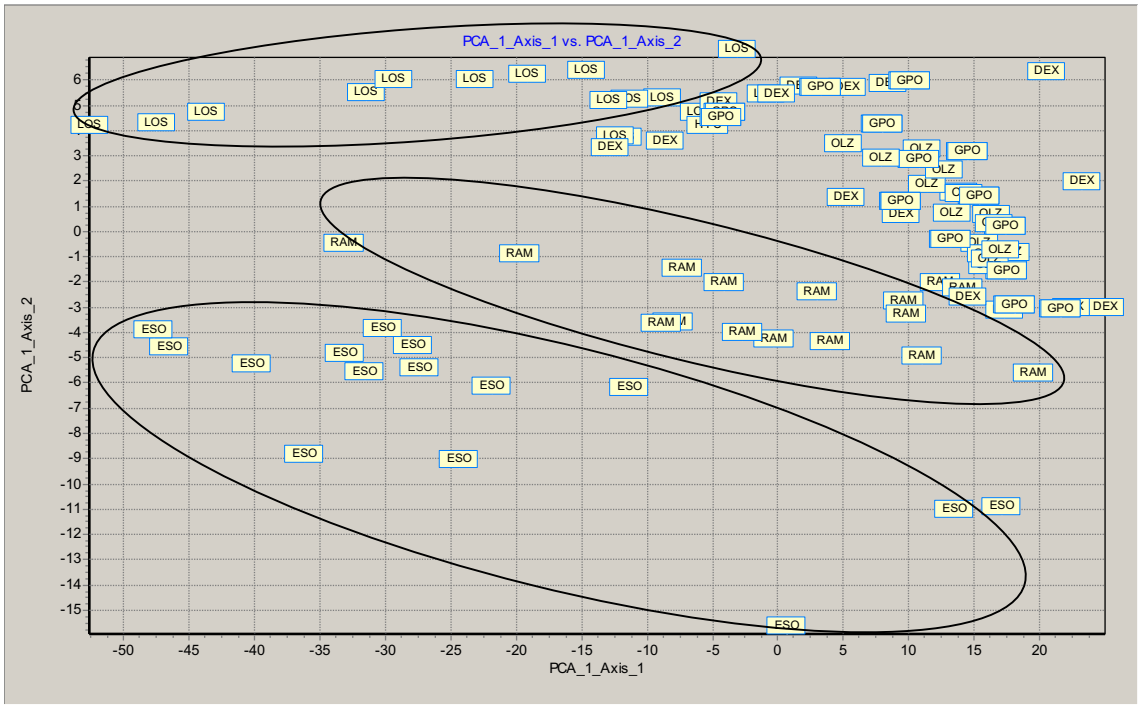

Fig.6. Principal Component Analysis of the results obtained by SERS analysis of the external side of omeprazole samples. PCA1=93.22%, PCA2=98.53%. From top to bottom, the circled clusters identify Losec, Rambaxy and Esopral.

Principal component (PC) 1 accounted for 93.22% of variation, with PC2 and PC3 being responsible for 5.31% and 0.65% respectively. PC1 and PC2 were therefore responsible for an accumulative 98.53% of the variation. The omeprazole generic medications were separated due to their individual association with the two main components.

Losec, Rambaxy and Esopral can be seen to partially separate over the value of PC2. Esopral is sparse at lower PC2 value, Rambaxy is located at a central PC2 value whilst Losec clusters at higher value of PC2. The cloud of data incorporating the overlap of other classes of samples is located in positive areas for both components. Predictive accuracy of the PCA model obtained was validated via cross validation and leave-one-out approach (Tab.1 and 2, respectively).

Table.1. Cross validation of the PCA from Fig.6 on SERS spectroscopy obtained from the surface analyses of the omeprazole generic medications.

Error rate			0.7786							
Values prediction			Confusion matrix							
Value	Recall	1-Precision		ESOP	RAM	LOS	DEX	HYC	OLZ	GPO	Sum
ESOP	0.4872	0.6667	ESOP	19	4	16	0	0	0	0	40
RAM	0.3415	0.7667	RAM	18	14	3	6	0	0	0	40
LOS	0.275	0.8778	LOS	20	5	11	2	0	0	2	40
DEX	0.375	0.6512	DEX	0	11	13	15	0	1	0	40
HYC	0	1	HYC	0	5	13	3	0	4	16	40
OLZ	0.025	0.875	OLZ	0	5	22	10	0	1	2	40
GPO	0.0513	0.9091	GPO	0	16	12	7	0	2	2	40
			Sum	57	60	90	43	0	8	22	280

The total error rate of the predictive model evaluated via cross validation accounted for 78%. GPO and Hycid presented the higher error rate, while the Dexcel showed the lowest error, but still high with a value prediction of 65%. From the confusion matrix, it is possible to notice that out of 40 specimens per each class of samples analysed, Esopral gave the best classification, with 19 samples correctly classified, corresponding to 47.5% of the total.

Table.2. Leave-one-out of the PCA from Fig.6 on SERS spectroscopy obtained from the surface analyses of the omeprazole generic medications.

Error rate			0.7987							
Values prediction			Confusion matrix							
Value	Recall	1-Precision		ESOP	RAM	LOS	DEX	HYC	OLZ	GPO	Sum
ESOP	0.3872	0.6922	ESOP	18	3	16	2	1	0	0	40
RAM	0.3815	0.7896	RAM	16	15	1	7	0	0	1	40
LOS	0.275	0.8898	LOS	19	4	10	2	0	3	2	40
DEX	0.475	0.6120	DEX	1	10	12	15	1	1	0	40
HYC	0.05	0.9601	HYC	1	4	14	4	1	4	12	40
OLZ	0.075	0.8965	OLZ	2	5	21	9	0	2	1	40
GPO	0.0613	0.9385	GPO	2	14	11	8	1	2	2	40
			Sum	59	55	85	47	4	12	18	280

Furthermore, the predictive accuracy of the model was also tested via leave-one-out validation. The cumulative error rate accounted for around 80%, with worse results in terms of predictions than the ones obtained from the cross validation. From the confusion matrix, it is also possible to observe that the correct classification for all the specimens analysed was quite low.

PCA of the SERS data obtained by analysis of the internal side of the omeprazole tablets shows a not clear distribution profiles for the classes of samples studied (Fig.7).

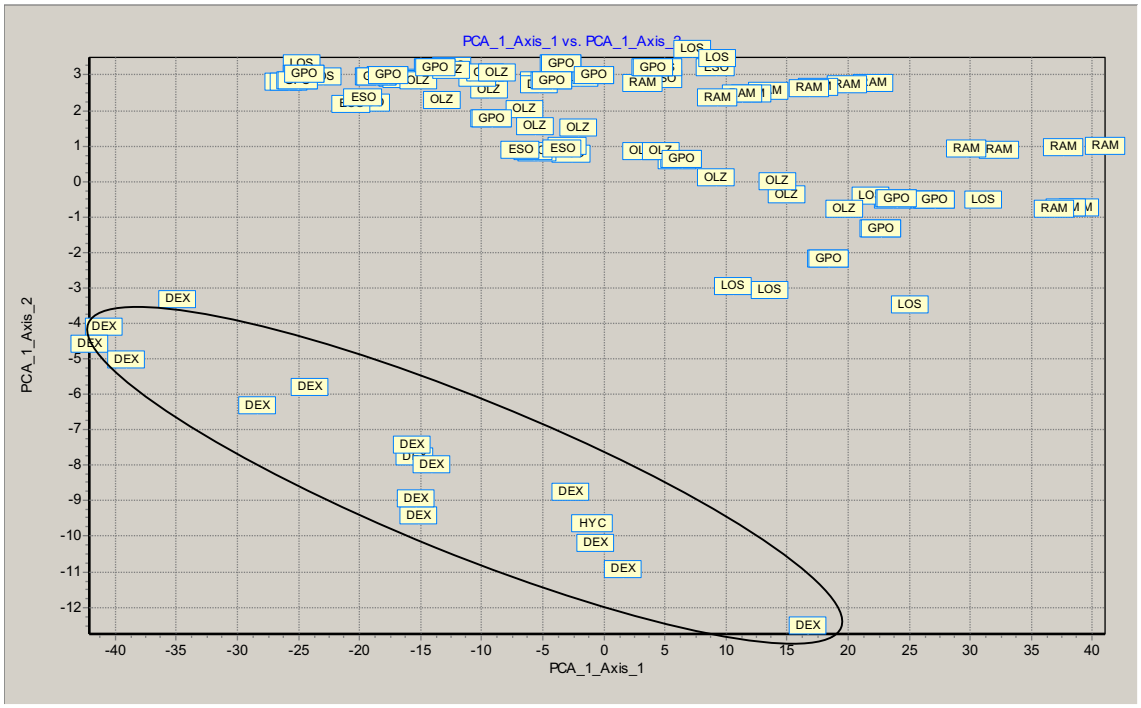

Fig.7. Principal Component Analysis of the results obtained by SERS analysis of the internal side of omeprazole samples. PCA1=95.83%, PCA2=99.59%.

Principal component (PC)1 accounted for 95.83% of variation, with PC2 and PC3 being responsible for 3.76% and 0.45% respectively. PC1 and PC2 were therefore responsible for an accumulative 99.59% of the variation. Only Dexcel samples resulted to cluster together in a sparse group but well separated over the values of PC2, in negative field, from the rest, all gathered together.

Predictive accuracy of the PCA model obtained was validated via cross validation and leave-one-out approach (Tab.3 and 4, respectively).

Table.3. Cross validation of the PCA from Fig.7 on SERS spectroscopy obtained from the internal analyses of the omeprazole generic medications.

Error rate			0.6977							
Values prediction			Confusion matrix							
Value	Recall	1-Precision		ESOP	RAM	LOS	DEX	HYC	OLZ	GPO	Sum
ESOP	0.4872	0.6167	ESOP	20	3	15	1	0	0	1	40
RAM	0.3415	0.6667	RAM	19	13	2	5	0	0	1	40
LOS	0.275	0.8078	LOS	21	4	12	2	0	0	1	40
DEX	0.375	0.5912	DEX	1	10	11	16	1	1	0	40
HYC	0	0.94	HYC	1	4	12	2	2	3	16	40
OLZ	0.025	0.884	OLZ	2	4	18	10	1	3	2	40
GPO	0.0513	0.8933	GPO	2	13	13	6	1	2	3	40
			Sum	66	51	83	42	5	9	24	280

The total error rate of the predictive model evaluated via cross validation accounted for 70%. The set of error rates was comparable with the ones originated from cross validation, with Hycid and GPO at the higher error rate, and Dexcel with the lowest error. From the confusion matrix, it is possible to notice that out of 40 specimens per each class of samples analysed, Esopral gave again the better classification.

Leave-one-out validation on this set of data reported a cumulative error rate accounted for 75%, with results comparable with the ones obtained from the cross validation. From the confusion matrix, it is also possible to observe the same trend in the classification.

Table.4. Leave-one-out of the PCA from Fig.7 on SERS spectroscopy obtained from the internal analyses of the omeprazole generic medications.

Error rate			0.7522							
Values prediction			Confusion matrix							
Value	Recall	1-Precision		ESOP	RAM	LOS	DEX	HYC	OLZ	GPO	Sum
ESOP	0.45133	0.6654	ESOP	18	5	14	1	1	1	0	40
RAM	0.3674	0.6874	RAM	18	11	2	5	3	1	0	40
LOS	0.376	0.7988	LOS	18	7	10	2	2	0	1	40
DEX	0.381	0.6123	DEX	0	11	11	14	1	1	2	40
HYC	0	0.9103	HYC	2	3	10	2	2	3	18	40
OLZ	0.035	0.9041	OLZ	4	4	16	8	1	3	4	40
GPO	0.0663	0.8103	GPO	4	11	14	6	0	3	2	40
			Sum	64	52	77	38	10	12	27	280

Chemometric assessments were performed on the all remainder set of experiments, both with Raman and by FT-IR, showing very similar results in terms of PCAs and correlated model validations.

Factor loading was also utilised, and each wavenumber gave factor values of 0.75 or above, with most giving value superseding 0.90. This suggests that each wavenumber, and therefore each PC, was accountable for the majority of variability in the data demonstrating a very similar representation to that of the PCA. In order to to identify significant wavenumbers that could be employed for distinct characterisation between the omeprazole generic medications, the factor loadings were plotted against the correspondent wavenumbers (Fig.8-10).

All the wavenumbers resulted to be clustered together for all the classes of samples with few exceptions. In the SERS analyses of the external sides of the sample (Fig.8), two main significant peaks identify sources of potential separation power. Losec and Rambaxy present a peak at 1500cm^{-1}; Rambaxy showed a peak at 1700cm^{-1}.

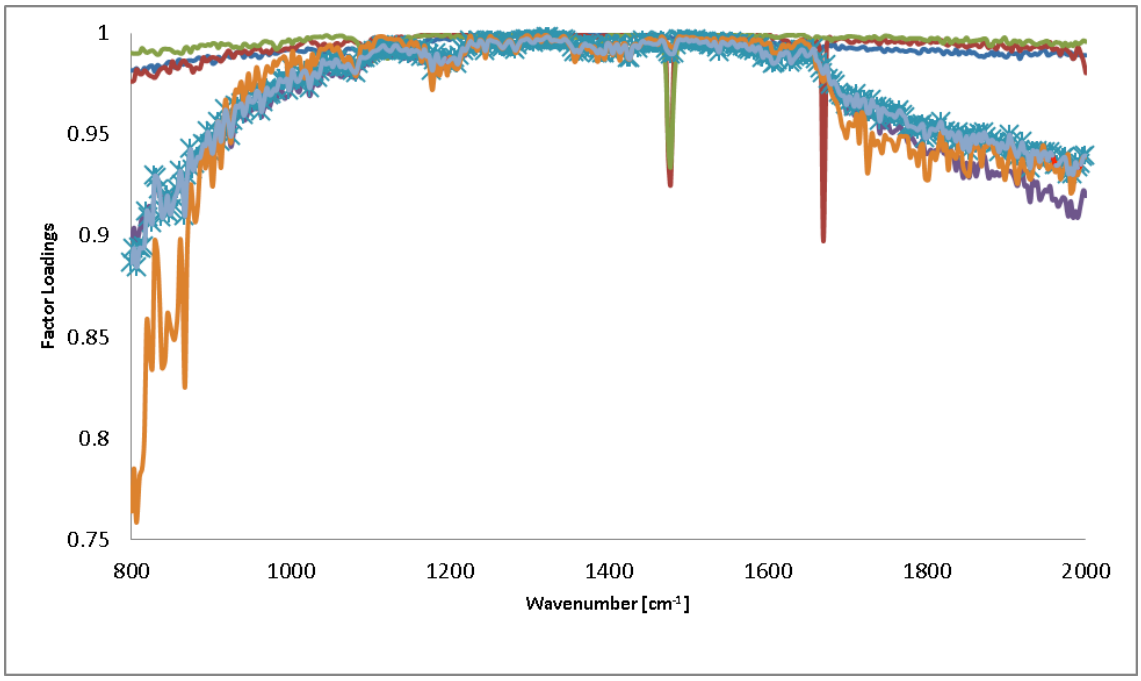

Fig.8. Factor Loadings of the omeprazole generic medications versus attributed wavenumbers in SERS analysis of external area of omeprazole generic medications. From top to bottom, Esopral (blue), Hycid (red), GPO (blue), Rambaxy (dark brown), Olzep (brown), Dexcel (purple), Losec (green).

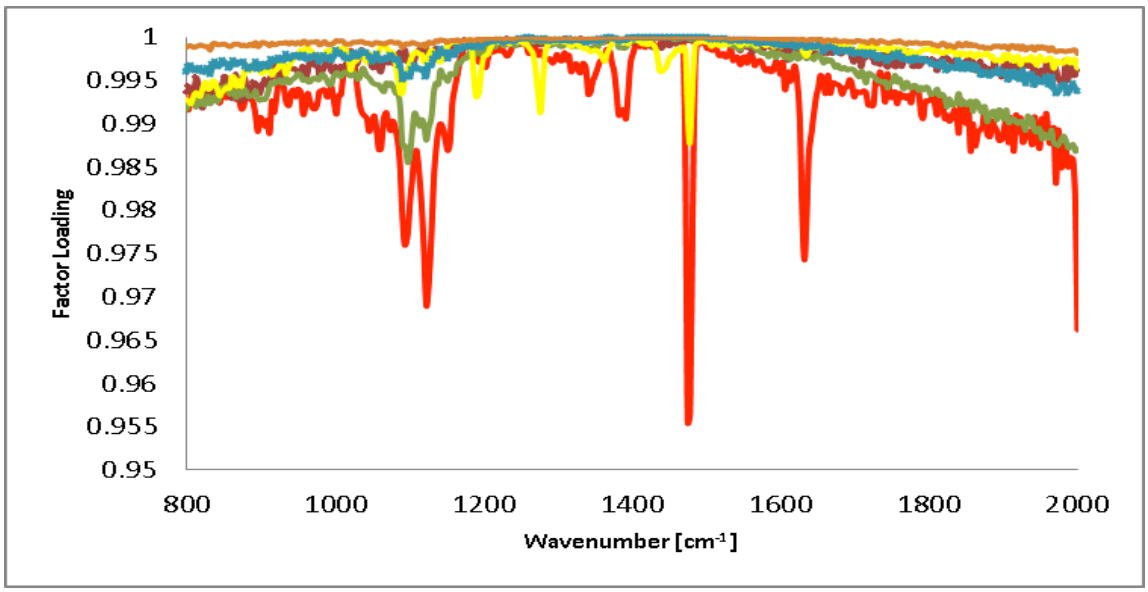

Fig.9. Factor Loadings of the omeprazole generic medications versus attributed wavenumbers in SERS analysis of internal areas of omeprazole generic medications. From top to bottom, Esopral (light blue), Hycid (red), GPO (blue), Rambaxy (dark brown), Olzep (brown), Dexcel (yellow), Losec (green).

In the SERS analyses of the internal sides of the sample (Fig.9), Hycid and GPO showed more characteristic spectra, with peaks at 1080, 1150, 1480, 1650cm^{-1}; Dexcel showed also characteristic peaks at 1220, 1290 and 1480cm^{-1} which could be used to separate them from the other omeprazole generic medications.

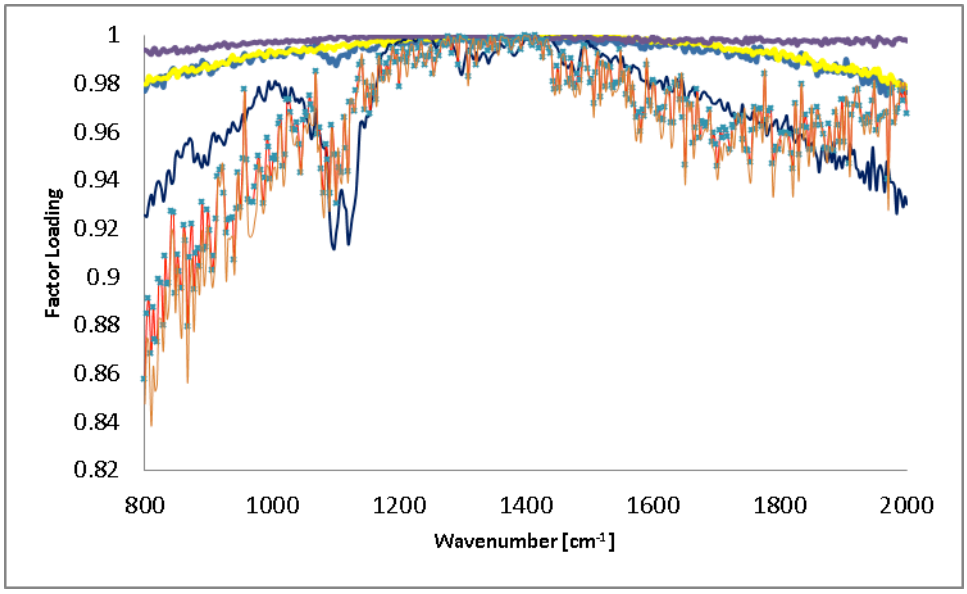

Fig.10. Factor Loadings of the omeprazole generic medications versus attributed wavenumbers in Raman analysis of external areas of omeprazole generic medications. From top to bottom, Esopral (blue), Hycid (red), GPO (blue), Rambaxy (dark brown), Olzep (brown), Dexcel (purple), Losec (green)

In the conventional Raman analyses of the surface sides of the samples (Fig.10), Esopral presented two peaks at 1090 and 1110cm^{-1}.

The other Raman analysis did not produce relevant representation with the majority of the wavenumbers all gathered close to Factor Loading 1.

6.3.2. FT-IR spectroscopy of omeprazole generic medications

The FT-IR spectra of the seven omeprazole generic medications salts have been overlaid in Fig.11. for the analysis of the external part of the samples and Fig.12 for the analysis of the internal sides. Both sides resulted to provide very similar spectra.

Starting at 3500cm^{-1}, broad peaks are observed for all seven omeprazole generic medications as a result of the hydroxyl O-H stretching from the excipients that overlap with the asymmetric stretching of N-H groups at 3300cm^{-1}.

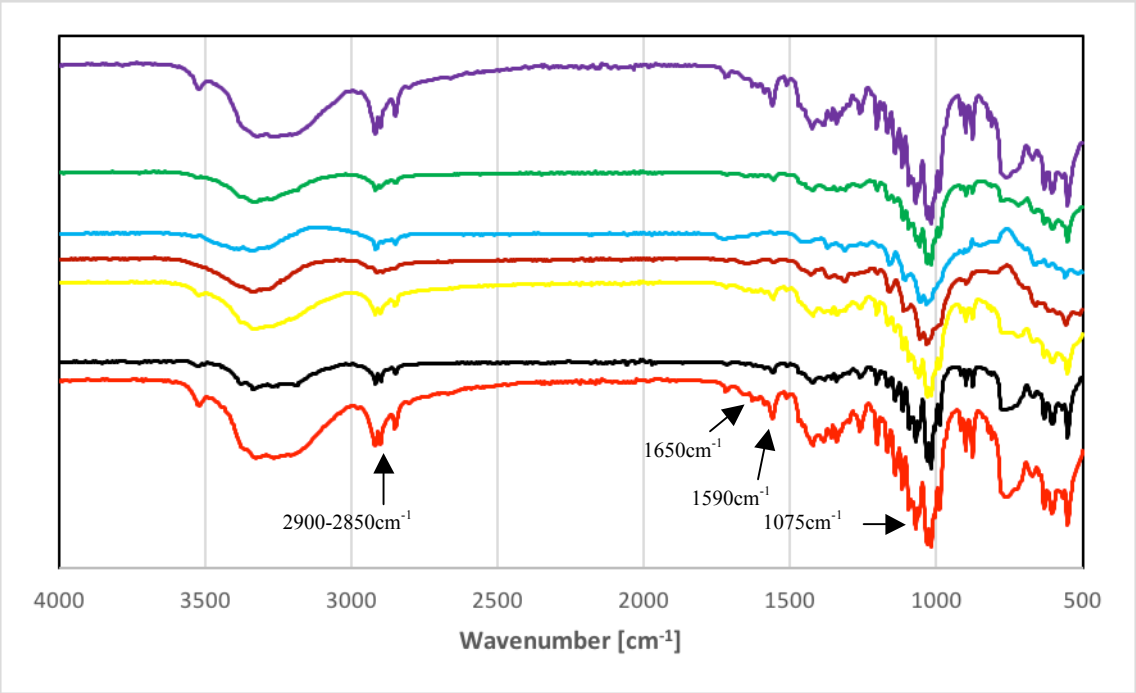

Fig.11. FT-IR analysis of omeprazole generic medications, external sides. Spectra have been off-set in order to avoid overlapping and facilitate observation. Rambaxy (black), Losec (yellow), Olsep (light blue), Esopral (green), Dexcel (brown). Hycid (red) and GPO (purple). Peak at 2900cm^{-1} could be accounted for by asymmetric C-H stretching; at 2850cm^{-1} peak due to the symmetric C-H stretching; at 1650cm^{-1} small but sharp peaks are recognised as a C=C aromatic stretching; at 1590cm^{-1} peak due to CN stretching; at 1075cm^{-1} peak due to the CS stretching

Between 2900 and 2850cm^{-1} a sharp dual set of peaks are seen for all the samples. The first at 2900cm^{-1} could be accounted for by asymmetric C-H stretching and the latter at 2850cm^{-1} by symmetric C-H stretching. Within the recognised 'fingerprint region' of the spectra, at 1650cm^{-1} small but sharp peaks are recognised as a C=C aromatic stretching and at 1590cm^{-1} as a CN stretching. The sharp peaks at 1075cm^{-1} are related to the CS stretching of the thiocarbonyl group.

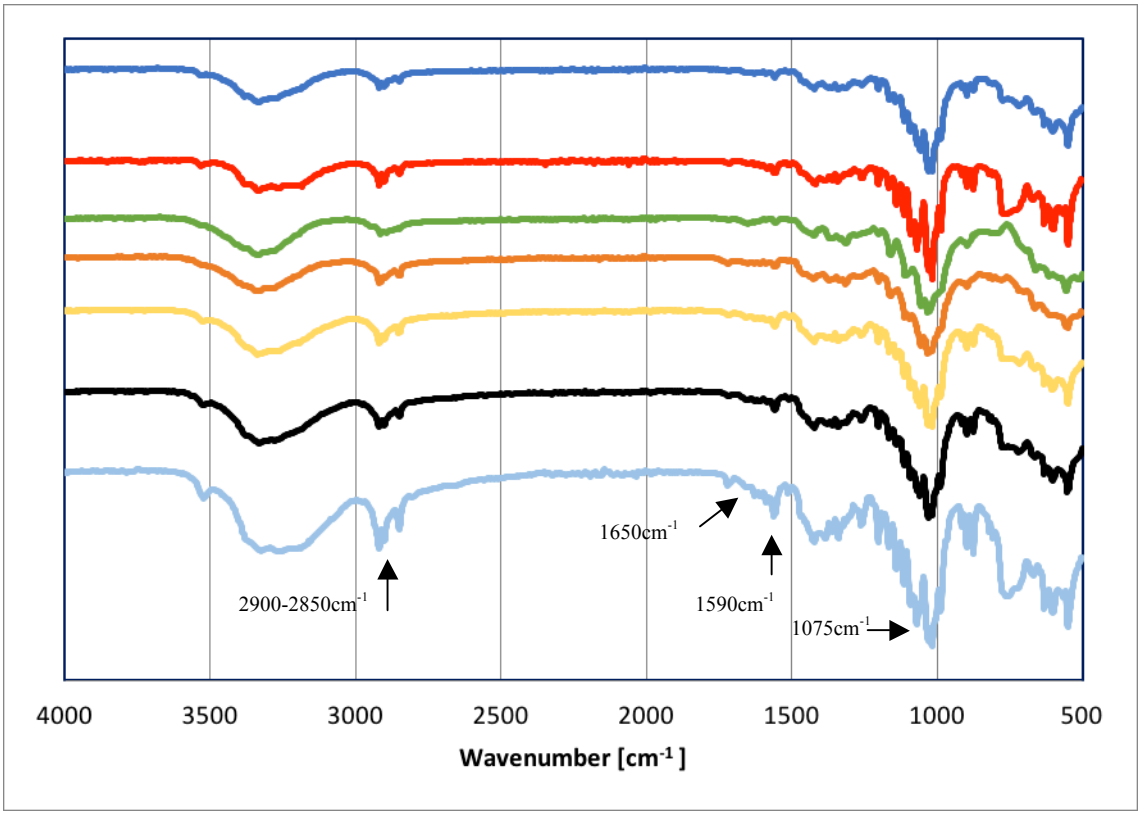

Fig.12. FT-IR analysis of omeprazole generic medications, internal sides. Spectra have been off-set in order to avoid overlapping and facilitate observation. Rambaxy (black), Losec (yellow), Olsep (light blue), Esopral (green), Dexcel (brown). Hycid (red) and GPO (purple). Peak at 2900cm^{-1} could be accounted for by asymmetric C-H stretching; at 2850cm^{-1} peak due to the symmetric C-H stretching; at 1650cm^{-1} small but sharp peaks are recognised as a C=C aromatic stretching at 1590cm^{-1} peak due to CN stretching; at 1075cm^{-1} peak due to the CS stretching

6.3.3. ^{1}H-NMR of omeprazole generic medications

In light of the similarity of the Raman and FT-IR spectra obtained which did not make possible to analytically discriminate amongst the seven different classes of samples, in order to gather additional information on the chemical structures of the samples the use of proton NMR was considered to elucidate more chemical diversities.

In its molecular structure, omeprazole presents two sources of structural differentiation when performing proton NMR. Firstly, omeprazole is a chiral molecule since it has a diastereogenic center located on the thiocarbonyl sulfur atom. The second source of diversity is that it presents tautomerisms.

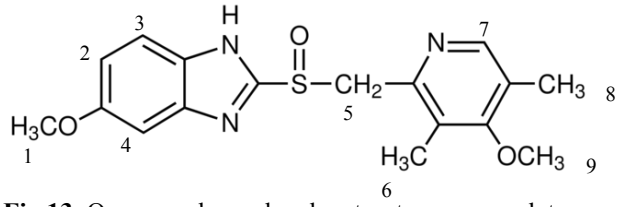

Fig.13. Omeprazole, molecular structure nomenclature used for peak assignment .

In the assignment of the NMR peaks it is important to follow clear conventions on the numbering system adopted to identify the location of the protons in the carbon backbone of the chemical structure. According to IUPAC nomenclature [20], for omeprazole, numbering starts from nitrogen atom of the NH in the benzimidazole moiety, however for instrument requirement the numbering adopted is as shown in Fig.13 and in Fig.14 is shown the NMR spectrum obtained from Olzep analysis, used to start the peak assignment. Proton nuclear magnetic resonance of the whole seven different omeprazole generic medications analysed is reported in Fig.15.

While spectroscopy gives information on the functional groups present in the compound, to go into the structure of the compounds NMR has been performed. Omeprazole molecule present two different aromatic moiety, benzimidazole and pyridinylic moieties.

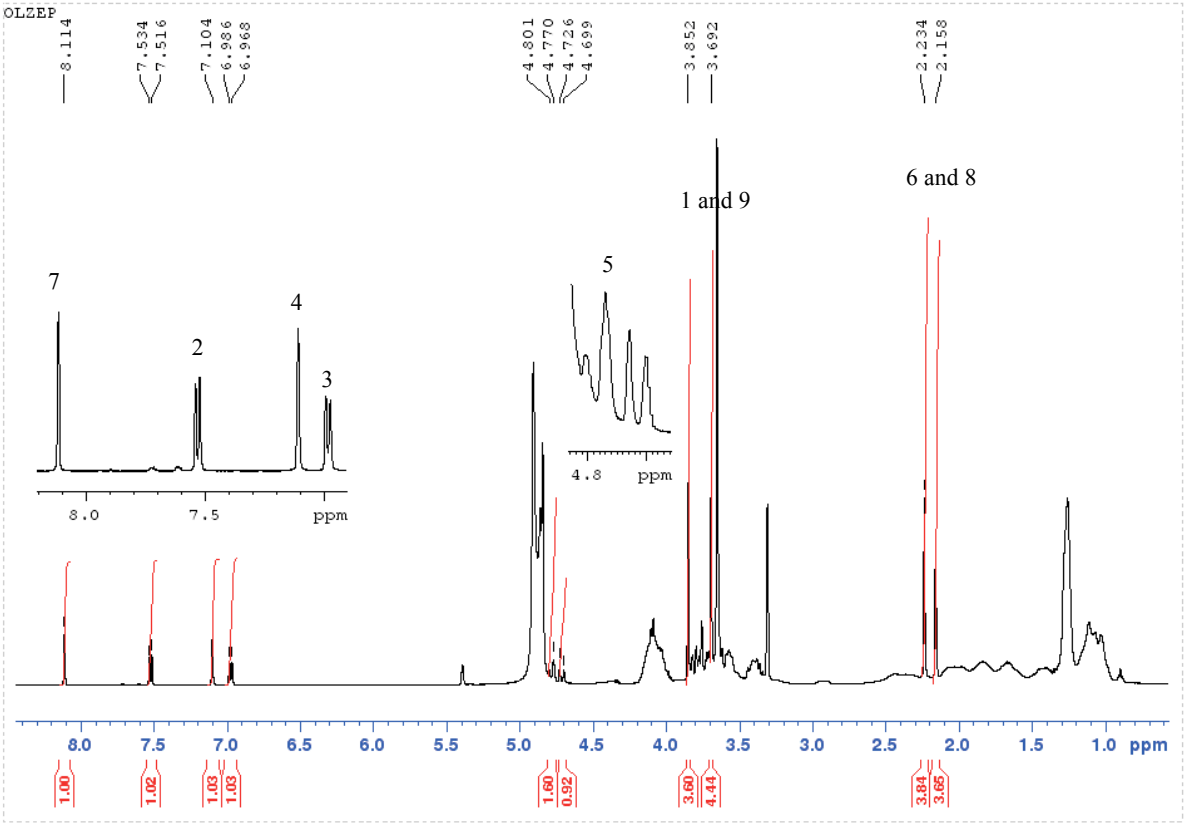

Fig.14. ^{1}H NMR of omeprazole OLZEP used to represent in details the peaks assignment (500 MHz, MeOD, 298K).

The NMR spectra for the specimens analysed (analyses kindly performed by Dr Steven Prior, University of Lincoln) resulted to be as expected similar but with few elements of differentiation. As reported by Claramunt et al in an assessment study conducted omeprazole standard, the peak at 8.2ppm (Fig.14) is related to the proton in position 7 in the pyridinylic moiety (Fig.13). This peak is present in all the seven different classes of samples.

Hycid and GPO showed two singlets at 7.6 and 7.7ppm, respectively, close to a doublet at 7.5ppm. While the doublet at 7.5ppm, present in each analyte's spectrum, is probably to assign to the protons in position 3 and 4, as reported by Claramunt et al, in the two aromatic moieties of the omeprazole molecule linked together, the two separate singlets are indicative of additional aromatic molecules present as impurities. The region between 6.5 and 4.75ppm is where the strongly de-shielded methyl/ methylene protons directly attached on the pyridinylic moieties could be found. The peaks located in the area of 5ppm are related to the hydroxyl groups present in the excipients, mainly

lactose, cellulose and starch for all the formulations studied (see chart of excipient reported in chapter 2.).

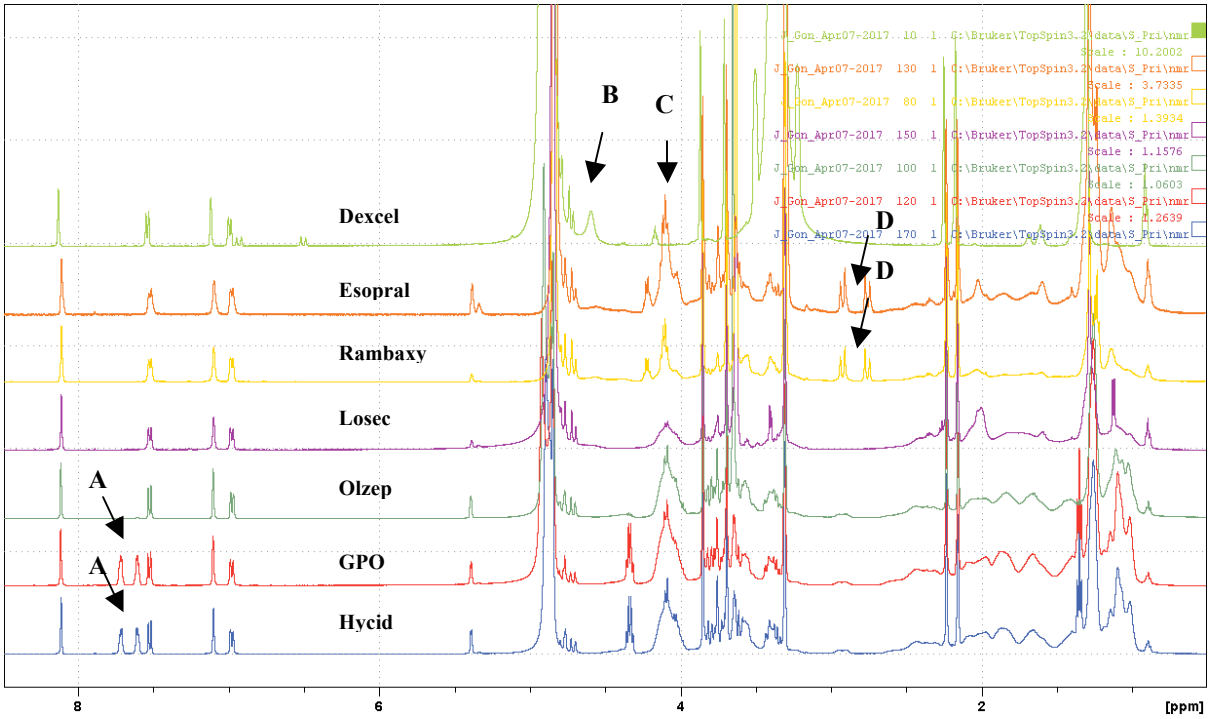

Fig.15. Nuclear Magnetic Resonance of the omeprazole generic medications. From top to bottom: Dexcel, Esopral, Rambaxy, Losec, Olzep, GPO, Hycid.. **A**: At 7.6 and 7.7ppm, Hycid and GPO present two singlets associated to aromatic compounds different than omeprazole structure; **B**: lactose; **C**: cellulose; **D**: aliphatic compound;

NMR analyses of the seven classes of omeprazole investigated present some remarkable differentiation (Fig.15). Olzep and Losec showed same formulation, with slightly different ratios. GPO and Hycid resulted to be identical in every way. GPO, Hycid, Olzep and Losec resulted to be very similar, with similar amount of both lactose and cellulose, however GPO and Hycid contain an extra aromatic compound, as indicated by the arrow A in Fig.15. Dexcel seemed to be the only one not containing cellulose, lacking the peaks indicated by arrow C in Fig.15. Rambaxy and Esopral, both isomers, contained the same chemicals, however Esopral contained significantly less API in the formulation. Both presented presence of lactose and cellulose. Esopral and Rambaxy contain another aliphatic compound, indicated with arrow D, with respect the other classes of samples. Hycid and GPO spectra presented elements of differentiation above 7ppm. At 7.6 and 7.7ppm, Hycid and GPO present two singlets associated to aromatic compounds different than omeprazole structure.

6.3.3.1. Hycid and GPO, internet samples

Two singlets have been identified in the spectra obtained from Hycid and GPO not found in the other spectra that can be related to additional aromatic substances not present in the other formulations. It could be speculated that these two aromatic compounds could be the two separated moieties, benzimidazole and pyridinylic moieties, of the omeprazole molecule that for unknown reason split or never linked together during the synthetic process. Another logical cause of the presence of these impurities is that they originated as contamination from the specific manufacturing process that Hycid and GPO have gone through. Notwithstanding the possible causes that have produced the two aromatic impurities, their presence in the composition of the final pharmaceutical products, ready to be used by patients, raises suspects on the quality of the medications themselves. These two set of samples have been both purchased off internet market from un-official websites, not regulated by any government authorization for the dispensing of medications. The origin of both websites where Hycid and GPO were purchased from are Asian regions. In this geographic area, between 1999 and 2004, the ratio of fraudulent medications boosted drastically, with an increased of forged medications sales range from 10% to more than 30% of the national legal market. Because of the anonymous nature of the online market, the risks of entry into the market of medications of uncertain quality is remarkably high, being the quality control performed by the government authorities significantly more difficult and consequently less effective.

The manufacturing production of medicines is extremely articulated but it can be divided into two principal stages: the primary manufacturing stage, where the active pharmaceutical ingredient (API) is manufactured, and the secondary manufacturing stage, where the API is mixed with other ingredients to prepare the final pharmaceutic formulation. Hycid and GPO impurities could have derived from one or both of these stages.

During the very last World Health Assembly, it was agreed to embrace the definition of ‘Substandard and Falsified medical products’ as official term to be used by the member states with a most recent change in terminology introducing the the classification of ‘substandard’ and ‘falsified’. The first, defines those official medical products that lack of reaching the required standards of quality and/or to meet the necessary specifications.

Falsified identifies those medical products that intentionally give false information on their nature, origin and structure. It is not clear at this stage of research if Hycid and GPO could be classified as substandard or, worse, as falsified, but it seems probable that the lack of quality control that has allowed to input into the market these medicinal, containing aromatic products, and as such usually biochemically active, suggests to address these products with caution.

6.4. Discussion

The analysis of the seven different omeprazole generic medications does not give significantly distinctive spectra when studied via conventional Raman spectroscopy and FT-IR, both internally and externally. Using SERS, however, it was possible to observe an improvement in the characterisation of the spectra, because of the nucleophilic nature of the omeprazole's chemical structure, especially on the analyses of the surfaces of the specimens. Although an omeprazole standard was not used in this study, previously published spectra compared to those obtained in this investigation shown that there are differences that can be accredited to the excipients added to the formulations [23, 24]. All the spectroscopic analyses obtained from the internal and external parts of the samples were quite alike although some differences in quality and resolution could be noticed, due to a possible coating effect on the tablets. Previous spectroscopic studies reported that the concentration of active ingredient in solid formulations resulted to be non-homogeneously spread across from external to internal areas. [25, 26]. SERS analyses of the internal side seem to show the same peaks but more pronounced than the external area. This could be related to the manufacturing processing, effectively giving a higher concentration at the internal area compared to that of the surfaces.

The spectra obtained from all the spectroscopic investigations did not produce a neat visual differentiation amongst the seven classes of samples investigated. It could be speculative to suggest that solid formulations often contain very low doses of API in comparison with the dosage of excipients used, which can present analytical challenges in terms of differentiation [26]. The weights of the tablets for all the different formulations were ranging between 399 and 410mg, leading to a ratio API/excipients of only 0.05. The limited differentiation in the results obtained from the spectroscopic

analyses was taken into consideration as a possible result obtained, because of the similar nature of the samples investigated.

Cellulose, lactose and starch are the main excipients used in all of the formulation studied and all present a high ratio of hydroxyl groups, which in the FT-IR analyses could have been masked with their broad peaks components of the omeprazole molecules, as reported in the chart of excipients in chapter 2. This is substantiated by the asymmetric stretching C-O-C and C-OH peaks found in the region around 1100cm^{-1} from the sugar rings of the excipients [27, 28]. Notwithstanding the great resemblances amongst the spectra of the seven different classes of analytes, the statistical analysis of these spectroscopic data allowed the minor differences to be significative, even though only very few differences could be effectively being exploited. There were limited regions of the spectra obtained that could be associated with the individual omeprazole core structure. This could be because both the core of the molecules for all the classes of omeprazole is the same and that the excipients employed in the manufacturing of the solid formulations, covering any of the more noticeable differences, are indeed the same or belonging to sugar classes with same functional groups.

The principal component analyses used within the Tanagra software generated score plots which meant to give visual clustering of the different omeprazole generic medicines but indeed only few separations could be observed. Throughout PCA analysis of all spectroscopic investigations, the omeprazole generic medications did not separate and clustered tightly, but they were rather spread in the same areas, overlapping. Only in the PCA algorithm obtained from the SERS analysis of the external areas, Losec, Rambaxy and Esopral showed to be clustered in separated groups, even if not tightly, presenting then the best separation power. This consideration is in line with previous articles that reported how the enhanced sensitivity of SERS over conventional Raman leads to an improved selectivity which would allow for better discriminatory abilities [29, 30]. The PCA results obtained ranged around 98-99% of variability for all the categories of set of experiments performed.

In this case, cross validation and leave-one-out used to validate the accuracy of the predictive models gave non-positive outcomes, with a considerable amount of error. The total error rates for all the PCAs executed were well over 75%, both from cross validation and from leave-one-out evaluation, showing that the multivariate analysis in

this study did not presented good discrimination power. There was no strong spectroscopic evidence in the factor loadings for omeprazole generic medications. This is not unexpected given spectral interferences from the excess bulk agents present in the formulation already mentioned. These results substantiate the fact that the classes of omeprazole studied are extremely similar.

Because of the need of additional information on the chemical structures of these samples to be able to discriminate differences amongst them, the use of NMR was considered to elucidate more chemical diversities. While Raman and FT-IR spectroscopies directly target each part of the molecular structure of a given compound providing information related to the functional groups present, NMR gives information on the chemical structure of the analyte. NMR analyses of the seven classes of omeprazole investigated present some remarkable point of discrimination. Olzep and Losec showed same formulation, with slightly different ratios. GPO, Hycid, Olzep and Losec resulted to be very similar, with similar amount of both lactose and cellulose. On the contrary, Dexcel seemed to be the only one not containing cellulose. Rambaxy and Esopral, both levo-isomers of omeprazole racemate, contained the same chemicals, however Esopral contained considerably less API in the formulation. Both presented presence of lactose and cellulose. Esopral and Rambaxy contain another aliphatic compound, not found in the other classes of samples. GPO and Hycid resulted to be identical in each other, however GPO and Hycid contain an extra aromatic compound. Two singlets were identified in their NMR spectra which could be due to aromatic impurities not otherwise found in the other formulations. Hycid and GPO were purchased from uncertified online websites, and the parcels received were both coming from Asian regions. Because of the nature of the impurities tracked in these two generic medications, it is plausible to consider them as suspicious and potentially fraudulent products.

6.5. Conclusions

In this study it was shown that Raman and FT-IR investigations with the aid of chemometric methods did not provide a valid analytical tool robust enough to differentiate the different generic PPIs studied.

Sixteen tablets of five different omeprazole generic medications and two different esomeprazole generic medications were analysed with each spectroscopic technique, both at their surface and after breaking, internally, in order to evaluate the possible coating effect influencing the response to the analytical examination. The spectroscopic results were then exposed to chemometric investigations. The predictive models produced by multivariate analysis were then submitted to validation via cross validation and leave-one-out processing. The validations showed that the prediction models were not successfully able to separate the PPIs analysed through spectroscopic investigations. This result could be attributed to the extreme similarity of the samples investigated in terms of active pharmaceutical ingredient and of excipients.

Further investigation by proton NMR of the backbone of the molecular structure of the omeprazole formulation highlighted the similarity of the samples, with GPO and Hycid being in effect the same product, probably manufactured by the same plant and then commercialised under two different marketing names. Olzep and Losec resulted nearly identical, showing a very similar NMR spectrum, as well.

NMR investigation allowed to notice in the internet samples GPO and Hycid the presence of aromatic impurities, not detected during the Raman and FT-IR analyses. The problem of the uncertain quality of medications purchased online has been previously emphasised in this study, showing how probable it could be to obtain low quality medications when bought from un-authorised online sources.

This results are particularly relevant, highlighting the fact that while spectroscopic techniques such as Raman and FT-IR, are typically used in standard quality control tests in the pharmaceutical industry, they may miss impurities in medications produced in different manufacturer plants under different quality regimes, opening the possibility to adopt NMR in the list of analytical technique that can help to assure the quality of medicines, tackling the problem of counterfeit and substandard medications.

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7. Antiepileptic and anti-allergic medications

7.1. Carbamazepine as an anti-convulsive medication

In the cure of epileptic conditions, as in all areas of medicine therapy, generic preparations usage is becoming more prominent as pressure to control drug costs increases. In common clinical practice, the interchangeability of proprietary and generic formulations is habitually assumed. However, in individual patients for medications with a narrow therapeutic range, switching to another formulation of an antiepileptic drug with different pharmacokinetics may lead to a diverse response to the treatment. Mayer et al reported how breakthrough seizures or toxicity may originate from switching from one antiepileptic to a generic equivalent version [1]. Rahman et al. evaluated the rate of drug adverse reports related to the antiepileptic generic medications usage in the period range between January 2004 and March 2015. Their conclusion was that proof of bioequivalence between original and generic preparations for the treatment of epilepsy does not imply that they are liberally interchangeable because of their narrow therapeutic index [2].

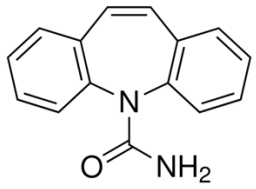

Fig.1. Carbamazepine, chemical structure

Nevertheless, the same authors reported that generic preparations with established bioequivalence to the original formulations used as reference can still be used, for example, at the beginning of the treatment or in not well-controlled patients with mid-range serum concentrations. The overall conclusion of their review was that the results demonstrate that current regulations for bioequivalence and the range of acceptability for interchangeability of generic preparations of carbamazepine are problematic [3]. A study conducted by the School of Medicine of the University of Miami on two children of age six, treated with carbamazepine, reported that after a switch from Tegretol, one main brand for carbamazepine, to a different generic carbamazepine, the serum levels of the drug increased to 22% and 41% per each patient respectively. This showed how,

despite the two formulations had been considered bioequivalent, the pharmacokinetics profile was different, leading to different rate of serum concentrations. The switch caused to the patients the insurgence of side effects such as lethargy, ataxia, slurred speech, and nystagmus. When dosage was adjusted and re-established the previous regime, symptoms of toxicity resolved [4]. It has been reported how, because of the potential for breakthrough seizures and adverse reaction related to generic substitution, prescribers need to be more attentive in their prescription-writing practices, prescribing per brand and not per generic name, to preclude unwanted substitution with generic medications [5]. In a dissolution test run on six different generic carbamazepine formulations, it has been reported how the generic formulation of carbamazepine presented extensively differences in their dissolution rate [6]. There is significative concern to recommend generic switching, and if necessary it should be performed on an individual patient basis with tight control on efficacy and adverse event throughout the changeover [7, 8]. In therapy conducted with carbamazepine, the evaluation of the rate of absorption is essential in light of the related side effects associated with different concentration as much as the effects of fluctuations and the flatness of the carbamazepine plasma concentration curve relative to the drug efficacy and tolerability [9]. Because of the narrow therapeutic index and the specific literature that highlight how important it is for the exact brand of carbamazepine to be employed in therapy, an investigation of these medications including Tegretol, the main original brand, and a generic carbamazepine, has been performed in order to evaluate if this technique could be able to differentiate the two different formulations.

7.2. Cetirizine and levocetirizine as anti-allergic medications

Histamine is one of the principal mediators involved in the pathophysiology of allergic conditions, including hay fever, rhinitis, urticaria, asthma and anaphylaxis [10]. Histamine, released from basophils and mast cells, exerts its actions interacting with one of four specific receptors, defined as H_1, H_2, H_3, H_4. In allergic disease, it is the H_1 receptor which is the pharmacological target to reduce the allergic reactions, although H_2 antihistamines may also play a therapeutic role. The main effects of the new generations of antihistamine are mediated via selective inhibition of peripheral H_1

receptors, with a reduced crossing of the blood–brain barrier, thus diminishing the sedative side-effect common with older antihistamines. H_1 antihistamines drugs remain first-line medications for the treatment of urticaria and allergic rhinoconjunctivitis. Newer generation of antihistamines, based on the use of the levorotary stereoisomers, rather than the whole racemic formulation, are preferred to their predecessors because of a better benefit-to-risk profile [10]. This second generation of antihistamines are not only more effective, but also present a reduced quota of undesired effects. Chang et al reported a clinical study conducted in South Corea where a challenge test was performed on side effects of different antihistamines [11]. A challenge test is a procedure in which patients are challenged by exposure to specified types of allergenes to determine whether they are reactive to them. In the study reported by Chang et al, urticaria resulted being induced by hydroxyzine and cetirizine (Fig.2), two first generation antihistamine, but not by levocetirizine, the levo stereoisomere of cetirizine.

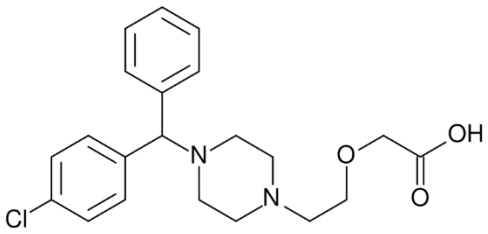

Fig. 2. Cetirizine, chemical structure

The challenge test conducted on levocetirizine resulted negative. Chang et al concluded that although the newer antihistamines are more expensive than the first generation, the cost is considerably offset by their higher potency and safety profile when used in therapeutic dosages [11]. A study conducted by Chih-Fang et al, instead, cetirizine seemed to show slight better results than the levo-isomeric formulation [12]. In the study, 74 paediatric patients affected by perennial allergic rhinitis, where split in three groups and treated with one of three treatment agents (cetirizine, levocetirizine and placebo) for twelve weeks. Nasal peak expiratory flow rate (nPEFR) and hematologic analysis in nasal smears were conducted and compared among the three groups. The results revealed that both cetirizine and levocetirizine improved the symptomatology in comparison with the placebo group, and cetirizine resulted to be more effective than levocetirizine. The eosinophil ratio in the nasal smears considerably declined among the

cetirizine treated group in comparison with the placebo group but there was no statistic significant in the levocetirizine groups [12]. Levocetirizine, which is not expose to racemization after administration, is quickly and extensively absorbed with a low rate of metabolism, resulted being a more potent antihistamine than loratadine in the treatment of human skin allergic reactions [13]. The levocetirizine's pharmacokinetic main features appear to be superior to the racemic ones. Its apparent that volume of distribution is smaller than that of dextrocetirizine, and the mostly hepatic clearance of levocetirizine is also significantly lower than that of dextrocetirizine, suggesting that the racemate should be replaced by the levo-stereoisomer [14].

In this work, cetirizine and levocetirizine, produced by the generic manufacturers Somex Pharma and Glenmark, respectively have been studied by Raman spectroscopy, in order to assess if this technique could be able to separate and differentiate the two different formulations.

7.3. Materials and methods

7.3.1 Materials

-Carbamazepine, 5H-Dibenzo[b,f]azepine-5-carboxamide, was obtained from obtained from Novartis, as brand name Tegretol, and from GenericUK manufacturer, as a generic.

–cetirizine, (2-(4-((4-Chlorophenyl) phenylmethyl)-1-piperazinyl) ethoxy) acetic acid, and levocetirizine, were obtained from the generic manufacturers Somex Pharma and Glenmark respectively.

7.3.2. Methods

Spectroscopic analysis was carried out by Raman spectroscopy, surface-enhanced Raman spectroscopy (SERS) and FT-IR. Raman analysis was performed on a FORAM 685-2 instrument with a laser operating at 532nm, to ensure high levels of sensitivity. The Raman instrument was also equipped with an integral video microscope. Analyses were performed between the wavenumbers 800 and 2000cm^{-1}.

A Perkin-Elmer Spectrum 100 was used for the FT-IR analysis. This was supported by a Motorola DSP56303 Digital Signal Processor and a near infrared detector. The FT-IR was equipped with a Perkin-Elmer Autoimage microscope, with a IR performance of 9000:1 p/p signal to noise ratio and a resolution greater than 10μm. The range of wavenumbers investigated was between 4000 and 500cm^{-1}.

Sample Preparation: eight tablets were taken for Raman analysis. To test the external side of the tablets using Raman spectroscopy the tablets were exposed to the laser at five points for each tablet, to achieve 40 repeated representative analyses for each amlodipine salt. To test the internal side, the tablets were broken in half and again exposed to the laser five times for each tablet.

SERS enhances the sensitivity of standard Raman scattering by depositing a metal colloid on the surface of the sample being analysed. For this part of the investigation, silver colloids were prepared as follows: silver nitrate was reduced using sodium citrate in water, and concentrated by centrifuging at 5000rpm. The eight tablets for SERS analysis were covered with 2μL of the prepared colloid solution and 2μL of NaCl 1M after analysis by normal Raman, and analysed immediately. To test the external side of the tablets using Raman spectroscopy the tablets were coated in the silver colloid preparation and exposed to the laser. This was again repeated five times for each tablet, with the laser directed at a variation of external sites, to achieve the 40 representative analyses for each amlodipine salt. To test the internal side, the tablets were broken in half prior to the addition of the silver colloid, and again exposed to the laser five times for each tablet.

Eight tablets were also taken for FT-IR analysis. To study the external side of the tablets by FT-IR the samples were prepared by scratching the surface of the tablets onto the ATR plate with a small spatula. This was done in order to attain adequate contact of the sample on the ATR crystal, so a successful spectrum could be produced. Due to the shape of the tablets this contact would not have been achieved if the tablet was kept intact, and would have resulted in poor transmittance. To study the internal side, the tablets were ground to ensure homogenisation and subjected to the aforementioned procedure. Each tablet was analysed individually using a scan cycle of 40.

Principal Component Analysis (PCA), Factor Loadings, Cross Validation, Leave-one-out were performed using Tanagra™ data mining software (University of Lyon, France). Analytical data were exported from the correspondent analytical apparatus to an excel spreadsheet and subsequently uploaded onto Tanagra.

7.4. Results and discussion

7.4.1. Raman spectroscopy of carbamazepine

Carbamazepine, whose IUPAC name is 5H-Dibenzo[b,f]azepine-5-carboxamide, presents a tricyclic aromatic molecular structure characterised by the presence of a carboxamide group (Fig.1).

In this study, eight tablets of 200mg Tegretol, from Novartis, and 200mg of generic carbamazepine, from GenericUK, were exposed to Raman and SERS analysis, as showed in the previous chapters, in order to evaluate the response of these samples to this analytical technique. Tablets have been tested on their surface and inside, to test possible coating interaction with the investigation and then the spectroscopic results were submitted to statistical analysis.

The spectra obtained from the traditional Raman analyses of the external and internal sides of the tablets of generic and branded carbamazepine are reported in Fig.3, while the SERS analyses are shown in Fig. 4. All the spectra resulted being not significantly differentiated. The analyses of the internal sides of the tablets performed by Raman produced identical spectra to the ones obtained from surface analyses. The same consideration could be express in reference to the SERS analyses. Structure specific peaks observed in the spectra were attributable to the molecule of carbamazepine and other signals related to the structure of the lactose used as a main excipient.

Analysing the spectra, at 873cm^{-1} it is possible to notice signals possibly corresponding to the C-C stretching of lactose, used as main excipients for the manufacturing of the tablets. The aromatic ring chain vibrations may be demonstrated by the signals visible at 985cm^{-1}, while the peak at 1027 and 1043cm^{-1} could be assigned to the carboxamide symmetrical stretching. The carbonyl group is linked to two well different nitrogen

substitute, one being a primary amine and the other a tertiary amine, the incorporated in the tricyclic structure.

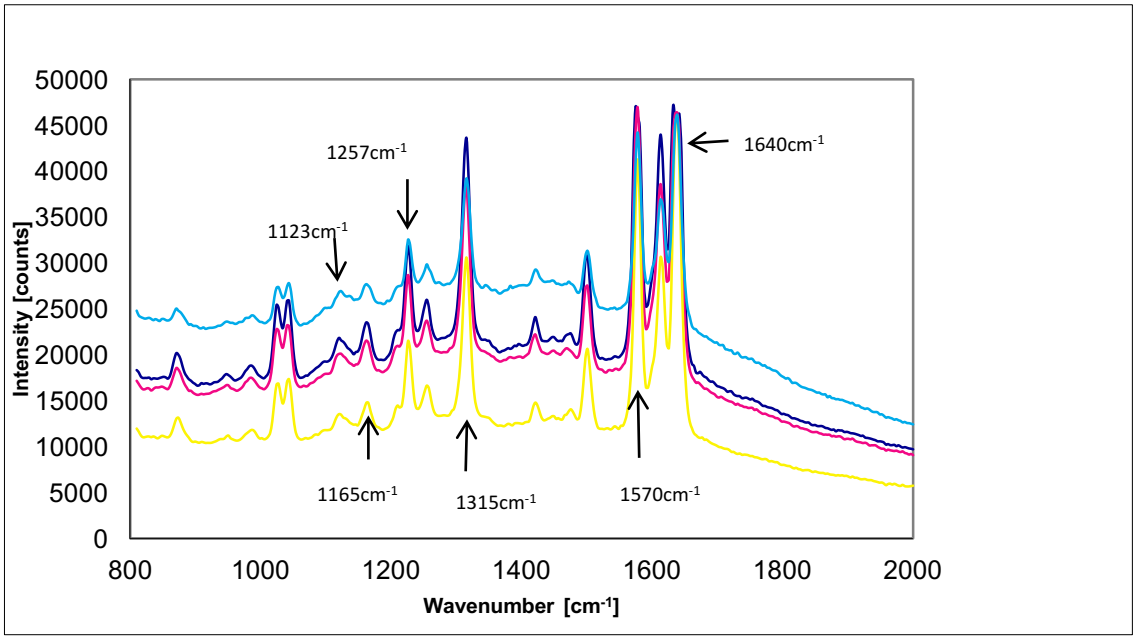

Fig. 3. Baseline corrected conventinal Raman comparison generic and Tegretol. Tegretol external (light blue line), carbamazepine generic external (dark blue line), Tegretol internal (yellow line), carbamazepine generic internal (purple line). At 1123cm^{-1} the vibrations C-O-H bending mode of lactose. At 1165cm^{-1} the NH_2 twisting peak group within the carboxamide group. At 1257cm^{-1} peak due to the =CH symmetric rocketing. At 1315cm^{-1} peak due the CH_2 in plane twist of the sugar excipient. At 1570cm^{-1} peak due to the aromatic C=C stretching of the three aromatic rings. At 1640cm^{-1} peak at carbonyl C=O unit stretching in the carboxamide group.

The diversity of the two different amines can generate the production of two distinctive emission signals [15]. Clearly visible is the intense band 1123cm^{-1} which could be corresponding to the vibrations C-O-H bending mode of lactose.

The NH_2 group within the carboxamide group is accounted for by the NH_2 twisting peak at 1165cm^{-1}. The peak at 1257cm^{-1} could be attributable to the =CH symmetric rocketing. The signal at 1315cm^{-1} could be related to the CH_2 in plane twist of the sugar excipient. Furthermore, at 1424cm^{-1} the CH_2 scissoring of the lactose could be noticed. The signals in the region of 1570cm^{-1} could be referred to the aromatic C=C stretching of the three aromatic rings, while the peak at 1613cm^{-1} may be related to the scissoring of the primary amine. The sharp signals in the region around 1640cm^{-1}are justified by the presence of carbonyl C=O unit in the carboxamide group.

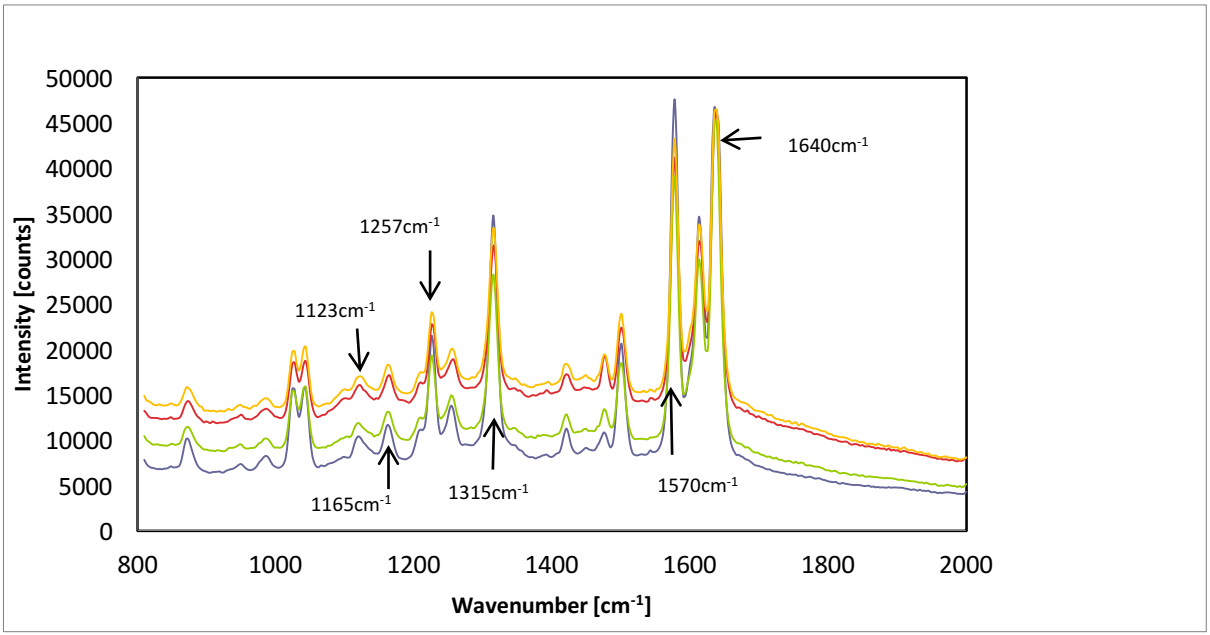

Fig. 4. Baseline corrected SERS comparison generic and Tegretol. Tegretol external (orange line), carbamazepine generic external (red line), Tegretol internal (green line), carbamazepine generic internal (blue line)(Baseline corrected). At 1123cm^{-1} the vibrations C-O-H bending mode of lactose. At 1165cm^{-1} the NH_2 twisting peak group within the carboxamide group. At 1257cm^{-1} peak due to the =CH symmetric rocketing. At 1315cm^{-1} peak due the CH_2 in plane twist of the sugar excipient. At 1570cm^{-1} peak due to the aromatic C=C stretching of the three aromatic rings. At 1640cm^{-1} peak at carbonyl C=O unit stretching in the carboxamide group.

As seen before, to aid the interpretation of the large amount of data generated by the Raman and SERS spectroscopic techniques, chemometric analysis was applied.

Chemometric measurements of the conventional Raman results, led to a Principal component (PC) 1 explained 94.47% of the variability, with PC2 and PC3 being accountable for 4.2% and 0.2% respectively. Based on the The Kaiser-Guttman criteria, which advises to only use PCs with variation greater than 1, PC3, being responsible for less than 1% of the variation was not taken into account. PC1 and PC2 are therefore responsible for an accumulative 98.67% of the variation. Visually it was not possible to cluster in defined separate groups the samples analysed. This observation confirms the overlapping of the spectra produced by normal Raman (Fig. 2). The lack of visual separation in the PCA results could give some information on the extremely high similarity in the composition of the two different classes of samples. The list of excipients, reported in chapter 3., reflects this similarity of the two different carbamazepine, prepared with the same bulk agents.

When the PCA model was submitted to validation, the cross validation generated an error rate around 86% and leave-one-out around 84% to reiterate that this

specific model was not producing a significant separation of the two classes of samples and more specific setting of the analytical approach was necessary.

Chemometric measurements of the SERS results, led to a PC1 which explained 95.63% of the variability, with PC2 and PC3 being accountable for 2.2% and 0.5% respectively. PC1 and PC2 are therefore responsible for an accumulative 97.83% of the variation. The PCA representation was very similar to the one obtained by conventional Raman and the subsequent validation gave similar error rates in terms of cross validation and leave-one-out, around 84% and 79%, respectively.

7.4.2. Raman spectroscopy of cetirizine

Conventional Raman and SERS spectroscopy of the cetirizine and levocetirizine samples are reported in Fig 5 and 6, respectively. The spectra obtained from the conventional Raman analyses of cetirizine and levocetirizine are reported in Fig. 5, while the correspondent SERS analyses are shown in Fig. 6. Cetirizine chemical structure is characterised by the presence of two phenyl rings, a piperazine ring and a carboxylic group (Fig.2). All the spectra resulted being not significantly differentiated one from each other. The analyses of the racemic cetirizine and the levo-isomer levocetirizine performed produced virtually identical spectra both in conventional Raman and in SERS. Structure specific peaks observed in the spectra were attributable to the molecule of cetirizine and other signals might be related to the structure the main excipients used in the formulation of the tablets analysed.

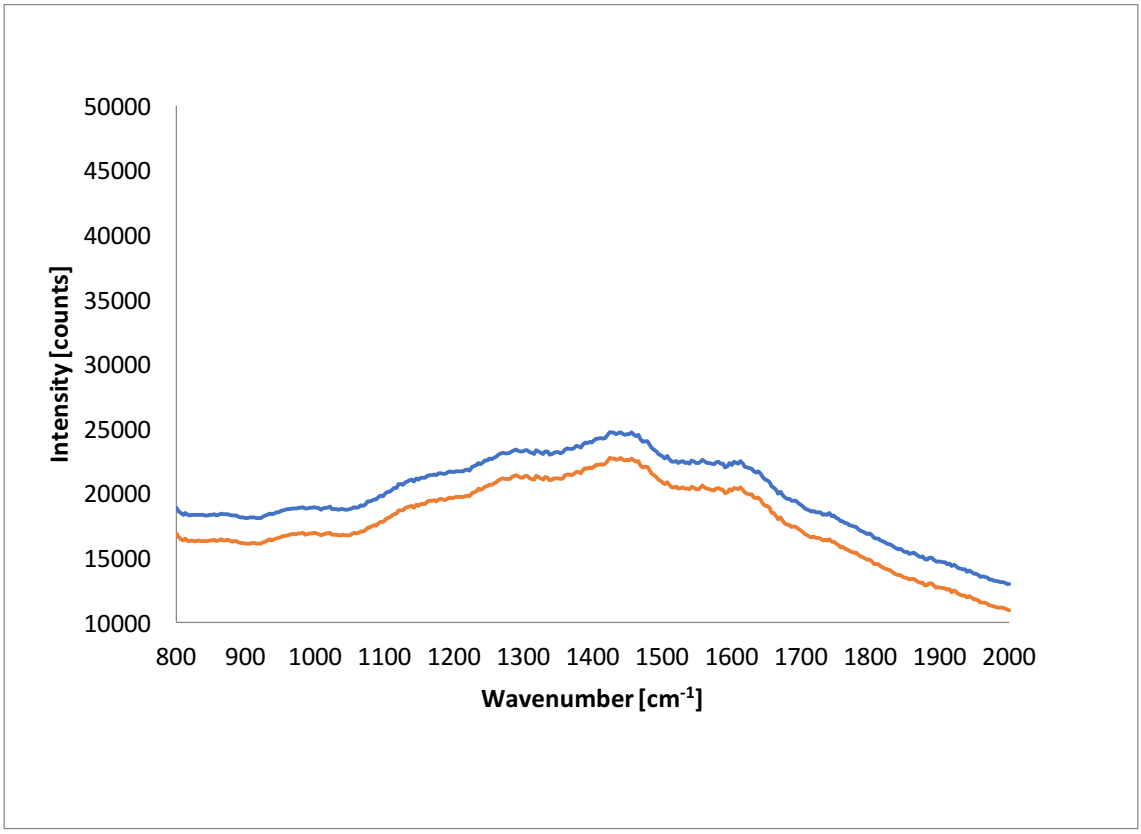

Fig. 5. Typical spectrum obtained from conventional Raman analyses of cetirizine (blue line) and levocetirizine (orange line)

A broad peak possibly attributable to the CN stretching of the piperazine is visible at 1050cm^{-1}. At 1424cm^{-1} the CH_2 scissoring of the lactose used as excipient could be noticed 1450cm^{-1}. A broad peak typical of C=C stretching of the phenyl ring are evident at 1630cm^{-1}.

This results were quite unexpected, considering that from the chemical structure (Fig. 2) cetirizine, as racemic or isomeric, is Raman active. Nevertheless, chemometric analysis was applied as aid for the interpretation of such spectrograms, both for the ones generated by normal Raman and for the others obtained by SERS.

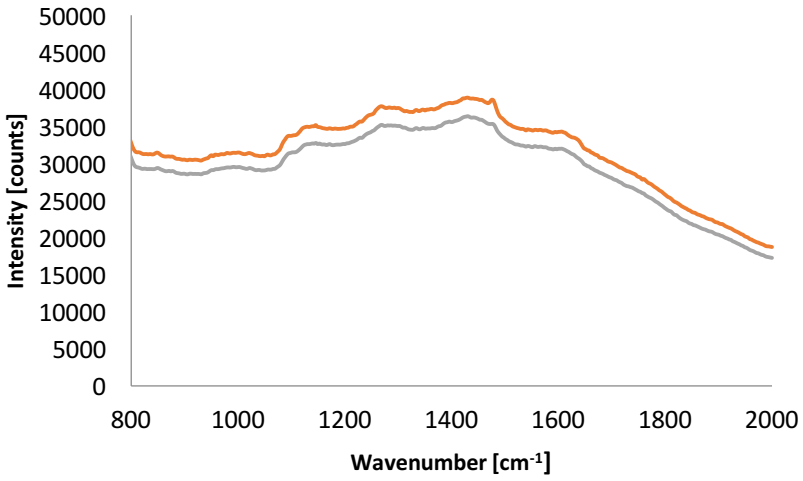

Fig. 6. Typical spectrum obtained from SERS analyses of cetirizine (grey line) and levocetirizine (orange line)

In conventional Raman, Principal component (PC) 1 explained 96.2% of the variation, with PC2 and PC3 being accountable for 2.2% and 0.6% respectively. PC1 and PC2 were consequently responsible for an accumulative 98.4% of the variability (Fig. 7).

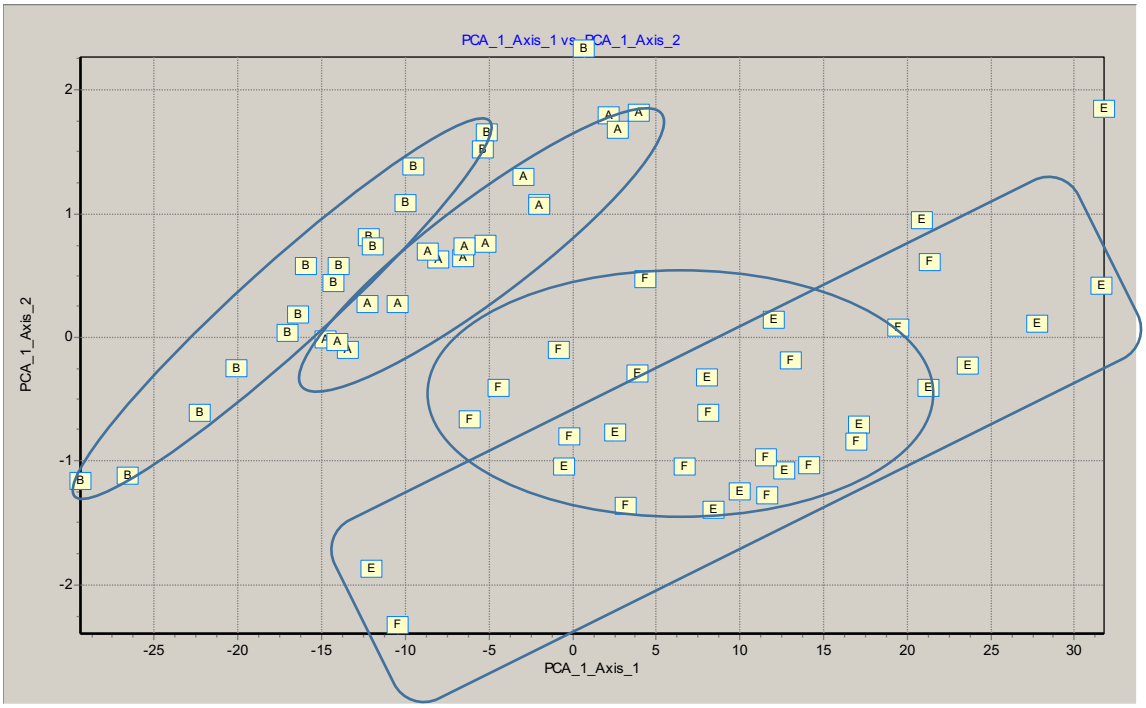

Fig 7. Principal Component Analysis scatterplot of the results obtained from conventional Raman spectroscopic analyses performed on cetirizine (A: cetirizine external; B: cetirizine internal) and levocetirizine (E: levocetirizine external; F: levocetirizine internal).

Visually it was possible to find clusters to separate the groups of the cetirizine samples, while the levocetirizine samples grouped in two overlapped clusters.. Nevertheless, these results were expected because of the overlapping spectra. Cross validation gave an error rate of around was of 88% and leave-one-out of 84%.

The method setting for the Raman analysis needs to be improved with different data acquisitions by adjusting the exposure time.

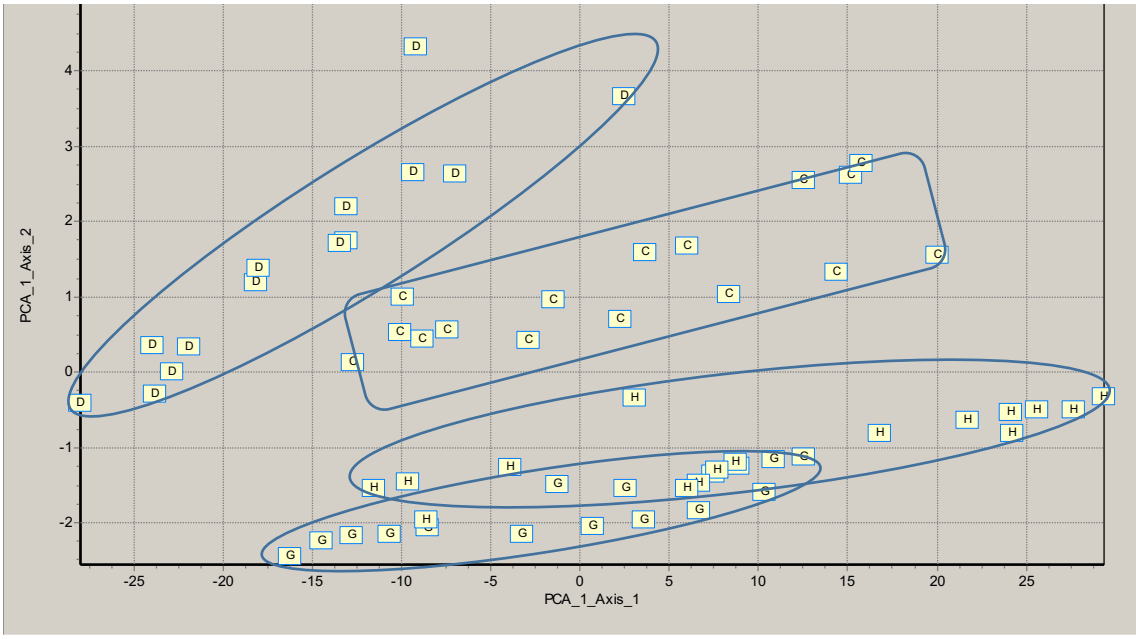

Fig 8. Principal Component Analysis scatterplot of the results obtained from SERS spectroscopic analyses performed on cetirizine (C: cetirizine external; D: cetirizine internal) and levocetirizine (G: levocetirizine external; H: levocetirizine internal).

In SERS, Principal component (PC) 1 explained 95.1% of the variation, with PC2 and PC3 being accountable for 1.8% and 0.5% respectively. PC1 and PC2 were consequently responsible for an accumulative 96.9% of the variability (Fig. 8).

Visually, the clusters of the cetirizine samples resulted to be seprated, while the levocetirizine samples grouped in two overlapped clusters, similarly to the conventional Raman model. Validation error rates of the model were very similar to the previous one, with cross validation which gave an error rate of around was of 82% and leave-one-out of 83%.

7.5. Conclusions

7.5.1. Raman spectroscopy of carbamazepine

It is supposed from the results obtained by the spectroscopic analyses of samples of carbamazepine, that there are not enough spectral differences between the two different classes samples of carbamazepine generic and branded to successfully separate the two different formulations. Nevertheless, both normal Raman and SERS technique provided peaks characteristic of the carbamazepine backbone. An improvement in the investigation of these sample is therefore required, concentrating on the analyses of the correspondent excipients under the same analytical condition to be able to interpreter the interferences deriving from the bulk agents used in the manufacturing of the tablets. An improvement in the analytical setting it is also required, to explore the possibility to increase the sensitivity of the method in order to detect more discriminant bands. Furthermore, it would be worth to consider to extend the range of generic carbamazepine manufactured by different companies to submit to examination in order to enhance the statistical value of the investigation.

7.5.2. Raman spectroscopy of cetirizine

Cetirizine and levocetirizine did not give a strong analytical response to the Raman investigations. This was an unexpected outcome, considering the structure of cetirizine, equipped with Raman sensitive functional groups. The chemometric of the spectroscopic instigations gave good report, but it was not supported by the validation of the predictive model. This negative results needs to be explored more in depth, giving a good opportunity to improve the development of the analytical setting for the analysis of medications in tablet form.

7.6. References

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8. Conclusion

A significant paradigm continuously frequented in pharmacology is the confliction between views on generic medications. Generic substitution has become common practise amongst healthcare professionals since generic medications can be used interchangeably with the original medicines. Despite this conventional normality, there are concerns in the healthcare sector, as well as amid patients, that not all generic preparations are equally clinically effective amongst themselves and their brand siblings.

Counterfeiting of medications is a serious and rapid growing danger for the patient's health and the pharmaceutical industry around the globe, with medications counterfeiting embracing not only replicas of branded drugs but also of generic medications. Fraudulent medications range from products without any active therapeutic ingredients to those with unsafe toxicological contents. Because of the anonymous nature of the internet web-sites, the risks of entry into the market of medications of uncertain quality is remarkably high, being the quality control performed by the government authorities significantly more difficult and consequently less effective.

The main aim of this research was to develop a non-destructive quick qualitative analytical methodology, a flagging system, to be able to discriminate differences in the chemical composition from generic medicines, that have been reported not presenting similar therapeutic equivalence in previous clinical comparison studies, originated from the same brand-name medications and also obtained from different sources, such as authorised pharmacies and non-authorised internet sellers.

This work has matched the aims initially set, generating novel methods of analysis to investigate differences in the chemical composition within groups of generic medications.

Digoxin, one of the main cardiac glycosides medication with narrow therapeutic index, has shown to have a strong analytical response under investigation by

voltammetric analysis. In this study we have investigated the suitability of solid electrodes as carbon glass, gold and silver electrodes in voltammetric analysis of this active pharmaceutical ingredient. The study of digoxin with Au and Ag electrodes in solution at different pHs, not reported to have been performed before, showed that both electrodes are suitable for the analytical determination of digoxin standard in solution in the range of concentrations from 3.92 to 14.81μg/mL, which considering that syrup of digoxin solution used in therapy are typically of a concentration of 50 μg/mL, resulted to be in the normal therapeutic regime. Micromole sensitivity has been achieved for gold and silver electrodes, showing that the use of these sensors could represent a preliminary analytical approach when the more accurate alternative choice is not necessary, being more practical and less environmental impacting than using mercury.

In the study of amlodipine, Raman spectroscopy and FT-IR have produced evidences to produce enough spectral differences, when coupled with chemometric methods, to successfully separate generics. The proposed method gives a level of sensitivity and selectivity never before achieved with alternative techniques, as well as being fast and requiring minimal sample preparation. This study presents a method that could have large applications in the pharmaceutical industry, distinguishing between three salt forms of a pharmacologically active compound, for which some argue can produce unrequited side effects. This is the first report to successfully identify and characterise amlodipine besylate, maleate and mesylate by Raman, SERS and FT-IR spectroscopy.

In the PPIs study it was shown that Raman and FT-IR investigations with the aid of chemometric methods did not provide an immediate valid analytical tool, robust enough to differentiate the different generic PPIs studied. The validations showed that the prediction models were not successfully able to successfully separate the PPIs analysed through spectroscopic investigations. These results could be attributed to the strong similarity of the samples investigated in terms of active pharmaceutical ingredient and of excipients showing that neither Raman nor FT-IR were analytically powerful enough to discriminate the subtle differences in the samples. Investigation by ^{1}H NMR of the backbone of the molecular structure of the omeprazole formulations

proved to be a valid and technique to utilise when the results from the Raman and FT-IR resulted not be adequate. By the use of ^{1}H NMR it was possible to discover that the samples obtained from unauthorised websites, showed aromatic impurities, not detected during the Raman and FT-IR analyses. These aromatic impurities were not found in the other omeprazole generic medications, obtained from authorised pharmacies. These results led to the consideration that while spectroscopic techniques such as Raman and FT-IR, are typically used in standard quality control tests in the pharmaceutical industry, they may miss impurities in medications produced in different manufacturer plants under different quality regimes.

This study has led to the creation and interpretation of novel knowledge, through a systematic acquisition and understanding of a substantial body of knowledge and through original research, and adjusting the project design in the light of unforeseen problems, conceptualizing, designing and implementing the research project for the generation of new knowledge.

9. Future work

Using the analytical approaches optimized, plan for future work will include:

1. Medications will be acquired through different internet websites and analysed in order to investigate on their authenticity. Internet pharmacy market is an up-coming concern for UK official authority. The Medicine and Health care products Regulatory Agency is strongly campaigning to raise attention on the risk of an unregulated pharmaceutical market. Recently worldwide, countries have granted permission to e-pharmacies to legitimate dispense medications through the internet market. Most of the e-pharmacies have passed all the strict regulations applied and have started to offer their service on-line, allowing patient to benefit of the convenience to buy online medications. Unfortunately, aside the legally recognised e-pharmacies, on the web operate unscrupulous online pharmacies that represent a major concern to the sector.

2. Use of Raman and FT-IR to possibly investigate on cardiac glycosides to differentiate generic and branded medications. At the present time, no study of this matter has been developed yet.

3. A structural comparison study of the three different salts of amlodipine currently used in cardio-vascular clinic, extended to more amlodipine generic medicines procured online from un-official websites in order to investigate the issue of fraudulent medications commercialised on line.

4. A study of omeprazole, esomeprazole generic medications characterized by Raman and FT-IR, and NMR showing how these two medications can be investigated with these three techniques, not mentioned in literature, so far. The study will be extended to more omeprazole generic medications procured online from un-official websites.

5. Clinical studies on anticonvulsants generic medications have reported having shown different therapeutic performances. Generic carbamazepine and Tegretol

as anticonvulsant will be analysed using the set of analytical methods shown before, extended to medications purchased from internet.

6. Extended the analysis of the aforementioned medications to other analytical techniques as X-Ray Fluorescence (XRF) and X-Ray Diffraction (XRD) spectroscopies in order to expand the characterization of these medications with the aim to find more differential elements able to discriminate genuine from fraudulent products. In fact, counterfeit medicines are often not only adulterated with organic chemical substances of different crystallographic structures but some samples may be contaminated by the presence of harmful mineral compounds. XRF and XRD spectroscopies are multi-elemental fast analytical methods which requires a minimal sample preparation. While XRF allows identification and quantitation of the inorganic compounds, X-ray diffraction has the ability to distinguish between various polymorphic structures both of the active principal ingredient and of the excipients allowing the crystalline phases in various pharmaceutical mixtures to be clearly resolved.

Glossary of terms

API

Active Pharmaceutical Ingredient, it is the molecule present in a pharmaceutical formulation responsible for the pharmacological activity exerted.

Bioavailability

It is the relative amount and the rate at which the API which enters into the systemic circulation.

Bioequivalence

Two o more pharmaceutical products are deemed to be bioequivalent when, administered at same molar dosage, their rate and extent of absorption lay within predetermined limits from those of the reference product.

Clinical Trial

Organised study of pharmaceutical products in human subjects with the object of acquire clinical information on the clinical, pharmacodynamic, pharmacokinetic, toxicological features, with the aim of determining their efficacy and safety.

C_{max}

This is the maximum drug concentration achieved in systemic circulation following drug administration.

C_{min}

This is the minimum drug concentration achieved in systemic circulation following multiple dosing at steady state.

GERD

Gastro-Esophageal Reflux Disease

Good Clinical Practices (GCP) Guidelines

Good clinical practices guidelines issued by national health authorities.

Narrow Therapeutic Index The therapeutic index (TI) is the range of doses at which a medication is effective without unacceptable adverse events. Drugs with a narrow TI (NTIDs) have a narrow window between their effective doses and those at which they produce adverse toxic effects (e.g. digoxin)

Pharmaceutical Alternatives

Pharmaceutical alternatives are drug products that contain the identical therapeutic moiety but not necessarily in the same amount.

Pharmaceutical Equivalents

Pharmaceutical equivalents are drug products that contain identical amount of identical active drug ingredient in identical dosage forms, but not necessarily containing the same inactive ingredients.

Pharmacodynamic Evaluation

Pharmaceutical evaluation is a measurement of the effect on patho- physiological process as a function of time, after administration of two different products to serve as a basis for bioequivalence assessment.

Pharmacokinetics

It refers to the absorption, distribution, metabolism and excretion the drug in the body.

PPIs

Proton Pump Inhibitors

Reference Product

It refers to the pharmaceutical product which is licensed by the relevant authorities as a reference for comparing with the generic product in bio-equivalence studies.

SERS

Surface-enhanced Raman spectroscopy

Steady State

Steady state is the state when the plasma concentration of drug at any time point during any dosing interval should be identical to the concentration at the same time during any other dosing interval.

Supra Bioavailability

It is the term used when the test product shows appreciable large bioavailability than the reference product.

$T_{1/2}$

Elimination half life of a drug is the time necessary to reduce the drug concentration in the blood, plasma, or serum to one-half of its initial concentration.

Therapeutic Equivalent

Therapeutic equivalents are drug products that contain the same active substance or therapeutic moiety and, clinically show the same efficacy and safety.

T_{max}

It is the time required to achieve maximum drug concentration in systemic circulation.