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Formulation and development of Florfenicol and Flunixin meglumine Injectable solution

تحضير وتطوير تركيبة محلول دوائي للحقن مكون من الفلورفنيكول والفلونكسين مغلومين

This thesis is submitted in partial fulfillment of the requirements for the degree of master in Industrial Pharmaceutical Technology from the Faculty of Graduate Studies at Birzeit University, Palestine.

By

Nidal Batrawi

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Supervisor

Dr. Hani Shtaya

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This thesis was defended successfully on August 26, 2017, and approved by:

Examination Committee members:

Name	Signature	Date
Dr. Hani Shtaya Head of committee	……..…	……..…
Dr. Moamal Qurt Internal Examiner	……..…	……..…
Dr. Fuad Al-Rimawi External Examiner	……..…	……..…

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Birzeit, August 2017

Nidal Batrawi

Contents

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List of abbreviations

IV	Intravenous
IM	Intramuscular
SC	Subcutaneous
GI	Gastro Intestinal
API	Active Pharmaceutical Ingredient
ICH	International Conference on Harmonization
USFDA	United States Food and Drug Administration
EMA	European Medicines Agency
INN	International Non-proprietary Name
CAS	Chemical Abstracts Service
UV	Ultraviolet
MICs	Minimum Inhibitory Concentrations
MBCs	Minimum Bactericidal Concentrations
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
Max.	Maximum
Abs.	Absorption
FDA	Food and Drug Administration
Flr	Florfenicol
Flx	Flunixin meglumine
BRD	Bovine Respiratory Disease
HPLC	High Performance Liquid Chromatography

BP	British Pharmacopeia
GMP	Good Manufacturing Practices
SAL	Sterility Assurance Level
CPPs	Critical Process Parameters
CQAs	Critical Quality Attributes
USP	United States Pharmacopeia
PDA	Photo Diode Array
LC	Liquid Chromatography
UPLC	Ultra-Performance Liquid Chromatography
RP-HPLC	Reversed Phase HPLC
RSD	Relative Standard Deviation
LOD	Limit of Detection
LOQ	Limit of Quantitation
ppm	Part Per Million
QbD	Quality by Design
AR	Analytical Reagent
NMP	N-methyl-2-pyrrolidone
GF	Glycerol Formal
PEG	Polyethylene Glycol 400
PG	Propylene Glycol
EA	Ethyl Alcohol
CA	Citric Acid
LA	Lactic Acid

WL	Wavelength
Conc.	Concentration
St	Standard
Sa	Sample
BN	Batch Number
rpm	Round Per Minute
NA	Not Applicable

ABSTRACT

The combination Florfenicol and Flunixin meglumine injectable solution (Flr&Flx) is an effective antimicrobial and non-steroidal anti-inflammatory for veterinary use.

A stable and high quality injectable solution of Flr and Flx in a mixture of excipients was developed, by performing various experimental studies of pre-formulation and formulation. Achieving that was not easy, as the great variation in the nature and behavior of the active substances in solubility and stability was a real challenge during this stage. Organic solvents and co-solvents were used to overcome the problem of variation in solubility of the active substances; a 1% citric acid was used in the formulation to solve the problem of Flx instability.

A 20% ethyl alcohol was incorporated in the formula to enhance syringeability and injectability of the solution by decreasing its viscosity.

It was very important during the formulation stage to carry out some tests, such as assay test, to evaluate the formulation; therefore, developing an analytical method for the quantitative determination of the active pharmaceutical ingredient was required, by which the decision is made to evaluate the developed formulation and its critical quality characteristics and to evaluate the drug product stability.

Developing an analytical method was another challenge, because of the difficulty of obtaining a method of simultaneous quantitative analysis of two components of different solubility and polarity. For that series of trials were performed, by using different RP-HPLC chromatographic conditions. Chromatographic parameters of Flr and Flx peaks were optimized, and the second challenge has been overcome,

where a novel, valid stability-indicating HPLC method for the simultaneous determination of florfenicol and flunixin in their combined pharmaceutical dosage form, was successfully obtained. Not only that, but also the analytical validation study has been published as a novel scientific research in reputed scientific journal "*Journal of Analytical Methods in Chemistry"*, with an impact factor of 1.8, the paper can be easily accessed at the link (https://doi.org/10.1155/2017/1529280). This is a strong indication of the quality and the strength of the research and the thesis generally.

An accelerated stability study was performed as a final stage of this product development, the study was conducted as a fast prediction, in short time of tests, and approved that the drug product is able to maintain its quality attributes during shelf-life and storage conditions.

All these studies were performed in accordance with the international regulations of pharmaceutical industry, such as International Conference on Harmonization (ICH), the United States Food and Drug Administration (USFDA), and the official compendia, such as the British Pharmacopeia (BP), the United States Pharmacopeia (USP). During the product development stage, beginning of pre-formulation till obtaining the final drug product, the concept of quality by design (QbD) was considered, and building the quality in the product was focused on. That was achieved by the identification and the knowledge of the critical process parameters (CPPs) and the finished product quality attributes (CQAs), and assuring product quality and stability using a reliable analytical method.

الملخّص بالعربية (Arabic abstract)

المستحضر المركب فلورفينيكول وفلونيكسين (سائل يعطى عن طريق الحقن)، هو علاج بيطري فعال، مضاد للميكروبات وللالتهابات غير-الستيرويدية.

لقد تم تطوير مستحضر الحقن فلورفينيكول وفلونيكسين بجودة عالية في تركيبة دوائية ثابتة مكونة من المادتين الفعالتين فلورفينيكول وفلونيكسين مغلومين، في خليط من المواد الاضافية الغير فعالة، وذلك بإجراء العديد من التجارب خلال مرحلة تشكيل التركيبة. ولم يكن هذا بالامر السهل، حيث شكل الاختلاف الكبير في طبيعة وسلوك المواد الفعالة من ناحية الذوبانية والثباتية تحدياً حقيقياً خلال هذه المرحلة. تم استخدام مذيبات عضوية و مساعدات اذابة للتغلب على مشكلة الاختلاف في الذوبانية للمواد الفعالة، وتم استخدام 1% من حمض الستريك في التركيبة وذلك لحل مشكلة عدم ثباتية مادة الفلونكسين.

تم ادخال 20% من الكحول الإيثيلي في التركيبة لتحسين خواص المستحضر من ناحية السحب والحقن وذلك بتخفيض لزوجة السائل.

وقد كان من المهم جدا خلال مرحلة التشكيل اجراء بعض الاختبارات، كاختبار التحليل الكمي للمواد الفعالة في المستحضر الجاهز، وذلك من اجل تقييم التركيبة. لذلك كان من الضروري ايجاد طريقة تحليل كمي، والتي من خلالها يتم تقييم التركيبة المطورة وخصائص جودتها المهمة وتقييم ثباتية المنتج النهائي، وهذا شكل تحدياً آخر وذلك لصعوبة الحصول على طريقة تحليل كمي بالتزامن لمكونين من مكونات تركيبة صيدلانية مختلفان من ناحية الذوبانية والقطبية.

لذلك فانه تم اجراء العديد من التجارب وذلك باستخدام مختلف الظروف الكروماتوغرافية لتقنية الكروماتوغرافيكي السائل (LC). وقد تم تحسين السمات الكروماتوغرافية لقمم الفلورفنيكول والفلونكسين، وبذلك تم التغلب على التحدي الثاني، حيث تم الحصول بنجاح على طريقة تحليل كروماتوغرافيكي سائل (جديدة)، مثبتة ومتخصصة في تقييم دراسة الثباتية والتحليل الكمي للفلورفنيكول والفلونيكسين بالتزامن.

ليس هذا فحسب، بل أيضا تم نشر دراسة التثبت التحليلي كبحث علمي جديد في مجلة علمية مشهورة "*مجلة طرق التحليل في الكيمياء*" ذات عامل تأثير 1.8، ويمكن الدخول بسهولة للبحث من خلال الرابط (https://doi.org/10.1155/2017/1529280). وهذا دليل قوي على جودة وقوة دراسة التثبت والأطروحة بشكل عام.

كذلك تم إجراء دراسة الثباتية المسَرَّعة كمرحلة أخيرة من دراسة تطوير المستحضر، وأجريت الدراسة كتنبؤ سريع في اقل وقت وعدد من الفحوصات. ولقد اثبتت دراسة الثباتية أن المستحضر المطور قادر على الحفاظ على سمات جودته خلال فترة الصلاحية وتحت ظروف التخزين.

وقد أجريت جميع هذه الدراسات وفقاً للمتطلبات الدولية لصناعة المستحضرات الصيدلانية، مثل المؤتمر الدولي للتنسيق (ICH)، وإدارة الغذاء والدواء الأمريكية (USFDA)، ودساتير الادوية العالمية الرسمية، مثل دستور الأدوية البريطاني (BP) ودستور للادوية الامريكي (USP.

خلال مرحلة تطوير المستحضر، من بداية مرحلة التشكيل حتى الحصول على المستحضر النهائي، تم الاخذ بعين الاعتبار مفهوم بناء الجودة خلال التصميم، وقد تحقق ذلك من خلال تحديد الخصائص المهمة لعملية التصنيع وخصائص جودة المنتج النهائي، وكذلك تم التأكد من جودة التركية المطورة للمنتج وثباتيته باستخدام طريقة تحليل موثوقة.

Chapter one

Introduction

1 INTRODUCTION

1.1 Parenteral dosage form

The term "*Parenteral*" is defined in the dictionary as non-enteral or non-oral, this term is used in the pharmaceutical convention to describe drug products administered by injection, an example for this route of administration is intravenous (IV), intramuscular (IM), subcutaneous (SC), and other routes such as intracardiac and intraspinal.

Parenteral (injectable) route of administration is one of the most effective routes, and there are various forms of pharmaceutical products administered by this route, such as but not limited, solutions, emulsions and suspensions.

The term "*Solution"* is defined as "liquid preparation that contains one or more chemical substances dissolved in a suitable solvent or mixture of mutually miscible solvents" [1], [2].

Veterinary parenteral dosage forms are including, aqueous organic solutions, oily solutions, emulsions, aqueous suspensions, oily suspensions, and sustained release implants [3].

These preparations must be sterile and pyrogen-free, and the injectable solutions are preferable to be isotonic, and easily syringeable [4].

Parenteral preparations are characterized from most other dosage forms by sterility requirement which considered a high level requirement in the pharmaceutical formulation.

To produce a sterile pharmaceutical product, the concept of quality by design regarding sterility requirement, as well as other requirements, should be in mind from the first stage of developing and optimizing the formulation and the manufacturing process.

In general, parenteral products are more expensive than other dosage forms, because of sterility requirements [1], [2].

1.2 Advantages of parenteral dosage form

- Useful in case of drugs that cause nausea and vomiting resulting from GI irritation.
- In case of uncooperative or unconscious animal.
- Provide rapid onset of action.
- Controlled time to onset of action can be achieved by injection site and formulation type.
- Suitable for products that are affected by the stomach acidic medium, or metabolized by the GI or the rumen enzymes.
- Useful when a rapid effect is required, and in case of emergency.
- Useful when requiring local effects [4].

1.3 Disadvantages of parenteral dosage form

- Manufacturing requirements are expensive.
- Once the dose is administered, it cannot be removed.
- May cause pain and or sepsis at the injection site.

- Potential for tissue damage or local irritation upon injection.
- Administration required trained person [4].

1.4 Veterinary drug products

Veterinary dosage forms are almost containing the same pharmaceutical ingredients as human dosage forms. Some of them are containing drugs not used in humans, where some API's have been developed specifically for animal use, such some antimicrobial agents classified under sulfonamides, fluoroquinolones, macrolides, and chloramphenicol derivatives [3].

Like in human drug products, formulation of animal preparations required knowledge and fundamentals of science in pharmaceutics, pre-formulation studies, technology, dosage form design, pharmaceutical operations and quality control [5].

In addition, the regulatory rules for manufacturing, approval and marketing veterinary preparations are subject to the same international regulatory rules as human preparations, such as the International Conference on Harmonization (ICH), the United States Food and Drug Administration (USFDA), the European Medicines Agency (EMA) [3], [4].

1.5 Drug substances

The physical and chemical properties of the drug substance should be determined and examined, where these properties can impact the performance of the final product and its manufacturability [6].

1.5.1 Florfenicol

1.5.1.1 General information

Florfenicol is a phenicol antibiotic, classified under the amphenicol group of antibiotics, which includes chloramphenicol and thiamphenicol. It is a fluorinated derivative of thiamphenicol, with the chemical name 2,2- dichloro-N-1-(fluoromethyl)-2-hydroxy-2-[(methylsulfonyl) phenyl] ethyl]-acetamide. The structure of florfenicol is shown in Figure1-1.

Florfenicol is indicated for the treatment of bovine respiratory disease (BRD) in cattle. In vitro, it is considered more effective than chloramphenicol against many pathogenic microorganisms; see mechanism of action under the next section.

Of the three types of phenicols, florfenicol is the only one approved for use in veterinary medicine [7]–[9].

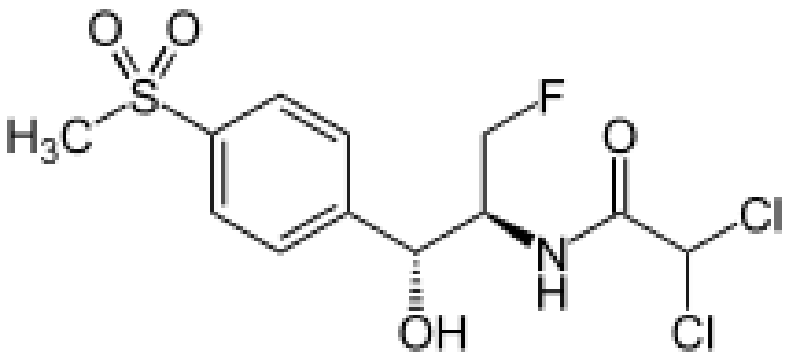


Figure 1-1 Chemical structure of florfenicol

Table 1-1 Chemical and physical properties of florfenicol

International Non-proprietary name (INN)	**Florfenicol**
Chemical name	2,2-dichloro-*N*- [1-(flouromethyl)-2-hydroxy-2-[4-(methylsulfonyl)phenyl]ethyl]-[*R*-(*R**,*S**)]-acetamide
Chemical Abstracts Service (CAS) registry number	76639-94-6
Molecular formula	$C_{12}H_{14}Cl_2FNO_4S$
Relative molecular mass	358.22
Physical description	White or almost white crystalline powder
Solubilities	Practically insoluble in water, very soluble in acetone and DMF. Soluble in ethanol (50 mg/mL), DMSO (100 mM), water (1.32 mg/mL, pH 7)
pH	4.5 to 6.5
pKa	10.73
Melting point	152^{o} C - 156^{o} C
Specific optical rotation	$[\alpha]_D^{20}$ -16^{o} to -19^{o} in methanol
UV absorption	The maximum absorption is at 266 nm in methanol

[10]

1.5.1.2 Mechanism of action:

Florfenicol is an antibiotic with a bacteriostatic and some bactericidal effect, it is acting as a protein synthesis inhibitor, where the synthesis of the susceptible bacteria protein is inhibited by binding to the 50S ribosomal subunits, causing the blocking of peptidyl transferase and inhibiting the transfer of amino-acids required for peptide and subsequent protein building.

As chloramphenicol and thiamphenicol, florfenicol site of action is the bacterial receptor. Florfenicol is considered bactericidal in the treatment of BRD, where when it is administered to achieve the MICs; the MBCs are very close to the MICs, in the treatment of some microorganisms such as Pasteurella haemolytica and Pasteurella multocida.

Florfenicol is a derivative of thiamphenicol, the only difference in their structures is that florfenicol contains a fluorine atom instead of the hydroxyl group at the C-3 location. This is an advantage making florfenicol more resistance than thiamphenicol and chloramphenicol to the inhibition by microorganisms resistant to plasmid transmissible that works by acetylating the hydroxyl group at the site C-3 in both thiamphenicol and chloramphenicol, and inhibit their binding to the 50S ribosomes [7].

1.5.2 Flunixin meglumine

1.5.2.1 General information

Flunixin meglumine is cyclo-oxygenase inhibitor analgesic, non-steroidal anti-inflammatory NSAID, used in animals to reduce pain and inflammation associated with serious and chronic disorders of endotoxic or septic shock and mastitis. Flunixin meglumine chemical name is 2-[[2-Methyl-3-(trifluoromethyl) phenyl] amino] pyridine-3-carboxylic acid, 1-deoxy-1- (methylamino)-D-glucitol [11]–[13]. The structure of flunixin meglumine is shown in Figure1-2

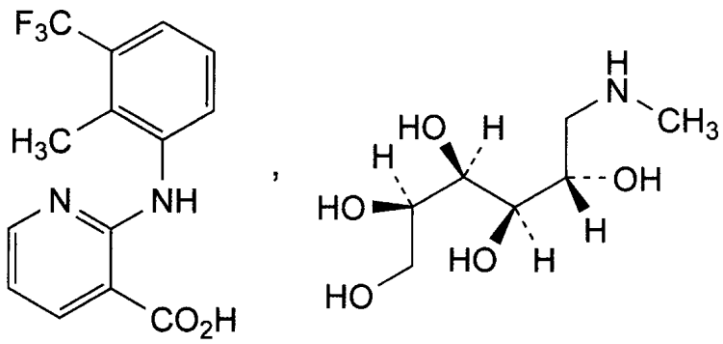


Figure 1-2 Chemical structure of flunixin meglumine

Table 1-2 Chemical and physical properties of flunixin meglumine

International Non-proprietary name (INN)	**Flunixin Meglumine**
Chemical name	2-[[2-Methyl-3-(trifluoromethyl) phenyl]amino]pyridine-3-carboxylic acid, 1-deoxy-1- (methylamino)-D-glucitol.
Chemical Abstracts Service (CAS) registry number	42461-84-7
Molecular formula	$C_{21}H_{28}F_3N_3O_7$.
Relative molecular mass	491.46
Physical description	White or almost white, crystalline powder
Solubilities	Freely soluble in water and in methanol, practically insoluble in acetone
pH	7.0 – 9.0
pKa	5.82
Melting point	137°C - 140°C
Specific optical rotation	$[\alpha]_D^{20}$ -9° to -12° in water
UV absorption	The max. abs. is at 252 nm in aqueous acid and 281 nm in aqueous alkali

[11][12]

1.5.2.2 Mechanism of action

Flunixin meglumine, like other NSAIDs, exhibits analgesic, anti-inflammatory and antipyretic activity, by blocking pain impulse generation by means of a peripheral action which inhibits the synthesis of prostaglandins. It is a very potent inhibitor of the cyclo-oxygenase, leading to decrease the formation of precursors of prostaglandins [12], [14].

1.6 Drug product

1.6.1 General information

Florfenicol and flunixin meglumine is a multidose injectable solution, contains 300 mg florfenicol and 16.5 mg flunixin as flunixin meglumine per mL.

This drug product combination is indicated to treat BRD associated with *Pasteurella haemolytica, Pasteurella multocida, Histophilus somni,* and *Mycoplasma bovis,* and to control BRD associated with pyrexia in beef and non-lactating dairy cattle [9].

There are many pharmaceutical companies, that produce veterinary medicines containing the antibacterial florfenicol injectable solution[15], [16], or the anti-inflammatory flunixin meglumine injectable solution [17]–[19], but the only product containing a combination of both API's is *Resflor Gold*® injectable solution, the brand name product by Intervet/Merck Animal Health Company [9], [20]. Moreover, by reviewing the literature, there is no generic product similar to the brand name drug produced yet.

Generic products are, according to the FDA, products similar to the brand name product with the same active ingredient, strength, dosage form, and route of administration [21].

The FDA approves Resflor Gold® injectable solution, as a New Animal Drug Application under the number NADA 141-299 [20].

In the literature, many research and comparisons proved the effectiveness of Flr and Flx combination in treating bovine respiratory disease (BRD) in cattle, compared to other drugs found in the market for the same purpose [22], [23].

1.6.2 Formulation development

Pharmaceutical dosage forms are consisting of both active pharmaceutical ingredients APIs, and excipients used to support formulation and production of the drug products [24].

Methodologies that are used in the formulation development of the veterinary preparations are the same as in human pharmaceutical preparations. These methodologies and techniques include the basics of pharmaceutical science, pre-formulation, pharmacokinetics, stability studies, manufacturing, control and sterility.

Pre-formulation is the studies that should be performed before the beginning of formulation development. It includes characterization of the physical and chemical properties of the drug substance [1].

Usually, in pre-formulation, physiochemical properties that should be initially determined are solubility and pKa, since they have the major role in determining

the initial formula. And as a first step after the drug substance characterization, it is necessary to develop a preliminary analytical method, which is required for drug quantitative analysis during this stage of development. This method could be a simple UV or HPLC method, where most pharmaceutical materials have UV absorbance [4].

1.6.3 General considerations for parenteral products formulation

1.6.3.1 Solubility

Solubility of the API in an aqueous solution is one of the main challenges during developing stage of a parenteral solution. There are various techniques to improve the solubility of poorly soluble drug, including pH alteration or the use of co-solvents or solubilizing or complexing agents [1].

1.6.3.2 Sterility

In parenteral preparations, the sterility requirement is mandatory and must be focused on at all formulation and process development stages. The regulators prefer the terminal sterilization technique for parenteral products sterilization, unless this choice is excluded, with justification, detailed information under manufacturing, section 1.6.4.

1.6.3.3 Endotoxins

Generally, parenteral products should be pyrogens free (fever causing substances) which come from microbial contamination. Endotoxins are a branch of pyrogens that are the residue of gram-negative bacteria [2].

For veterinary parenteral products, a special requirements for endotoxins test is applied, as recommended by the British Pharmacopeia BP, where the test is depending on dose volume and animal body weight, the test is required for the drug product when the single dose volume is 15 mL or more per animal and is equal to 0.2 mL or more per kilogram of animal body weight [25].

1.6.3.4 Other considerations

During the formulation of parenteral preparations, parameters such as stability, viscosity and syringeability of the drug product have to be considered. It is necessary also to carry out a comparison with the innovator product regarding these parameters. Syringeability is the ability of the parenteral solution to be easily withdrawn from the vial by a syringe and suitable needle [26], [27].

1.6.4 Manufacture

1.6.4.1 Processing

Parenteral drug products should be prepared under rigorous, good manufacturing practices (GMP) by a well designed manufacturing process, to guarantee the sterility of the drug product. Sterilization process is defined as “the complete destruction of all living organisms or their spores”[3].

Parenteral products are generally manufactured by two methods, terminal sterilization or aseptic processing.

Terminal sterilization is defined as “a final manufacturing step applied on the product in its final container, to obtain a sterility assurance level, SAL, of at least

10^{-6}, where, the product, container, and closure are not pre-sterilized, but they are with lowest bioburden at the filling stage".

Aseptic processing is defined as "a process in which the product is sterilized separately and is filled into pre-sterilized container and closure in a controlled environment" (Figure 1-3).

Product solution can be sterilized by filtration through 0.2 µm filter or less and filled into a pre-sterilized container sterilized by steam, dry heat, gas, or radiation.

Aseptic processing is generally used when heat used in terminal sterilization may affect the final product quality.

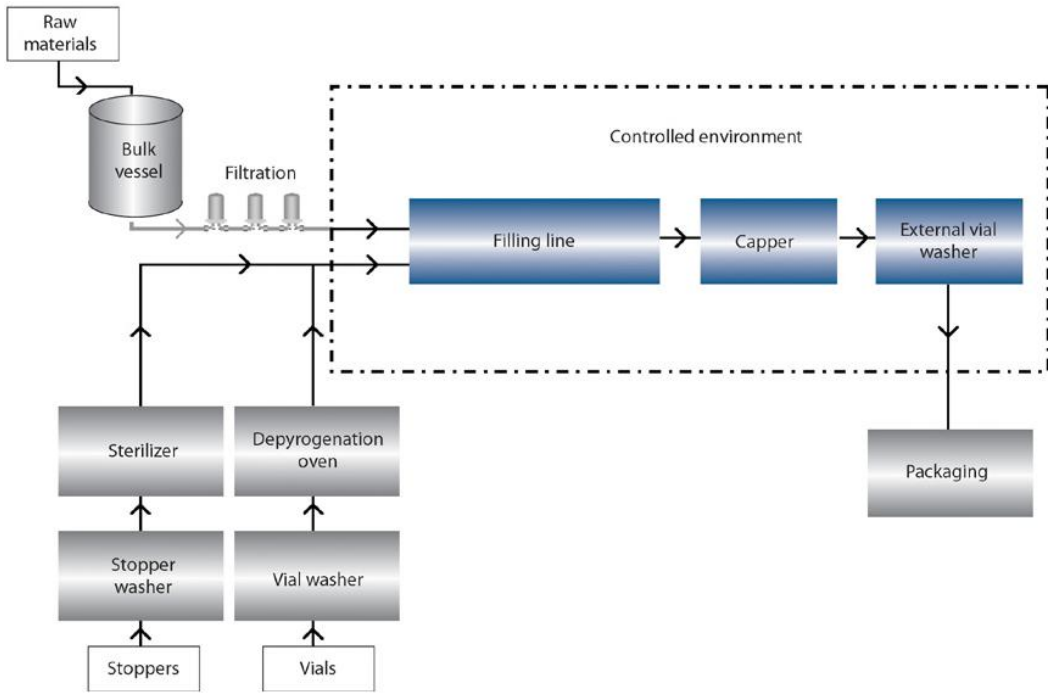


Figure 1-3 Liquid aseptic processing

In 1991, new regulations for the preparation of human and veterinary sterile drug products using terminal sterilization and aseptic processing were proposed by the USFDA.

Terminal sterilization method is preferred by the FDA as the first choice for parenteral preparation manufacturing, unless this method may impact the product quality. In this case, aseptic processing can be used, and manufacturers have to justify the reason for not using terminal sterilization technique [4].

Although the regulators are preferring the terminal sterilization method, most parenterals are manufactured using the aseptic processing, and this mostly due to the adverse effect of heat on the drug products [1].

1.6.4.2 Critical process parameters (CPPs):

The critical process parameters CPPs are defined in ICH-Q8 as "process parameters whose variability has an impact on critical quality attributes CQAs and therefore should be monitored or controlled to ensure the process produces the desired quality". Thus, CPPs should be controlled to ensure the intended final quality of the medicinal product.

Process parameters are features of the production system, related to the equipment or the manufacturing process, such as temperature, time and mixing speed; whereas the quality attributes are features related to the product, such as assay, pH, viscosity, homogeneity and sterility. CPPs may vary based on the product type, properties of the starting materials in the formulation and desired quality of the product [6], [28].

1.6.4.3 Critical quality attributes (CQAs):

The critical quality attributes CQAs are defined in ICH-Q8 as “A physical, chemical, biological or microbiological property or characteristic that should be within an appropriate range, to ensure the desired product quality”.

CQAs are usually related to the API, excipients, in-process materials and final product. Drug product's CQAs as potency, stability, impurity, drug release and microbiological attributes may impact the final product quality, efficacy and safety. Drug product CQAs may derive from the predetermined desired quality of the drug product, and they are useful during the drug product development stage. Potential critical quality attributes can be changed after the selection of formulation and manufacturing procedure.

During the development stage, critical quality attributes can be determined using quality risk management and trials that define the degree of their variation effect on the finished product quality [6], [28].

1.6.5 Selection of excipients

Excipients are defined as inactive ingredients that are used in the pharmaceutical formulation to stabilize the active ingredient and the drug product dosage form, and to improve some required properties of the medicinal preparation, such as compressibility of the API in tablet formulation and bioavailability of the drug product, and to support certain functions of the drug product such as controlled release and targeting of the active pharmaceutical ingredient, etc...

The function of excipient can be solvent/co-solvent such as PEG, propylene glycol and glycerol, surface active agent such as polysorbates, chelating agent such as EDTA, antioxidant such as BHT or antimicrobial preservative such as benzyl alcohol, etc.

When selecting excipients for parenteral perpetrations, the following points should be considered:

- Excipient effect on the final product quality, stability, and efficacy.
- The acceptable amount of excipient to be added.
- The function of excipient.
- Route of administration.
- Compatibility of excipients with each other, with the drug substance and with the container.
- Dose volume and whether the product is administered as single or multidose. Where, parenteral products should not include antimicrobial preservatives, as recommended by the USP, except the multidose products.
- Safety of the excipient usage and its permissible concentration in the formulation.
- Whether the innovator product containing this excipient is approved.
- Cost and availability of the excipient.
- Whether the excipient manufactured according to the official pharmacopeial standards [3].

Development of parenteral drug products usually requires particular considerations regarding the choice and the quality of excipients to be used.

Excipients play a major role in the finished pharmaceutical preparation; the final characteristics of the product such as safety, bioavailability, efficacy and stability are mostly dependent on the chosen excipients.

The correct selection of excipients is very important for the development of the drug product and for the enhancement of its intended quality and performance [24], [29].

The selection of excipients type and quality should be justified, and the function, concentration and properties of the selected excipients, which can affect the product quality or manufacturability, have to be discussed. It is very important to prove the ability of excipients to perform their intended use, and to maintain their function during the product shelf life [6].

1.6.6 Container and closure

1.6.6.1 Selection of the primary packing material

Container and closure for parenteral preparations should be inert with the product, and not altering the product quality. Glass vials should be clear, colorless or light amber to ease check of its contents.

The suitable glass containers types for parenteral preparations are type I, II, and III, and type I is the most resistant to chemical interaction. (Figure 1-4) [2].

Figure 1-4 Primary packing materials for parenteral preparations

The glass containers are generally more suitable than plastic containers, as they can easily sterilized and depyrogenated, and they are inert with the product.

Glass vials are sealed with rubber stoppers to permit the product solution withdrawal by syringe [3].

Rubber stoppers should be pre-sterilized using steam autoclave, where it cannot hold the depyrogenation process. A little siliconization of the rubber stoppers is require facilitating their automatic processing after vial filling with the drug product, they can be purchased pre-siliconized, washed and contained in autoclavable bags [1].

1.6.6.2 Container/Closure Integrity

It is very necessary to assure container closure integrity of the vials, throughout the shelf life of parenteral products.

Generally, this is performed by conducting sterility tests as part of the stability study at 12 month intervals, but this method alone is insufficient to assure the integrity of container closure system.

Further test, called media immersion tests, is usually carried out by most manufacturers, where sterile media filled vials are immersed in a contaminated solution and subjected to pressure [1].

1.7 Quantitative analysis:

1.7.1 Chromatography:

Chromatography is the analytical technology used to effectively separate and analyze multi components in a mixture, and is also defined as a technology by which analytes are separated by distribution or differential migration between two phases, on the basis of the analyte physicochemical properties. One of the two phases known as stationary phase or column, which is fixed, the other is the mobile phase, which moves by pressure force in a fixed flow rate, carrying the analyte through the column causing separation of the analytes from each other [30], [31].

There are various types of chromatography used in the quantitative and qualitative analysis, one of these is the High Performance Liquid Chromatography (HPLC), which is an advanced instrument and considered as the method of choice for many quantitative and qualitative analysis in the pharmaceutical field, (Figure 1-5).

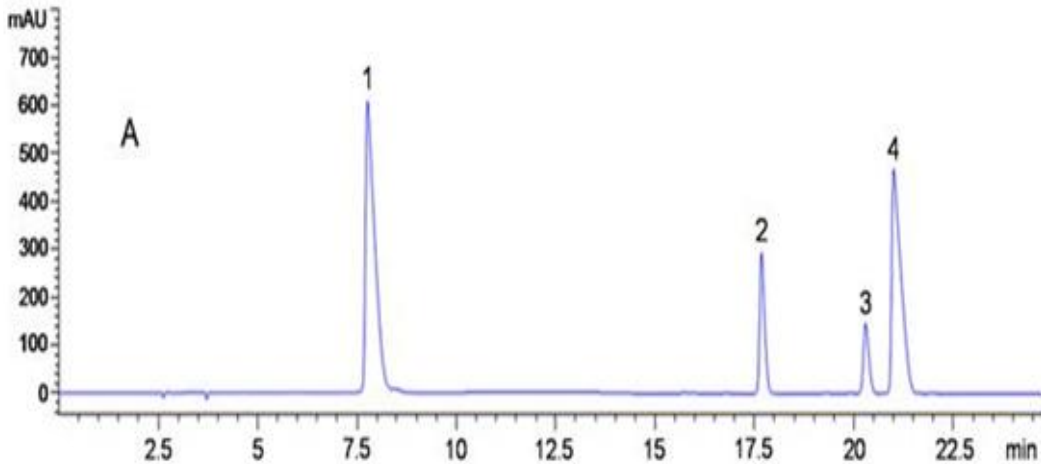


Figure 1-5 HPLC Chromatogram showing efficient separation of four components in a mixture.

HPLC methods are very specific and able to accurately and precisely analyze the intended analyte in a mixture of other components. HPLC instruments are used in many fields such as pharmaceutical, biomedical, and clinical analysis, in addition to many other applications including food, cosmetics and chemical industries.

The efficiency, availability and reliability of the HPLC equipment, make it the most preferable analytical technique in the pharmaceutical industry, in whole drug product developing stages and quality control testing.

A typical high performance liquid chromatography HPLC instrument consists of pump, injector, column, detector, and data handling system (Figure 1-6).

For pharmaceutical analysis, a more developed system is required which is almost consists of multi-solvent pump, degassing system, autosampler, column oven and PDA detector, all controlled by computerized data system software used for data processing and evaluation [30].

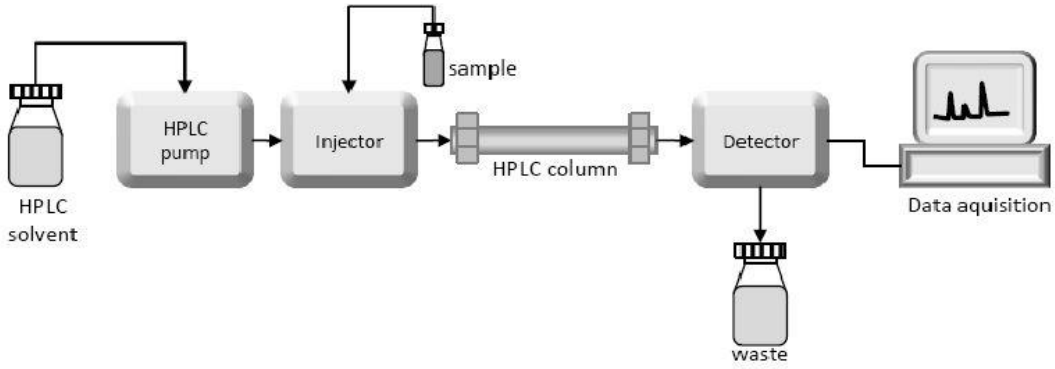


Figure 1-6 Typical HPLC system diagram

In the 1960s, the principles of HPLC were established; the development in stationary phase packing materials was continued to develop the reverse-phase HPLC technology in the 1970s.

During the 1980s, additional automated and computerized techniques were developed and added to the HPLC systems.

Many other techniques were added later including developing microcolumns, HILIC columns, specialized detectors, photo diode array PDA detection system and autosamplers, coupled with integrated data acquisition system, this led to high rise of speed and efficiency of the instrument (Figure 1-7) [3].

Recently the highly developed LC system is the Ultra-Performance Liquid Chromatography UPLC, which was introduced by Waters Corporation in 2004.

It was a real revolution in chromatography, where great advances have been added to instrumentation and stationary phase to obtain valuable improvement in speed, resolution, and sensitivity [32].

UPLC system was developed to reduce chromatographic analysis time to 9 times less than the HPLC system and to enhance peak resolution 2 times and sensitivity 3 times [30].

HPLC includes two separation modes, reversed phase and normal phase, in the normal phase the stationary phase is polar and the mobile phase is non-polar and vice versa in the RP-HPLC mode, where the stationary phase is non-polar and the mobile phase is polar.

Nowadays, the RP-HPLC is the most commonly used in pharmaceutical industry, where about 75% of quantitative analysis is performed by this mode [26].

Figure 1-7 Advanced HPLC instrument

1.7.2 Analytical method development and optimization

Before the beginning of formulation studies, a reliable analytical method should be available to be used for evaluating these studies.

Usually, most parameters that are considered in the formulation stage are required to be evaluated by accurate analytical method such as HPLC methods. It is necessary to generate new analytical method for the analysis of the main analyte in the drug products, when there is no method available in the official pharmacopeia or in the literature.

Candidate method has to be specific and able to analyze the intended analytes individually and separate excipients and degradation materials from the major analyte.

The method is preferable to be economical with a minimal analysis time. For this purpose, analytical methods such as UV spectrophotometry and titration methods are not appropriate; they cannot be considered specific or stability indicating methods.

HPLC methods are considered stability indicating, and hence they are the methods of choice in the field of pharmaceutical analysis [26], [33].

In the early development stage and with conjunction with developing a primary method, it is recommended to generate an orthogonal analytical method that works in different separation mechanism. Orthogonal methods are almost used to support the primary method and to assure that it still reliable to separate all main components [26].

Generally, the following steps should be followed to generate a new HPLC assay method:

- Reviewing the literature and the pharmacopoeias for similar analytes determination methods.
- Determination of the physical and chemical properties of the analyte including structure, polarity, pka, UV spectra, solubility and stability in solution.
- Knowledge of product formulation and strength.
- Starting the experimental HPLC method development, depending on the classical approach for that, to obtain isocratic elution method, as preferable in pharmaceutical analysis, or gradient elution method as a second choice. And this includes selection of mobile phase, selection of stationary phase, and other HPLC methods parameters such as detection wavelength, sample preparation, and injection volume, etc.
- Optimization of the generated method regarding chromatographic conditions acceptance criteria (Table 1-3) [26], [30], [34].

Table 1-3 System suitability parameters

Chromatographic parameter	Acceptance Criteria
Tailing factor, T	≤ 2.0
Resolution, R	> 2
Number of theoretical plates, N	> 2000
%RSD (n = 6)	≤ 2.0%

[34]

1.7.3 Analytical method validation

Analytical method validation is an experimental laboratory study aim to prove the suitability of the developed method is for its intended use.

Compendial methods required verification of its suitability for a specific formulation. The validated assay procedure will be used by the quality control unit, for analyzing both drug substance and drug product, to be released for manufacturing or marketing.

Many activities are involved in the analytical method during the drug product life cycle (Figure 1-8).

The method should be revalidated, if any change occurs in the API source, manufacturing, drug product formulation or in the method settings, the degree of revalidation depends on the nature of the change.

Figure 1-8 Life cycle of the analytical method

1.7.3.1 Validation parameters

Typical validation parameters (performance characteristics) that have to be evaluated are accuracy, precision, specificity, limit of quantitation LOQ, limit of detection LOD, linearity, range, and robustness. Using qualified instruments, these performance characteristics should be validated in accordance with the ICH guidelines.

1.7.3.1.1 Accuracy

Accuracy of the analytical procedure is defined as the closeness of its obtained results to the true value. Accuracy is performed by spiking specific amounts of the analyte into a placebo (mixture of excipients) to obtain three concentration levels around the test concentration. The prepared solutions are tested using the analytical method by nine determinations (three replicates of each concentration level). The percentage recovery and relative standard deviation RSD measures are used to evaluate the accuracy results.

1.7.3.1.2 Precision

Precision of the analytical procedure is defined as the closeness of its obtained results to each other. Precision is performed at two levels, repeatability and intermediate-precision. Repeatability, or method precision, is determined by six assay determinations at the 100% concentration levels on the same day. The RSD of obtained results is calculated to evaluate repeatability results.

Intermediate-precision or ruggedness is determined by performing repeatability test by another analyst on a different day and using different equipment.

The RSD of combined results obtained by both analysts is calculated to evaluate intermediate-precision results.

1.7.3.1.3 Specificity

Specificity of the analytical procedure is defined as its ability to separate the analyte from other expected interfering materials such as excipients, impurities or degradants. Specificity is performed by analyzing samples which are prepared by

spiking the drug product or drug substance with known amounts of potential impurities, degradants and excipients.

The method should demonstrate that the assay results are not affected by the presence of these extraneous materials. In case of degradation materials are not available; a forced degradation study may be conducted by submitting the drug product and drug substance to stress conditions of light, heat, hydrolysis, and oxidation. Forced degradation study solutions should be analyzed using the developed method and the degradation materials peaks should be adequately separated from that of the main analytes. Stress testing should be terminated if 5-20% degradation is obtained, or after the end of maximum recommended time if no degradation is observed. Assay methods that will be used for evaluating stability studies, should be stability indicating methods, in which the method has adequate specificity to measure the analyte in the presence of all other materials, including degradation materials, impurities and excipients.

1.7.3.1.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

Limit of detection (LOD) is the lowest amount of an analyte that can be detected, but not necessarily quantitated as a perfect amount.

Limit of quantitation (LOQ) is the lowest amount of an analyte in a sample that can be determined with suitable precision. LOD and LOQ are commonly expressed as the concentration in percentage (%) or part per million (ppm) of analyte in sample. These limits are mostly required for analytical methods applicable to impurities and degradation materials determination.

There are several approaches for calculating the LOD and the LOQ, the most common two approaches are the signal to noise approach and the standard deviation of response and slope approach.

1.7.3.1.4.1 Signal to noise approach

The limit of detection (LOD) is the concentration that gives a signal to noise ratio of approximately 3:1, while the limit of quantification (LOQ) is the concentration that gives a signal to noise ratio of approximately 10:1 with %RSD (n=3) of less than 10%. (Figure 1-9).

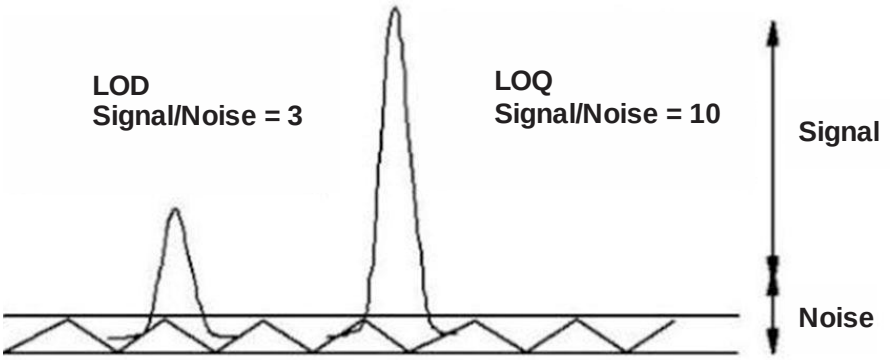


Figure 1-9 Signal to Noise ratio

1.7.3.1.4.2 Standard deviation of response and slope approach

LOD and LOQ are estimated by calculating the residual standard deviation of response (σ) and slope (S) of the regression line for low linear concentrations of samples containing the analyte. LOD and LOQ can be approximated by this approach using the following equations:

$$LOD = 3.3 \times \sigma / S \qquad LOQ = 10 \times \sigma / S$$

1.7.3.1.5 Linearity

Linearity of an analytical procedure is its ability to obtain a directly proportional relationship between the analyte concentration and its response within a given range. Linearity is determined by analyzing solutions of different concentrations using the developed method, and plotting the response versus concentration.

The obtained regression line is analyzed mathematically regarding correlation coefficient, slope and y-intercept measures; by evaluating these measures the linearity and range of the analytical procedure can be determined.

The range of the analytical method is defined as the interval between upper and lower concentration of the analyte at which the method is accurate, precise and linear. The range is usually derived from linearity results.

1.7.3.1.6 Robustness

Robustness of the analytical method is the capacity to withstand minor, intentional changes of its setting including flow rate, pH of the mobile phase, organic composition of the mobile phase and column temperature. Robustness is performed by applying little deliberate changes of the chromatographic conditions. Sample and standard solutions are analyzed for each change. Obtained data for each case is evaluated by calculating %RSD and percent of recovery.

1.7.3.2 Acceptance criteria of the method validation parameters

Before starting the validation of an analytical method it is recommended to determine the acceptable range of each validation parameter, as guidance to the researcher who is performing the experimental study. Typical acceptance criteria for analytical method validation parameters are shown in table 1-4.

Table 1-4 Typical acceptance criteria for method validation parameters

Parameter	Measure	Limits
Specificity	Peaks interference	None
Robustness	Percent of Recovery	97.0 - 103.0
	%RSD	Max 3.0%
LOD	S/N ratio	First concentration of S/N ratio ≥ 3
LOQ	S/N ratio	First concentration of S/N ratio ≥ 10 and %RSD ≤ 10%
Linearity	Correlation Coefficient	Min 0.9990
	Y-Intercept	± 3.0% of the mean
Range	Derived from Linearity	Min 80% – 120% around the test concentration.
Precision	%RSD	Max 2.0%
Accuracy	Percent of Recovery	98.0 - 102.0
	%RSD	Max 2.0%

[26], [35]–[37]

1.8 Stability study

1.8.1 General information

Stability of any pharmaceutical drug product, human or veterinary, defined as "the ability of a drug formulation in a specific container/closure system to remain within its physical, chemical, microbiological and toxicological initial specifications through its shelf life".

Through stability studies, the effect of the storage conditions on the quality of a drug is evaluated by submitting the drug product to accelerated conditions of temperature and humidity for a given period.

The purpose of performing stability studies on the developed pharmaceutical drug products is part of the drug product development to achieve the QbD concept of developing a drug product formulation, producing a product meets its predetermined specification, and quality attributes [38].

1.8.2 Types

Three types of stability testing are performed at three stages over drug product life-cycle, the purpose of each type is shown in Table 1-5 [38], [39].

Table 1-5 Stability studies types

Stage	Stability type	Objective
Development stage	Accelerated stability tests	To provide a fast prediction of how the drug formulation can maintain its quality in short time of tests, and prediction of its shelf-life and best storage conditions
Registration stage	Both accelerated and Long term studies	For the registration of dosage form and to determine shelf-life and storage conditions
After registration stage	On-going real-time stability studies	To assure that no changes of the product quality occurred during manufacturing process that may affect the stability of the product

1.8.3 Considerations

The following points should be considered when performing stability studies:

- Drug samples during stability study should be stored in special equipment called stability chamber with controlled temperature and humidity, in which we can simulate the climatic conditions according to the target zone to be studied (Figure 1-10). By using these chambers we can evaluate the product stability based on accelerated or real-time environmental conditions [38].

Figure 1-10 Stability chamber with controlled temperature and humidity

- Stability study should be evaluated using a valid stability-indicating analytical method, which provides a high degree of analytical confidence

and can specifically detect any degradation products may produced during the study or during shelf life.

- Release methods, such as spectrophotometric methods, titration methods and non stability-indicating HPLC methods are not allowable to be used for evaluating stability testing.
- It is necessary to prepare the drug product at the same strength as to be marketed. And any overages should be justified and should not exceed the 5% for antibiotics and 3% for non-antibiotic chemicals according to the Center for Veterinary Medicine.
- Tested product should be in its finished container and closure system as in market [39].

1.8.4 Selection of Batches

A minimum of three pilot batches of the drug product should be used for the study, and of the same formulation, primary packaging as intended for marketing. Two batches at least should be pilot scale batches and the third can be smaller. Different batches of the drug substance, recommended to be used in manufacturing the three batches.

1.8.5 Container Closure System

Stability study of the dosage form should be carried out on its finished primary and secondary packaging container and closure system, in which the product should be marketed [40].

1.8.6 Tests to be evaluated by stability study

Stability testing should cover all tests indicating the quality of drug product, which may affected during storage. The tests should involve physical, chemical, and microbiological tests. The following tests should be carried out during stability study for injectable solutions [39], [41]:

- Appearance, colour, clarity.
- Particulate matter (for solutions).
- API(s) assay.
- Degradation products.
- Antimicrobial preservative content.
- Sterility.
- Bacterial endotoxins.
- pH (aqueous preparations only).
- Syringeability, where appropriate.
- Stability after first opening (for multi dose only).

1.8.7 Testing Frequency

The testing frequency is scheduled depending on the proposed shelf life:

Generally, testing should be carried out initially, then every 3 months during the first year, and every 6 months over the second year, then annually to the end of the proposed shelf life (Table 1-5).

If justified, number of tests and testing frequency can be reduced according to matrixing or bracketing design [40].

Table 1-6 Stability study testing frequency

Proposed expiry date	Testing Schedule
6 months	0 (Initial), 3 and 6 months.
1 year	0 (Initial), 3, 6, 9, 12 months.
More than 1 year	0 (Initial), 3, 6, 9, 12, 18, 24, 36, 48 and 60 months.

1.8.8 Storage Conditions

It is well known that temperature and humidity are the main storage conditions that most drugs are sensitive for, therefore stability of drug products should be evaluated under appropriate tolerances of these two conditions, and this includes the stability during use (after reconstitution of the product).

The following stability cases are depending on the product storage conditions:

1.8.8.1 General case

Table 1-7 General case storage condition for stability studies

Study type	Storage condition	Duration
Long term	L1 : 25°C ± 2°C/60% RH ± 5% RH L2: 30°C ± 2°C/65% RH ± 5% RH	12 months
Intermediate	30°C ± 2°C/65% RH ± 5% RH	6 months
Accelerated	40°C ± 2°C/75% RH ± 5% RH	6 months

If justified the manufacturer can decide whether long term stability testing is carried out at L1 or L2, since the later may save time in case of failure at the accelerated condition, and no need for intermediate condition.

Product is considered stability failed if "significant change" occurs at any time during the 6 months' testing of the accelerated study, and in this case, intermediate conditions have to performed and evaluated against "significant change" criteria. In general, "significant change" according to ICH Guideline Q1A(R2), is defined as: "A 5% change in assay from its initial value; or failure to meet the acceptance criteria for potency when using biological or immunological procedures; or any degradation product's exceeding its acceptance criterion; or failure to meet the acceptance criteria for appearance, physical attributes, and functionality test (e.g., color, phase separation, resuspendibility, caking, hardness, dose delivery per actuation); however, some changes in physical attributes (e.g., softening of suppositories, melting of creams) may be expected under accelerated conditions; and, as appropriate for the dosage form: Failure to meet the acceptance criterion for pH; or failure to meet the acceptance criteria for dissolution for 12 dosage units".

1.8.8.2 Drug products intended for storage in a refrigerator (2°C -8°C)

In this case, product is considered stability failed if significant change occurs within the first 3 months testing at the accelerated storage condition, and here it is unnecessary to continue through 6 months testing.

Table 1-8 Stability studies drug products intended for storage in a refrigerator

Study type	Storage condition	Duration
Long term	5°C ± 3°C	12 months
Accelerated	25°C ± 2°C/60% RH ± 5% RH	6 months

In addition, if "significant change" occurs during the 3 and the 6 months of the accelerated stability testing, the proposed shelf life must be based on the real time data available from the long-term storage conditions.

1.8.8.3 Drug products intended for storage in a freezer

Table 1-9 Stability studies drug products intended for storage in a freezer

Study type	Storage condition	Duration
Long term	- 20°C ± 5°C	12 months

In this case, the proposed shelf life depends only on the real time data obtained at the long-term storage conditions [40].

1.8.9 Stability study protocol

Before the execution of stability study, a study protocol is demanded to be prepared and approved, which is a written plan contains a full description of what, where, why and how the stability study will be conducted.

The protocol should contain [38], [39]:

- Drug product description.
- The formulation.

- The type of dosage form.
- The proposed container and closure system.
- The climatic zones or regions where the product to be marketed.
- Sampling procedure.
- Testing frequency.
- Number of samples.
- Storage conditions (storage period, type, temperatures, humidity and packaging).
- Testing methods.
- List of equipment.
- Acceptance criteria.

1.8.9.1 Stability study report

After finishing the stability study, a study report should be prepared, which is a written document contains a full description of how the stability study was conducted and evaluated [42].

The final report should contain:

- Batches description.
- Times during storage.
- Storage conditions.
- Tabulated data and results.
- Statistics and calculations.
- Summary of results against acceptance criteria.
- Conclusion.

Chapter two

Objectives and Significance of the study

2 OBJECTIVES AND SIGNIFICANCE OF THE STUDY

2.1 Objectives of the study

- Producing a high quality, safe and effective veterinary drug product, important for the protection of animal health and for maintaining livestock productivity. Where veterinary medicinal products play an important role in controlling and protecting animal health, so thus protecting health of human who is the main consumer of animal products of meat and milk.
- Producing a veterinary pharmaceutical preparation, with a high quality and competitive price necessary for livestock farmers who are working within narrow profit margins in Palestine.
- Produce a pharmaceutical preparation using the right pharmaceutical basics and a sound scientific methodology, in accordance with the official pharmaceutical requirements and regulations.
- Performing the required pre-formulation and formulation studies.
- Developing a valid stability-indicating analytical method, in which we can confidentially evaluate the product quality during the stability study.
- Carrying out a stability study, to ensure that the drug formulation can maintain its quality during its proposed shelf life.
- Publishing the research study or part of it in an international scientific journal, to enrich the scientific research in this field in Palestine.

2.2 Significance of the study

- Parenteral dosage forms are the favorite and the most commonly used pharmaceutical dosage form in veterinary medicine, and the combination therapy of drug products is widely used in this field.
- (Flr&Flx) is a combination injectable solution of the powerful antibiotic *Florfenicol* for the treatment of bovine respiratory disease (BRD) and the fast-acting non-steroidal anti-inflammatory drug (NSAID) *Flunixin meglumine* to reduce BRD associated fever.
- Bovine respiratory disease (BRD) in cattle producing meat and milk is a main cause of the high economic loss in livestock around the world and in our country, due to high mortality and weight loss caused by the spread of such disease. Therefore, the production of such pharmaceutical preparation will greatly help to fight this disease, and thus reduce significant economic losses, especially for small livestock farmers.
- Due to high therapeutic efficacy of this combination, it is highly desirable locally.
- By reviewing the literature, this combination will be the first generic product similar to the brand name product *Resflor Gold*® injectable solution, produced by Intervet/Merck Animal Health Company [9], [20].
- Currently it is found only as the brand product (Resflor gold), which sold at a high price. The cost of the drugs is a critical point for most farmers in our country, where there is no medical insurance covering animal health like human, therefore producing this product locally with a high quality

and good competitive price will help a wide range of livestock farmers in our country.

- This combination has not been found in any pharmaceutical pharmacopoeia, and according to the literature, there was no published stability-indicating chromatographic method for the simultaneous quantitative determination of florfenicol and flunixin till I did that. Therefore, the analytical part of this thesis had the novelty to develop and publish a new, valid stability-indicating analytical method.

Chapter three

Methodology

3 METHODOLOGY

The first point of working strategy was to obtain one or more initial formula, to be evaluated by a reliable analytical method, which is to be validated later. Then the validated method is to be used for the evaluation of an accelerated stability study of the developed formulation.

3.1 Materials and reagents

All materials used in the study were of pharmaceutical grade (Table 3-1), and all reagents were of analytical grade (Table 3-2). These materials and reagents were purchased from reliable sources and donated by the Advanced Veterinary Co. Ltd.

Table 3-1 List of materials

No.	Material	Function	Manufacturer / Supplier
1.	Florfenicol	API	Hubei Longxiang Pharmaceutical Co., Ltd
2.	Flunixin meglumine	API	Qilu Synva Pharmaceutical Co. Ltd
3.	N-methyl-2-pyrrolidone	Solvent	Gadot
4.	Glycerol formal	Vehicle / Stabilizer	Elementis
5.	Propylene glycol	Preservative	Dow Chemical Co.
6.	Polyethylene glycol 400	Vehicle / Stabilizer	OXITENO
7.	Ethyl alcohol	Vehicle	Commercial alcohols
8.	Citric acid	Stabilizer	Merck
9.	Florfenicol	Reference standard	Sigma-Aldrich
10.	Flunixin meglumine	Reference standard	Sigma-Aldrich

Table 3-2 List of reagents

No.	Reagent	Grade	Function	Supplier
1.	Acetic acid, glacial	AR	Buffering / acidifying agent	Merck
2.	Acetonitrile	HPLC	Organic solvent	Sigma-Aldrich
3.	Buffer solution pH 4.0	AR	Standard solution	J.T.Baker
4.	Buffer solution pH 7.0	AR	Standard solution	Merck
5.	Distilled water	----	Solvent	-----
6.	Fluid thioglycolate media	USP	Culture media	Acumedia
7.	Hydrochloric acid (37.0%)	AR	Acidifying agent	Merck
8.	Hydrogen peroxide 35%	AR	Oxidizing agent	Carlo Erba
9.	Methanol	HPLC	Organic solvent	Sigma-Aldrich
10.	Peptone water	USP	Diluent	Acumedia
11.	Phosphoric acid	AR	Buffering / acidifying agent	Carlo Erba
12.	Sodium hydroxide pellets	AR	Alkalizing agent	Merck
13.	Tryptic soy agar	USP	Culture media	Acumedia

3.2 Instruments and equipment

The assay method development and validation tests, and all assay tests to evaluate formulation and stability of the final product, was conducted using Dionex-Ultimate 3000 HPLC system, equipped with LPG-3400SD pump, WPS-3000SL autosampler, TCC-3000 column oven, Phenomenex Luna C18 (5μm × 25cm × 4.6mm id) column and DAD-3000 UV–VIS diode array detector (Figure 3-1).

Chromeleon Data system Software (Version 6.80 DU10A Build 2826 (171948)) was used for data processing and evaluation.

All other instruments that were used in the study were highly qualified analytical instruments (Table 3-3).

Glassware including volumetric flasks, pipettes, cylinders, beakers, HPLC autosampler vials were all made of class-A glass with accurate volumes.

Figure 3-1 Dionex-Ultimate 3000 HPLC system

Table 3-3 List of instruments and equipment

No.	Instrument	Model	Manufacturer/Supplier
1.	Climatic chamber	KBF 240	Binder
2.	Digital balance	205 A SCS	Precisa
3.	Filtration system	28	Millipore
4.	HPLC #1	Ultimate-3000	Dionex
5.	HPLC #2	D-7000	Merck-Hitachi
6.	Incubator	Isotherm	ESCO
7.	pH meter	827 pH lab	Metrohm
8.	Steam autoclave #1	STE-18L	MRC
9.	Steam autoclave #2	ELV 5075	Tuttnauer
10.	Viscometer	DV1MLVTJ0	Brookfield
11.	Water bi-distiller	Aquatron A4000D	*Bibby Sterilin* Ltd.

3.3 Formulation

3.3.1 Pre-formulation

Before starting the experimental formulation, the CQAs and desired final quality and properties of the finished product such as assay, sterility, stability, safety, viscosity and syringeability were defined.

Final formulation is containing florfenicol 300 mg per mL and flunixin meglumine 27.4 mg per mL, equivalent to flunixin 16.5 mg per mL, in a mixture of excipients.

3.3.2 Selection of excipients

Depending on literature survey, characterization of the two active materials and the knowledge of their physiochemical properties such solubility and chemical stability, a number of excipients were selected to support formulation and to achieve the required final chemical, physical and microbiological properties of the developed drug product. These optional excipients include solubilizing agents, antimicrobial preservative, acidifying agents, stabilizers, complexing agent and viscosity reducing solvents.

Since the developed product is parenteral solution, solubility of the active materials is critical.

Flunixin meglumine have a good solubility in water and florfenicol solubility is better in organic solvents such as N-methyl-2-pyrrolidone (NMP), polyethylene glycol and glycerol formal.

On the basis of the two API's solubility and according to similar products containing florfenicol, NMP was found to be a main solvent in such formulation; especially that it can dissolve not only florfenicol but also flunixin meglumine as found experimentally. Other optional solvents that were selected to be used in the formulation include water, ethanol, propylene glycol (PG), glycerol formal (GF) and polyethylene glycol 400 (PEG). NMP with the candidate solvents constitute about 80% of the formulation.

Ethyl alcohol (EA) and water were selected as vehicle to reduce the drug product viscosity to appropriate level adequate for acceptable syringeability and injectability. Citric acid (CA) was used to stabilize Flunixin meglumine, while lactic acid (LA) was used as acidifying agent in formulation containing water.

3.3.3 Formulation trials

Following are formulation trials that were conducted to choose the final candidate formula and manufacturing procedure, Table 3-4 summaries these formulation trials, where trials were conducted according to the quantities and total volume specified in Table 3-4, and using different manufacturing procedures.

The main goal was to design a formula of the same pharmaceutical dosage form of the reference drug product, containing the same active materials, strength, and similar as possible to the declared excipients of the reference drug product.

The final quality properties of the developed drug product were considered during the formulation, where each trial of the following trials was performed to fulfill an improvement on certain property of the final formulation.

Table 3-4 Summary of formulation trials that were conducted to choose the final candidate formula and manufacturing procedure

Quantities of materials per 100 mL										
Material / Formula	Flr	Flx	NMP	PEG	GF	LA	CA	EA	Water	PG
Unit	g	g	mL	mL	mL	mL	g	mL	mL	mL
FF1	30.0	2.74	30.0	20.0	18.0	0.0	0.0	0.0	0.0	15.0
FF2	30.0	2.74	30.0	8.0	30.0	0.0	0.0	0.0	0.0	15.0
FF3	30.0	2.74	30.0	8.0	15.0	0.0	0.0	0.0	15.0	15.0
FF4	30.0	2.74	30.0	8.0	12.5	2.5	0.0	0.0	15.0	15.0
FF5	30.0	2.74	30.0	8.0	20.0	0.0	0.0	0.0	10.0	15.0
FF6	30.0	2.74	30.0	8.0	17.5	2.5	0.0	0.0	10.0	15.0
FF7	30.0	2.74	30.0	8.0	10.0	0.0	0.0	20.0	0.0	15.0
FF8	30.0	2.74	30.0	8.0	30.0	0.0	1.0	0.0	0.0	15.0
FF9	30.0	2.74	30.0	8.0	10.0	0.0	1.0	20.0	0.0	15.0

3.3.3.1 Formula 1 (FF1)

This experiment was conducted with the least number of excipients, as a starting point for the formulation.

Florfenicol and flunixin meglumine were dissolved completely in N-methyl-2-pyrrolidone (NMP). While continuous mixing, a mixture of about 80% glycerol formal (GF) and polyethylene glycol 400 (PEG) was added, followed by the addition of propylene glycol (PG). Then the total volume was accurately completed by GF.

3.3.3.2 Formula 2 (FF2)

This formulation trial is similar to the previous one except the difference in the ratio of PEG and GF, to examine the effect of reducing the amount of PEG on the viscosity of the formulation.

3.3.3.3 Formula 3 (FF3)

The aim of this experiment was to examine the effect of using 15% of water at neutral pH on the viscosity and stability of the formulation.

Florfenicol and flunixin meglumine were dissolved completely in NMP, then and while continuous mixing a mixture of about 80% GF and PEG was added followed by the addition of a mixture of water and PG, then the total volume was accurately completed by GF.

3.3.3.4 Formula 4 (FF4)

This experiment was formulated as FF3 except the use of lactic acid (LA) to examine the effect of low pH on the stability of the aqueous formulation containing 15% water.

3.3.3.5 Formula 5 (FF5)

This experiment was formulated as FF3 except the use of 10% of water instead of 15%, to examine its effect on the viscosity and stability of the formulation.

3.3.3.6 Formula 6 (FF6)

This experiment was formulated as FF5 except the use of lactic acid to examine the effect of low pH on the stability of the aqueous formulation containing 10% water.

3.3.3.7 Formula 7 (FF7)

The aim of this experiment was to examine the effect of ethyl alcohol (EA) on the viscosity and stability of the formulation.

Florfenicol and flunixin meglumine were dissolved completely in NMP, then and while continuous mixing a mixture of about 80% GF and PEG was added followed by the addition of a mixture of PG and ethanol, then the total volume was accurately completed by GF.

3.3.3.8 Formula 8 (FF8)

The aim of this experiment was to examine the effect of using citric acid (CA) on the stability of the active materials.

Citric acid was completely dissolved in NMP. Flunixin meglumine was then added while mixing till completely dissolved, then florfenicol was added and mixed till completely dissolved.

And while continuous mixing, a mixture of about 80% GF and PEG was added followed by the addition of PG. Then the total volume was accurately completed by GF.

3.3.3.9 Formula 9 (FF9)

The aim of this experiment was to examine the effect of citric acid on the stability of the active materials, in the presence of ethyl alcohol.

Citric acid was completely dissolved in NMP. Flunixin meglumine was then added while mixing till completely dissolved, then florfenicol was added and mixed till completely dissolved.

And while continuous mixing, a mixture of about 80% GF and PEG was added followed by the addition of a mixture of PG and ethanol. Then the total volume was accurately completed by GF.

3.3.4 Packing materials

The final drug product was filled in 100-mL amber glass vials, type II as the primary packaging material, each vial was closed with rubber stopper and aluminum cap, and is to be labelled and contained in a well designed and elegant carton box as secondary packaging material.

3.4 Analysis

Chemical and physical tests that used in the study to evaluate the formulation and the stability of the drug product were selected on the basis of the type of the dosage form and formulation, and on the basis of the required quality properties of the finished product.

All tests were pharmacopeial methods and performed using qualified and calibrated analytical instruments. Assay method was developed and validated as shown in sections 3.4.4. and 3.4.5.

Results were finally evaluated and compared against the predetermined specification and acceptance criteria as illustrated in chapter 4.

3.4.1 Physical tests

3.4.1.1 Appearance test

During the study, the appearance of the product was checked visually, and was compared with that of the patent product.

Acceptance criteria: The product is a clear light yellow solution.

3.4.1.2 Viscosity

The viscosity was measured using a Brookfield viscometer DVI, spindle type # (LV-02) # 62. The viscosities of the candidate formulation were compared with that of the reference product (*Resflor gold injectable solution*). The tests were carried out with two rotation speeds of 50 and 100 RPM, at 25^0C.

3.4.1.2.1 Acceptance criteria

Syringeability and injectability are the two factors that are affected by the viscosity of parenteral products. No acceptance criteria found in literature or in any official reference for viscosity of parenteral preparations witch suit syringeability and injectability.

Therefore, viscosity can be accepted when good syringeability and injectability are obtained.

3.4.2 Chemical tests

3.4.2.1 Assay

A fast and robust stability-indicating analytical method for the simultaneous determination of florfenicol and flunixin was developed and validated in accordance with the official and international requirements see section 3.4.4 and section 3.4.5. Following are the developed method chromatographic conditions, and sample preparation procedure:

3.4.2.1.1 Chromatographic conditions

Mobile phase was prepared by mixing 600 mL acetonitrile with 400 mL of water, and then adjusted to pH 2.8 using 2M phosphoric acid. The chromatographic conditions were run as shown in Table 3-5.

Table 3-5 HPLC chromatographic conditions of the developed method

Chromatographic conditions	
Flow rate	1.0 mL/min
Wavelength (λ)	268 nm
Stationary phase	RP18e, 5 µm, 250 x 4.6 mm
Column temperature	25°C
Injection volume	20 µL
Run time	10 minutes.

3.4.2.1.2 Preparation of standard solutions

A standard solution of florfenicol (1.2 mg/mL) and flunixin meglumine (0.1096 mg/ mL) was prepared by dissolving an accurately weighed amount of florfenicol 300 mg and 27.4 mg of flunixin meglumine in 50 mL of mobile phase, and then 5 mL of the resulting solution was diluted to 25 mL with the same solvent.

3.4.2.1.3 Preparation of sample solution

A sample solution was prepared with a concentration equivalent to that in standard solution by transferring 1 mL of the drug injectable solution, which contains 300 mg of florfenicol and 27.4 mg of flunixin meglumine, with about 40 mL of the mobile phase into a 50-mL volumetric flask, the volume was completed to mark by the same solvent, and then 5 mL of the resulting solution was diluted to 25 mL with the same solvent.

3.4.2.1.4 Calculation

$$\% \text{ Assay} = \frac{\text{Peak area of sample}}{\text{Peak area of standard}} \times 100$$

3.4.2.1.5 Acceptance criteria

90.0% to 110.0% of the labelled amounts of florfenicol and flunixin (as meglumine).

3.4.3 Microbiological tests

3.4.3.1 Sterility

Injectable preparations should be sterile; the sterility of the developed product was tested using the membrane filtration method, under United States Pharmacopeia (USP).

3.4.4 Assay method development and optimization

With regard to the physical and chemical properties of the analytes and the information obtained from the literature, analytical method was developed to select a preliminary reversed phase HPLC-UV chromatographic conditions, including detection wavelength, mobile phase, stationary phase and sample preparation procedure. For that, series of trials were performed, by using different compositions of mobile phase, different types of stationary phase and column lengths, with different pH values and buffering agents.

Final developed method was used for solution stability test and system suitability test as a part of the analytical method development and validation.

3.4.4.1 System Suitability

System suitability parameters for the developed method were performed using six replicates of a standard solution containing both florfenicol and flunixin, to verify the analytical system performance. The %RSD for both florfenicol and flunixin, and all system suitability parameters such as the column efficiency, the tailing factors and the resolution values, were calculated.

3.4.4.2 Solution Stability

The stability of solutions was performed by the assay analysis at regular intervals for 16 hours. The solution was tested every 2 hours from the initial to 16 hours. The percent of recovery and RSD for both active ingredients florfenicol and flunixin were calculated to evaluate the stability of the prepared solutions.

3.4.5 Analytical method validation

3.4.5.1 Instrumentation

Liquid chromatography assay method development and validation analysis were conducted using Dionex-Ultimate 3000 HPLC system, other equipment and tools were listed under section 3.2. All instruments and glassware used in the study were qualified and well calibrated.

3.4.5.2 Chemicals and reagents

Active materials florfenicol and flunixin meglumine working reference standards were purchased from Sigma Aldrich. All active materials and excipients were purchased from reliable commercial sources. The acetonitrile used were of HPLC grade and water was obtained by double distillation.

Other reagents such as phosphoric acid, hydrochloric acid, sodium hydroxide, and hydrogen peroxide were purchased from Merck and Sigma Aldrich.

3.4.5.3 Chromatographic conditions

See section 3.4.2.1.1.

3.4.5.4 Preparation of standard solutions

See section 3.4.2.1.2.

3.4.5.5 Preparation of sample solution

See section 3.4.2.1.3.

3.4.5.6 Validation parameters

The method was validated as per ICH and FDA guidelines for specificity, linearity and range, accuracy, precision, LOQ, LOD, and robustness [34], [43].

3.4.5.6.1 Specificity

Forced degradation study was conducted by exposing samples of the drug substance and drug product to various stress conditions of hydrolysis, oxidation, photo and thermal, the time and condition are illustrated in Table 3-6.

Stressed samples were analyzed occasionally; related peaks were checked for the retention times, peaks interference, and separation factors. The purity and homogeneity of Flr and Flx peaks were verified by purity check using PDA and by matching the peak spectra at peak start, peak top and peak end.

Table 3-6 Stress conditions applied for drug substance and drug product

Stress type	Conditions	Time
Acid hydrolysis	1N HCl; at 40°C	2 days
Base hydrolysis	0.02N NaOH; at RT	2 hours
Oxidative/solution	0.2% H_2O_2 at 40°C; protected from light	7 days
Thermal	75°C	14 days
Photo-degradation	UV light	3 days

3.4.5.6.2 Linearity

To evaluate linearity and range of the analytical method, seven different concentrations of florfenicol (480, 720, 960, 1200, 1440, 1680 and 1920 µg/mL) and flunixin meglumine (43.8, 65.8, 87.7, 109.6, 131.5, 153.4 and 175.4 µg/mL) were prepared. Three injections from each concentration were analyzed under the same conditions.

3.4.5.6.3 Accuracy

The accuracy of the analytical method was performed on three spiked concentration levels (80%, 100% and 120%) around the test concentration (florfenicol 1200 µg/mL and flunixin meglumine 109.6 µg/mL), by nine determinations (three replicates of each concentration).

The percentage recovery and RSD were calculated for each of the replicate samples.

3.4.5.6.4 Precision

Precision of the analytical method was performed at two levels, repeatability and intermediate-precision. Repeatability, or method precision, was established by six assay determinations at the 100% concentration levels on the same day. The RSD of obtained results was calculated to evaluate repeatability results.

Intermediate-precision or ruggedness was established by doing repeatability test by another analyst on a different day and using different equipment. The RSD of combined results obtained by both analysts was calculated to evaluate intermediate-precision results.

3.4.5.6.5 LOD and LOQ

Signal to noise approach was used to determine LOD and LOQ of florfenicol and flunixin, where different diluted solutions of florfenicol and flunixin were analyzed using the developed analytical assay method, and signal to noise ratio was measured.

The concentration that gives a signal to noise ratio of about 3:1 was determined to obtain the limit of detection (LOD).

The concentration that gives a signal to noise ratio of about 10:1 was determined to obtain the limit of quantification (LOQ), with %RSD ($n=3$) of less than 10%.

3.4.5.6.6 Robustness

Robustness was performed by applying little deliberate changes of the method conditions as follow:

- pH of mobile phase: ±0.2
- Temperature: ±5°C
- Flow rate: ±0.1 mL/min
- Wavelength: ±2 nm
- Mobile phase composition, organic composition ±5%

Sample and standard solutions were analyzed for each change. Change was made to evaluate its effect on the method.

Obtained data for each case was evaluated by calculating %RSD and percent of recovery.

3.5 Accelerated stability study

The study was conducted in accordance with ICH guidelines regarding selection of batches, storage conditions and testing frequency. The study results were calculated and evaluated.

3.5.1 Selection of batches

Three pilot batches of the candidate formulation of the developed drug product were prepared for the study with the same strength as the product to be marketed and without any overage.

3.5.2 Container

100-mL amber glass vial (type-II) contained in a carton box.

3.5.3 Storage conditions and testing Frequency

Sufficient samples of each batch, in its final packaging, were retained and stored at two controlled storage conditions; samples were periodically tested according to the testing program (Table 3-7).

Table 3-7 Storage conditions and testing frequency

	Temperature	Humidity	Testing frequency
Accelerated conditions	40° C	75%	0, 3, and 6 months
Normal conditions	25° C	60%	

3.5.4 Test methods

The physical, chemical and microbiological tests specified under analysis section 3.4, were performed in accordance with the testing frequency program for the retained samples.

3.5.5 Acceptance criteria

Product is considered stable if “significant change” didn’t occur at any time during the 6 months’ testing of the accelerated stability study,

In general, “significant change” according to ICH Guideline Q1A(R2), is defined as:“A 5% change in assay from its initial value, and /or any degradation products exceeding its acceptance criterion, and/or failure to meet the acceptance criteria for appearance and physical properties”.

Chapter Four

Results and Discussion

4 RESULTS AND DISCUSSION

4.1 Pre-formulation and formulation development

The quality of drug products samples obtained by trials listed under section 3.3 was evaluated, regarding chemical and physical properties using analytical tests detailed under section 3.4.

4.1.1 Experiments

As a starting point, experiment FF1 was somewhat satisfactory except for the viscosity of the solution, where some modifications were required to decrease the viscosity by decreasing the amount of PEG as done in experiment FF2, but this was not effective.

The choice of incorporating viscosity decreasing agents in the formulation, such as water and ethyl alcohol, was used concurrently in experiments FF3, FF4, FF5, FF6 and FF7.

A 15% of water was used in experiments FF3 and FF4 at two different pH values using lactic acid as acidifying agent, the viscosity was decreased but unfortunately, there was some turbidity in the solution after a few days of the preparation, due to precipitation of florfenicol.

In experiments FF5 and FF6 the water percentage was reduced to 10%, the viscosity was good in both of the experiments but some precipitation occurred in experiment FF6 which has the acidic pH.

So, FF5 experiment showed the best results of product viscosity of all experiments that used water for that purpose.

But in experiment FF7, where ethyl alcohol was used, the results regarding viscosity of the solution were better than those obtained by FF5; therefore FF7 formula was selected to be submitted for other quality parameters evaluation.

Samples of experiment FF7 were analyzed regarding the assay of florfenicol and flunixin using the developed HPLC assay method, the results for florfenicol was good but there was a significant loss in the flunixin assay.

In experiments FF8, citric acid was used in the formulation to stabilize the flunixin meglumine and was used in FF9 in the presence of ethyl alcohol, both experiments have good results for both florfenicol and flunixin assay.

Formula FF9, which have the best results regarding the viscosity of the solution and the assay of the active materials, was selected to be the candidate formula.

Samples of FF9 were retained for couple of weeks, at room temperature as fast evaluation and stability prediction of the formula before submitting other samples of it for accelerated conditions required for the official accelerated stability study.

The obtained results of analysis after 2 weeks were excellent for both physical appearance and chemical assay tests.

4.1.2 Candidate formula FF9

4.1.2.1 Composition per 100 mL

Materials with their quantities and functions of the candidate formula FF9 are illustrated in table 4-1.

Table 4-1 Materials and quantities per 100 mL of the candidate formula FF9

Material	Quantity	Unit	Function
Florfenicol	30	g	API
Flunixin meglumine	2.74	g	API
N-methyl-2-pyrrolidone	30.0	mL	Solvent
Polyethylene glycol 400	8.0	mL	Vehicle / Stabilizer
Glycerol formal	10.0	mL	Vehicle / Stabilizer
Propylene glycol	15.0	mL	Antimicrobial preservative
Ethyl alcohol	20.0	mL	Vehicle
Citric acid	1.0	g	Stabilizer

4.1.2.2 Procedure

Dissolve citric acid completely in NMP. Then add flunixin meglumine while continuous mixing till completely dissolved, add florfenicol and mixed well till completely dissolved.

Add, while mixing, a mixture of about 80% GF and PEG, followed by the addition of a mixture of PG and ethyl alcohol.

Then accurately complete the total volume to 100 mL by GF.

4.2 Analytical method development and optimization

Following are the HPLC parameters and chromatographic conditions that were used for developing the candidate analytical method:

4.2.1 Selection of mobile phase

According to the analytes physicochemical properties, a mixture of acetonitrile and water 50% : 50% v/v was selected initially as the mobile phase, adjusted to pH 4.2 with diluted acetic acid, and a flow rate of 1.0 mL/min.

4.2.2 Selection of detection wavelength (λ)

Using the PDA-UV a WL of 268 nm was selected as the optimum wavelength.

4.2.3 Selection of stationary phase

On the basis that the method will be used for separation of two analytes from each other, and also from their degradants, the RP18e stationary phase with a 250 mm length was initially selected as the column of choice.

A standard solution containing both drug substances Flr and Flx was analyzed using these isocratic chromatographic conditions.

First successful effort of eluting the two analytes simultaneously has established as shown in Figure 4-1, the florfenicol peak symmetry and column efficiency were good, but the flunixin peak eluted with poor symmetry and column efficiency.

This required carrying out some modifications in the mobile phase composition and its pH value.

Therefore, the ratio of the mobile phase components was changed to be acetonitrile and water 60% : 40% v/v and the pH was reduced to 3.0 by diluted acetic acid.

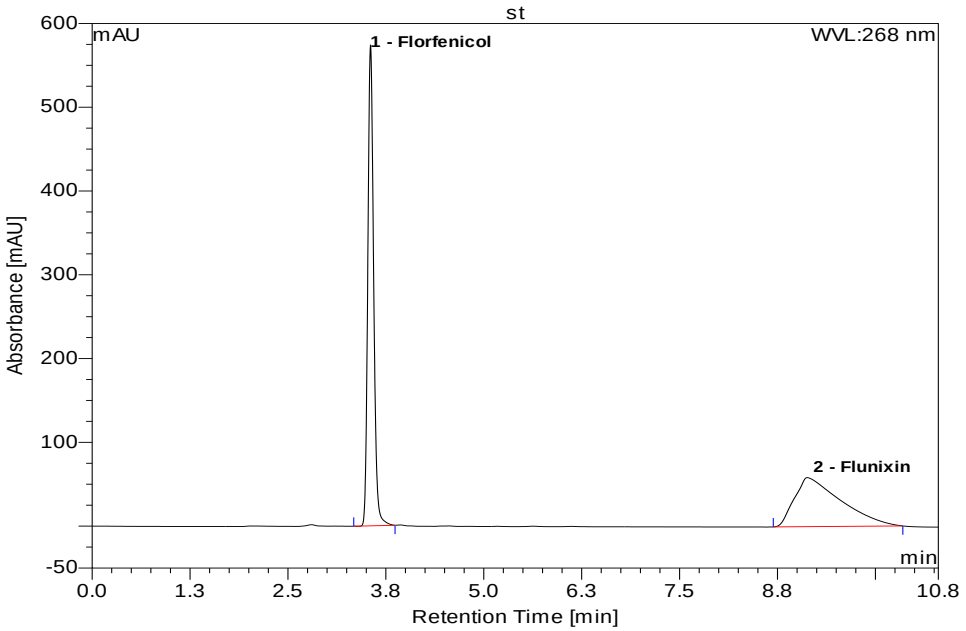


Figure 4-1 Chromatogram of the first eluted analytes

Good flunixin peak symmetry and column efficiency have obtained, but unfortunately, the florfenicol peak was affected (Figure 4-2).

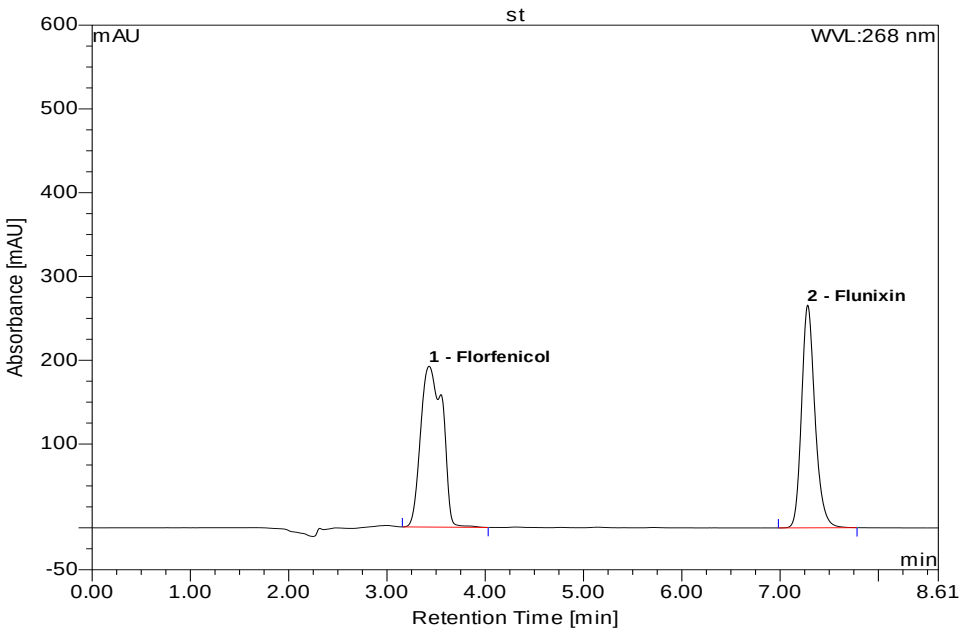


Figure 4-2 Chromatogram of the optimized flunixin peak

Additional chromatographic conditions were altered to optimize the florfenicol peak, where the pH of the same mobile phase was reduced to 2.8 by diluted phosphoric acid. As result of that, a satisfying analytical method was obtained as shown in Figure 4-3, the resolution (R) and other system suitability parameters of the obtained peaks of florfenicol and flunixin were calculated.

Placebo (mixture of excipients) was analyzed using the developed method and it did not show any response (Figure 4-6). Figure 4-7 is the chromatograms overlay of the standard, sample and placebo peaks, indicating the selectivity of the developed analytical method.

Sample of the developed formulation florfenicol and flunixin injectable solution was compared with that of the reference innovator product (*Resflor gold injectable solution*), showing good results (Figure 4-4 and 4-5).

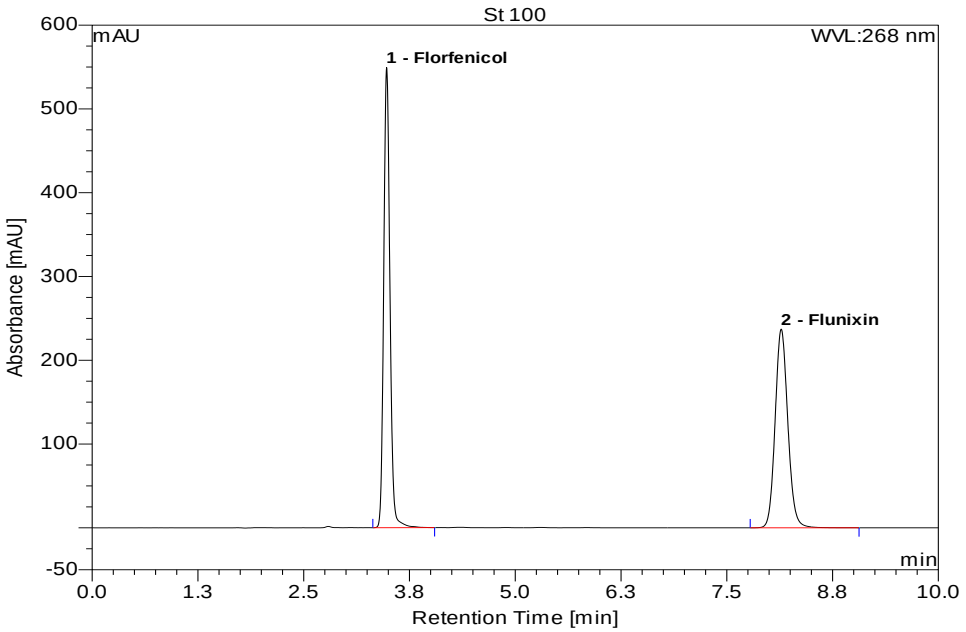


Figure 4-3 Chromatogram of florfenicol and flunixin standard solution using the developed method.

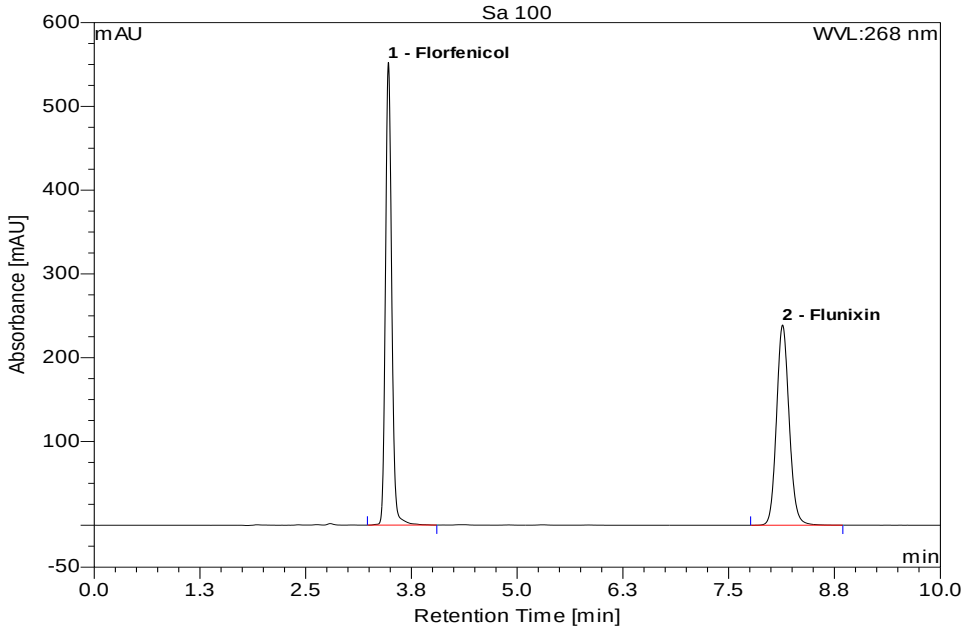


Figure 4-4 Chromatogram of florfenicol and flunixin injectable solution sample using the developed method.

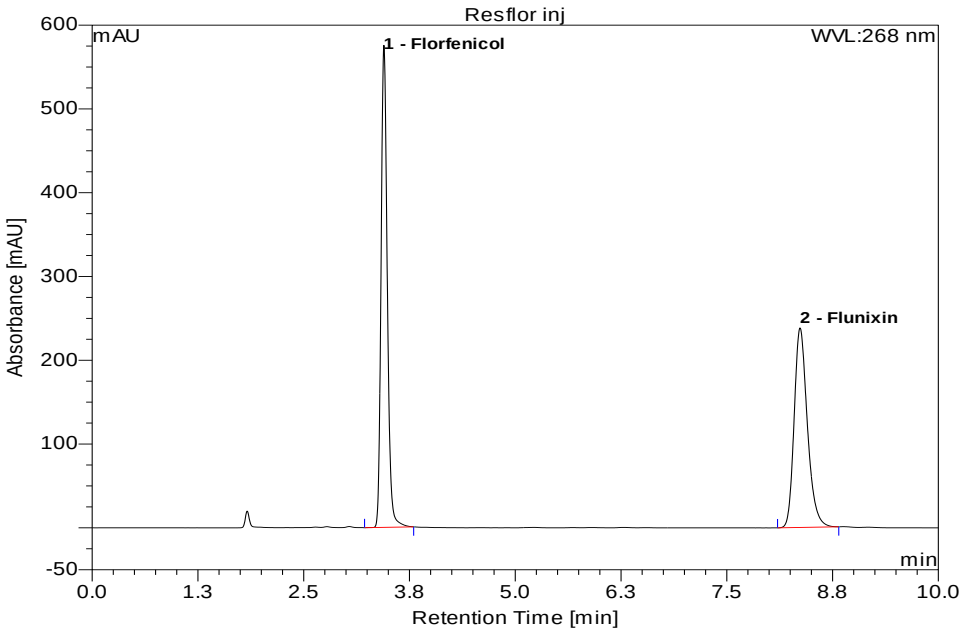


Figure 4-5 Chromatogram of the reference innovator product (Resflor gold injectable solution) using the developed method.

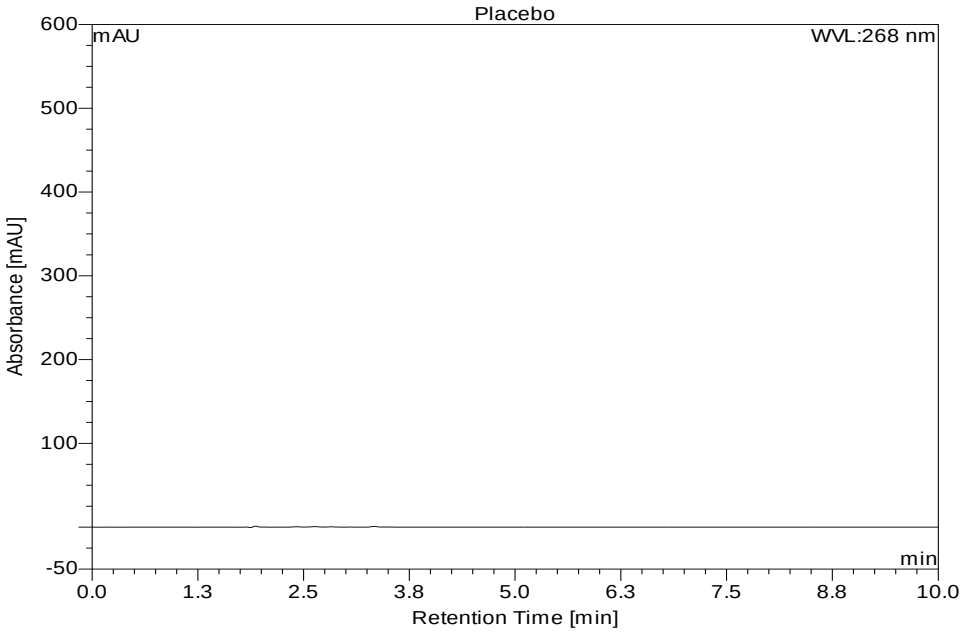


Figure 4-6 Chromatogram of the placebo solution.

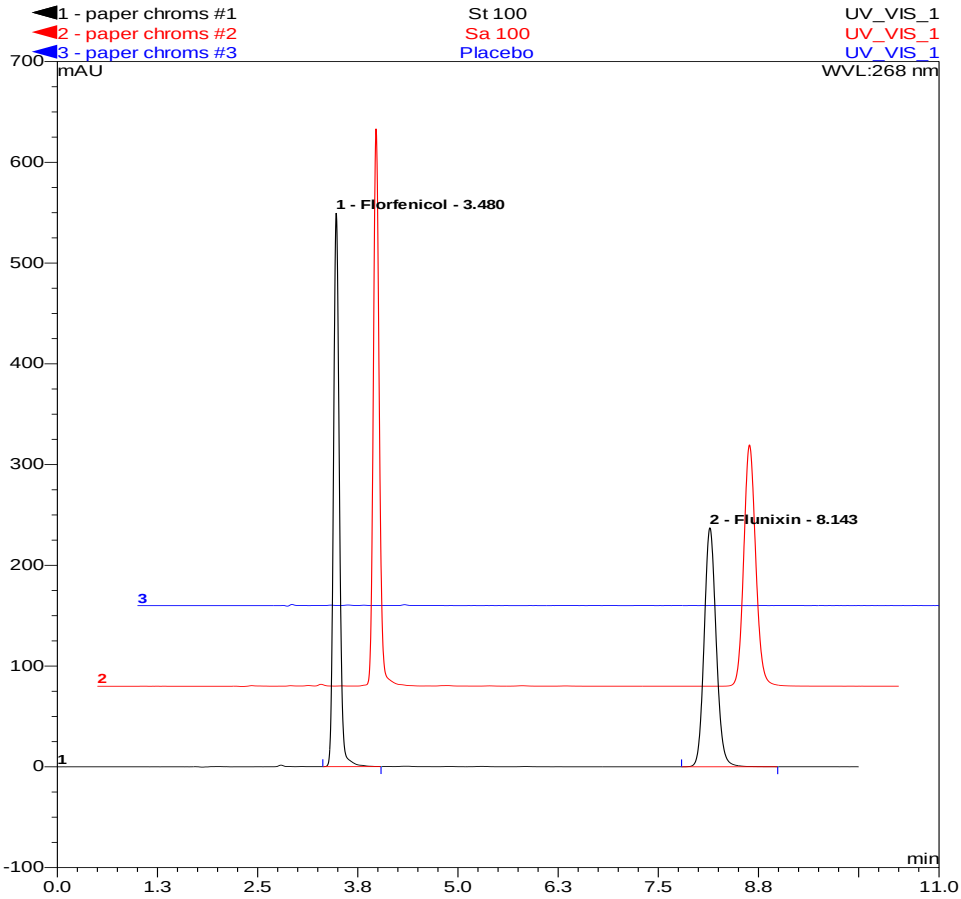


Figure 4-7 Chromatograms overlay of the standard, sample and placebo solutions.

4.2.4 System Suitability

The method shows that the %RSD values are not more than 2.0% for both florfenicol and flunixin, and all the values for the system suitability parameters such as the column efficiency, the tailing factors and the resolution values, as presented in Table 4-2**,** are within limits.

Table 4-2 System suitability parameters of the current method.

	Florfenicol	Flunixin	Acceptance Criteria
Capacity factor, K’	1.1	3.8	1 - 20
Tailing factor, T	1.10	1.12	≤ 2.0
Resolution, R	4.4		> 2.0
Number of theoretical plates, N	11500	14700	> 2000
%RSD (n = 6)	0.19	0.22	≤ 2.0%

4.2.5 Solution Stability

The assay analysis of the prepared solutions was performed at regular intervals. The percent of recovery was within the range of 98.0% to 102.0% and RSD was not more than 2.0% for both active ingredients florfenicol and flunixin, indicating a good stability of sample and standard solutions for 16 hrs.

4.3 Analytical method validation

4.3.1 Specificity and stability indicating study

Stress testing study of the drug product (Table 3-6), was conducted to induce force degradation and determine degradation pathways, and help evaluate the stability of the drug substance and to validate the specificity of the analytical method.

The basic condition applied on the active drug substances for 2 hours induced the hydrolysis of florfenicol, caused assay loss of about 26% and produced degradative materials (Fr1) and (Fr2) of about 23% and 4.5% respectively, while no degradation observed for flunixin.

The acidic condition applied on the active drug substances for 2 days induced the hydrolysis of florfenicol, caused assay loss of about 10.5% and produced a degradative material (Fr3) of about 11%, while no degradation observed for flunixin.

The oxidative condition applied on the active drug substances for 7 days induced the oxidation of flunixin, and caused assay loss of about 61% and produced a degradative material (Fx1) of about 14.5%, while no degradation observed for florfenicol.

The thermal condition applied on the active drug substances for 14 days induced the degradation of florfenicol, and caused assay loss of about 7.5% and produced a degradative material (Fr4) of about 8%, while no degradation observed for flunixin.

There was no evidence of any degradation of the drug product samples that were exposed to the photo stress conditions. These results are summarized in Table 4-3.

Results showed no interference between the chromatographic peaks of florfenicol and flunixin and the excipients, impurities and degradation products under the various stress conditions (Figure 4-8, 4-9, 4-10, and 4-11).

The spectra of all the peaks were checked using PDA showing perfect purity.

Table 4-3 The results of stress testing of Flr and Flx under various conditions.

Stress type	Detectable change	Degradation	
		Name	Percentage
Basic hydrolysis	26% florfenicol assay loss		
	Degradation	Fr1	23.0%
		Fr2	4.5%
Acid hydrolysis	10.5% florfenicol assay loss		
	Degradation	Fr3	11.0%
Oxidative/solution	61% flunixin assay loss		
	Degradation	Fx1	14.5%
Thermal	7.5% florfenicol assay loss		
	Degradation	Fr4	8.0%
Photo-degradation	No change		

Forced degradation study solutions were analyzed using the developed method and the degradative materials peaks were adequately separated from that of Flr and Flx. The optimized chromatographic method conditions were given under section 3.4.2.1.1.

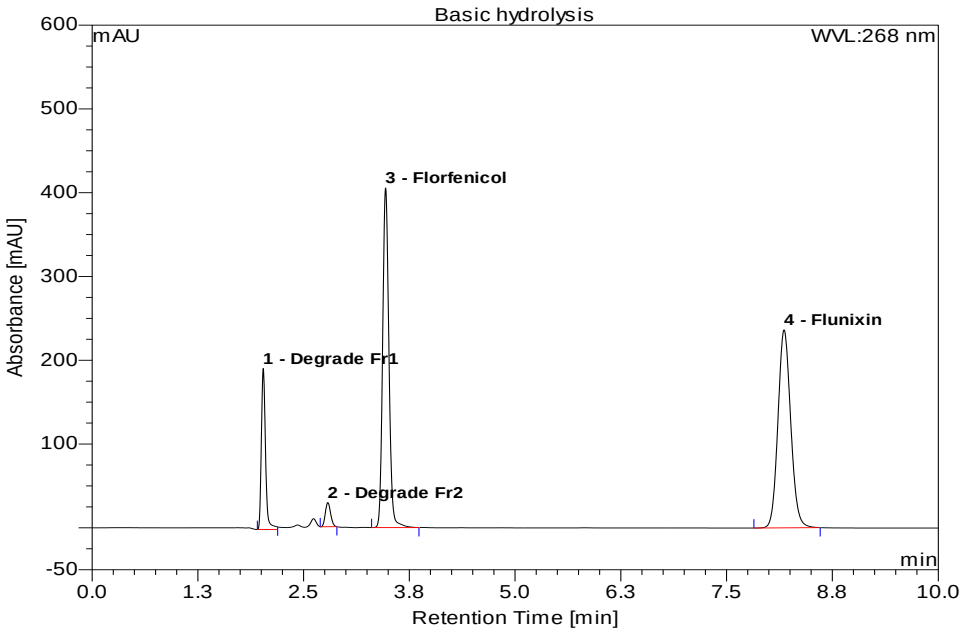


Figure 4-8 Chromatogram of stress testing of Flr and Flx under basic hydrolysis condition of 0.02N NaOH, at RT for 2 hours.

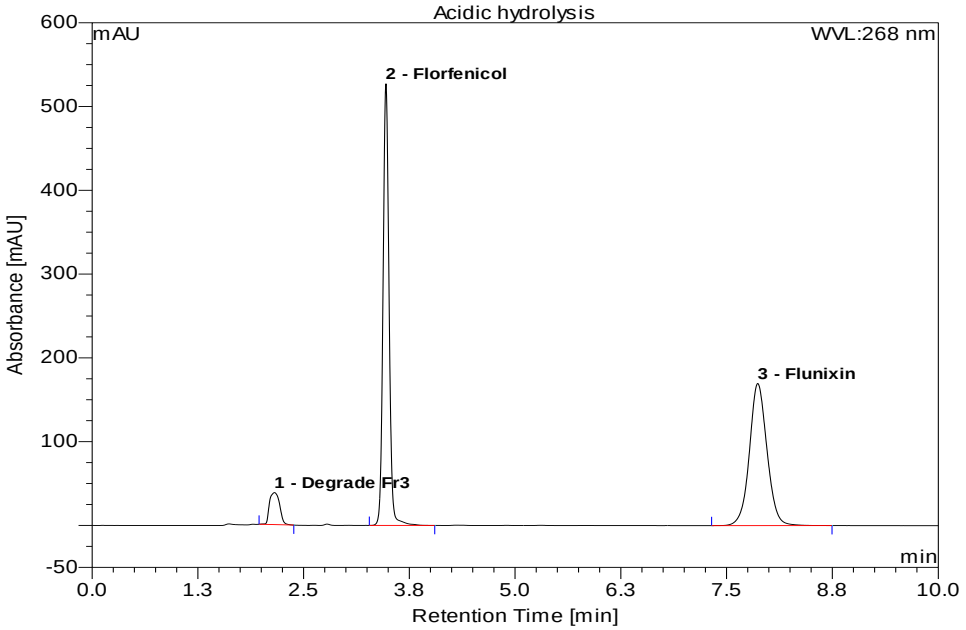


Figure 4-9 Chromatogram of stress testing of Flr and Flx under acidic hydrolysis condition of 1N HCl, at 40°C for 2 days.

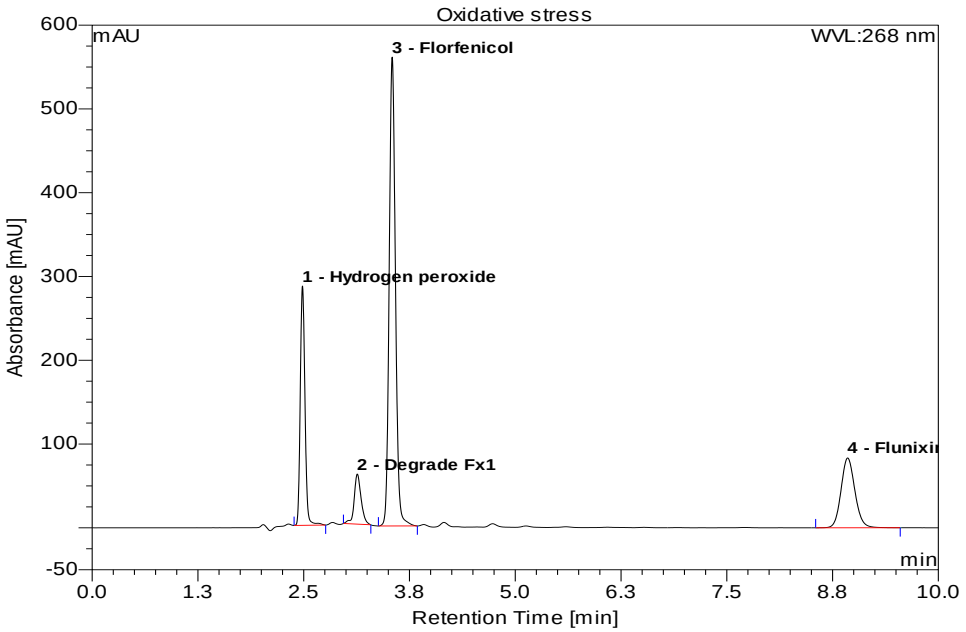


Figure 4-10 Chromatogram of stress testing of Flr and Flx under oxidative condition of 0.2% H_2O_2 at 40°C; protected from light for 7 days.

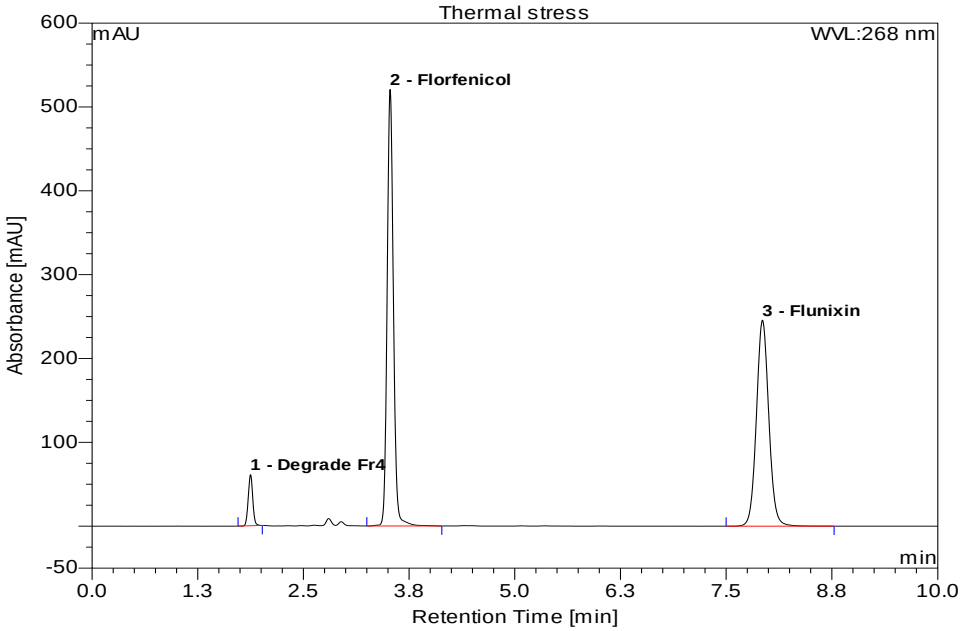


Figure 4-11 Chromatogram of Flr and Flx under thermal stress testing condition of 75°C for 14 days.

The spectra of both Flr and Flx peaks showed perfect purity (Figure 4-12 and 4-13), indicating high purity and homogeneity of the peaks.

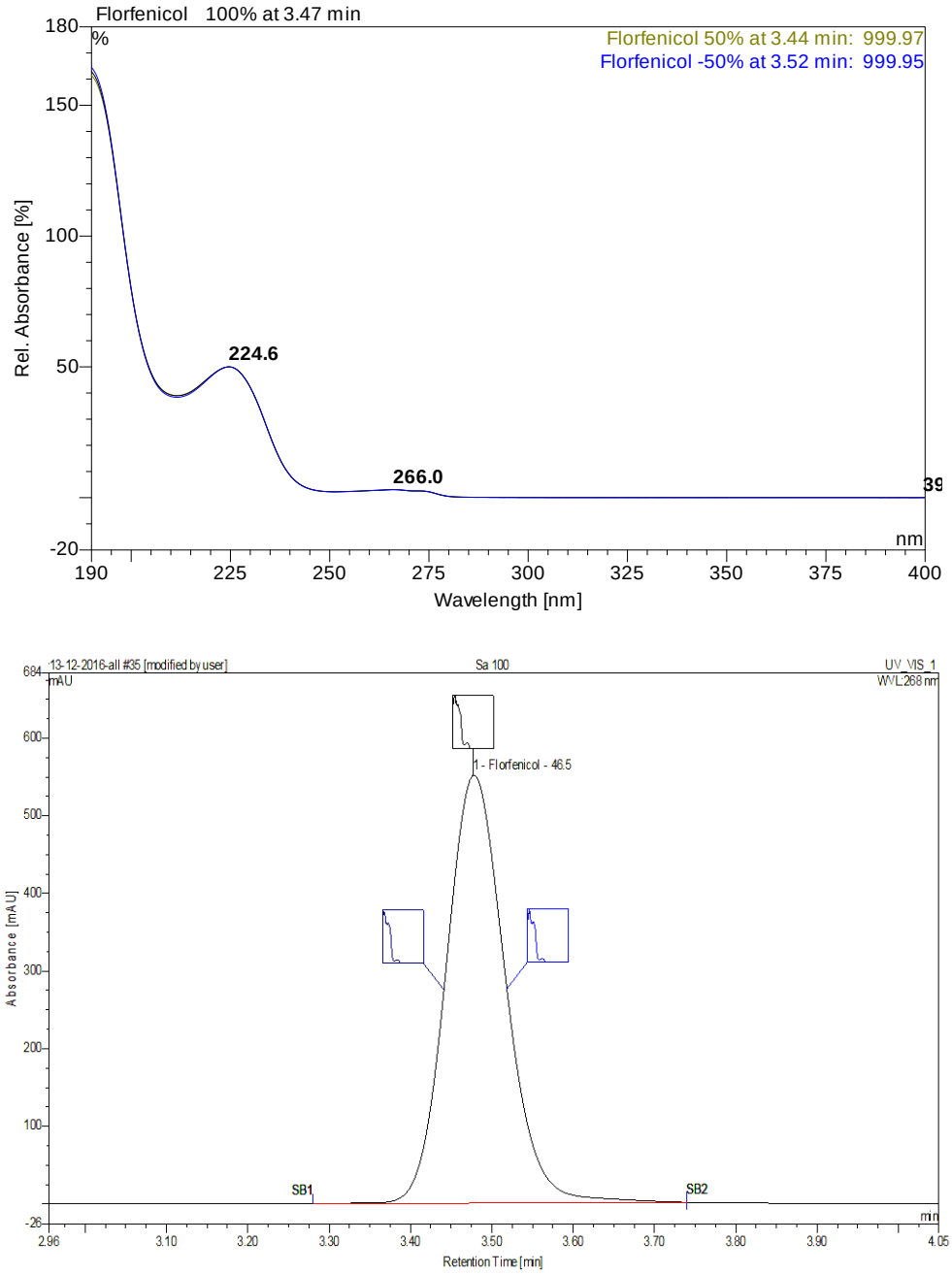


Figure 4-12 Peak purity spectra of Florfenicol.

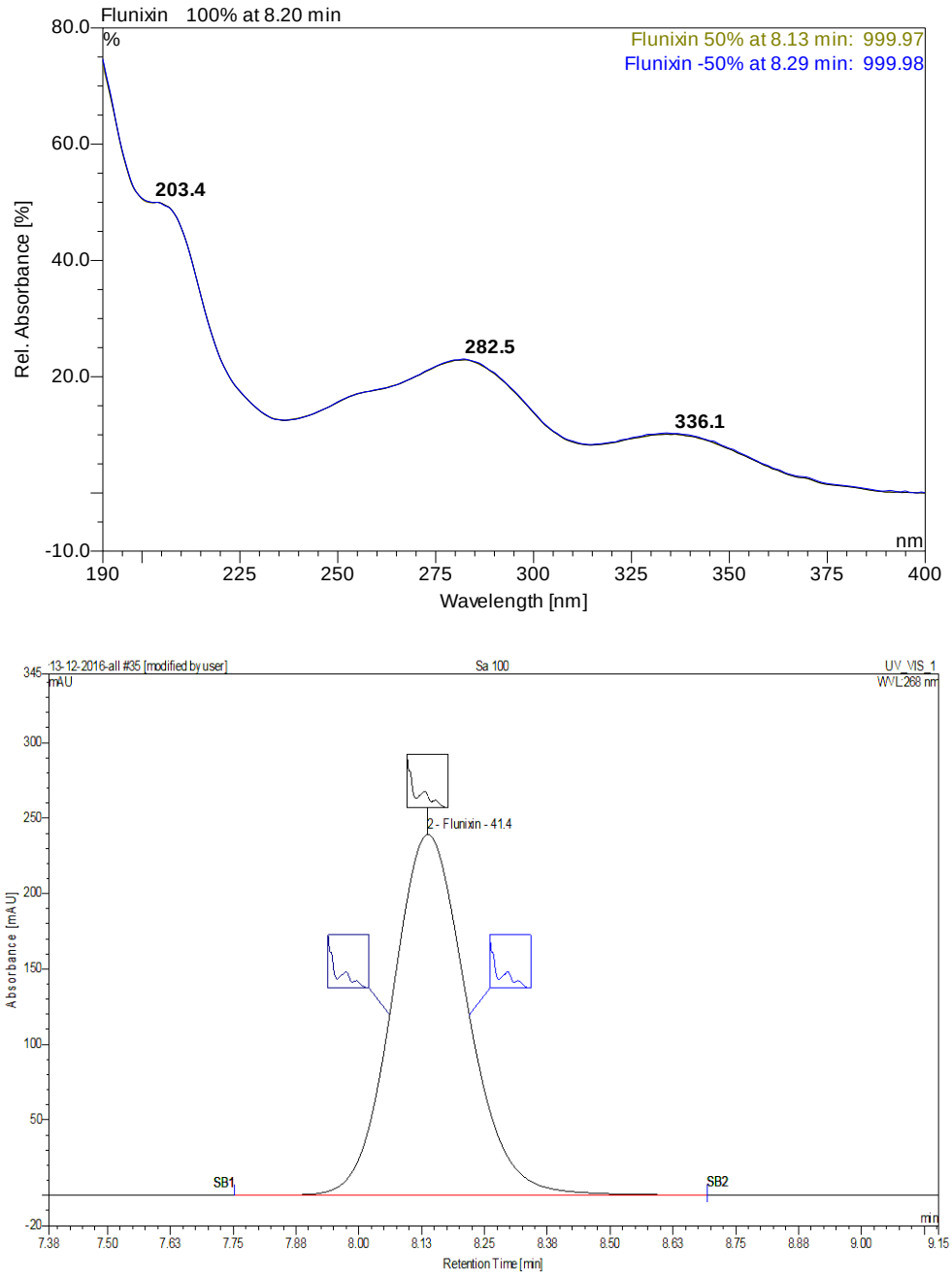


Figure 4-13 Peak purity spectra of Flunixin meglumine.

4.3.2 Linearity and Range:

By analyzing the obtained regression lines for both Flr and Flx linearity peaks, the linearity of the method was observed in the concentration range of 480 µg/mL to 1920 µg/mL for florfenicol, and 43.8 µg/mL to 175.4 µg/mL for flunixin, demonstrating its suitability for analysis as shown in Figure 4-14 and Figure 4-15. The goodness-of-fit (R^2) was found to be 0.9997 for each of Flr and Flx respectively, indicating a linear relationship between the concentration of analyte and area under the peak, as shown in Table 4-4.

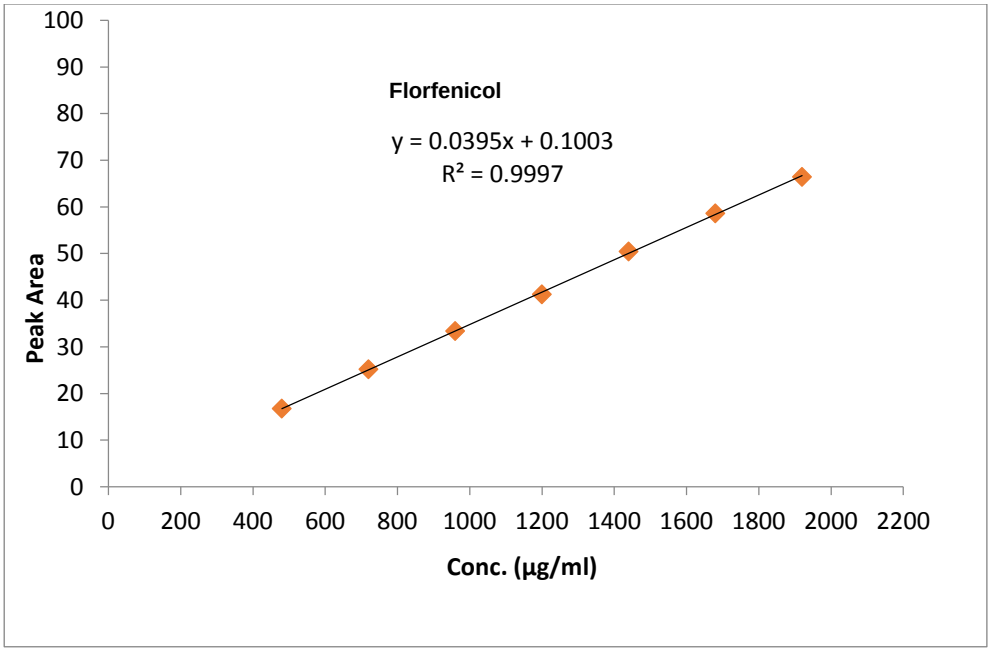


Figure 4-14 Linearity and range of florfenicol (peak area as a function of concentration)

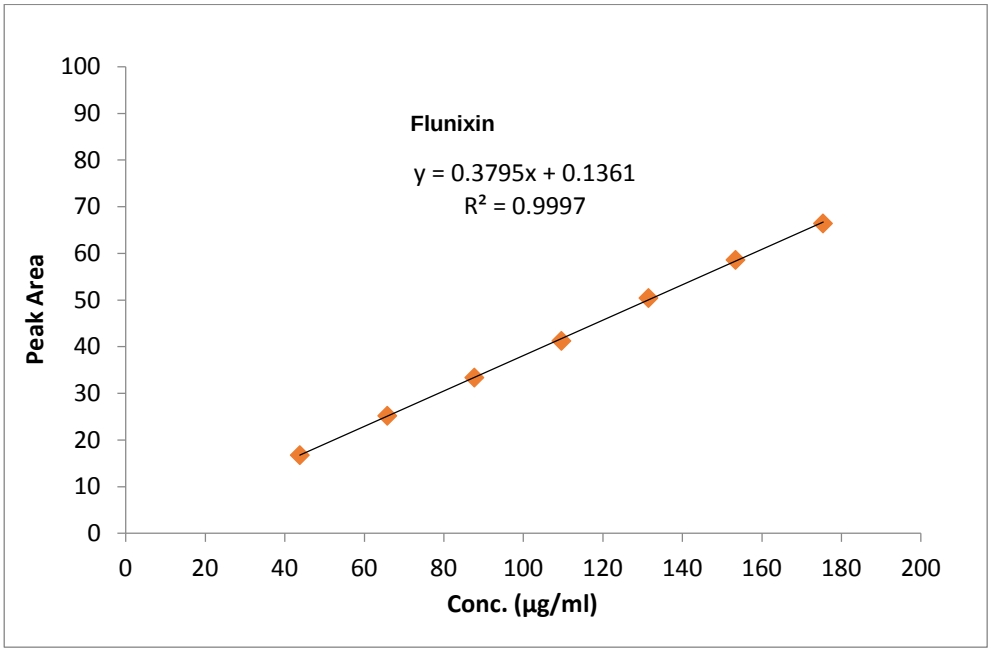


Figure 4-15 Linearity and range of flunixin meglumine (peak area as a function of concentration)

Table 4-4 Regression analysis of florfenicol and flunixin.

API	Linearity range (µg/mL)	(R^2)	Linearity equation	Y-intercept
Flr	480 to 1920	0.9997	y = 0.0395x + 0.1003	0.10
Flx	43.8 to 175.4	0.9997	y = 0.3795x + 0.1361	0.13

An overlay of the peaks obtained by the linearity study, for both Flr and Flx, shown in Figure 4-16.

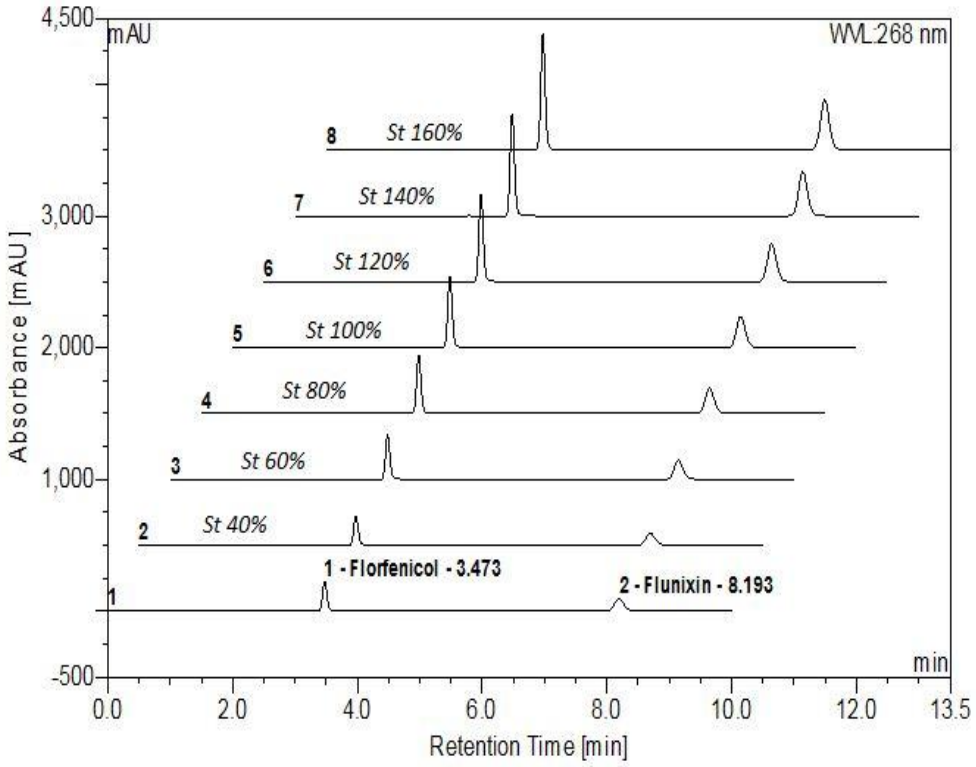


Figure 4-16 Chromatogram overlay of the linearity study solutions.

4.3.3 Accuracy:

The percentage recovery and RSD were calculated for both active ingredients florfenicol and flunixin; all the results are within limits.

Acceptable accuracy was within the range of 98.0% to 102.0% recovery and not more than 2.0% RSD, as demonstrated in Table 4-5.

Table 4-5 Evaluation of the accuracy of the method developed in this study.

API	Spiked level (µg/mL)	Replicate No.	Recovery (µg/mL)	% Mean recovery	% RSD
Flr	960.0	1	960.79	99.84	0.22
		2	957.58		
		3	956.90		
	1200.0	1	1214.99	101.10	0.15
		2	1213.30		
		3	1211.35		
	1440.0	1	1431.03	99.43	0.07
		2	1432.83		
		3	1431.33		
Flx	87.7	1	87.59	99.62	0.23
		2	87.21		
		3	87.29		
	109.6	1	110.72	100.83	0.18
		2	110.49		
		3	110.33		
	131.5	1	130.55	99.31	0.03
		2	130.62		
		3	130.60		

4.3.4 Precision:

The results of repeatability and intermediate-precision testing showed that the method is precise within the acceptable limits.

The RSD were calculated for both active ingredients florfenicol and flunixin; all the results are within limits. Precision was not more than 2.0% RSD, as demonstrated in Table 4-6.

Table 4-6 Evaluation of precision of the method developed in this study.

		API	Flr	Flx
		Spiked amount (µg/mL)	1200	109.6
			Recovery (µg/mL)	
Intermediate Precision (Ruggedness)	Repeatability (Method Precision)	Replicate No.	Day 1	
		1	1214.99	110.72
		2	1213.30	110.49
		3	1211.35	110.33
		4	1210.77	110.26
		5	1209.64	110.09
		6	1208.75	110.10
		Mean recovery	1211.47	110.33
		%RSD	0.19	0.22
			Day 2	
		1	1204.72	110.29
		2	1198.82	113.07
		3	1200.00	110.95
		4	1202.35	110.27
		5	1200.59	111.19
		6	1202.35	108.61
		Mean recovery	1206.47	110.53
		%RSD	0.47	0.92

4.3.5 Robustness:

The robustness of the method was examined using the minor modifications, as shown in section 3.4.5.6.6.

The results of Robustness testing showed that little change of method conditions, such as pH of the mobile phase, composition of the mobile phase, temperature, flow rate and wavelength does not affect the method significantly, and so it is robust within the acceptable limits.

Percent of recovery was within the range of 97.0% to 103.0% and RSD was not more than 3.0% for both active ingredients florfenicol and flunixin.

4.3.6 Limit of Detection & Limit of Quantification (LOD & LOQ):

The analytical method, using signal to noise approach, showed a LOD of 0.60 & 0.20 µg/mL for florfenicol and flunixin respectively.

And showed a LOQ of 2.4 µg/mL with a RSD (*n=3*) of 2.4% for florfenicol & 0.40 µg/mL with a RSD (*n=3*) of 2.6% for flunixin.

4.4 Viscosity

The viscosity of the candidate formulation was compared with that of the reference product (*Resflor gold injectable solution*).

The optimized formula FF9 showed viscosity average of 25.2 mpa.s, which is excellent compared with 87.0 mpa.s for the Reference product (Table 4-7), indicating a good syringeability and injectability of the developed product.

Table 4-7 Viscosity results of the developed product compared with that of the reference product

Speed (rpm)	Resflor gold		FF9	
	%Torque	Viscosity (mpa.s)	%Torque	Viscosity (mpa.s)
100	29.2	87.6	9.1	27.6
50	14.4	86.4	3.8	22.8
Average		**87.0**		**25.2**

4.5 Accelerated stability study

Depending on the obtained results from samples of the candidate formula FF9 after 2 weeks, as a fast stability prediction and evaluation of the formulation, the official accelerated stability study was performed on three pilot batches stored for 6 months under two storage conditions, as recommended by ICH guidelines, and tested as detailed under section 3.5. The tested quality parameters were assay, physical appearance, viscosity, and sterility.

Results of the accelerated stability testing after 6 months, under different storage conditions, didn't show any significant change (Table 4-8), and didn't show any produced impurities, indicating that there is no excipients incompatibility, and that the candidate formula FF9 is stable and qualified to be the final drug product formulation for the developed drug product florfenicol and flunixin meglumine injectable solution.

Table 4-8 Accelerated stability study results

Storage condition	BN	Month	Assay %		Color	Clarity	Viscosity (mpa.s)	Sterility
			Flr	Flx				
25C/60% RH	A	0	99.3	99.0	Light yellow	Clear	25.2	Sterile
		3	102.5	98.9	Light yellow	Clear	NA	NA
		6	99.8	98.9	Light yellow	Clear	29.4	Sterile
	B	0	99.1	98.7	Light yellow	Clear	25.8	Sterile
		3	102.4	98.9	Light yellow	Clear	NA	NA
		6	99.6	99.4	Light yellow	Clear	27.5	Sterile
	C	0	99.7	98.9	Light yellow	Clear	26.1	Sterile
		3	102.8	98.7	Light yellow	Clear	NA	NA
		6	100.2	101.0	Light yellow	Clear	28.7	Sterile
40C/75% RH	A	0	99.3	99.0	Light yellow	Clear	25.2	Sterile
		3	102.3	100.8	Light yellow	Clear	NA	NA
		6	98.8	97.9	Light yellow	Clear	29.7	Sterile
	B	0	99.1	98.7	Light yellow	Clear	25.8	Sterile
		3	102.3	101.3	Light yellow	Clear	NA	NA
		6	98.6	97.3	Light yellow	Clear	26.5	Sterile
	C	0	99.7	98.9	Light yellow	Clear	26.1	Sterile
		3	101.1	102.3	Light yellow	Clear	NA	NA
		6	98.7	97.2	Light yellow	Clear	28.6	Sterile
Acceptance criteria			**±5% of initial**		**Light yellow**	**Clear**	**NA**	**Sterile**

Chapter Five

Conclusion

5 CONCLUSION

A generic parenteral solution drug product containing florfenicol and flunixin meglumine for veterinary use was successfully formulated, using a mixture of organic solvents. The product was examined after submitting to storage conditions recommended for accelerated stability testing, showing excellent stability and maintenance of its quality properties. The developed product quality attributes were compared to that of the reference product, showing that the product can reliably used for its intended use.

A fast, simple and robust stability-indicating HPLC method has been developed and validated for the simultaneous analysis of florfenicol and flunixin in a pharmaceutical formulation. Forced degradation study was performed, the obtained degradants were effectively separated using the developed analytical method, indicating that the method of analysis is qualified and reliable to demonstrate and detect any expected change or any potential degradation in the drug product during stability studies and product shelf life, and can be used for routine quality control analysis. The method is robust enough to reproduce accurate and precise results under different chromatographic conditions.

Chapter Six

Appendixes

6 APPENDIXES

6.1 Excipients profile

6.1.1 N-methyl-2-pyrrolidone [44], [45].

Nonproprietary Names

BP: Methylpyrrolidone.

Synonyms:

N-methyl-2-pyrrolidone, 1-Methyl-2-pyrrolidone, methylazacyclopentan-2-one, N-methylpyrrolidone, 1-methylpyrrolidone, 1-methylpyrrolidinone, and methylpyrrolidone.

Chemical Name:

1-Methylpyrrolidin-2-one.

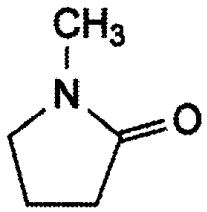
Empirical formula:

C_5H_9NO

Molecular weight:

99.1 g/mol

Chemical structure:



Description

Clear, colourless liquid.

Solubility

Miscible with water, alcohol, ketones, polyethylene glycols, and other solvents such as ethyl acetate, chloroform, and benzene.

Incompatibilities

With strong oxidizing agents, strong acids, bases, strong reducing agents.

Applications and use:

NMP has been used as an excipient in topical pharmaceutical preparations in human medicine and in cosmetics. It is used as a solubilizing agent in veterinary medicines intended for parenteral and topical application.

Storage conditions

Protected from light.

6.1.2 Glycerol Formal [46], [47].

Nonproprietary Names

BP: Glycerol formal.

Synonyms:

Glycerol formal, Methylidinoglycerol; Glicerinformal; Sericosol.

Chemical Name:

Mixture of 1,3-dioxan-5-ol and (1,3-dioxolan-4-yl)methanol.

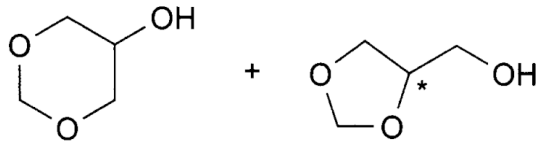
Empirical formula:

$C_4H_8O_3$

Molecular weight:

104.1 g/mol

Chemical structure:



Description

Clear, colourless liquid.

Solubility

Miscible with water and with ethanol (96 per cent).

Applications and use:

As a solvent in oral, dermal and injectable products.

Storage conditions

Under nitrogen, in an airtight container.

6.1.3 Polyethylene Glycol 400 [48][24].

Nonproprietary Names

BP: Macrogols

JP: Macrogol400

PhEur: Macrogols

USP-NF: Polyethylene Glycol

Synonyms:

Carbowax; Carbowax Sentry; Lipoxol; Lutrol E; macrogola; PEG; PluriolE and polyoxyethylene glycol.

Chemical Name:

α-Hydro-ω-hydroxypoly (oxy-1,2-ethanediyl)

Empirical formula:

$HOCH_2(CH_2OCH_2)_{8.7}CH_2OH$

Molecular weight:

380-420 g/mol

Description

Polyethylene glycol 400 occurs as clear, colorless or slightly yellow-colored, viscous liquids, with a slight but characteristic odor and a bitter, slightly burning taste.

Solubility

Polyethylene glycol 400 is soluble in water, acetone, alcohols, benzene, glycerin, and glycols.

Applications and use:

Polyethylene glycol 400 is widely used in a variety of pharmaceutical formulations, in parenteral, topical, ophthalmic, and oral preparations. Used as ointment base; plasticizer; and solvent; it is used as the vehicle for parenteral dosage forms, and can be used either as suspending agents or to adjust the viscosity and consistency of other suspending vehicles.

Incompatibilities

The chemical reactivity of polyethylene glycols is mainly confined to the two terminal hydroxyl groups, which can be either esterified or etherified. However, all grades can exhibit some oxidizing activity owing to the presence of peroxide impurities and secondary products formed by autoxidation. Liquid and solid polyethylene glycol grades may be incompatible with some coloring agents.

Stability and storage conditions

Polyethylene glycols are chemically stable in air and in solution, Polyethylene glycols should be stored in well-closed containers in a cool, dry place. Stainless steel, aluminum, glass, or lined steel containers are preferred for the storage of liquid grades.

6.1.4 Propylene glycol [24].

Nonproprietary Names

BP, JP, PhEur, USP: Propylene Glycol

Synonyms:

1,2-Dihydroxypropane; E1520; 2-hydroxypropanol; methyl ethylene glycol; methyl glycol; propane-1,2-diol; propylenglycolum.

Chemical Name:

1,2-Propanediol

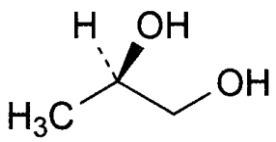
Empirical formula:

$C_3H_8O_2$

Molecular weight:

76.09 g/mol

Chemical structure:



Description

Propylene glycol is a clear, colorless, viscous, practically odorless liquid, with a sweet, slightly acrid taste resembling that of glycerin

Solubility

Miscible with acetone, chloroform, ethanol (95%), glycerin, and water; soluble at 1 in 6 parts of ether; not miscible with light mineral oil or fixed oils, but will dissolve some essential oils

Incompatibilities

Propylene glycol is incompatible with oxidizing reagents such as potassium permanganate

Applications and use:

Propylene glycol is widely used as a solvent, extractant, and preservative in a variety of parenteral and nonparenteral pharmaceutical formulations. Used as disinfectant; humectant; plasticizer; solvent; stabilizing agent; water-miscible cosolvent.

Storage conditions

Propylene glycol is hygroscopic and should be stored in a well-closed container, protected from light, in a cool, dry place.

6.1.5 Ethyl alcohol [24].

Nonproprietary Names

BP: Ethanol (96%)

JP: Ethanol

PhEur: Ethanol (96 per cent)

USP: Alcohol

Synonyms:

Ethanolum (96 per centum); ethyl alcohol; ethyl hydroxide; grain alcohol; methyl carbinol.

Chemical Name:

Ethanol

Empirical formula:

C_2H_6O

Molecular weight:

46.07 g/mol

Chemical structure:

Description

A clear, colorless, mobile, and volatile liquid with a slight, characteristic odor and burning taste.

Solubility

Miscible with chloroform, ether, glycerin, and water (with rise of temperature and contraction of volume).

Incompatibilities

In acidic conditions, ethanol solutions may react vigorously with oxidizing materials.

Mixtures with alkali may darken in color owing to a reaction with residual amounts of aldehyde.

Organic salts or acacia may be precipitated from aqueous solutions or dispersions. Ethanol solutions are also incompatible with aluminum containers and may interact with some drugs.

Applications and use:

Ethanol and aqueous ethanol solutions of various concentrations are widely used in pharmaceutical formulations and cosmetics. Although ethanol is primarily used as a solvent, it is also employed as a disinfectant, and in solutions as an antimicrobial preservative.

Storage conditions

Aqueous ethanol solutions may be sterilized by autoclaving or by filtration and should be stored in airtight containers, in a cool place.

6.1.6 Citric Acid, anhydrous [24].

Nonproprietary Names

BP, USP: Anhydrous citric acid

Synonyms:

Citric Acid; Aciletten; Citretten; Citro; 2-Hydroxy-1,2,3-propanetricarboxylic acid; «beta»-Hydroxytricarballylic acid; Anhydrous citric acid.

Chemical Name:

2-Hydroxypropane-1,2,3-tricarboxylic acid.

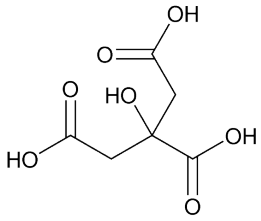
Empirical formula:

$C_6H_8O_7$

Molecular weight:

192.1 g/mol

Chemical structure:



Description

White or almost white, crystalline powder, colourless crystals or granules.

Solubility

Very soluble in water, freely soluble in ethanol (96 per cent).

Incompatibilities

Citric acid is incompatible with potassium tartrate, alkali and alkaline earth carbonates and bicarbonates, acetates, and sulfides. Incompatibilities also include oxidizing agents, bases, reducing agents, and nitrates. It is potentially explosive in combination with metal nitrates. On storage, sucrose may crystallize from syrups in the presence of citric acid.

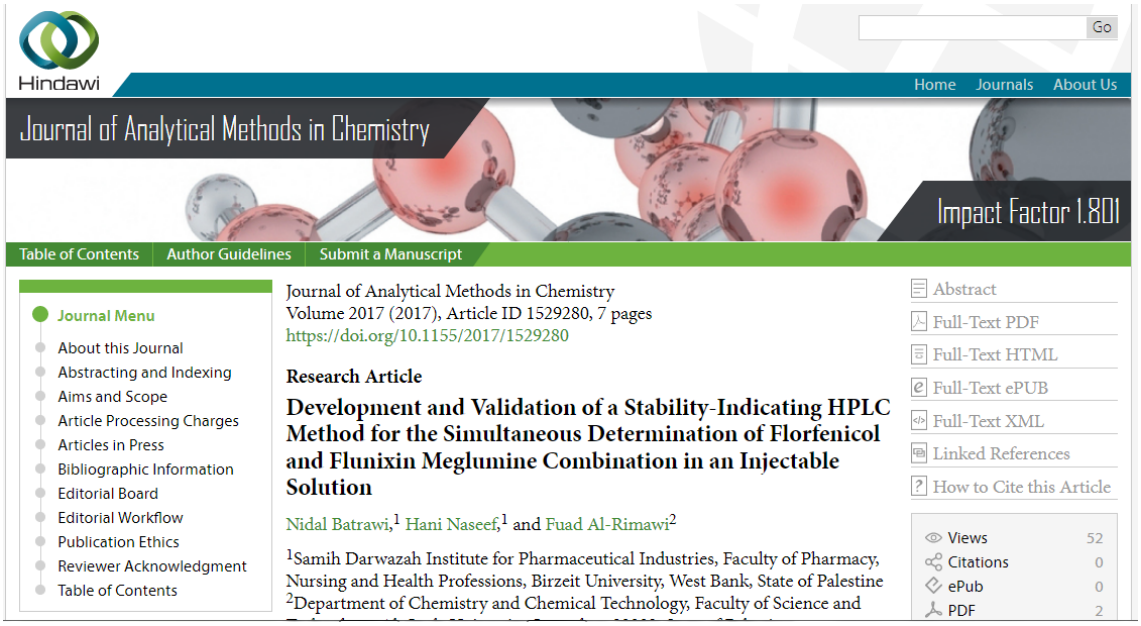
Applications and use:

Citric acid is widely used in pharmaceutical formulations and food products, primarily to adjust the pH of solutions. Used as acidifying agent; antioxidant; buffering agent; chelating/sequestering agent; flavor enhancer; preservative.

Storage conditions

Stored in a well-closed container, protected from light, in a cool, dry place.

6.2 Published part



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Research Article

Development and Validation of a Stability-Indicating HPLC Method for the Simultaneous Determination of Florfenicol and Flunixin Meglumine Combination in an Injectable Solution

Nidal Batrawi,[1] Hani Naseef,[1] and Fuad Al-Rimawi[2]

[1]Samih Darwazah Institute for Pharmaceutical Industries, Faculty of Pharmacy, Nursing and Health Professions, Birzeit University, West Bank, State of Palestine
[2]Department of Chemistry and Chemical Technology, Faculty of Science and

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Development and Validation of a Stability-Indicating HPLC Method for the Simultaneous Determination of Florfenicol and Flunixin Meglumine Combination in an Injectable Solution

Nidal Batrawi,[1] Hani Naseef,[1] and Fuad Al-Rimawi[2]

[1]*Samih Darwazah Institute for Pharmaceutical Industries, Faculty of Pharmacy, Nursing and Health Professions, Birzeit University, West Bank, State of Palestine*
[2]*Department of Chemistry and Chemical Technology, Faculty of Science and Technology, Al-Quds University, Jerusalem 20002, State of Palestine*

Correspondence should be addressed to Nidal Batrawi; nidbat@yahoo.com

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The combination of the powerful antimicrobial agent florfenicol and the nonsteroidal anti-inflammatory flunixin meglumine is used for the treatment of bovine respiratory disease (BRD) and control of BRD-associated pyrexia, in beef and nonlactating dairy cattle. This study describes the development and validation of an HPLC-UV method for the simultaneous determination of florfenicol and flunixin, in an injectable preparation with a mixture of excipients. The proposed RP-HPLC method was developed by a reversed phase- (RP-) C18e (250 mm × 4.6 mm, 5 μm) column at room temperature, with an isocratic mobile phase of acetonitrile and water mixture, and pH was adjusted to 2.8 using diluted phosphoric acid, a flow rate of 1.0 mL/min, and ultraviolet detection at 268 nm. The stability-indicating method was developed by exposing the drugs to stress conditions of acid and base hydrolysis, oxidation, photodegradation, and thermal degradation; the obtained degraded products were successfully separated from the APIs. This method was validated in accordance with FDA and ICH guidelines and showed excellent linearity, accuracy, precision, specificity, robustness, LOD, LOQ, and system suitability results within the acceptance criteria.

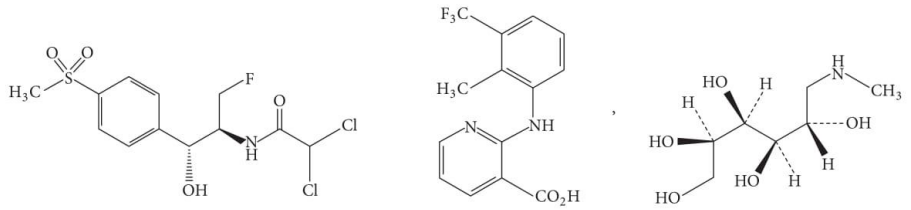
1. Introduction

Florfenicol and flunixin meglumine combination (Flr&Flx) is an effective antimicrobial and nonsteroidal anti-inflammatory for veterinary use, indicated for treatment of bovine respiratory disease (BRD) associated with *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*, and control of BRD-associated pyrexia in beef and nonlactating dairy cattle.

Florfenicol is a broad-spectrum, primarily bacteriostatic antibiotic, effective against wide range of pathogenic strains of microorganisms including many Gram-negative and Gram-positive bacteria [1].

Flunixin is cyclooxygenase inhibitor analgesic anti-inflammatory used to reduce the hemodynamic inflammation caused by endotoxin and to reduce mortality associated with endotoxemic shock [2, 3]. The structure of florfenicol and flunixin is shown in Figure 1.

By reviewing the literature, there are many analytical methods for individual determination of Flr or Flx or in combination with other drugs in a pharmaceutical formulation, but none of these methods include stability-indicating analytical method for the simultaneous determination of both Flr and Flx, in the presence of degradation materials [4–11]. The objective of this study is therefore to develop and validate a simple and fast RP-HPLC method using UV-PDA detector to simultaneously quantify florfenicol and flunixin in a medicinal formulation. The developed method is a validated stability-indicating method, which provides a high degree of analytical confidence that it can be used for the assay test of both active ingredients in a single run and can specifically detect any potential degradants that may produce during stability testing or during product shelf life. This method was validated in accordance with the requirements of FDA, ICH, and USP guidelines [12–16].



Florfenicol Flunixin meglumine

Figure 1: Chemical structure of florfenicol and flunixin meglumine.

Table 1: HPLC chromatographic conditions of the current method.

Chromatographic conditions	
Flow rate	1.0 mL/min
Wavelength (λ)	268 nm
Stationary phase	RP18e, 5 μm, 250 × 4.6 mm
Column temperature	25°C
Injection volume	20 μL
Run time	10 minutes

2. Materials and Methods

2.1. Instrumentation. Liquid chromatography method development and validation analysis were conducted using Dionex-Ultimate 3000 HPLC system, equipped with LPG-3400SD pump, WPS-3000SL autosampler, TCC-3000 column oven, DAD-3000 UV–VIS diode array detector, and Phenomenex Luna C18 (5 μm × 25 cm × 4.6 mm id) column. Chromeleon Data system Software (Version 6.80 DU10A Build 2826 (171948)) was used for data processing and evaluation. The used double-distilled water was prepared by Aquatron equipment model A 4000D.

2.2. Chemicals and Reagents. Active materials, florfenicol and flunixin meglumine, working reference standards with a purity of (>99%) were purchased from Sigma Aldrich. The finished injectable solution samples and all active materials and excipients were gifted by the Advanced Veterinary Manufacturing Company (Palestine). The acetonitrile used was of HPLC grade and water was obtained by double distillation. Other reagents such as phosphoric acid, hydrochloric acid, sodium hydroxide, and hydrogen peroxide were purchased from Merck and Sigma Aldrich.

2.3. Chromatographic Conditions. Mobile phase was prepared by mixing 600 mL acetonitrile with 400 mL of water and then adjusted to pH 2.8 using 2 M phosphoric acid. The chromatographic conditions were run as shown in Table 1.

2.4. Preparation of Standard Solutions. A standard solution of florfenicol (1.2 mg/mL) and flunixin meglumine (0.1096 mg/mL) was prepared by dissolving an accurately weighed amount of florfenicol 300 mg and 27.4 mg of flunixin meglumine in 50 mL of mobile phase, and then 5 mL of the resulting solution was diluted to 25 mL by the same solvent.

Table 2: Stress conditions applied for drug substance and drug product.

Stress type	Conditions	Time
Acid hydrolysis	1 N HCl; at 40°C	2 days
Base hydrolysis	0.02 N NaOH; at RT	2 hours
Oxidative/solution	0.2% H_2O_2 at 40°C; protected from light	7 days
Thermal	75°C	14 days
Photodegradation	UV light	3 days

2.5. Preparation of Sample Solution. A sample solution was prepared with a concentration equivalent to that in standard solution by transferring 1 mL of the drug injectable solution, which contains 300 mg of florfenicol and 27.4 mg of flunixin meglumine, with about 40 mL of the mobile phase into a 50 mL volumetric flask; the volume was completed to mark by the same solvent, and then 5 mL of the resulting solution was diluted to 25 mL by the same solvent.

2.6. Method Validation. The method was validated as per ICH and FDA guidelines for specificity, linearity and range, accuracy, precision, LOQ, LOD, and robustness [12, 15].

2.6.1. Specificity. Forced degradation study was conducted by exposing samples of the drug substance and drug product to various stress conditions of hydrolysis, oxidation, photodegradation, and thermal stress; the time and conditions are illustrated in Table 2. Stressed samples were analyzed occasionally; related peaks were checked for the retention times, peaks interference, spectra purity, and separation factors.

2.6.2. Linearity. To evaluate linearity and range of the method, seven different concentrations of florfenicol (480, 720, 960, 1200, 1440, 1680, and 1920 μg/mL) and flunixin meglumine (43.8, 65.8, 87.7, 109.6, 131.5, 153.4, and 175.4 μg/mL) were prepared. Three injections from each concentration were analyzed under the same conditions.

2.6.3. Accuracy. The accuracy of the assay method was performed on three spiked concentration levels (80%, 100%, and 120%) around the test concentration (florfenicol 1200 μg/mL and flunixin meglumine 109.6 μg/mL), by nine determinations (three replicates of each concentration). The percentage recovery and RSD were calculated for each of the replicate samples.

2.6.4. Precision. Precision was performed at two levels, repeatability and intermediate precision. Repeatability, or method precision, was established by six assay determinations at the 100% concentration levels on the same day. The RSD of obtained results was calculated to evaluate repeatability results.

Intermediate precision or ruggedness was established by doing repeatability test by another analyst on a different day and using different equipment. The RSD of combined results obtained by both analysts was calculated to evaluate intermediate-precision results.

2.6.5. LOD and LOQ. LOD and LOQ of florfenicol and flunixin using this method were determined by analyzing different dilute solutions of florfenicol and flunixin and measuring signal-to-noise ratio. The limit of detection (LOD) is the concentration that gives a signal-to-noise ratio of approximately 3 : 1, while the limit of quantification (LOQ) is the concentration that gives a signal-to-noise ratio of approximately 10 : 1 with % RSD ($n = 3$) of less than 10%.

2.6.6. Robustness. Robustness was performed by applying little deliberate changes of the following method conditions:

(i) pH of mobile phase: ±0.2

(ii) Temperature: ±5°C

(iii) Flow rate: ±0.1 mL/min

(iv) Wavelength: ±2 nm

(v) Mobile phase composition, organic composition ±5%

Sample and standard solutions were analyzed for each change. Change was made to evaluate its effect on the method. Obtained data for each case was evaluated by calculating % RSD and percent of recovery.

3. Results and Discussion

3.1. Method Development and Optimization. With regard to the physical and chemical properties of the analytes and the information obtained from the literature, analytical method was developed to select a preliminary reversed phase HPLC-UV chromatographic conditions, including detection wavelength, mobile phase, stationary phase, and sample preparation procedure. For that, series of trials were performed, such as different compositions of mobile phase and different types of stationary phase and column lengths, with different pH values and buffering agents.

On the basis that the method will be used for separation of two analytes from each other, and also from their degradants, the RP18e stationary phase with a 250 mm length was initially selected. According to the analytes physicochemical properties, a mixture of acetonitrile and water 50% : 50% v/v was selected as the mobile phase, adjusted to pH 4.2 with diluted acetic acid and a flow rate of 1.0 mL/min.

Using these isocratic chromatographic conditions, first successful effort of eluting the analytes simultaneously has been established; the florfenicol peak symmetry and column efficiency were good, but the flunixin peak eluted lately with poor symmetry and column efficiency.

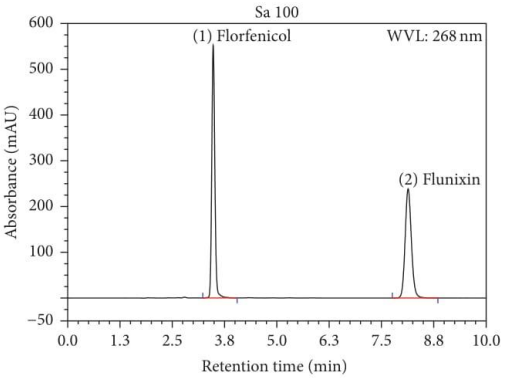


Figure 2: Chromatogram of Flr and Flx in drug product using the developed method in this study.

This required carrying out some modifications in the mobile phase composition and its pH value. Therefore, the ratio of the mobile phase components was changed to be acetonitrile and water 60% : 40% v/v and the pH reduced to 3.0 by diluted acetic acid.

Good flunixin peak symmetry and column efficiency were obtained, but the florfenicol peak was affected.

Additional chromatographic conditions were altered to optimize the florfenicol peak, where the pH of the same mobile phase was reduced to 2.8 by diluted phosphoric acid. As a result of that, a satisfying analytical method was obtained as shown in Figure 2, the resolution (R) and other system suitability parameters of the obtained peaks of florfenicol and flunixin were excellent, as illustrated in Table 7.

Using the PDA-UV a WL of 268 nm was selected as the optimum wavelength. Placebo (mixture of excipients) did not show any response. Forced degradation study solutions were analyzed using the developed method and the degradative materials peaks were adequately separated from that of Flr and Flx (Figures 3, 4, 5, and 6). The optimized conditions were given in Table 1.

3.2. Specificity and Stability-Indicating Study. Specificity is the ability of the analytical method to measure the active ingredient response in the presence of other excipients and its potential degradants. Forced degradation was carried out to evaluate the specificity and stability-indicating properties of the method, by exposing samples of the drug substance and drug product to stress conditions of hydrolysis, oxidation, photodegradation, and thermal degradation as detailed under Section 2.6.1.

Stress testing of the drug product was performed to induce force degradation and determine degradation pathways and help evaluate the stability of the drug substance and also validate specificity of the analytical procedures.

The basic condition applied on the active drug substances for 2 hours induced the hydrolysis of florfenicol causing assay loss of about 26% and degradative materials (Fr1) and (Fr2) of about 23% and 4.5%, respectively, while no degradation was observed for flunixin.

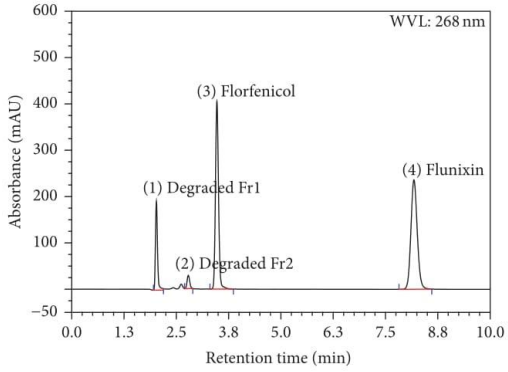


Figure 3: Chromatogram of stress testing of Flr and Flx under basic hydrolysis condition of 0.02 N NaOH, at RT for 2 hours.

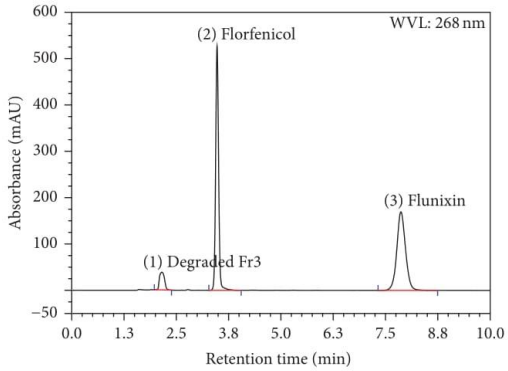


Figure 4: Chromatogram of stress testing of Flr and Flx under acidic hydrolysis condition of 1 N HCl, at 40°C for 2 days.

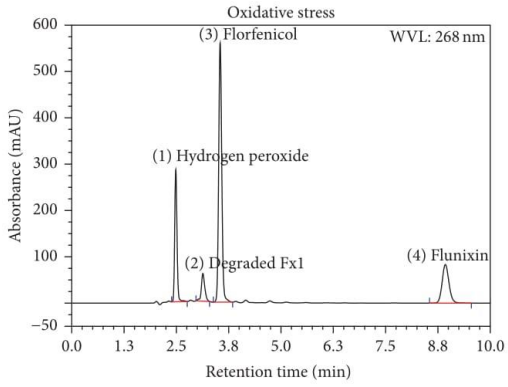


Figure 5: Chromatogram of stress testing of Flr and Flx under oxidative condition of 0.2% H_2O_2 at 40°C, protected from light for 7 days.

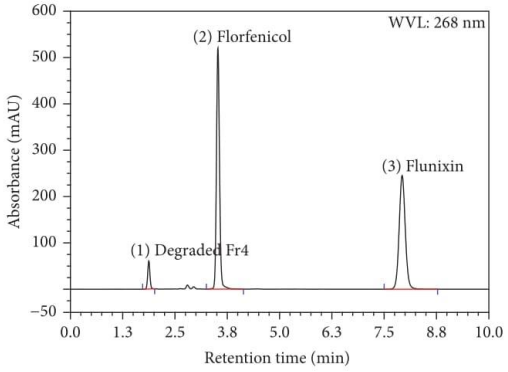


Figure 6: Chromatogram of Flr and Flx under thermal stress testing condition of 75°C for 14 days.

The acidic condition applied on the active drug substances for 2 days induced the hydrolysis of florfenicol causing assay loss of about 10.5% and degradative material (Fr3) of about 11%, while no degradation was observed for flunixin.

The oxidative condition applied on the active drug substances for 7 days induced the oxidation of flunixin causing assay loss of about 61% and degradative material (Fx1) of about 14.5%, while no degradation was observed for florfenicol.

The thermal condition applied on the active drug substances for 14 days induced the degradation of florfenicol causing assay loss of about 7.5% and degradative material (Fr4) of about 8%, while no degradation was observed for flunixin.

There was no evidence of degradation of the drug product exposed to stress condition of the photodegradation type. These results are summarized in Table 3.

Results showed no interference between the chromatographic peaks of florfenicol and flunixin and the excipients, impurities, and degradation products under the various stress conditions (Figures 3, 4, 5, and 6). The spectra of all the peaks were checked using PDA showing perfect purity.

It is concluded that method of analysis is qualified and reliable to demonstrate and detect any expected change in the drug product assay during stability studies.

3.3. Linearity and Range. The linearity of an analytical method can be defined as the ability of the method to obtain test results that are directly proportional to the analyte concentration, within a given range. The linearity of the method was observed in the concentration range of 480 μg/mL to 1920 μg/mL for florfenicol and 43.8 μg/mL to 175.4 μg/mL for flunixin demonstrating its suitability for analysis. The goodness of fit (R^2) was found to be 0.9997 for each of Flr and Flx, respectively, indicating a linear relationship between the concentration of analyte and area under the peak, as shown in Table 4.

3.4. Accuracy. The accuracy of an analytical procedure expresses the closeness of results obtained by that method

Table 3: The results of stress testing of Flr and Flx under various conditions.

Stress type	Detectable change	Degradation	
		Name	Percentage
Basic hydrolysis	26% florfenicol assay loss Degradation	Fr1 Fr2	23.0% 4.5%
Acid hydrolysis	10.5% florfenicol assay loss Degradation	Fr3	11.0%
Oxidative/solution	61% flunixin assay loss Degradation	Fx1	14.5%
Thermal	7.5% florfenicol assay loss Degradation	Fr4	8.0%
Photodegradation	No change		

Table 4: Regression analysis of florfenicol and flunixin.

API	Linearity range (μg/mL)	(R^2)	Linearity equation	Y-intercept
Flr	480 to 1920	0.9997	$y = 0.0395x + 0.1003$	0.10
Flx	43.8 to 175.4	0.9997	$y = 0.3795x + 0.1361$	0.13

Table 5: Evaluation of the accuracy of the method developed in this study.

API	Spiked level (μg/mL)	Replicate number	Recovery (μg/mL)	% mean recovery	% RSD
Flr	960.0	1	960.79	99.84	0.22
		2	957.58		
		3	956.90		
	1200.0	1	1214.99	101.10	0.15
		2	1213.30		
		3	1211.35		
	1440.0	1	1431.03	99.43	0.07
		2	1432.83		
		3	1431.33		
Flx	87.7	1	87.59	99.62	0.23
		2	87.21		
		3	87.29		
	109.6	1	110.72	100.83	0.18
		2	110.49		
		3	110.33		
	131.5	1	130.55	99.31	0.03
		2	130.62		
		3	130.60		

to the true value. The results of accuracy testing showed that the method is accurate within the acceptable limits. The percentage recovery and RSD were calculated for both active ingredients florfenicol and flunixin; all the results are within limits. Acceptable accuracy was within the range of 98.0% to 102.0% recovery and not more than 2.0% RSD, as demonstrated in Table 5.

3.5. Precision. Precision of an analytical method is defined as "the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions," and it is normally expressed as the relative standard deviation.

Table 6: Evaluation of precision of the method developed in this study.

API	Flr	Flx
Spiked amount (μg/mL)	1200	109.6
Intermediate precision (ruggedness)		
Replicate number	Recovery (μg/mL)	
Repeatability (method precision)		
	Day 1	
1	1214.99	110.72
2	1213.30	110.49
3	1211.35	110.33
4	1210.77	110.26
5	1209.64	110.09
6	1208.75	110.10
Mean recovery	1211.47	110.33
% RSD	0.19	0.22
	Day 2	
1	1204.72	110.29
2	1198.82	113.07
3	1200.00	110.95
4	1202.35	110.27
5	1200.59	111.19
6	1202.35	108.61
Mean recovery	1206.47	110.53
% RSD	0.47	0.92

The results of repeatability and intermediate-precision testing showed that the method is precise within the acceptable limits. The RSD were calculated for both active ingredients florfenicol and flunixin; all the results are within limits. Precision was not more than 2.0% RSD, as demonstrated in Table 6.

3.6. Robustness. The robustness of the method was examined using the minor modifications, as shown in Section 2.6.6. The results of robustness testing showed that little change of method conditions, such as pH of the mobile phase, composition of the mobile phase, temperature, flow rate, and wavelength, does not affect the method significantly, and so it

Table 7: System suitability parameters of the current method.

	Florfenicol	Flunixin	Acceptance criteria
Tailing factor, T	1.10	1.12	≤2.0
Resolution, R	4.4		>2.0
Number of theoretical plates, N	11500	14700	>2000
% RSD ($n = 6$)	0.19	0.22	≤2.0%

is robust within the acceptable limits. Percent of recovery was within the range of 97.0% to 103.0% and RSD was not more than 3.0% for both active ingredients, florfenicol and flunixin.

3.7. Limit of Detection and Limit of Quantification (LOD and LOQ). The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, while the limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision. The method showed a LOD of 0.60 and 0.20 μg/mL for florfenicol and flunixin, respectively, and showed a LOQ of 2.4 and 0.40 μg/mL for florfenicol and flunixin, respectively, with a RSD ($n = 3$) of 2.4% and 2.6% for florfenicol and flunixin, respectively.

3.8. System Suitability. System suitability parameters were performed using six replicates of a standard solution containing both florfenicol and flunixin, to verify the analytical system performance. The method shows that the % RSD values are not more than 2.0% for both florfenicol and flunixin, and all the values for the system suitability parameters such as the column efficiency, the tailing factors, and the resolution values, as presented in Table 7, are within limits.

3.9. Solution Stability. The stability of solutions was performed at room temperature, by the assay analysis at regular intervals. The solution was tested every 2 hours from the beginning to 16 hours. The percent of recovery was within the range of 98.0% to 102.0% and RSD was not more than 2.0% for both active ingredients, florfenicol and flunixin, indicating a good stability of sample and standard solutions for 16 hrs.

4. Conclusion

A fast, simple, accurate, precise, and linear stability-indicating HPLC method has been developed and validated for the simultaneous analysis of florfenicol and flunixin in a pharmaceutical formulation. The method is stability indicating and reliable to detect and quantify any potential degradation in the drug product during stability studies and can be used for routine quality control analysis. The method is robust enough to reproduce accurate and precise results under different chromatographic conditions.

Conflicts of Interest

The authors declare no conflicts of interest in publication of this research.

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