Development and validation of chiral separation of some enantiomeric drugs by HPLC and LC-MS

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In

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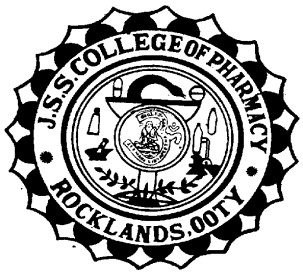
Submitted

By

B. GOWRAMMA, M. Pharm.,



JUNE 2012

J.S.S. COLLEGE OF PHARMACY
ROCKLANDS, OOTACAMUND- 643 001
TAMILNADU, INDIA

CERTIFICATE

This is to certify that the thesis entitled **"Development and validation of chiral separation of some enantiomeric drugs by HPLC and LC-MS"** is a record of research work done by Mrs. B. Gowramma at J.S.S. College of Pharmacy, Ootacamund – 643 001, Tamilnadu, India during the years 2008 – 2012 under my supervision and that the thesis had not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title previously. I also certify that the thesis represents independent work done by the candidate.

Dr. S. N. Meyyanathan

Supervisor

CERTIFICATE

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Dr. B. Duraiswamy

Co-Guide

J.S.S. MAHAVIDYAPEETHA

J.S.S. COLLEGE OF PHARMACY, OOTACAMUND

Off campus, J.S.S. University, Mysore

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Dr. K. Elango

Principal i/c

P. B. No.20, "Rocklands", Ootacamund – 643 001, The Nilgiris, Tamil Nadu, India
Phone: +91-423-2443393/2443847; Fax: +91-423-2442937; E-mail: jaspharm@hotmail.com;
website: www.jsscpooty.org

DECLARATION

I hereby declare that the thesis entitled **"Development and validation of chiral separation of some enantiomeric drugs by HPLC and LC-MS"** submitted by me for the award of degree of **Doctor of Philosophy** of the Tamilnadu Dr. M.G.R. Medical University, Chennai is a record of research work done by me at J.S.S. College of Pharmacy, Ootacamund – 643 001, Tamilnadu, India during the years 2008 – 2012 under the supervision of Dr. S. N. Meyyanathan and that the thesis had not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title previously.

Mrs. B. GOWRAMMA

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GOWRAMMA. B

CONTENTS

1. INTRODUCTION

This thesis deals with the studies carried out by the writer in this laboratory for the past four years on the development and validation of chiral separation of some enantiomeric drugs by HPLC and LC-MS methods. Before discussing the experimental procedures adopted and the results obtained, a brief introduction on chirality, need for chiral separation methods and validation would be discussed in detail. The literature on the selected racemic drugs for chiral separation, namely, Rosiglitazone, Pioglitazone, Zaltoprofen and Valganciclovir Hydrochloride would also be reviewed here.

A chiral molecule is defined as a molecule that is not super imposable on its mirror image. The two mirror image forms of a chiral molecule are called enantiomers. The separation of chiral compounds has been of great interest because the majority of bioorganic molecules are chiral. The chirality of enantiomeric molecule is caused by the presence of one or more chirality elements (chirality axis, chirality plane or chirality centre) in their structure. The biological activity of chiral substances often depends upon their stereo selectivity, since living body is a highly chiral environment which shows different biological response to one pair of enantiomers in the drugs. A large percentage of commercial and investigational compounds are enantiomers and many of them show significant enantioselective differences in their pharmacokinetics and pharmacodynamics. The development of analytical methods for the quantitative analysis of chiral materials and for the assessment of enantiomeric purity is extremely challenging due to the fact that enantiomers possess virtually identical physiochemical properties. Chirality is a major concern in the modern pharmaceutical industry[1-5]. This interest can be attributed largely to a heightened awareness that enantiomers of racemic drugs may have different pharmacological activities, as well as different pharmacokinetic and pharmacodynamics effects.

Chirality in drug therapy[6,7]

Many of the compounds associated with living organisms are chiral, for example DNA, enzyme, antibody and hormones. Thus biology is very sensitive to chirality and the activity of drugs also depends on which enantiomer is used. Most drugs consist of chiral molecules and since a drug must match the receptor in the cell, it is often only one of the enantiomer that is of interest. In certain cases the other enantiomer may be harmful (Table 1).

Drug	R enantiomer	S enantiomer
Propranolol	Beta blocker	Inactive
Labetalol	RR-Beta Blocker	SR-alpha blocker
Amino acids	(-) tasteless/bitter	(+) sweet
Epinephrine	(-) 10 times active	(+) less active
Barbiturates	R (+) convulsant	S (-) depressant
Ibuprofen	Less active	1.4 times active
Omeprazole	Less active	S (-) omeprazole (Superior)

Table 1. Pharmacologic properties of different isomers

The advantages of using single enantiomeric drugs are

- The total dose could be reduced
- Dose response relationship would be simpler. i.e., simplification of drug monitoring
- A source of inter object variability would be minimized
- Toxicity from inactive stereoisomers would be minimized
- Elimination of reduction of pharmacokinetic complexities that may arise from different metabolism, protein binding, transport or excretion of enantiomers.

Estimation of R and S enantiomers in their formulations

The body is highly chiral selective; it will interact with each racemic drug differently and metabolize each enantiomer by a separate pathway to produce different pharmacological activity. One isomer may thus produce the desired therapeutic activities, while the other may be inactive or produce unwanted side effects.

Even when side effects are not serious, the inactive enantiomer must be metabolized and thus represents an unnecessary burden for the organism. One chiral form of the drug naproxen has 28 times the anti-inflammatory activity of the chiral relative. One isomer of dopamine, used to treat Parkinson's disease, acts on nerve cells to control tremor, while the other is toxic to nerve cells. Racemic mixtures of the drug thalidomide were marketed to pregnant women in the 1960s to counter morning sickness. Therapeutic activity, however, comes exclusively from the (R) - enantiomer. After the thalidomide tragedy, the USFDA, in 1992, issued a guideline that for chiral drugs, only it's therapeutically active isomer is brought into market and that each enantiomer of the drug should be studied separately for its pharmacologic and metabolic pathways. It was discovered only after several hundred birth defects had resulted from administration of thalidomide that the (S)-enantiomer is teratogenic. In addition, a rigorous justification is required for market approval of a racemate of chiral drugs. By then, consideration of chirality has become an integral part of drug research & development and of the regulatory process.

Methods of separation of enantiomers

Single enantiomeric drug is obtained by following methods

a. Chiral synthesis
b. Achiral synthesis followed by indirect resolution
c. Chromatographic chiral separation

a. Chiral Synthesis

Chiral synthesis requires a stereo specific reagent or chiral reagent with high purity. The chiral purity must be monitored throughout the synthesis to avoid racemization. The advantages are apparent in the long term due to the lack of wastage of the unwanted enantiomer and the ability to scale up the reaction to production size.

b. Achiral Synthesis followed by indirect resolution

Achiral synthesis will tend to be quicker and cheaper than a chiral synthesis, however the resolution steps are time consuming and difficult. The options for resolution are

i. Crystallization
ii. Enzymatic reaction
iii. Diastereoisomer formation followed by crystallization.

In crystallization method a supersaturated solution of the racemic mixture is treated with a crystal of one enantiomer (or an isomorphous substance) where upon this form is precipitated. The major problem of this method is that it requires chiral selector of a very high degree of enantioselectivity. The second method is based on certain bacteria and moulds when they grow in a dilute solution of racemic modification, destroy one enantiomer more rapidly than the other, e.g., Penicillium glaucam (a mould), when grown in a solution of racemic ammonium tartrate attacks the (+) form and leaves the (-) form.

The disadvantages of this method are

- Dilute solutions must be used and so the amounts obtained will be small
- One form of isomer is destroyed
- It is necessary to find a micro-organism which will attack only one of the enantiomers

The third method is based on in converting the enantiomers of a racemic mixture into diastereoisomer by treating racemic with an optically active

substance and the diastereoisomer there by produced are separated by fractional crystallization.

c. Chromatographic chiral separations[8]

The chromatography is a fascinating separation tool for efficient enantiomeric separation (Fig. 1). Separation of enantiomers by liquid chromatography needs specific conditions that differ from separations of any other mixtures. Enantiomers exhibit the same physical and chemical properties as long as they only interact with achiral compounds. They differ only in the handedness of their sterochemical arrangement. Therefore chromatographic system must show a specific kind of selectivity directed toward the geometry of the enantiomers. This implies that chiral component either in the mobile or the stationary phase must be able to interact with the enantiomers to form diastereoisomer that are different for both enantiomers.

Enantiomers can be separated by using HPLC by either chiral stationary phase or chiral mobile phase additives or chiral derivatisation. Out of these, the chiral stationary phase and chiral techniques are most popular and can be used for the routine estimations.

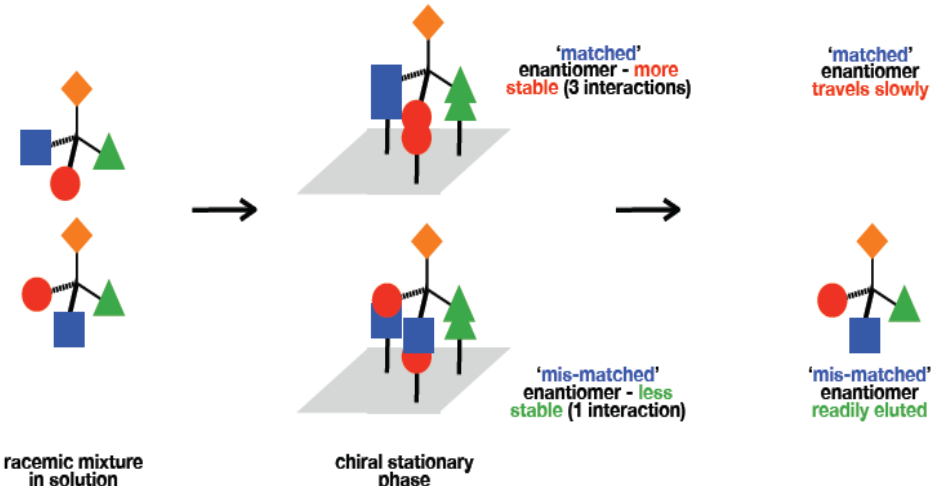

Figure 1. Resolution of enantiomers by chiral chromatography

Most of the pharmaceutical and pharmacological studies of stereo selectivity of chiral drugs were involved precolumn derivatisation of the enatiomers with normal phase mode of chromatography.

The methods of separating chiral compounds by HPLC are;

- Chiral stationary phase method,
- Chiral mobile phase additive method and
- Derivatisation method.

Chiral HPLC columns are made by immobilizing single enantiomers onto the stationary phase. Resolution relies on the formation of transient diastereoisomer on the surface of the column packing. The compound which forms the most stable diastereoisomer will be most retained whereas the opposite enantiomer will form a less stable diastereoisomer and will elute first. To achieve discrimination between enantiomers there needs to be a minimum of three points of interaction to achieve chiral recognition.

The forces that lead to this interaction are very weak and require careful optimization by adjustment of the mobile phase and temperature to maximize selectivity. Chromatography is a multi step method where the separation is a result of the sum of large number of interactions. Typically a free energy of interaction difference of only 0.03 KJ/mol between the enantiomers and the stationary phase will lead to resolution.

The intermolecular forces involved with chiral recognition are polar/ionic interactions, pi-pi interactions, hydrophobic effects and hydrogen bonding. These can be augmented by the formation of inclusion complexes and binding to specific sites such as peptide or receptor sites in complex phases. The analyst may manipulate the intermolecular forces by choosing suitable mobile phases for instance polar interactions may be controlled by pH.

The effect of temperature is important in chiral HPLC. Lower temperature will recognize chiral recognition, but as it alters the kinetics of mass transfer, it may actually makes the chromatography worse by broadening peaks.

The type of column used for separating class of enantiomer is often very specific. This combined with the high cost of chiral columns, makes the choice of which column to use seem at first bewildering. Fortunately taking time to study the structure of chiral phases and visualizing the potential interactions with the analyte can narrow down the choice significantly. There should be a special correlation between the solvent and the solutes in order to establish a difference in the behaviour of the enantiomers and to separate them in columns are

- Strong interactions such as co-ordinate bonds, covalent bonds, hydrogen bonds, etc to yield short lived diastereoisomer.
- Close proximity of the bonds to the respective asymmetric carbons.
- More than a single bond to prevent free rotation and to increase the interaction between the adjacent solvent-solute molecules while two respective asymmetric carbons are brought to proximity.
- To minimize the non-contributing associative forms which do not bring the respective asymmetric centers to proximity.

Chiral stationary phases[9]

Great efforts have been devoted to the development of better methodology for enantioselective chromatography during the past decade and have resulted in a new chiral stationary phases pioneered by Pirkle chiral agents were derivatized and immobilized on the surface of the support (silica gel mostly) and served as the in-situ chiral discriminators during the chromatographic process. The preference of chiral stationary phases lies in the inherent advantages of any chromatographic separation, such as the speed of analysis and the possibility to analyze or purify the enantiomers in complex mixtures. Moreover analytical chromatographic systems can be adapted to preparative separations in which pure enantiomers can be collected. In addition

to their distinct practical applicability chiral stationary phases can uniquely contribute to studies of the nature of molecular recognition. Since the differential retention of enantiomers in the chromatographic system employing chiral stationary phases can be attributed only to chiral discrimination by chiral sites, these interactions can be isolated and explored.

Types of Chiral Stationary Phases (CSP)

Chiral stationary phases may be classified according to their interaction mechanism with the solute. A scheme for classification was proposed by lrwing Wainer.

Type I chiral stationary phases are those which differentiate enantiomers by the formation of complexes based on attractive interactions. There may be hydrogen bonds, interactions and dipole stacking.

Type II chiral stationary phases are those which involve a combination of attractive interactions and inclusion complexes to produce a separation. Most type II phases are based on cellulose derivatives.

Type III chiral stationary phases rely on the solute entering into chiral cavities to form inclusion complexes. The classic inclusion complex columns are crown ethers and helical polymers such as poly triphenyl methyl methacrylate.

Type IV chiral stationary phases separate by means of diasteroisomeric metal complexes. This technique is also known as Chiral Ligand Exchange chromatography.

Type V chiral stationary phases are proteins where separations rely on combination of hydrophobic and polar interactions.

Chiral mobile phase additives

Chiral mobile phases have the following advantages over chiral stationary phases.

- Can use standard columns namely silica, C_8, C_{18} columns.
- High bonding capacity is possible
- Solute character may be modified i.e ion pairing

- Wide range of additives available

The disadvantages are

- The removal of the chiral selector after chromatography
- Separations are difficult to develop
- May be expensive on a large scale without recycling of additives

Chiral derivitization

Chiral derivatisation involves reaction of an enantiomerically pure chiral derivatising agent to form two diasteriomeric derivatives. The diastereomers can be separated using either conventional reversed phases or normal phase. It is important that no racemization occurs during the derivatisation reaction and the chiral derivatising agent should be optically pure (99%). The most common reactions of chiral derivatising agents involve the formation of diasteriomeric amides, carbamates and urea's (Table 2).

Functional groups	Derivative
Amino groups	Amides, Carbonates, urea, thiourea
Hydroxyl groups	Esters, carbamates, carbonates
Carboxyl Groups	Esters, amides
Epoxides	Isothiocyanates, olefins
Thiols	Thioesters

Table 2. Functional groups and their derivatives for chiral analysis

Chiral derivatisation has been applied to both reversed phase and normal phase liquid chromatography. The key to chiral analysis is the ability to react on optically active target molecule with an optically active reagent. In addition to chiral compounds the use of achiral reagent can increase the selectivity of chiral stationary phase towards a chiral analyte. Some compounds do not have distinct energy binding sites to obtain adequate resolution in a CSP and with chiral reagent allows their separation.

General considerations in choosing a derivatising reagent are:

- The derivatising agents must be stable
- The derivatising agent and by products formed during derivatisation should not be detectable or must be separated from the analyte.
- The analyte must be reactive with derivatising agent under convenient conditions
- If possible, reagents should be non-toxic

The procedure should be adaptable to automation with an achiral reagent often enhances enantiomeric separation on pirkle columns. The use of an achiral reagent coupled with a CSP column is usually preferred to derivatization with a chiral reagent, because an optically pure derivatizing reagent is not required. If the analyte has an easily derivatizable functional group that is near the stereogenic center, resolution of the derivatized enantiomers is more likely (Fig. 2). The most easily derivatizable groups are alcohol, amines and acids. A chiral derivation for enhanced enantio selectivity can be applied to amino acids, amines, amino alcohols, alcohols and thiols. Derivatives provide additional interactions to achieve formation of a suitable diasteromeric compound between the CSP and analytes.

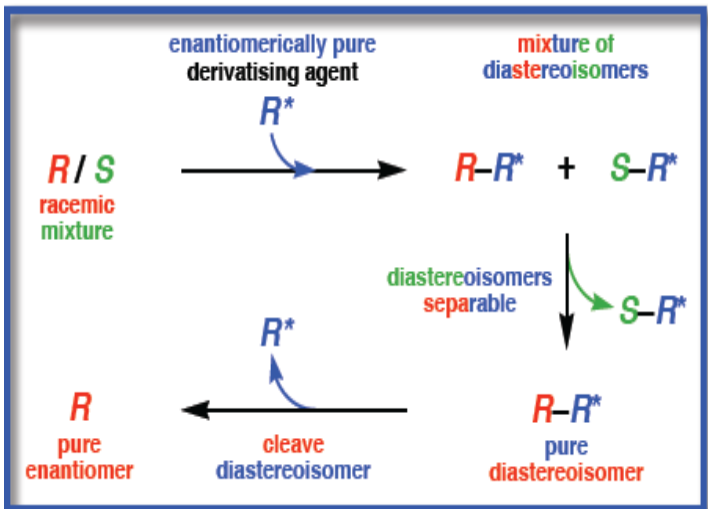

Figure 2. Chiral derivatising agents

Chromatographic methods[10-11]

This is a method of separation, where the individual components are separated and analyzed. In this technique two or more components are separated by a dynamic differential migrational process, in a system consisting of two phases, one of which moves continuously in a given direction in which the individual components exhibit different mobilities due to the difference in their adsorption or partition or molecular size etc. Most reliable and widely used chromatographic techniques for the separation and estimation of enantiomers in chiral drugs are high performance liquid chromatography and liquid chromatography - mass spectrometry.

a. High performance liquid chromatography

This technique employs a liquid mobile phase and a very finely divided stationary phase for separation. In order to obtain satisfactory flow rates the mobile phase is pressurized to several hundred pounds per square inch or more. HPLC is used to separate a mixture of components using a variety of chemical and physical interactions between the substance being analyzed and the chromatographic columns.

Recently, several techniques have been developed, which combine two or more instruments into one. One of the earliest of these hybrid areas involves the coupling of a mass spectrometer (MS) with gas chromatography (GC), (GC – MS) or HPLC (LC-MS).

b. Liquid chromatography – mass spectrometry[12]

This is one of the most commonly used hyphenated techniques. This creates an ideal analytical tool for the laboratory. An HPLC column can separate almost any mixture that can be dissolved and a mass spectrometer can ionize the separated peak solution and provide a molecular weight for each peak component. One of the most widely used

liquid insertion interfaces for HPLC – MS is the thermospray, which works best in reverse phase chromatography. HPLC – MS with thermospray interface represents one of the ultimate analytical hyphenated procedures for the detection and identification of metabolites and drugs in the body fluid.

Sometimes two similar instruments are used in the analysis such as TANDEM Mass spectrometry, which involves coupling of one mass spectrometer to a second one, in which the first one isolates the molecular ions of various components of a mixture and the second one serves for fragmentation of the molecular ions produced by the first one. The latest and recent methods involves, potentially more powerful combination of GC – FTIR – MS, in which these three instruments are operated in series. The GC effluent is usually split, so that about 2% goes directly to the more sensitive MS and the remaining 98% goes into the FTIR. The MS & IR spectra obtained is used, each for its separate library search.

Forced degradation studies in chiral stability indicating method development

Forced degradation studies typically involve the exposure of representative sample of the drug substance or drug product to the relevant stress conditions of light, heat, humidity, acid/base hydrolysis and oxidation. These experiments play an important role in the drug development process to facilitate: stability indicating method development, drug formulation design, selection of storage conditions and packaging, better understanding of the potential liabilities of the drug molecule chemistry and the resolution of stability related problem[13-15]. Forced degradation on the drug substance and product will also provide following information.

- To determine structural transformations of the drug substance and drug product.
- To detect low concentration of potential degradation products.

- To detect unrelated impurities in presence of the desired product and product-related degradants.
- To separate product related degradants from those derived from excipients and intact placebo.
- To elucidate possible degradation path-way.
- May be useful in determining whether accidental exposures to condition other than normal range.
- Reveal the thermolytic, hydrolytic, oxidative and photolytic degradation mechanism of the drug substance and drug product.

According to the ICH and FDA guidance documents, forced degradation studies is conducted to fulfill three main purposes: to provide a stability assessment of the drug substance and drug product; to elucidate possible degradation pathways and to investigate the stability indicating power of the analytical procedures applied for the drug substance and drug product.

Experimental Design to Forced Degradation Studies- Study Protocol[16-17]

A general protocol for conducting forced degradation studies, shown in the Fig. 3 is arranged according to the type of test material (drug substance and drug product) and the type of degradation (hydrolysis, oxidation, etc).

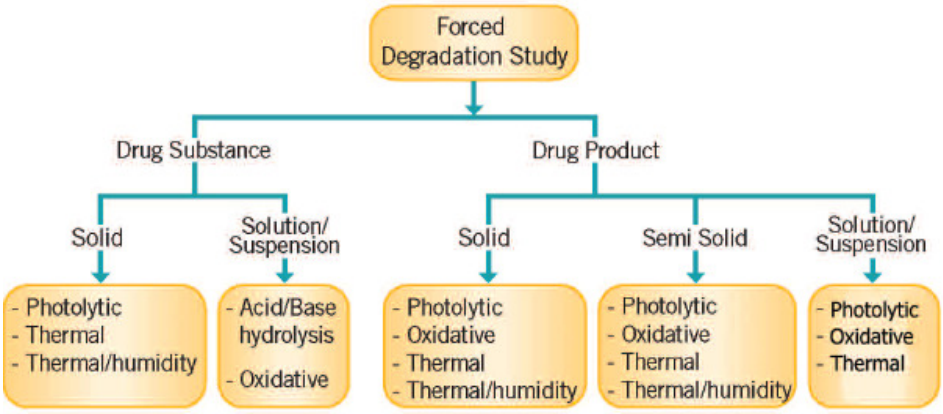

Figure 3. An illustrative flow diagram showing the different forced degradation conditions used for drug substance and drug products

Conditions for stress testing

The initial experiment should be focused on determining the conditions that degrade the drug by approximately 10%. The different stress conditions and exposure time generally employed for forced degradation are summarized in Table 3. The concentration of drug in stressed sample solution may affect the target level of degradation that is ultimately achieved. A more dilute sample concentration generally yields more extensive degradation than does a more concentrated solution. Therefore, lowering the drug concentration may help to increase degradation when necessary.

Degradation Type	Experimental Condition	Storage Condition	Sampling Time
Hydrolysis	Control API (no acid or base)	40 °C, 60 °C	1, 3, 5 days
	0.1N HCl	40 °C, 60 °C	1, 3, 5 days
	0.1N NaOH	40 °C, 60 °C	1, 3, 5 days
	Acid Control (no API)	40 °C, 60 °C	1, 3, 5 days
	Base Control (no API)	40 °C, 60 °C	1, 3, 5 days
	pH: 2, 4, 6, 8	40 °C, 60 °C	1, 3, 5 days
Oxidative	3% H_2O_2	25 °C, 40 °C	1, 3, 5 days
	Peroxide Control	25 °C, 40 °C	1, 3, 5 days
	Azobisisobutyronitrile (AIBN)	40 °C, 60 °C	1, 3, 5 days
	AIBN Control	40 °C, 60 °C	1, 3, 5 days
Photolytic	Light, 1 X ICH	NA	1, 3, 5 days
	Light, 3 X ICH	NA	1, 3, 5 days
	Light control	NA	1, 3, 5 days
Thermal	Heat Chamber	60 °C	1, 3, 5 days
	Heat Chamber	60 °C / 75% RH	1, 3, 5 days
	Heat Chamber	80 °C	1, 3, 5 days
	Heat Chamber	80 °C / 75% RH	1, 3, 5 days
	Heat Control	Room Temp.	1, 3, 5 days

Table 3. Conditions generally applied for forced degradation studies

timeline for conducting studies

ICH guideline makes no mention of any regulatory requirement for forced degradation studies at phase I or phase II of development. There are good

reasons for initiating forced degradation studies on drug substances at phase I. The most important reason is to support the development of a preliminary method that would be highly discriminating due to its ability to detect most of all the potential degradation products. Such a method would have stability indicating power and would require only minimal validation at this stage. Forced degradation studies on drug substance and drug product should be completed prior to registration stability studies and it would be useful to have identified major degradants by that time.

Validation of analytical method[18-19]

Validation is a process that confirmation or establishment by laboratory studies that a method developed is accurate, precise and rugged. In simple terms, validation of an analytical procedure is to demonstrate that the procedure developed is suitable for its intended purpose and it works in a reproducible manner when carried out by the same or different persons, in the same or different laboratories, using different brands of reagents and equipments etc.

The various validation performance parameters are

- Accuracy,
- Precision (repeatability and reproducibility),
- Linearity and range,
- Limit of detection (LOD)/limit of quantification (LOQ),
- Selectivity/ specificity,
- Ruggedness/robustness,
- Stability and
- System suitability

Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method

should be established across its range. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals. Accuracy is calculated from the test results as the percentage of analyte recovered by the assay.

Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (co - efficient of variation) of a series of measurements. The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimate of standard deviation or relative standard deviation. The precision determinations permit an estimate of the reliability of single determination and are commonly in the range of 0.3 to 3% for assays.

Specificity

The International Conference on Harmonisation (ICH) documents define specificity as the ability to assess unequivocally the analyte into the presence of components that may be expected to be present, such as impurities, degradation products and matrix components.

In case of assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, it can be done by spiking the substance or product with appropriate levels of impurity or recipients and demonstrating that the assay results are unaffected by the presence of this extraneous material.

If impurity or degradation product standards are unavailable specificity may be demonstrated by comparing test results of samples containing impurities or degradation product to a well characterized procedure. This comparison should include sample stored under relevant stress conditions Eg. Light, heat, humidity, acid/base hydrolysis and oxidation.

Selectivity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the same matrix. Selectivity in HPLC is usually expressed by the minimum resolution factor (Rs) of two neighbouring peaks and peak purity. The peak purity can be checked by subtracting two chromatograms of the sample obtained at two different wavelengths. If the peak is pure, the absorption ratio at the two wavelengths should be exactly same from the beginning to end of the peak.

The selectivity of analytical method is determined by comparing test results from the analyses of samples containing impurities or degradation products or placebo ingredients with those obtained from the analyses of samples without impurities or degradation products or placebo ingredients.

Linearity and range

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. It should be established across the range of the analytical procedure. Linearity is usually expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentration of analyte.

The range of an analytical method is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to the determined with a suitable level of precision, accuracy and linearity using the method written. The range is normally expressed in the same unit as test result. (eg. percent/ppm).

Limit of detection

Limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitative, under the stated experimental conditions. The detection limits is usually expressed as the concentration of analyte (eg. percent/ppm) in the sample.

Limit of quantification

Limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. It is expressed as the concentration of analyte (eg. percent, ppb) in the sample.

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperature and different days etc. Ruggedness is normally expressed as the lack of influence on the test results of operational and environmental variable of the analytical method.

Robustness

The robustness of analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. A good practice is to vary

important parameters in method systematically and measure their effect on separation. Such parameters include mobile phase composition and pH, mobile phase additives, column temperature, flow rate etc.

Stability

Stability of sample, standard and reagents used in analytical methods are required for a responsible time to generate reproducible and reliable results. For example 24 hours stability is desired for solutions and reagents that need to be prepared for each analysis. Long term column stability is critical for method ruggedness since even a best HPLC column will eventually degrade and loose its initial performance.

System suitability tests

System suitability tests ensure that the method developed can generate results of acceptable accuracy and precision. The USP defines parameters that can be used to determine system suitability prior to analysis. These parameters include column efficiency (N), peak asymmetry factor (As), resolution (Rs), capacity factor (K´) and/or separation factor (α) and relative standard deviation (RSD) of peak area.

2. AIM AND OBJECTIVES

The number of racemic drugs and drug formulations introduced into the market by pharmaceutical industries has been increasing at an alarming rate. These drugs or formulations may be either new entities or partial structural modifications of the existing ones or novel dosage forms (controlled/sustained release formulations) or multi component dosage forms. Many of the prescribed drugs in clinical practice are marketed as racemate. The pharmacokinetics and metabolism of the individual enantiomers of several racemic drugs have been documented to exhibit stereo selective differences in addition to the inherent pharmacological or toxicological differences between the enantiomers.

Most synthetic drugs developed in the past were not chiral, though some were. Drugs developed from natural products are largely chiral. Currently, about 40% of the drugs in use are known to be chiral. In the early 1980s analytical chiral separation was a rather difficult task. Preparative, synthetic and separation methods were not as advanced as today. Nevertheless, it was clear that chiral drugs should be enantio separated and that each enantiomer should be used separately. Nowadays, enantiomers are considered distinctly different compounds, as enantiomers of drug substances may have distinct biological interactions and consequently, profoundly different pharmacological, pharmacokinetic or toxicological activities. Therefore it is very important to develop stereo selective analytical methods, to characterize the individual drugs with respect to issue associated with pharmacology, toxicology and pharmacokinetics during the drug development processes.

Separation of enantiomers is a common problem in stereo chemical research as well as in the preparation of biologically active compounds, in particular drugs. Fewer methods of enantiomeric analysis include some non-chromatographic methods like polarimetry, NMR, isotopic dilution and enzyme technique are also available. But the disadvantages of the above methods are their insensitivity and inability to separate enantiomers. HPLC and LC-MS methods are used for the separation and quantification of vast

number of racemic drugs candidates. Thus there is a need for newer techniques, which helps, in rapid, accurate and precise estimation of R and S enantiomers in racemic mixtures. All these facts combined suggest that, newer and rapid analytical methods are required for the analysis of enantiomers in chiral drugs.

Numerous chiral separation studies of drug formulations have been previously reported. There are no reports, however, of such studies for the separation, quantification and stress degradation behavior of the enantiomers of rosiglitazone, pioglitazone, zaltoprofen and valganciclovir hydrochloride in pharmaceutical dosage forms. The purpose of this study was, therefore, to develop chiral separation methods for the selected drug candidates.

Thus this work is very keen in development and validation of newer rapid, accurate, precise, sensitive and reliable analytical methodologies for the analysis of enantiomers in racemic drugs**.**

3. REVIEW OF LITERATURE

Several investigations have been carried out in the past on method development of chiral separation and chiral stability indicating HPLC method for analysis of enantiomers in pharmaceutical formulation. A survey of literature was carried out in such investigations. In what follows, some of the important investigations are discussed.

S. Eto and coworkers[20] have reported high performance liquid chromatographic method for direct separation of 5-(p-hydroxyphenyl)-5-phenylhydantoin enantiomers using a chiral tris(4-methylbenzoate) column. After simple purification of the incubation mixture of phenytoin in isolated rat hepatocytes, 5-(p-hydroxyphenyl)-5-phenylhydantoin (p-HPPH), which formed as a major metabolite was readily resolved to each enantiomer by direct high performance liquid chromatography on a cellulose tris(4-methylbenzoate) column, with a mobile phase of ethanol-water. It was also observed that the formation of S-(-)-p-HPPH was dominant and the S/R ratio was 11.5.

Y. Hassan and coworkers[21] have reported enantiomeric separation of ketamine hydrochloride in pharmaceutical formulation and human serum by chiral liquid chromatography. A commercially available microcrystalline cellulose triacetate (CA-1) chiral stationary phase was used for the enantiomeric resolution of ketamine [(±)-2-(2-chlorophenyl)-2-methylaminocyclohexanone]. A simple isocratic and direct liquid chromatographic resolution of racemic ketamine was accomplished without derivatization, using pure ethanol as eluent with flow rate of 1 ml/min and at 25°C. The enantiomeric elution order was determined by chromatographing the racemic ketamine and separate the enantiomers under the similar conditions. The capacity factor (k) for the first eluted peak was 2.2; stereochemical separation factor (α) and stereochemical resolution factor (R) were 2.32 and 3.38, respectively. This method has been applied to determine and identify ketamine enantiomers in human serum and a pharmaceutical

dosage form.

D.T. Witte[22] has reported high performance liquid chromatography for direct and indirect method for enantiomeric separation of chiral drugs. The final conclusion is that both the indirect and the direct separation methods provide various options to achieve separation and quantitation of enantiomers. Which of the two methods would be the best choice is greatly dependent on the chemical structure of the solute. For reasons of practice and reliability direct separation methods if available should be preferred over indirect methods. For biological samples an exhaustive clean up step is required before a direct and/or indirect separation method can be used. For direct methods this clean-up step is the only way to prevent the expensive column from being damaged. For indirect methods the clean up step may be less exhaustive depending on the kind of chiral derivatising agent applied.

J.W. Kelly and coworkers[23] have reported liquid chromatographic separation of praziquantel enantiomers in serum using cellulose based chiral stationary phase**.** R(−) and S(+) praziquantel (PZQ) in human serum were resolved and quantified using a stereospecific LC method. Each enantiomer and the internal standard 2-methylamino-5-chlorobenzophenone were isolated from serum using a solid-phase extraction procedure on a cyanopropyl column. Recoveries of 98.8, 93.3 and 35.4% were obtained for R(−) PZQ, S(+) PZQ, and the internal standard, respectively. A cellulose-based chiral analytical column (Chiralcel OD) was used with a mobile phase consisting of hexane-2-propanol (70:30, v/v). Linear calibration curves were obtained in the concentration range 50–1000 ng/ml for each enantiomer in serum. The detection limit for each enantiomer in serum using UV detection at 212 nm was 5 ng/ml (S/N = 3). The limit of quantitation of each enantiomer was 25 ng/ml.

Makoto Tanaka and coworkers[24] have reported direct HPLC separation of enantiomers of pantoprazole and other benzimidazole sulfoxides using cellulose-based chiral stationary phases in reversed-phase mode. A direct, isocratic and simple reversed-phase HPLC method was described for the separation of enantiomers of the proton pump inhibitor, rac-pantoprazole

(PAN) using cellulose-based chiral stationary phases (Chiralcel OD-R and Chiralcel OJ-R). Some structurally related chiral benzimidazole sulfoxides, rac-omeprazole (OME) and rac-lansoprazole (LAN) were also studied. Chiralcel OJ-R was successful in the resolution of enantiomers of rac-PAN and rac-OME, while Chiralcel OD-R was most suitable for resolving the enantiomers of rac-LAN. Highest enantio selectivity to rac-PAN and rac-OME was achieved on Chiralcel OJ-R by using acetonitrile as an organic modifier, whereas methanol afforded better resolution of rac-LAN on Chiralcel OD-R than acetonitrile. Increases in buffer concentration and column temperature decreased retention and did not improve the resolution of the enantiomers on both columns. Using a mixture of 50 mM sodium perchlorate solution and acetonitrile as a mobile phase at a flow rate of 0.5 ml/min, maximum separation factors of 1.26 and 1.13 were obtained for the enantiomers of rac-PAN and rac-OME using a Chiralcel OJ-R column, while maximum separation factor of 1.16 was obtained for the enantiomers of rac-LAN using a Chiralcel OD-R column.

Madhusudhan Siluveru and coworker[25] have reported enantiomeric HPLC separations of chiral local anesthetics using cellulose based chiral stationary phases. Enantiomeric resolution of four racemic local anesthetics bupivacaine, mepivacaine, prilocaine and etidocaine were investigated on cellulose based chiral stationary phases. The phases studied were cellulose tris-3,5-dimethyl phenylcarbamate (Chiralcel OD) and cellulose trisbenzoate (Chiralcel OJ). Baseline separation (Rs>1.5) was achieved for bupivacaine and prilocaine enantiomers on Chiralcel OD using hexane-ethyl alcohol (99:1, v/v) and for etidocaine enantiomers on Chiralcel OJ using hexane-methanol (98:2, v/v). Etidocaine and mepivacaine enantiomers only gave partial separations (Rs ≤ 0.81) on Chiralcel OD. The effects of various protic and aprotic organic modifiers were investigated on resolution and retention of the enantiomers.

Ulrike Selditz and coworkers[26] have reported direct enantiomeric separation of mianserin and 6-azamianserin derivatives using chiral stationary phases. The direct enantiomeric separation of mianserin and 6-azamianserin and some of their derivatives, respectively, by means of HPLC using two

different chiral selectors was investigated. For the cellulose-based Chiralcel OD column, a strong dependence of the lipophilicity of the compounds tested on the retention behaviour was observed. To some extent, this was also found for the enantiomeric separation on the amylose-based Chiralpak AD column. In some cases a complementary behaviour of these two phases was observed, racemic mixtures that could not be separated by one column could be resolved by the other one.

Kumar V Penmetsa and coworkers[27] have reported development of reversed phase chiral HPLC methods using mass spectrometry compatible mobile phases. The majority of chiral HPLC separations are performed in the normal phase mode using alcohol-modified hexane mobile phases. Normal phase chiral HPLC methods are not routinely coupled with electrospray ionization mass spectrometry (ESI-MS) because of the mobile phase incompatibility. In this study, they investigated the use of ESI-MS compatible mobile phases for chiral HPLC methods. This would enable the sensitivity and selectivity of LC/MS to be applied to chiral HPLC analyses. They used a commercially available reversed phase chiral HPLC column (chiralcel OD-R) that permits the use of aqueous organic-modified mobile phases. This paper describes the development of direct, isocratic and simple reversed-phase chiral HPLC methods for the separation of enantiomers of benzoin, indapamide, 2-phenylbutyric acid, 3-phenylbutyric acid, trans-2-phenylcyclopropane-1-carboxylic acid, verapamil hydrochloride and pindolol. In addition, they also demonstrate that the reversed phase chiral HPLC methods developed in this study can be directly coupled with ESI-MS without any modifications. Examples of reversed phase chiral high performance liquid chromatography-mass spectrometry (RP Chiral-LC/MS) methods are shown for indapamide and pindolol.

E. Belloli and coworkers[28] have reported direct separation of the stereoisomers of methoxy tetra hydro naphthalene derivatives, new agonist and antagonist ligands for melatonin receptors, by liquid chromatography on cellulose chiral stationary phases. Analytical HPLC methods using derivatized

cellulose chiral stationary phases were developed for the direct separation of the stereoisomers of disubstituted tetralin derivatives with two chiral centers, new agonist and antagonist ligands for melatonin receptors. The separations were made using normal phase methodology with a mobile phase consisting of n-hexane–alcohol (methanol, ethanol, 1-propanol or 2-propanol) in various proportions, and a silica-based cellulose tris-3,5-dimethylphenylcarbamate (Chiralcel OD-H), or tris-methyl benzoate (Chiralcel OJ). The effects of concentration of various aliphatic alcohols in the mobile phase were studied. A better separation was achieved on cellulose carbamate phase compared with the cellulose ester phase. The effects of structural features of the solutes on the discrimination between the stereoisomers were examined. Baseline separation (Rs > 1.5) was easily obtained in many cases.

Bao Hai Shao and coworkers[29] have reported enantioseparation of racemic naproxen esters on cellulose tris(4-methylbenzoate) chiral stationary phase. Several kinds of racemic naproxen ester were successfully separated on CTMB chiral stationary phase with hexane-ethanol (98:2, v/v) as the mobile phase. The influence of mobile phase composition and structure of racemic naproxen ester on chiral separation was studied and the chiral recognition mechanism of CTMB was discussed.

N. Suzuki and coworkers[30] have reported direct chiral separation of troglitazone stereoisomers using reversed phase high performance liquid chromatography. A simple HPLC method for the direct chiral separation of troglitazone stereoisomers was developed. The separation was performed on a reversed phase cellulose derivertized chiral column (Chiralcel OJ-R) using a mobile phase consisting of methanol–acetic acid (1000:1, v/v) at a flow rate of 0.5 ml/min. The peak areas of stereoisomers separated from 0.13 to 0.75 mg/ml of troglitazone had good linearity, with correlation coefficients >0.999 in the reversed phase mode. The repeatability of the ratios of stereoisomers isolated from 0.5 mg/ml of troglitazone had a relative standard deviation of 0.1–0.2%. The relative sensitivities of the four isomers at UV 285 nm were similar, as each response factor was within the range of 0.99–1.01. Troglitazone racemized at the

chiral center of the thiazolidine ring in methanol solution, but was found to be stable for 24 hrs in methanol–acetic acid (1000:1, v/v). This method was applied to the stereoisomeric analysis of troglitazone in pharmaceutical formulations and used to evaluate the constancy of the stereoisomer ratio in the manufacturing process and stability testing.

Chang Kee Lim and coworker[31] have reported current developments in LC-MS for pharmaceutical analysis. The current developments in liquid chromatography-mass spectrometry (LC-MS) and its applications to the analysis of pharmaceuticals are reviewed. Various mass spectrometric techniques, including electrospray and nanospray ionization, atmospheric pressure chemical ionization and photo ionization and their interface with liquid chromatographic techniques are described. These include high performance liquid chromatography, capillary electrophoresis and capillary electrochromatography and the advantages and disadvantages of each technique are discussed. The applications of LC-MS to the studies of *in vitro* and *in vivo* drug metabolism, identification and characterization of impurities in pharmaceuticals, analysis of chiral impurities in drug substances and high-throughput LC-MS-MS systems for applications in the "accelerated drug discovery" process are described.

H.Y. Aboul Enein and coworkers[32] have reported enantiomeric resolution of some human aldosterone synthase [CYP II B2] inhibitors on derivatized polysaccharides chiral stationary phases. Aldosterone synthase (CYP 11 B2) is a mitochrondrial cytochrome P450 enzyme catalyzing the last steps of aldosterone production in the adrenal cortex. A new pharmacological approach for the treatment of the aldosterone-induced effects in congestive heart failure and all forms of hyperaldosteronism could be achieved through the use of (CYP 11 B2) inhibitors. The chiral resolution of some of active compounds, namely 1-(4-pyridyl(methyl)tetralin (I)), 7-chloro-1-(1-imidazolyl)tetralin (II) and 5-hydroxy-2-(4-pyridylmethyl) indane (III), on various polysaccharide derivative chiral stationary phases, namely Chiralcel OD, OJ, OC and Chiralpak AD and AS, in normal phase mode was achieved.

The mobile phase used was hexane/2-propanol/triethylamine (9 : 1 : 0.1, v/v/v). The flow rate of the mobile phase was 0.8 ml/min and the wavelengths of detection of compounds I, II and III were set at 288, 271 and 254 nm, respectively. The chromatographic parameters: retention factor (k), selectivity (α) and resolution factor (R_s) were calculated. The chiral recognition mechanisms between these analytes and chiral selectors are discussed.

Chiyo Yamamoto and coworker[33] have reported chiral separation by HPLC using polysaccharide-based chiral stationary phases. Chiral separation by high-performance liquid chromatography (HPLC) using a chiral stationary phase (CSP) is one of the most efficient methods for separating enantiomers, not only on an analytical scale, but also on a preparative scale and in the past two decades, many CSPs have been developed. Polysaccharides such as cellulose, amylose and chitin are the most abundant optically active polymers on the earth and can be readily modified to carbamates and esters through the reaction with isocyanates and acid chlorides, respectively. The CSPs based on polysaccharide derivatives are some of the most popular ones and can separate a wide range of chiral compounds.

B.M. Rao and coworkers[34] have reported a validated chiral LC method for the enantioselective analysis of levetiracetam and its enantiomer R-alpha ethyl-2-oxo pyrrolidine acetamide on amylose based stationary phase. A new, simple chiral HPLC method was developed for the enantiomeric separation of levetiracetam, [(S)-alpha-ethyl-2-oxo-pyrrolidine acetamide], an antiepileptic drug in pharmaceutical formulations and in bulk materials. Enantiomeric separation was achieved on a chiralpak AD-H column using a mobile phase consisting of hexane and isopropanol in the ratio (90:10, v/v) at a flow rate of 1.0 ml/min. The resolution between the enantiomers was found to be not less than 7 in the optimized method. Interestingly, unwanted enantiomer, namely R-alpha-ethyl-2-oxo-pyrrolidine acetamide ((R)-enantiomer) was eluted prior to its mirror image in the developed method. The developed method was found to be selective in the presence of related impurities of Levetiracetam, namely N-(1-carbamoyl-propyl)-4-chloro-butyramide (Imp-1) and 1-ethyl-2-oxo-1-

pyrrolidine acetic acid (Imp-2) and also under exposed conditions of UV light and 60^0C. The limit of detection (LOD) and limit of quantification (LOQ) of (R)-enantiomer were found to be 900 and 2250 ng/ml, respectively, for 10 µl injection volume. The method precision for (R)-enantiomer at limit of quantification level was within 8% R.S.D. Calibration curve for (R)-enantiomer was linear over the studied ranges (2250-9000 ng) with correlation coefficient greater than 0.998. The active pharmaceutical ingredient was extracted from its finished dosage form (tablet) using isopropanol. The percentage recoveries of (R)-enantiomer were ranged from 94.2 to 102.6 and from 93.5 to 104.1 in spiked bulk and formulation samples of Levetiracetam, respectively. Levetiracetam sample solution and mobile phase are found to be stable for at least 48 h. The developed method was found to be rugged and robust. The proposed method was found to be suitable and accurate for the quantitative determination of (R)-enantiomer in bulk drugs and commercial formulations. Chiralcel OD-H column can also be used as an alternative column for the above purpose.

M.K. Srinivasu and coworkers[35] have reported a validated chiral LC method for the enantiomeric separation of zolmitriptan key intermediate, ZTR-5. A new and accurate chiral liquid chromatographic method was described for the enantiomeric separation of ZTR-5 [(4S)-4-(4-aminobenzyl)-2-oxazolidinone, (S)-isomer], a key intermediate of zolmitriptan in bulk drugs. The enantiomers of ZTR-5 were baseline resolved on a chiralpak AD-H (250 mm × 4.6 mm, 5 µm) column using a mobile phase system containing hexane: ethanol (70:30, v/v). The resolution between the enantiomers was not less than four and interestingly distomer was eluted prior to eutomer. The limit of detection and limit of quantification of (4R)-4-(4-aminobenzyl)-2-oxazolidinone [(R)-isomer] were found to be 250 and 750 ng/ml, respectively, for 10 µl injection volume. The percentage recovery of (R)-isomer ranged from 92.0 to 105.6 in the bulk drug samples of ZTR-5. The validated method yielded good results regarding precision, linearity, accuracy and ruggedness. The proposed method was found to be suitable and accurate for the quantitative determination of (R)-isomer in bulk drug samples of ZTR-5.

M.K. Srinivasu and coworkers[36] have reported a validated chiral liquid chromatographic method for the enantiomeric separation of rivastigmine hydrogen tartarate, a cholinesterase inhibitor. A new and accurate chiral liquid chromatographic method was developed for the enantiomeric resolution of rivastigmine hydrogen tartarate, (-) S-N-ethyl-3-[(1-dimethyl-amino)ethyl]-N-methylphenyl-carbamate hydrogen tartarate, a cholinesterase inhibitor in bulk drugs. The enantiomers of rivastigmine hydrogen tartarate were baseline resolved on a Chiralcel OD-H (250 mm x 4.6 mm, 5 µm) column using a mobile phase system containing hexane: isopropanol: trifluoroacetic acid (80:20:0.2, v/v/v). The resolution between the enantiomers was not less than four and interestingly distomer was eluted prior to eutomer in the developed method. The presence of trifluoroacetic acid in the mobile phase has played an important role in enhancing chromatographic efficiency and resolution between the enantiomers. The developed method was extensively validated and proved to be robust. The limit of detection and limit of quantification of (R)-enantiomer were found to be 500 and 1500 ng/ml, respectively for 10 µl injection volume. The percentage recovery of (R)-enantiomer was ranged from 95.2 to 104.3 in bulk drug samples of rivastigmine hydrogen tartarate. Rivastigmine hydrogen tartarate sample solution and mobile phase were found to be stable for at least 48 hrs. The proposed method was found to be suitable and accurate for the quantitative determination of (R)-enantiomer in bulk drugs. Chiralcel OJ-H column can also be used as an alternative for the above purpose.

A. Bielejewska and co-workers[37] have reported HPLC separation of linezolid enantiomers using polysaccharide-based chiral stationary phases. Separation of the enantiomers of linezolid has been compared on derivatized cellulose and amylose chiral stationary phases (Chiracel OD and Chiralpak AD) with mixtures of hexane with 1-propanol, 2-propanol, or ethanol as mobile phases. It was found that use of a small amount of water as mobile phase additive can improve or prevent enantioseparation, depending on the column. The order of elution of the linezolid enantiomers was different on the Chiracel OD and Chiralpak AD columns and baseline resolution was achieved on the

Chiralpak AD column only. Reversal of the order of elution of the enantiomers on changing from the propanols to ethanol was observed on both columns. The effect of temperature (in the narrow range 15–35°C) on retention and enantio selectivity was studied for both columns and a variety of mobile phases. The standard enthalpy (ΔH°) and entropy (ΔS°) changes for solute transfer between the mobile and stationary phases were also estimated.

P. Wang and coworkers[38] have reported direct enantiomeric resolutions of chiral triazole pesticides by high performance liquid chromatography. Cellulose-tris (3,5-dimethylphenylcarbamate; CDMPC) was synthesized and coated on aminopropyl silica to prepare chiral stationary phase (CSP). Normal phase high performance liquid chromatography (HPLC) methods for the resolutions of five chiral triazole pesticides, diniconazole, tebuconazole, hexaconazole, triadimefon and flutriafol, on the CSP were developed. Several operating parameters such as mobile phase composition, modifier and column temperature were studied for the optimization of the resolutions. Better separations were achieved using 2% isobutanol for diniconazole, 2% ethanol for tebuconazole, 2% iso-propanol for hexaconazole, 1% n-butanol for triadimefon and 2% n-propanol for flutriafol as modifiers in n-hexane at 0°C with the resolution factors (Rs) of 1.62, 1.66, 2.46, 1.68 and 1.98, respectively. Low temperature was better for the resolutions. Validation of the methods included linearity and precision.

D.B. Pathare and coworkers[39] have reported validated chiral liquid chromatographic method for the enantiomeric separation of pramipexole dihydrochloride monohydrate. A chiral liquid chromatographic method was developed for the enantiomeric resolution of pramipexole dihydrochloride monohydrate, (S)-2-amino-4,5,6,7-tetra-hydro-6-(propylamino) benzothiazole dihydrochloride monohydrate, a dopamine agonist in bulk drugs. The enantiomers of pramipexole dihydrochloride monohydrate were resolved on a Chiralpak AD (250 mm x 4.6 mm, 10 µm) column using a mobile phase system containing n-hexane: ethanol: diethylamine (70:30:0.1, v/v/v). The resolution between the enantiomers was found not less than eight. The presence of

diethylamine in the mobile phase has played an important role in enhancing chromatographic efficiency and resolution between the enantiomers. The developed method was extensively validated and proved to be robust. The limit of detection and limit of quantification of (R)-enantiomer were found to be 300 and 900 ng/ml, respectively for 20 µl injection volume. The percentage recovery of (R)-enantiomer was ranged from 97.3 to 102.0 in bulk drug samples of pramipexole dihydrochloride monohydrate. Pramipexole dihydrochloride monohydrate sample solution and mobile phase were found to be stable for at least 48 h. The proposed method was found to be suitable and accurate for the quantitative determination of (R)-enantiomer in bulk drugs.

R.V.S. Nirogi and coworkers[40] have reported chiral high performance liquid chromatographic method for enantioselective analysis of zaltoprofen. A simple reversed phase chiral HPLC method has been developed and validated for direct separation of the enantiomers of zaltoprofen. Separation of the enantiomers was tested on numerous commercial chiral HPLC columns. Separation was best (resolution, $R_S = 3$) on a Chiralcel OJ-RH stationary phase. Typical retention times of the S and R enantiomers were approximately 14 and 16 min, respectively. Mobile phase composition was systematically studied to find the optimum chromatographic conditions. Validation of the method under the conditions selected showed it was selective and precise and the detector response was a linear function of zaltoprofen enantiomer concentration.

R. Nirogi and coworkers[41] have reported enantiomeric separation of Linezolid by chiral reversed phase liquid chromatography. A chiral liquid chromatographic method is developed for the enantiomeric resolution of Linezolid, (S)-N-[[-3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5 oxazolidinyl] methyl] acetamide, an antibiotic in bulk drugs. The enantiomers of Linezolid are resolved on a Chiralcel OJ-RH column using a mobile phase system containing 150 mM di-sodium hydrogen phosphate buffer (pH 4.5): acetonitrile (86:14, v/v). The resolution between the enantiomers is found to be two. The developed method is extensively validated and proved to be robust. The limit of detection and limit of quantification of (R)-enantiomers are found to be 94

and 375 ng/ml, respectively, for 10 µl injection volume. The percentage recovery of (R)-enantiomer is ranged from 98.9 to 102.9 in bulk drug samples of Linezolid. Linezolid sample solution and mobile phase are found to be stable for at least 48 hrs. The proposed method is found to be suitable and accurate for the quantitative determination of (R)-enantiomer in bulk drugs.

V.P. Rane and coworker[42] have reported the development and validation of chiral LC method for the enantiomeric separation of duloxetine on amylose based stationary phase. A simple, rapid and robust liquid chromatography method was developed and validated for the enantiomeric separation of duloxetine in bulk drug substance. The enantiomers of duloxetine were resolved on a chiralpak AD-H (amylose based stationary phase) column using a mobile phase consisting of n-hexane-ethanol-diethyl amine (80:20:0.2, v/v/v) at a flow rate of 1.0 ml/min. The resolution between the enantiomers was found to be not less than 2.8 in optimized method. The presence of diethyl amine in the mobile phase played an important role in enhancing chromatographic efficiency and resolution between the enantiomers. The developed method was extensively validated and proved to be robust. The calibration curve for (R)-enantiomer showed excellent linearity over the concentration range of 750 ng/ml (LOQ) to 7500 ng/ml. The limit of detection and quantitation for (R)-enantiomer were 250 and 750 ng/ml, respectively. The percentage recovery of the (R)-enantiomer ranged between 98.3% and 101.05% in bulk drug samples of duloxetine. The proposed method was found to be suitable and accurate for quantitative determination of (R)-enantiomer in bulk drug substance.

Maha A Sultan and coworkers[43] have reported chiral stability indicating HPLC method for analysis of arotinolol in pharmaceutical formulation and human plasma. An enantioselective stability-indicating high performance liquid chromatographic method was developed for the analysis of arotinolol in standard solution. The degradation behaviour of arotinolol was investigated under different stress conditions recommended by International Conference on Harmonization (ICH). Resolution of the drug and complete separation from its degradation products were successfully achieved on a chirobiotic V column,

using UV detector set at 315 nm, polar organic mobile phase (POM) consisting of methanol: glacial acetic acid: triethylamine, (100:0.02:0.03, v/v/v), and a flow rate of 1 ml/min. The drug was subjected to oxidation, hydrolysis, photolysis, and heat to apply stress conditions. The drug was found to degrade in alkaline, acidic, oxidative conditions and when exposed to heat. The drug was stable to sunlight. The method reported here has also been successfully applied to pharmaceutical formulation and to human plasma that spiked with stock solutions of arotinolol enantiomers. Arotinolol enantiomers were recovered from plasma by using liquid–liquid extraction procedure with ethyl ether. The method was highly specific, where degradation products and co formulated compounds did not interfere and was sensitive with good precision and accuracy and was linear over the range of 50–400 ng/ml ($R^2 > 0.9981$) with a detection limit of 20 ng/ml for each enantiomer. The mean extraction efficiency for arotinolol was in the ranges 96–104 % for each enantiomer. The mean relative standard deviation (RSD) of the results of within-day precision and accuracy of the drug were < 7.1 %. There was no significant difference between inter- and intra-day studies for each enantiomer which confirmed the reproducibility of the assay. The overall recoveries of arotinolol enantiomers from pharmaceutical formulations were in the ranges 97.6–101.8 %.

L. Peng and coworkers[44] have reported reversed phase chiral HPLC and LC/MS analysis with tris (chloromethyl phenylcarbamate) derivatives of cellulose and amylose as chiral stationary phases. Three polysaccharide-derived chiral stationary phases (CSP) were evaluated for the resolution of more than 200 racemic compounds of pharmaceutical interest in the reversed-phase (RP) separation mode. The population of test probes was carefully evaluated in order to ensure that it covers as completely as possible all structural diversity of chiral pharmaceuticals. RP showed the highest potential for successful chiral resolution in HPLC and LC/MS analysis when compared to normal phase and polar organic separation modes. Method development consisted of optimizing mobile phase eluting strength, nature of organic modifier, nature of additive and column temperature. The newer CSPs, cellulose tris (3-chloro-4-

methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate), were compared to the commonly used cellulose tris(3,5-dimethylphenylcarbamate) in regard to their ability to provide baseline resolution. Comparable success rates were observed for these three CSPs of quite complimentary chiral recognition ability. The same method development strategy was evaluated for LC/MS analysis. Diethylamine as additive had a negative effect on analyte response with positive ion mode electrospray (ESI(+)) MS(/MS) detection, even at very low concentration levels (e.g., 0.025 %). Decreasing the organic modifier (acetonitrile or methanol) content in the mobile phase often improved enantioselectivity. The column temperature had only a limited effect on chiral resolution and this effect was compound dependent. Ammonium hydrogen carbonate was the preferred buffer salt for chiral LC with ESI(+) MS detection for the successful separation and detection of most basic pharmaceutical racemic compounds. Ammonium acetate is a viable alternative to ammonium hydrogen carbonate. Aqueous formic acid with acetonitrile or methanol can be successfully used in the separation of acidic and neutral racemates. Cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate) emerge as CSPs of wide applicability in either commonly used separation modes rivaling such well established CSPs as cellulose tris(3,5-dimethylphenylcarbamate). Screening protocols including these two new CSPs in the preferentially screened set of chiral columns have higher success rates in achieving baseline resolution in shorter screening time.

Ch Surya Naga Malleswara Rao and coworkers[45] have reported a validated LC method for the determination of chiral purity of (S)-2-azido-3-methylbutanoic acid: a key raw material of valganciclovir hydrochloride. A simple and accurate normal phase liquid chromatographic method was developed for the determination of chiral purity of (S)-2-azido-3-methylbutanoic acid, S-enantiomer used as key starting raw material in the manufacturing of valganciclovir hydrochloride bulk drug. Chromatographic separation between (S)-2-azido-3-methylbutanoic acid and its opposite

enantiomer (R)-2-azido-3-methylbutanoic acid, R-enantiomer was achieved using a chiralpak IA column using a mobile phase containing n-hexane, ethanol, isopropyl alcohol and tri-fluoro acetic acid (98:1.5:0.5:0.1 v/v/v/v). The resolution between the two enantiomers was found to be more than 2.0. The limit of detection (LOD) and limit of quantification (LOQ) of the R enantiomer was 0.15 and 0.5 µg/ml, respectively, for 10 µl injection volumes. The percentage recoveries of the R-enantiomer ranged from 96.5 to 105.3 in the samples of (S)-2-azido-3- methylbutanoic acid. The test solution and mobile phase was observed to be stable up to 24 hrs after the preparation. The developed method was validated as per International Conference on Harmonization guidelines in terms of LOD, LOQ, precision, linearity, accuracy, robustness and ruggedness.

S. Vyas and coworkers[46] have reported development and validation of a stability indicating method for the enantioselective estimation of omeprazole enantiomers in the enteric coated formulations by high performance liquid chromatography. Omeprazole is widely prescribed in the form of enteric coated formulations, due to the rapid degradation of the drug in the acidic condition of the stomach. In the current article, they are reporting the development and complete validation of a stability indicating chiral high performance liquid chromatography (HPLC) method for the enantioselective analysis of omeprazole in the enteric-coated formulations. A precise and sensitive enantiomeric separation of omeprazole was obtained on Chiralcel OD-H analytical column (250 mm × 4.6 mm, 5 µm particle size) using normal phase chromatography. The analysis was performed under UV detection at 301 nm wavelength. During method development, the addition of methanol to the mobile phase helped in getting the sharp peaks. The developed method showed linear response over a wide concentration range of 0.39-800 µg/ml and the regression coefficients value (r^2) was obtained more than 0.999 for (S)- and (R)-omeprazole. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) for (R)-omeprazole were found to be 0.39 and 0.78 µg/ml, respectively for 5 µl injection volumes. The percentage recovery of (R)-

omeprazole ranged from 93.5 to 104 in spiked formulation samples and omeprazole sample solution and mobile phase were found to be stable for at least 24 hrs at room temperature. The proposed method was found to be suitable and accurate for the quantitative determination of undesired enantiomer in the enteric coated omeprazole formulations.

K. Zhang and coworkers[47] have reported a validated chiral liquid chromatographic method for the enantiomeric separation of safinamide mesilate, a new anti-parkinson drug. A enantioselective reversed-phase high performance liquid chromatographic method was developed for the enantiomeric resolution of safinamide mesilate, 2(S)-[4-(3-fluorobenzyloxy)benzylamino] propionamide methanesulfonate, a neuroprotectant with antiparkinsonian and anticonvulsant activity for the treatment of Parkinson disease. The enantiomers of safinamide mesilate were baseline resolved on a Chiralcel OD-RH (150 mm × 4.6 mm, 5 µm) column using a mobile phase system containing 300 mM sodium dihydrogen phosphate buffer (pH 3.0): methanol: acetonitrile (65:25:10, v/v/v). The resolution between the enantiomers was not less than 3.0. The pH value of buffer solution in the mobile phase has played a key role in enhancing chromatographic efficiency and resolution between the enantiomers. The developed method was validated and proved to be robust. The limit of detection and limit of quantification of (R)-enantiomer were found to be 15 and 50 ng/ml, respectively, for 20 µl injection volumes. The percentage recovery of (R)-enantiomer was ranged from 94.2 to 103.7 in bulk drug samples of safinamide mesilate. The sample solution and mobile phase were found to be stable at least for 48 hrs. The final optimized method was successfully applied to separate (R)-enantiomer from safinamide mesilate and was proven to be reproducible and accurate for the quantitative determination of (R)-enantiomer in bulk drugs.

M. Dousa and coworker[48] have reported rapid determination of ambrisentan enantiomers by enantioselective liquid chromatography using cellulose based chiral stationary phase in reverse phase mode. A sensitive, specific and rapid high performance liquid chromatography (HPLC) method

for the determination of ambrisentan enantiomers has been developed and validated. Six chiral columns were tested in a reversed phase system. Excellent enantioseparation with the resolution more than 2.5 was achieved on Chiralcel OZ-3R (cellulose 3-chloro-4-methylphenylcarbamate) using mixture of 20 mM sodium formate (pH 3.0) with acetonitrile (55:45, v/v). Validation of the HPLC method including linearity, limit of detection, limit of quantification, precision, accuracy and selectivity was performed according to the International Conference on Harmonisation (ICH) guidelines. The method has an advantage of a very quick chromatographic separation (less than 6 min) and therefore is highly suitable for routine determination of (R)-ambrisentan in enantiopure active pharmaceutical ingredient (S)-ambrisentan.

4. PLACE OF RESEARCH WORK

Research work was carried out at the Department of Pharmaceutical Analysis, J.S.S. College of Pharmacy, Ootacamund – 643 001, Tamilnadu, India and at ISO 9001-2000 certified Centre for Advanced Drug Research and Testing (CADRAT), J.S.S. College of Pharmacy, Ootacamund – 643 001, Tamilnadu, India

5. SCOPE AND PLAN OF WORK

Chirality is a major concern in the modern pharmaceutical industry. This interest can be attributed largely to a heightened awareness that enantiomers of a racemic drug may have different pharmacological activities, as well as different pharmacokinetic and pharmacodynamic effects.

HPLC and LC-MS methods have proven to be the best methods for the direct separation and analysis of enantiomers. They are more versatile than GC method because they can separate a wide variety of nonvolatile compounds. HPLC and LC-MS methods are considered to be most suitable since these methods are powerful, rugged and are extremely specific, linear, precise, accurate, sensitive and rapid.

The present study therefore, aims in developing the validated HPLC and LC-MS analytical methods for the chiral separation of the following selected drugs:

- Rosiglitazone
- Pioglitazone
- Zaltoprofen
- Valganciclovir Hydrochloride

PLAN OF WORK

The project was carried out in the following stages:

Stage I: Literature survey

Stage II: Development of HPLC methods and optimization of chromatographic conditions such as

- Selection of wavelength,
- Selection of initial separation conditions,
- Nature and selection of stationary phase,

- Nature of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate) and
- Sensitivity

Stage III: Development of LC-MS methods for the selected drug candidates and optimization of chromatographic conditions such as

- Selection of mass range,
- Nature and selection of stationary phase,
- Nature of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate) and
- Sensitivity

Stage IV: Estimation of R and S enantiomers in racemic drugs by HPLC and LC-MS methods

Stage V: Forced degradation studies on the selected racemic drugs using acid, alkaline, neutral, oxidative, photolytic and quantification of enantiomers by HPLC in different stress conditions.

Stage VI: Validation of the developed methods as per ICH guidelines using various validation parameters like

- Accuracy,
- Precision,
- Linearity and range,
- Limit of detection (LOD) / Limit of quantitation (LOQ),
- Selectivity / specificity,
- Robustness / ruggedness,
- Stability and
- System suitability

6. MATERIALS AND METHODS

6.1 Reagents and chemicals used

Methanol, n-hexane, acetonitrile, diethylamine, formic acid, glacial acetic acid, ammonium acetate, orthophosphoric acid, sodium perchlorate, potassium dihydrogen ortho phosphate, hydrochloric acid, hydrogen peroxide, perchloric acid and sodium hydroxide were supplied by Qualigens fine chemicals and S.D. Fine chemicals. Water (HPLC grade) was obtained from Milli Q RO system. All the reagents and chemicals used were of HPLC and analytical grade.

Reference standards of enantiomers of Rosiglitazone, Pioglitazone, Zaltoprofen and Valganciclovir Hydrochloride were procured from Sigma Aldrich limited, Mumbai, India. Working standard of Rosiglitazone RS (98.67%) was obtained as gift sample from Sun Pharmaceutical Industries Ltd., Bharuch, Gujarat, India. Working standard of Pioglitazone RS (99.37%) was obtained as gift sample from Micro Labs Limited, Hosur, Tamil Nadu, India. Working standard of Zaltoprofen RS (99.20%) was obtained as gift sample from Shanghai Titanchem Co Ltd., China. Working standard of Valganciclovir Hydrochloride RS (99.30%) was obtained as gift sample from Dr. Reddy's Lab, Hyderabad, India.

6.2 Formulations used

Commercially available tablets Rosiglitazone, Pioglitazone, Zaltoprofen and Valganciclovir Hydrochloride were purchased commercially from the local market, Ooty, Tamilnadu, India.

Reglit Tablets (2 mg of Rosiglitazone RS) of Dr. Reddy's Laboratories, Enselin Tablets (4 mg of Rosiglitazone RS) of Torrent Pharma Limited, Rosicon Tablets (4 mg of Rosiglitazone RS) of Alembic Limited, Rosinorm Tablets (8 mg of Rosiglitazone RS) of Glaxo SmithKline Limited, Pepar Tablet (15 mg of Pioglitazone RS) of Glenmark (Healtheon), Diavista Tablet (15 mg of Pioglitazone RS) of Dr. Reddy's Laboratories, Piozulin Tablet (30 mg of Pioglitazone RS) of Cadila Pharmaceuticals Limited, Piolem Tablet (30 mg of Pioglitazone RS) of Cipla Pharmaceutical Limited, Zaltokin-80 Tablet (80 mg of Zaltoprofen RS) of Ipca Laboratories Limited and Valcyte Tablet (450 mg of Valganciclovir Hydrochloride RS) of Roche Palo Alto llc were used.

6.3 Instruments used

1. Sartorius single pan balance (BS R223S)
2. Systronics pH meter, µ pH system 361
3. Sonicator (Bandelin Electronics, Berlin)
4. Shimadzu liquid chromatographic system equipped with LC – 10 AT VP solvent delivery system (pump), SPD M-10A photodiode array detector and rheodyne 7725i injector with 20 µl loop volume. Class VP 6.01 data station for data collection and processing (Shimadzu technologies, Japan).
5. Water's liquid chromatographic system equipped with waters 1515 isocratic solvent delivery system with a waters 2487 dual wavelength UV absorbance detector and rheodyne 7725i injector with 50 µl loop volume. Breeze 3.3 data station was used for data collection and processing.
6. API 3000 LC-MS/MS with auto injector and Analyst 1.31 data solution
7. Shimadzu 1700 UV-VIS spectrophotometer
8. Chiral columns of different make such as ACI cellu 1 (150 x 4.6 mm i.d., particle size 5 µ) and Lux 5µ 1 (Cellulose tris (3, 5-dimethylphenylcarbamate, 250 x 4.6 mm i.d., particle size 5 µ)
9. Analytical columns of different make such as Phenomenex C_{18} (250 × 4.6 mm i.d., particle size 5µ), Princeton C_{18} (150 × 4.6 mm i.d., particle size 5µ), Princeton C_8 (250 × 4.6 mm i.d., particle size 5µ), Hibar Purospher Star C_{18} (250 mm x 4.6 mm i.d., 5µ), Hypersil C_4 (250 × 4.6 mm i.d., particle size 5µ) and Princeton SPHER (100 × 4.6 mm i.d., particle size 5µ)

6.4 Method development and optimisation of chromatographic conditions for HPLC and LC MS methods

Proper selection of the chromatographic methods depends upon the nature of the sample (chirality, ionic or neutral molecule), its molecular weight and solubility. For the present study, direct and indirect chiral HPLC and LC-MS methods were considered as they are more suitable for the separation of enantiomers, because they are extremely specific, linear, precise, accurate, sensitive and rapid methods.

Chromatographic conditions for HPLC and LC-MS like

- Selection of detection wavelength/mass range (LC-MS)
- Nature of stationary phase
- Nature and ratio of mobile phase were optimised.

6.4.1 Selection of detection wavelength/mass range

The sensitivity of the HPLC method that uses UV/Visible detection depends upon the proper selection of the wavelength. An ideal wavelength is one that gives good response for all the components to be detected.

UV spectrums of 10 µg/ml of standard drugs in selected solvents were recorded individually. These solutions were scanned in the UV region of 200 - 400 nm and the UV spectrums were recorded (Fig. 4-7).

The mass range of the selected drugs were determined in SCAN mode. 10 µg/ml of standard drugs in selected solvents were injected into the MS system and scanned for the fragmentation pattern. The peak corresponding to m/z ratio equivalent to (M+H/M-H) was selected for further analysis in SIM mode.

6.4.2 Nature of stationary phase

Different chiral stationary phases of various dimensions viz., ACI cellu 1 and Lux 5µ 1 were used for the separation of the enantiomers of Rosiglitazone, Pioglitazone and Zaltoprofen in pharmaceutical formulations by HPLC and LC-MS methods. For indirect chiral chromatography, reverse phase stationary phases of various dimensions (C_4, C_8 and C_{18}) were used for the separation of Valganciclovir HCl enantiomers in pharmaceutical formulation by HPLC and LC-MS methods.

6.4.3 Nature of the mobile phase

Based on sample solubility, stability and suitability various mobile phase compositions were tried to achieve good separation and resolution with sharp peaks for HPLC methods and LC-MS methods. Acetonitrile was used for the initial separation conditions. When acetonitrile was substituted with other solvents, the solvents to buffer ratios were calculated using solvent strength. Various ratios of water or buffers and different organic solvents like acetonitrile and methanol were tried to get good separation and peak resolution. The different chromatographic conditions were maintained for the selected drugs such as mobile phase pH, solvent strength, addition of peak modifiers, flow rate and solvent ratio on the peak

resolution and symmetry were studied. The resulting chromatograms were recorded and the chromatographic parameters such as capacity factor, asymmetry factor, resolution and column efficiency were calculated. The conditions that gave good resolution, symmetry and efficiency were selected for the estimation of enantiomers in formulations. Volatile buffers at very low concentrations were used for LC-MS methods to achieve proper separation and resolution.

6.5 Forced degradation studies

The specificity of the method can be demonstrated through forced degradation studies conducted on the sample using acid, alkaline, oxidative, thermal, photolytic and UV degradations. In order to establish whether the analytical method and the assay were stability indicating, pure active pharmaceutical ingredient (API) was stressed under various conditions to conduct forced degradation studies.

As the enantiomers are freely soluble and stable in methanol, methanol was used as a co-solvent in all the forced degradation studies. The solutions were prepared by dissolving the API in methanol and further the degradation was carried out in various degradation medias viz., hydrogen peroxide (oxidation), distilled water (neutral degradation), hydrochloric acid (acidic hydrolysis), sodium hydroxide (basic hydrolysis) and sunlight (photolysis).

6.6 Optimized chromatographic conditions

Based on the above studies, the following optimized chromatographic conditions were selected for the chiral separation and estimation of enatiomers in selected formulations.

6.6.1 Direct chiral HPLC Chromatographic conditions for Rosiglitazone

Stationary Phase : Lux 5µ cellulose 1 (150 mm x 4.6 mm i.d., 5 µ)
Mobile phase : Methanol: 0.1% Diethyl amine (60:40)
Detection wavelength : 220 nm
Flow rate : 1.0 ml/min
Sample injector : Auto sampling unit with 100 µl loop volume
Injection volume : 100 µl
Retention time : R-Rosiglitazone : 1.4 min S-Rosiglitazone : 2.6 min
Instrument used : Shimadzu LC 2010AT vp HPLC (Auto sampler)
Data station : Class VP 6.01 data station

6.6.2 Preparation of standard solution

The stock solutions containing 1 mg/ml of R and S form of Rosiglitazone were prepared in methanol. These stock solutions were stored in light resistant containers.

Aliquots of R-Rosiglitazone (3 - 7 µg/ml) and S-Rosiglitazone (9 - 21 µg/ml) were prepared in the mobile phase for analysis.

6.6.3 Preparation of sample solution

Twenty tablets were weighed; the average weight was determined and finely powdered. The powder equivalent to 5 mg of R and S form of Rosiglitazone (equivalent to 10 mg of racemic rosiglitazone) was accurately weighed and transferred into a 10 ml volumetric flask. To this 5 ml of mobile phase was added and sonicated for 10 min. The resulting solution was made up to 10 ml with mobile phase and filtered using whatmann filter paper No. 42. The components R and S enantiomers of rosiglitazone from four different formulations (Reglit Tablets containing 2 mg of Rosiglitazone, Enselin Tablets containing 4 mg of Rosiglitazone, Rosicon Tablets containing 4 mg of Rosiglitazone and Rosinorm Tablets containing 8 mg of Rosiglitazone) were extracted in mobile phase containing 10 µg/ml of each enantiomer (theoretical value).

The blank, standard and sample solutions were analysed by the optimized chromatographic conditions, the chromatograms were recorded and are shown in Fig. 8-13.

6.7 Stress degradation studies of Rosiglitazone enantiomers

Stress degradation studies were performed by subjecting the standard drug solution (1mg/ml of rosiglitazone RS in methanol) to various degradation media such as acidic medium, basic medium, neutral medium, oxidation and photo degradation studies. Depending on the extent of degradation observed, the studies were prolonged by certain variations in the concentrations of the degradation medium. The studies were performed at room temperature and in certain cases it was extended to 24 hrs at room temperature.

6.7.1 Acid degradation

1 ml of standard drug solution was taken into 10 ml volumetric flask and volume was made up with 0.1 M hydrochloric acid. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was further diluted to

10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 1 M hydrochloric acid and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 7, Fig. 14-15.

6.7.2 Basic degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 0.1 M sodium hydroxide. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was further diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 1 M sodium hydroxide and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 8, Fig. 16-17.

6.7.3 Degradation in neutral condition

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with water. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further the standard solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 9, Fig. 18-19.

6.7.4 Oxidation degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 3% hydrogen peroxide. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were

recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 30% hydrogen peroxide and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 10, Fig. 20-21.

6.7.5 Photo degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with water. The solution was exposed to sunlight for 24 hrs. 1 ml aliquots of the samples were withdrawn at 12 and 24 hrs, diluted with mobile phase and analysed by the optimised chromatographic conditions.

Photo degradation in powder form was carried out by exposing 25 mg of the enantiomers in petridish to sunlight for 24 hrs. 10 mg of the samples were withdrawn at 12 and 24 hrs, suitably diluted with mobile phase and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 11, Fig. 22-24.

6.8 Validation

Linearity

Standard solutions of 3 - 7 µg/ml of R-Rosiglitazone and 9 - 21 µg/ml of S-Rosiglitazone were analyzed to check the linearity of response (Table 4 and Fig. 27).

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of R and S Rosiglitazone in samples were confirmed by comparing the retention time and spectra of the standards.

Precision

Six injections at three different concentrations of R-rosiglitazone (3, 5, 7 µg/ml) and S-rosiglitazone (9, 15, 21 µg/ml) enantiomers were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 5).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at single level by adding a known quantity

of Rosiglitazone R and S enantiomers to the drug products of pre analyzed samples and the mixtures were reanalyzed. The average recoveries obtained from each sample were shown in Table 6.

Ruggedness and Robustness

The ruggedness of the proposed method was determined by carrying out the experiment on different instruments of Shimadzu HPLC (LC 2010AHT) and Water's breeze HPLC (Water's 1515 pump, Water's 2487 Dual wavelength detector) by different operators. Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines.

Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed HPLC method obtained were shown in Table 12.

6.9 LC-MS/MS Chromatographic conditions for Rosiglitazone

LC conditions

Stationary Phase	: ACI cellu 1 (150 x 4.6 mm i.d., 5 µ)
Mobile phase	: 0.025% formic acid (pH 6): Acetonitrile (15:85)
Flow rate	: 0.5 ml/min
Injection volume	: 10 µl using auto injector
Column oven temp	: 30^{o}C
System	: API 3000 LC-MS/MS with auto injector
Data station	: Analyst 1.31 data solution

MS/MS Conditions

Scan type	: MRM
Polarity	: Positive
Probe Temperature	: 510 ^{o}C
Q1 Mass	: 357.43
NEB	: 12
Cur	: 10
CAD	: 4.00
EP	: -8
Detection	: R and S Rosiglitazone – m/z 358

Retention time : R-Rosiglitazone : 3.09 min S-Rosiglitazone : 5.3 min

6.9.1 Preparation of standard solution

The stock solutions containing 1 mg/ml of R and S form of Rosiglitazone were prepared in methanol. These stock solutions were stored in light resistant containers.

Aliquots of R-Rosiglitazone (30 - 70 ng/ml) and S-Rosiglitazone (90 - 210 ng/ml) were prepared in the mobile phase for analysis.

6.9.2 Preparation of sample solution

Twenty tablets were weighed; the average weight was determined and finely powdered. The powder equivalent to 5 mg of R and S form of Rosiglitazone (equivalent to 10 mg of racemic rosiglitazone) was accurately weighed and transferred into a 10 ml volumetric flask. To this 5 ml of mobile phase was added and sonicated for 10 min. The resulting solution was made up to 10 ml with mobile phase and filtered using whatmann filter paper No. 42. The components R and S enantiomers of rosiglitazone from one formulation (Reglit Tablets containing 2 mg of Rosiglitazone) were extracted in mobile phase containing 10 µg/ml of each enantiomer (theoretical value).

The standard and sample solutions were analysed by the optimized chromatographic conditions, the chromatograms were recorded and are shown in Fig. 29-30.

6.10 Validation

Linearity

Standard solutions of 30 - 70 ng/ml of R-Rosiglitazone and 90 - 210 ng/ml of S-Rosiglitazone were analyzed to check the linearity of response (Table 13 and Fig. 31).

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of R and S Rosiglitazone in samples were confirmed by comparing the retention time and spectra of the standards.

Precision

Six injections at three different concentrations of R-Rosiglitazone (30, 50, 70 ng/ml) and S-Rosiglitazone (90, 150, 210 ng/ml) enantiomers were made and

analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 14).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at single level by adding a known quantity of Rosiglitazone R and S enantiomers to the drug products of pre analyzed samples and the mixtures were reanalyzed. The average recoveries obtained from each sample were shown in Table 15.

Ruggedness and Robustness

The ruggedness of the proposed method was determined by carrying out the experiment on different operators. Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines.

Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed method obtained were shown in Table 16.

6.11 Direct chiral HPLC Chromatographic conditions for Pioglitazone

Stationary Phase	: ACI Cellu 1 (150 x 4.6 mm i.d., 5 µ)
Mobile phase	: n-Hexane : n-Propyl alcohol (80:20)
Detection wavelength	: 233 nm
Flow rate	: 1.0 ml/min
Sample injector	: Auto sampling unit with 100 µl loop volume
Injection volume	: 100 µl
Retention time	: R-Pioglitazone : 3.6 min S-Pioglitazone : 4.6 min
Instrument used	: Shimadzu LC 2010AT vp HPLC (Auto sampler)
Data station	: Class VP 6.01 data station

6.11.1 Preparation of standard solution

The stock solutions containing 1 mg/ml of R and S form of Pioglitazone were prepared in methanol. These stock solutions were stored in light resistant containers.

Aliquots of R-Pioglitazone (5 - 11 µg/ml) and S-Pioglitazone (4 - 10 µg/ml) were prepared in the mobile phase for analysis.

6.11.2 Preparation of sample solution

Twenty tablets were weighed; the average weight was determined and finely powdered. The powder equivalent to 5 mg of R and S form of Pioglitazone (equivalent to 10 mg of racemic Pioglitazone) was accurately weighed and transferred into a 10 ml volumetric flask. To this 5 ml of mobile phase was added and sonicated for 10 min. The resulting solution was made up to 10 ml with mobile phase and filtered using whatmann filter paper No. 42. The components R and S enantiomers of Pioglitazone from four different formulations (Pepar Tablets containing 15 mg of Pioglitazone, Diavista Tablets containing 15 mg of Pioglitazone, Piozulin Tablets containing 30 mg of Pioglitazone and Piolem Tablets containing 30 mg of Pioglitazone) were extracted in mobile phase containing 10 µg/ml of each enantiomer (theoretical value).

The blank, standard and sample solutions were analysed by the optimized chromatographic conditions, the chromatograms were recorded and are shown in Fig. 32-37.

6.12 Stress degradation studies of Pioglitazone enantiomers

Stress degradation studies were performed by subjecting the standard drug solution (1mg/ml of Pioglitazone RS in methanol) to various degradation media such as acidic medium, basic medium, neutral medium, oxidation and photo degradation studies. Depending on the extent of degradation observed, the studies were prolonged by certain variations in the concentrations of the degradation medium. The studies were performed at room temperature and in certain cases it was extended to 24 hrs at room temperature.

6.12.1 Acid degradation

1 ml of standard drug solution was taken into 10 ml volumetric flask and volume was made up with 0.1 M hydrochloric acid. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was further diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 1 M hydrochloric acid and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised

chromatographic conditions. The chromatograms were recorded and are shown in Table 20, Fig. 38-39.

6.12.2 Basic degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 0.1 M sodium hydroxide. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was further diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 1 M sodium hydroxide and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 21, Fig. 40-41.

6.12.3 Degradation in neutral condition

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with water. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further the standard solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 22, Fig. 42-43.

6.12.4 Oxidation degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 3% hydrogen peroxide. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 30% hydrogen peroxide and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised

chromatographic conditions. The chromatograms were recorded and are shown in Table 23, Fig. 44-45.

6.12.5 Photo degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with water. The solution was exposed to sunlight for 24 hrs. 1 ml aliquots of the samples were withdrawn at 12 and 24 hrs, diluted with mobile phase and analysed by the optimised chromatographic conditions.

Photo degradation in powder form was carried out by exposing 25 mg of the enantiomers in petridish to sunlight for 24 hrs. 10 mg of the samples were withdrawn at 12 and 24 hrs, suitably diluted with mobile phase and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 24, Fig. 46-48.

6.13 Validation

Linearity

Standard solutions of 5-11 µg/ml of R-Pioglitazone and 4-10 µg/ml of S-Pioglitazone were analyzed to check the linearity of response (Table 17, Fig. 51).

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of R and S Pioglitazone in samples were confirmed by comparing the retention time and spectra of the standards.

Precision

Six injections at three different concentrations of R-Pioglitazone (5, 8, 11 µg/ml) S-Pioglitazone (4, 7, 10 µg/ml) enantiomers were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 18).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at single level by adding a known quantity of Pioglitazone R and S enantiomers to the drug products of pre analyzed samples and the mixtures were reanalyzed. The average recoveries obtained from each sample were shown in Table 19.

Ruggedness and Robustness

The ruggedness of the proposed method was determined by carrying out the experiment on different instruments of Shimadzu HPLC (LC 2010AHT) and Water's breeze HPLC (Water's 1515 pump, Water's 2487 Dual wavelength detector) by different operators. Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines.

Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed HPLC method obtained were shown in Table 25.

6.14 LC-MS/MS chromatographic conditions for Pioglitazone

LC conditions

Stationary Phase	: ACI cellu 1 (150 x 4.6 mm i.d., 5 µ)
Mobile phase	: 10 mM ammonium acetate: Acetonitrile (5:95)
Flow rate	: 0.5 ml/min
Injection volume	: 10 µl using auto injector
Column oven temp	: 35^{o}C
System	: API 3000 LC-MS/MS with auto injector
Data station	: Analyst 1.31 data solution

MS/MS Conditions

Scan type	: MRM
Polarity	: Positive
Probe Temperature	: 510 ^{o}C
Q1 Mass	: 357.43
NEB	: 20.00
Cur	: 24
CAD	: 5.00
EP	: -10
Detection	: R and S Pioglitazone– m/z 357
Retention time	: R-Pioglitazone : 1.28 min S-Pioglitazone : 2.58 min

6.14.1 Preparation of standard solution

The stock solutions containing 1 mg/ml of R and S form of Pioglitazone were prepared in methanol. These stock solutions were stored in light resistant containers.

Aliquots of R-Pioglitazone (50 - 110 ng/ml) and S-Pioglitazone (40 - 100 ng/ml) were prepared in the mobile phase for analysis.

6.14.2 Preparation of sample solution

Twenty tablets were weighed; the average weight was determined and finely powdered. The powder equivalent to 5 mg of R and S form of Pioglitazone (equivalent to 10 mg of racemic Pioglitazone) was accurately weighed and transferred into a 10 ml volumetric flask. To this 5 ml of mobile phase was added and sonicated for 10 min. The resulting solution was made up to 10 ml with mobile phase and filtered using whatmann filter paper No. 42. The components R and S enantiomers of Pioglitazone from one formulation (Pepar Tablets containing 15 mg of Pioglitazone) was extracted in mobile phase containing 10 µg/ml of each enantiomer (theoretical value).

The standard and sample solutions were analysed by the optimized chromatographic conditions, the chromatograms were recorded and are shown in Fig. 53-54.

6.15 Validation

Linearity

Standard solutions of 50 - 110 ng/ml of R-Pioglitazone and 40 - 100 ng/ml of S-Pioglitazone were analyzed to check the linearity of response (Table 26 and Fig. 55).

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of R and S Pioglitazone in samples were confirmed by comparing the retention time and spectra of the standards.

Precision

Six injections at three different concentrations of R-Pioglitazone (40, 70, 100 ng/ml) and S-Pioglitazone (50, 80, 110 ng/ml) enantiomers were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 27).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at single level by adding a known quantity

of pioglitazone R and S enantiomers to the drug products of pre analyzed samples and the mixtures were reanalyzed. The average recoveries obtained from each sample were shown in Table 28.

Ruggedness and Robustness

The ruggedness of the proposed method was determined by carrying out the experiment on different operators. Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines.

Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed method obtained were shown in Table 29.

6.16 Direct chiral HPLC Chromatographic conditions for Zaltoprofen

Stationary Phase	: ACI Cellu 1 (150 x 4.6 mm i.d., 5 µ)
Mobile phase	: Acetonitrile: 25 mM Sodium Perchlorate (80:20)
Detection wavelength	: 254 nm
Flow rate	: 1.0 ml/min
Sample injector	: Auto sampling unit with 100 µl loop volume
Injection volume	: 100 µl
Retention time	: S-Zaltoprofen : 3.1 min R-Zaltoprofen : 4.5 min
Instrument used	: Shimadzu LC 2010AT vp HPLC (Auto sampler)
Data station	: Class VP 6.01 data station

6.16.1 Preparation of standard solution

The stock solutions containing 1 mg/ml of S and R form of Zaltoprofen were prepared in methanol. These stock solutions were stored in light resistant containers.

Aliquots of S-Zaltoprofen (2 - 4 µg/ml) and R-Zaltoprofen (3 - 5 µg/ml) were prepared in the mobile phase for analysis.

6.16.2 Preparation of sample solution

Twenty tablets were weighed; the average weight was determined and finely powdered. The powder equivalent to 5 mg of S and R form of Zaltoprofen (equivalent to 10 mg of racemic Zaltoprofen) was accurately weighed and transferred into a 10 ml volumetric flask. To this 5 ml of mobile phase was added and sonicated for 10 min. The resulting solution was made up to 10 ml with mobile phase and filtered using whatmann filter paper No. 42. The components S and R enantiomers of

Zaltoprofen from one formulation (Zaltokin Tablets containing 80 mg of Zaltoprofen) was extracted in mobile phase containing 10 µg/ml of each enantiomer (theoretical value).

The blank, standard and sample solutions were analysed by the optimized chromatographic conditions, the chromatograms were recorded and are shown in Fig. 56-58.

6.17 Stress degradation studies of Zaltoprofen enantiomers

Stress degradation studies were performed by subjecting the standard drug solution (1mg/ml of Zaltoprofen RS in methanol) to various degradation media such as acidic medium, basic medium, neutral medium, oxidation and photo degradation studies. Depending on the extent of degradation observed, the studies were prolonged by certain variations in the concentrations of the degradation medium. The studies were performed at room temperature and in certain cases it was extended to 24 hrs at room temperature.

6.17.1 Acid degradation

1 ml of standard drug solution was taken into 10 ml volumetric flask and volume was made up with 0.1 M hydrochloric acid. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was further diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 1 M hydrochloric acid and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 33, Fig. 59-60.

6.17.2 Basic degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 0.1 M sodium hydroxide. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was further diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 1 M sodium hydroxide and the solution was kept at room

temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 34, Fig. 61-62.

6.17.3 Degradation in neutral condition

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with water. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further the standard solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 35, Fig. 63-64.

6.17.4 Oxidation degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 3% hydrogen peroxide. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 30% hydrogen peroxide and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 36, Fig. 65-66.

6.17.5 Photo degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with water. The solution was exposed to sunlight for 24 hrs. 1 ml aliquots of the samples were withdrawn at 12 and 24 hrs, diluted with mobile phase and analysed by the optimised chromatographic conditions.

Photo degradation in powder form was carried out by exposing 25 mg of the enantiomers in petridish to sunlight for 24 hrs. 10 mg of the samples were withdrawn at 12 and 24 hrs, suitably diluted with mobile phase and analysed by the optimised

chromatographic conditions. The chromatograms were recorded and are shown in Table 37, Fig. 67-69.

6.18 Validation

Linearity

Standard solutions of 2 - 4 µg/ml of S-Zaltoprofen and 3 - 5 µg/ml of R-Zaltoprofen were analyzed to check the linearity of response (Table 30, Fig. 72).

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of S and R Zaltoprofen in samples were confirmed by comparing the retention time and spectra of the standards.

Precision

Six injections at three different concentrations of S-Zaltoprofen (2, 3, 4 µg/ml) and R-Zaltoprofen (3, 4, 5 µg/ml) enantiomers were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 31).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at single level by adding a known quantity of Zaltoprofen S and R enantiomers to the drug products of pre analyzed samples and the mixtures were reanalyzed. The average recoveries obtained from each sample were shown in Table 32.

Ruggedness and Robustness

The ruggedness of the proposed method was determined by carrying out the experiment on different instruments of Shimadzu HPLC (LC 2010AHT) and Water's breeze HPLC (Water's 1515 pump, Water's 2487 Dual wavelength detector) by different operators. Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines.

Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed HPLC method obtained were shown in Table 38.

6.19 LC-MS/MS Chromatographic conditions for Zaltoprofen

Stationary Phase	: ACI cellu 1 (150 x 4.6 mm i.d., 5 µ)
Mobile phase	: 0.1% Ammonia solution: Acetonitrile (10: 90)
Flow rate	: 0.5 ml/min
Injection volume	: 10 µl using auto injector
Column oven temp	: 30°C
System	: API 3000 LC-MS/MS with auto injector
Data station	: Analyst 1.31 data solution

MS/MS Conditions

Scan type	: MRM
Polarity	: Positive
Probe Temperature	: 400 °C
Q1 Mass	: 298
NEB	: 10.00
Cur	: 8.00
CAD	: 6.00
EP	: 10.00
Detection	: S and R Zaltoprofen– m/z 299
Retention time	: S- Zaltoprofen – 2.48 min; R- Zaltoprofen – 3.99 min

6.19.1 Preparation of standard solution

The stock solutions containing 1 mg/ml of S and R form of Zaltoprofen were prepared in methanol. These stock solutions were stored in light resistant containers.

Aliquots of S-Zaltoprofen (18 - 54 ng/ml) and R-Zaltoprofen (22 - 66 ng/ml) were prepared in the mobile phase for analysis.

6.19.2 Preparation of sample solution

Twenty tablets were weighed; the average weight was determined and finely powdered. The powder equivalent to 5 mg of R and S form of Zaltoprofen (equivalent to 10 mg of racemic Zaltoprofen) was accurately weighed and transferred into a 10 ml volumetric flask. To this 5 ml of mobile phase was added and sonicated for 10 min. The resulting solution was made up to 10 ml with mobile phase and filtered using whatmann filter paper No. 42. The components R and S enantiomers of Zaltoprofen from one formulation (Zaltokin Tablets containing 80 mg of Zaltoprofen)

was extracted in mobile phase containing 10 µg/ml of each enantiomer (theoretical value).

The standard and sample solutions were analysed by the optimized chromatographic conditions, the chromatograms were recorded and are shown in Fig. 74-75.

6.20 Validation

Linearity

Standard solutions of 18 - 54 ng/ml of S-Zaltoprofen and 22 - 66 ng/ml of R-Zaltoprofen were analyzed to check the linearity of response (Table 39 and Fig. 76).

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of R and S Zaltoprofen in samples were confirmed by comparing the retention time and spectra of the standards.

Precision

Six injections at three different concentrations of S-Zaltoprofen (18, 36, 54 ng/ml) and R-Zaltoprofen (22, 44, 66 ng/ml) enantiomers were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 40).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at single level by adding a known quantity of Zaltoprofen R and S enantiomers to the drug products of pre analyzed samples and the mixtures were reanalyzed. The average recoveries obtained from each sample were shown in Table 41.

Ruggedness and Robustness

The ruggedness of the proposed method was determined by carrying out the experiment on different operators. Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines.

Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed method obtained were shown in Table 42.

6.21 Indirect chiral HPLC Chromatographic conditions for Valganciclovir Hydrochloride

Stationary Phase	: Hibar Purospher Star C_{18} (250 mm x 4.6 mm i.d., 5 µ)
Mobile phase	: 25mM Ammonium acetate (pH 3.5): Methanol (90:10)
Detection wavelength	: 210 nm
Flow rate	: 1.0 ml/min
Sample injector	: Auto sampling unit with 100 µl loop volume
Injection volume	: 100 µl
Retention time	: R-Valganciclovir Hydrochloride : 9.07 min
	S-Valganciclovir Hydrochloride : 10.10 min
Instrument used	: Shimadzu LC 2010AT vp HPLC (Auto sampler)
Data station	: Class VP 6.01 data station

6.21.1 Preparation of standard solution

The stock solutions containing 1 mg/ml of R and S form of Valganciclovir Hydrochloride were prepared in methanol. These stock solutions were stored in light resistant containers.

Aliquots of R-Valganciclovir Hydrochloride (10 - 30 µg/ml) and S-Valganciclovir Hydrochloride (8 - 24 µg/ml) were prepared in the mobile phase for analysis.

6.21.2 Preparation of sample solution

Twenty tablets were weighed; the average weight was determined and finely powdered. The powder equivalent to 5 mg of R and S form of Valganciclovir Hydrochloride (equivalent to 10 mg of racemic Valganciclovir Hydrochloride) was accurately weighed and transferred into a 10 ml volumetric flask. To this 5 ml of mobile phase was added and sonicated for 10 min. The resulting solution was made up to 10 ml with mobile phase and filtered using whatmann filter paper No. 42. The components R and S enantiomers of Valganciclovir Hydrochloride from one formulation (Valcyte Tablets containing 450 mg of Valganciclovir Hydrochloride) was extracted in mobile phase containing 10 µg/ml of each enantiomer (theoretical value).

The blank, standard and sample solutions were analysed by the optimized chromatographic conditions, the chromatograms were recorded and are shown in Fig. 77-79.

6.22 Stress degradation studies of Valganciclovir Hydrochloride enantiomers

Stress degradation studies were performed by subjecting the standard drug solution (1mg/ml of Valganciclovir Hydrochloride RS in methanol) to various degradation media such as acidic medium, basic medium, neutral medium, oxidation and photo degradation studies. Depending on the extent of degradation observed, the studies were prolonged by certain variations in the concentrations of the degradation medium. The studies were performed at room temperature and in certain cases it was extended to 24 hrs at room temperature.

6.22.1 Acid degradation

1 ml of standard drug solution was taken into 10 ml volumetric flask and volume was made up with 0.1 M hydrochloric acid. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was further diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 1 M hydrochloric acid and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 46, Fig. 80-81.

6.22.2 Basic degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 0.1 M sodium hydroxide. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was further diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 1 M sodium hydroxide and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised

chromatographic conditions. The chromatograms were recorded and are shown in Table 47, Fig. 82-83.

6.22.3 Degradation in neutral condition

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with water. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further the standard solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 48, Fig. 84-85.

6.22.4 Oxidation degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with 3% hydrogen peroxide. The solution was kept at room temperature for 2 hrs and analysed after 2 hrs. 1 ml of solution was diluted to 10 ml with mobile phase. The solutions were injected and the chromatograms were recorded. Further, to increase the extent of degradation, the standard drug solution was treated with 30% hydrogen peroxide and the solution was kept at room temperature for 24 hrs. 1 ml aliquots of the solutions were withdrawn at 1, 2, 4, 8, 12 and 24 hrs. The solutions were further diluted and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 49, Fig. 86-87.

6.22.5 Photo degradation

1 ml of standard stock solution was taken into 10 ml volumetric flask and volume was made up with water. The solution was exposed to sunlight for 24 hrs. 1 ml aliquots of the samples were withdrawn at 12 and 24 hrs, diluted with mobile phase and analysed by the optimised chromatographic conditions.

Photo degradation in powder form was carried out by exposing 25 mg of the enantiomers in petridish to sunlight for 24 hrs. 10 mg of the samples were withdrawn at 12 and 24 hrs, suitably diluted with mobile phase and analysed by the optimised chromatographic conditions. The chromatograms were recorded and are shown in Table 50, Fig. 88-90.

6.23 Validation

Linearity

Standard solutions of 10 - 30 µg/ml of R-Valganciclovir Hydrochloride and 8 - 24 µg/ml of S-Valganciclovir Hydrochloride were analyzed to check the linearity of response (Table 43, Fig. 93).

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of R and S Valganciclovir Hydrochloride in samples were confirmed by comparing the retention time and spectra of the standards.

Precision

Six injections at three different concentrations of R-Valganciclovir Hydrochloride (10, 20, 30 µg/ml) and S-Valganciclovir Hydrochloride (8, 16, 24 µg/ml) enantiomers were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 44).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at single level by adding a known quantity of Valganciclovir Hydrochloride R and S enantiomers to the drug products of pre analyzed samples and the mixtures were reanalyzed. The average recoveries obtained from each sample were shown in Table 45.

Ruggedness and Robustness

The ruggedness of the proposed method was determined by carrying out the experiment on different instruments of Shimadzu HPLC (LC 2010AHT) and Water's breeze HPLC (Water's 1515 pump, Water's 2487 Dual wavelength detector) by different operators using different columns of similar types. Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines.

Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed HPLC method obtained were shown in Table 51.

6.24 LC-MS/MS Chromatographic conditions for Valganciclovir Hydrochloride

Stationary Phase	**:** Princeton SPHER (100 X 4.6 mm, i.d., 5µ)
Mobile phase	: 10 mM Ammonium formate (pH 9): Acetonitrile (25: 75)
Flow rate	: 0.5 ml/min
Injection volume	: 10 µl using auto injector
Column oven temp	: 20^{o}C
System	: API 3000 LC-MS/MS with auto injector
Data station	: Analyst 1.31 data solution

MS/MS Conditions

Scan type	: MRM
Polarity	: Positive
Probe Temperature	: 450 ^{o}C
Q1 Mass	: 354
NEB	: 15
Cur	: 7.00
CAD	: 5.00
EP	: -10
Detection	: R and S Valganciclovir Hydrochloride– m/z 355
Retention time	: R-Valganciclovir Hydrochloride: 1.05 min S-Valganciclovir Hydrochloride: 2.02 min

6.24.1 Preparation of standard solution

The stock solutions containing 1 mg/ml of R and S forms of Valganciclovir Hydrochloride were prepared in methanol. These stock solutions were stored in light resistant containers.

Aliquots of R-Valganciclovir Hydrochloride (150 - 350 ng/ml) and S-Valganciclovir Hydrochloride (120 - 280 ng/ml) were prepared in the mobile phase for analysis.

6.24.2 Preparation of sample solution

Twenty tablets were weighed; the average weight was determined and finely powdered. The powder equivalent to 5 mg of R and S form of Valganciclovir Hydrochloride (equivalent to 10 mg of racemic Valganciclovir Hydrochloride) was accurately weighed and transferred into a 10 ml volumetric flask. To this 5 ml of

mobile phase was added and sonicated for 10 min. The resulting solution was made up to 10 ml with mobile phase and filtered using whatmann filter paper No. 42. The components R and S enantiomers of Valganciclovir Hydrochloride from one formulation (Valcyte Tablets containing 150 mg of Valganciclovir Hydrochloride) was extracted in mobile phase containing 10 µg/ml of each enantiomer (theoretical value).

The standard and sample solutions were analysed by the optimized chromatographic conditions, the chromatograms were recorded and are shown in Fig. 95-96.

6.25 Validation

Linearity

Standard solutions of 150 - 350 ng/ml of R-Valganciclovir Hydrochloride and 120 - 280 ng/ml of S-Valganciclovir Hydrochloride were analyzed to check the linearity of response (Table 52 and Fig. 97).

Specificity

The specificity of the method was ascertained by analyzing the standards and the samples. The peaks of R and S Valganciclovir Hydrochloride in samples were confirmed by comparing the retention time and spectra of the standards.

Precision

Six injections at three different concentrations of R-Valganciclovir Hydrochloride (150, 250, 350 ng/ml) and S-Valganciclovir Hydrochloride (120, 200, 280 ng/ml) enantiomers were made and analyzed to examine the precision of the method. The mean peak area, standard deviation and % RSD were calculated (Table 53).

Accuracy

Accuracy of the method was determined by recovery experiments. The recovery of the method was determined at single level by adding a known quantity of Valganciclovir R and S enantiomers to the drug products of pre analyzed samples and the mixtures were reanalyzed. The average recoveries obtained from each sample were shown in Table 54.

Ruggedness and Robustness

The ruggedness of the proposed method was determined by carrying out the experiment on different operators using different columns of similar types. Robustness of the method was determined by making small changes in the chromatographic conditions as stated in ICH guidelines.

Limit of detection and Limit of quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed method obtained were shown in Table 55.

7. RESULTS AND ANALYSIS

7.1 Optimisation of chromatographic conditions

Optimisation of chromatographic conditions is necessary for a proper separation and estimation of the enantiomers present in the selected drugs. Conditions like detection wavelength, nature of stationary phase, pH and composition of mobile phase, selection of interface, strength of mobile phases used, peak modifiers and flow rate were optimised to get the best possible resolution. The procedure adopted and an elaborate discussion on optimization and optimized conditions for the analysis of drug candidates, are presented in chapter 6. With the optimized conditions, standard and sample solutions were injected and the chromatograms were recorded. The optimized conditions used for estimation provided a well defined separation between R and S enantiomers and degradation products formed during stress degradation studies.

7.1.1 Selection of wavelength

The sensitivity of a HPLC method that uses UV detection depends upon the proper selection of the wavelength. The standard solutions were scanned from 200 to 400 nm and the UV spectra obtained were recorded. From these spectrums, 220, 233, 254 and 210 nm was selected for the method development of rosiglitazone, pioglitazone, zaltoprofen and valganciclovir hydrochloride enantiomers in the formulations. The selected drug candidates gave good peak response at the wavelengths selected (Fig. 4-7).

7.1.2 Initial chromatographic conditions

Acetonitrile, methanol and n-hexane were selected as organic phases in most of the mobile phases to elute the enantiomers from the stationary phase because of its favourable UV transmittance, low viscosity and better solubility for the selected drugs. The mixed standard solutions were chromatographed using the initial chromatographic conditions. To improve the resolution of the peaks, chromatographic variables like mobile phase, nature of stationary phase and composition of mobile phase were optimized. The procedure adopted and discussed on optimization is presented in chapter 6.

7.1.3 Estimation of enantiomers in formulations by HPLC and LC-MS methods

The estimation of selected drug formulations by HPLC and LC-MS methods were carried out using the optimized chromatographic conditions. The standard and sample solution chromatograms were recorded.

The peak area of the standard and sample solutions was recorded. From linearity curve of peak area versus concentration of standard, the concentration of each enantiomer in sample was estimated. The percentage of individual enantiomers found in formulations, mean and standard deviation for the formulations were calculated and are reported. The results of analysis show that the amount of each enantiomer was in good agreement with the label claim of the formulations.

7.2 Direct reverse phase chiral HPLC method for separation of Rosiglitazone enantiomer

Optimisation of the method was carried out using various concentrations of methanol while keeping the concentration of the aqueous phase constant. A solvent combination of diethylamine: methanol (40:60 v/v) gave a satisfactory separation of the enantiomers of interest. This optimized mobile phase separated R-Rosiglitazone at 1.8 min and S-Rosiglitazone at 2.2 min respectively (Fig. 8-13).

7.3 Stress degradation studies

The standard solution of Rosiglitazone (100 µg/ml) was subjected to various stress conditions and the following results were obtained. With the optimized chromatographic conditions the enantiomers were well separated from the degradants.

7.3.1 Acidic degradation

The chromatogram indicates that 9.46 % of R-Rosiglitazone and 13.57% of S-Rosiglitazone were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 7, Fig. 14-15. The drug degraded may be due to the reaction between amino group and acid.

7.3.2 Basic degradation

The chromatogram indicates that 24.96 % of R -Rosiglitazone and 74.89% of S-Rosiglitazone were degraded after 24 hrs. The mean peak area, %

degradation and retention time of the drug and degradants are given in Table. 8, Fig. 16 – 17. The drug degraded may be due to the reaction between oxygen in thiazolidine-2,4-dione and alkali.

7.3.3 Neutral degradation

The chromatogram indicates that 4.24 % of R-Rosiglitazone and 6.46% of S-Rosiglitazone were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 9, Fig. 18 – 19. The drug degraded may be due to the instability in water.

7.3.4 Oxidative degradation

The chromatogram indicates that 24.96 % of R -Rosiglitazone and 74.80% of S-Rosiglitazone were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table.10, Fig. 20 – 21. The drug degraded may be due to the reaction between carbonyl group and oxidizing agent.

7.3.5 Photolytic degradation:

The chromatogram indicates that 0.19 % of R-Rosiglitazone and 0.30% of S-Rosiglitazone drug in powder form were degraded. It was found that 0.79 % of R-Rosiglitazone and 2.05 % of S-Rosiglitazone drug in solution form were degraded. The mean peak area, % degradation and retention time of the drug and degradants are given in Table.11, Fig. 22 - 24. The drug slightly degraded may be due to the instability under sunlight.

The bar charts of decrease in the concentrations of R and S Rosiglitazone with various stress conditions were presented in Figure 25 – 26. The R and S enantiomers were found to degrade in alkaline, acidic, neutral, oxidative conditions and slightly degraded when exposed to sunlight.

7.4 Validation

The real goal of validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method. The components of validation methods and procedure adopted for the method validation were discussed in detail in the earlier chapter. This section deals with the discussions of the results obtained.

The calibration curves of R-Rosiglitazone and S-Rosiglitazone were linear in the range of 3-7 µg/ml and 9-21 µg/ml respectively (Fig. 27 and Table 4). Linear regression equation and correlation coefficient are shown in Table 12.

The precision of the method was demonstrated by reproducibility studies. The mean, standard deviation and % RSD were calculated and are presented in Table 5. The % RSD values of less than 2% revealed that the methods were precise.

The accuracy of the optimized methods was determined by absolute recovery experiments. An analysis of the results showed that the percentage recovery values were close to 100 % thus establishing that the developed method is accurate and reliable (Table 6).

Detection limits and quantification limits of R-Rosiglitazone and S-Rosiglitazone were found to be 0.596 µg/ml and 1.80 µg/ml respectively (Table 12).

No marked changes in the chromatogram occurred on changing the instrument, operator and chromatographic conditions indicated that the developed method was rugged and robust.

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions and are presented in Table 12. The values obtained demonstrated the suitability of the system for the analysis of rosiglitazone enantiomers in pharmaceutical formulations.

7.5 LC-MS method for separation of Rosiglitazone enantiomer

In the spectral investigation by LC/MS in the SCAN mode, standard solution of rosiglitazone showed major peak at m/z of 359, which was assigned to the [M+H] ion of rosiglitazone (Fig. 28). Optimization of the method was carried out using various concentrations of acetonitrile while keeping the aqueous phase constant. A solvent combination of 0.025% formic acid (pH 6): acetonitrile (15: 85 % v/v) gave a satisfactory separation of the enantiomers of interest. This optimized mobile phase separated R-Rosiglitazone at 3.09 min and S-Rosiglitazone at 5.3 min respectively. The typical chromatograms of the standard and the sample solutions are shown in Fig. 29 – 30.

The calibration curves of R-Rosiglitazone and S-Rosiglitazone were linear in the range of 30–70 ng/ml and 90–210 ng/ml respectively (Fig. 31, Table 13). Linear regression equation and correlation coefficient are shown in Table 16.

The precision of the method was demonstrated by reproducibility studies. The mean, standard deviation and % RSD were calculated and are presented in Table 14. The % RSD values of less than 2% revealed that the methods were precise.

The accuracy of the optimized method was determined by absolute recovery experiments. The percentage recovery values for R and S Rosiglitazone was found to be between 25.24 % and 75.49 %. An analysis of the results showed that the percentage recovery values were close to 100 % thus establishing that the developed method is accurate and reliable (Table 15).

Detection limits and quantification limits of R-Rosiglitazone and S-Rosiglitazone were found to be 0.2086 ng/ml and 0.6324 ng/ml respectively (Table 16).

No marked changes in the chromatogram occurred on changing the operator and chromatographic conditions indicating that the developed method was rugged and robust.

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions and are presented in Table 16. The values obtained demonstrated the suitability of the system for the analysis of R-Rosiglitazone and S-Rosiglitazone in combined form in pharmaceutical formulation.

7.6 Direct normal phase chiral HPLC method for separation of Pioglitazone enantiomer

Optimisation of the method was carried out using various concentrations of n-propanol while keeping the concentration of other phase constant. A solvent combination of n-hexane: n-propyl alcohol (80:20 v/v) gave a satisfactory separation of the enantiomers of interest. This optimized mobile phase separated R-Pioglitazone at 3.6 min and S-Pioglitazone at 4.6 min respectively (Fig. 32-37).

7.7 Stress degradation studies

The standard solution of Pioglitazone (100 µg/ml) was subjected to various stress conditions and the following results were obtained. With the optimized chromatographic conditions the enantiomers were well separated from the degradants.

7.7.1 Acidic degradation

The chromatogram indicates that 13.87 % of R-Pioglitazone and 12.22% of S-Pioglitazone were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 20, Fig. 38-39. The drug degraded may be due to the reaction between amino group and acid.

7.7.2 Basic degradation

The chromatogram indicates that 52% of R-Pioglitazone and 5.82% of S-Pioiglitazone were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 21, Fig. 40 – 41. The drug degraded may be due to the reaction between oxygen in thiazolidine-2,4-dione and alkali.

7.7.3 Neutral degradation

The chromatogram indicates that 3.75 % of R-Pioglitazone and 0.6% of S-Pioglitazone were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 22, Fig. 42 – 43. The drug degraded may be due to the instability in water.

7.7.4 Oxidative degradation

The chromatogram indicates that 7.86 % of R -Pioglitazone and 4.51% of S-Pioglitazone were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table.23, Fig. 44 – 45. The drug degraded may be due to the reaction between carbonyl group and oxidizing agent.

7.7.5 Photolytic degradation

The chromatogram indicates that 0.215 % of R-Pioglitazone and 0.24% of S-Pioglitazone drug in powder form were degraded. It was found that 0.266 % of R-Pioglitazone and 0.143 % of S-Pioglitazone drug in solution form were degraded. The mean peak area, % degradation and retention time of the drug

and degradants are given in Table.24, Fig. 46 - 48. The drug slightly degraded may be due to the instability under sunlight.

The bar charts of decrease in the concentrations of R and S Pioglitazone with various stress conditions were presented in Fig. 49 – 50. The R and S enantiomers were found to degrade in alkaline, acidic, neutral, oxidative conditions and slightly degraded when exposed to sunlight.

7.8 Validation

The calibration curves of R-Pioglitazone and S-Pioglitazone were linear in the range of 5 – 11 µg/ml and 4 – 10 µg/ml respectively (Fig. 51 and Table 17). Linear regression equation and correlation coefficient are shown in Table 25.

The precision of the method was demonstrated by reproducibility studies. The mean, standard deviation and % RSD were calculated and are presented in Table 18. The % RSD values of less than 2% revealed that the methods were precise.

The accuracy of the optimized methods was determined by absolute recovery experiments. An analysis of the results showed that the percentage recovery values were close to 100 % thus establishing that the developed method is accurate and reliable (Table 19).

Detection limits and quantification limits of R-Pioglitazone and S-Pioglitazone were found to be 1.4 µg/ml and 4.26 µg/ml respectively (Table 25).

No marked changes in the chromatogram occurred on changing the instrument, operator and chromatographic conditions indicated that the developed method was rugged and robust.

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions and are presented in Table 25. The values obtained demonstrated the suitability of the system for the analysis of Pioglitazone enantiomers in pharmaceutical formulations.

7.9 LC-MS method for separation of Pioglitazone enantiomer

In the spectral investigation by LC/MS in the SCAN mode, standard solution of Pioglitazone showed major peak at m/z of 357, which was assigned to the [M+H] ion of Pioglitazone (Fig. 52). Optimization of the method was carried out using various concentrations of acetonitrile while keeping the

aqueous phase constant. A solvent combination of 10 mM ammonium acetate: acetonitrile (5:95 v/v) gave a satisfactory separation of the enantiomers of interest. This optimized mobile phase separated R-Pioglitazone at 1.28 min and S-Pioglitazone at 2.58 min respectively. The typical chromatograms of the standard and the sample solutions are shown in Fig. 53 – 54.

The calibration curves of R-Pioglitazone and S-Pioglitazone were linear in the range of 50–110 ng/ml and 40 - 100 ng/ml respectively (Fig. 55, Table 26). Linear regression equation and correlation coefficient are shown in Table 29.

The precision of the method was demonstrated by reproducibility studies. The mean, standard deviation and % RSD were calculated and are presented in Table 27. The % RSD values of less than 2% revealed that the methods were precise.

The accuracy of the optimized method was determined by absolute recovery experiments. The percentage recovery values for R and S Pioglitazone was found to be between 55.06 % and 45.19 %. An analysis of the results showed that the percentage recovery values were close to 100 % thus establishing that the developed method is accurate and reliable (Table 28).

Detection limits and quantification limits of R-Pioglitazone and S-Pioglitazone were found to be 0.139 ng/ml and 0.421 ng/ml respectively (Table 29).

No marked changes in the chromatogram occurred on changing the instrument, operator and chromatographic conditions indicating that the developed method was rugged and robust.

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions and are presented in Table 29. The values obtained demonstrated the suitability of the system for the analysis of R-Pioglitazone and S-Pioglitazone in combined form in pharmaceutical formulation.

7.10 Direct normal phase HPLC method for separation of Zaltoprofen enantiomer

Optimisation of the method was carried out using various concentrations of acetonitrile while keeping the concentration of aqueous phase constant. A solvent combination of acetonitrile: 25 mM sodium perchlorate (80:20 v/v) gave

a satisfactory separation of the enantiomers of interest. This optimized mobile phase separated S-Zaltoprofen at 3.1 min and R-Zaltoprofen at 4.5 min respectively (Fig. 56-58).

7.11 Stress degradation studies

The standard solution of Zaltoprofen (100 µg/ml) was subjected to various stress conditions and the following results were obtained. With the optimized chromatographic conditions the enantiomers were well separated from the degradants.

7.11.1 Acidic degradation

The chromatogram indicates that 7.75 % of S-Zaltoprofen and 8.45% of R-Zaltoprofen were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 33, Fig. 59-60. The drug degraded may be due to the reaction between methyl group and acid.

7.11.2 Basic degradation

The chromatogram indicates that 14.53 % of S-Zaltoprofen and 13.69 % of R-Zaltoprofen were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 34, Fig. 61 – 62. The drug degraded may be due to the reaction between oxygen in dibenzo[b,f]thiepin-2-acetic acid and alkali.

7.11.3 Neutral degradation

The chromatogram indicates that 1.49 % of S-Zaltoprofen and 6.14 % of of R-Zaltoprofen were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 35, Fig. 63 – 64. The drug degraded may be due to the instability in water.

7.11.4 Oxidative degradation:

The chromatogram indicates that 36.11 % of S-Zaltoprofen and 49.93 % of of R-Zaltoprofen were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table.36, Fig. 65 – 66. The drug degraded may be due to the reaction between carbonyl group and oxidizing agent.

7.11.5 Photolytic degradation:

The chromatogram indicates that 0.21 % of S-Zaltoprofen and 0.24% of R-Zaltoprofen drug in powder form were degraded. It was found that 0.98 % of S-Zaltoprofen and 1.61 % of R-Zaltoprofen drug in solution form were degraded. The mean peak area, % degradation and retention time of the drug and degradants are given in Table.37, Fig. 67 - 69. The drug slightly degraded may be due to the instability under sunlight.

The bar charts of decrease in the concentrations of S and R Zaltoprofen with various stress conditions were presented in Fig. 70 – 71. The S and R enantiomers were found to degrade in alkaline, acidic, neutral, oxidative conditions and slightly degraded when exposed to sunlight.

7.12 Validation

The calibration curves of S- Zaltoprofen and R- Zaltoprofen were linear in the range of 2 – 4 µg/ml and 3 – 5 µg/ml respectively (Fig. 72 and Table 30). Linear regression equation and correlation coefficient are shown in Table 38.

The precision of the method was demonstrated by reproducibility studies. The mean, standard deviation and % RSD were calculated and are presented in Table 31. The % RSD values of less than 2% revealed that the methods were precise.

The accuracy of the optimized methods was determined by absolute recovery experiments. An analysis of the results showed that the percentage recovery values were close to 100 % thus establishing that the developed method is accurate and reliable (Table 32).

Detection limits and quantification limits of S-Zaltoprofen and R-Zaltoprofen were found to be 4.16 ng/ml and 12.61 ng/ml respectively (Table 38).

No marked changes in the chromatogram occurred on changing the instrument, operator and chromatographic conditions indicated that the developed method was rugged and robust.

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions and are presented in Table 38. The values obtained

demonstrated the suitability of the system for the analysis of Zaltoprofen enantiomers in pharmaceutical formulation.

7.13 LC-MS method for separation of Zaltoprofen enantiomer

In the spectral investigation by LC/MS in the SCAN mode, standard solution of Zaltoprofen showed major peak at m/z of 299, which was assigned to the [M+H] ion of Zaltoprofen (Fig. 73). Optimization of the method was carried out using various concentrations of acetonitrile while keeping the ammonia phase constant. A solvent combination of 0.1% ammonia solution: acetonitrile (10: 90, v/v) gave a satisfactory separation of the enantiomers of interest. This optimized mobile phase separated S-Zaltoprofen at 2.4 min and R-Zaltoprofen at 3.9 min respectively. The typical chromatograms of the standard and the sample solutions are shown in Fig. 74 – 75.

The calibration curves of S-Zaltoprofen and R-Zaltoprofen were linear in the range of 18-54 ng/ml and 22 - 66 ng/ml respectively (Fig. 76, Table 39). Linear regression equation and correlation coefficient are shown in Table 42.

The precision of the method was demonstrated by reproducibility studies. The mean, standard deviation and % RSD were calculated and are presented in Table 40. The % RSD values of less than 1% revealed that the methods were precise.

The accuracy of the optimized methods was determined by absolute recovery experiments. The percentage recovery values for S and R Zaltoprofen was found to be 45.63 % and 56.04 %. An analysis of the results showed that the percentage recovery values were close to 100 % thus establishing that the developed method is accurate and reliable (Table 41).

Detection limits and quantification limits of S- Zaltoprofen and R-Zaltoprofen were found to be 0.016 ng/ml and 0.051 ng/ml respectively (Table 42).

No marked changes in the chromatogram occurred on changing the instrument, operator and chromatographic conditions indicating that the developed method was rugged and robust.

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions and are presented in Table 42. The values obtained

demonstrated the suitability of the system for the analysis of S and R Zaltoprofen in combined form in pharmaceutical formulation.

7.14 Indirect normal phase HPLC method for separation of Valganciclovir Hydrochloride enantiomer

Optimisation of the method was carried out using various concentrations of methanol while keeping the concentration of aqueous phase constant. A solvent combination of 25mM Ammonium acetate (pH 3.5): Methanol (90:10 v/v) gave a satisfactory separation of the enantiomers of interest. This optimized mobile phase separated R-Valganciclovir Hydrochloride at 9.07 min and R-Valganciclovir Hydrochloride at 10.1 min respectively (Fig. 77-79).

7.15 Stress degradation studies

The standard solution of Valganciclovir Hydrochloride (100 µg/ml) was subjected to various stress conditions and the following results were obtained. With the optimized chromatographic conditions the enantiomers were well separated from the degradants.

7.15.1 Acidic degradation

The chromatogram indicates that 12.59% of R-Valganciclovir Hydrochloride and 14.51 % of S-Valganciclovir Hydrochloride were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 46, Fig. 80-81. The drug degraded may be due to the reaction between amino group and acid.

7.15.2 Basic degradation

The chromatogram indicates that 54.77% of R-Valganciclovir Hydrochloride and 44.87 % of S-Valganciclovir Hydrochloride were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table. 47, Fig. 82-83. The drug degraded may be due to the reaction between hydroxyl group and alkali.

7.15.3 Neutral degradation

The chromatogram indicates that 10.07% of R-Valganciclovir Hydrochloride and 6.16% of S-Valganciclovir Hydrochloride were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug

and degradants are given in Table. 48, Fig. 84 – 85. The drug degraded may be due to the instability in water.

7.15.4 Oxidative degradation:

The chromatogram indicates that 20.63% of R-Valganciclovir Hydrochloride and 14.74% of S-Valganciclovir Hydrochloride were degraded after 24 hrs. The mean peak area, % degradation and retention time of the drug and degradants are given in Table.49, Fig. 86 – 87. The drug degraded may be due to the reaction between carbonyl group and oxidizing agent.

7.15.5 Photolytic degradation:

The chromatogram indicates that 3.65% of R-Valganciclovir Hydrochloride and 1.62% of S-Valganciclovir Hydrochloride drug in powder form were degraded. It was found that 1.62% of R-Valganciclovir Hydrochloride and 8.59% of S-Valganciclovir Hydrochloride drug in solution form were degraded. The mean peak area, % degradation and retention time of the drug and degradants are given in Table.50, Fig. 88 - 90. The drug degraded may be due to the instability under sunlight.

The bar charts of decrease in the concentrations of R and S Valganciclovir Hydrochloride with various stress conditions were presented in Fig. 91 – 92. The R and S enantiomers were found to degrade in alkaline, acidic, neutral, oxidative conditions and when exposed to sunlight.

7.16 Validation

The calibration curves of R-Valganciclovir Hydrochloride and S-Valganciclovir Hydrochloride were linear in the range of 10 – 30 µg/ml and 8 – 24 µg/ml respectively (Fig. 93 and Table 43). Linear regression equation and correlation coefficient are shown in Table 51.

The precision of the method was demonstrated by reproducibility studies. The mean, standard deviation and % RSD were calculated and are presented in Table 44. The % RSD values of less than 1% revealed that the methods were precise.

The accuracy of the optimized methods was determined by absolute recovery experiments. An analysis of the results showed that the percentage

recovery values were close to 100 % thus establishing that the developed method is accurate and reliable (Table 45).

Detection limits and quantification limits of R-Valganciclovir Hydrochloride and S-Valganciclovir Hydrochloride were found to be 0.417 µg/ml and 1.264 µg/ml respectively (Table 51).

No marked changes in the chromatogram occurred on changing the instrument, operator and chromatographic conditions indicated that the developed method was rugged and robust.

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions and are presented in Table 51. The values obtained demonstrated the suitability of the system for the analysis of Valganciclovir Hydrochloride enantiomers in pharmaceutical formulations.

7.17 LC-MS method for separation of Valganciclovir Hydrochloride enantiomer

In the spectral investigation by LC/MS in the SCAN mode, standard solution of Valganciclovir Hydrochloride showed major peak at m/z of 355, which was assigned to the [M+H] ion of Valganciclovir Hydrochloride (Fig. 94). Optimization of the method was carried out using various concentrations of acetonitrile while keeping the aqueous phase constant. A solvent combination of 10 mM Ammonium formate (pH 9): Acetonitrile (25: 75 v/v) gave a satisfactory separation of the enantiomers of interest. This optimized mobile phase separated R-Valganciclovir Hydrochloride at 1.5 min and S-Valganciclovir Hydrochloride at 2.02 min respectively. The typical chromatograms of the standard and the sample solutions are shown in Fig. 95 – 96.

The calibration curves of R-Valganciclovir Hydrochloride and S-Valganciclovir Hydrochloride were linear in the range of 150 - 350 ng/ml and 120 - 280 ng/ml respectively (Fig. 97, Table 52). Linear regression equation and correlation coefficient are shown in Table 55.

The precision of the method was demonstrated by reproducibility studies. The mean, standard deviation and % RSD were calculated and are presented in Table 53. The % RSD values of less than 0.5% revealed that the methods were precise.

The accuracy of the optimized method was determined by absolute recovery experiments. An analysis of the results showed that the percentage recovery values were close to 100 % thus establishing that the developed method is accurate and reliable (Table 54).

Detection limits and quantification limits of R-Valganciclovir Hydrochloride and S-Valganciclovir Hydrochloride were found to be 0.041 ng/ml and 0.125 ng/ml respectively (Table 55).

No marked changes in the chromatogram occurred on changing the instrument, operator and chromatographic conditions indicating that the developed method was rugged and robust.

The column efficiency, resolution and peak asymmetry were calculated for the standard solutions and are presented in Table 55. The values obtained demonstrated the suitability of the system for the analysis of R-Valganciclovir Hydrochloride and S-Valganciclovir Hydrochloride in combined form in pharmaceutical formulation.

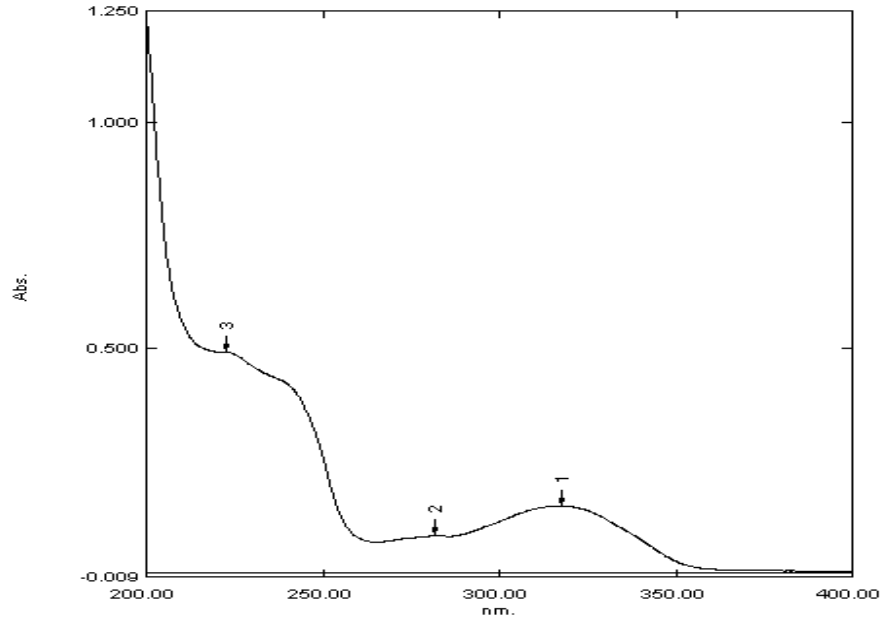

Figure 4: Typical UV spectra of standard Rosiglitazone

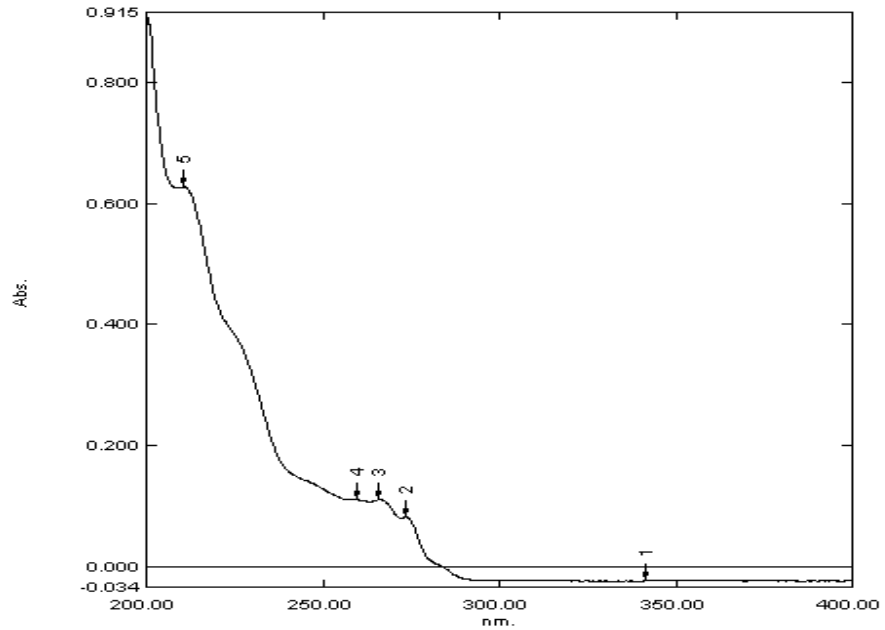

Figure 5: Typical UV spectra of standard Pioglitazone

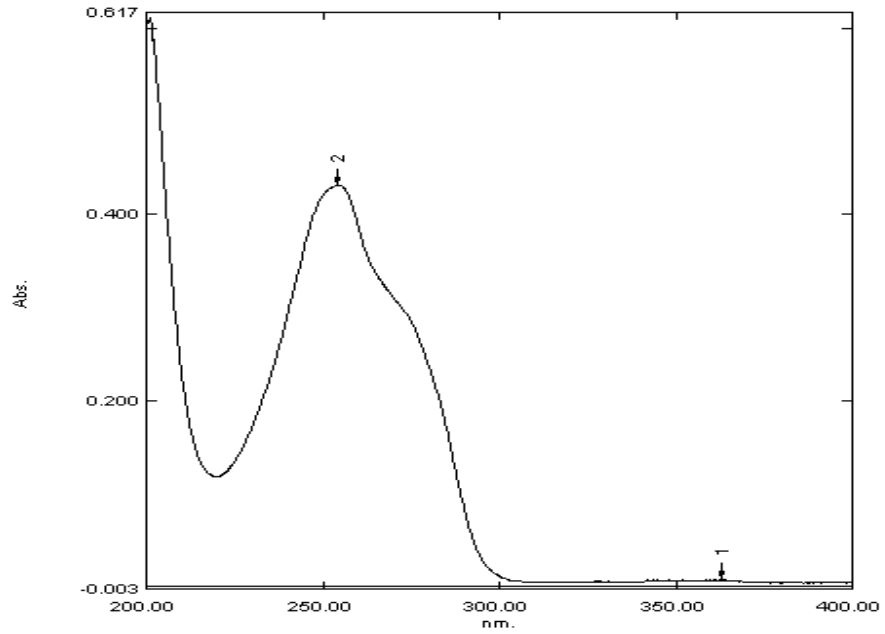

Figure 6: Typical UV spectra of standard Zaltoprofen

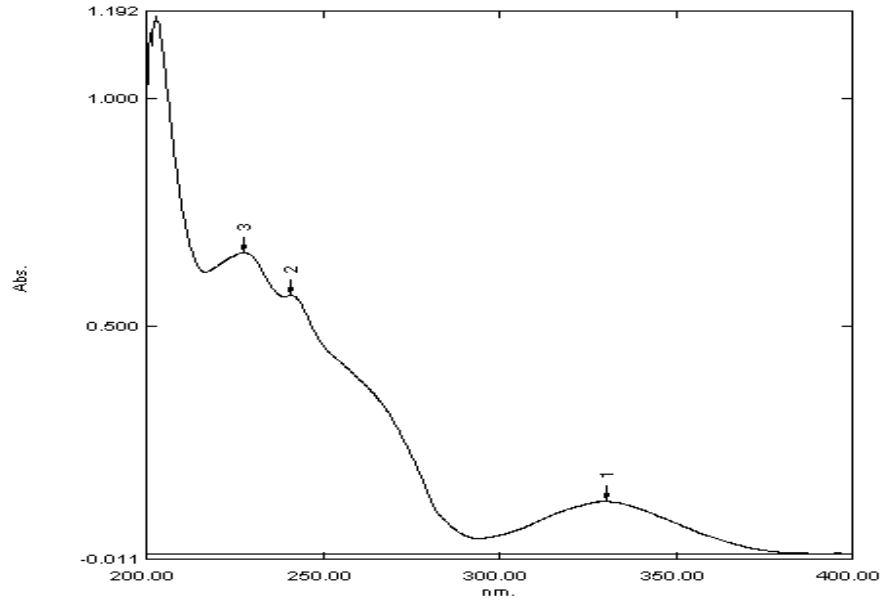

Figure 7: Typical UV spectra of standard Valganciclovir Hydrochloride

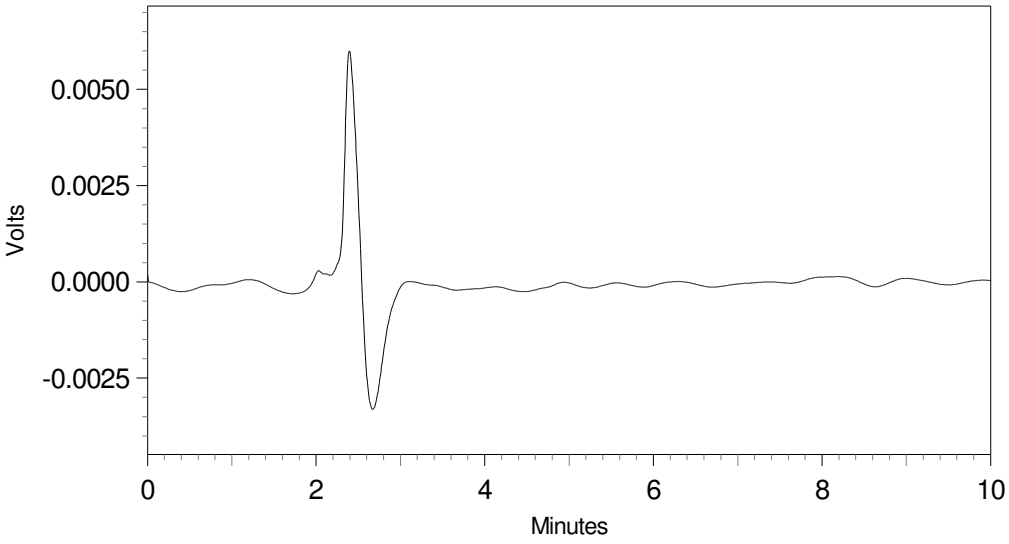

Figure 8: Typical HPLC chromatogram of Rosiglitazone (R & S) blank solution

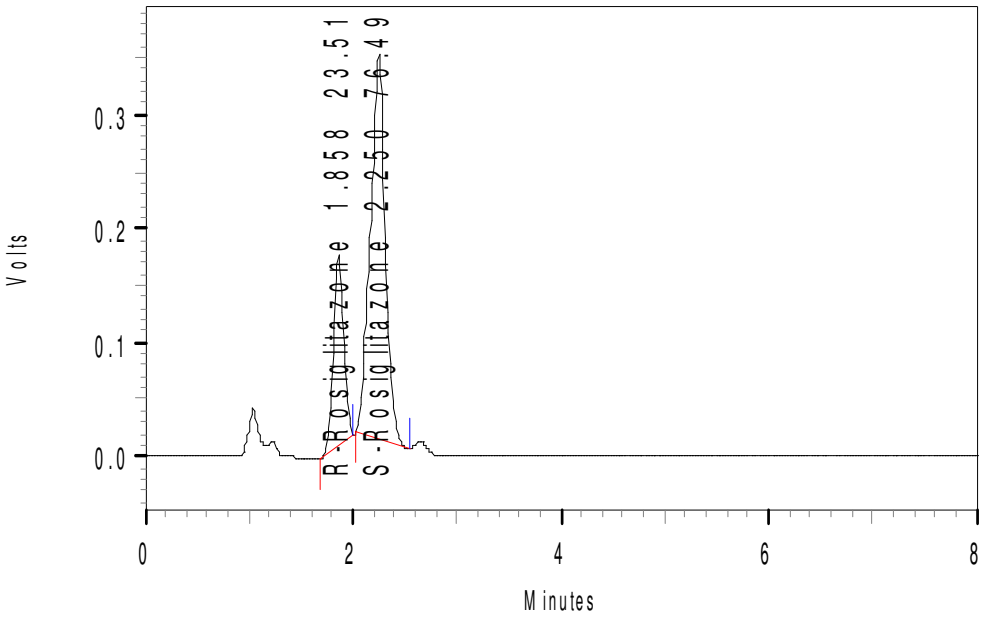

Figure 9: Typical HPLC chromatogram of Rosiglitazone (R & S) standard solution

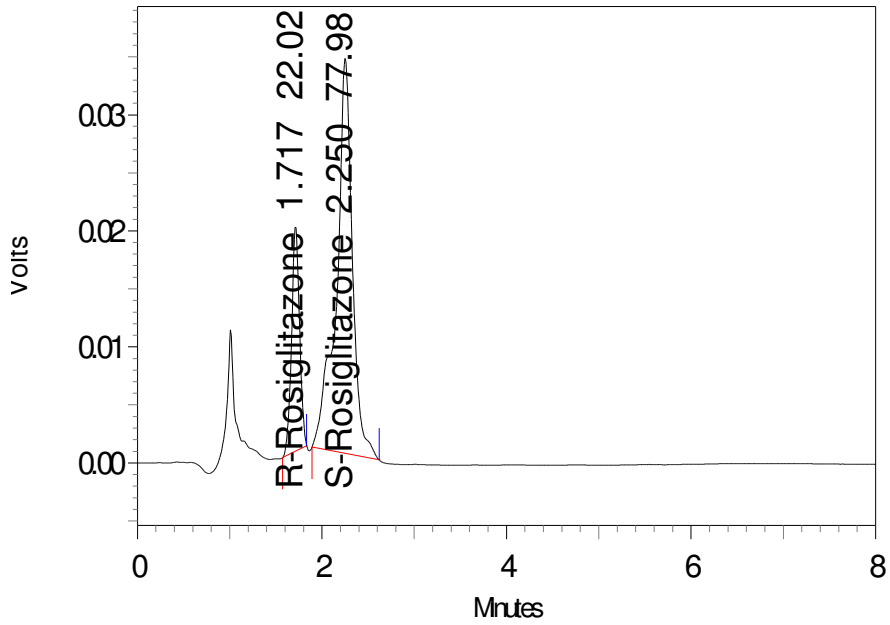

Figure 10: Typical HPLC chromatogram of sample I containing Rosiglitazone (RS)

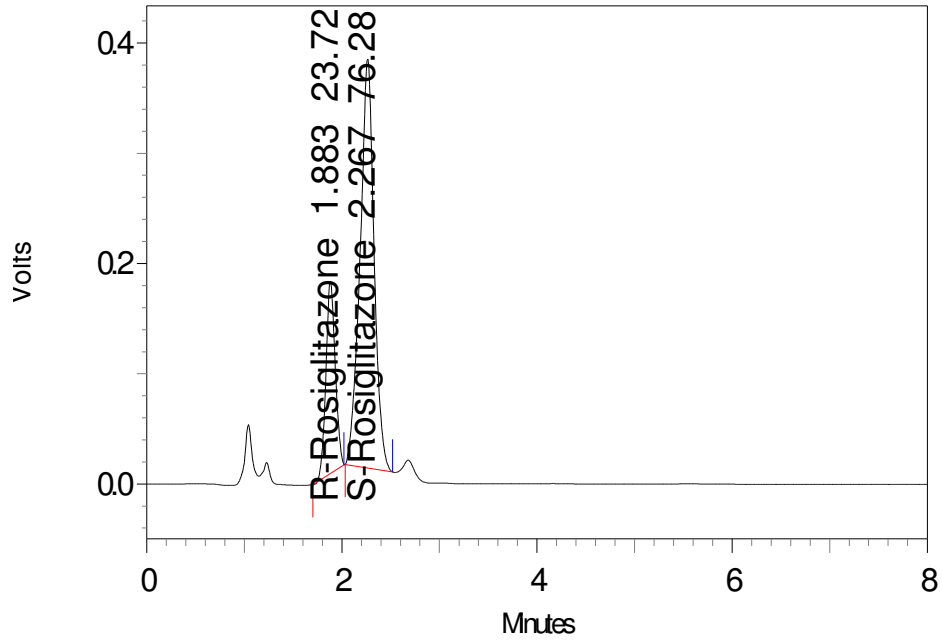

Figure 11: Typical HPLC chromatogram of sample II containing Rosiglitazone (RS)

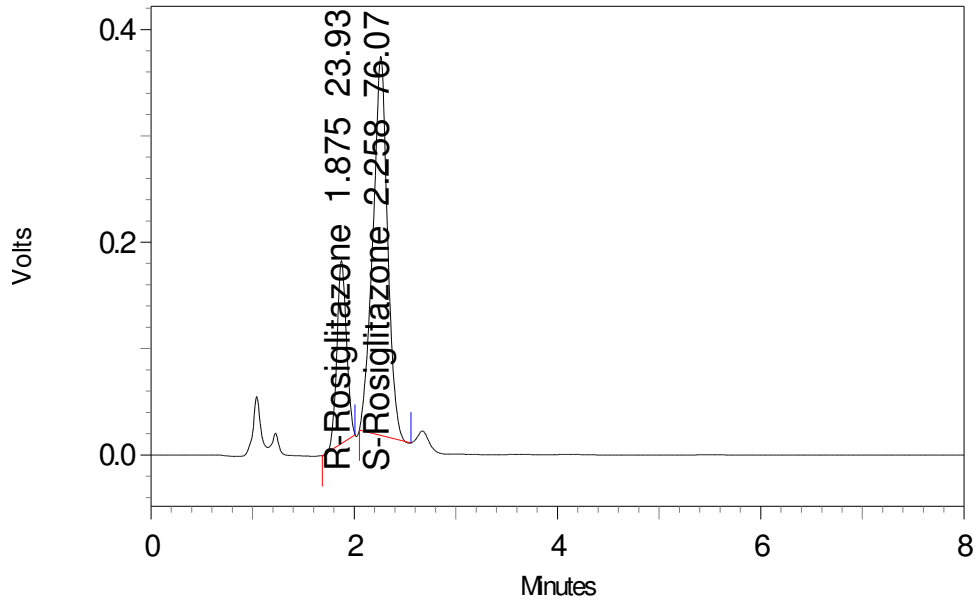

Figure 12: Typical HPLC chromatogram of sample III containing Rosiglitazone (RS)

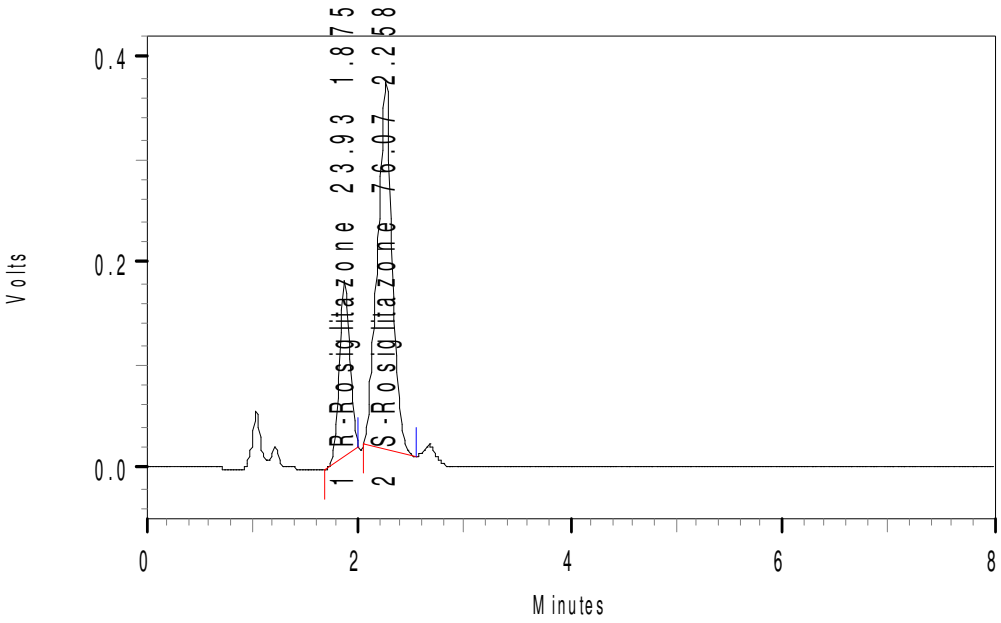

Figure 13: Typical HPLC chromatogram of sample IV containing Rosiglitazone (RS)

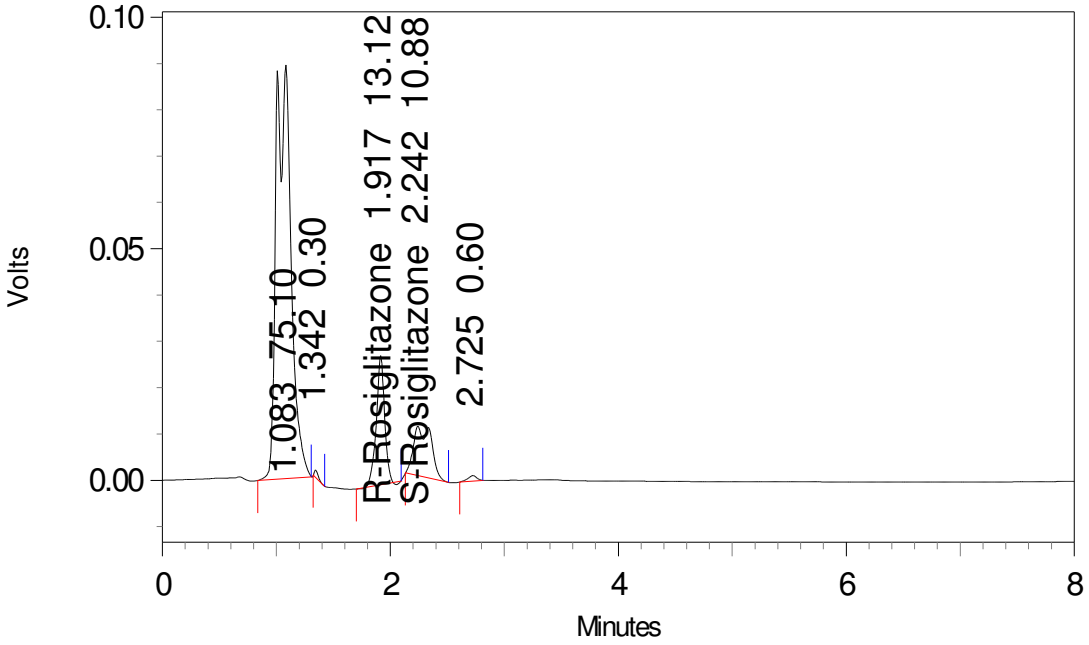

Figure 14: Typical HPLC chromatogram of acid degradation sample of Rosiglitazone (R&S) with 1N HCl at 0 hr

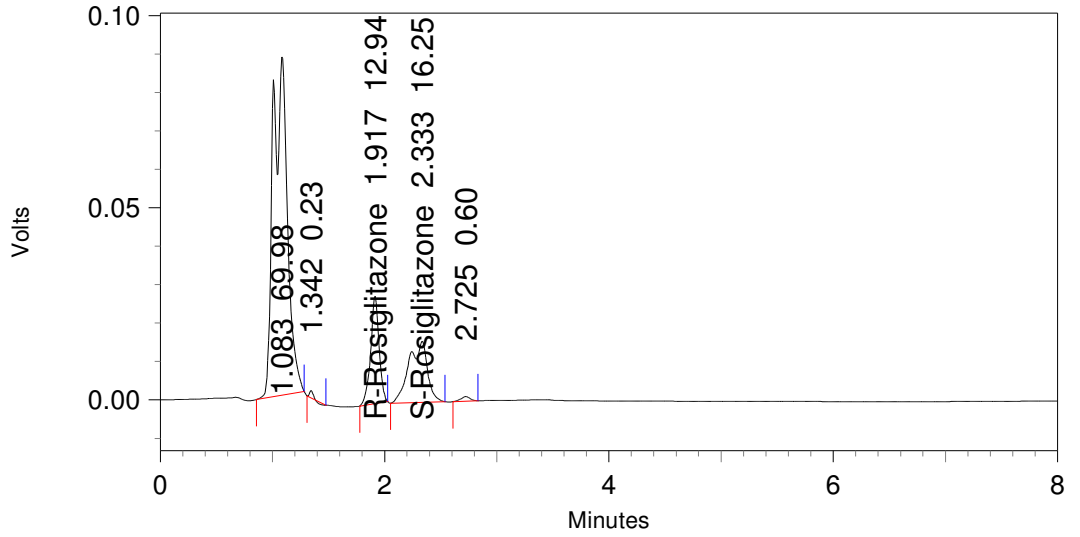

Figure 15: Typical HPLC chromatogram of acid degradation sample of Rosiglitazone (R&S) with 1N HCl at 24 hrs

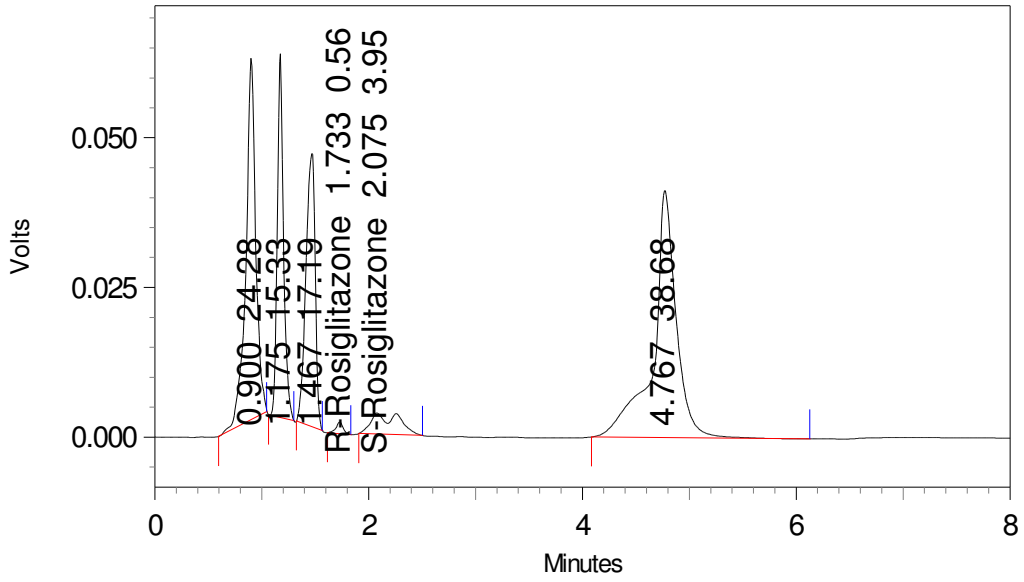

Figure 16: Typical HPLC chromatogram of basic degradation sample of Rosiglitazone (R&S) with 1N NaOH at 0 hr

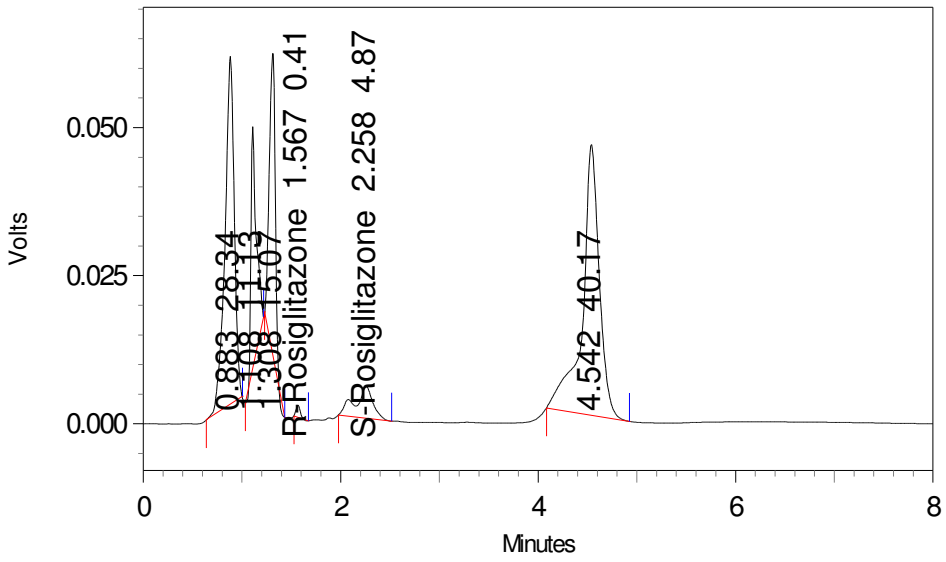

Figure 17: Typical HPLC chromatogram of basic degradation sample of Rosiglitazone (R&S) with 0.1N NaOH at 24 hrs

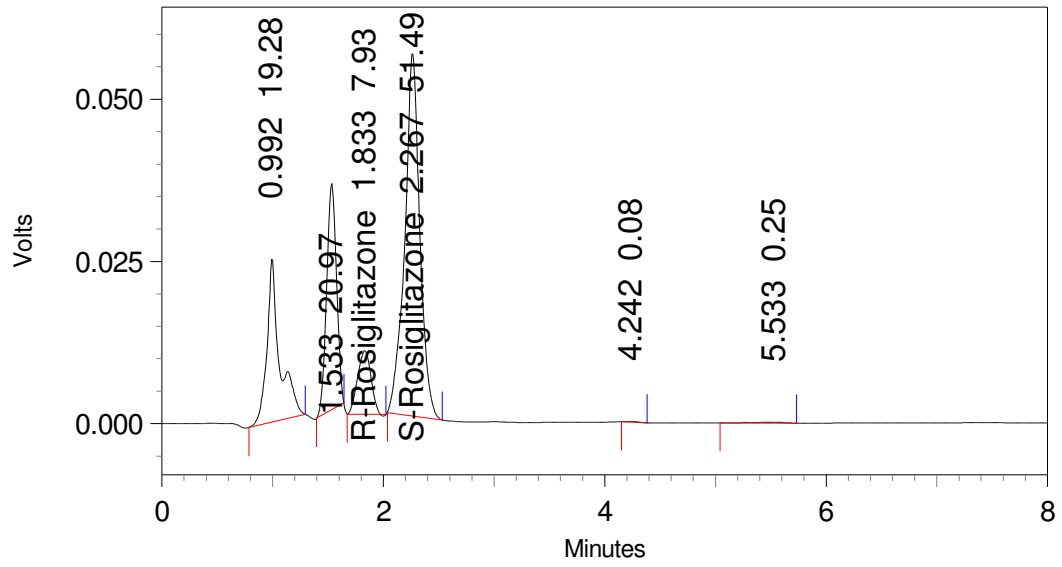

Figure 18: Typical HPLC chromatogram of neutral degradation sample of Rosiglitazone (R&S) with water at 0 hr

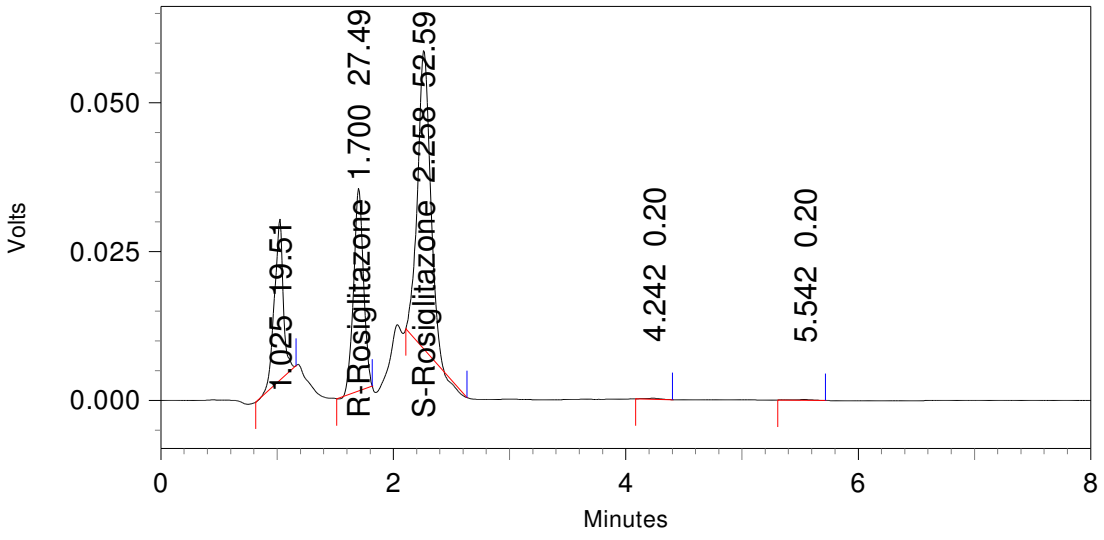

Figure 19: Typical HPLC chromatogram of neutral degradation sample of Rosiglitazone (R&S) with water at 24 hrs

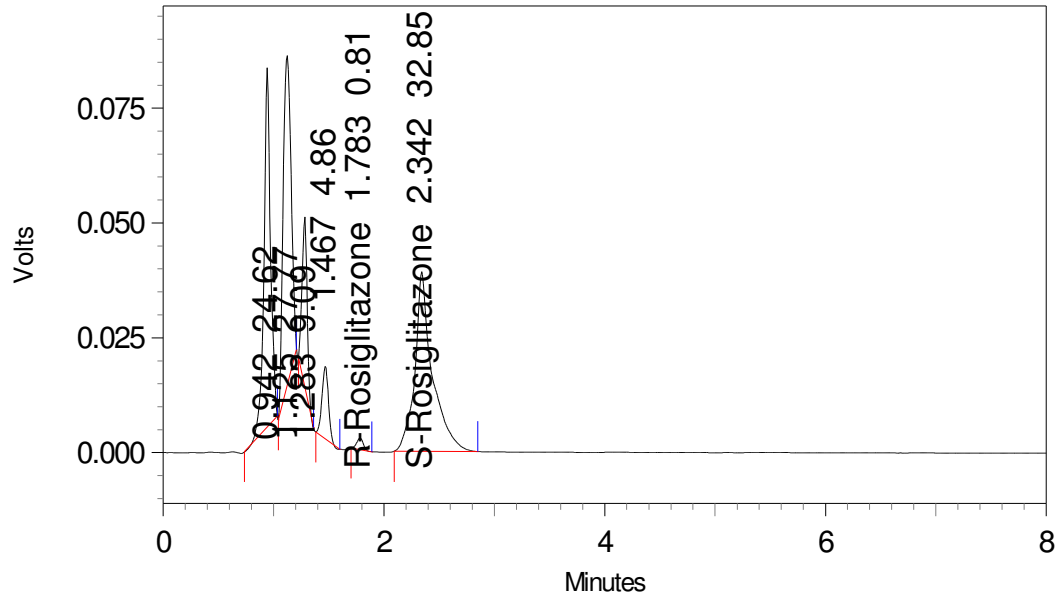

Figure 20: Typical HPLC chromatogram of oxidative degradation sample of Rosiglitazone (R&S) with hydrogen peroxide at 0 hr

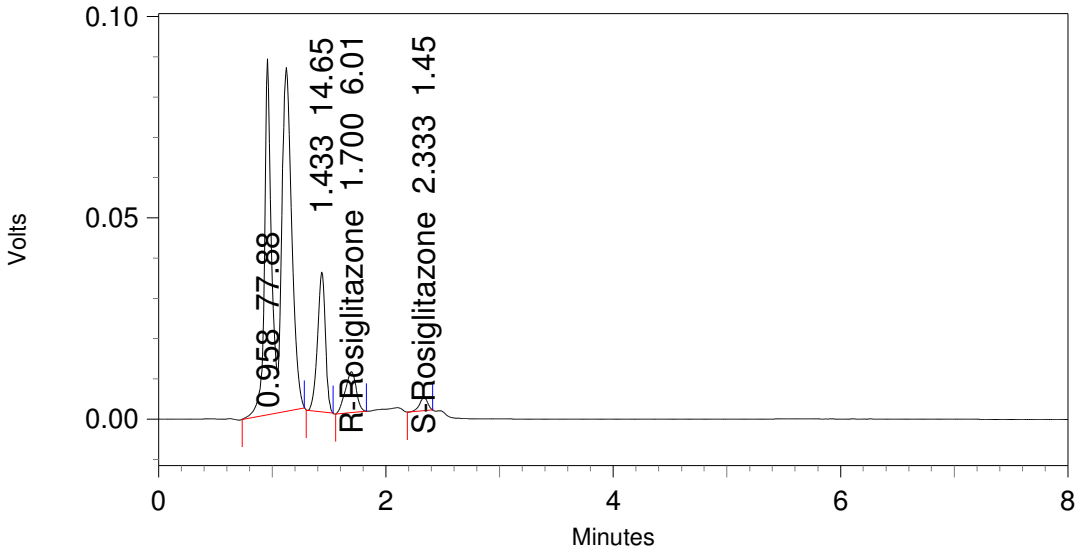

Figure 21: Typical HPLC chromatogram of oxidative degradation sample of Rosiglitazone (R&S) with hydrogen peroxide at 24 hrs

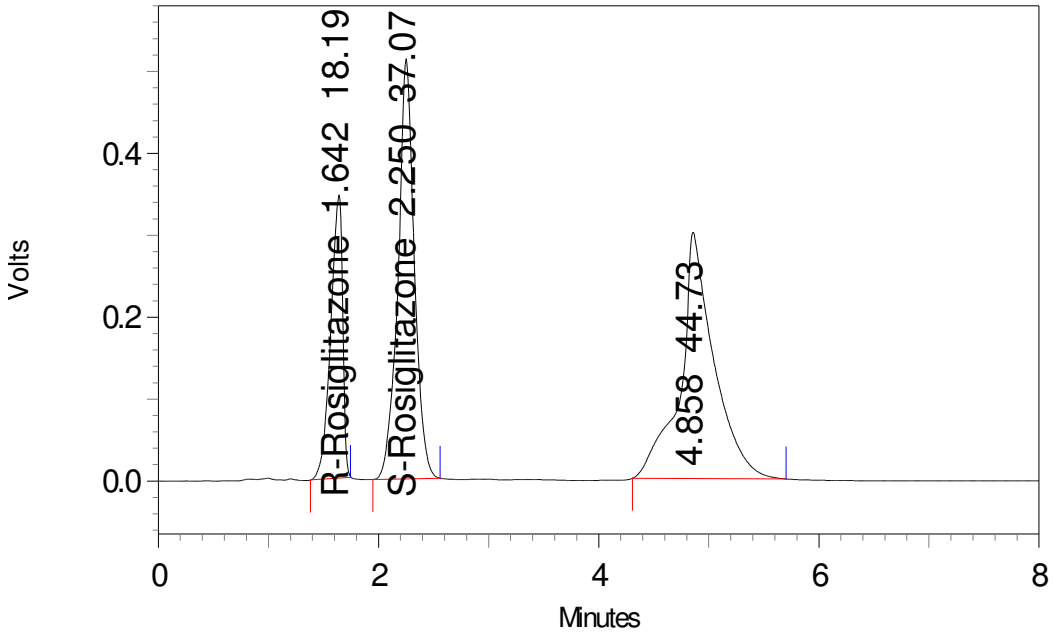

Figure 22: Typical HPLC chromatogram of photo degradation sample of Rosiglitazone (R&S) with sunlight at 0 hr

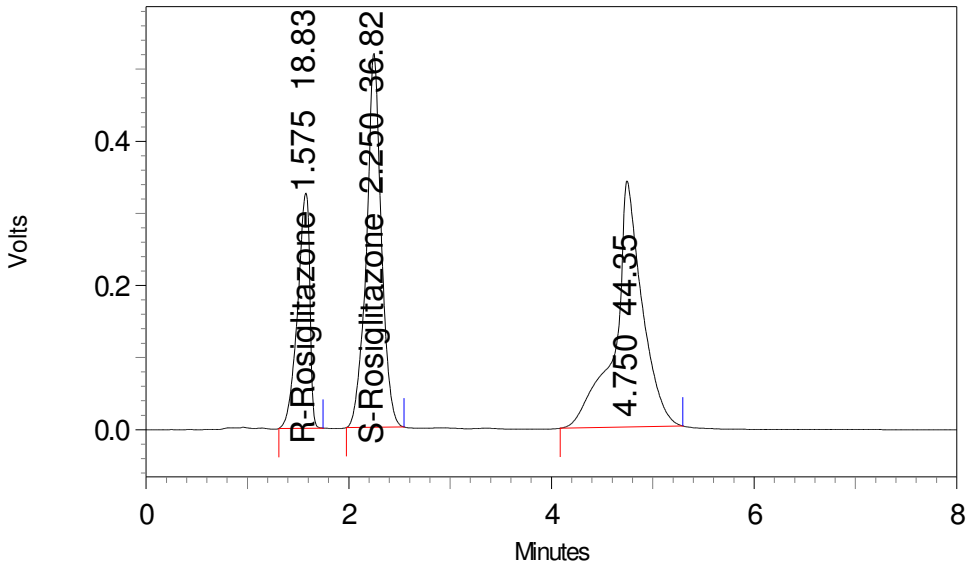

Figure 23: Typical HPLC chromatogram of photo degradation sample of Rosiglitazone (R&S) with sunlight at 24 hrs (Powder form)

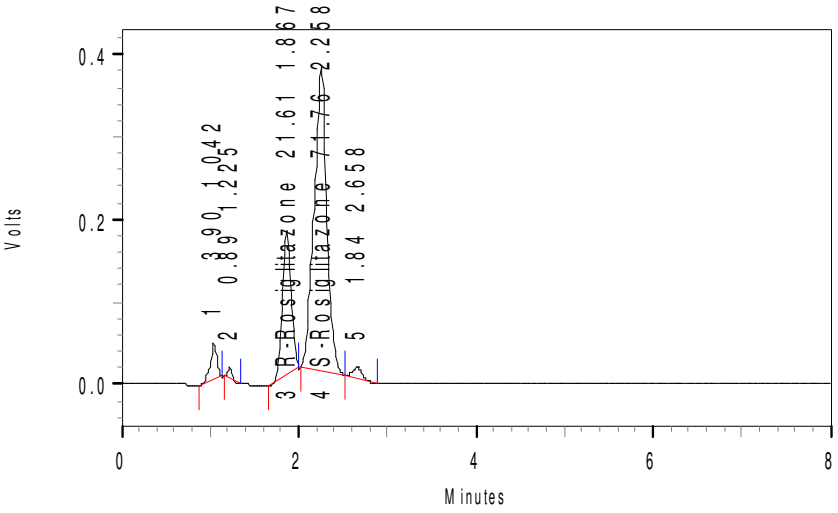

Figure 24: Typical HPLC chromatogram of photo degradation sample of Rosiglitazone (R&S) with sunlight at 24 hrs (Solution form)

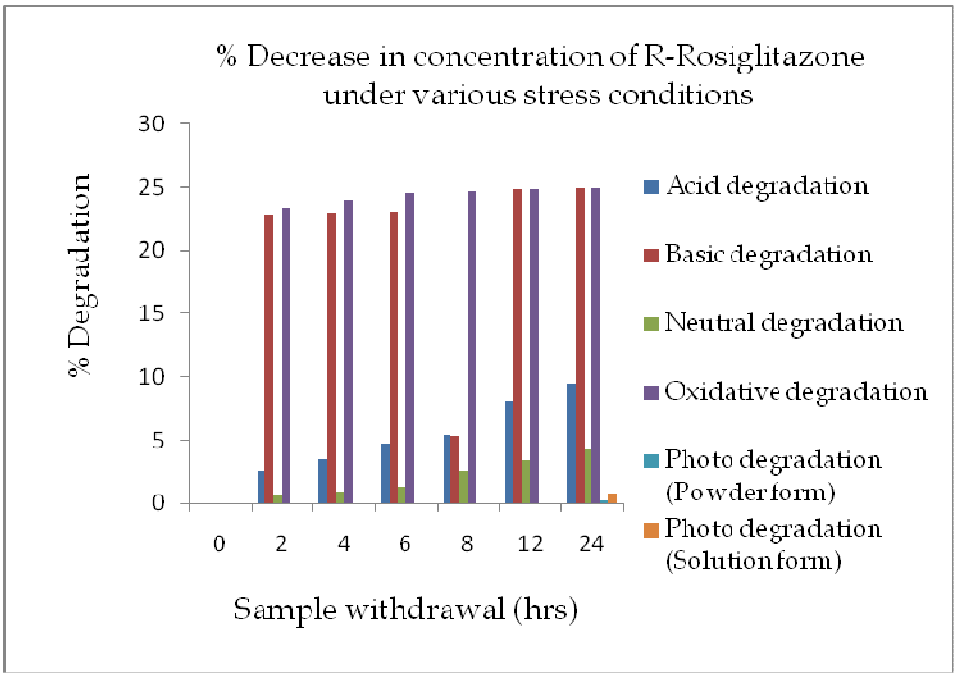

Figure 25: Graphical representation of % decrease in concentration of R-Rosiglitazone under various stress conditions

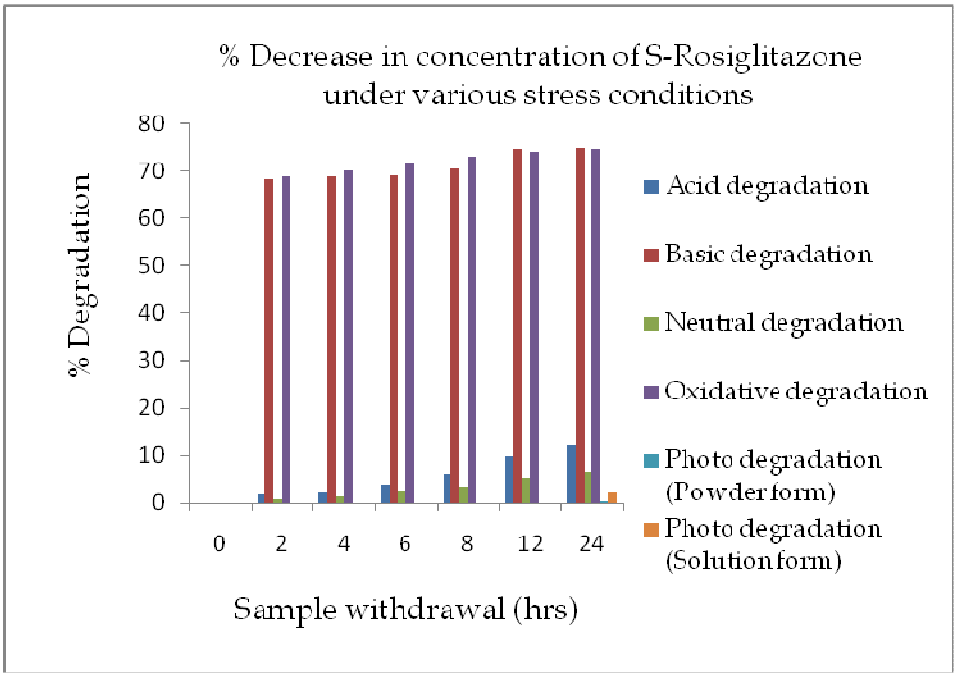

Figure 26: Graphical representation of % decrease in concentration of S-Rosiglitazone under various stress conditions

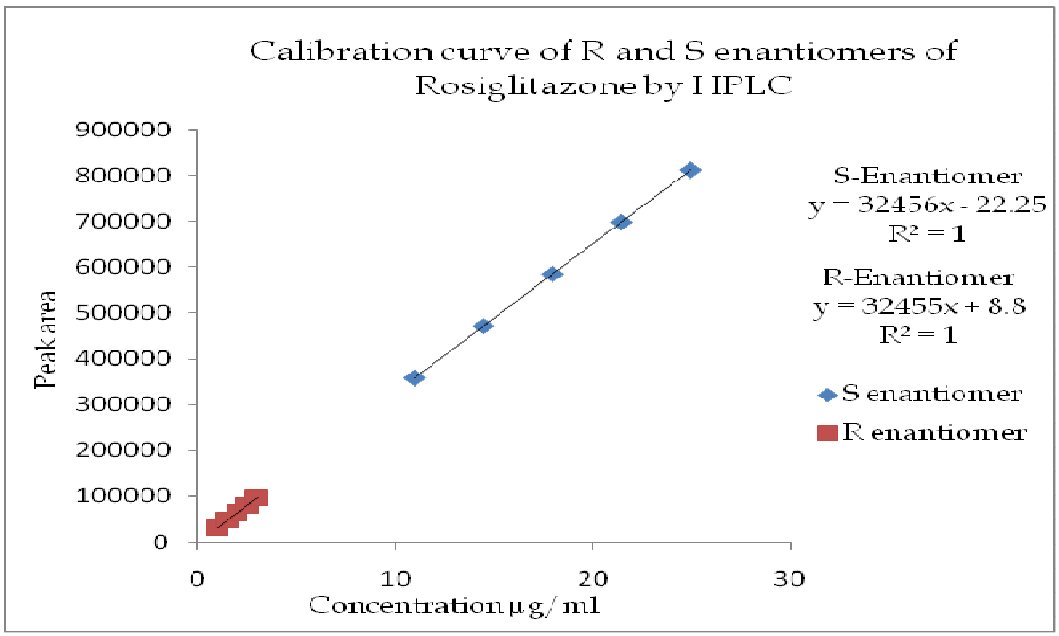

Figure 27: HPLC calibration curve of R and S Rosiglitazone enantiomers by HPLC

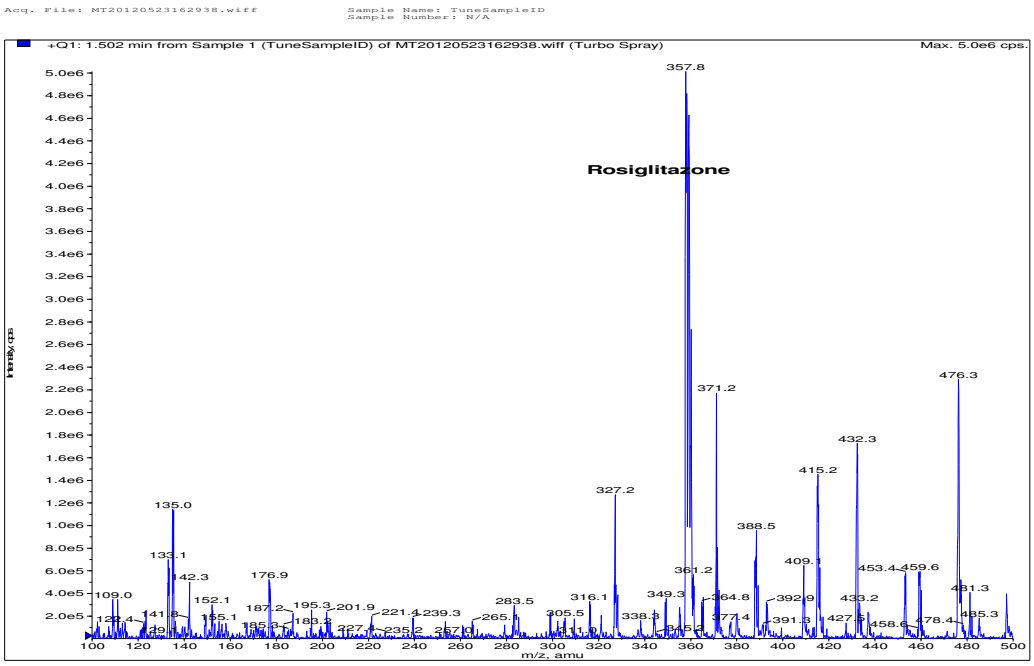

Figure 28: Typical MS chromatogram of Rosiglitazone

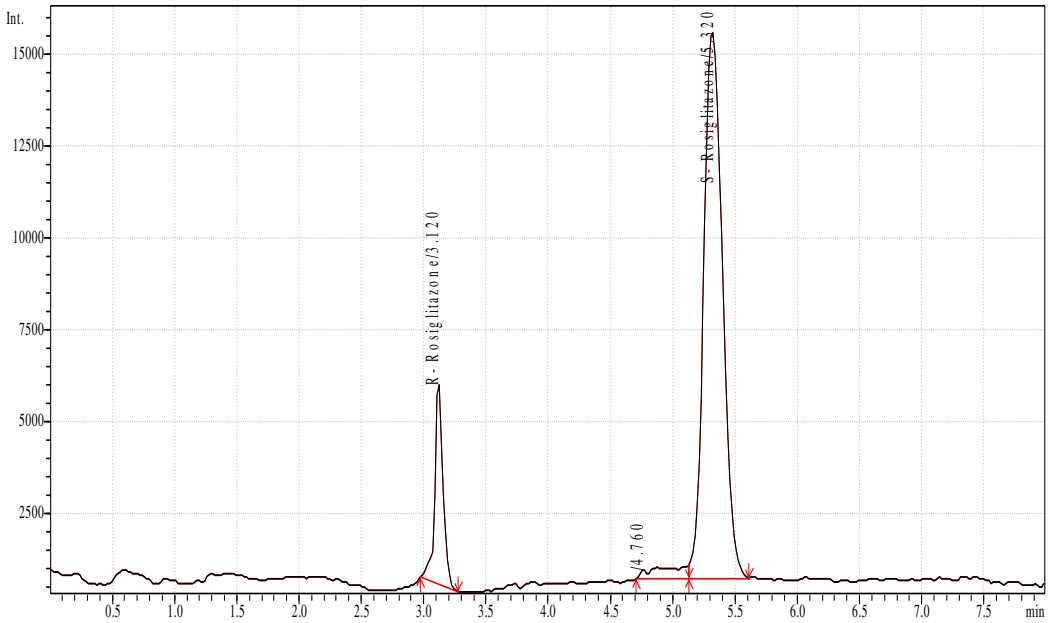

Figure 29: Typical LC-MS chromatogram of Rosiglitazone standard solution

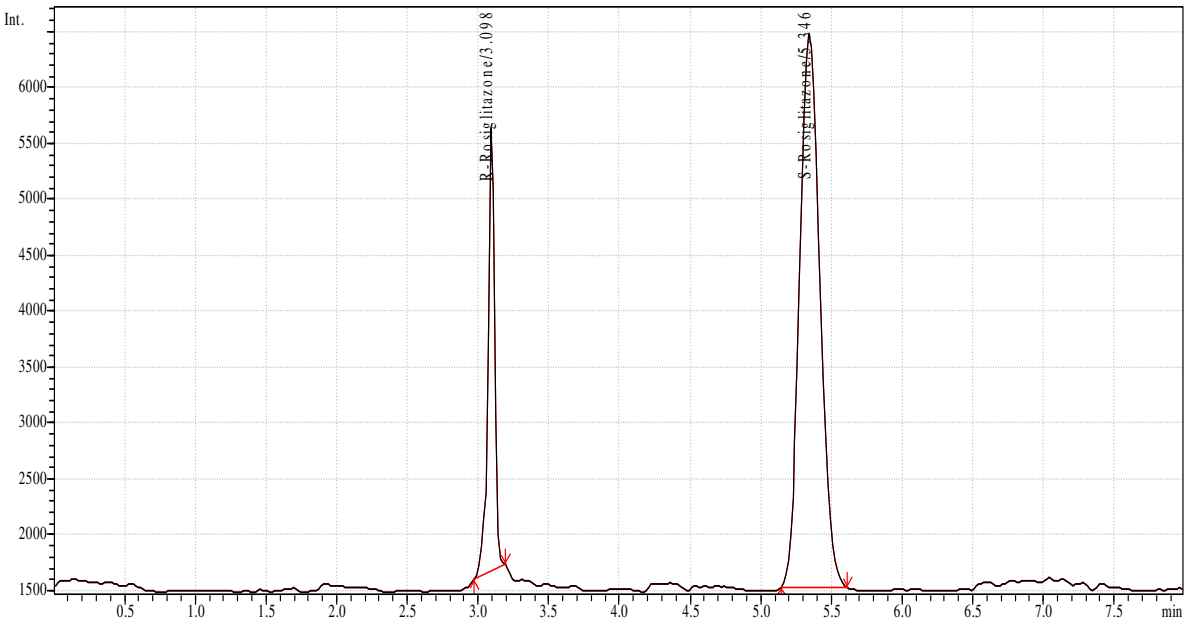

Figure 30: Typical LC-MS chromatogram of sample I containing Rosiglitazone

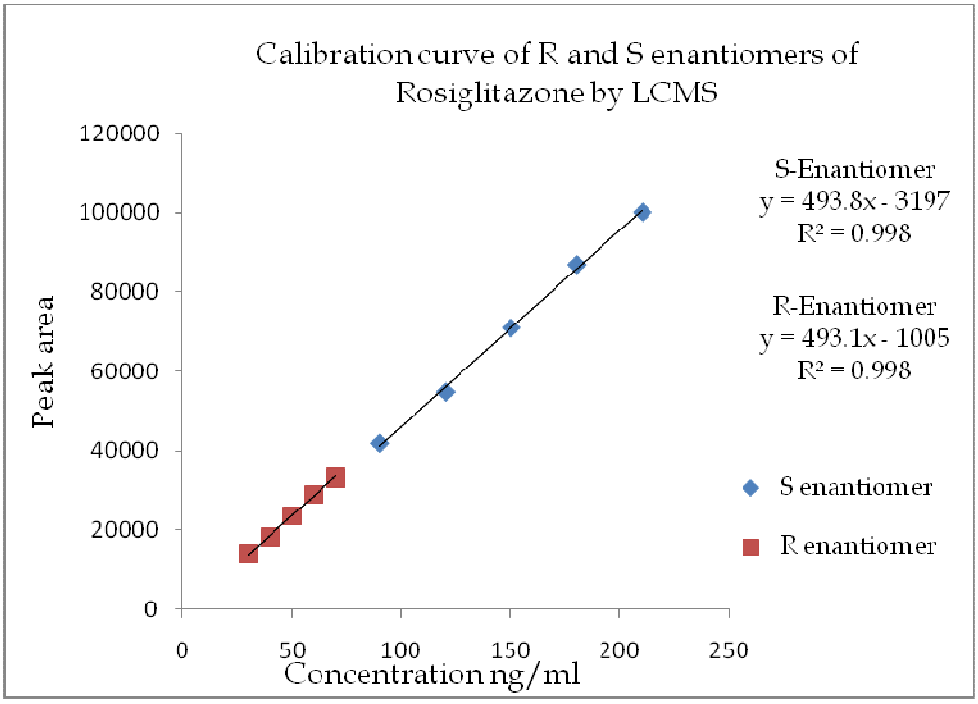

Figure 31: LC-MS Calibration curve of Rosiglitazone

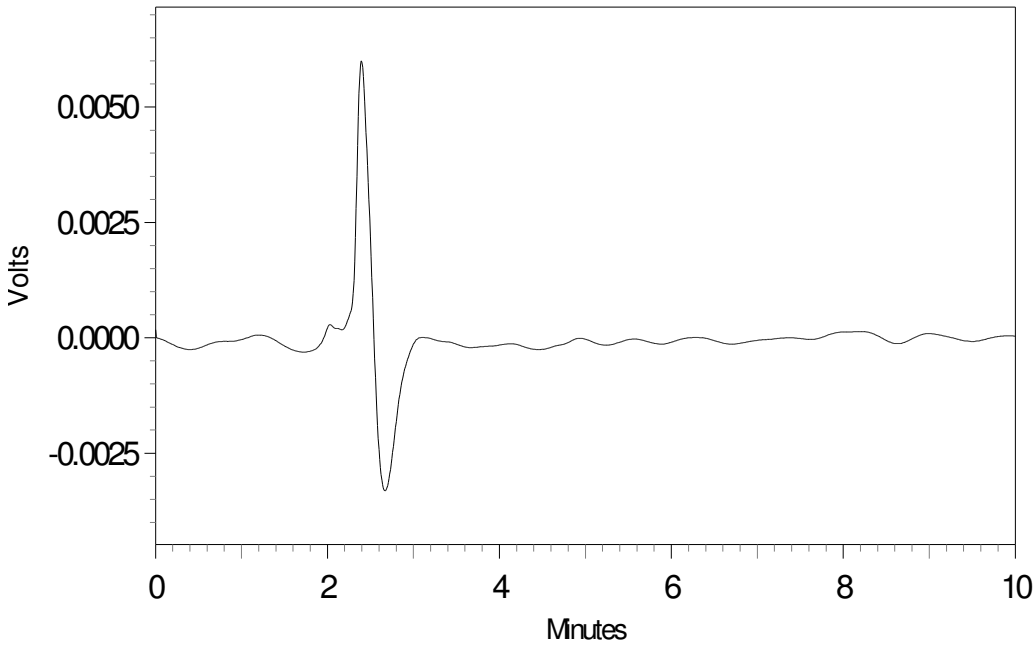

Figure 32: Typical HPLC chromatogram of Pioglitazone blank solution

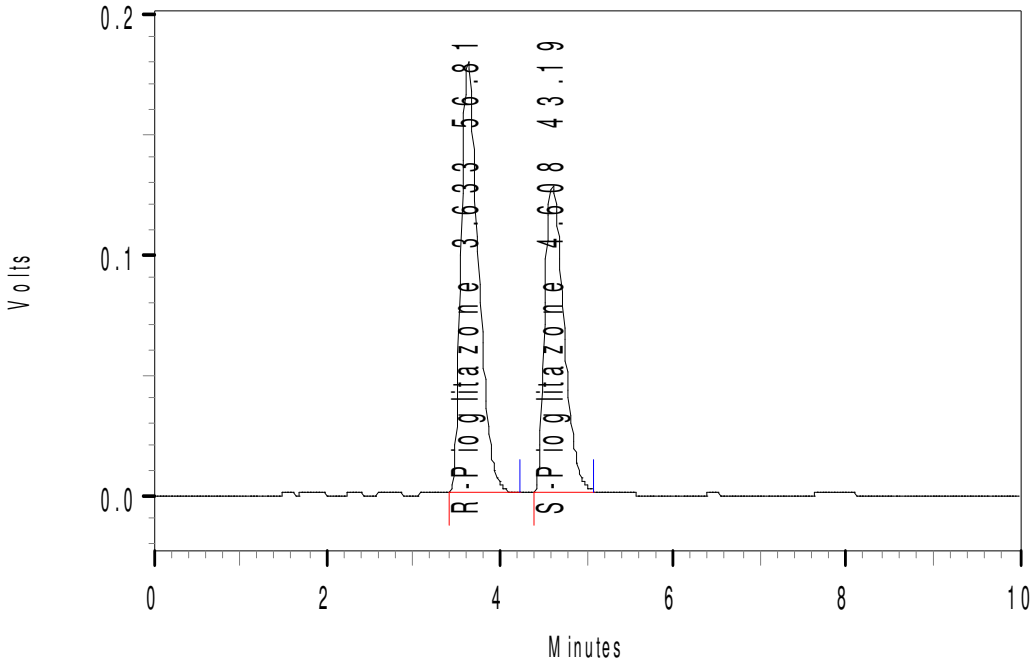

Figure 33: Typical HPLC chromatogram of Pioglitazone standard solution

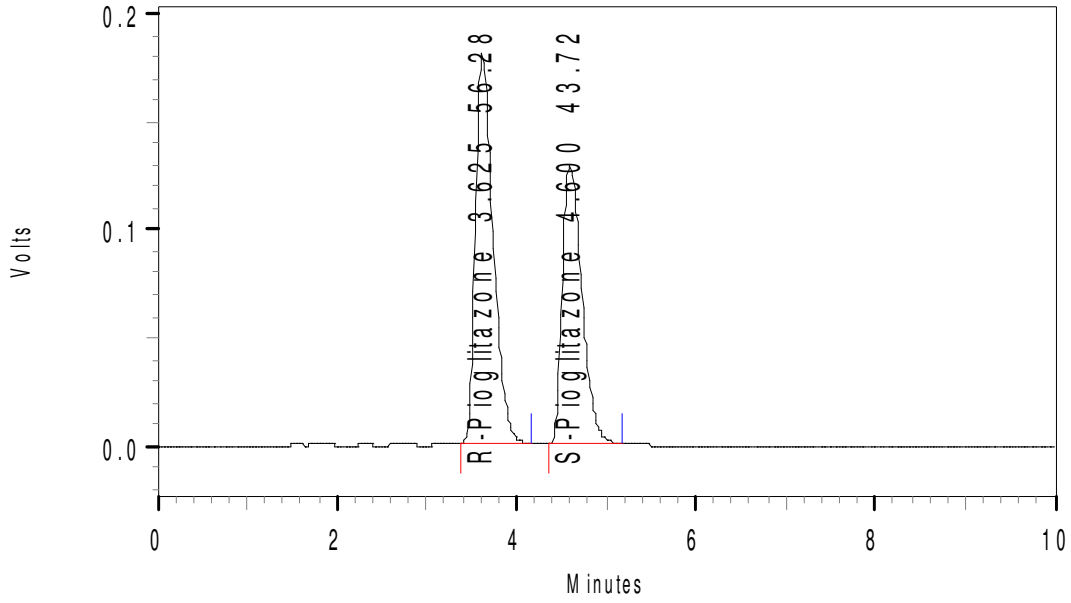

Figure 34: Typical HPLC chromatogram of sample I containing Pioglitazone

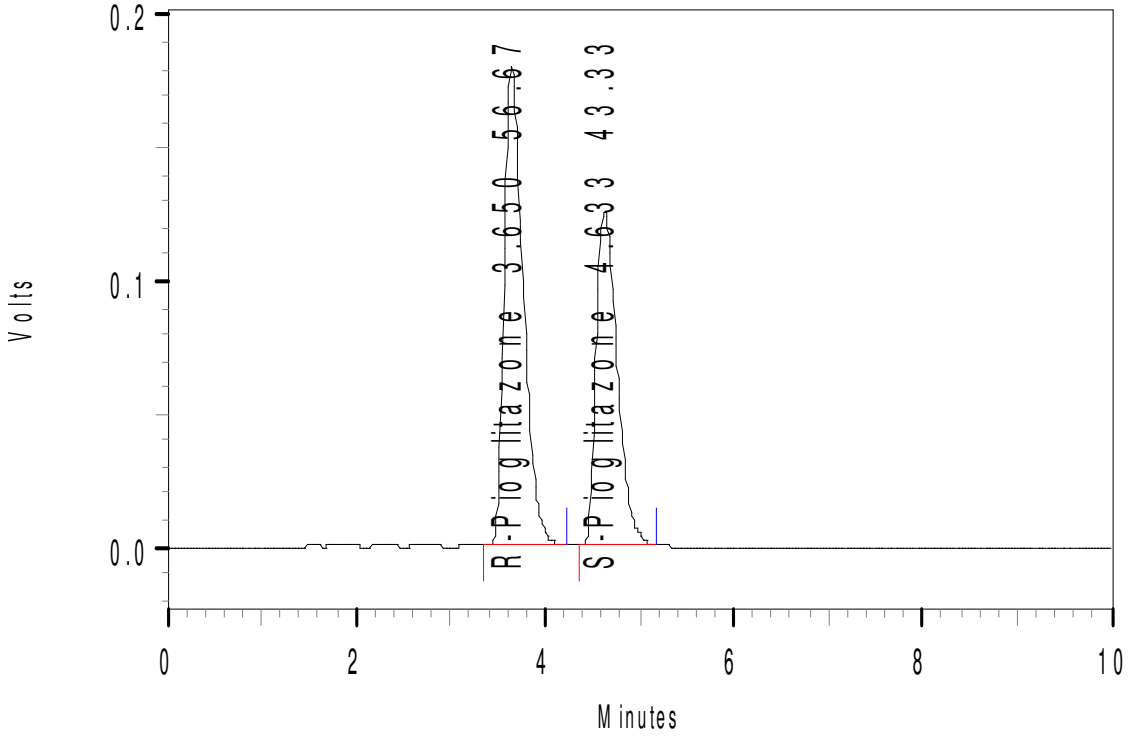

Figure 35: Typical HPLC chromatogram of sample II containing Pioglitazone

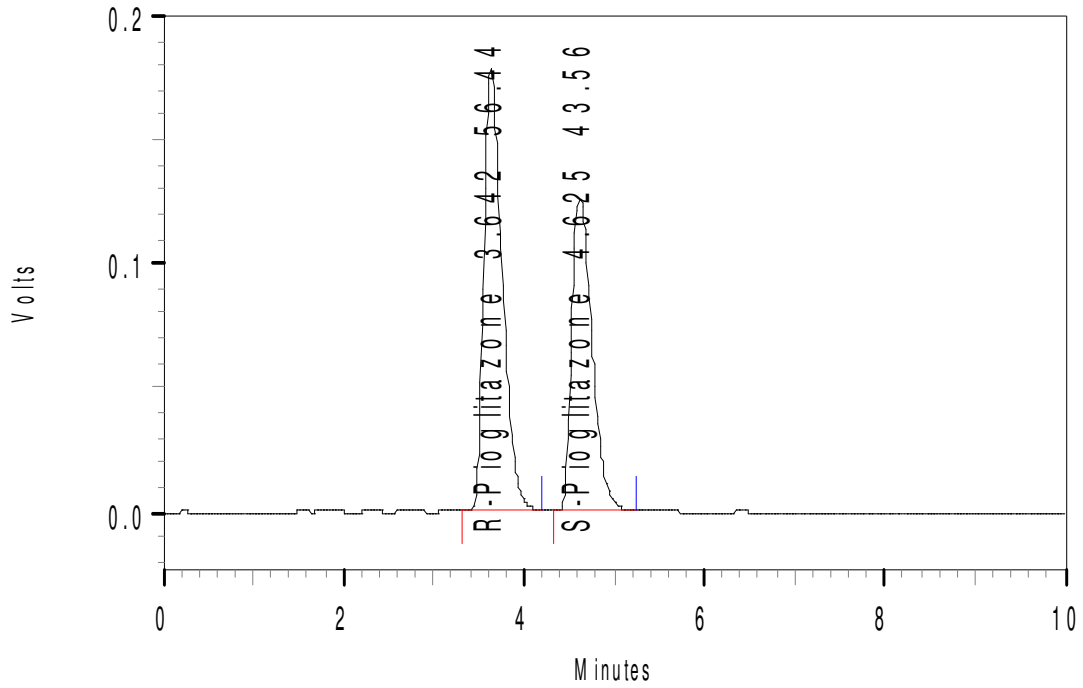

Figure 36: Typical HPLC chromatogram of sample III containing Pioglitazone

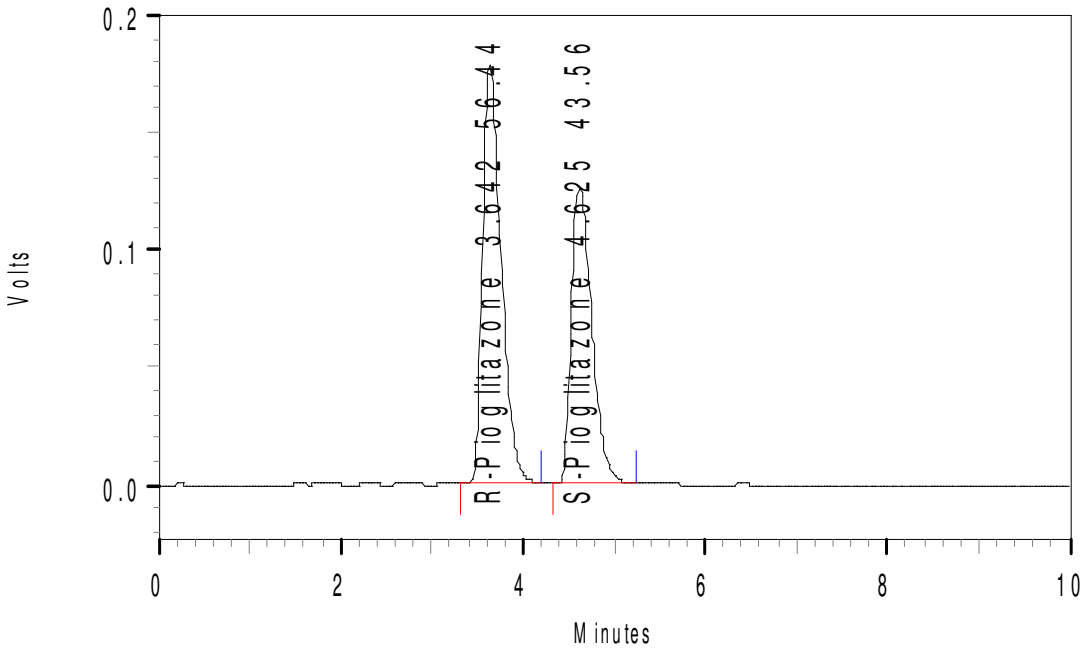

Figure 37: Typical HPLC chromatogram of sample IV containing Pioglitazone

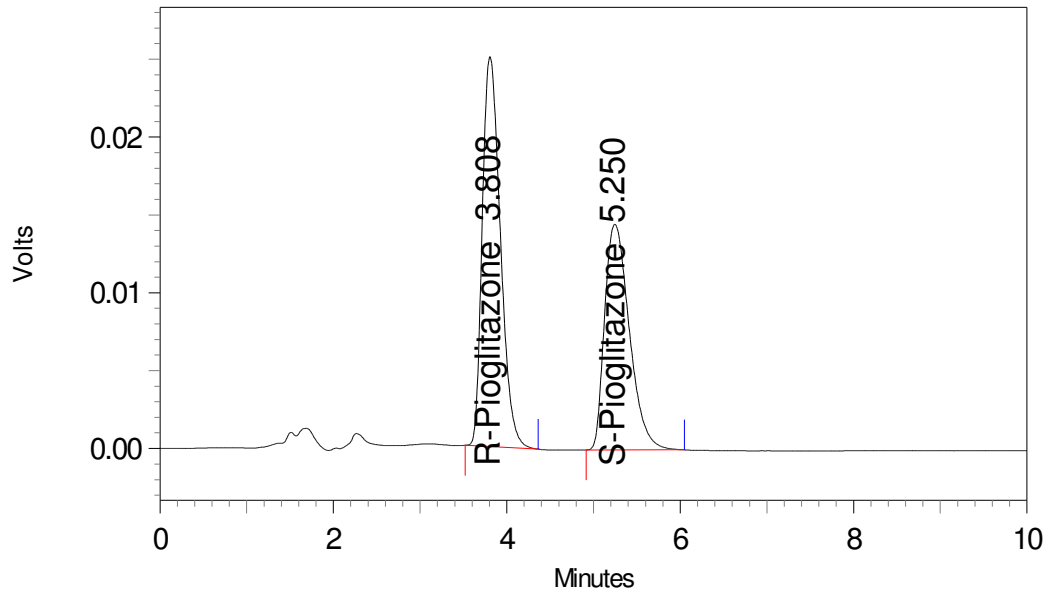

Figure 38: Typical HPLC chromatogram of acid degradation sample of Pioglitazone (R&S) with 0.1N HCl at 0 hr

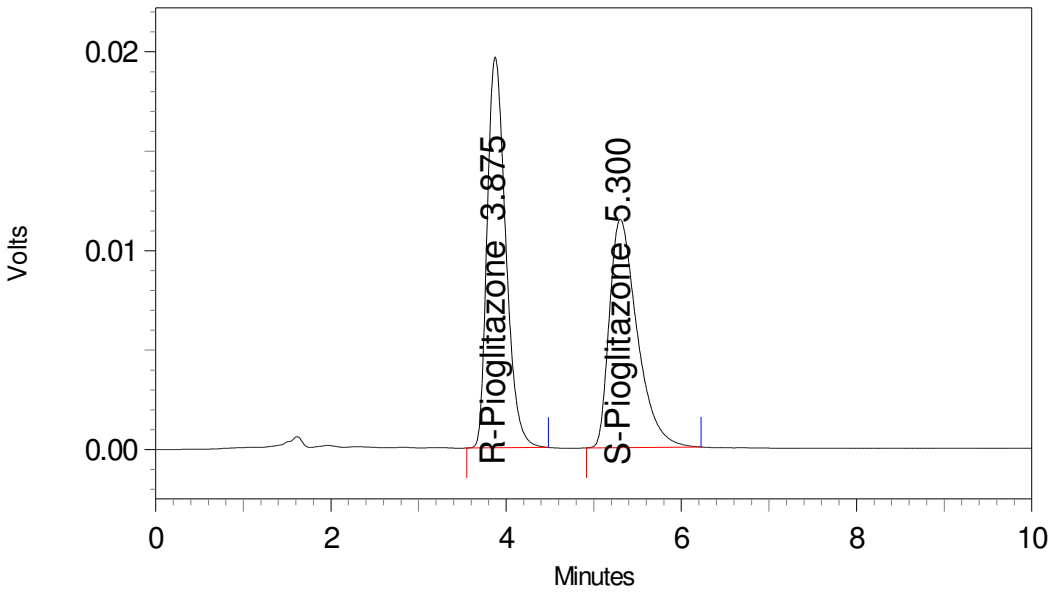

Figure 39: Typical HPLC chromatogram of acid degradation sample of Pioglitazone (R&S) with 0.1N HCl at 24 hrs

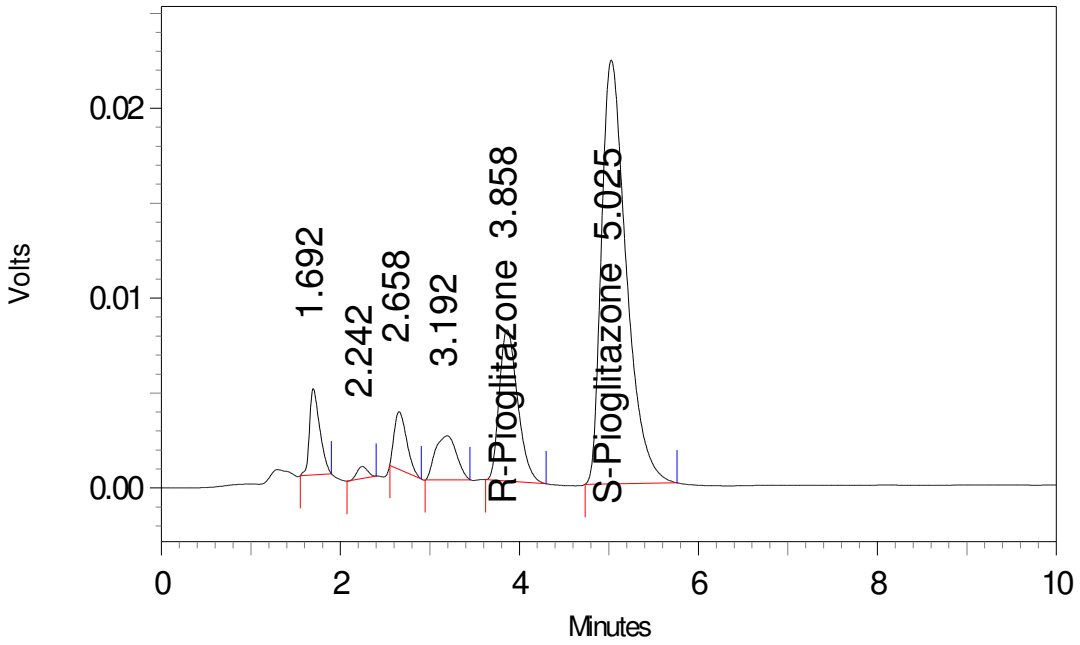

Figure 40: Typical HPLC chromatogram of basic degradation sample of Pioglitazone (R&S) with 0.1N NaOH at 0 hr

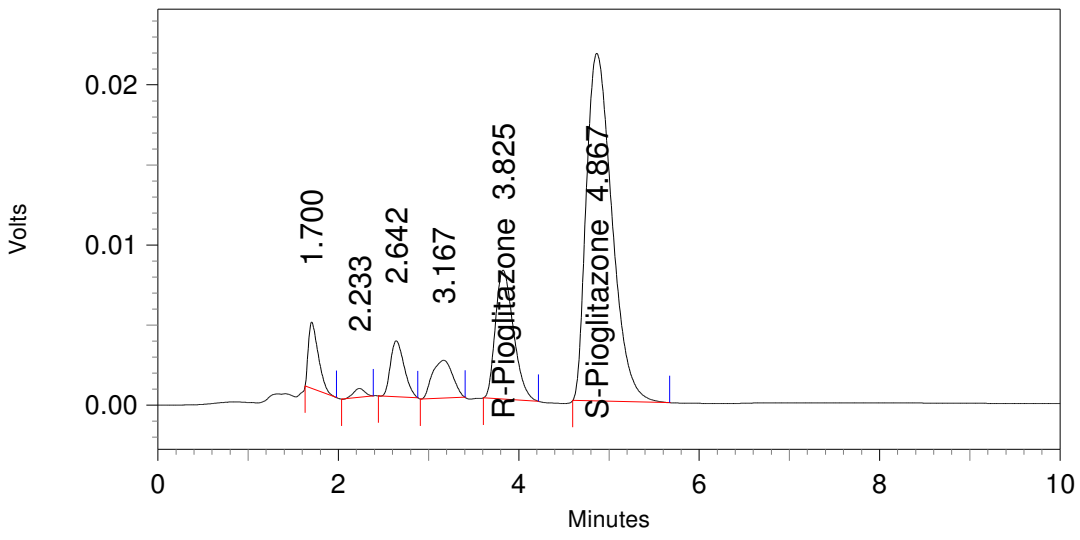

Figure 41: Typical HPLC chromatogram of basic degradation sample of Pioglitazone (R&S) with 0.1N NaOH at 24 hrs

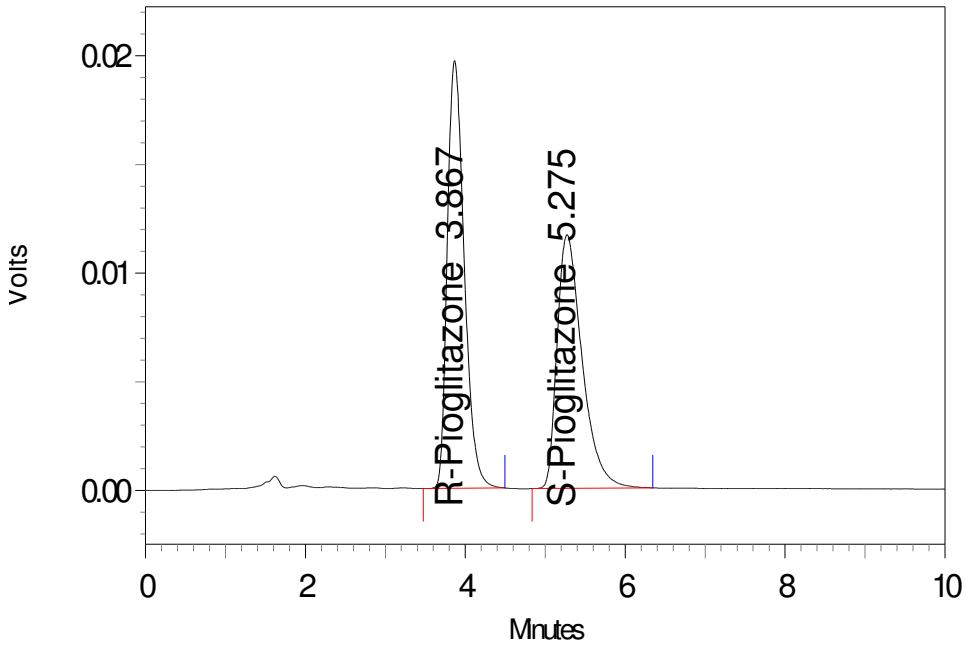

Figure 42: Typical HPLC chromatogram of neutral degradation sample of Pioglitazone (R&S) with water at 0 hr

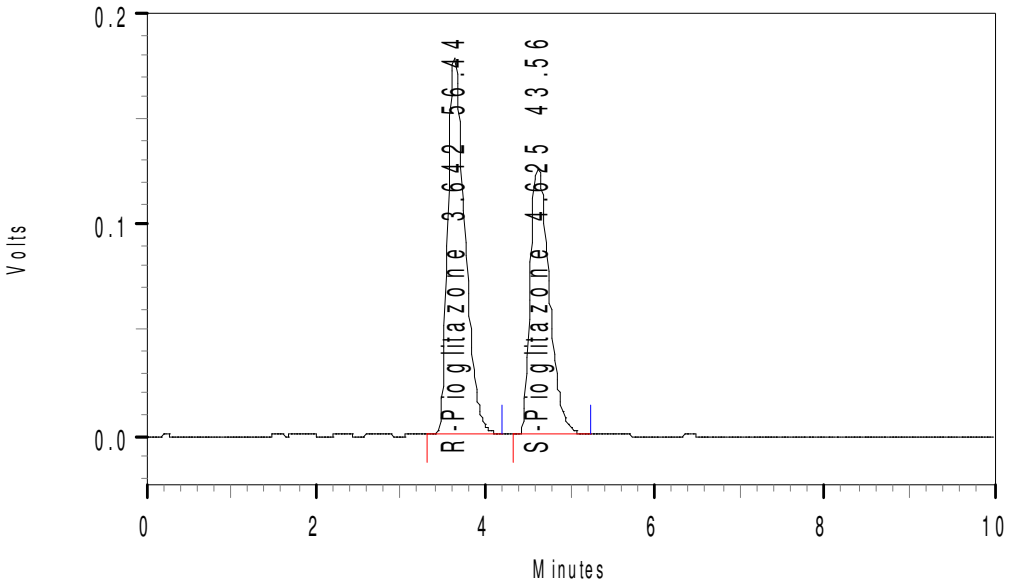

Figure 43: Typical HPLC chromatogram of neutral degradation sample of Pioglitazone (R&S) with water at 24 hrs

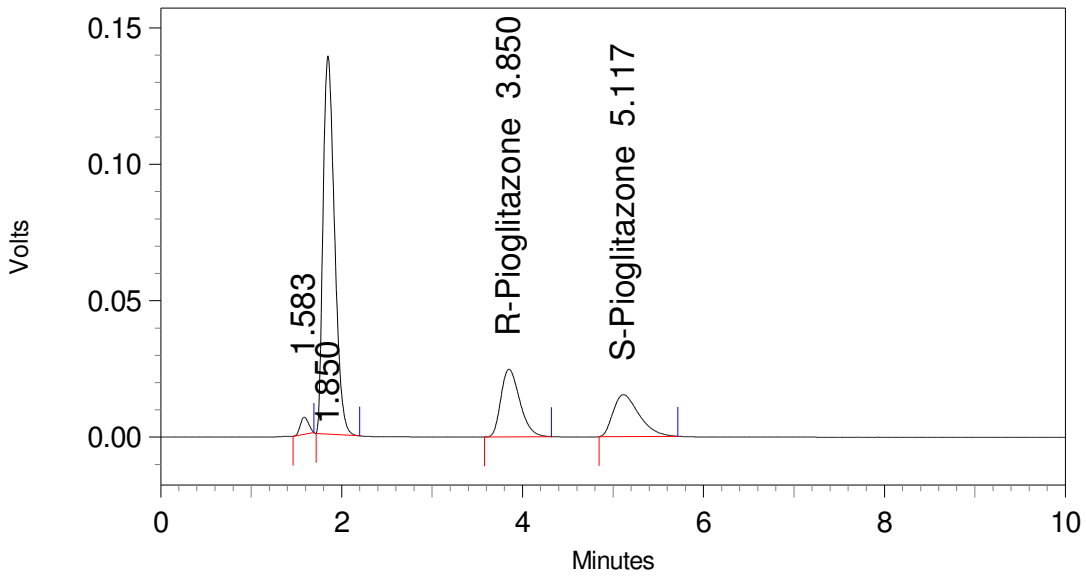

Figure 44: Typical HPLC chromatogram of oxidative degradation sample of Pioglitazone (R&S) with 3% H_2O_2 at 0 hr

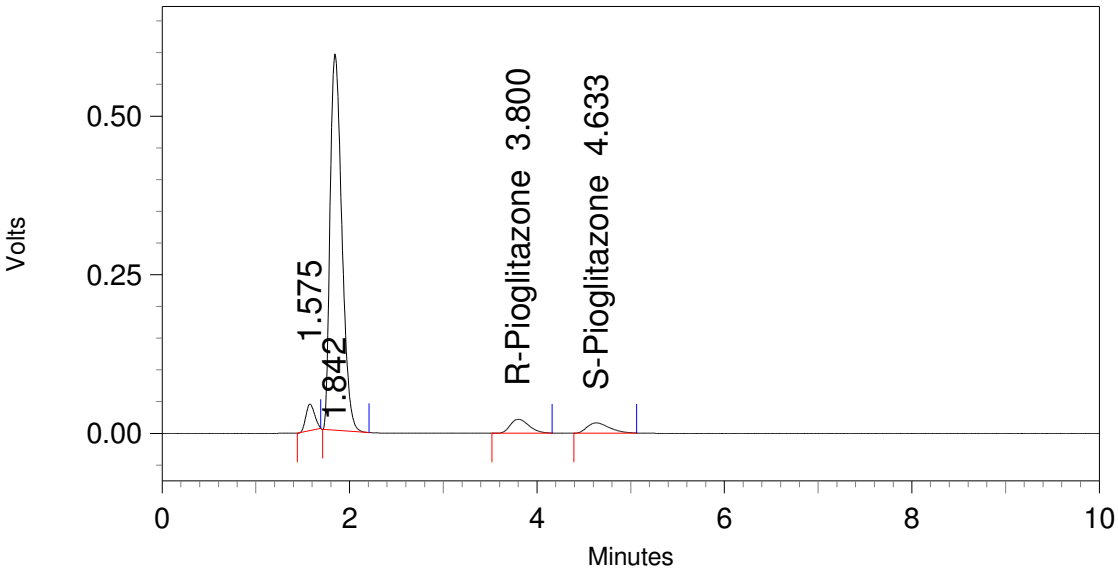

Figure 45: Typical HPLC chromatogram of oxidative degradation sample of Pioglitazone (R&S) with 3% H_2O_2 at 24 hrs

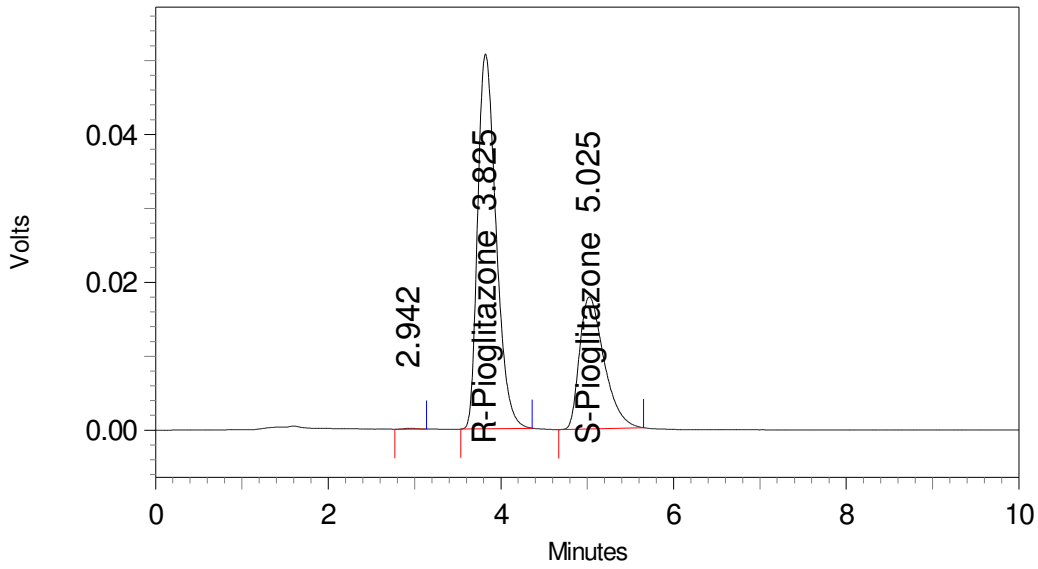

Figure 46: Typical HPLC chromatogram of photo degradation sample of Pioglitazone (R&S) with sunlight at 0 hr

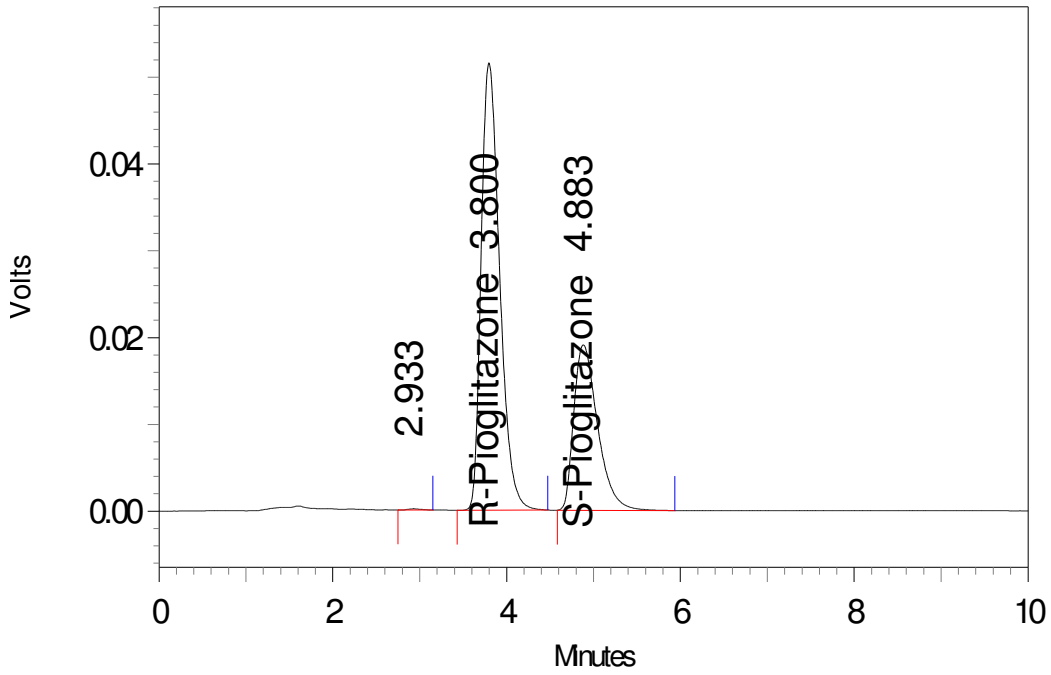

Figure 47: Typical HPLC chromatogram of photo degradation sample of Pioglitazone (R&S) with sunlight at 24 hrs (Solution form)

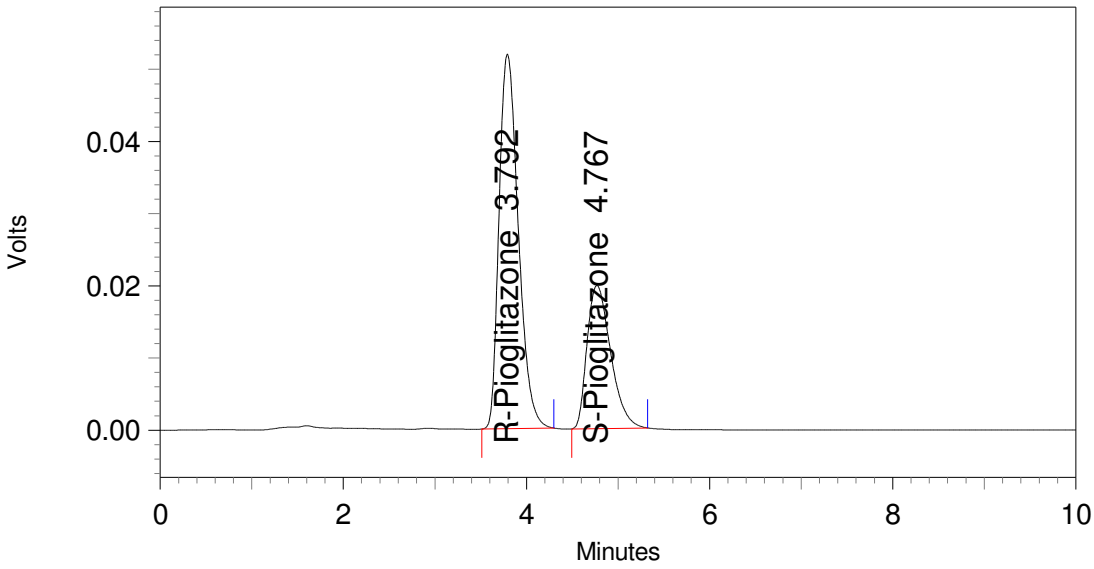

Figure 48: Typical HPLC chromatogram of photo degradation sample of Pioglitazone (R&S) with sunlight at 24 hrs (Powder form)

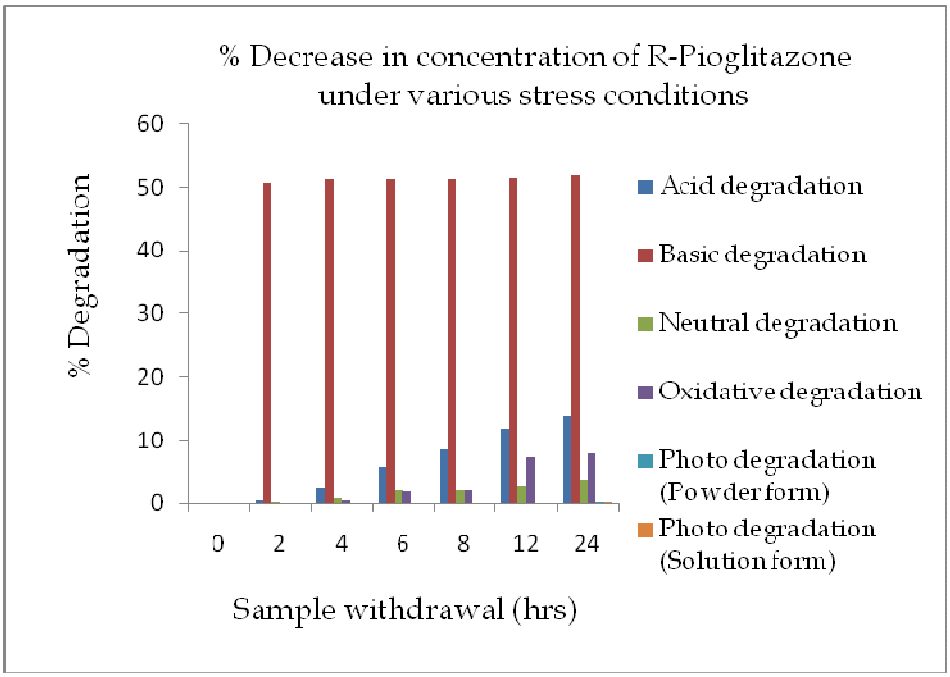

Figure 49: Graphical representation of % decrease in concentration of R-Pioglitazone under various stress conditions

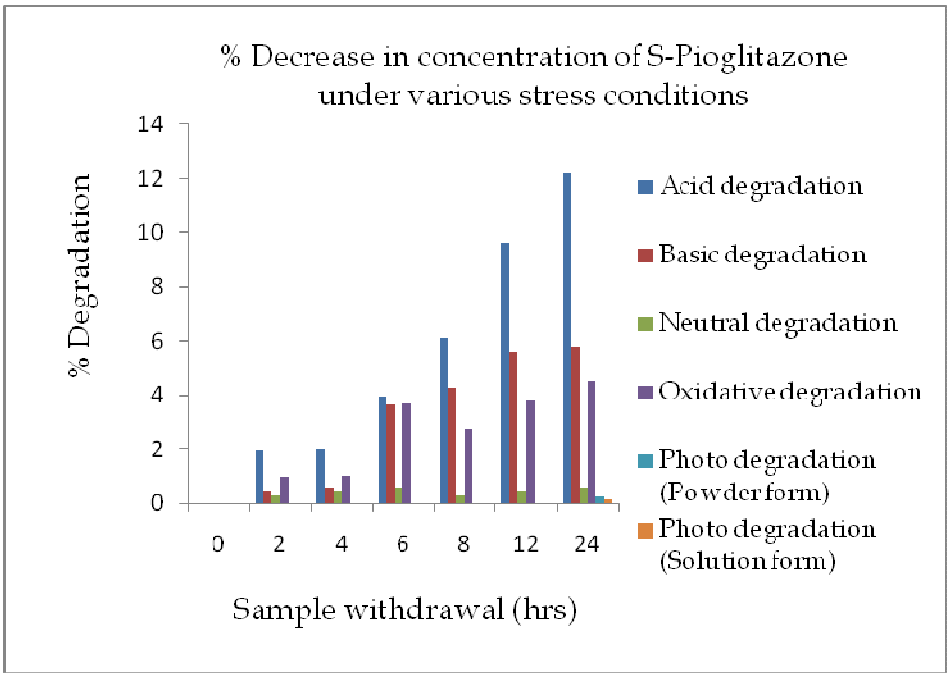

Figure 50: Graphical representation of % decrease in concentration of S-Pioglitazone under various stress conditions

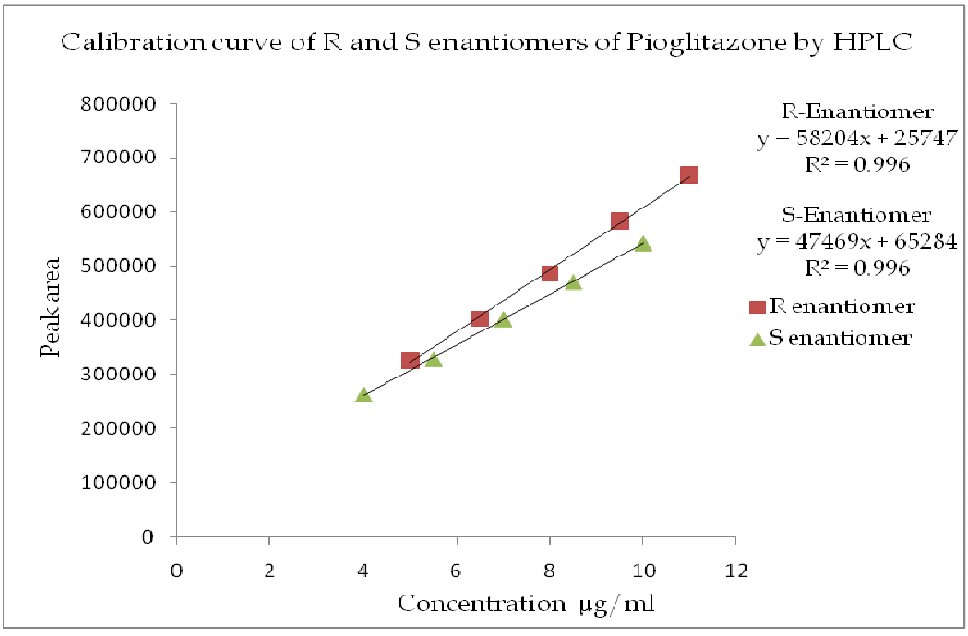

Figure 51: Calibration curve of R and S Pioglitazone enantiomers by HPLC

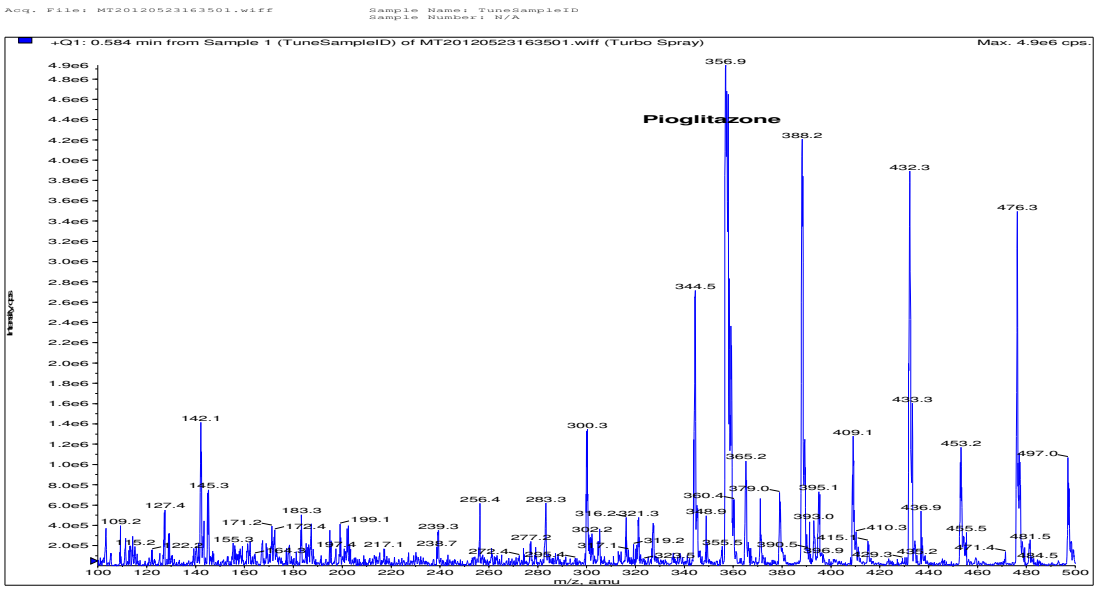

Figure 52: Typical MS chromatogram of Pioglitazone

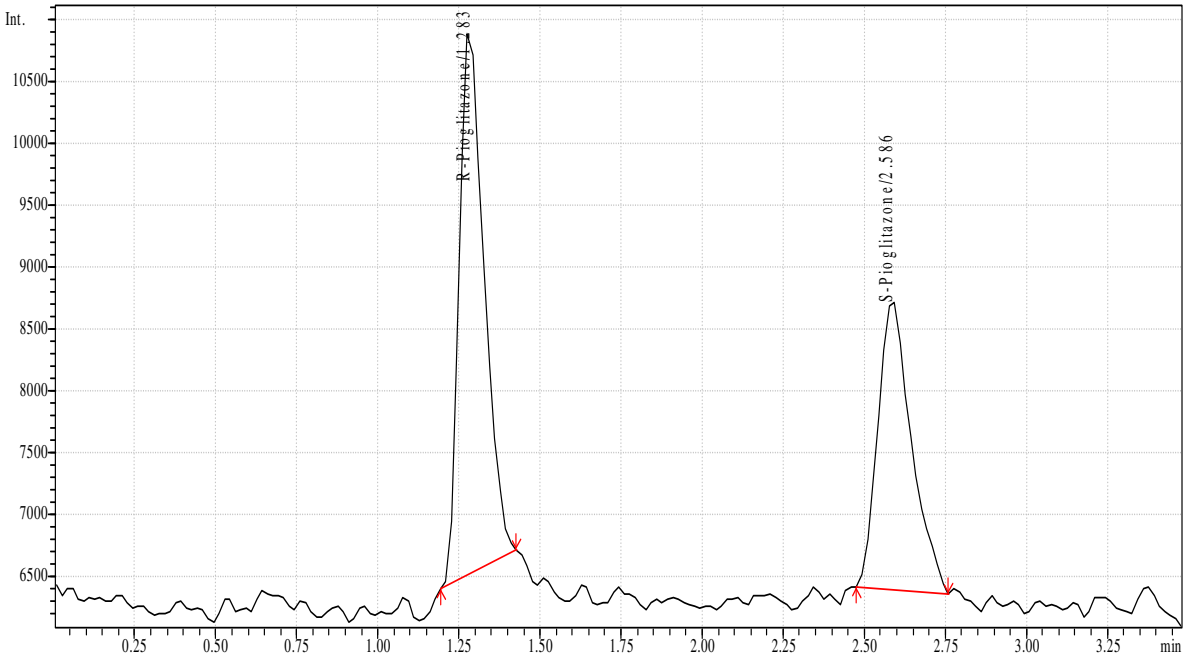

Figure 53: Typical LC-MS chromatogram of Pioglitazone standard solution

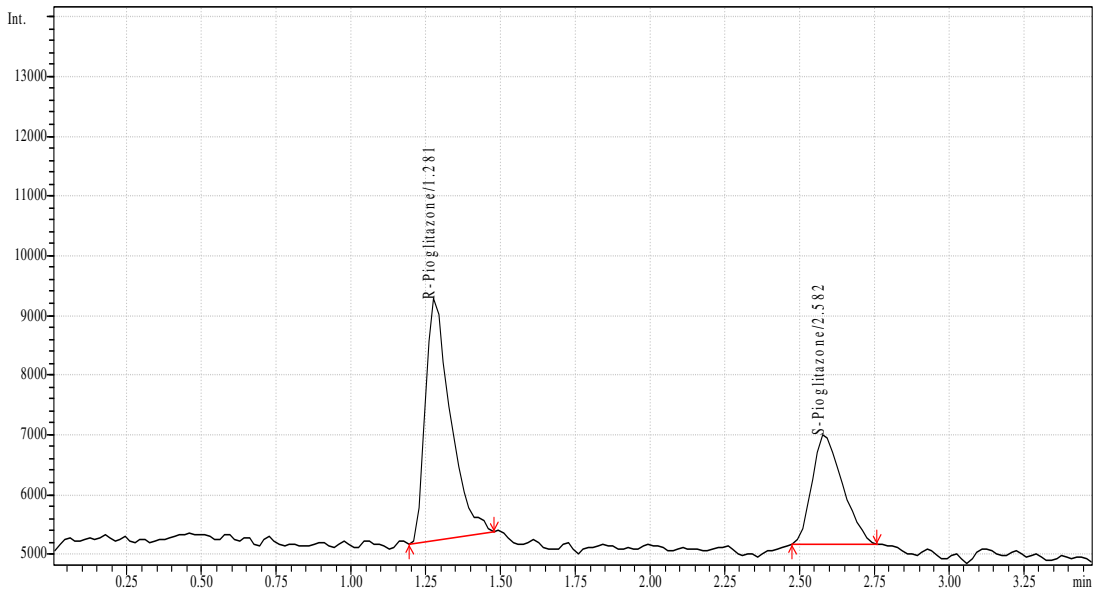

Figure 54: Typical LC-MS chromatogram of sample I containing Pioglitazone

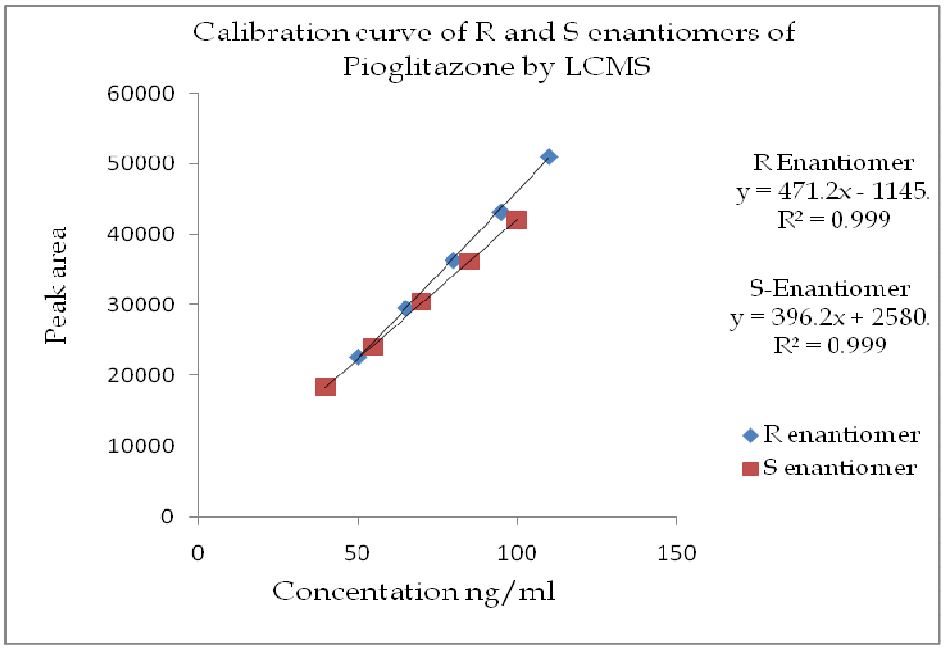

Figure 55: Calibration curve of R and S Pioglitazone enantiomers by LC-MS

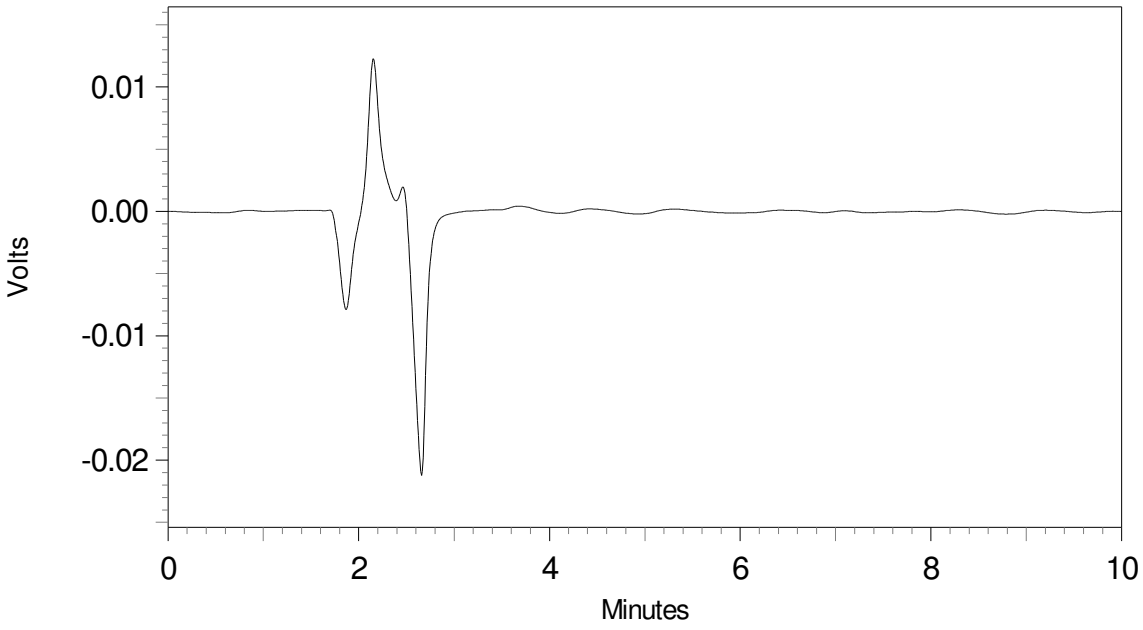

Figure 56: Typical HPLC chromatogram of Zaltoprofen blank solution

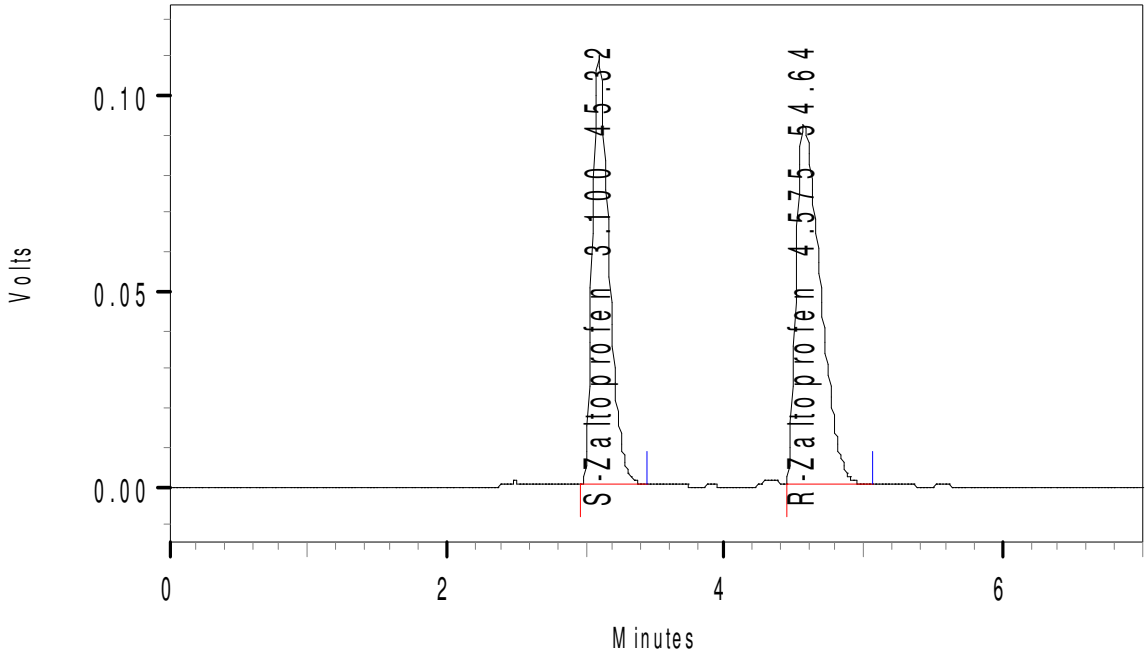

Figure 57: Typical HPLC chromatogram of Zaltoprofen standard solution

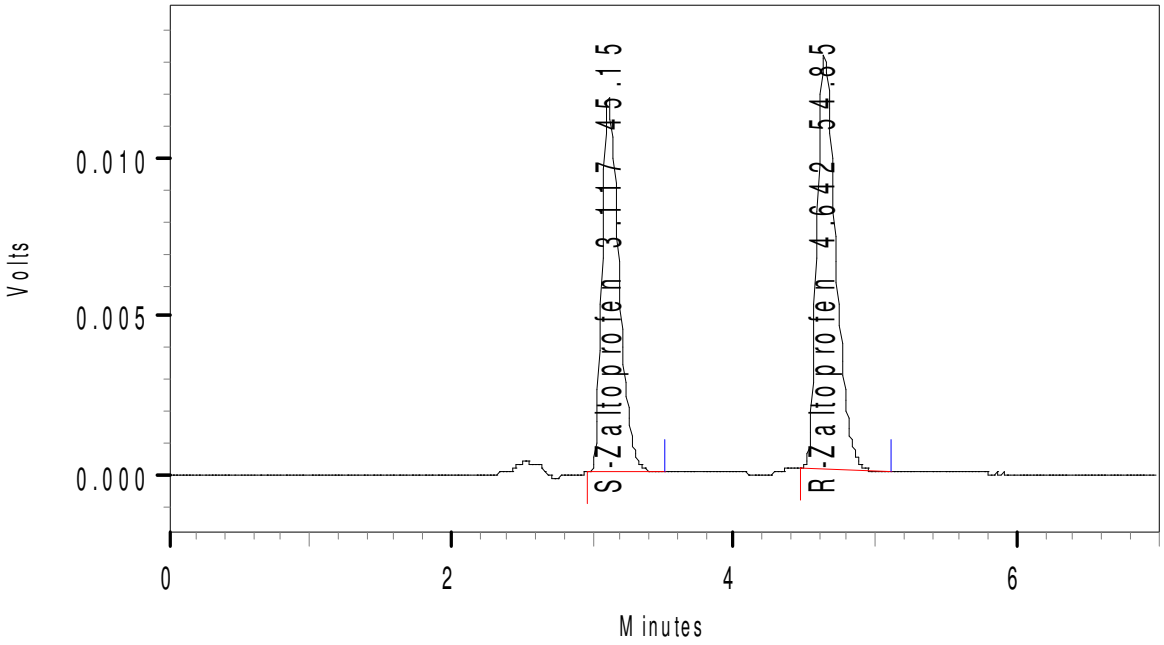

Figure 58: Typical HPLC chromatogram of sample I containing Zaltoprofen

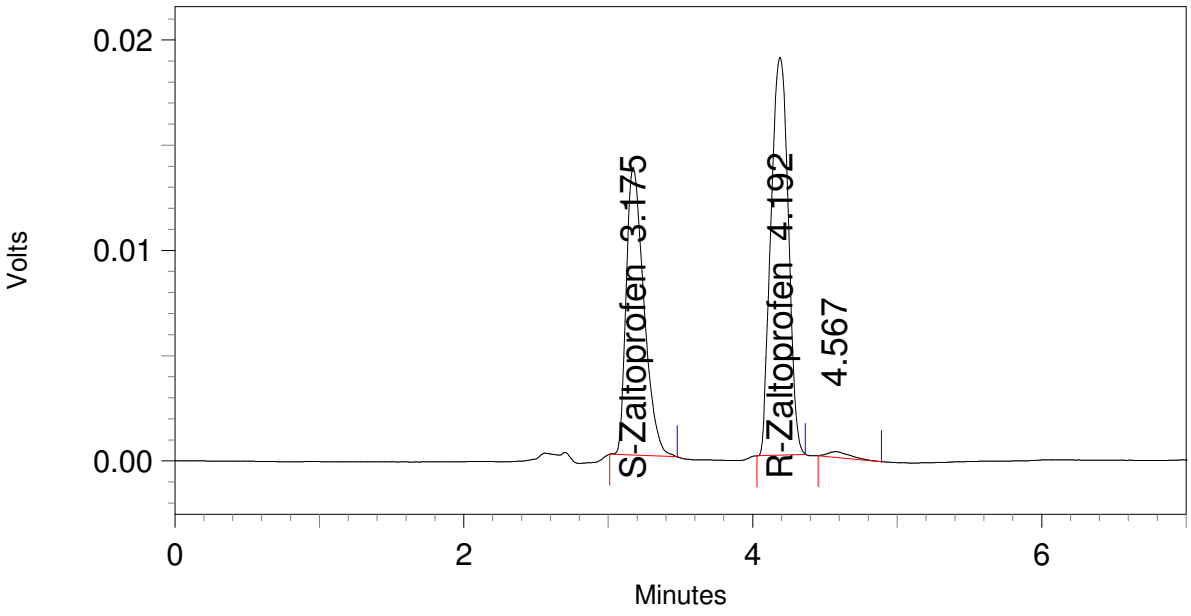

Figure 59: Typical HPLC chromatogram of acid degradation sample of Zaltoprofen (R&S) with 0.1N HCl at 0 hr

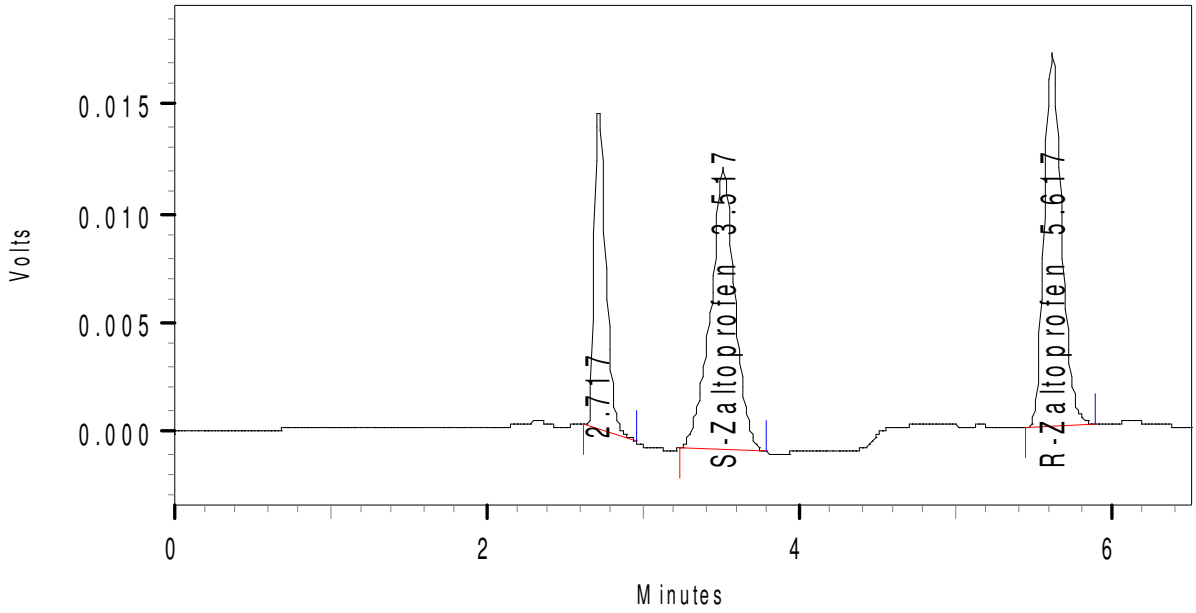

Figure 60: Typical HPLC chromatogram of acid degradation sample of Zaltoprofen (R&S) with 0.1N HCl at 24 hrs

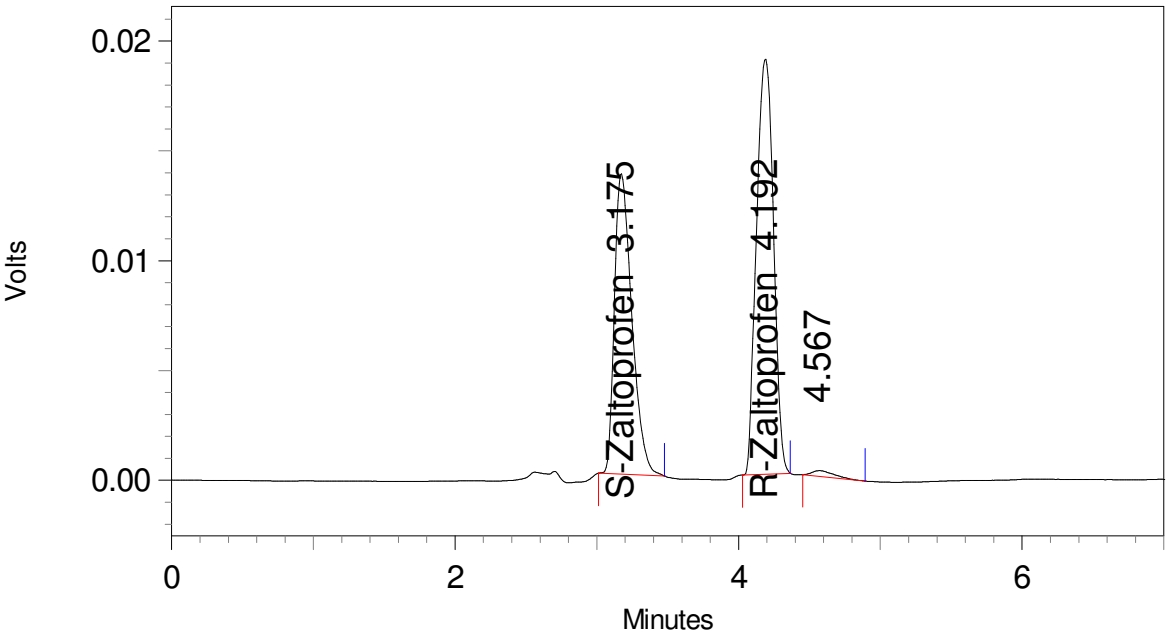

Figure 61: Typical HPLC chromatogram of basic degradation sample of Zaltoprofen (R&S) with 0.1N NaOH at 0 hr

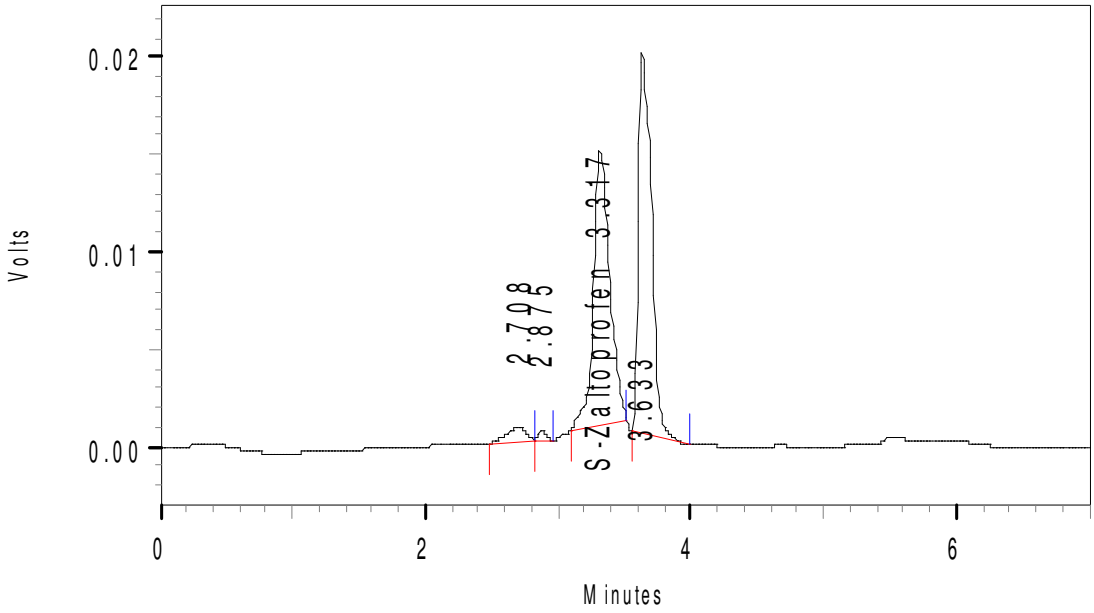

Figure 62: Typical HPLC chromatogram of basic degradation sample of Zaltoprofen (R&S) with 0.1N NaOH at 24 hrs

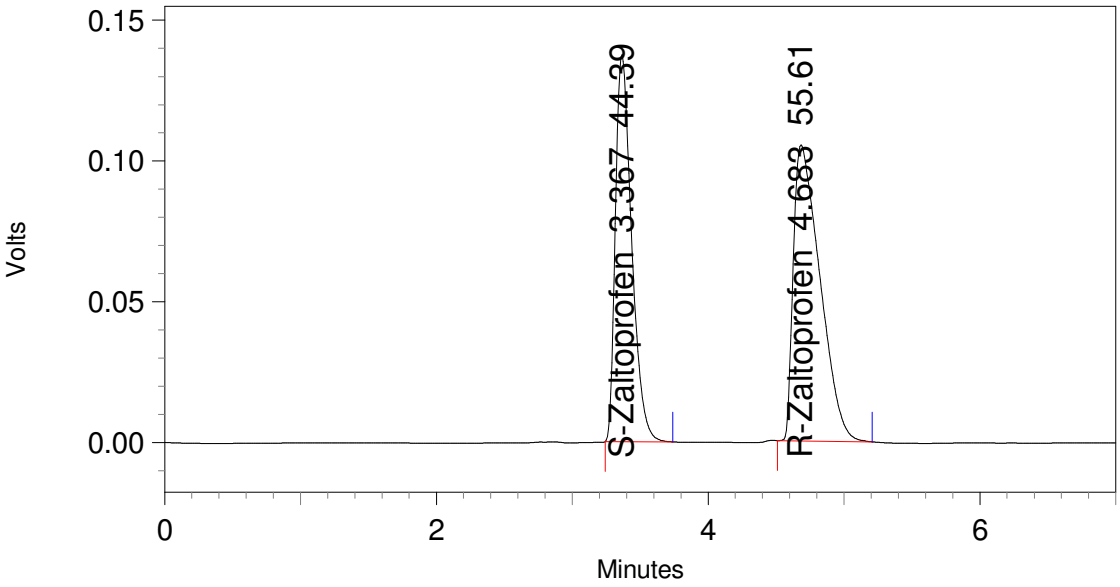

Figure 63: Typical HPLC chromatogram of neutral degradation sample of Zaltoprofen (R&S) with water at 0 hr

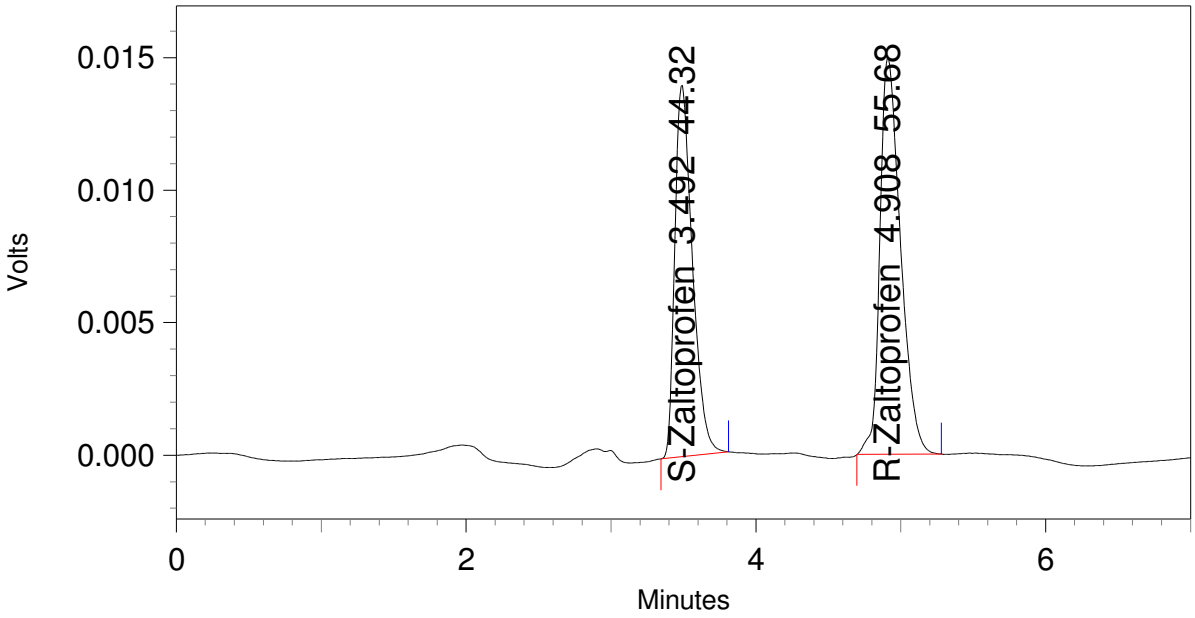

Figure 64: Typical HPLC chromatogram of neutral degradation sample of Zaltoprofen (R&S) with water at 24 hrs

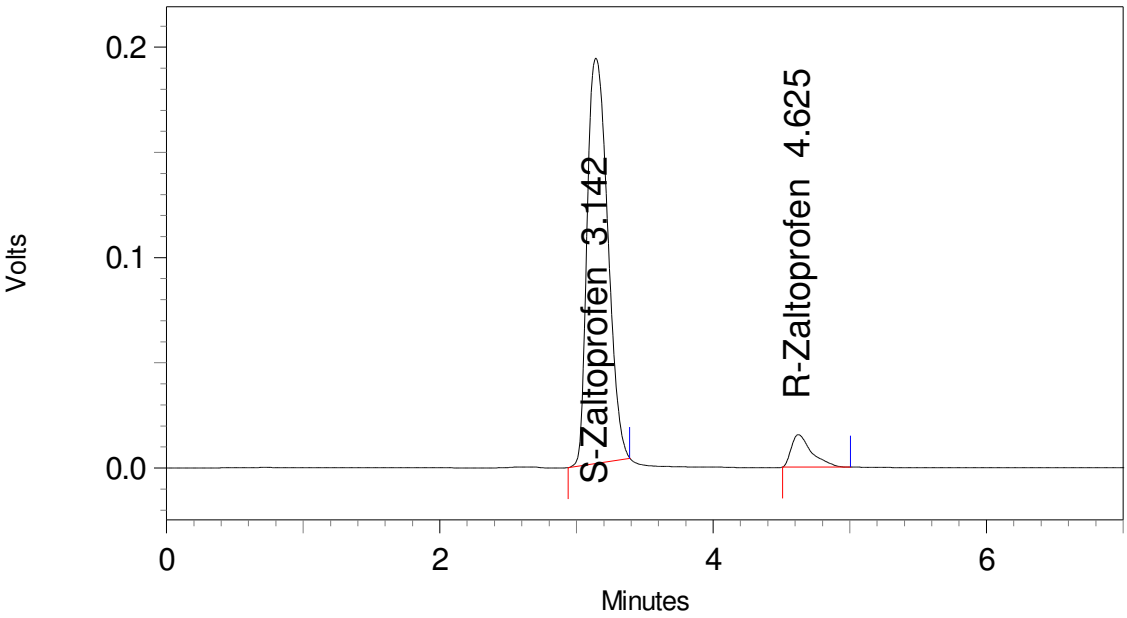

Figure 65: Typical HPLC chromatogram of oxidative degradation sample of Zaltoprofen (R&S) with 3% H_2O_2 at 0 hr

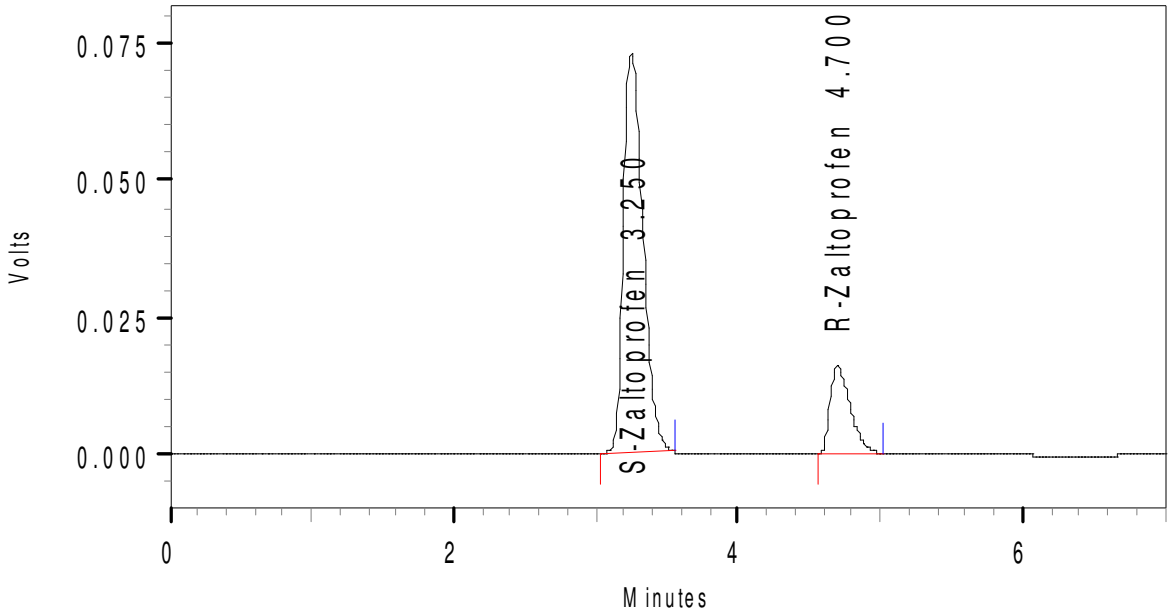

Figure 66: Typical HPLC chromatogram of oxidative degradation sample of Zaltoprofen (R&S) with 3% H_2O_2 at 24 hrs

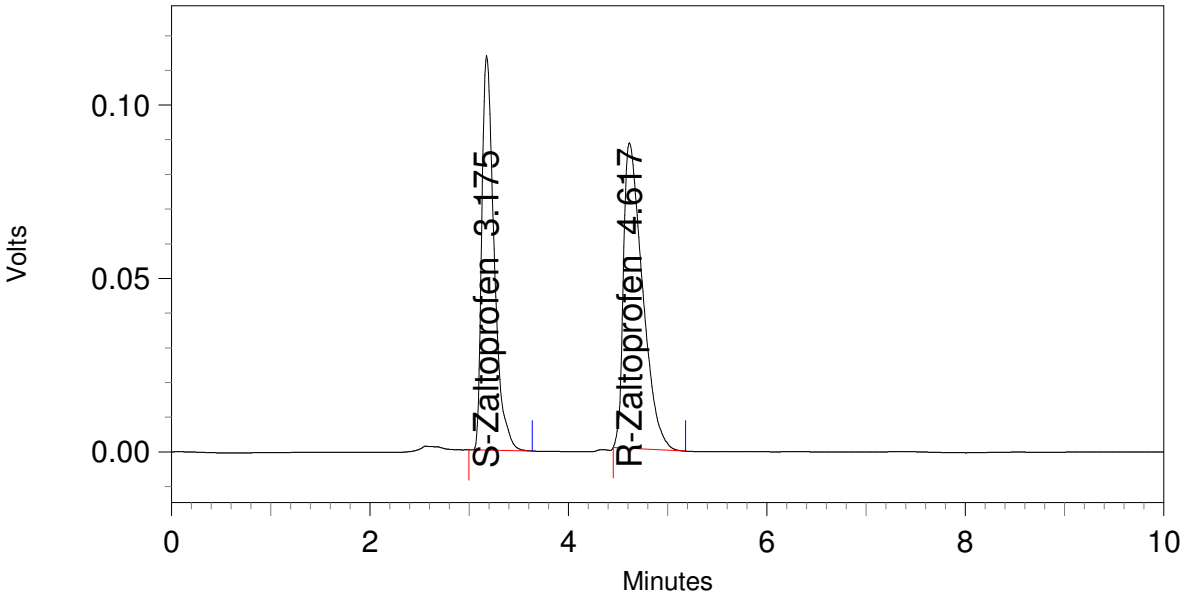

Figure 67: Typical HPLC chromatogram of photo degradation sample of Zaltoprofen (R&S) with sunlight at 0 hr

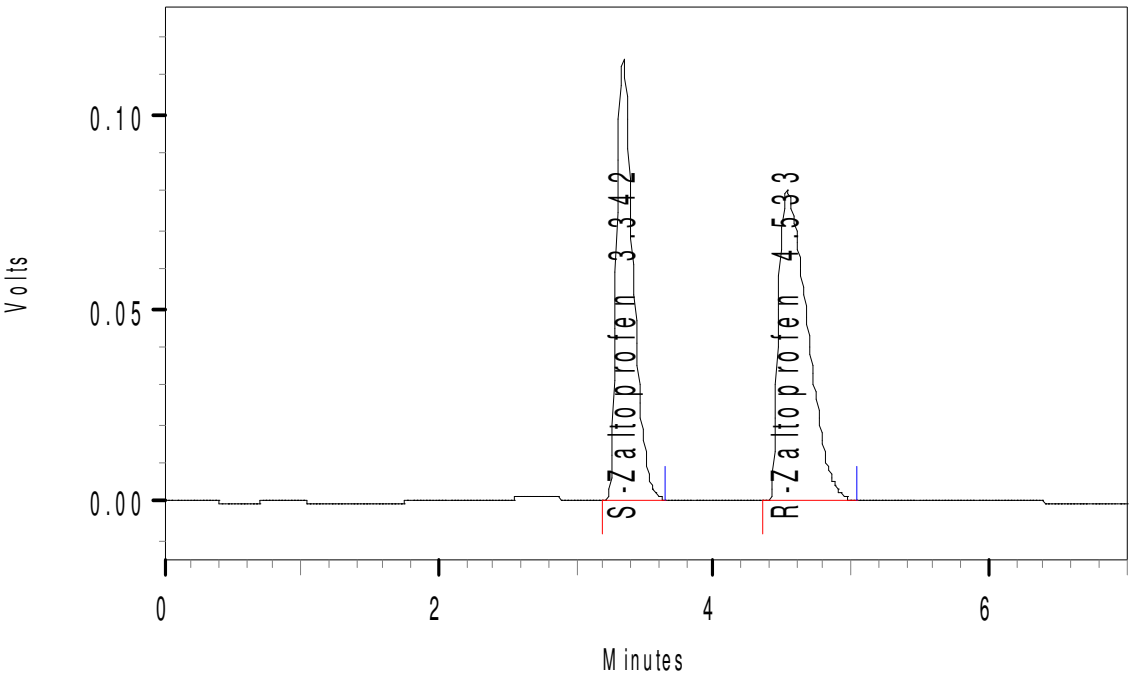

Figure 68: Typical HPLC chromatogram of photo degradation sample of Zaltoprofen (R&S) with sunlight at 24 hrs (Powder form)

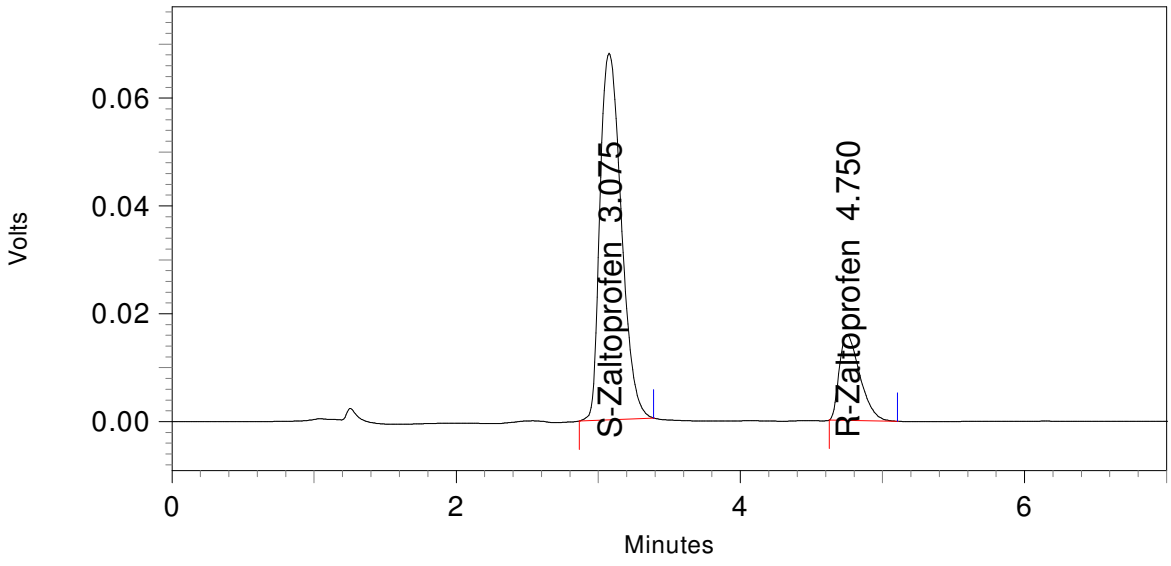

Figure 69: Typical HPLC chromatogram of photo degradation sample of Zaltoprofen (R&S) with sunlight at 24 hrs (Solution form)

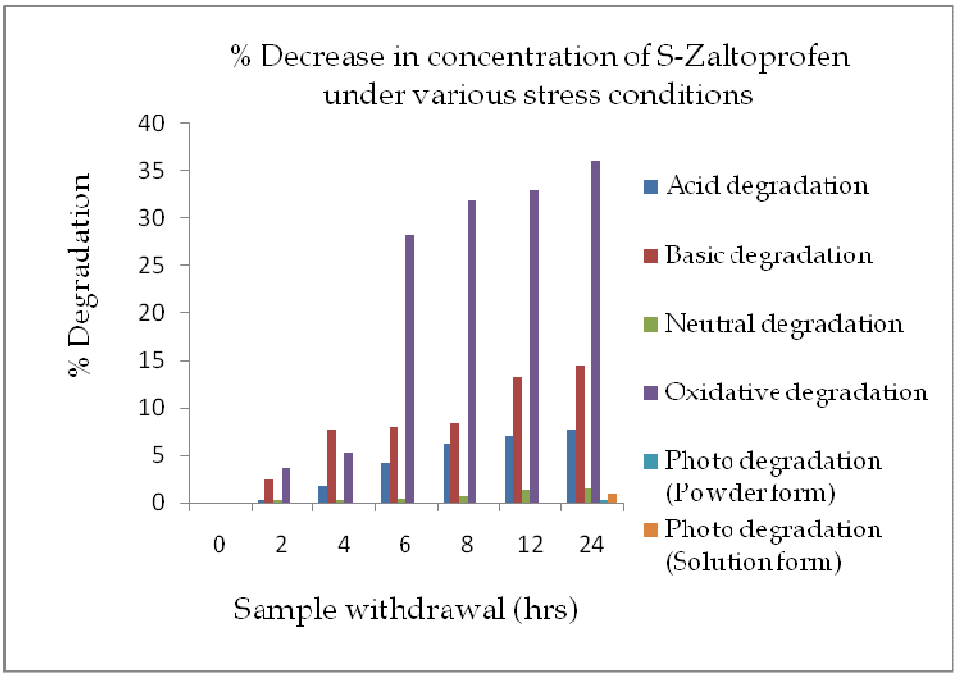

Figure 70: Graphical representation of % decrease in concentration of S-Zaltoprofen under various stress conditions

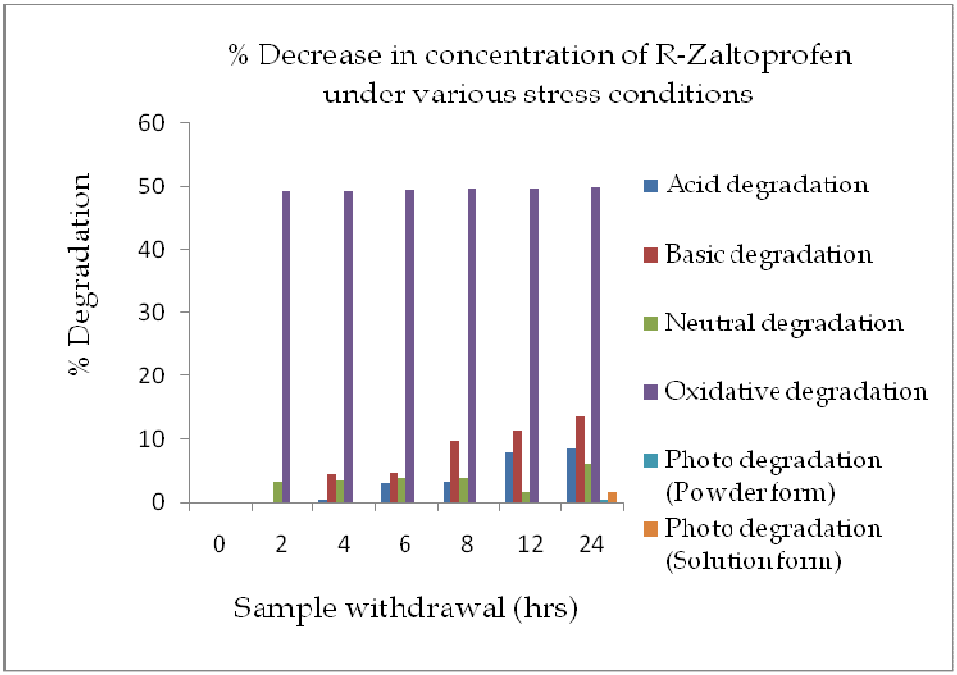

Figure 71: Graphical representation of % decrease in concentration of R-Zaltoprofen under various stress conditions

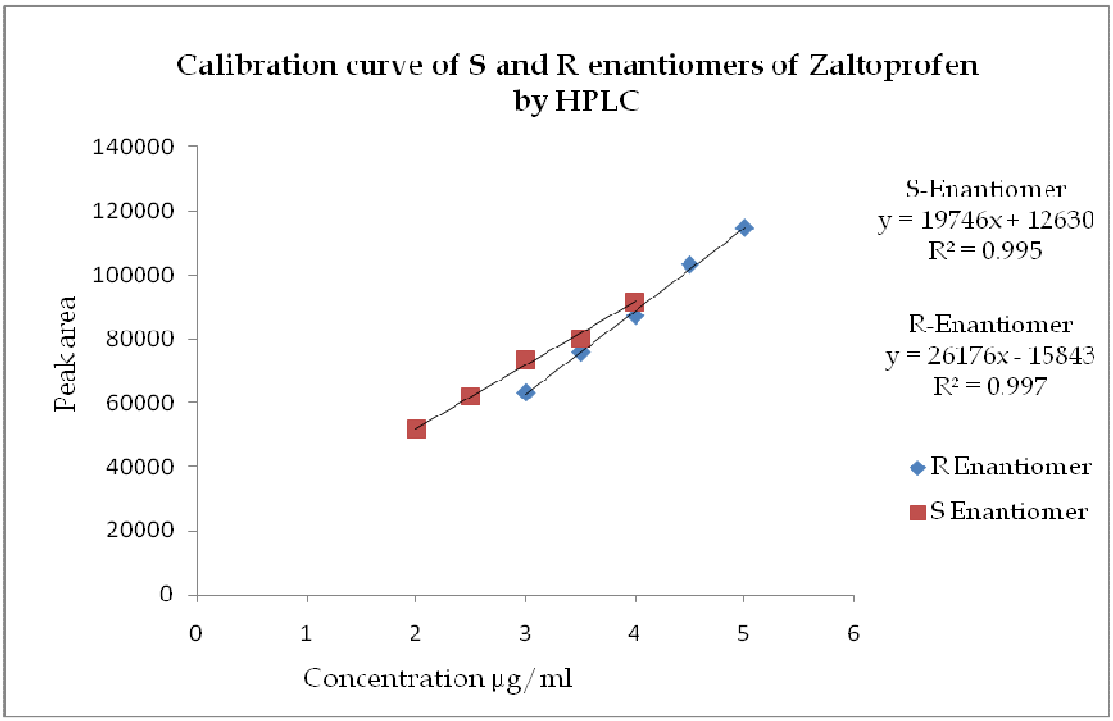

Figure 72: HPLC calibration curve of Zaltoprofen (R&S)

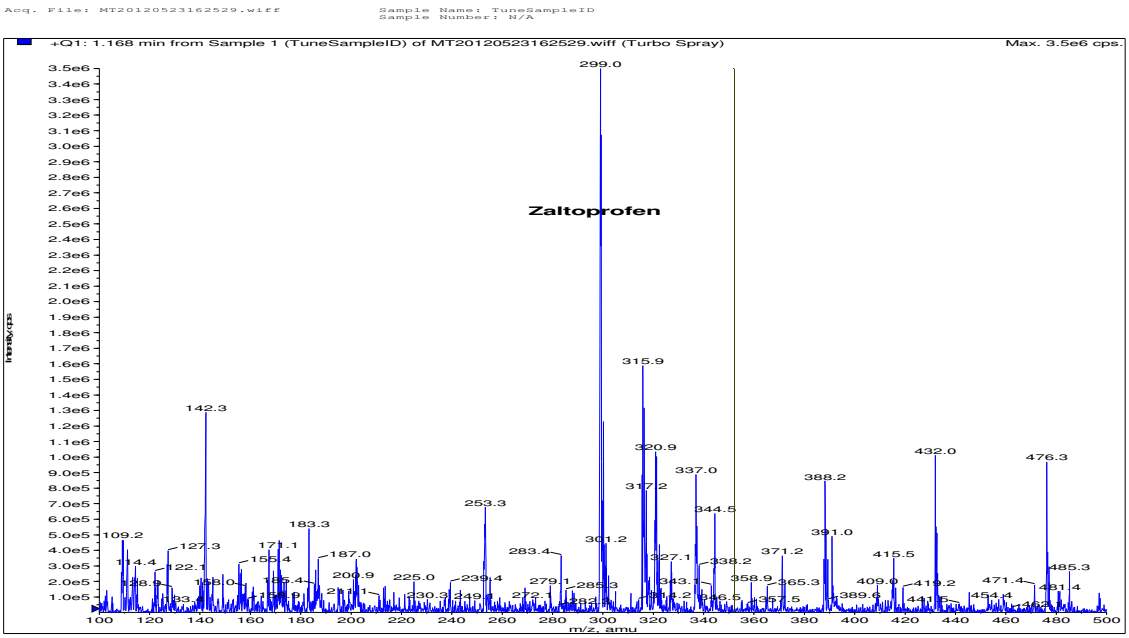

Figure 73: Typical MS chromatogram of Zaltoprofen

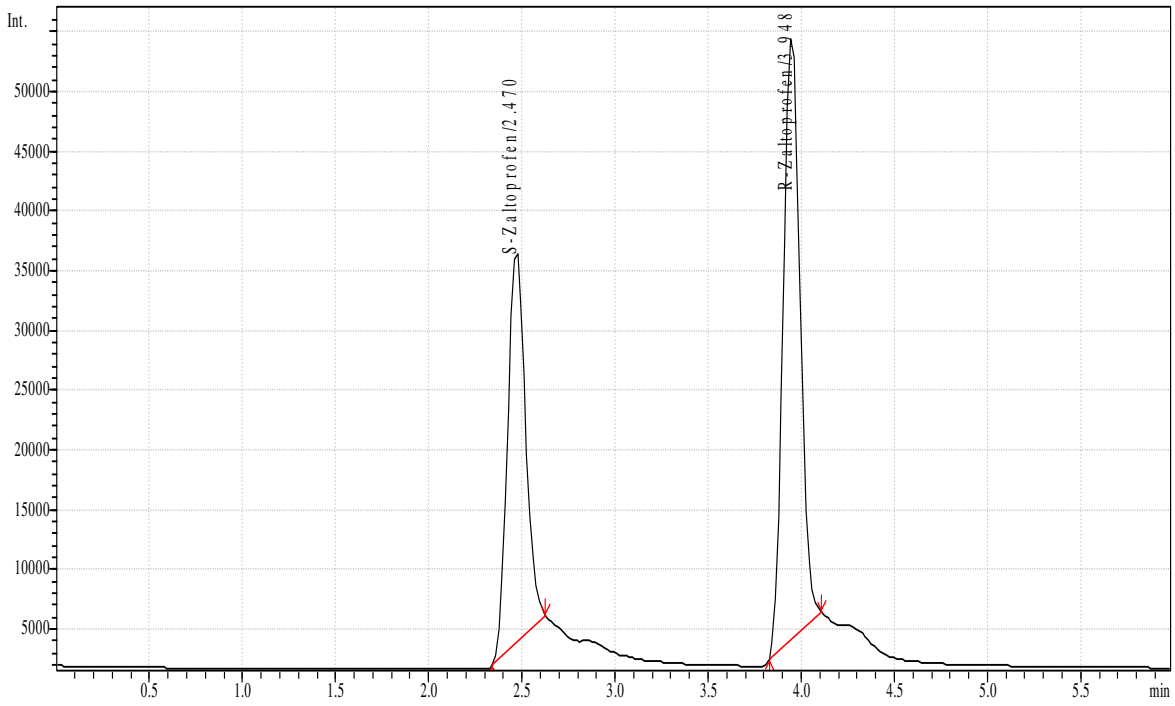

Figure 74: Typical LC-MS chromatogram of Zaltoprofen standard solution

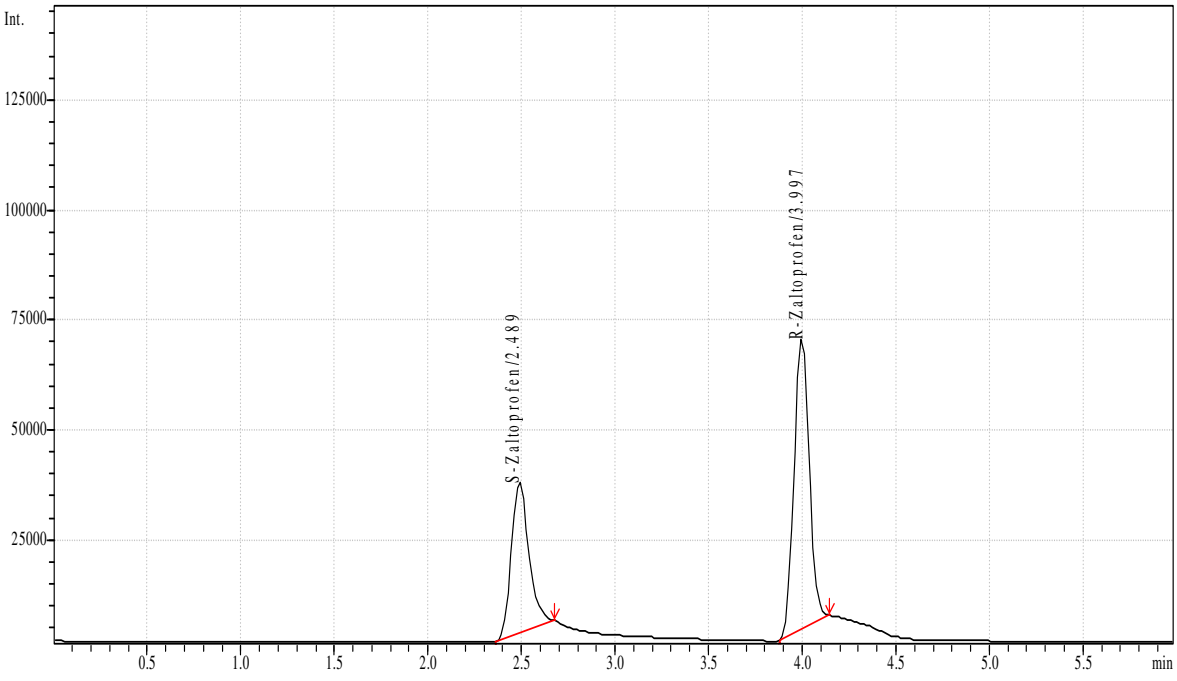

Figure 75: Typical LC-MS chromatogram of Zaltoprofen sample solution

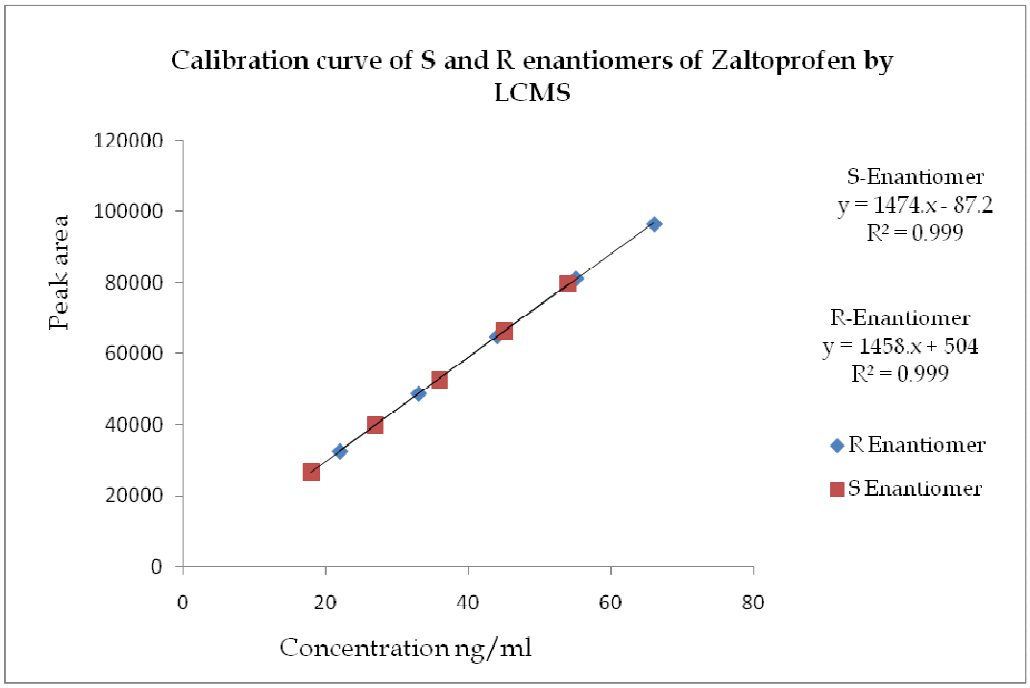

Figure 76: LC-MS Calibration curve of Zaltoprofen

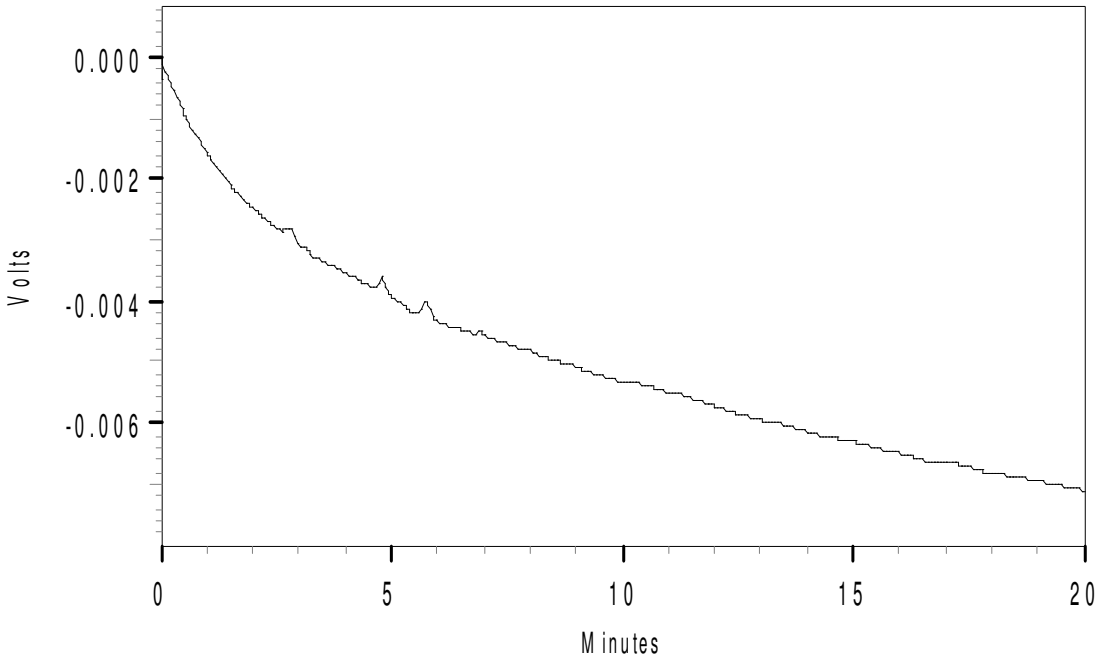

Figure 77: Typical HPLC chromatogram of Valganciclovir hydrochloride blank solution

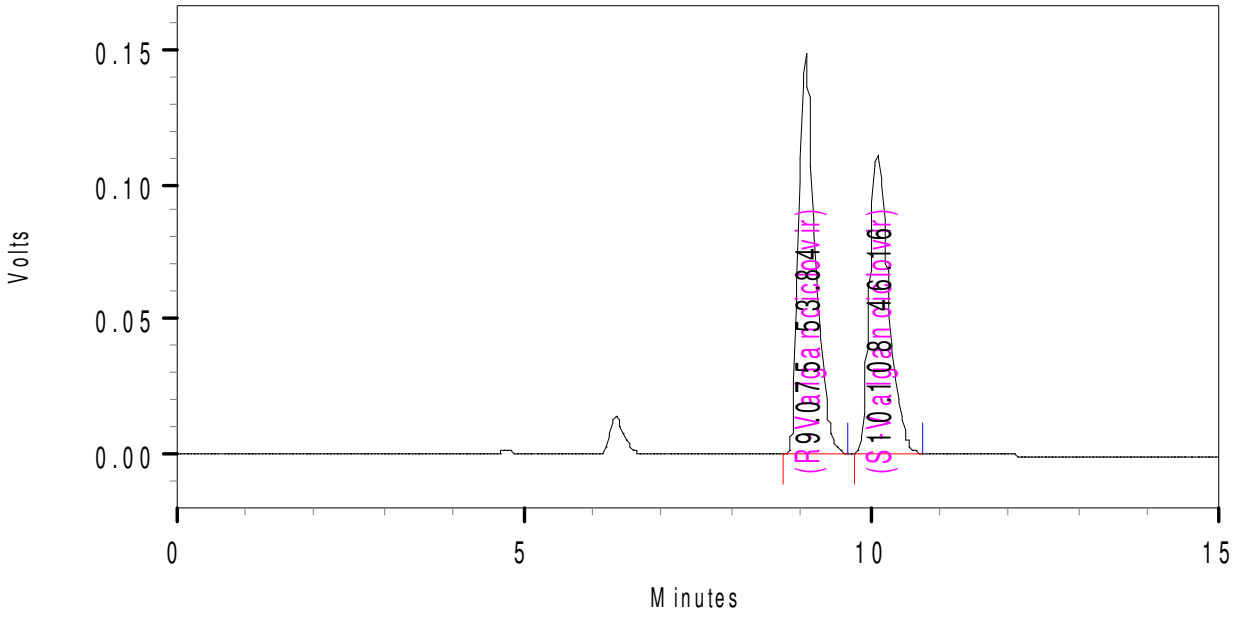

Figure 78: Typical HPLC chromatogram of Valganciclovir hydrochloride standard solution

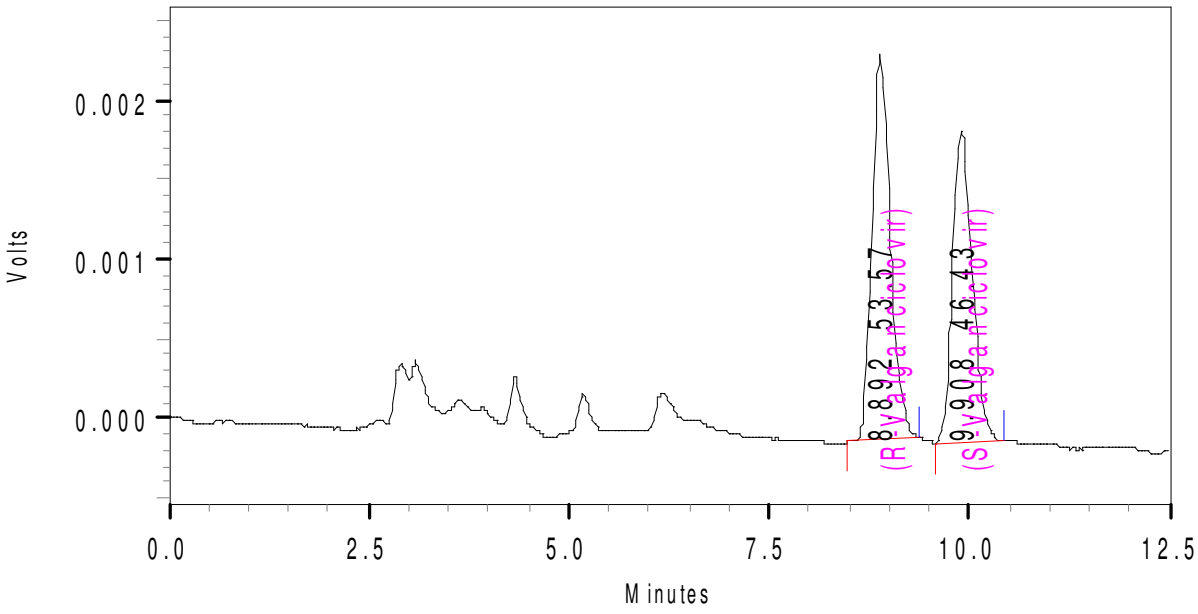

Figure 79: Typical HPLC chromatogram of sample I containing Valganciclovir hydrochloride

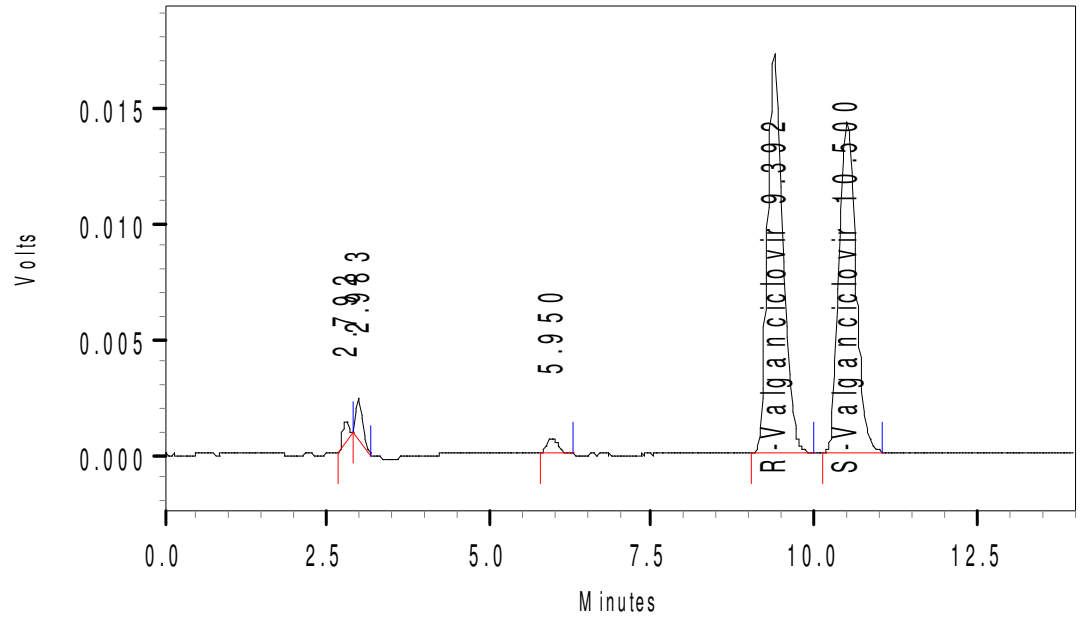

Figure 80: Typical HPLC chromatogram of acid degradation sample of Valganciclovir hydrochloride (R&S) with 0.1N HCl at 0 hr

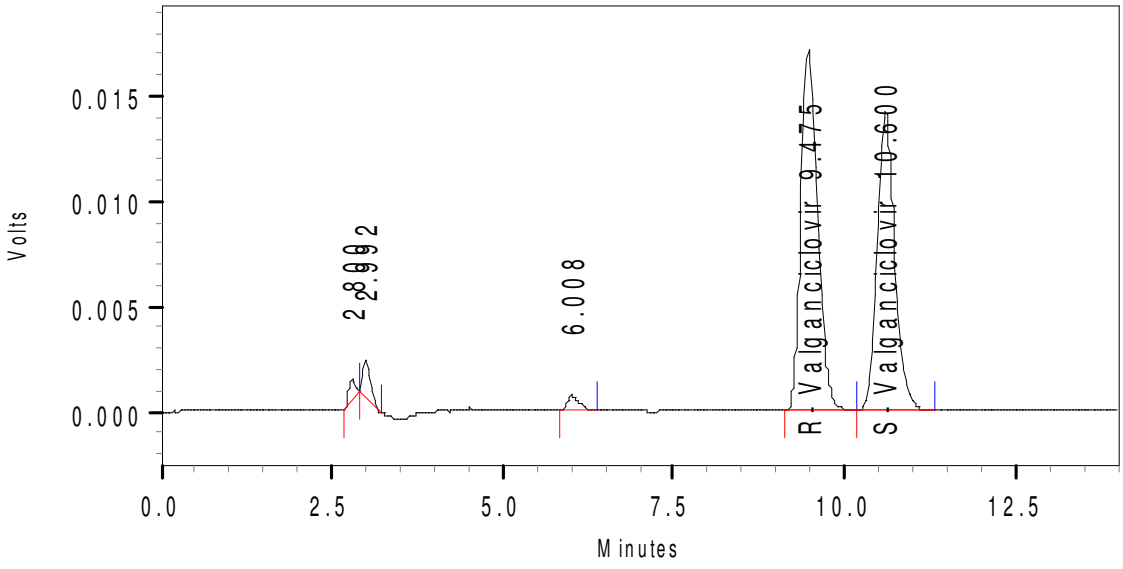

Figure 81: Typical HPLC chromatogram of acid degradation sample of Valganciclovir hydrochloride (R&S) with 0.1N HCl at 24 hrs

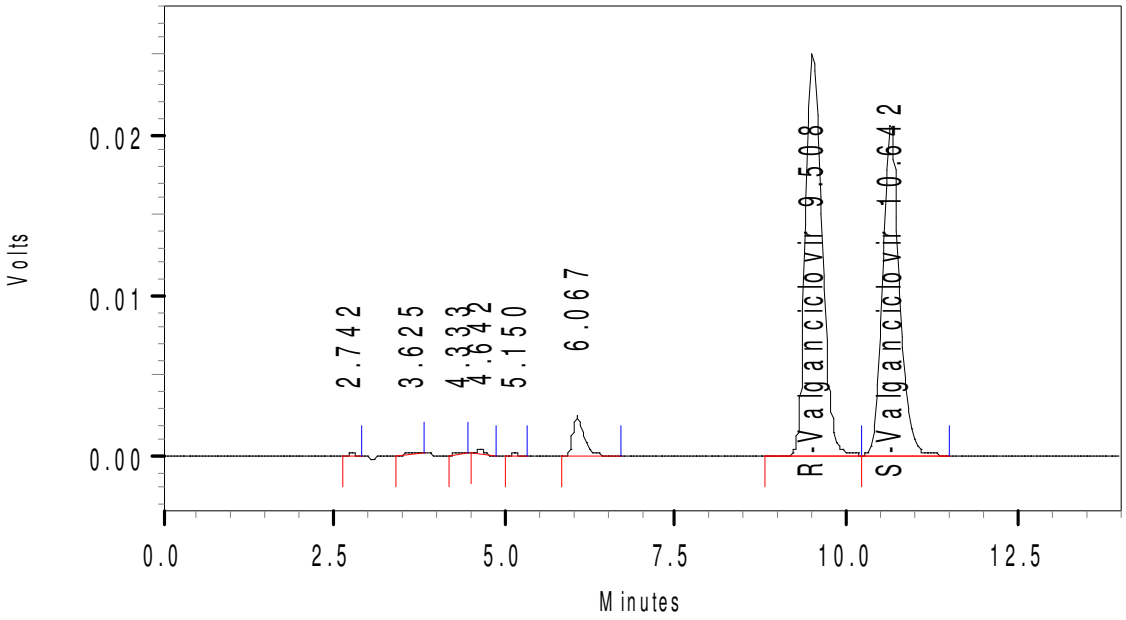

Figure 82: Typical HPLC chromatogram of basic degradation sample of Valganciclovir hydrochloride (R&S) with 0.1N NaOH at 0 hr

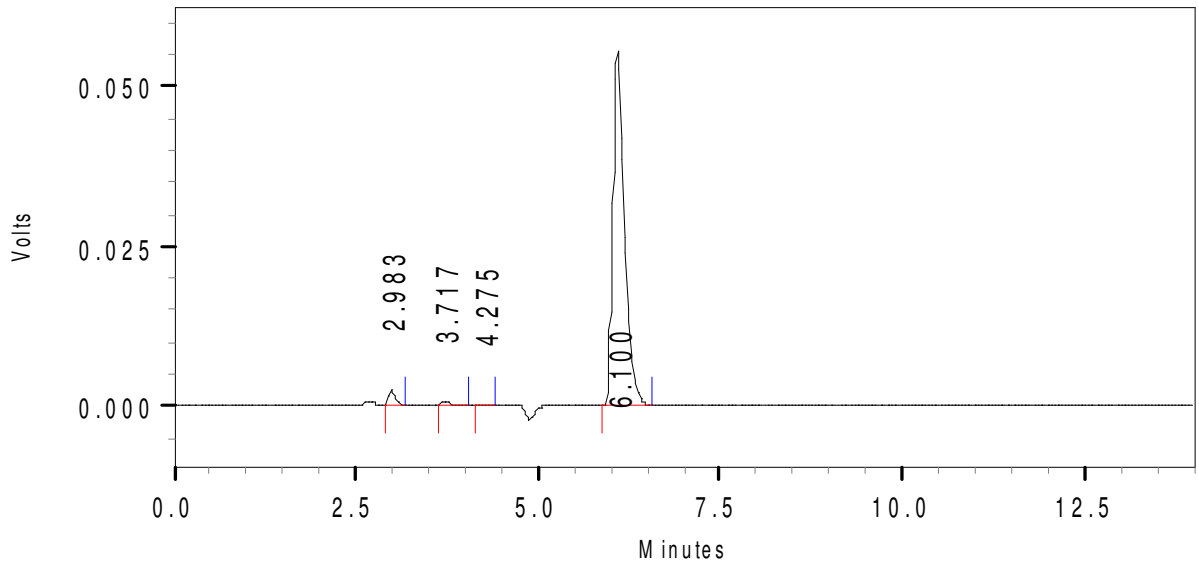

Figure 83: Typical HPLC chromatogram of basic degradation sample of Valganciclovir hydrochloride (R&S) with 0.1N NaOH at 24 hrs

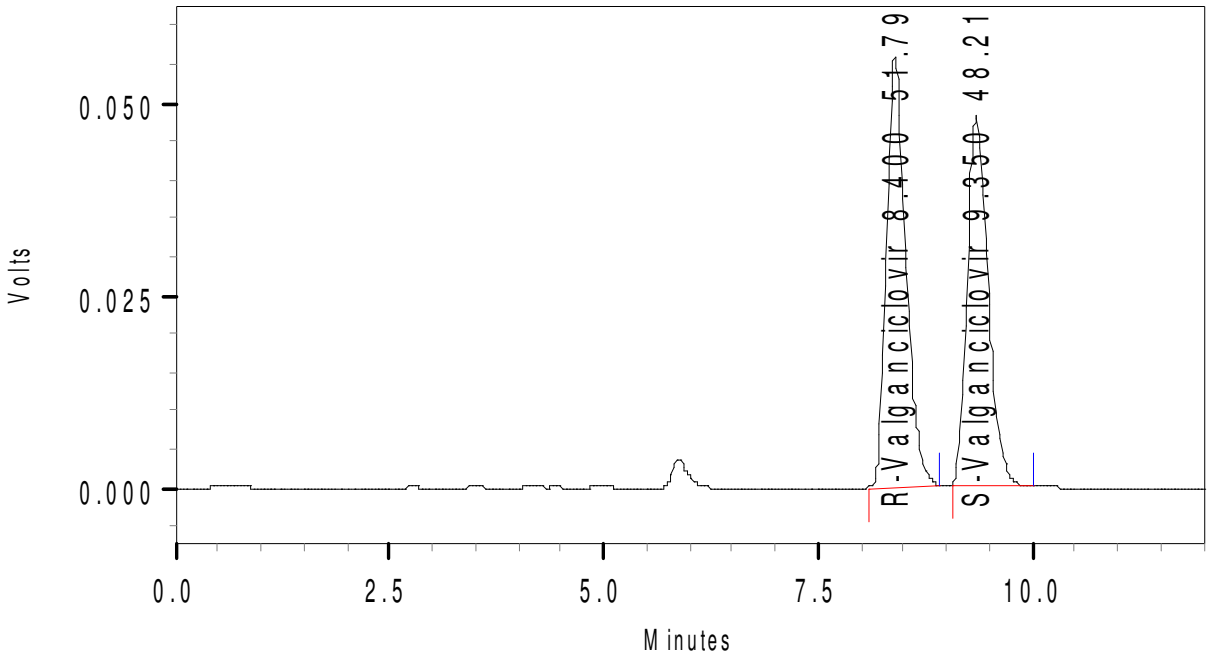

Figure 84: Typical HPLC chromatogram of neutral degradation sample of Valganciclovir hydrochloride (R&S) with water at 0 hr

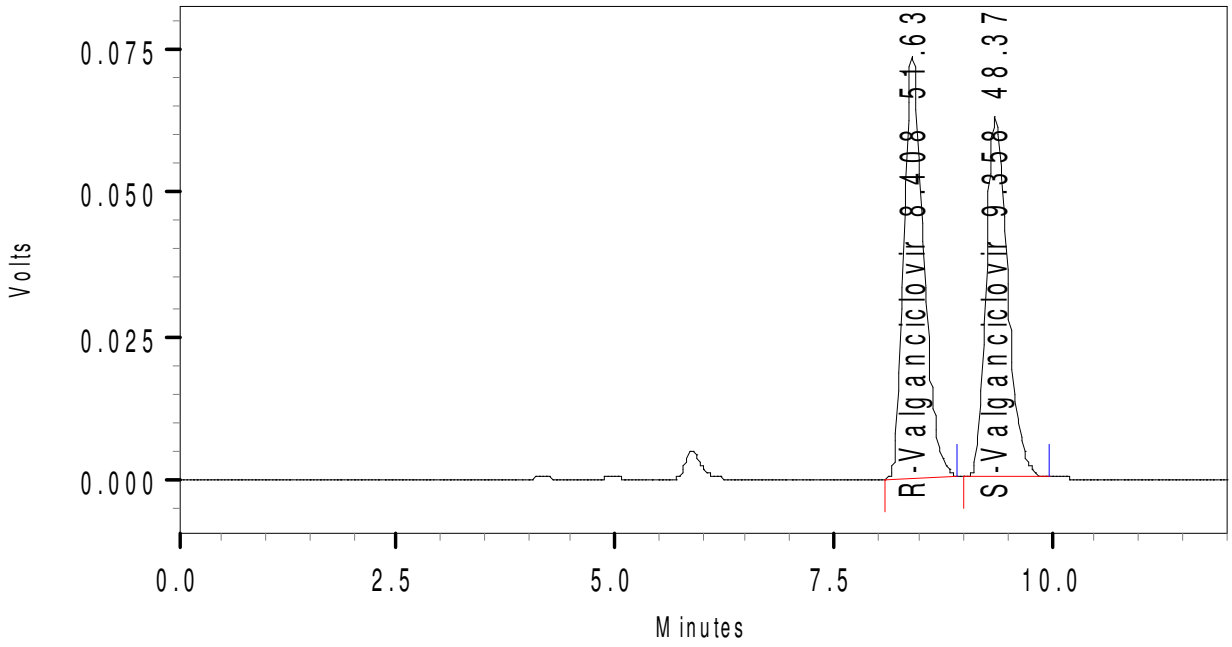

Figure 85: Typical HPLC chromatogram of neutral degradation sample of Valganciclovir hydrochloride (R&S) with water at 24 hrs

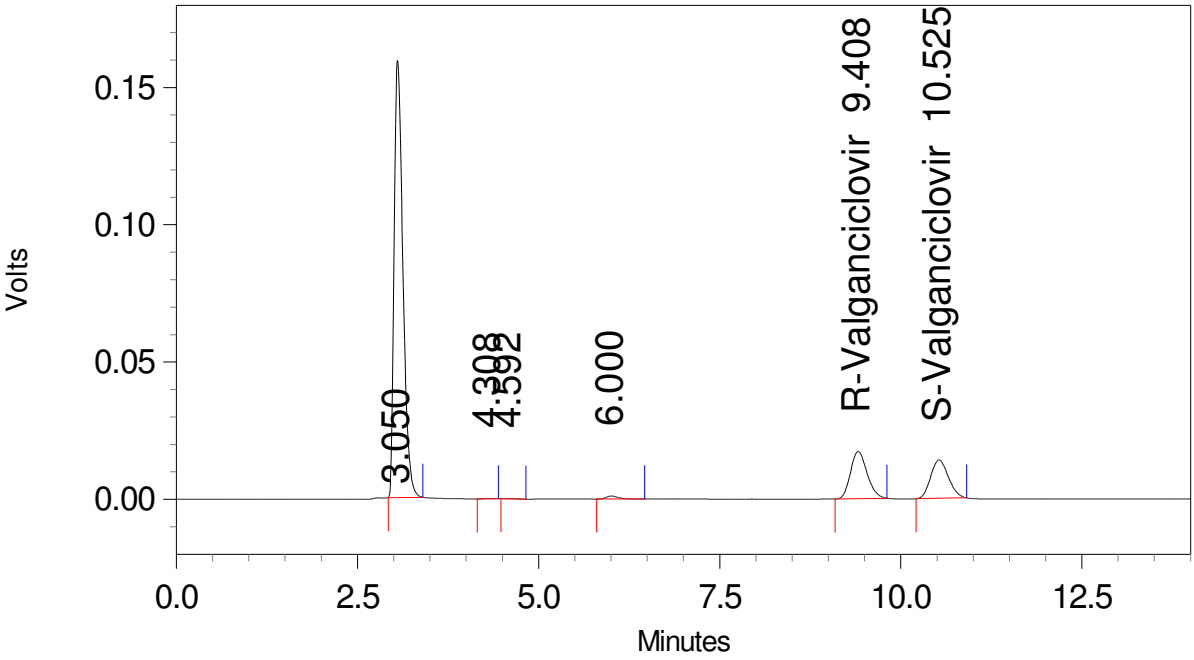

Figure 86: Typical HPLC chromatogram of oxidative degradation sample of Valganciclovir hydrochloride (R&S) with 3% H_2O_2 at 0 hr

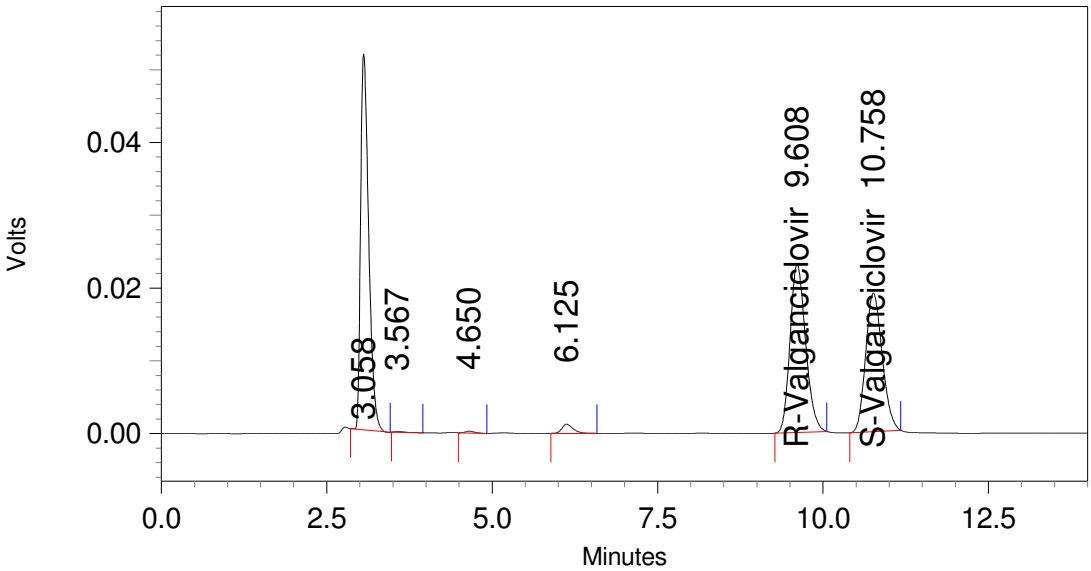

Figure 87: Typical HPLC chromatogram of oxidative degradation sample of Valganciclovir hydrochloride (R&S) with 3% H_2O_2 at 24 hrs

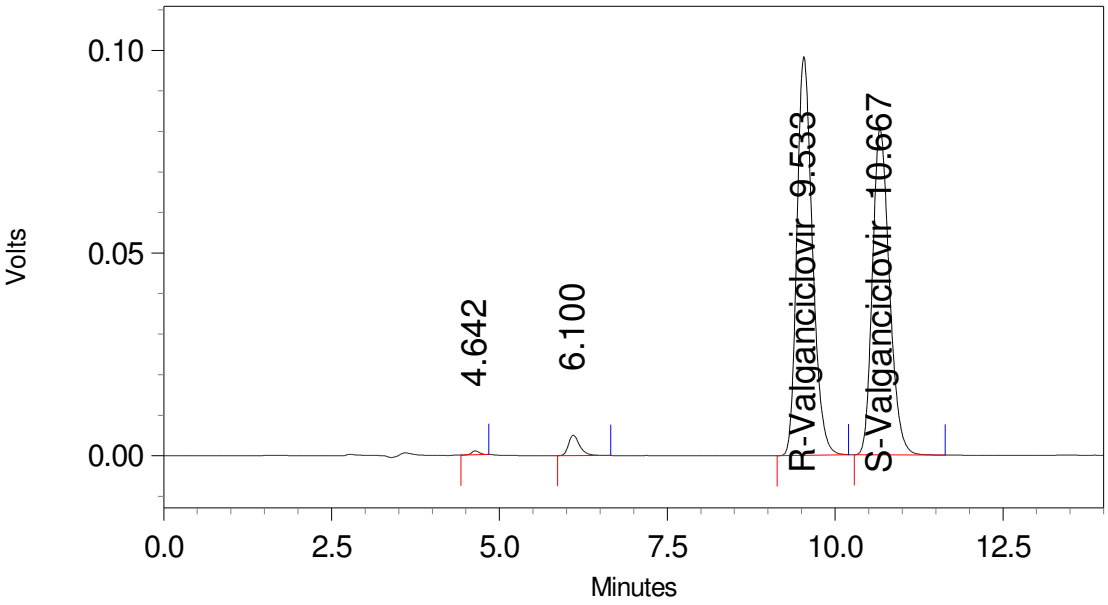

Figure 88: Typical HPLC chromatogram of photo degradation sample of Valganciclovir hydrochloride (R&S) with sunlight at 0 hr

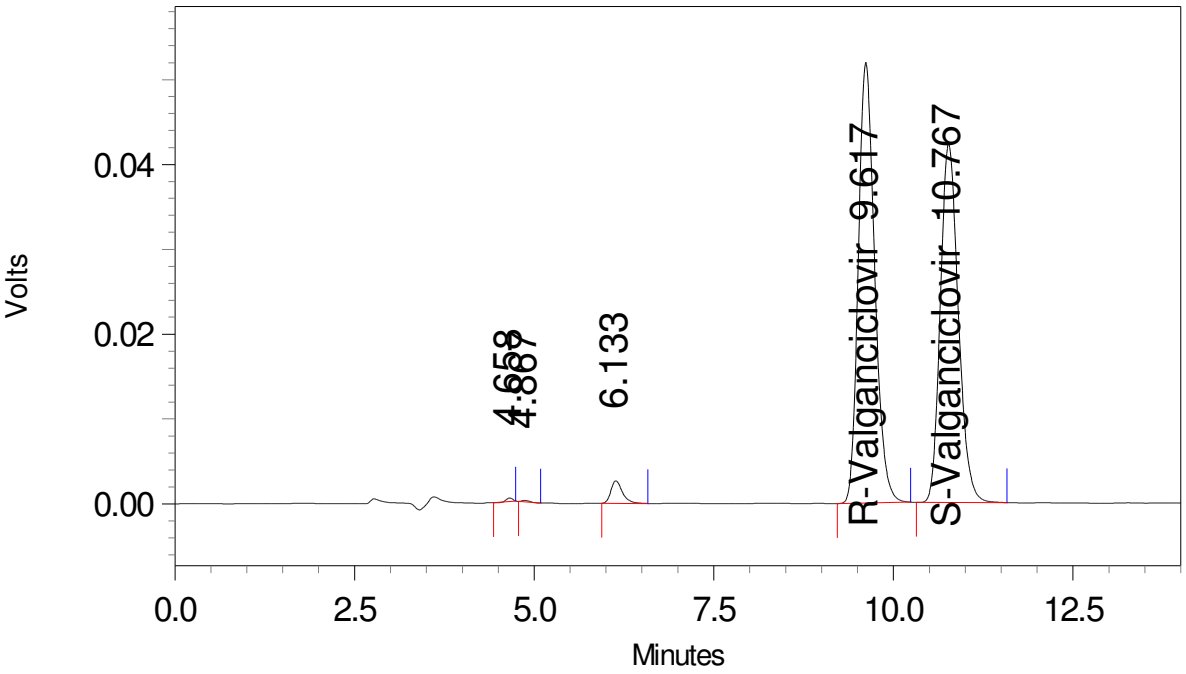

Figure 89: Typical HPLC chromatogram of photo degradation sample of Valganciclovir hydrochloride (R&S) with sunlight at 24 hrs (Powder form)

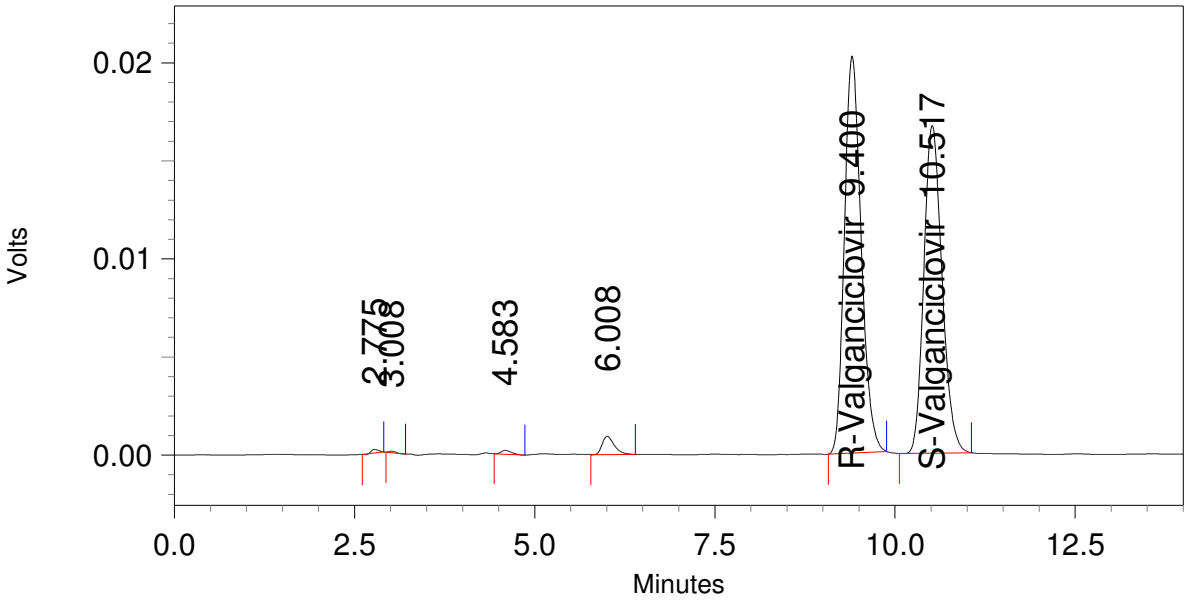

Figure 90: Typical HPLC chromatogram of photo degradation sample of Valganciclovir hydrochloride (R&S) with sunlight at 24 hrs (Solution form)

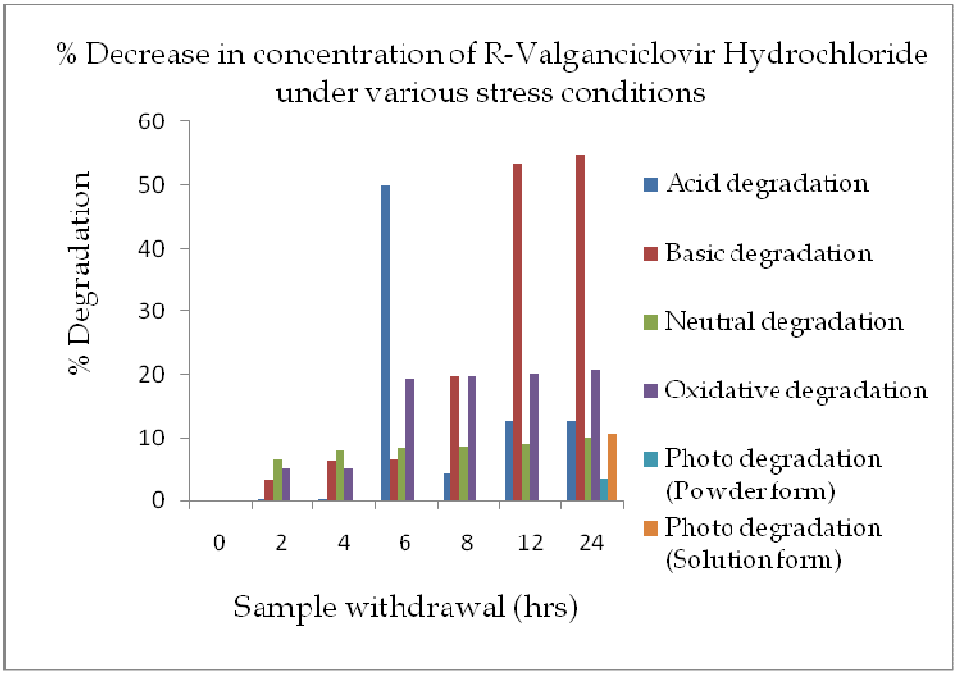

Figure 91: Graphical representation of % decrease in concentration of R-Valganciclovir hydrochloride under various stress conditions

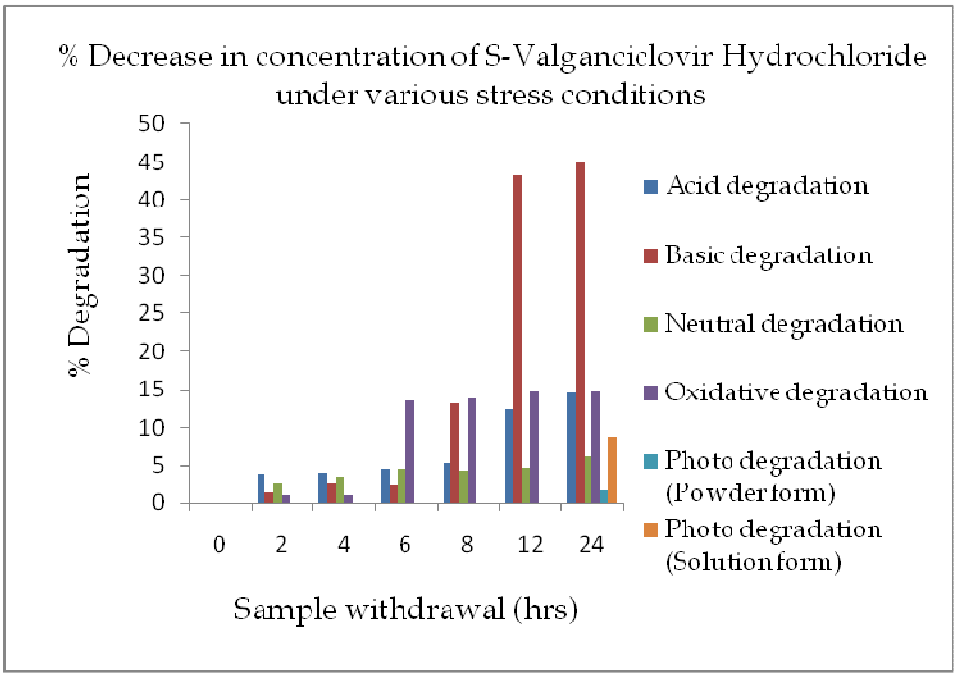

Figure 92: Graphical representation of % decrease in concentration of S-Valganciclovir hydrochloride under various stress conditions

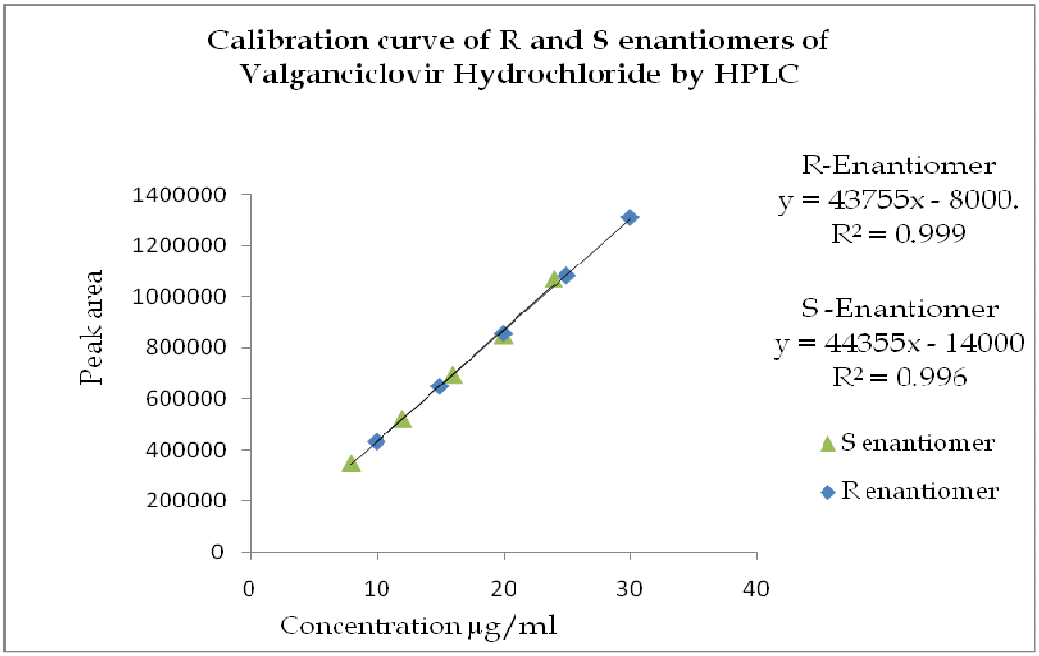

Figure 93: Linearity and range of R and S Valganciclovir hydrochloride by HPLC

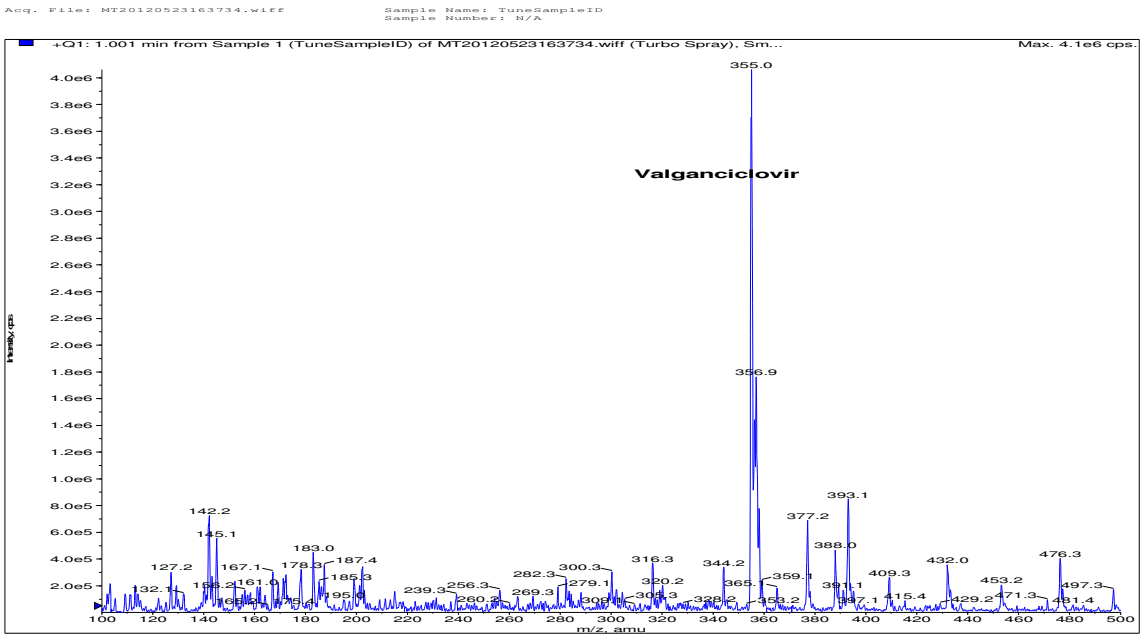

Figure 94: Typical MS chromatogram of Valganciclovir hydrochloride

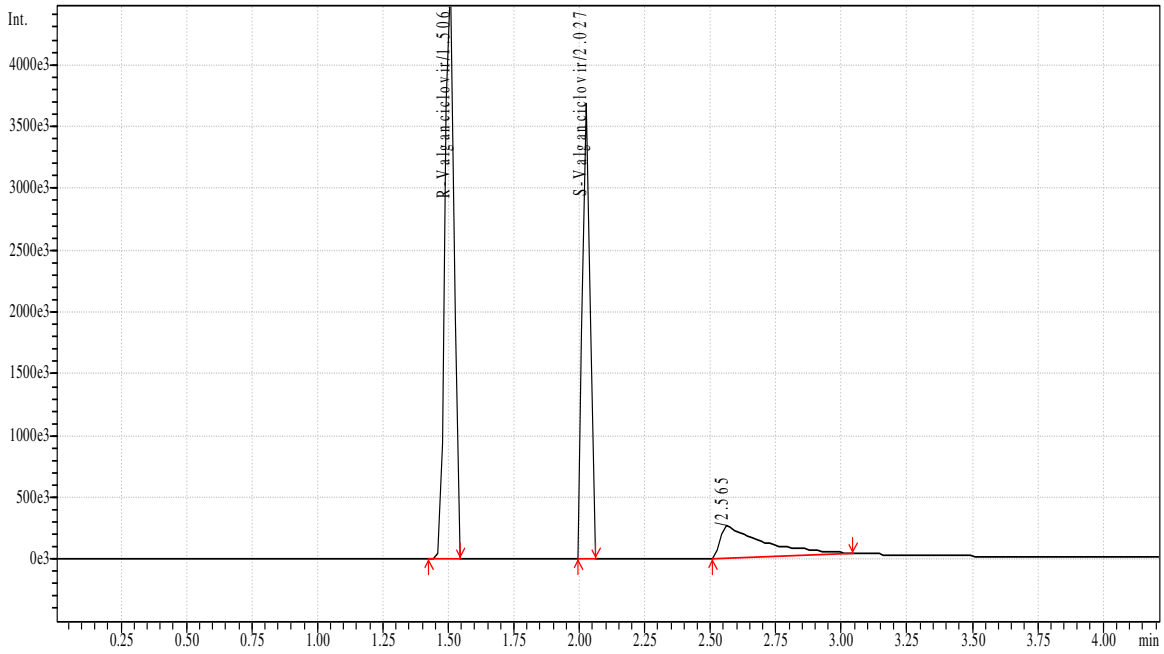

Figure 95: Typical LC-MS chromatogram of Valganciclovir hydrochloride standard solution

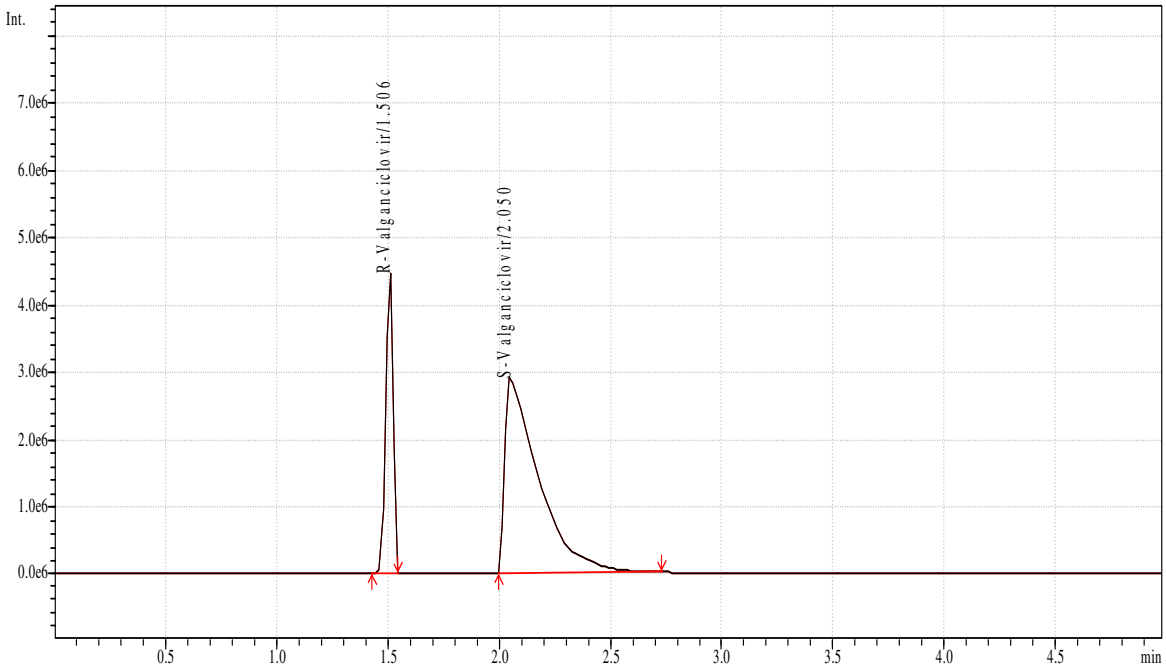

Figure 96: Typical LC-MS chromatogram of Valganciclovir hydrochloride sample solution

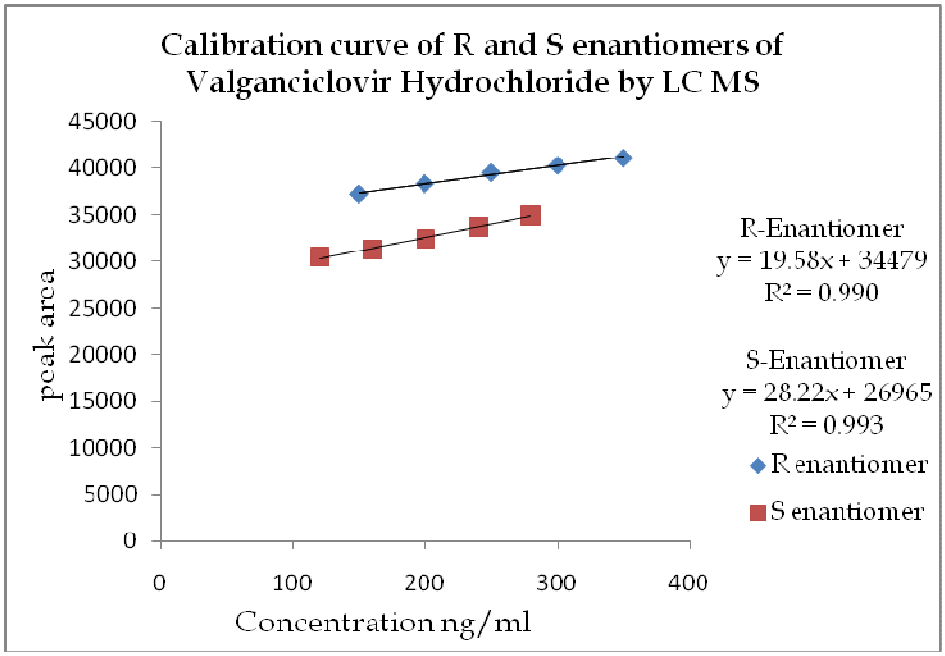

Figure 97: LC-MS Calibration curve of Valganciclovir Hydrochloride

Table 4: Linearity and range for R and S Rosiglitazone enantiomers by HPLC

S. No	R - Rosiglitazone		S - Rosiglitazone	
	Concentration µg/ml	Peak area	Concentration µg/ml	Peak area
1	3	97365	9	292094
2	4	129820	12	389460
3	5	162278	15	486833
4	6	194733	18	584198
5	7	227188	21	681564

Table 5: Precision studies for R and S Rosiglitazone enantiomers by HPLC

S. No	R - Rosiglitazone			S - Rosiglitazone		
	3 µg/ml	5 µg/ml	7 µg/ml	9 µg/ml	15 µg/ml	21 µg/ml
1	97365	162278	227188	292094	486833	681564
2	97002	165786	221895	297654	486578	689654
3	96999	165431	227643	294352	497652	689987
4	97765	165765	226112	290081	493211	686543
5	96432	164087	227965	290354	495432	680054
6	97978	164999	226345	288699	489978	689455
Mean	97256.83	164724.33	226191.33	292205.66	491614	686209.5
SD	566.00	1354.67	2224.18	3302.82	4571.23	4387.46
% RSD	0.581	0.822	0.983	1.133	0.929	0.639

Table 6: Results of analysis of drug products and recovery studies for R and S Rosiglitazone enantiomers by HPLC

Sample	Label claim (mg)	Amount present (mg/tablet) ± % RSD*			% Label Claim			% Recovery ± % RSD*
		R&S Rosiglitazone	R Rosiglitazone	S Rosiglitazone	R&S Rosiglitazone	R Rosiglitazone	S Rosiglitazone	
Sample I	2	2.01±0.7892	0.51±0.6492	1.51±0.2454	100.44±0.6155	25.24±0.5955	75.20±0.2008	99.05±0.3225
Sample II	4	4.04±0.8072	1.04±0.3248	3.04±0.1592	100.53±0.1778	25.24±0.4325	75.29±0.6034	97.88±0.5441
Sample III	4	4.03±0.4358	1.01±0.7922	2.99±0.83436	100.33±0.9173	25.23±0.4156	75.10±0.9016	96.25±0.4956
Sample IV	8	8.14±0.3086	2.04±0.6393	6.02±1.0193	100.21±0.4727	25.23±0.2575	74.98±0.2397	99.76±0.8809

* n=3

Sample I: Reglit Tablets containing 2 mg of Rosiglitazone (RS)

Sample II: Enselin Tablets containing 4 mg of Rosiglitazone (RS)

Sample III: Rosicon Tablets containing 4 mg of Rosiglitazone (RS)

Sample IV: Rosinorm Tablets containing 8 mg of Rosiglitazone (RS)

Table 7: Stress degradation studies of Rosiglitazone RS (Acidic degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area(S)	% drug present (R)	% drug present (S)	% degradation (R Rosiglitazone)	% degradation (S Rosiglitazone)
0.1 N HCl							
1	0	811385	2434156	25	75	0	0
2	2	764649	2336141	23.56	71.98	1.44	3.02
1 N HCl							
1	0	812438	2455321	25	75	0	0
2	2	728919	2313567	22.43	70.67	2.57	4.33
3	4	699997	2172593	21.54	70.43	3.46	4.57
4	6	660025	2040210	20.31	69.87	4.69	5.13
5	8	636301	1900660	19.58	67.45	5.42	7.55
6	12	553107	1663711	17.02	65.65	7.98	9.35
7	24	505011	1362690	15.54	61.43	9.46	13.57

Table 8: Stress degradation studies of Rosiglitazone RS (Basic degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area(S)	% drug present (R)	% drug present (S)	% degradation (R Rosiglitazone)	% degradation (S Rosiglitazone)
0.1 N NaOH							
1	0	834211	2532111	25	75	0	0
2	2	793502	2486533	23.78	73.65	1.22	1.35
1 N NaOH							
1	0	834435	2573297	25	75	0	0
2	2	72262	222813	2.16	6.49	22.83	68.51
3	4	67542	208262	2.02	6.069	22.97	68.93
4	6	64972	200119	1.94	5.83	23.05	69.17
5	8	51156	156902	19.68	4.57	5.36	70.43
6	12	3931	12125	0.11	0.35	24.88	74.66
7	24	1114	3437	0.03	0.10	24.96	74.89

Table 9: Stress degradation studies of Rosiglitazone RS (Neutral degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area(S)	% drug present (R)	% drug present (S)	% degradation (R Rosiglitazone)	% degradation (S Rosiglitazone)
Water							
1	0	832342	2434177	25	75	0	0
2	2	814030	2405291	24.45	74.11	0.55	0.89
3	4	803044	2390361	24.12	73.65	0.88	1.35
4	6	788061	2350765	23.67	72.43	1.33	2.57
5	8	747443	2318959	22.45	71.45	2.55	3.55
6	12	721474	2264109	21.67	69.76	3.33	5.24
7	24	691177	2224513	20.76	68.54	4.24	6.46

Table 10: Stress degradation studies of Rosiglitazone RS (Oxidative degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area(S)	% drug present (R)	% drug present (S)	% degradation (R Rosiglitazone)	% degradation (S Rosiglitazone)
3% H_2O_2							
1	0	843298	2765865	25	75	0	0
2	2	819583	2547380	24.29	69.07	1.09	5.92
30% H_2O_2							
1	0	844567	2731156	25	75	0	0
2	2	53918	217070	1.59	5.96	23.40	69.04
3	4	33569	170839	0.99	4.69	24.01	70.31
4	6	12879	128029	0.38	3.51	24.61	71.48
5	8	9647	68730	0.28	1.88	24.71	73.11
6	12	4504	31807	0.13	0.87	24.86	74.12
7	24	1188	7150	0.04	0.19	24.96	74.80

Table 11: Stress degradation studies of Rosiglitazone RS (Photo degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Rosiglitazone)	% degradation (S Rosiglitazone)
Photolytic degradation exposed to sunlight for 24 hrs							
1	0	846543	2743765	25	75	0	0
2	Powder form	839796	2732789	24.80	74.70	0.19	0.30
3	Solution form	819865	2668768	24.21	72.95	0.79	2.05

Table 12: System suitability studies for estimation of R and S Rosiglitazone enantiomers by HPLC

S. No.	Parameters	R - Rosiglitazone	S – Rosiglitazone
1	Linearity range	3 – 7 µg/ml	9 - 21 µg/ml
2	Regression equation Y = mx + c	y = 32455x + 8.8	y = 32456x - 22.25
3	Correlation coefficient	1.0	1.0
4	Theoretical plate/meter	40074	45503
5	Resolution factor	0.6	
6	Asymmetric factor	1.02	1.01
7	LOD (µg/ml)	0.596	0.596
8	LOQ (µg/ml)	1.80	1.80

Table 13: Linearity and range for R and S Rosiglitazone enantiomers by LC-MS

S. No	R – Rosiglitazone		S – Rosiglitazone	
	Concentration ng/ml	Peak area	Concentration ng/ml	Peak area
1	30	13980	90	41921
2	40	18329	120	54786
3	50	23670	150	70892
4	60	28952	180	86825
5	70	33325	210	99980

Table 14: Precision studies for R and S Rosiglitazone enantiomers by LC-MS

S. No	R – Rosiglitazone			S – Rosiglitazone		
	30 ng/ml	50 ng/ml	70 ng/ml	90 ng/ml	150 ng/ml	210 ng/ml
1	13980	23670	33325	41921	70892	99980
2	13985	23587	33387	41865	70799	99498
3	14191	23602	33211	41893	70853	99987
4	13889	23591	33305	41975	70875	99643
5	13897	23685	33427	41906	70902	99454
6	13995	23732	33298	41942	70823	99455
Mean	13989.5	23644.5	33325.5	41917	70857.33	99669.5
SD	108.99	59.86	75.32	38.48	40.27	252.89
% RSD	0.779	0.253	0.226	0.091	0.0564	0.253

Table 15: Results of analysis of drug products and recovery studies for R and S Rosiglitazone enantiomers by LC-MS

Sample	Label claim (mg)	Amount present (mg/tablet) ± % RSD*			% Label Claim			% Recovery ± % RSD*
		R&S Rosiglitazone	R Rosiglitazone	S Rosiglitazone	R&S Rosiglitazone	R Rosiglitazone	S Rosiglitazone	
Sample I	2	2.01±0.3468	0.50±0.8481	1.50±0.6987	100.73±0.8303	25.24±0.9394	75.49±0.3635	98.65±0.7641

* n=3

Sample I: Reglit Tablets containing 2 mg of Rosiglitazone (RS)

Table 16: System suitability studies for estimation of R and S Rosiglitazone enantiomers by LC-MS

S. No	Parameters	R - Rosiglitazone	S - Rosiglitazone
1	Linearity range	30 -70 ng/ml	90 - 210 ng/ml
2	Regression equation Y = mx + c	y = 493.1x - 1005	y = 493.8x - 3197
3	Correlation coefficient	0.998	0.998
4	Resolution factor	1.8	
5	Asymmetric factor	1.02	1.01
6	LOD (ng/ml)	0.2086	0.2086
7	LOQ (ng/ml)	0.6324	0.6324

Table 17: Linearity and range for R and S Pioglitazone enantiomers by HPLC

S. No	R – Pioglitazone		S – Pioglitazone	
	Concentration µg/ml	Peak area	Concentration µg/ml	Peak area
1	5	326557	4	263294
2	6.5	403118	5.5	328278
3	8	485951	7	402187
4	9.5	583230	8.5	471246
5	11	668029	10	542827

Table 18: Precision studies for R and S Pioglitazone enantiomers by HPLC

S. No	R – Pioglitazone			S – Pioglitazone		
	5 µg/ml	8 µg/ml	11 µg/ml	4 µg/ml	7 µg/ml	10 µg/ml
1	326557	485791	668029	263294	402357	539980
2	324579	485951	658721	267532	419067	542827
3	319986	496321	659906	268907	413265	541875
4	317520	494608	664752	270054	410985	547824
5	329875	493703	670351	260891	409975	538798
6	324135	489972	671446	261437	402187	549840
Mean	323775.33	491057.66	665534.16	265352.5	409639.33	543524
SD	4454.37	4523.26	5349.45	3973.97	6519.50	4392.76
% RSD	1.375	0.921	0.803	1.497	1.591	0.808

Table 19: Results of analysis of R and S Pioglitazone enantiomers and recovery studies for R and S Pioglitazone enantiomers by HPLC

Sample	Label claim (mg)	Amount present (mg/Tablet) ± % RSD*			% Label Claim			% Recovery ± % RSD*
		R&S Pioglitazone	R Pioglitazone	S Pioglitazone	R&S Pioglitazone	R Pioglitazone	S Pioglitazone	
Sample I	15	15.11±0.2514	8.34±0.3985	6.75±0.4529	100.70±0.9357	55.66±0.6707	45.03±0.6498	100.04±0.9214
Sample II	15	15.22±0.5207	8.47±0.7629	6.74±0.6578	101.48±0.4783	56.50±0.9869	44.97±0.1879	98.40±0.5153
Sample III	30	30.45±0.4404	16.88±0.8908	13.56±0.8496	101.51±0.3469	56.29±0.6373	45.21±0.3255	97.14±0.9632
Sample IV	30	30.39±0.9759	16.87±0.5108	13.52±0.3483	101.31±0.6801	56.23±0.2871	45.08±0.5141	99.88±0.5386

* n=3

Sample I: Pepar tablets containing 15 mg of Pioglitazone (RS)

Sample II: Diavista tablets containing 15 mg of Pioglitazone (RS)

Sample III: Piozulin tablets containing 30 mg of Pioglitazone (RS)

Sample IV: Piolem tablets containing 30 mg of Pioglitazone (RS)

Table 20: Stress degradation studies of Pioglitazone RS (Acidic degradation)

S. No	Time (hrs)	Mean peak area (R)	Mean peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Pioglitazone)	% degradation (S Pioglitazone)
0.1 N HCl							
1	0	297654	234780	55	45	0	0
2	2	288765	228765	53.35	43.84	1.65	1.16
1 N HCl							
1	0	289875	230087	55	45	0	0
2	2	287654	220765	54.57	43.17	0.43	1.93
3	4	276543	220003	52.47	43.02	2.53	1.98
4	6	259876	209876	49.30	41.04	5.7	3.96
5	8	245432	198765	46.56	38.87	8.44	6.13
6	12	227898	180987	43.24	35.39	11.76	9.61
7	24	216789	167654	41.13	32.78	13.87	12.22

Table 21: Stress degradation studies of Pioglitazone RS (Basic degradation)

S. No	Time (hrs)	Mean peak area (R)	Mean peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Pioglitazone)	% degradation (S Pioglitazone)
1 N NaOH							
1	0	297654	247804	55	45	0	0
2	2	45675	765	8.44	0.14	46.56	44.86
0.1 N NaOH							
1	0	295644	248984	55	45	0	0
2	2	23265	246554	4.33	44.56	50.67	0.44
3	4	20113	245543	3.74	44.38	51.26	0.62
4	6	20021	228876	3.72	41.36	51.27	3.63
5	8	19321	225422	3.59	40.74	51.41	4.26
6	12	18765	217898	3.49	39.38	51.51	5.62
7	24	16123	216789	2.99	39.18	52.00	5.82

Table 22: Stress degradation studies of Pioglitazone RS (Neutral degradation)

S. No	Time (hrs)	Mean peak area (R)	Mean peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Pioglitazone)	% degradation (S Pioglitazone)
Water							
1	0	278968	224567	55	45	0	0
2	2	278087	223218	54.82	44.72	0.17	0.27
3	4	275432	222241	54.30	44.53	0.70	0.47
4	6	268968	221567	53.02	44.39	1.97	0.60
5	8	268587	223218	52.95	44.72	2.04	0.27
6	12	265432	222241	52.33	44.53	2.66	0.47
7	24	259968	221567	51.25	44.39	3.75	0.60

Table 23: Stress degradation studies of Pioglitazone RS (Oxidative degradation)

S. No	Time (hrs)	Mean peak area (R)	Mean peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Pioglitazone)	% degradation (S Pioglitazone)
30% H_2O_2							
1	0	354567	292345	55	45	0	0
2	2	0	0	0	0	55	45
3% H_2O_2							
3	0	359807	294789	55	45	0	0
4	2	359625	288251	54.97	44.19	0.027	0.99
5	4	357220	288090	54.60	43.97	0.39	1.02
6	6	348338	270523	53.24	41.29	1.75	3.70
7	8	346573	276543	52.97	42.21	2.02	2.78
8	12	312345	270021	47.74	41.22	7.25	3.78
9	24	308338	265223	47.13	40.48	7.86	4.51

Table 24: Stress degradation studies of Pioglitazone RS (Photo degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area (S)	% drug present (R)	% degradation (S)	% degradation (R Pioglitazone)	% degradation (S Pioglitazone)
Photolytic degradation exposed to sunlight for 24 hrs							
1	0	2556183	1965366	55	45	0	0
2	Powder form	2546183	1954732	54.78	44.76	0.215	0.24
3	Solution form	2543792	1959084	54.73	44.85	0.266	0.143

Table 25: System suitability studies for estimation of R and S pioglitazone enantiomers by HPLC

S. No.	Parameters	R - Pioglitazone	S - Pioglitazone
1	Linearity range	5 – 11 µg/ml	4 - 10 µg/ml
2	Regression equation Y = mx + c	y = 58204x + 25747	y = 47469x + 65284
3	Correlation coefficient	0.996	0.996
4	Theoretical plate/meter	54082	54365
5	Resolution factor	1.85	
6	Asymmetric factor	1.02	1.01
7	LOD (µg/ml)	1.4	1.4
8	LOQ (µg/ml)	4.26	4.26

Table 26: Linearity and range for R and S Pioglitazone enantiomers by LC-MS

S. No	R – Pioglitazone		S - Pioglitazone	
	Concentration ng/ml	Peak area	Concentration ng/ml	Peak area
1	50	22570	40	18470
2	65	29556	55	24170
3	80	36345	70	30558
4	95	43187	85	36258
5	110	51095	100	42148

Table 27: Precision studies for R and S Pioglitazone enantiomers by LC-MS

S. No	R – Pioglitazone			S - Pioglitazone		
	50 ng/ml	80 ng/ml	110 ng/ml	40 ng/ml	70 ng/ml	100 ng/ml
1	22570	36345	51095	18470	30558	42148
2	22579	36451	51221	18532	30467	42827
3	22586	36421	51206	18907	30265	42875
4	22570	36458	51152	18054	30985	42724
5	22675	36403	51201	18891	30975	42798
6	22695	36442	51246	18437	30487	42840
Mean	22612.5	36420	51186.83	18548.5	30622.83	42702
SD	56.83	41.96	54.55	319.13	293.31	276.12
% RSD	0.251	0.115	0.106	1.720	0.957	0.646

Table 28: Results of analysis of drug products and recovery studies for R and S Pioglitazone enantiomers by LC-MS

Sample	Label claim (mg)	Amount present (mg/Tablet) ± % RSD*			% Label Claim			% Recovery ± % RSD*
		R&S Pioglitazone	R Pioglitazone	S Pioglitazone	R&S Pioglitazone	R Pioglitazone	S Pioglitazone	
Sample I	15	15.03±0.2804	8.25±0.4909	6.77±0.7895	100.25±0.5969	55.06±0.1543	45.19±0.8146	101.12±0.5601

* n=3

Sample I: Sample I: Pepar tablets containing 15 mg of Pioglitazone (RS)

Table 29: System suitability studies for estimation of R and S Pioglitazone enantiomers by LC-MS

S. No	Parameters	R - Pioglitazone	S - Pioglitazone
1	Linearity range	50-110 ng/ml	40 - 100 ng/ml
2	Regression equation Y = mx + c	y = 471.2x - 1145	y = 396.2x + 2580
3	Correlation coefficient	0.999	0.999
4	Resolution factor	**1.8**	
5	Asymmetric factor	1.02	1.01
6	LOD (ng/ml)	0.139	0.139
7	LOQ (ng/ml)	0.421	0.421

Table 30: Linearity and range for S and R Zaltoprofen enantiomers by HPLC

S. No	S – Zaltoprofen		R - Zaltoprofen	
	Concentration µg/ml	Peak area	Concentration µg/ml	Peak area
1	2	51663	3	63143
2	2.5	61995	3.5	75772
3	3	73475	4	87253
4	3.5	80364	4.5	103325
5	4	91844	5	114806

Table 31: Precision studies for S and R Zaltoprofen enantiomers by HPLC

S. No	S – Zaltoprofen			R - Zaltoprofen		
	2 µg/ml	3 µg/ml	4 µg/ml	3 µg/ml	4 µg/ml	5 µg/ml
1	51663	73475	91844	63143	87253	114806
2	52889	75603	93567	64786	87989	110098
3	51999	73995	92456	64245	88695	110432
4	52678	74887	92994	64001	87943	113765
5	53998	75213	93989	64390	87004	114985
6	52654	74978	92054	65987	89908	111987
Mean	52646.83	74691.83	92817.33	64425.33	88132	112678.83
SD	809.06	798.50	851.66	940.96	1055.68	2154.55
% RSD	1.536	1.069	0.917	1.461	1.197	1.912

Table 32: Results of analysis of drug products and recovery studies for S and R Zaltoprofen enantiomers by HPLC

Sample	Label claim (mg)	Amount present (mg/Tablet) ± % RSD*			% Label Claim			% Recovery ± % RSD*
		R&S Zaltoprofen	S Zaltoprofen	R Zaltoprofen	R&S Zaltoprofen	S Zaltoprofen	R Zaltoprofen	
Sample I	80	79.24±0.5397	35.56±0.3452	43.67±0.7145	100.46±0.6747	45.08±0.2127	55.37±0.5342	98.04±0.4478

* n=3

Sample I: Zaltoken Tablets containing 80 mg of Zaltoprofen (RS)

Table 33: Stress degradation studies of Zaltoprofen RS (Acidic degradation)

S. No	Time (hrs)	Mean peak area (S)	Mean peak area (R)	% drug present (S)	% drug present (R)	% degradation (S Zaltoprofen)	% degradation (R Zaltoprofen)
1N HCl							
1	0	322345	394567	45	55	0	0
2	2	0	0	0	0	55	45
0.1N HCl							
3	0	320483	399807	45	55	0	0
4	2	318251	399625	44.68	54.97	0.31	0.025
5	4	308090	397220	43.25	54.64	1.74	0.35
6	6	290523	378338	40.79	52.04	4.20	2.95
7	8	276543	376573	38.83	51.80	6.16	3.19
8	12	270021	342345	37.91	47.09	7.08	7.90
9	24	265223	338338	37.24	46.54	7.75	8.45

Table 34: Stress degradation studies of zaltoprofen RS (Basic degradation)

S. No	Time (hrs)	Mean peak area (S)	Mean peak area (R)	% drug present (S)	% drug present (R)	% degradation (S Zaltoprofen)	% degradation (R Zaltoprofen)
1N NaOH							
1	0	197865	270234	45	55	0	0
2	2	15432	19961	3.51	4.06	41.49	50.94
0.1N NaOH							
3	0	198754	249786	45	55	0	0
4	2	187864	249612	42.53	54.96	2.46	0.038
5	4	164567	230076	37.25	50.66	7.74	4.33
6	6	163210	228765	36.95	50.37	8.047	4.62
7	8	161498	205432	36.56	45.23	8.43	9.76
8	12	140023	198965	31.70	43.81	13.29	11.19
9	24	134562	187609	30.46	41.31	14.5352	13.69

Table 35 : Stress degradation studies of Zaltoprofen RS (Neutral degradation)

S. No	Time (hrs)	Mean Peak area (S)	Mean Peak area (R)	% drug present (S)	% drug present (R)	% degradation (S Zaltoprofen)	% degradation (R Zaltoprofen)
Water							
1	0	100897	128715	45	55	0	0
2	2	100234	121229	44.70	51.80	0.295	3.20
3	4	100164	121072	44.67	51.73	0.325	3.26
4	6	99876	120054	44.54	51.29	0.455	3.70
5	8	99436	119876	44.34	51.22	0.651	3.78
6	12	98056	117476	43.73	53.45	1.27	1.54
7	24	97549	114329	43.50	48.85	1.49	6.14

Table 36: Stress degradation studies of Zaltoprofen RS (Oxidative degradation)

S. No	Time (hrs)	Mean Peak area (S)	Mean Peak area (R)	% drug present (S)	% drug present (R)	% degradation (S Zaltoprofen)	% degradation (R Zaltoprofen)
30% H_2O_2							
1	0	549678	674932	45	55	0	0
2	2	45385	16543	3.72	1.35	41.28	53.65
3% H_2O_2							
1	0	549160	672362	45	55	0	0
2	2	503476	71897	41.25	5.88	3.74	49.12
3	4	485432	70453	39.77	5.76	5.22	49.23
4	6	204583	69764	16.76	5.71	28.23	49.29
5	8	158765	67531	13.01	5.52	31.99	49.47
6	12	145732	63659	11.94	5.21	33.058	49.79
7	24	108435	61916	8.88	5.06	36.11	49.93

Table 37: Stress degradation studies of Zaltoprofen RS (Photo degradation)

S. No	Time (hrs)	Mean Peak area (S)	Mean Peak area (R)	% drug present (S)	% drug present (R)	% degradation (S Zaltoprofen)	% degradation (R Zaltoprofen)
Photolytic degradation exposed to sunlight for 24 hrs							
1	0	606628	741434	45	55	0	0
2	Powder form	603797	735772	44.79	54.58	0.21	0.42
3	Solution form	593416	719730	44.02	53.39	0.98	1.61

Table 38: System suitability studies for estimation of S and R Zaltoprofen enantiomers by HPLC

S. No.	Parameters	S - Zaltoprofen	R - Zaltoprofen
1	Linearity range	2 - 4 µg/ml	3- 5 µg/ml
2	Regression equation Y = mx + c	y = 19746x + 12630	Y = 26176X - 15843
3	Correlation coefficient	0.995	0.997
4	Theoretical plate/meter	56411	54089
5	Resolution factor	1.85	
6	Asymmetric factor	1.02	1.01
7	LOD (ng/ml)	4.16	4.16
8	LOQ (ng/ml)	12.61	12.61

Table 39: Linearity and range for S and R Zaltoprofen enantiomers by LC-MS

S. No	S – Zaltoprofen		R – Zaltoprofen	
	Concentration ng/ml	Peak area	Concentration ng/ml	Peak area
1	18	26531	22	32425
2	27	39784	33	48673
3	36	52674	44	64775
4	45	66401	55	81124
5	54	79572	66	96425

Table 40: Precision studies for S and R Zaltoprofen enantiomers by LC-MS

S. No	S – Zaltoprofen			R - Zaltoprofen		
	18 ng/ml	36 ng/ml	54 ng/ml	22 ng/ml	44 ng/ml	66 ng/ml
1	26531	52647	69572	32425	64775	96425
2	26489	52603	69401	32586	64989	95987
3	26499	52535	69856	32585	64995	95892
4	26488	52457	69494	32591	63943	95908
5	26432	52413	69889	32590	63974	95985
6	26410	52478	69834	32587	64908	95887
Mean	26474.83	52522.16	69674.33	32560.66	64597.33	96014
SD	45.05	89.88	210.84	66.50	501.26	206.27
% RSD	0.170	0.171	0.303	0.204	0.775	0.218

Table 41: Results of analysis of drug products and recovery studies for S and R Zaltoprofen enantiomers by LC-MS

Sample	Label claim (mg)	Amount present (mg/Tablet) ± % RSD*			% Label Claim			% Recovery ± % RSD*
		R&S Zaltoprofen	S Zaltoprofen	R Zaltoprofen	R&S Zaltoprofen	S Zaltoprofen	R Zaltoprofen	
Sample I	80	80.20±0.4253	35.99±0.8783	44.20±0.9212	101.67±0.2735	45.63±0.7758	56.04±0.9591	99.54±0.6083

* n=3

Sample I: Zaltokin Tablets containing 80 mg of Zaltoprofen (RS)

Table 42: System suitability studies for estimation of S and R Zaltoprofen enantiomers by LC-MS

S. No	Parameters	S - Zaltoprofen	R - Zaltoprofen
1	Linearity range	18 - 54 ng/ml	22 - 66 ng/ml
2	Regression equation Y = mx + c	Y= 1474X – 87.2	Y = 1458X + 504
3	Correlation coefficient	0.999	0.999
4	Resolution factor	1.8	
5	Asymmetric factor	1.02	1.01
6	LOD (ng/ml)	0.016	0.016
7	LOQ (ng/ml)	0.051	0.051

Table 43: Linearity and range for R and S Valganciclovir hydrochloride enantiomers by HPLC

S. No	R-Valganciclovir Hydrochloride		S-Valganciclovir Hydrochloride	
	Concentration µg/ml	Peak area	Concentration µg/ml	Peak area
1	10	433553	8	346843
2	15	650330	12	520264
3	20	867106	16	693685
4	25	1083883	20	867106
5	30	1300660	24	1040528

Table 44: Precision studies for R and S Valganciclovir hydrochloride enantiomers by HPLC

S. No	R-Valganciclovir Hydrochloride			S-Valganciclovir Hydrochloride		
	10 µg/ml	20 µg/ml	30 µg/ml	8 µg/ml	16 µg/ml	24 µg/ml
1	433553	867106	1300660	346843	693685	1040528
2	434897	870011	1310098	345990	694532	1051020
3	429987	869021	1309877	339987	689975	1041237
4	437654	858769	1310123	345678	679999	1050768
5	429679	865432	1281122	339870	698987	1041235
6	439876	866534	1292233	345678	689975	1040211
Mean	434274.33	866145.5	1300685.5	344007.66	691192.16	1044166.5
SD	4079.39	3980.09	11969.30	3188.56	6424.61	5227.05
% RSD	0.939	0.459	0.920	0.926	0.929	0.500

Table 45: Results of analysis of R and S Valganciclovir hydrochloride enantiomers and recovery studies for R and S Valganciclovir hydrochloride enantiomers by HPLC

Sample	Label claim (mg)	Amount present (mg/Tablet) ± % RSD*			% Label Claim			% Recovery ± % RSD*
		R&S Valganciclovir Hydrochloride	R Valganciclovir Hydrochloride	S Valganciclovir Hydrochloride	R&S Valganciclovir Hydrochloride	R Valganciclovir hydrochloride	S Valganciclovir hydrochloride	
Sample I	450	453.27±0.1488	242.25±0.6692	232.19±0.4375	100.72±0.8231	53.83±0.4114	46.88±0.5643	101.38±0.7541

* n=3

Sample I: Valcyte tablets containing 450 mg of Valganciclovir hydrochloride (RS)

Table 46: Stress degradation studies of Valganciclovir hydrochloride RS (Acidic degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Valganciclovir Hydrochloride)	% degradation (S Valganciclovir Hydrochloride)
0.1 N HCl							
1	0	2316597	2056732	55	45	0	0
2	2	2158439	1738907	51.24	38.02	3.76	6.98
1 N HCl							
1	0	2323033	2099658	55	45	0	0
2	2	2316228	1924878	54.84	41.25	0.16	3.74
3	4	2313429	1918473	54.77	41.15	0.23	3.88
4	6	218060	1890851	5.16	40.52	49.84	4.47
5	8	2141370	1853221	50.69	39.72	4.30	5.28
6	12	1794454	1522318	42.48	32.63	12.51	12.37
7	24	1790850	1422719	42.40	30.49	12.59	14.51

Table 47: Stress degradation studies of Valganciclovir hydrochloride RS (Basic degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Valganciclovir Hydrochloride)	% degradation (S Valganciclovir Hydrochloride)
1 N NaOH							
1	0	3897654	3298765	55	45	0	0
2	2	3768493	3074831	53.17	43.50	1.83	1.5
0.1 N NaOH							
1	0	3908862	3379978	55	45	0	0
2	2	3668749	3279978	51.62	43.66	3.37	1.33
3	4	3461955	3185877	48.71	42.41	6.28	2.58
4	6	3450848	3200686	48.55	42.61	6.44	2.38
5	8	2509435	2391494	35.31	31.83	19.69	13.16
6	12	101830	129088	1.43	1.718	53.56	43.28
7	24	9346	9760	0.22	0.12	54.77	44.87

Table 48: Stress degradation studies of Valganciclovir hydrochloride (Neutral degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Valganciclovir Hydrochloride)	% degradation (S Valganciclovir Hydrochloride)
Water							
1	0	2333858	1909520	55	45	0	0
2	2	2059735	1803435	48.54	42.50	6.46	2.5
3	4	1996509	1766093	47.05	41.62	7.95	3.38
4	6	1983779	1722811	46.75	40.60	8.35	4.4
5	8	1975292	1729176	46.55	40.75	8.45	4.25
6	12	1954499	1713900	46.06	40.39	8.94	4.61
7	24	1906549	1648128	44.93	38.84	10.07	6.16

Table 49: Stress degradation studies of Valganciclovir hydrochloride RS (Oxidative degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Valganciclovir Hydrochloride)	% degradation (S Valganciclovir Hydrochloride)
30 % H_2O_2							
1	0	2574268	2199856	55	45	0	0
2	2	2398754	1975874	51.25	40.41	3.74	4.81
3 % H_2O_2							
1	0	2482969	2031520	55	45	0	0
2	2	2249569	1988180	49.83	44.04	5.17	0.96
3	4	2249575	1988185	49.82	44.03	5.18	0.97
4	6	1612575	1419806	35.72	31.45	19.28	13.55
5	8	1596323	1406263	35.36	31.15	19.64	13.85
6	12	1574653	1371501	34.88	30.38	20.12	14.62
7	24	1190922	1366084	34.37	30.26	20.63	14.74

Table 50: Stress degradation studies of Valganciclovir hydrochloride RS (Photo degradation)

S. No	Time (hrs)	Mean Peak area (R)	Mean Peak area (S)	% drug present (R)	% drug present (S)	% degradation (R Valganciclovir Hydrochloride)	% degradation (S Valganciclovir Hydrochloride)
Photolytic degradation exposed to sunlight for 24 hrs							
1	0	2646168	2090775	55	45	0	0
2	Powder form	2470367	2015042	51.35	43.37	3.65	1.62
3	Solution form	2135378	1691669	44.38	36.41	10.62	8.59

Table 51: System suitability studies for estimation of R and S Valganciclovir hydrochloride enantiomers by HPLC

S. No.	Parameters	R- Valganciclovir hydrochloride	S- Valganciclovir hydrochloride
1	Linearity range	10 – 30 µg/ml	8 - 24 µg/ml
2	Regression equation Y = mx + c	Y= 43755X – 8000	Y = 44355X - 14000
3	Correlation coefficient	0.999	0.996
4	Theoretical plate/meter	14589	14553
5	Resolution factor	2.02	
6	Asymmetric factor	1.02	0.99
7	LOD (µg/ml)	0.417	0.417
8	LOQ (µg/ml)	1.264	1.264

Table 52: Linearity and range for R and S Valganciclovir hydrochloride enantiomers by LC-MS

S. No	R- Valganciclovir hydrochloride		S- Valganciclovir hydrochloride	
	Concentration ng/ml	Peak area	Concentration ng/ml	Peak area
1	150	37286	120	30526
2	200	38408	160	31333
3	250	39604	200	32494
4	300	40380	240	33720
5	350	41196	280	34978

Table 53: Precision studies for R and S Valganciclovir hydrochloride enantiomers by LC-MS

S. No	R- Valganciclovir hydrochloride			S- Valganciclovir hydrochloride		
	150 ng/ml	250 ng/ml	350 ng/ml	120 ng/ml	200 ng/ml	280 ng/ml
1	37286	39604	41196	30526	32494	34978
2	37397	39611	41198	30541	32503	34999
3	37387	39784	41177	30543	32406	35082
4	37294	39501	41203	30238	32511	34979
5	37489	39609	41222	30339	32697	35194
6	37389	39613	41233	30437	32698	34983
Mean	37373.66	39620.33	41204.83	30437.33	32551.5	35035.83
SD	75.22	91.17	19.95	125.84	119.25	87.03
% RSD	0.201	0.230	0.0484	0.413	0.366	0.248

Table 54: Results of analysis of drug products and recovery studies for R and S Valganciclovir hydrochloride enantiomers by LC-MS

Sample	Label claim (mg)	Amount present (mg/Tablet) ± % RSD*			% Label Claim			% Recovery ± % RSD*
		R&S Valganciclovir hydrochloride	R Valganciclovir hydrochloride	S Valganciclovir hydrochloride	R&S Valganciclovir hydrochloride	R Valganciclovir hydrochloride	S Valganciclovir hydrochloride	
Sample I	450	452.33±0.5658	240.93±0.7479	232.61±0.855	100.51±0.2403	53.54±0.1064	46.97±0.4172	100.43±0.5208

* n=3

Sample I: Valcyte Tablets containing 450 mg of Valganciclovir hydrochloride (RS)

Table 55: System suitability studies for estimation of R and S Valganciclovir hydrochloride enantiomers by LC-MS

S. No	Parameters	R- Valganciclovir hydrochloride	S- Valganciclovir hydrochloride
1	Linearity range	150 - 350 ng/ml	120 - 280 ng/ml
2	Regression equation Y = mx + c	Y= 19.38X + 34479	Y = 28.22X + 26965
3	Correlation coefficient	0.990	0.993
4	Resolution factor	1.8	
5	Asymmetric factor	1.02	1.01
6	LOD (ng/ml)	0.041	0.041
7	LOQ (ng/ml)	0.125	0.125

8. DISCUSSION

For the development of direct and indirect chiral HPLC and LC-MS methods, preparation of standard and sample solutions required less time and no tedious extraction procedures involved. Run time required for recording chromatograms of the developed HPLC and LC-MS methods were less than 15 and 10 minutes respectively. This demonstrates that the developed methods are rapid and accurate. The degradation behaviour of Rosiglitazone, Pioglitazone, Zaltoprofen and Valganciclovir Hydrochloride and were investigated under different stress conditions recommended by International Conference on Harmonization (ICH). The methods developed in the present study are therefore suitable for the quality control of raw materials, formulations and dissolution studies.

9. SUMMARY AND CONCLUSION

This thesis deals with the studies carried out by the writer in the laboratory for the past four years on the development and validation of chiral separation of some enantiomeric drugs by HPLC and LC-MS methods.

Thesis begins with a brief introduction of the reasons for analysing enantiomeric drugs and introduction to the analytical methods used like HPLC and LC-MS. The methods used for the quantitative analysis of enantiomers and the introduction to the chiral separation are given.

Thesis deals with the aim and objective of the present work, the reasons for analysing the enantiomers and need for newer analytical methods for the estimation of enantiomers in pharmaceutical formulation are briefly discussed.

Thesis deals with the reviews of literature on the analytical methods available for the estimation of the drug candidates in pharmaceutical formulations are explained.

Thesis deals with the materials and instruments used in the experimental procedures adopted. It describes in detail the procedure adopted for the optimisation of the chromatographic conditions for the chiral separation of drugs by HPLC and LC-MS methods and for the estimation of enantiomers in the selected drug candidates are presented and discussed. Forced degradation studies on the selected racemic drugs using acid, alkaline, neutral, oxidative and photolytic degradations and quantification of enantiomers by HPLC in different stress conditions are presented and discussed.

The results obtained are presented and discussed. HPLC and LC-MS chromatograms of the standard and sample solutions, calibration curves for the selected analytes are also presented. The results of the experiments carried out to check the accuracy, reproducibility of the methods carried out are presented and discussed in detail. System suitability studies and forced degradation studies carried out for various methods developed are also presented and discussed.

The following are some of the salient features and conclusions made for the present study.

- Four racemic drugs namely Rosiglitazone, Pioglitazone, Zaltoprofen and Valganciclovir Hydrochloride for which there were no HPLC and LC-MS methods reported for the estimation of enantiomers in pharmaceutical formulations and their stability in different stress conditions were selected for the present study after thorough literature survey.
- The chromatographic conditions like detection wavelength/mass range, nature and composition of mobile phase, nature of stationary phase, peak modifiers, flow rate etc were optimised for the best possible separation and quantification of the analytes. Forced degradation studies on the selected racemic drugs using acid, alkaline, neutral, oxidative and photolytic degradations were performed.
- The developed HPLC and LC-MS methods were validated for their transferability to other laboratories, in terms of specificity, selectivity, accuracy, precision, linearity and range, detection and quantification limits, ruggedness, robustness and system suitability. The validation studies carried out revealed that the developed methods satisfy the ideal characteristics of the analytical methods.
- The direct and indirect chiral HPLC and LC-MS methods developed in the present study for the estimation were found to be simple, rapid, accurate, precise, specific, linear and rugged. They are thus suitable for the estimation of enantiomers in raw materials and formulations. The newly developed analytical methods can be used in the following fields:

    - Research institutions,
    - Academic institutes,
    - Quality control department in industries,
    - Approved testing laboratories,
    - Biopharmaceutics & bioequivalence studies and
    - Clinical & pharmacokinetic studies after suitable modification.

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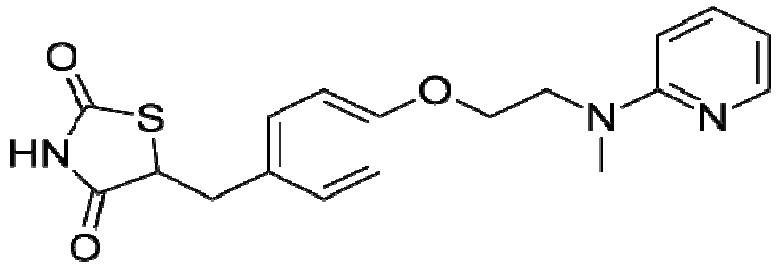
DRUG PROFILES

1. ROSIGLITAZONE

Rosiglitazone (RS)-5-[4-(2-[methyl (pyridin-2-yl) amino] ethoxy) benzyl] thiazolidine-2,4-dione is official in Martindale Extra Pharmacopoeia.

The structural formula of Rosiglitazone is



Rosiglitazone is an oral hypoglycemic agent used in Type II diabetes. It targets insulin resistance. It is highly selective and potent against for the peroxisome proliferators activated receptor gamma (PPAR gamma).The activation of PPAR-γ nuclear receptor receptors is said to regulate the transcription of insulin responsive genes, involved in the control of glucose production, transport and utilization. The usual dosage available is 2 mg, 4 mg and 8 mg tablets.

Molecular formula : $C_{18}H_{19}N_3O_3S$

Molecular weight : 357.43

Properties : Soluble in methanol, DMF and acetonitrile

Melting point : 125-128 ^oC

pKa **:** 6.1 and 6.8

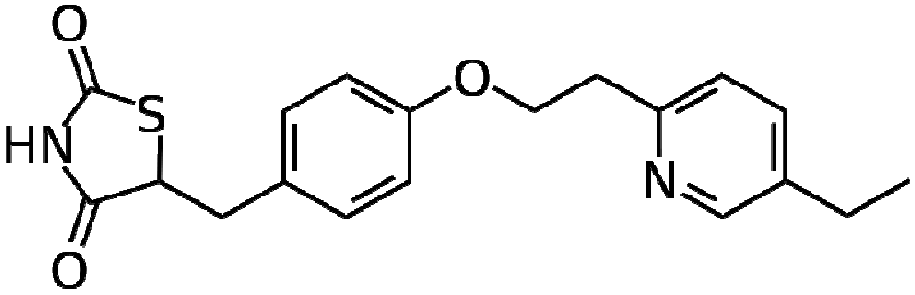
Therapeutic category : Antidiabetic

Dosage and administration: Rosiglitazone may be administered at a starting dose of 4 mg either as a single daily dose or in 2 divided doses. For patients who respond inadequately following 8 to 12 weeks of treatment, as determined by reduction in fasting plasma glucose (FPG), the dose may be increased to 8 mg daily.

2. PIOGLITAZONE

Pioglitazone (RS)-5-({4-[2-(5-ethylpyridin-2-yl) ethoxy] phenyl} methyl)-1,3-thiazolidine-2,4-dione is official in Martindale Extra Pharmacopoeia.

The structural formula of Pioglitazone is



Pioglitazone is used for the treatment of diabetes mellitus type 2. Pioglitazone selectively stimulates nuclear receptor peroxisone proliferator-activated receptor gamma (PPAR-gamma). It modulates the transcription of the insulin-sensitive genes involved in the control of glucose and lipid metabolism in the lipidic, muscular tissues and in the liver.

Molecular formula : $C_{19}H_{20}N_2O_3S$

Molecular weight : 356.44

Properties : Soluble in methanol, DMF and acetonitrile

Melting point : 125-128 ^oC

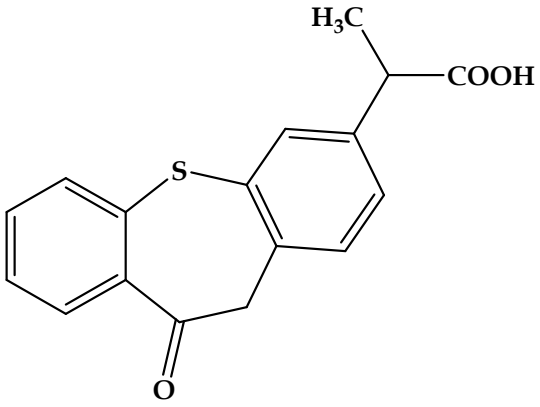
pKa **:** 5.2 and 6.8

Therapeutic category : Antidiabetic

Dosage and administration: Pioglitazone is supplied in oral tablets containing 15, 30 or 45 mg of pioglitazone base. 15-30 mg once daily increased in increments if necessary. Max dose: 45 mg/day. It may be taken with or without food.

3. ZALTOPROFEN

Zaltoprofen is (±)-10,11-Dihydro-α-methyl-10-oxodibenzo[b,f]thiepin-2-acetic acid.



Molecular Formula : $C_{17}H_{14}O_3S$

Molecular Weight : 298.35

Properties **:** Less soluble in water freely soluble in organic solvents like methanol, acetone etc.

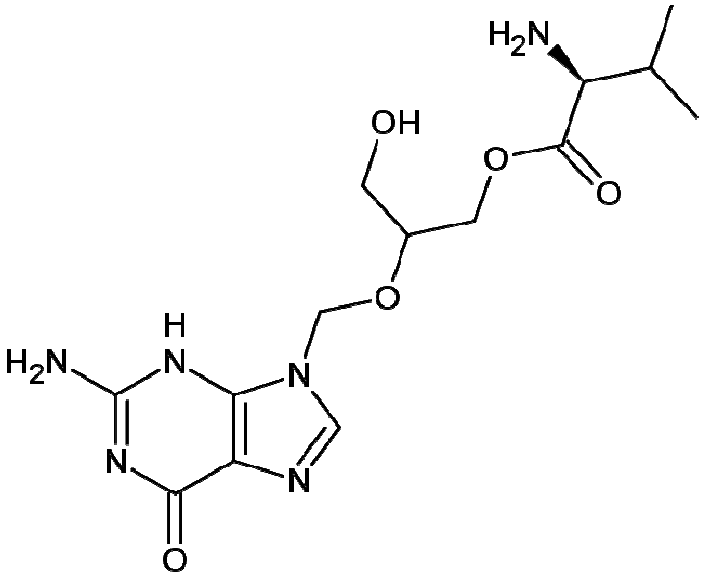
Melting point : 130 ± 0.8 ^{0}C

Therapeutic category : Analgesic

Dosage and administration: Adults: One tablet (80 mg) three times a day to be taken orally.

4. VALGANCICLOVIR HYDROCHLORIDE

Valganciclovir hydrochloride is 2-[(2-amino-6-oxo-6,9-dihydro-3H-purin-9-yl) methoxy]-3-hydroxy propyl (2S)-2-amino-3-methyl butanoate. Valganciclovir HCl falls under the category of anti-retroviral drug. Its structure is



Molecular formula : $C_{14}H_{22}N_6O_5$

Molecular weight : 354.36

Properties **:** Soluble in Water, Methanol

Melting point : 85-92℃

pKa : 7.6

Therapeutic category: Anti HIV agent

Dose : 900 mg twice daily for first three weeks (HIV patients)

LIST OF PAPERS PUBLISHED

1. B. Gowramma, B. Babu, S. N. Meyyanathan and K. Elango. New chiral normal phase HPLC method for determination of pioglitazone enantiomers on its marketed formulation. J Pharmacy Research. 2012; 5(1): 72-74.
2. B. Gowramma, B. Babu, S. N. Meyyanathan and K. Elango. Chiral HPLC method for the enantioselective analysis of rosiglitazone maleate in formulations. J Pharmacy Research. 2012; 5(1): 79-81.