In the Name of Allah, the Almighty

Quality Assessment of Some Commonly Used Enantiomeric Drugs in Bangladesh

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Dedicated to My Parents

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Abstract

This thesis describes rapid, accurate, precise, less time consuming analytical methods with excellent resolution for separation and determination of chiral drugs. By performing significant number of trial and error methods using a large number of polar and non-polar solvent mixtures as mobile phase, new and suitable chiral HPLC methods were developed for the separation of common enantiomeric drugs such as omeprazole, esomeprazole, rabeprazole, pantoprazole, salbutamol, levosalbutamol, ibuprofen, dexibuprofen, ofloxacin, levofloxacin, citalopram, *S*-citalopram, carvedilol and propranolol which are commonly prescribed in pharmaceutical formulations in Bangladesh. The chiral separation was achieved on different chiral columns, such as, Chiralcel OD-H, Chiralpak AGP, Chiralcel IC, Lux cellulose-3 and Chiral CD-PH (250 x 4.6 mm, 5 µm particle size, Daicel Chemical Industries Ltd., Tokyo, Japan) for various chiral drugs. This thesis presents suitable chiral HPLC methods for the respective drugs which have been validated according to the guidelines of the United States of Pharmacopeia (USP) and International Conference on Harmonization (ICH). For all enantiomeric drugs, the calibration curve showed good linearity with coefficient of determination (r^2) values of ≤0.995. The percentage recovery for all drugs was found to be within the limit (97%-103%) and also the percentage of relative standard deviation (%RSD) of repeatability and intermediate precision was within the acceptable limit (%RSD ≤2). It showed that all the proposed methods met the system suitability criteria with resolution (> 2) though it is very difficult to separate enantiomers having similar physical and chemical properties. The limit of detection (LOD) and the limit of quantitation (LOQ) were also evaluated. Others parameters such as, capacity factor and selectivity were also determined for each method. Finally, all methods have been applied for quantitative determination of enantiomers of the respective drugs and calculated percentage purity of enantiomeric drugs.

The current research presents the enantiomeric quantitation and purity profiles of the following enantiomeric formulations of different manufacturers of Bangladesh:

I. Omeprazole of twenty-six pharmaceutical companies and *S*-omeprazole (esomeprazole) of twenty-six companies,
II. Rabeprazole of sixteen companies,
III. Pantoprazole of twenty companies,

IV. Salbutamol of ten companies and levosalbutamol of twelve companies,
V. Ibuprofen of twelve companies and dexibuprofen of twelve companies,
VI. Citalopram of two companies and *S*-citalopram of nine companies,
VII. Ofloxacin of three companies and levofloxacin of nine companies,
VIII. Carvedilol of five companies, and
IX. Propranolol of six companies.

Thus, all the proposed chiral HPLC methods can be used for routine analysis of the respective drugs for the enantiomeric determination in bulk as well as in pharmaceutical formulations of Bangladesh by simultaneous quantification of (*S*)- and (*R*) enantiomers.

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LIST OF ABBREVIATION

Abbreviation	Elaboration
HPLC……	High Performance Liquid Chromatography
MeOH……...	Methanol
H_2O……..	Water
ACN…….	Acetonitrile
NaOAc…….	Sodium acetate
NH_4OAc……	Ammonium acetate
IPA……	Isopropylalcohol
EtOH…….	Ethanol
Hx…….	*n*-Hexane
Hp…….	Heptane
$NH_4H_2PO_4$……	Ammonium dihydrogen phosphate
$Na_2H_2PO_4$…….	Sodium dihydrogen phosphate
DEA……..	Diethylamin
TEA……...	Triethylamin
AA…….	Acetic acid
MTBE……	Methyl *tert* butyl ether
EP……..	Enantiomeric purity

Chapter 1: Introduction

Chapter 1 INTRODUCTION

1.1. Overview

A chiral molecule is a molecule having at least one asymmetric carbon. Carbon is not the only atom that can act as an asymmetric center. Sulfur, phosphorus and nitrogen can sometimes form chiral molecules such as omeprazole, cyclophosphamide and methaqualone. Chiral molecules exhibit optical activity, so enantiomers are also sometimes called optical isomers. A large number, however, are still marketed as racemic mixtures. Only about one-third drugs are administered as pure enantiomers. The enantiomeric forms of a drug can differ markedly in potency, toxicity, and behavior in biological systems. In the early 1980s analytical chiral separation was a rather difficult task, and preparative synthetic and separation methods were not as advanced as today. Nevertheless, it was clear that chiral drugs should be enantioseparated and that each enantiomer should be used separately. Now-a-days, 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 (Vermeulen and Koppele, 1993). In racemate, there must be presence equal portions of (50/50) of *S* and *R* enantiomers in chiral molecule (Davankov, 1997). This mixture is optically inactive due to the rotation of one molecule exactly cancelling the opposite rotation of its enantiomer. Enantiomers are chiral molecules that are mirror images but non-superimposable to one another (Boyle, 2005). In single enantiomer, only one enantiomer is present in a sample.

1.2. Basic concepts of chirality

Chirality (also sometimes called stereoisomerism or dissymmetry) is a property of an object which is non-superimposable with its mirror image. The word chiral is derived from Greek word '*cheir'*, which means 'handedness'. When a molecule cannot be superimposed on its mirror image, this molecule and its image are called chiral. It is like a pair of hands which are otherwise appear identical but in fact are non-superimosible on each other as demonstrated in figure 1.1 (Nguyen *et al.*, 2006). A chiral molecule contains at least one chiral center or asymmetric center, which is a central carbon atom to which four different atoms (or group of atoms) are attached. Carbon is not the only atom that can act as an asymmetric center. Sulfur,

phosphorus and nitrogen can sometimes form chiral molecules such as omeprazole, cyclophosphamide and methaqualone. (McConathy and Owens, 2003).

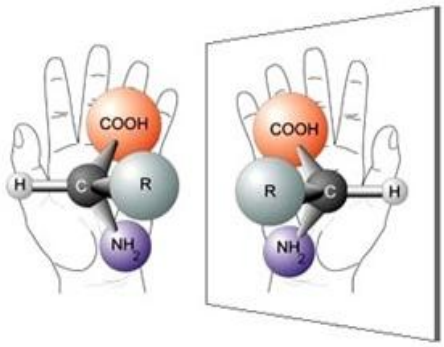


Figure 1.1: Non-superimposible mirror images (enantiomers) of a chiral molecule.

1.3. Basic terminology of stereochemistry

Isomers

Compounds that have the same molecular formula but differ in the way the constituent atoms are linked together.

Stereoisomers

Compounds having the same molecular formula but with the atoms in a different three-dimensional arrangement. Stereoisomers can be divided into two distinct categories, enantiomers and diastereomers.

Enantiomers

Compounds that contain the same atoms linked together in the same way but in a different three-dimensional arrangement. Enantiomers have identical physical properties, but rotate the plane of polarised light in opposite directions.

Diastereoisomers

Diastereomers are a type of a stereoisomer. Diasteoreomers are defined as non-mirror image non-identical stereoisomers. Hence, they occur when two or more stereoisomers of a compound have different configurations at one or more (but not all) of the equivalent (related) stereocenters and are not mirror images of each other.

Achiral

An entity, such as a molecule, is achiral if it is superposable with its mirror image.

Chiral

This is not superposable with its mirror image, as applied to molecules, conformations, as well as macroscopic objects, such as crystals.

Homochiral

Isometric molecules are homochiral if they have the same sense of chirality, that is, if they are all *R* or all *S*.

Chiral centre

Atoms, usually carbon, attached to four different substitutions that could be swapped to create a new stereoisomer.

Racemate

A mixture of all possible stereoisomers of a compound in equal proportions. It does not have optical activity.

Stereoselective

It relates primarily to one specific stereoisomer. A biological reaction is stereospecific if either the substrate or its binding is chiral.

1.4. Nomenclature

Chiral molecules have the ability to rotate plane polarized light. When two enantiomers of a chiral compound rotate plane polarize light in right and left direction, they may be classified as dextrorotary (*d*-isomer) and levorotary (*l*-isomer) respectively. Racemic mixture is denoted with sign (±) or (*dl*) or with prefix *rac*. (Nguyen *et al.*, 2006). According to the Sequence Rule, (Cahn *et al.*, 1956) depending on ordering the priority to the substituents attached to the asymmetric atom, the configuration is indicated by use of prefixes *R* and *S*. If the counting from the highest atomic number or highest mass to the lowest one, goes in a clockwise direction, the configuration is designated as *R* (Latin: *Rectus* means right); otherwise if counting goes in a counter clockwise direction, the configuration is designated as *S* (Latin: *Sinister* means left).

1.5. Biological activity of chiral molecule

The body with its numerous homochiral compounds being amazingly chiral selector, will interact with each racemic drug differently and metabolize each enantiomer by a separate

pathway to generate different pharmacological activity. Thus, one isomer may produce the desired therapeutic activities, while the other may be inactive or, in worst cases, produce undesired or toxic effects. (Ariens, 1983; Drayer, 1986; Landoni and Soraci, 2001; Davies and Teng, 2003; Mehvar and Brocks, 2004; Patocka and Dvorak, 2004).

In pharmacology area, only racemic drugs will be examined and their activity can be divided into three main groups. The majority of racemic pharmaceuticals have one major bioactive enantiomer (called eutomer), the other is inactive or less active (distomer) or toxic or can exert other desired or undesired pharmacological properties. The second category is intended to drugs where the two enantiomers are equally active and have the same pharmacodynamics. The last one is racemic drugs having only one eutomer, but the distomer could be transformed in body into its bioactive antipode by chiral inversion (Ariens, 1983; Drayer, 1986; Davies and Teng, 2003; Waldeck, 2003).

1.5.1. Group 1. Racemic drugs with one major bioactive enantiomer

In this group, there are a number of cardiovascular drugs that are widely used for the treatment of hypertension, heart failure, arrhythmias, and other diseases. Among these are the β-adrenergic blocking agents, calcium channel antagonists and angiotensin-converting enzyme (ACE) inhibitors.

Levorotary isomer of all β-blockers is more potent in blocking β-adrenoceptors than their dextrorotary isomer, such as *S*-(-)-propranolol is 100 times more active than its *R* (+)-antipode (Barrett and Cullum, 1968; Rahn *et al.*, 1974; Stoschitzky, Zernig and Lindner, 1998). A number of β-blockers are still marketed in racemic form such as acebutolol, atenolol, alprenolol, betaxolol, carvedilol, metoprolol, labetalol, pindolol, sotalol, etc, except timolol and penbutolol, which are used as single *l*-isomer. However, it has been demonstrated that *d*, *l*- and *d*-propranolol can inhibit the conversion of thyroxin (T4) to triiodothyronin (T3), contrary to its *l*-form (Harrower *et al.*, 1977; Wiersinga and Touber, 1977; Stoschitzky *et al.*, 1998). Therefore, single *d*- propranolol might be used as a specific drug without β-blocking effects to reduce plasma concentrations of T3 particularly in patients suffering from hyperthyroidism in which racemic propranolol cannot be administered because of contraindications for β-blocking drugs (Stoschitzky *et al.*, 1992). It is to be noted that for a

racemic drug, each enantiomer possesses its own pharmacological activities that can be null, similar, different or opposite.

Many calcium channel antagonists are used under racemic form such as verapamil, nicardipine, nimodipine, nisoldipine, felodipine, mandipine etc, except diltiazem is a diastereoisomer with two pairs of enantiomers. For example, the pharmacological potency of *S*-(-)-verapamil is 10-20 times greater than its *R*-(+)- antipode in terms of negative chromotropic effect on AV conduction and vasodilatator in man and animals (Satoh *et al.*, 1980; Echizen *et al.*, 1988). On the other hand, verapamil has another possible application in cancer chemotherapy as a modifier of multidrug resistance. Unfortunately, for this purpose, verapamil must be used at high concentrations leading to high cardiotoxicity. However, it was later found that *R*- (+)-verapamil has far less cardiotoxicity than *S*-(-)-verapamil. Therefore, the *R*-enantiomer would be pref- erable as a modifier of multidrug resistance in cancer chemotherapy, while the *S*-enantiomer or the racemate would be preferable as a calcium channel blocker for cardiovascular therapy (*Industry Overview. An international symposium on chirality.*, 1999).

All ACE inhibitors such as captopril, benazepril, enalapril, idapril are chiral compounds under diastereoisomeric form and most of them are marketed as single isomer. Valsartan, an angiotensin II receptor antagonist, is used as a single *S*-enantiomer and the activity of the *R*-enantiomer is clearly lower than the *S*-enantiomer (Patocka and Dvorak, 2004). Albuterol (salbutamol), salmeterol and terbutaline are sympathomimetic drug selective β_2-adrenoceptor agonists mainly used as bronchodilators in the treatment of asthma. They are longtime marketed as racemate. Pharmacologically, only their *l*-isomer or *R*- (-)-isomer is effective and the other inactive *d*-or *S*- (+)-isomer may be responsible for the occasional unpleasant side- effects associated with the drug. The U.S. Food and Drug Administration recently approved a chiral switch drug, levalbuterol (the pure *l*-isomer of albuterol) as a preservative free nebulizer solution. However, some clinical studies recently reported that it is neither safer nor more effective than a same dose of racemic albuterol. In contrast, levalbuterol may cost as much as 5 times more than its racemate (Asmus and Hendeles, 2000; Nowak, 2003). In neurology and psychiatry, many pharmaceuticals used are chiral compounds and most of them are marketed as racemates. Hypnotics such as hexobarbital, secobarbital, mephobarbital, pentobarbital, thiopental, thiohexital are racemic compounds

and overall, only *l*-isomer is hypnotic or sedative, the other is either inactive or excitative. For example, *S*-(-)-secobarbital is more potent as anesthetics than *R*-(+)-secobarbital i.e. it causes a smoother more rapid anesthetic effect (Drayer, 1986; Harris and Ho, 1988). Ketamine is an intravenous anesthetic. The (+)-isomer is more potent and less toxic than it *S*-(-)-antipode, but unfortunately, ketamine is still used as racemic drug (Williams and Lee, 1990; Katzung, 2004). Isoflurane is an inhalational general anesthetic widely used in surgical operations as a racemic mixture of its two optical isomers. The (+) isomer of isoflurane is more effective than the (-) isomer at inhibiting currents induced by the bath application of acetylcholine. In the treatment of depression, *S*-(+)-citalopram is over 100-fold more potent as a selective serotonin reuptake inhibitor than *R*-(-)-enantiomer (Rentsch, 2002). Methadone, a central acting analgesic with high affinity for μ-opiod receptors, has been used to treat opiate dependence and cancer pain. Methadone is a chiral synthetic compound used in therapy under racemic mixture. In human, *R*-(-)-methadone is about fold more potent as an analgesic than its *S*-(+)- antipode (Olsen *et al.*, 1977; Mohler and Richards, 1983; Powell, Ambre and TI, 1988; Harris and Ho, 1988; Jamali, Mehvar *et al.*, 1989; Williams and Lee, 1990; Scott, 1993; Robin *et al.*, 1995; Eichelbaum, 1995; Pham-Huy *et al.*, 1997; Lin and Lu, 1997; Flaih *et al.*, 1999; Burke and Henderson, 2002; Marzo and Heftmann, 2002; Mehvar *et al.*, 2002; Rentsch, 2002; McConathy and Owens, 2003; Hutt and Valentova, 2003; Rajkumar, 2004; Somogyi *et al.*, 2004; He *et al.*, 2004; Katzung, 2004; Brocks and Mehvar, 2010; Zhang *et al.*, 2018).

The list of racemic drugs with one eutomer is long. It includes anticonvulsants such as mephenytoine, ethosuximide; antiarrhythmics and local anesthetics such as propafenone, disopyramide, prilocaine, tocainide; antibiotics such as ofloxacin, moxalactam; anticoagulants such as warfarine, acenocoumarol; antihistaminics such as terfenadine, loratadine; antihyperlipidemic such as atorvastatin; psychostimulants such as amphetamine, metamphetamine; proton pump inhibitors such as omeprazole, pantoprazole, lansoprazole, etc. (Olsen *et al.*, 1977; Rentsch, 2002; Waldeck, 2003). Some of these racemates recently undergo chiral switch to single enantiomer such as levofloxacin (from ofloxacin), levalbuterol (from albuterol), escitazolam (from citalopram), esomeprazole (from omeprazole), dexketoprophen (from ketoprophen), dexmethylphenidate (from methylphenidate), etc.

1.5.2. Group 2. Racemic drugs with equally bioactive enantiomers

There are only some racemic drugs that could belong to this group such as cyclophosphamide (antineoplastic), flecainide (antiarrhythmic), fluoxetine (antidepressant) (Davies and Teng, 2003).

1.5.3. Group 3. Racemic drugs with chiral inversion

There are two kinds of drug chiral inversion: unidirectional and bidirectional inversion. (Marzo and Heftmann, 2002). Unidirectional enzyme mediated inversion was previously described only with 2-arylpropionate nonsteroidal anti-inflammatory drugs (NSAID), namely ibuprofen, ketoprofen, fenprofen, benoxaprophen, etc. For this group, only *S*-enantiomer is active i.e. has an analgesic and anti-inflammatory effect. For example, *S*-ibuprofen is over 100-fold more potent as an inhibitor of cyclooxygenase I than (*R*)-ibuprofen. In the body, only inactive *R*-enantiomer can undergo chiral inversion by hepatic enzymes into the active *S*-enantiomer and not *vice-versa* (Landoni and Soraci, 2001; Marzo and Heftmann, 2002). Bidirectional chiral inversion or racemization should be represented by 3-hydroxy-benzodiazepines (oxazepam, lorazepam, temazepam) and thalidomide in which *R* and *S* enantiomer can racemize *in vitro* by aqueous solution. However, *in vivo* this phenomenon could occur with thalidomide, but not with hydroxyl-benzodiazepines because of the differences in substituents around their chiral carbon. Some authors (Pham-Huy *et al.*, 2002) have found for the first time the difference in *R*- and *S*-oxazepam concentrations in treated rabbit serum. They explained that the chiral inversion by tautomerization of oxazepam cannot occur *in vivo* because each enantiomer is transported by protein (albumin) with different affinity. The binding affinities of the enantiomers to albumin may inhibit the attack of hydroxyl ions (water) and thus retard the epimerization and racemization *in vivo.* Therefore, *R*- and *S*-oxazepam concentrations can be found different in the serum of these treated rabbits. On the other hand, (Marzo and Heftmann, 2002; He *et al.*, 2004) have also demonstrated that the *in vitro* chiral inversion of these benzodiazepine enantiomers was temperature-dependent and was inhibited by lowering temperature of aqueous solution to about 10°C (Pham-Huy *et al.*, 2002; He *et al.*, 2004). The *S*-(+)-oxazepam enantiomer is 100-200 fold more potent as a tranquilizer and

sedative than *R*-(-)-oxazepam ((Mohler and Richards, 1983). Thalidomide is a former racemic sedative withdrawn from the market in the 1960s due to severe teratogenic effects (phocomelia, amelia). However, there is renewed interest in restricted use of thalidomide because of its immunomodulatory (Pham-Huy *et al.*, 1997), anti-angiogenic, and anti-inflammatory effects (Davies and Teng, 2003). Moreover, it strongly inhibits the tumor necrosis factor α (TNF-α). Thalidomide gave spectacular results in the treatment of erythema nodosum leprosum, aptosis, Behcet's syndrome and has been assayed for organ transplantation, some autoimmune diseases such as chronic lupus erythema- tosus, rheumatoid arthritis, some forms of cancer, etc. (Pham-Huy *et al.*, 1997; Davies and Teng, 2003). Single thalidomide enantiomers and its derivative, N-hydroxythalidomide, were also synthetized by asymmetric technique in order to study their individual biological and chemical activities (Flaih *et al.*, 1999; Robin *et al.*, 1995). It seems that a multitude of its pharmacological activities could be due not only to the mother molecule but also to its numerous chiral and achiral metabolites. Because of this *in vivo* interconversion of thalidomide, it is difficult to determine exactly the pharmacological effect of each enantiomer.

The main pharmacological potency observed from two isomers of some current racemic drugs is gathered in the table 1.1.

Table 1.1: Comparison of isomer potency of some racemic drugs.

Main pharmacological effects of drugs	**Isomer potency***
ß-Adrenoceptor blocking drugs (ß-blockers): propranolol, acebutolol, atenolol, alprenolol, betaxolol, carvedilol, metoprolol, labetalol, pindolol, sotalol, etc.	*l* > *d* (*d* = inactive) Ex: *S*-(-)-propranolol > *R*-(+)-propranolol
Calcium channel antagonists: verapamil, nicadipine, nimodipine, nisoldipine, felodipine, mandipine etc.	*l* > *d*, Ex: *S*-(-)-verapamil > *R*-(+)-verapamil
ß-Adrenoceptor agonists: Brochodilators: Albuterol (salbutamol, salmeterol and terbutaline	*l* > *d* (*d* = inactive) Ex: *R*-(-)-Albuterol > *S*-(+)-Albuterol
Hynotics, sedatives: hexobarbital, secobarbital, mephobarbital, pentobarbital, thiopental, thiohexital	*l* > *d*, Ex: *S*-(-)secobarbital > *R*-(+)-secobarbital

Anesthetics: Ketamine, isoflurane	*d* > *l*, Ex: *S*- (+)-Ketamine > *R*- (-)-Ketamine *S* (+)-isoflurane >*R* (-)-isoflurane
Centralacting analgesic (μ-opiod receptors): Methadone	*d* > *l*, Ex: *R*-(-)-Methadone >*S*-(+)-Methadone
Analgesics, Anti-inflammatory: (NSAID): ibuprofen, ketoprofen, benoxaprophen, etc.	Ex: *S*-(+)-ibuprofen > *R*-(-)-ibuprofen
Tranquilizers: 3-hydroxy benzodiazepines: oxazepam, lorazepam, temazepam	*d* > *l*, Ex: *S*-(+)-oxazepam > *R*-(-)-oxazepam

1.6. Importance of chiral separation

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. The body being amazingly chiral selective, will interact with each racemic drug differently and metabolize each enantiomer by a separate pathway to produce different pharmacological activity. Thus, one isomer may produce the desired therapeutic activities, while the other may be inactive or, in worst cases, produce unwanted effects. Consider the tragic case of the racemic drug of n-phthalyl-glutamic acid imide that was marketed in the 1960's as the sedative Thalidomide. Its therapeutic acitivity resided exclusively in the *R*- (+)-enantiomer. It was discovered only after several hundred births of malformed infants that the *S*- (+)- enantiomer was teratogenic. The U.S. Food and Drug Administration, in 1992, issued a guideline that for chiral drugs only its therapeutically active isomer be brought to market, and that each enantiomer of the drug should be studied separately for its pharmacological and metabolic pathways. In addition, a rigorous justification is required for market approval of a racemate of chiral drugs. Presently, a majority of commercially available drugs are both synthetic and chiral. However, a large number of chiral drugs are still marketed as racemic mixtures. Nevertheless, to avoid the possible undesirable effects of a chiral drug, it is imperative that only the pure, therapeutically active form be prepared and marketed. Hence, there is a great

need to develop the technology for analysis and separation of racemic drugs. Current methods of enantiomeric analysis include such non-chromatographic techniques as polarimetry, nuclear magnetic resonance, isotopic dilution, calorimetry, and enzyme techniques. The disadvantages of these techniques are the need for pure samples, and no separation of enantiomers is involved. Quantitation, which does not require pure samples, and separation of enantiomers, can be done simultaneously by either gas chromatography (GC) or high performance liquid chromatography (HPLC). Chiral HPLC has proven to be one of the best methods for the direct separation and analysis of enatiomers. It is more versatile than chiral GC because it can separate a wide variety of nonvolatile compounds. It provides fast and accurate methods for chiral separation, and allows on-line detection and quantitation of both mass and optical rotation of enantiomers if appropriate detection devices are used (Davankov *et al.,* 1983).

Enantioselective separations have been realised in all possible separation techniques, including gas chromatography, column liquid chromatography, thin-layer chromatography, supercritical fluid chromatography, as well as electromigration methods, counter current liquid chromatography and liquid-liquid extractions. Numerous review papers and special monographs describe the technical details as well as the achievements and potential of these important modern separation techniques (Davankov *et al.*, 1983; Armstrong *et al.*, 1988; Pirkle and Pochapsky, 1989; Ahuja, 1991; Schreier *et al.*, 1995).

1.7. Basic considerations in method development for chiral compounds

Cost considerations, availability of equipment, and know-how play important roles in the selection process for an appropriate method. Paper chromatography (PC) and thin-layer chromatography (TLC) have been used where cost considerations outweigh other factors. PC is used very rarely these days; however, TLC can be a very useful qualitative technique that entails minimal costs (Davankov *et al.*, 1983). It can also provide good indications as to which HPLC method would be most suitable for resolving enantiomers. Of course, it can also be used as an independent technique with limitations of resolution and low precision. Commonly used methods for separation of enantiomers today can be classified broadly into the following four categories:

- Gas chromatography (GC)
- High-performance liquid chromatography (HPLC)
- Supercritical fluid chromatography (SFC), and

• Capillary electrophoresis (CE)

Since HPLC methods are generally favored for a variety of reasons, some basic information on selecting a suitable method for HPLC has been included in this chapter. A basic understanding of chiral discrimination by various chiral stationary phases (CSPs) has been provided to help method development. A strategy for fast method development is also provided in this chapter.

1.8. Chiral chromatography

Chiral column chromatography is a variant of column chromatography in which the stationary phase contains a single enantiomer of a chiral compound rather than being achiral. The two enantiomers of the same analyte compound differ in affinity to the single-enantiomer stationary phase and therefore they exit the column at different times. Chromatographic methods are considered the most useful for chiral separation. There are two approaches: indirect, which utilizes derivatizing agents, and direct, which uses chiral stationary phases or chiral mobile phase additives.

1.8.1. Direct chromatographic methods

1.8.1.1. Direct chromatographic methods (Chiral mobile phase additives) (Kolodiazhnyi, 2016)

Direct separation of enantiomers on an achiral column using a chiral mobile phase additive is applied only in HPLC. In GC the mobile phase is an inert carrier gas, where the possibility of selective interactions with the analyte or the stationary phase is minimal. However, in HPLC, the mobile phase is a dynamic part of the system that influences both analyte and stationary phase interactions. Many racemic mixtures can be separated on conventional achiral LC columns by using an appropriate chiral mobile phase additive. Additives such as α, β and γ-cyclodextrins have been successful. Advantages of this technique are as follows:

- less expensive conventional LC columns can be used
- a wide variety of possible additives are available, and
- different selectivities from the chiral phases can be obtained.

However, the problems with this technique include:

- many chiral additives are costly, and sometimes, have to be synthesized
- the mode of operation is complex, and

- incovenient for preparative applications because the chiral additive must be removed from the enantiomeric solutes.

1.8.1.2. Direct chromatographic methods (Chiral stationary phases)

Enantiomeric separation by using chiral HPLC stationary phases (CSPs) is based on the formation of transient diastereomeric analyte-CSP complexes between the enantiomers and the chiral molecule that is an integral part of the stationary phase. At present, there are over a hundred CSPs for HPLC that are commercially available.

There are five major classes of HPLC-CSPs based on the type of analyte-CSP complexes formed which are showed in table 1.2.

Table 1.2: Classification of chiral stationary phases (CSPs).

Type	Description	Examples	Mode (modifiers)
1	Pirkle-type (p-donor or p-acceptors)	DNB-phenylglycine, DNB-leucine, naphthylalanine	Normal phase (polar)
2	Attractive interactions followed by inclusion (derivatized cellulose)	Chiralcel OA, OB, OD, OF, OJ	Normal phase (polar)
3	Inclusion (cyclodextrins, polyacrylates, polyacrylamides, crown ethers)	Cyclobond I, II, III; Chiralpak OP, OT; Chiralcel CR	Reversed phase (aqueous acetonitrile or methanol)
4	Ligand exchange	Proline, hydroxyproline	Reversed phase (aqueous buffer)
5	Proteins	Albumin, glycoprotein	Reversed phase (aqueous buffer)

1.8.2. Indirect chromatographic method (Kolodiazhnyi, 2016)

In the indirect method, a racemic mixture is made to react with a chiral reagent to form a pair of diastereomers and then chromatographed using an achiral column. Because diastereomers possess different physiochemical properties, they can be separated in an achiral environment. The advantages of the indirect approach are the following:

- less expensive, i.e., conventional chromatographic columns can be used
- flexible because various achiral columns and mobile phase conditions, as in HPLC, can be used

- numerous types of derivatization chemistry are available and the cost of each reagent may be less expensive than for a chiral column, and
- different selectivities can be achieved.

On the other hand, the disadvantages of this method are:

- long analysis time that include sample preparation and verification of the derivatization chemistry
- inconvenience, specifically in preparative chromatography, when reversal of derivatization is needed to recover the pure enantiomers
- the need to synthesize non-commercially available pure derivatizing reagent, and
- biased results for enantiomeric composition due to partial racemization of derivatizing agent or unequal reaction rates.

1.9. Advantages of cellulose based CSPs

The cellulose-based CSPs generally are of two types: the coated and the bonded. The coated cellulose-based CSPs consisting of the low-molecular-weight cellulose benzoate or phenyl carbamate showed higher chiral recognition than the covalently bonded CSPs for most racemates. The major reason was considered to be an optimal secondary and supermolecular structure for the chiral recognition mechanism of polysaccharide derivatives under coated conditions (Okamoto and Kaida, 1994; Okamoto and Ikai, 2008). However, the coated CSPs can only be used with a limited range of solvents as mobile phases such as alkanes, alcohols, acetonitrile, or aqueous solvents including alcohols or acetonitrile because CSPs may dissolve in 'strong' solvents such as tetrahydrofuran (THF) and chloroform ($CHCl_3$). Such dissolution would damage or destroy the CSPs. This has limited the application range of the coated CSPs on separation and preparation of chiral compounds, because the solubility of the sample in the mobile phase is very important to increase the amount of racemates loaded on CSPs, especially on a preparative large-scale separation (Franco *et al.*, 1997).

The bonded CSPs were prepared by covalently bonding cellulose derivates to silica gel. They can be applied to a wider range of resolving conditions than the coated type. The fixation can affect the conformation of cellulose derivates and make it difficult to obtain optimal supermolecular structure. This results in lower chiral recognition ability of the bonded-type

CSPs. However, the fixation improves versatility in the solvent selection, and allows the use of some solvents that cannot usually be applied on the coated CSPs as mobile phases or sample dissolving reagents (Kasuya *et al.*, 2002).

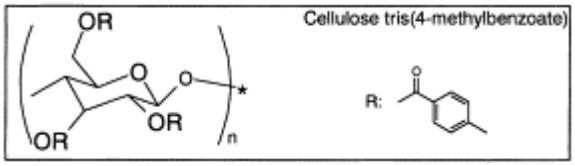
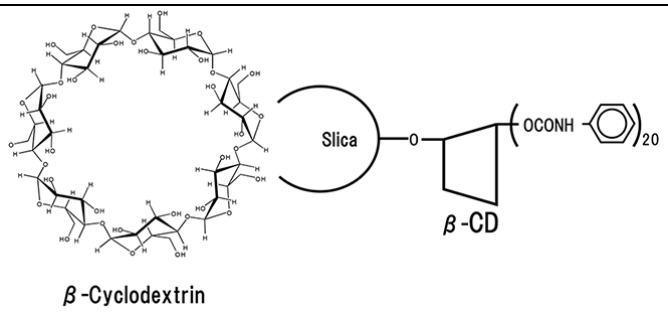
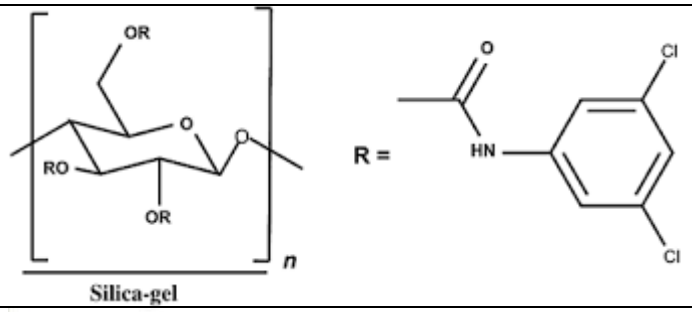
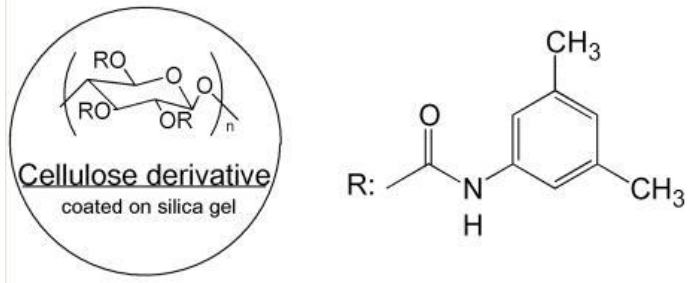
The commercial cellulose-based CSPs including the coated and the bonded CSPs currently in use are summarized in table 1.3.

Table 1.3: Commercial cellulose-based CSPs.

No.	**Chemical name**	**Commercial product** (Qiu *et al.*, 2013)	**Type**
1	Cellulose-*tris*-(3,5-dimethylphenyl-carbamate)	Chiralcel OD-H; Chiralcel OD; Chiralcel OD-RH; Chiralcel OD-R; Lux Cellulose-1; Kromasil CelluCoatTM	Coating
2	Cellulose-*tris*-phenyl-carbamate	Chiralcel OC	Coating
3	Cellulose-*tris*-(4-fluorophenyl-carbamate)	Chiralcel OF	Coating
4	Cellulose-tris(4-chloro-3-methylphenyl-carbamate)	Chiralcel OX-H; Lux Cellulose-4	Coating
5	Cellulose-tris(3-chloro-4-methylphenylcarbam ate	Chiralcel OZ-H; Chiralcel OZ-RH; Lux Cellulose-2	Coating
6	Cellulose-*tris*-(4-methylphenylcarbamate)	Chiralcel OG	Coating
7	Cellulose-*tris*-(4-methylbenzoate)	Chiralcel OJ-H; Chiralcel OJ; Chiralcel OJ-RH; Lux Cellulose-3	Coating
8	Cellulose-tris- benzoate	Chiralcel OB-H Chiralcel OB	Coating
9	Cellulose-tris-acetate	Chiralcel OA	Coating
10	Mricocrystalline cellulose-tris-acetate	Chiralcel CA-1	Coating
11	Cellulose-tris- cinnamate	Chiralcel OK	Coating
12	Cellulose-tris-(3,5- dimethyl-phenylcarba mate)	Chiralpak IB	Bonding
13	Cellulose-tris-(3,5- dichloro-phenylcarbamate)	Chiralpak IC	Bonding

In this study, the following CSPs are used which are stated in table 1.4.

Table 1.4: Structure of common CSPs.

CSP	Structure
Lux cellulose-3 [Cellulose tris(4-methylbenzoate)]	
CD-PH	
Chiralpak IC [Cellulose tris(3,5-dichlorophenyl carbamate)]	
Chiralcel OD-H [Cellulose tris(3,5-dimethylphenyl carbamate)]	

1.10. Mechanism for chiral separations by HPLC

To develop an optimum method, it is important to understand the mechanism of chiral separation. Our understanding of chiral separations with some of the systems is quite good, while it remains poor for protein and cellulose stationary phases. The separation basis with various chiral stationary phases is discussed below in their respective group; some general comments are included here. A number of chiral recognition models have been proposed to account for optical resolutions by HPLC; these are often based upon the three-point interaction

rule advanced by Dalgliesh in 1952. He arrived at this conclusion from paper chromatographic studies of certain aromatic amino acids. He assumed that the hydroxyl groups of the cellulose were hydrogen-bonded to the amino carboxyl groups of the amino acid. A third interaction was caused, according to these views, by the aromatic ring substituents. It led to the postulation that three simultaneously operating interactions between an enantiomer and the stationary phase are needed for chiral discrimination. However, this is not always necessary as steric discrimination also could result from steric interactions. Chiral separations also are possible through reversible diastereomeric association between an enantiomeric solute and a chiral environment that is introduced into the column. Because chromatographic resolutions are possible under a variety of conditions, it might be concluded that the necessary difference in association can be obtained by many types of molecular inter actions. The association, which may be expressed quantitatively as an equilibrium constant, will be a function of the magnitudes of the binding as well as the repulsive interactions involved. The latter are usually steric, although dipole-dipole repulsions also could occur, whereas various kinds of binding interactions can operate. These include hydrogen bonding, electrostatic and dipole-dipole attractions, charge–transfer interaction, and hydrophobic interaction (in aqueous systems). CSPs, where steric fit is of primary importance, include those based upon inclusion phenomena, such as cyclodextrin and crown ether phases. It is possible to construct chiral cavities for the preferential inclusion of only one enantiomer. Molecular imprinting techniques are very interesting in this respect. The idea is to create rigid chiral cavities in a polymer network in such a way that only one of two enantiomers will find the environment acceptable. Here, interaction between derivatives of mandelic acid and CSP (Whelk-O1) are illustrated below in figure 1.2.

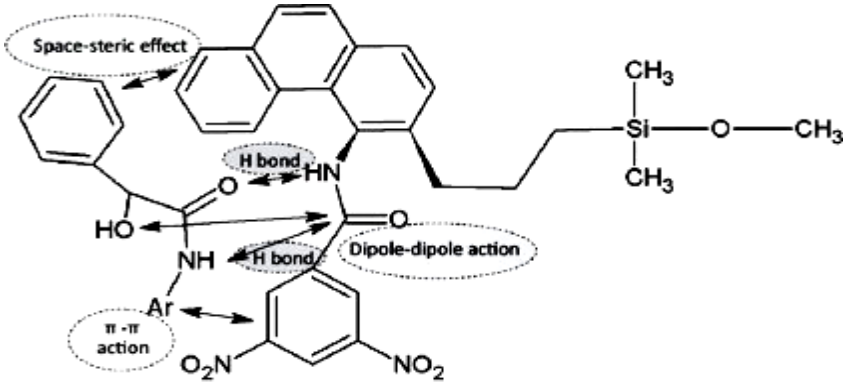


Figure 1.2: Interaction between derivatives of mandelic acid and CSP (Whelk-O1).

1.11. The drugs selected for the study

Very common racemic drugs available in Bangladesh are selected for quality assessment by chiral chromatography. Purpose of this work is described below:

1.11.1. Omeprazole

Omeprazole is the most popular proton-pump inhibitor (PPI) mainly used to reduce gastric acid secretion by targeting the gastric acid pump, H^+, K^+, adenosine triphosphatase (ATPase) in the canalicular membrane of the parietal cell (Horn, 2000). It is also used to treat diseases like dyspepsia, gastroesophageal reflux disease (GERD), laryngopharyngeal reflux, and Zollinger-Ellison syndrome (Sachs, 1997; DeVault and Castell, 2005). Omeprazole, 6-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)methanesulfinyl]-1*H*-1,3-benzodiazole possesses an asymetric sulfoxide moiety exhibiting (*S*)- and (*R*)- omeprazole an equimolar mixture (1:1) of the two (Vyas *et al.*, 2011). (*S*)- omeprazole received approval with more pronounced inhibition of acid secretion and less inter-patient variation than racemate omeprazole (Shin *et al.*, 2009). Literature survey revealed that (*S*)-omeprazole provides more effective acid control than twice the dose of racemic omeprazole (Röhss *et al.*, 2002). Because of (*S*)- omeprazole is metabolized more slowly compared to racemic omeprazole in human liver by CYP 2C19 and CYP 3A4 (Röhss *et al.*, 2002; Vyas *et al.*, 2011). For this, attention is now focused on purity of the active single enantiomer. Therefore, analytical methods of chiral drugs are highly needed.

1.11.2. Rabeprazole

Rabeprazole sodium which is chemically described as monosodium (*RS*)-2-({[4-(3-methoxypropoxy)-3-methyl- pyridin-2-yl] methyl} sulfinyl)-1H-benzimidazolide is one of the latest proton-pump inhibitors (PPIs) developed for suppression of gastric acid secretion by H+/Na+-ATPase. Like other PPIs, rabeprazole possesses an asymmetric sulfoxide center. Although it is clinically administered as a mixture of *R*-(+) and *S*-(-) rabeprazole, some studies suggested higher therapeutic effects of *R*-(+)-rabeprazole (Bodhankar *et al.*, 2006; Pai and Pai, 2007).

1.11.3. Pantoprazole

The Pantoprazole sodium is widely used as proton pump inhibitors (PPIs). The drug irreversibly inhibits proton pump function, they block final step in acid production and effective inhibit acid

secretion. Pantoprazole sodium is also used as anti ulcer. Pantoprazole sodium is racemic mixture of [+] *S*- and [-] *R*-pantoprazole sodium. Pantoprazole sodium chemically known as sodium 5-(difluoromethoxy)-2–[[(3,4-dimethoxy-2-pyridinyl) methyl] sulfinyl]-1H-benzimidazole sesquihydrate, and chemical formula is $C_{16}H_{14}F_2N_3NaO_4S$, $1^1/_2\ H_2O$. It has been demonstrated in animal that the [+]-*S*-form is biologically active. Several different methods have been reported for qualitative and quantitative analysis of pantoprazole sodium. This study introduces an improved HPLC method with short analysis time, high sensitivity for determination of rabeprazole enantiomers in commercial tablets using immobilized cellulose-based stationary phase.

1.11.4. Propranolol

Propranolol, is chemically (±)-1-Isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride, is a medication of the beta blocker type (Singh *et al.*, 2004). It is used to treat high blood pressure, a number of types of irregular heart rate, thyrotoxicosis, capillary hemangiomas, performance anxiety, and essential tremors (Singh *et al.*, 2004). It is also used to prevent migraine headaches, and to prevent further heart problems in those with angina or previous heart attacks. It is usually given orally as a racemic mixture [i.e., 50:50 mixtures of (*S*)- (–)-and (*R*)-(+)-enantiomers]. (*S*)-(–)-enantiomer of propranolol hydrochloride has been found biologically active (Drake, 1988). From the literature survey, to measure propranolol several methods are presently available.

1.11.5. Carvedilol

The compound carvedilol ((±)-1-(carbazol-4-yloxy)-3-[[2-(*O*-methoxy- phenoxy)ethyl]amino]-2-propanol), is administered clinically as a racemic mixture of the *R*(+)- and *S*(−)-enantiomers for two complementary pharmacological effects, vasodilation and β-blockade (Borbe, 1990). In the racemate, only the *S*-enantiomer of carvedilol exerts β-blocking effects, no substantial difference between the enantiomers could be seen with respect to α-blockade. To date, many methods are developed for the enantioselective analysis of carvedilol.

In this study, separation of carvedilol enantiomers is accomplished by a new commercial CSP based upon cellulose tris-(3,5-dichlorophenylcarbamate) polymer immobilized on silica. Cellulose and amylose derived chiral stationary phase in the chiral columns are extensively

applicable to separate enantiomers and have the good capability for the chiral resolution (Yang *et al.*, 2004; Cirilli *et al.*, 2008; Raghuram *et al.*, 2009; Ozkırımlı *et al.*, 2011; VK *et al.*, 2011).

1.11.6. Ibuprofen

Ibuprofen, (2*R*)-2-[4-(2-methylpropyl)phenyl]propanoic acidis a medication in the nonsteroidal anti-inflammatory drug (NSAID) class that is used for treating pain, fever, and inflammation. This includes painful menstrual periods, migraines, and rheumatoid arthritis. It may also be used to close a patent ductus arteriosus in premature baby. It can be used by mouth or intravenously. It typically begins working within an hour.

Racemic ibuprofen, which contains equal quantities of *R*-(-)-ibuprofen and *S*-(+)-ibuprofen, has been used as an anti-inflammatory and analgesic agent for over 30 years. Although the *S*- (+)-enantiomer is capable of inhibiting cyclooxygenase (COX) at clinically relevant concentrations, *R*- (-)-ibuprofen is not a COX inhibitor. The two enantiomers of ibuprofen are therefore different in terms of their pharmacological properties and may be regarded as two different 'drugs' (Evans, 2001).

1.11.7. Salbutamol

Salbutamol, 2-tert-butylamino-l-(4-hydroxy-3-hydroxymethyl) phenylethanol is a short-acting beta-2 adrenergic agonist primarily used in the treatment of asthma and chronic obstructive pulmonary disease (COPD). It is the most widely used short-acting β_2-agonist, consists of a racemic mixture of equal amounts of two enantiomers, (*R*)-salbutamol and (*S*)-salbutamol. (Penn *et al.*, 1996; Page and Morley, 1999). The bronchodilator effects of salbutamol are attributed entirely to (*R*)-salbutamol (levosalbutamol), while (*S*)-salbutamol has been shown to possess bronchospastic and pro-inflammatory effects both *in vitro* and *in vivo* studies (Jantikar *et al.*, 2007). Literature survey revealed that levosalbutamol has 150 times greater affinity for the beta-2-receptor than the *S*-enantiomer. *S*-enantiomer possesses toxicity.

1.11.8. Ofloxacin

Levofloxacin, (3*S*)-9-Fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7H-[1,4] oxazino[2,3,4-ij] quinoline-6-carboxylic acid) (the optically active form of ofloxacin) is one of the most promising fluoroquinolone drugs, and its antibacterial activity is substantially higher than the activity of other drugs of the fluoroquinolone family. As of 2007, the Infectious

Disease Society of America (IDSA) and the American Thoracic Society recommended levofloxacin and other respiratory fluoroquinolines as first line treatment for community acquired pneumonia when comorbidities such as heart, lung, or liver disease are present or when in-patient treatment is required (Mandell *et al.*, 2007). Levofloxacin also plays an important role in recommended treatment regimens for ventilator-associated and healthcare-associated pneumonia (File, 2010). It is one of the enantiomer of ofloxacin. Ofloxacin is a racemic mixture of (*R*)-ofloxacin and (*S*)-ofloxacin (levofloxacin). The *S*-enantiomer shows 8–128 times higher activity than the *R*-form against Gram-positive and Gram-negative bacteria (Hayakawa *et al.*, 1986; Fujimoto and Mitsuhashi, 1990). Recent, separation of enantiomers has become very important in analytical chemistry, especially in the pharmaceutical and biological fields, because some stereoisomers of racemic drugs have quite different pharmacokinetics and different pharmacological or toxicological effects (Maier *et al.*, 2001; Nguyen *et al.*, 2006). Therefore, enantiomers selective analysis of levofloxacin is important for the drug quality control as well as for the design and development of new chiral method. Current chiral HPLC method, direct chiral separations using CSPs are more widely used and are more predictable, in mechanistic terms, than those using chiral additives in the mobile phase (Koppenhoefer *et al.*, 1994). Chiral HPLC is more versatile than chiral GC for enantiomeric separation because it can separate a wide variety of nonvolatile compounds. It provides fast and accurate methods for chiral separation, and allows on-line detection and quantitation of both mass and optical rotation of enantiomers if appropriate detection devices are used (Boehme *et al.*, 1982; Purdie and Swallows, 1989). A new commercial CSP is now available, based upon cellulose tris-(3,5- dichlorophenylcarbamate) polymer immobilized on silica. Immobilised polysaccharide based stationary phases play a significant role in the chiral separation science. Cellulose and amylose derived chiral stationary phase in the chiral columns are extensively applicable to separate enantiomers and have the good capability for the chiral resolution (Maier *et al.*, 2001; Yang *et al.*, 2004; Cirilli *et al.*, 2008; Raghuram *et al.*, 2009; Ozkırımlı *et al.*, 2011; Sharma and Singh, 2013).

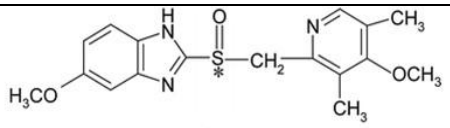
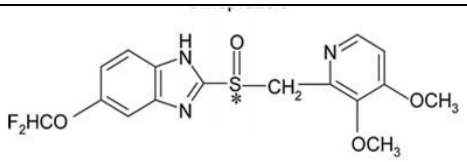
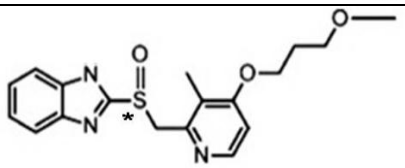
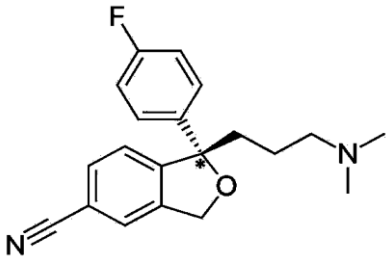
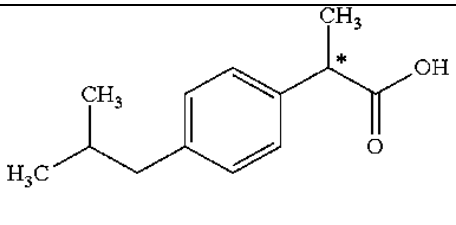
To the best of our knowledge, this work is the first attempt of employing cellulose tris-(3,5-dichlorophenylcarbamate (IC column) as a chiral selector to separate ofloxacin, offering a promising alternative for ofloxacin enantiomers quantitation.

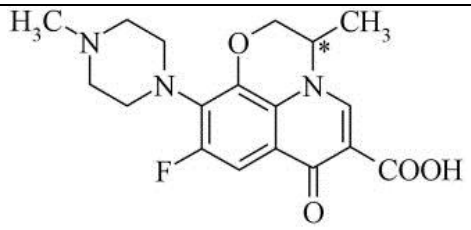
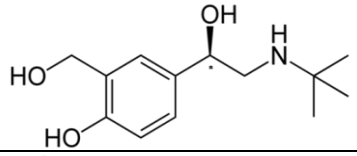
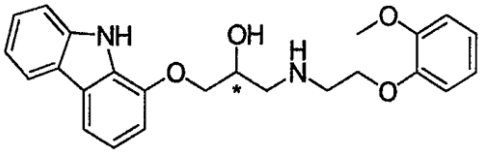
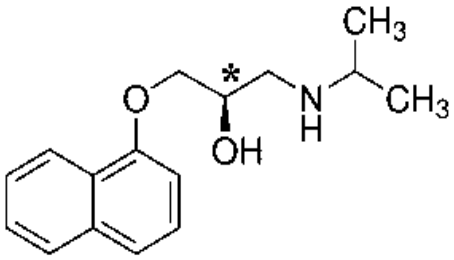
1.11.9. Citalopram

Citalopram (1-[3-(dimethylamino) propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile) is a selective serotonin reuptake inhibitor used as an antidepressant drug. Citalopram possesses one asymmetric carbon, giving a pair of enantiomers, (*S*)-citalopram, pharmacologically effective and (*R*)-citalopram is considered to be inactive (Hyttel and Larsen, 2009). Citalopram is also applied to treat panic, anxiety and obsessive compulsive disorders of pathological laughing and crying (Mork *et al.*, 2003).

These common drugs are stated in table 1.5.

Table 1.5: Common enantiomeric drugs available in Bangladesh.

Racemic mixture	Single-enantiomer	Activity	Structures
Omeprazole	Esomeprazole	In acid related treatment	
Pantoprazole		In acid related treatment	
Rabeprazole		In acid related treatment	
Citalopram	Escitalopram	Used as antidepressant drug	
Ibuprofen	Dexibuprofen	Used as active NSAID	

Ofloxacin	Levofloxacin	Approved for the treatment of bacterial infections. Dextrofloxacin is inactive	
Salbutamol	Levosalbutamol	Used as bronchodilator	
Carvedilol	*S*-carvedilol	Beta-blocker, used to treat heart failure and hypertension (high blood pressure)	
Propranolol	*S*-propranolol	Beta-adrenergic blocking agent that is used for treating high blood pressure, heart pain (angina), abnormal rhythms of the heart, and some neurologic conditions.	

1.12. Techniques involved in enantiomeric purity

For determination of enantiomeric purity various techniques are used. One is non-separative and other is separative methods. In non-separative methods polarimetry, circular dichroism, nuclear magnetic resonance (NMR), isotopic dilution, X-ray crystallography and enzyme techniques are involved. Disadvantages of these methods are:

- requirement of large amount of sample.
- error measurements of enantiomeric purity estimations
- difficult to control concentrations and that solvent properties

For these reasons, non-separative methods are not so useful for determination of enantiomeric purity.

Today the most common techniques used for determination of enantiomeric purity are separative methods, mainly chromatographic chiral separation techniques, in which gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary

electrophoresis (CE) are widely employed for their high efficiency and sensitivity (Kim *et al.*, 2017).

Among them, chiral HPLC has proven to be one of the best methods for the direct separation and analysis of enatiomers (Boehme *et al.*, 1982; Purdie and Swallows, 1989). It is more versatile than chiral GC because it can separate a wide variety of nonvolatile compounds. It provides fast and accurate methods for chiral separation. Direct chiral separations using CSPs are more widely used and are more predictable than those using chiral additives in the mobile phase (Koppenhoefer *et al.*, 1994).

1.13. Enantiomeric purity

The US Food and Drug Administration and other regulatory agencies have made it mandatory for the manufacturers to investigate each enantiomer of the chiral drug individually (Rauws and Groen, 1994). According to the International Conference on Harmonization (ICH) guidelines, chiral identity, enantiomeric impurity and chiral assay tests may be needed in drug substance and product specifications (Branch, 2005). Chromatographic methods have dominated separation of enantiomers during the past several decades, especially by HPLC (Hiep *et al.*, 1998; Brandsteterova and Wainer, 1999; Campanero *et al.*, 1999; Bortocan *et al.*, 2000). In order to quantitatively determine it is important to measure the enantiomeric composition and diastereomeric ratio for a chiral molecule. In an enantiomerically enriched mixture, the excess of one enantiomer over the other is called enantiomeric excess (ee) and this, as a percentage, is usually used to state the enantiomeric purity of a compound. A chiral molecule containing only one enantiomeric form is regarded as optically pure or enantiopure or enantiomerically pure. Determination of enantiomeric excess (ee) of chiral compounds is performed by chiral HPLC (Mehta, 1988; Sanchez, Diaz and Pareja, 1996; Andersson *et al.*, 2003; Matthijs *et al.*, 2004; Andersson *et al.*, 2007).

1.14. Determination of enantiomeric purity or enantiomeric excess (ee %)

Determination of Enantiomeric purity or enantiomeric excess (ee %)

= % of major enantiomer - % of minor enantiomer (for single enantiomer) (*Illustrated Glossary of Organic Chemistry*).

= 50% - (% of major enantiomer) for racemate (1:1 mixture of enantiomers) (*Optical Purity and all that....*).

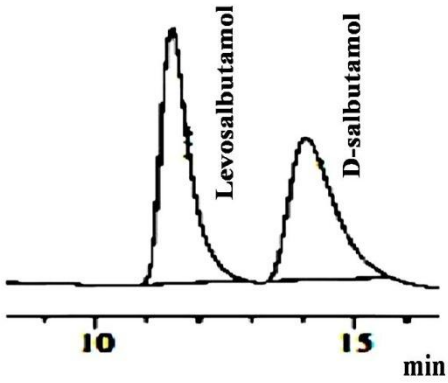
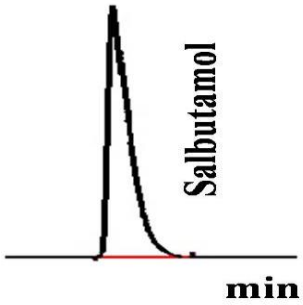
1.15. Comparison between chiral and high performance liquid chromatography (HPLC)

HPLC is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. Chromatography is used to separate proteins, nucleic acids, or small molecules in complex mixtures.

In chiral chromatography, chiral molecules are separated in a mixture. In this chromatography, the chiral stationary phase can be prepared by attaching a suitable chiral compound to the surface of an achiral support such as silica gel, which creates a Chiral Stationary Phase (CSP). Many common chiral stationary phases are based on oligosaccharides such as cellulose or cyclodextrin (in particular with β-cyclodextrin, a seven sugar ring molecule). As with all chromatographic methods, various stationary phases are particularly suited to specific types of analytes. Table 1.6 shows the comparison between chiral and high performance liquid chromatography (HPLC).

Table 1.6: Comparison between chiral and high performance liquid chromatography (HPLC).

Parameters	**Chiral HPLC**	**HPLC**
Principle	Column chromatography in which the stationary phase contains a single enantiomer of a chiral compound rather than being achiral	Formerly referred to as high-pressure liquid chromatography, is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture.
Stationary phase	Cellulose or cyclodextrin	Silica or modified silica
Separation mechanism	Requires a minimum of 3 interactions through: H-bonding, π-π interactions, Dipole stacking	Separation mode based on polarity

Chromatography of salbutamol		
Mobile phase	Chiral CD-PH column, MP: disodium hydrogen phosphate: Methanol (50:50) (Present work)	Ref.: (Chitlange *et al.*, 2011)

1.16. Pharmaceutical evaluation

The chiral drugs have dominated the pharmaceutical business since 1990's. Among the all marketed drugs, more than 40% are currently sold in a single enantiomeric form (Mylari *et al.*, 1991). The market share of chiral drugs is expected to rise sharply in the near future as approximately 80% of total drugs under development are chiral. About 70% of the new small-molecule drugs which received the approval by Food & Drug Administration in 2007 were chiral (Tsuji and Ishikawa, 1994). Past two decades have witnessed a significant expansion in the production of chiral drug molecules. A worldwide sinerio of emergence of single enantiomeric drugs over couple of years is given in table 1.7.

Table 1.7: Annual distribution of FDA-approved drugs from 1992 to 2008 (Mylari *et al.*, 1991; Tsuji and Ishikawa, 1994)**.**

Year	Racemic (%)	Chiral (%)	Achiral (%)
1992	21	44	35
1993	16	45	39
1994	38	38	24
1995	21	46	33
1996	9	41	50
1997	24	30	46
1998	15	50	35
1999	19	50	31
2000	3	67	30
2001	0	72	28
2002	6	58	36
2003	0	76	24
2004	6	76	18
2005	5	63	32
2006	10	55	35
2007	5	68	27
2008	5	63	32

Besides development of new pharmaceuticals in single enantiomeric form, chiral technology (i.e. chirotechnology) has a crucial role in re-evaluation and remarketing of single enantiomeric forms of existing racemic drugs (called Racemic switching) which permits additional years of market exclusivity (patent protection). According to Frost & Sullivan, worldwide revenues from chiral compounds destined for the drug industry amounted to $4.8 billion in 1999 and will be triple to $14.9 billion by 2009, with the average annual growth of 12% (Bell *et al.*, 1995). The global growth in revenues of pharmaceutical sector driven by terotechnology over last 10 years is given in table 1.8.

Today chiral technology is mainly driven by pharmaceutical industries. Besides pharmaceuticals, the ‘chirality’ is receiving attention from several business sectors such as biochemicals, agrochemicals, aroma and flavour compounds, dyes and pigments and polymers. The industrial demand of enantiopure chemicals is therefore expected to show explosive growth in the coming years (Mylari *et al.*, 1991).

Table 1.8: The global growth in revenues of pharmaceutical sector from chiral technology (Bell *et al.*, 1995)**.**

Year	Revenue ($ Billions)	Annual growth (%)
1999	4.80	-
2000	5.40	12.5
2001	6.10	13.0
2002	7.00	14.8
2003	7.74	10.6
2004	8.57	10.8
2005	9.53	11.1
2006	10.61	11.3
2007	11.85	11.7
2008	13.28	12.1

1.17. Literature review

In the past, several investigations have been carried out on method development of chiral separation for analysis of enantiomers in pharmaceutical formulation. A survey of literature was carried out in such investigations. Some of the important investigations are discussed below.

1.17.1. Omeprazole

Bonato and Paias (2004) have reported enantioselective analysis of omeprazole in pharmaceutical formulations by chiral high-performance liquid chromatography and capillary electrophoresis. They developed two sensitive and simple assay procedures based on high-performance liquid chromatography and capillary electrophoresis (CE) for the enantioselective analysis of omeprazole in pharmaceutical formulations. Rac-omeprazole and (*S*)-omeprazole were extracted from commercially available tablets using methanol: NaOH (2.5 mol L^{-1}): (90:10, v/v). Chiral hplc separation of omeprazole was obtained on a chiralpak ad column using hexane: ethanol (40:60, v/v) as the mobile phase and detection at 302 nm. The resolution of omeprazole enantiomers by CE was carried out using 3% sulfated β-cyclodextrin in 20 mmol L^{-1} phosphate buffer, pH 4.0 and detection at 202 nm. In this analysis, they used chiralcel od-h, chiralcel ob-h, chiralpak ad and chiralpak as columns which were evaluated

under normal phase conditions using hexane/ethanol or hexane/2-propanol as mobile phases. The chiralcel oj-r column was evaluated under reversed phase conditions, using acetonitrile: H_2O or acetonitrile: $NaClO_4$ solution. The chiral agp column was used with mobile phases consisting of a phosphate buffer solution supplemented with acetonitrile. chiralpak ad, chiralcel od-h, chiralcel ob-h, chiralpak as, chiralcel oj-r, chiral agp showed resolution 3.3, 2.3, 1.1, 2.0,0.5, 3.3 respectively. Among the chiral columns evaluated, only chiralcel oj-r did not show complete resolution of omeprazole. The chiralpak ad column was selected for development of the method due to its stability and high resolving ability. Calibration curves were linear over the concentration range of 25 to 150 μg mL^{-1} for both methods, with correlation coefficients better than 0.99.

Hancu *et al.* (2015) have reported chiral separation of the enantiomers of omeprazole by capillary electrophoresis. They reported the systematic screening of different native and derivatized, charged and uncharged cyclodextrin (CD) derivatives for chiral separation, by capillary electro-phoresis, of the enantiomers of omeprazole, the most frequently used proton-pump inhibitor. To optimize the analytical and electrophoretic conditions the effect of buffer composition, concentration, and pH, chiral selector type and concentration, potential, temperature, and injection volume were investigated. Baseline chiral separation was achieved by use of phosphate buffer at pH 2.5 and randomly methylated β-CD as chiral selec-tor for omeprazole. For omeprazole, chiral interactions were observed when β-CD, HP-β-CD, and RAMEB were used over the pH range 2.0–4.0 The effect of buffer concentration was studied in the range 25–100 mM. The optimum buffer concentration was 50 mMfor all separations. The effect of the CD concentration was studied in the range 10–40 mM for uncharged CD and 1–10 mM for charged CD. For native β-CD the maximum concentration was 20 mM, because of their poor solubility in aqueous solutions. Chiral resolution increased with increasing CD concentration until the optimum CD concentration was reached and maximum resolution was achieved; further increasing the CD concentration then resulted in reduced resolution. RAMEB was the best CD for separation of the enantiomers of omeprazole. The optimum CD concentrations were 20 mM for separation of omeprazole.

Mahmoud and Ahmed (2016) have reported a high-throughput 96-microwell plate fluorometric method. It was developed and validated to determine omeprazole (OMZ) in its dosage forms.

The method was based on the charge-transfer (CT) sensitized fluorescence reaction of OMZ with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). This fluorescence reaction provided a new approach for simple, sensitive and selective determinations of OMZ in pharmaceutical preparations. In the present method, the fluorescence reaction was carried out in 96-microwell plates as reaction vessels in order to increase the automation of the methodology and the efficiency of its use in quality control laboratories. All factors affecting the fluorescence reaction were carefully studied and the conditions were optimized. The stoichiometry of the fluorescence reaction between OMZ and DDQ was determined and the reaction mechanism was suggested. Under the optimum conditions, the linear range was 100-6000 ng/ml with the lowest LOD of 33 ng/ml. Analytical performance of the proposed assay, in terms of accuracy and precision, was statistically validated and the results were satisfactory; RSD was <2.6 % and the accuracy was 98.6-101.6 %. The method was successfully applied to the analysis of OMZ in its dosage forms; the recovery values were 98.26-99.60 ± 0.95-2.22 %.

Gallinella *et al.* (2016) have reported a simple analytical high-performance liquid chromatographic (HPLC) method that was applied for the enantiomeric excess determination of esomeprazole (*S*)-OME), the enantiopure active ingredient contained in drug products, in the presence of its potential organic impurities. The enantioselective separation was accomplished on the immobilized type Chiralpak ID-3 chiral stationary phase (CSP) under reversed phase conditions. The results were evaluated and compared with those obtained by the official enantioselective method of European Pharmacopoeia used as the reference for checking the enantiomeric excess of (*S*)-OME. It has been established that the use of the Chiralpak ID-3 CSP allows the determination of the enantiomeric purity of (*S*)-OME without any interference coming from its chiral and achiral related substances.

Hussein *et al.* (2016) have reported reverse-phase chromatographic condition. They showed a simple and precise reversed-phase high performance liquid chromatographic (HPLC) assay for the measurement of omeprazole level in human plasma, using lansoprazole as an internal standard (IS). Plasma samples containing omeprazole were spiked with the IS then extracted with tert butyl methyl ether and reconstituted in mobile phase. The compounds of interest were efficiently separated on Atlantis C18 column at room temperature and detected with photodiode array detector set at 302 nm. The mobile phase consisted of 0.05 M dibasic sodium phosphate

(pH = 7.0, adjusted with phosphoric acid) and acetonitrile (60:40, v/v) and was delivered at a flow rate of 1.0 ml/min. The relationship between omeprazole concentration in plasma and peak height ratio of omeprazole/ IS was linear (R^2 = 0.9992) in the range of 0.01 – 1.20 µg/ml and the intra- and interday≥ coefficient of variations (CV) and accuracy were ≤5.0% and ≤10.1%, ≥90% and ≥92%, respectively. Mean extraction recovery of omeprazole and the IS from plasma samples both was 91% and 91%, respectively. In processed samples, omeprazole was stable for at least 24 hours at room temperature (≥91%) and 48 hours at -20 º C (≥89%). In unprocessed samples it was stable at least 12 weeks at -20ºC (≥96%) and after 3 cycles of freeze and thaw (≥96%). Further, it was successfully applied to measure omeprazole level in samples obtained from a healthy volunteer.

Olsson *et al.* (2006) have reported a method of capillary electrophoresis (NACE) for enantiomeric determination of omeprazole and its metabolite 5-hydroxyomeprazole. This study demonstrated the development and validation of a non-aqueous capillary electrophoresis (NACE) method. Heptakis-(2,3-di-O-methyl-6-O-sulfo)-beta-cyclodextrin (HDMS-beta-CD) was chosen as the chiral selector in an ammonium acetate buffer acidified with formic acid in methanol. Parameters such as CD concentration, concentration of buffer electrolyte, voltage and temperature were studied in order to optimize both the enantioresolution and migration times. An experimental design was utilized for method optimization, using software Modde 5.0. Validation of the developed method showed good linearity, which was tested over a concentration range of 2.5-500 microM. The regression coefficients for *S*-omeprazole, *S*-5-hydroxyomeprazole, *R*-omeprazole and *R*-5-hydroxyomeprazole were between 0.996 and 0.997. The limits of detection (LOD) for the four enantiomers were in the range from 45 to 51 microM and the limits of quantification (LOQ) were between 149 and 170 microM with UV detection at 301 nm. Using a reduced temperature of 16 degrees gave improved resolution values, reproducibility and also decreased the occurrence of current loss within the capillary. RSD values for peak migration time were calculated to be between 0.41 and 1.48% using an inter-day study.

Nozal *et al.* (2004) have reported Chiral separation of omeprazole with supercritical fluid chromatography. A study of the enantiomeric separation of omeprazole and several related benzimidazoles, using supercritical fluid chromatography (SFC), on the amylose based column

Chiralpak AD is presented in this work. The effect of the organic modifier as well as temperature on the retention and enantioresolution was investigated. Alcohol-type modifiers provided the best results, allowing the enantiomeric separation of all the compounds studied with resolutions that were in most cases higher than 2, and analysis times lower than 10 minutes. An investigation of the temperature effect revealed that the isoelution temperature was below the working temperature range in only two cases, and hence it was better to work at the highest temperature permitted.

Tanaka *et al.* (1995) have reported a direct, isocratic, and simple reversed-phase HPLC method 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 raclansoprazole (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 enantioselectivity 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. Increase 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.

El-Sherif *et al.* (2006) have reported a simple sensitive, selective and accurate reversed-phase high performance liquid chromatographic method. They developed and validated the method for the quantitative determination of lansoprazole, omeprazole and pantoprazole sodium sesquishydrate in the presence of their acid-induced degradation products. The three compounds were monitored at 280 nm using Nova-Pak C_{18} column and a mobile phase consisting of 0.05 M potassium dihydrogen phosphate: methanol: acetonitrile (5:3:2 v/v/v). Linearity ranges were 2-20 2-36 µg/mL, 2-36 µg/mL and 0.5-20 2-36 µg/mL for lansoprazole, omeprazole and pantoprazole, respectively. The corresponding recoveries were 100.61+/-0.84%, 100.50+/-

0.80% and 99.78+/-0.88%. The minimum detection limits were 0.55, 0.54 and 0.03 2-36 μg/mL for lansoprazole, omeprazole and pantoprazole, respectively. The method could be successfully applied to the determination of pure, laboratory prepared mixtures and pharmaceutical dosage forms. The results obtained were compared with the reported methods for lansoprazole and pantoprazole or the official U.S.P method for omeprazole.

Espinosa *et al.* (2007) have reported an alytical methodology for the determination of omeprazole. Omeprazole, a gastric acid pump inhibitor, dose-dependently controls gastric acid secretion; the drug has greater antisecretory activity than histamine H_2-receptor antagonists. Omeprazole has been determined in formulations and biological fluids by a variety of methods such as spectrophotometry, high-performance liquid chromatography with ultraviolet detection and liquid chromatography coupled with tandem mass spectrometry.

Mahmoud (2009) has developed new sensitive kinetic spectrophotometric methods for determination of omeprazole in dosage forms. New rapid, sensitive, and accurate kinetic spectrophotometric methods were developed, for the first time, to determine omeprazole (OMZ) in its dosage forms. The methods were based on the formation of charge-transfer complexes with both iodine and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The variables that affected the reactions were carefully studied and optimized. The formed complexes and the site of interaction were examined by UV/VIS, IR, and NMR techniques, and computational molecular modeling. Under optimum conditions, the stoichiometry of the reactions between OMZ and the acceptors was found to be 1:1. The order of the reactions and the specific rate constants were determined. The thermodynamics of the complexes were computed and the mechanism of the reactions was postulated. The initial rate and fixed time methods were utilized for the determination of OMZ concentrations. The linear ranges for the proposed methods were 0.10–3.00 and 0.50–25.00 μg/mL with the lowest LOD of 0.03 and 0.14 μg/mL for iodine and DDQ, respectively. Analytical performance of the methods was statistically validated; RSD was <1.25% for the precision and <1.95% for the accuracy. The proposed methods were successfully applied to the analysis of omeprazole in its dosages forms; the recovery was 98.91-100.32% ± 0.94-1.84, and found to be comparable with that of reference method.

El-Kommos *et al.* (2015) have reported a systematic comprehensive review of literature for the analysis of the most recent antiulcer drugs and proton pump inhibitors (PPIs) by different chromatographic and electrophoretic methods are presented. The review includes literature from 1985 until 2015 on eight proton pump inhibitors; namely: omeprazole (OMZ), lansoprazole (LAN), pantoprazole (PAN), rabeprazole (RAB), esomeprazole (EMZ), dexlansoprazole (DLAN), dexrabeprazole (DRAB), and tenatoprazole (TNB). The investigated literature survey covers most chromatographic and electrophoretic methods used for the assay of these drugs in pure forms, in combined mixtures, in pharmaceutical dosage forms, and in biological fluids.

Belaz *et al.* (2008) have developed multimilligram enantioresolution of sulfoxide proton pump inhibitors by liquid chromatography on polysaccharide-based chiral stationary phase. The enantiomers of sulfoxide proton pump inhibitors – omeprazole, lansoprazole, and rabeprazole were enantiomerically separated by liquid chromatography at multimilligram scale on a polysaccharide based chiral stationary phase using normal and polar organic conditions as mobile phase. The values of the recovery and production rate were significant for each enantiomer; better results were achieved using a solid phase injection system. However, this system was applied just for the enantiomeric separation of omeprazole to demonstrate the applicability of this injection mode at milligram scale. The chiroptical characterization of the compounds was performed using a polarimeter and a circular dichroism detector. The higher enantiomeric purity obtained for the isolated enantiomers suggests that the methods here described should be considered as a simple and rapid way to obtain enantiomeric pure standards for analytical purpose.

Vyas *et al.* (2011) have reported the development and complete validation of a stability indicating chiral high-performance liquid chromatographic (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 h 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.

Estevez *et al.* (2014) have developed a capillary electrophoresis method for determination of enantiomeric purity and related substances of esomeprazole in raw material and pellets. They have showed a capillary electrophoresis method using CDs for quality control of esomeprazole (ESO) in terms of enantiomeric purity and related substances in raw material and pellets was developed. ESO is the *S*-enantiomer of omeprazole (OMZ). Several parameters were evaluated, including type and concentration of buffer and CD, concentration of additives and electrolyte pH. Resolution between the enantiomers of OMZ obtained for each parameter tested was calculated and the presence of the main related substance such as OMZ sulfone was carefully monitored. The optimized system consisted of 100 mM tris-phosphate buffer pH 2.5 with 20 mM 2-hydroxypropyl-β-CD, 1 mM sodium dithionite, temperature at 15°C, voltage at 28 kV, and UV detection at 301 nm. Once optimized, the electrophoretic system was validated according to ICH guidelines. The limits of detection and quantification for *R*-OMZ were 0.6 μg/mL (0.06% w/w of ESO) and 2.0 μg/mL (0.2% w/w of ESO), respectively. A mean concentration of *R*-OMZ <0.2% limit established by the United States Pharmacopeia (USP) was found in the raw material and six-pellet samples of ESO. No other impurities were found in the samples under these conditions. Therefore, the developed method was found to be appropriate not only for enantiomeric quality control of ESO but also for the analysis of ESO and the main related substance in raw material and pharmaceutical formulations as well as for stability indicating studies.

Somagoni *et al.* (2011) have reported enantiomeric separation and determination of stereospecific drug release from marketed racemic omeprazole by chiral HPLC. The objective of carrying out this research work was to investigate the effect of chirality on stereospecific dissolution of omeprazole enantiomers from various marketed racemic omeprazole products.

Omeprazole is used for the treatment of gastro-duodenal ulcers and symptomatic gastro-oesophageal reflux. Dissolution of various marketed products was performed using USP type I apparatus in 0.1 N HCl for 2 h and in pH 6.8 phosphate buffer for 1h at 100 rpm. The separation of enantiomers was done using a chiral HPLC method on chiral AGP column (100 x 4.6 mm i.d.). The wavelength for UV detection was set at 210 nm. The mobile phase was 10 mM phosphate buffer with 5 % acetonitrile adjusted to pH 6.5 at a flow rate of 0.9 ml/min with an injector valve fitted to a 50 μL volume sample loop. The retention times for *R* and *S* enantiomers of omeprazole were 5 and 7.5 min, respectively. The dissolution of *S* enantiomer of Ocid-20 and Omee was found to be significantly more compared to their *R* enantiomer at 5 and 10 min dissolution time points after which there was no stereospecific discrimination in the dissolution. From the *S*/*R* ratios of different racemic omeprazole marketed products it was concluded that at 5 and 10 min dissolution time points there was a stereospecific drug release between the *S* and *R* enantiomers with the brands Ocid-20 and Omee ($p < 0.05$) but no stereospecificity was observed with Omez-20 ($p > 0.05$).

1.17.2. Pantoprazole

Tanaka *et al.* (1995) 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 and rac-lansoprazole 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.

Tanaka and Yamazaki (1996) reported a direct stereoselective reversed-phase HPLC method for the determination of the enantiomers of a proton pump inhibitor, pantoprazole (PAN), in human serum. The enantiomers were separated with high resolution on a cellulose-based chiral stationary phase (Chiralcel OJ-R) following on-line solid phase sample cleanup with a column-switching device. A mixture of acetonitrile and 50 mM sodium perchlorate was used as the mobile phase at a flow rate of 0.5 mL/min. Pantoprazole enantiomers were detected by monitoring the column effluent with UV light at a wavelength of 290 nm. The calibration curve for each enantiomer was linear from 0.1 to 5.0 μg/mL. Under these conditions, the determination of pantoprazole enantiomers in human serum can be achieved with satisfactory selectivity, sensitivity, precision, and accuracy. The described procedure is very simple and rapid since labor-intensive sample preparation is not required. The method was applied to the analyses of the serum samples obtained from a volunteer who received an 80 mg oral dose of racemic PAN. The samples showed the (+)/ (−) isomer ratios ranging from 0.74 to 1.03 up to 6h after dosing, indicating that there is only a small difference in the concentrations of (+)- and (−)-PAN.

Espinosa *et al.* (2007) have reported about omeprazole, a gastric acid pump inhibitor, dose-dependently controls gastric acid secretion; the drug has greater antisecretory activity than histamine H_2-receptor antagonists. Omeprazole has been determined in formulations and biological fluids by a variety of methods such as spectrophotometry, high-performance liquid chromatography with ultraviolet detection and liquid chromatography coupled with tandem mass spectrometry. The overview includes the most relevant analytical methodologies used in its determination since the origin still today.

Jiao *et al.* (2017) have reported the development and validation of an enantioselective LC-MS/MS method for the simultaneous determination of pantoprazole enantiomers in human plasma. Pantoprazole enantiomers and the internal standard were extracted from plasma using acetonitrile. Chiral separation was carried on a Chiralpak IE column using the mobile phase consisted of 10 mM ammonium acetate solution containing 0.1% acetic acid–acetonitrile

(28:72, *v*/v). MS analysis was performed on an API 4000 mass spectrometer. Multiple reactions monitoring transitions of *m/z* 384.1→200.1 and 390.1→206.0 were used to quantify pantoprazole enantiomers and internal standard, respectively. For each enantiomer, no apparent matrix effect was found, the calibration curve was linear over 5.00–10,000 ng/mL, the intra- and inter-day precisions were below 10.0%, and the accuracy was within the range of –5.6% to 0.6%. This method was applied to the stereoselective pharmacokinetic studies in human after intravenous administration of *S*- (–) -pantoprazole sodium injections. No chiral inversion was observed during sample storage, preparation procedure and analysis. While *R*- (+) -pantoprazole was detected in human plasma with a slightly high concentration, which implied that *S*-(–) -pantoprazole may convert to *R*-(+)-pantoprazole in some subjects.

Cass *et al.* (2002) have reported the use of multidimensional HPLC by coupling a RAM column with a chiral polysaccharide column to the analysis of pantoprazole in human plasma by direct injection. The enantiomers from the plasma samples were separated with high resolution on a tris-(3,5-dimethoxyphenylcarbamate) of amylose phase after clean-up by a RAM BSA octyl column. Water was used as solvent for the first 5 min in a flow-rate of 1.0 ml/min for the elution of the plasmatic proteins and then acetonitrile-water (35:65 v/v) for the transfer and analysis of pantoprazole enantiomers, which were detected by UV at 285 nm. Analysis time was 28 min with no time spent on sample preparation. A good linear relationship was obtained in the concentration range of 0.20 to 1.5 μg/mL for each enantiomer. Inter and intra-day precision and accuracy were determined by one low (0.24 μg/mL), one medium (0.70 μg/mL) and one high (1.3 μg/mL) plasma concentration and gave a C.V. varying from 1.80 to 8.43% and accuracy from 86 to 92%. Recoveries of pantoprazole enantiomers were in the range of 93.7-101.2%. The validated method was applied to the analysis of the plasma samples obtained from ten Brazilian volunteers who received an 80 mg oral dose of racemic pantoprazole and was able to quantify the enantiomers of pantoprazole in all clinical samples analyzed.

Ashour and Omar (2016) have reported a simple and rapid reversed-phase high-performance liquid chromatographic method for the direct determination of pantoprazole in pharmaceutical dosage forms. Lansoprazole was used as internal standard. The chromatographic separation of pantoprazole and lansoprazole was achieved on a Nucleodur C_8 column (250 × 4.6 mm i.d., 5 μm particle size) using the photodiode array detector at 280 nm. The optimized mobile phase

was consisted of a mixture of 0.1 M ammonium acetate solution and methanol (42:58, v/v), pumped at a flow rate 1.0 mL min^{-1}. The retention times for pantoprazole and lansoprazole were 8.10 and 11.15 min, respectively. Linearity range was 3.06–1243.0 μg mL^{-1} with limit of detection value of 0.78 μg mL^{-1}. The precision of the method was demonstrated using intra- and inter-day assay RSD% values which were less than 2.07%, while the recovery was 99.07–103.95%. No interference from any components of pharmaceutical dosage forms or degradation products was observed. According to the validation results, the proposed method was found to be specific, accurate, precise and could be applied to the quantitative analysis of pantoprazole in tablets.

El-Kommos *et al.* (2015) have reported a systematic comprehensive review of literature for the analysis of the most recent antiulcer drugs and proton pump inhibitors (PPIs) by different chromatographic and electrophoretic methods are presented. The review includes literature from 1985 until 2015 on eight proton pump inhibitors; namely: omeprazole (OMZ), lansoprazole (LAN), pantoprazole (PAN), rabeprazole (RAB), esomeprazole (EMZ), dexlansoprazole (DLAN), dexrabeprazole (DRAB), and tenatoprazole (TNB). The investigated literature survey covers most chromatographic and electrophoretic methods used for the assay of these drugs in pure forms, in combined mixtures, in pharmaceutical dosage forms, and in biological fluids.

Gosavi *et al.* (2006) have reported a simple, rapid, and reliable HPTLC method that has been established for determination of pantoprazole sodium sesquihydrate in tablets. Identification and determination were performed on aluminum-backed silica gel 60 F_{254} TLC plates previously washed with methanol. The mobile phase was methanol-water-ammonium acetate, 4 + 1 + 0.5 (v/*v*). Calibration plots were established the dependence of response (peak area) on the amount chromatographed. The spots were scanned at λ = 290 nm. The suitability of this HPTLC method for quantitative determination of compound was proved by validation in accordance with the requirements of pharmaceutical regulatory standards. The method was used for determination of the compound in commercial pharmaceutical dosage forms. The method is simple, reproducible and accurate and is a more effective option than other chromatographic techniques in routine quality control.

Altınöz and Süslü (2005) have reported a simple, sensitive, and selective square-wave voltammetric method that was developed for the determination of pantoprazole. The influence of the nature of the supporting electrolyte solution, pH, modulation amplitude, frequency, and scan increment was examined by square-wave voltammetric method for pantoprazole. The best results were obtained in Britton-Robinson buffer of pH 5.0. The peak currents were measured with hanging mercury drop electrode at −987 mV vs. Ag/AgCI. The calibration curve for pantoprazole was found as linear at a concentration range from 0.15 to 25.23 μg mL^{-1}. The limit of detection and the limit of quantification of pantoprazole were 0.048 μg mL^{-1} and 0.15 μg mL^{-1}, respectively. The validation parameters of the proposed method were evaluated. The method was applied to the pharmaceutical formulation and to both spiked plasma and the plasma of patients orally administered pantoprazole. A spectrophotometric method reported in the literature was utilized as a comparison method. There were no significant differences between the results obtained by the two methods.

1.17.3. Rabeprazole

Khashaba *et al.* (2017) studied the effect of adding transition metals to the electrolyte containing proton pump inhibitors, such as rabeprazole sodium (RAB sodium), on the voltammetric response of pencil graphite electrode. Both square-wave adsorptive stripping voltammetry (SWAdSV) and cyclic voltammetry (CV) were utilized to elucidate and confirm the possible complexation reaction that could occur between RAB sodium and cobalt as a transition metal. The current signal due to the oxidation process was a function of the concentration of RAB sodium, pH of the medium, cobalt concentration, scan rate, frequency, and deposition time at the electrode surface. This phenomenon could be used for the determination of RAB sodium using CV and SWAdSV. The oxidation peaks current linearly varied with the concentration over the range of 0.05–9 × 10^{-9} M and 0.2–8.5 × 10^{-7} M for SWAdSV and CV, respectively. The limits of detection were found to be 0.015 × 10^{-9}M and 0.06 × 10^{-7} M for SWAdSV and CV, respectively. The validity of using these methods for the determination of RAB sodium in its pharmaceutical formulation and human urine samples was evaluated.

Cao *et al.* (2016) have developed a sensitive, rapid and stable HPLC-MS/MS method and validated for the determination of rabeprazole enantiomers and their four metabolites in beagle dog plasma using esomeprazole as the internal standard. The analytes and the internal standard were extracted

from plasma samples by liquid–liquid extraction and separated on a Chiral-HSA column using acetonitrile 10 mmol L^{-1} ammonium acetate as the mobile phase by gradient elution. The method was validated with respect to sensitivity, specificity, linearity, precision, accuracy and especially the stability of analytes under various conditions, and was successfully applied in evaluating the pharmacokinetic profiles of racemic rabeprazole, the pure enantiomers and their metabolites in beagle dogs after single intravenous administration of (*R*)-rabeprazole sodium injection (at 0.33, 1 and 3 mg kg^{-1}), (*S*)-rabeprazole sodium injection (at 1 mg kg^{-1}) and racemic rabeprazole sodium injection (at 2 mg kg^{-1}). The two enantiomers showed different profiles of pharmacokinetic parameters. The AUC_{0-t} and $t_{1/2}$ values of (*R*)-rabeprazole were higher and the clearance (CL) value of (*R*)-rabeprazole was lower than that of (*S*)-rabeprazole. Compared to (*S*)-rabeprazole, the higher absorption and slower elimination of (*R*)-rabeprazole explain why (*R*)-rabeprazole is more effective than the racemate.

El-Kommos *et al.* (2015) developed different chromatographic and electrophoretic methods for analysis of proton pump inhibitors (PPIs). Here, they applied a systematic comprehensive review of literature for the analysis of the most recent antiulcer drugs and proton pump inhibitors (PPIs) by different chromatographic and electrophoretic methods are presented. The review included literature from 1985 until 2015 on eight proton pump inhibitors; namely: omeprazole (OMZ), lansoprazole (LAN), pantoprazole (PAN), rabeprazole (RAB), esomeprazole (EMZ), dexlansoprazole (DLAN), dexrabeprazole (DRAB), and tenatoprazole (TNB). The investigated literature survey coverd most chromatographic and electrophoretic methods used for the assay of these drugs in pure forms, in combined mixtures, in pharmaceutical dosage forms, and in biological fluids.

Simpemba *et al.* (2014) worked with simultaneous determination of rabeprazole and its two active metabolites in human urine by liquid chromatography with tandem mass spectrometry and its application in a urinary excretion study. Here, they described a simple and rapid liquid chromatography with tandem mass spectrometry method for the determination of rabeprazole and its two active metabolites, rabeprazole thioether and desmethyl rabeprazole thioether, in human urine using donepezil as the internal standard. The sample preparation procedure involved a simple dilution of urine sample with methanol (1:3, v/v). The chromatographic separation was achieved on a Hedera ODS-2 C_{18} column using a mixture of methanol/10

mmol/L ammonium acetate solution (containing 0.05% formic acid; 55:45, v/v) as the mobile phase. The method was validated over the concentration ranges of 0.15–100 ng/mL for rabeprazole, 0.30–400 ng/mL for rabeprazole thioether, and 0.05–100 ng/mL for desmethyl rabeprazole thioether. The established method was highly sensitive with a lower limit of quantification of 0.15 ng/mL for rabeprazole, 0.30 ng/mL for rabeprazole thioether, and 0.05 ng/mL for desmethyl rabeprazole thioether. The intra- and interbatch precision was <4.5% for the low, medium, and high quality control samples of all the analytes. The recovery of the analytes was in the range 95.4–99.0%. The method was successfully applied to a urinary excretion profiles after intravenous infusion administration of 20 mg rabeprazole sodium in healthy volunteers.

1.17.4. Salbutamol

Rosales-Conrado *et al.* (2013) optimized a direct chiral LC-UV method for the determination of salbutamol (SAL) β_2-agonist in environmental water. Two commercially available columns were evaluated: teicoplanin Chirobiotic-T™ (150 2.1 mm i.d., 5 mm) and vancomycin Chirobiotic-V™ (150 2.1 mm i.d., 5 mm). Finally, teicoplanin chiral stationary phase was selected for SAL enantiomer resolution. In order to preserve its integrity and maintain the column performance for longer time, the use of additives such as triethylamine (TEA) in the mobile phase was avoided. Experimental design was applied to simultaneously evaluate the influence of several parameters involved in enantiomer separation and to establish the conditions for acceptable resolution and performance in short analysis time. Optimum mobile phase was methanol–20 mM ammonium acetate buffer at pH 4.5 (98:2, v/v). A solid-phase extraction procedure for sample pre-concentration and clean-up allowed the determination of chiral SAL residues in natural water samples spiked at low concentrations in the range 1.0–20 ng/ mL. Reproducible recoveries, between 77 and 98%, were obtained and matrix effect was negligible. Injection of sample solutions at low elution strength permitted the SAL enantioresolution in the natural water complex matrix with satisfactory sensitivity and precision.

Halabi *et al.* (2004) developed a fast, reliable and specific method for the screening, confirmation, determination and quantitation of salbutamol enantiomers and validated. The described procedure includes a single robust chiral HPLC determination employing a teicoplanin stationary phase. The method was evaluated for specificity, robustness, linearity,

precision and accuracy. Under the chromatographic conditions of the method, known impurities were separated from the active principle.

Yang *et al.* (2012) presented a heart-cut two-dimensional high-performance liquid chromatography method for enantiomeric determination of salbutamol, salmeterol and atenolol in urine. It involves the use of two separations in a liquid chromatography–liquid chromatography achiral–chiral coupling. Target compounds were previously separated in a primary column (KinetexTM HILIC, 2.6 lm, 150 9 2.1 mm I.D.) with a mixture of methanol: acetonitrile: ammonium acetate buffer (5 mM, pH 6) 90:5:5 (v/v/v) as mobile phase at a flow rate of 0.40 mL min^{-1}. Enantiomeric separation was carried out by transferring peak of each compound through a switching valve to a vancomycin chiral column (ChirobioticTM V, 2.6 lm, 150 9 2.1 mm I.D.) using methanol: ammonium acetate buffer (2 mM, pH 4) 97:3 (v/v) as mobile phase at a flow rate of 0.50 mL min^{-1}. Ultraviolet detection was done at 227 nm. The method was applied to determine target analytes in urine samples after enzymatic hydrolysis with β-glucuronidase from helix pomatia, followed by a solid-phase extraction procedure using Isolute HCX mixed-mode cartridges. Extraction recoveries ranged from 82 to 90 % in urine samples. Detection limits were 0.091–0.095 lg for each enantiomer of atenolol and between 0.058 and 0.076 and 0.18–0.14 lg for enantiomers of salbutamol and salmeterol, respectively (3 mL of urine). Intraday and interday reproducibilities of enantiomeric ratio and enantiomeric fraction, expressed as relative standard deviation, were between 1.9 and 9.0 %.

Adams and Stewart (1993) developed a chiral high performance liquid chromatographic method for the simultaneous assay of *S* (+) and *R* (−) albuterol in human serum. The assay utilizes solid-phase extraction on a silica column as a sample clean-up step. The chiral separation was accomplished under isocratic conditions using a sumichiral OA 4700 column and a mobile phase consisting of 350:410:40:2 v/v/v/v hexane/methylene chloride/absolute methanol/trifluoroacetic acids at a flow rate of 1.0 mL/min. The enantiomers were measured using fluorescence detection set at 228 nm excitation and an emission filter of >280nm. Racemic atenolol was used as internal standard. Drug to internal standard peak height ratios were linear over a 2–20 ng/mL range for each enantiomer. The limit of detection of each analyte was 2.0 ng/mL (S/N = 3). The lowest quantifiable level of each enantiomer was 3 ng/mL.

Bernal *et al.* (1996) described a method to determine salbutamol sulphate and six impurities: 5-formyl-saligenin, salbutamol ketone, salbutamol bis ether, isopropyl salbutamol, desoxysalbutamol sulphate and salbutamol aldehyde, using reverse phase high performance liquid chromatography (RP-HPLC) with diode array detection (DAD). The best separation was achieved using a gradient of 0.1 M ammonium acetate pH 3.0 and acetonitrile. When the procedure was applied to the analysis of tablets and cough syrups, the versatility of the HPLC method was higher than one based on supercritical fluid chromatography (SFC). When using the later method, the excipient difficulted the identification and quantification of some compounds in cough syrup samples.

Gotti *et al.* (2000) stated a statistical experimental design that was used for the optimization and for robustness evaluation of a capillary electrophoretic method for the enantioresolution of salbutamol. Dermatan sulfate was used as chiral selector. The goal of the study was to obtain an efficient and fast separation. An eight-run Plackett–Burman matrix was used during the optimization process for the screening of the factors and to adjust the experimental domain under study. Response surface methodology was adopted after the screening phase to obtain information about how the factors percentage of chiral selector, pH and voltage affected the considered responses resolution and analysis time. The Derringer desirability function, which makes it possible to combine results obtained for properties measured on different scales, was used to simultaneously optimize the two responses. Robustness testing was carried out using a Plackett–Burman matrix. The method was found robust as regards the response resolution while voltage and chiral selector were found to be critical factors for the robustness of analysis time response. The proposed CE method permitted the complete enantioseparation of racemic salbutamol and was applied to its chiral resolution in spiked urine samples.

Caira *et al.* (1999) have isolated the (*R*)-enantiomer of albuterol via resolution of albuterol acetonide with (2*S*,3*S*)-di-*O*-benzoyl- or (2*S*,3*S*)-di-*O*-toluoyltartaric acid. The absolute configuration of the resolved acetonide was assessed by ^{1}H NMR analysis of its (*R*)-Mosher's ester, and confirmed by an X-ray crystal structure determination of the (*R*)-phenylethylurea derivative of the (*S*)-enantiomer.

Bergés *et al.* (1999) have developed liquid chromatographic procedure with fluorimetric detection for chiral separation and quantification of salbutamol enantiomers in urine samples. The extraction of free salbutamol from urine has been considered using liquid–liquid and solid-phase procedures. The effect of pH, salting-out effect and organic solvent has been studied in liquid–liquid extraction from aqueous and urine samples. For solid-phase extraction, different mechanisms (polar, non-polar, cation-exchange and interactions with a polymeric phase) have been tested and the effect of the urine matrix on the extraction recoveries has been considered. Bond-Elut Certify™ extraction cartridges provided the best specificity and good recoveries for salbutamol in urine. The sample was acidified, applied to the preconditioned cartridges and, after a washing step, salbutamol enantiomers were eluted with a mixture of chloroform and 2-propanol (80:20, v/v) containing 2% ammonia. Atenolol was used as external standard. Enantioselective separation was accomplished with a Chirex™3022 stationary phase (urea type silica-bonded chiral phase) using a mobile phase containing hexane–dichloromethane–methanol–trifluoroacetic acid (250:218:31:1, v/v) and fluorimetric detection with excitation and emission wavelengths set at 230 and 309 nm, respectively. The method proposed was rapid, selective and sensitive, and found to be useful to differentiate between an authorized and a prohibited use of the drug in doping control.

Kim and Kim (1998) have developed a stereospecific HPLC method for the resolution of the enantiomers of salbutamol in human urine. After solid-phase extraction and derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate, the diastereomeric derivatives were resolved (Rs=1. 83) on 5 μm octadecylsilane column using 35% acetonitrile in 0.05M ammonium acetate buffer (pH=6) as a mobile phase with electrochemical detection. The diastereomeric derivatives were formed within 30 min. The detection limit of each enantiomer was 20 ng/ml (S/N=3).

Aboul-enein and Serignese (1995) described a direct, isocratic, and simple chromatographic method for the resolution of racemic albuterol using the α_1-acid glycoprotein chiral stationary phase (AGP-CSP) under reverse phase conditions. The effect of various organic modifiers, temperature, and phosphate buffer ionic strength on the separation factor (α) and stereochemical resolution factor (R_s) has been studied. The enantiomeric separation of albuterol was also achieved using a urea-type CSP of (*S*)-indoline-2-carboxylic acid and (*R*)-1-(α-naphthyl)

ethylamine, known as Chirex 3022, running in the normal phase mode. The effect of different organic acids added to the mobile phase was examined and the chiral recognition mechanism was discussed. Solid phase extraction with C_{18} Sep-Pak cartridges was applied as a clean-up step to determine the enantiomeric ratio between (−) -*R* and (+)-*S*-albuterol in pharmaceutical formulations and in human plasma.

1.17.5. Ibuprofen

Valderrama *et al.* (2009) described the determination of ibuprofen (IBU) enantiomers by chiral high-performance liquid chromatography. The methodology was based on chiral recognition of ibuprofen by a chiral column based on cellulose tris-(4-methylbenzoate) coated on silica gel (Chiralcel OJ-H). The mobile phase was *n*-hexane–2- propanol–trifluoroacetic acid (98:2:0.1, v/v/v). The flow rate was 1.0 mL/min, and UV detection was 254 nm. The samples of ibuprofen were prepared in *n*-hexane in the concentration range 50-100% of (S)-IBU 1×10^{-3} mol/L. Calibration and validation method were performed with six and nine samples, respectively. Goodness-of-fit measures represented by correlation coefficient, y-intercept, and slope of the regression line were 0.9836, 21373, 2162, respectively. Average of the relative error of the proposed method was 3.0%, 0.9% (S)-IBU selectivity, and 2162% (S)-IBU-1 sensitivity. The minimum concentration difference between two samples that could be determined in the linear dynamic range was 0.4% (S)-IBU. Limits of detection and quantification were 8.1 and 27.0% (S)-IBU, respectively. These results indicate that the proposed method can be employed for determination of the enantiomeric composition of IBU.

Oliveira *et al.* (2005) developed a simple and rapid solid-phase microextraction method for the enantioselective analysis of ibuprofen in urine. The sampling was made with a polydimethylsiloxane-divinylbenzene coated fiber immersed in the liquid sample. After desorptioning from the fiber, ibuprofen enantiomers were analyzed by HPLC using a Chiralpak AD-RH column and UV detection. The mobile phase was made of methanol-pH 3.0 phosphoric acid solution (75:25, v/v), at a flow rate of 0.45 mL/min. The mean recoveries of SPME were 19.8 and 19.1% for (-)-*R*-ibuprofen and (+)-(*S*)-ibuprofen, respectively. The method was linear at the range of 0.25-25 microg/mL. Within-day and between-day assay precision and accuracy were below 15% for both ibuprofen enantiomers at concentrations of 0.75, 7.5 and 20

microg/mL. The method was tested with urine quality control samples and human urine fractions after administration of 200 mg rac-ibuprofen.

Zheng *et al.* (2008) developed a rapid, sensitive and stereoselective HPLC method based on chiral column analysis and fully validated for the simultaneous determination of the two enantiomers of ibuprofen in human plasma. Using this method, a chiral pharmacokinetic study of two different ibuprofen tablets, i.e. dexibuprofen tablets and racemic ibuprofen tablets, was carried out on 20 healthy Chinese male volunteers according to a single-dose (400 mg), two-way, cross-over randomized design. When a non-chiral calculation method' was used, the statistical analysis showed no significant difference for the pharmacokinetic parameters ($AUC_{0-\infty}$, AUC_{0-t}, C_{max}, and t_{max}) between the two oral formulations, suggesting that they were pharmaceutically bioequivalent. Considering that the pharmacological activity of ibuprofen resides exclusively in the *S*- (+)-enantiomer, and that the unidirectional inversion of the *R*- (−) to the *S*-(+)-enantiomer is incomplete and might be race-dependent, the pharmacokinetic parameters for only the *S*-(+)-enantiomer were further compared and the inversion ratio calculated. It was found that only 25% of *R*- (−)-ibuprofen in the racemic ibuprofen tablets was inverted into *S*- (+)-ibuprofen in the Chinese population, which suggested that dexibuprofen might possess a much stronger pharmacological activity than that of racemic ibuprofen when administered at the same dose.

Gowramma *et al.* (2011) developed a simple and precise validated method for the separation of ibuprofen enantiomers. In this study, direct method utilizing a chiral stationary phase (CSP) was used for the separation of ibuprofen enantiomers. Enantiomeric separation was achieved on Lux 5µ cellulose 1 column as stationary phase and mobile phase consisting of perchloric acid (pH 2) and acetonitrile in the ratio (50:50, v/v) at a flow rate of 1.0 ml/min and UV detection at 254 nm. Sustained release tablets of ibuprofen enatiomers were formulated by wet granulation method, the percentage release of the formulation was carried on dissolution apparatus using phosphate buffer pH 6.8 and quantified. The retention times of (*R*)- and (*S*)- enantiomers were 6.3 and 10.4 min, respectively. Linearity was performed in the range of 0.5-3.0 µg/ml.

Szeitz *et al.* (2010) developed a modified ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method and validated for the quantitation of ibuprofen

enantiomers in human plasma. Ibuprofen and flurbiprofen (internal standard) were extracted from human plasma at acidic pH, using a single-step liquid-liquid extraction with methyl-tert-butyl ether. The enantiomers of ibuprofen and flurbiprofen were derivatized to yield the corresponding diastereomers. Chromatographic separation was achieved using a phenyl column with a run time of 20 min. The method was validated for accuracy, precision, linearity, range, limit of quantitation (LOQ), limit of detection (LOD), selectivity, absolute recovery, matrix effect, dilution integrity, and evaluation of carryover. Accuracy for (*R*)-ibuprofen ranged between -11.8% and 11.2%, and for (*S*)-ibuprofen between –8.6% and –0.3%. Precision for (*R*)-ibuprofen was ≤ 11.2%, and for (*S*)-ibuprofen ≤ 7.0%. The calibration curves were linear with r^2 for (*R*)-ibuprofen ≥ 0.988 and for (*S*)-ibuprofen ≥ 0.990. The range of the method was 50 to 5000 ng/mL with the LOQ of 50 ng/mL, and LOD of 1ng/mL, for (*R*)- and (*S*)-ibuprofen requiring 100 µL of sample. The method was applied successfully to a pharmacokinetic study with the administration of a single oral dose of ibuprofen capsules to human subjects.

Awad *et al.* (2012) developed a high-performance liquid chromatography–diode array detector (HPLC-DAD) method and validated for the quantitation of dexibuprofen in dexibuprofen tablets using ovomucoid chiral stationary phase (Ultron ES-OVM). The mobile phase was composed of 0.025 M potassium phosphate dibasic (pH 4.5)-methanol-ethanol (85:10:5 v/v/v). The method was validated for specificity, linearity, range, accuracy, precision and robustness. The method was enantiomerspecific for the determination of dexibuprofen [*S*-(+)-isomer ibuprofen] in the presence of *R*-isomer ibuprofen in bulk drug, pharmaceutical dosage form and under stress degradation. The method was linear over the range 15–35 mg/mL with r 2 = 0.9995; accuracy and precision were acceptable with %RSD < 2.0%. The method was found to be specific, precise, accurate, robust and stability-indicating, and can be successfully applied for the routine analysis of dexibuprofen in bulk drug and pharmaceutical dosage form.

Przejczowska-Pomierny *et al.* (2017) developed a direct fluorometric high performance liquid chromatography (HPLC) method and validated for the analysis of ibuprofen enantiomers in mouse plasma (100 µl) and tissues (brain, liver, kidneys) using liquid–liquid extraction and 4-tertbutylphenoxyacetic acid as an internal standard. Separation of enantiomers was accomplished in a Chiracel OJ-H chiral column based on cellulose tris(4-methylbenzoate) coated on 5 µm silica gel, 250 x 4.6 mm at 22 °C with a mobile phase composed of *n*-hexane, 2-

propanol, and trifluoroacetic acid that were delivered in gradient elution at a flow rate of 1 ml min^{-1}. A fluorometric detector was set at: λexcit. = 220 nm and λemis. = 290 nm. Method validation included the evaluation of the selectivity, linearity, lower limit of quantification (LLOQ), within run and between run precision and accuracy. The LLOQ for the two enantiomers was 0.125 μg ml^{-1} in plasma, 0.09 μg g^{-1} in brain, and 0.25 μg g^{-1} in for liver and kidney homogenates. The calibration curves showed good linearity in the ranges of each enantiomers: from 0.125 to 35 μg ml^{-1} for plasma, 0.09–1.44 μg g^{-1} for brain, and 0.25–20 μg g^{-1} for liver and kidney homogenates. The method was successfully applied to a pharmacokinetic study of ibuprofen enantiomers in mice treated i.v. with 10 mg kg^{-1} of racemate.

Ferrari *et al.* (2016) presented a thermodynamic study of racemic ibuprofen separation by liquid chromatography using cellulose-based stationary phase. They studied the effect of temperature change (from 288.15 to 308.15^{o}K) on the ibuprofen resolution was studied. A column (mm) packed with tris (3,5-dimethylphenylcarbamate) was used to obtain the thermodynamic parameters, such as enthalpy change, entropy change, variation enthalpy change, variation entropy change, and isoenantioselective temperature. The mobile phase was a combination of hexane (99%), isopropyl alcohol (1%), and TFA (0.1%), as an additive. The conditions led to a selectivity of 1.20 and resolution of 4.55. The first peak, *R*-(−)-ibuprofen, presented an enthalpy change of 7.21 kJ/mol and entropy change of 42.88 kJ/K·mol; the last peak, *S*-(+)-ibuprofen, has an enthalpy change of 8.76 kJ/mol and 49.40 kJ/K·mol of entropy change.

1.17.6. Ofloxacin

Sun *et al.* (2009) developed a new chiral liquid-chromatographic method for the enantiomeric separation of ofloxacin with a fluorescence detector. The enantiomers of ofloxacin were baseline resolved on a Chiralcel OD-H (250 mm x 4.6 mm, 5 microm) column using a mobile-phase system containing hexane-ethanol-methanol-acetic acid-diethylamine (70/20/10/0.45/0.05, v/v/v/v/v). The presence of diethylamine in the mobile phase has played an important role in enhancing the chromatographic efficiency and resolution between enantiomers.

Fang *et al.* (2013) performed a direct semi-preparative high performance liquid chromatography (HPLC) enantioseparation of ofloxacin on chemically immobilized cyclodextrin derivative–mono (6A-azido-6A-deoxy)-per(p-chlorophenyl carbamoylated) β-CD chiral stationary phase. Conditions for semi-preparative separations were established using a 250 × 4.6 mm i.d. column and subsequently extended to a 250 × 10.0 mm i.d. column that enabled separations on a milligram scale. Optimization of the chromatographic conditions (mobile phase and column load) with respect to better efficiency, resolution and peak retention resulted in a system capable of separating up to 304 mg of (–) -(*S*)-ofloxacin and 56 mg of (+) -(*R*)-ofloxacin of the racemate over 6 h. The purities of the separated enantiomers were determined by HPLC. Moreover, both separated enantiomers were characterized by mass spectrometry; then, the absolute configuration of the products was clearly confirmed by polarimetry.

Dat *et al.* (2015) developed a ligand exchange HPLC method for enantioseparation of ofloxacin enantiomers by a using C_8 stationary phase. Chromatographic separation was performed on a Zorbax-300SB-C_8 column eluted with the mobile phase consisting of 15 % methanol in water containing 10mM phenylalanin and 5 mM $CuSO_4$ at flow rate of 0.8 mL/min. The method was simple, rapid and suitable for routine analytical studies of ofloxacin enantiomers.

Bi *et al.* (2011) developed a simple and accurate method for the separation and determination of ofloxacin enantiomers by ionic liquid-assisted ligand-exchange high performance liquid chromatography. Both achiral and chiral ionic liquids were tested for their efficiency of ofloxacin enantiomeric separation. The effects of ligands, concentration of metal ion, organic modifier, pH of the mobile phase, and temperature were also investigated and evaluated. Optimal conditions were obtained on a conventional C_{18} column, where the mobile phase consisted of methanol/water (20:80, v/v) (containing 4.0 mmol L^{-1}amino acid ionic liquid and 3.0 mmol L^{-1} copper sulfate) at a flow rate of 0.5 mL min^{-1}. Under this condition, the ofloxacin enantiomers could be baseline separated within 14 minutes; the proposed method was used to analyze different commercial ofloxacin medicines.

Salama *et al.* (2014) described a novel economic thin-layer chromatographic procedure for stereoselective separation of racemic mixtures of each of zopiclone and ofloxacin, and

determination of their enantiomers: eszopiclone, (+)-(*S*)-zopiclone, and levofloxacin, (−)-(*S*)-ofloxacin. The method was based on using normal plates and hydroxypropyl-β-cyclodextrin (HP-β-CD) as chiral mobile phase additive (CMPA). The spots were detected under UV lamp 254 nm, followed by densitometric measurements at 304 and 330 nm for (+)-(*S*)-zopiclone and (−)-(*S*)-ofloxacin, respectively. The mobile phase enabling successful resolution of the drugs was ethanol–acetonitrile–glacial acetic acid–diethylamine–distilled water containing 0.5% HP-β-CD (4:2:3:1:1, by volume), pH 4, for zopiclone and ethanol: acetonitrile: glacial acetic acid: diethylamine: distilled water containing 0.3% HP-β-CD (4:4:3:2:1 by volume), pH 4.5, for ofloxacin at 25 ± 2°C. All variables affecting the resolution, such as concentration of different chiral selectors, temperatures and pH, were investigated, and the conditions were optimized. Furthermore, some thermodynamic parameters were calculated. The procedure provided a linear response over the concentration range of 1-4 and 2-7 μg $spot^{-1}$ for determination of pure active isomers, (+)-(*S*)-zopiclone and (−)-(*S*)-ofloxacin, respectively, with acceptable precision (relative standard deviation [% RSD]).

Yan and Row (2007) developed a sensitive, simple, and accurate method for determination of levofloxacin and its (*R*)-enantiomer to determine the chiral impurity of levofloxacin in Cravit tablets material by ligand-exchange high performance liquid chromatography. The effects of different kinds of ligands, concentration of ligands in mobile phase, organic modifier, pH of mobile phase, and temperature on enantioseparation were investigated and evaluated. Chiral separation was performed on a conventional C_{18} column, where the mobile phase consisted of a methanol–water solution (containing 10 mmol L^{-1} L-leucine and 5 mmol L^{-1} copper sulfate) (88:12, v/v) and its flow-rate was set at 1.0 mL min^{-1}. The conventional C_{18} column offered baseline separation of two enantiomers with a resolution of 2.4 in less than 20 min. Thermodynamic data ($\Delta\Delta H$ and $\Delta\Delta S$) obtained by *Van't Hoff* plots revealed the chiral separation as an enthalpy-controlled process. The standard curves showed excellent linearity over the concentration range from 0.5 to 400 mg L^{-1} for levofloxacin and its (*R*)-enantiomer. The linear correlation equations are: $y = 1.33 \times 10^5 x + 6297$ ($r = 0.9991$) and $y = 1.34 \times 10^5 x + 3565$ ($r = 0.9997$), respectively. The relative standard deviation (RSD) of the method was below 2.3% ($n = 3$).

Liang *et al.* (2015) developed a chiral ligand-exchange high-performance liquid chromatography method for the enantioseparation of ofloxacin and its six related substances termed impurities A, B, C, D, E, and F. The separation was performed on a conventional C18 column. Different organic modifiers, copper salts, amino acids, the ratio of Cu^{2+} to amino acid, pH of aqueous phase, and column temperature were optimized. The optimal mobile phase conditions were methanol-water systems consisting of 5 mmol/L copper sulfate and 10 mmol/L L-isoleucine (L-Ile). Under such conditions, good enantioseparation of ofloxacin and impurities A, C, E, and F could be observed with resolutions (*RS*) of 3.54, 1.97, 3.21, 3.50, and 2.12, respectively. On the relationship between the thermodynamic parameters and structures of analytes, the mechanism of chiral recognition was investigated. It was concluded that ofloxacin and impurities A, C, E, and F were all enthalpically driven enantioseparation and that low column temperature was beneficial to enantioseparation.

Yan and Row (2007) developed a sensitive and simple method for the determination of ofloxacin enantiomers in human urine by ligand exchange high performance liquid chromatography. Chiral separation was performed on a C_{18} column, where the mobile phase consisted of a methanol-water solution (containing 1.2 mmol L^{-1} L-phenylalanine and 1.0 mmol L^{-1} copper sulphate) (15:85, v/v) and its flow rate was set at 1.0 mL min^{-1}. After centrifugation, the human urine was injected into a C_{18} column directly. Baseline separation of ofloxacin enantiomers in human urine were obtained with a resolution of 3.24 in less than 25 min, and no interference by the protein or endogenous compounds were observed. The effects of different separation conditions were investigated and the concentration of ligand and pH of the mobile phase play a critical role in the enantioseparation. The standard curves showed excellent linearity over the concentration range from 0.8 to 400 µg mL^{-1} for ofloxacin enantiomers. The linear correlation equations are: Y=171.11X+ 504.13 (r=0.9995) and Y=169.01X+631.59 (r=0.9994) for (*S*)-ofloxacin and (*R*)-ofloxacin, respectively. The average recovery of ofloxacin enantiomers from human urine samples was more than 96%. The procedure developed was successfully applied to investigate the stereoselectivity and pharmacokinetics of ofloxacin enantiomers in the human body.

Garcia *et al.* (2002) described a sensitive HPLC method with fluorescence detection for the analysis of ofloxacin (OFL) enantiomers in plasma samples. Plasma samples were prepared by

adding phosphate buffer (pH 7.4), then extracted with trichloromethane. *S*-OFL and *R*-OFL were separated on a reversed-phase column with water-methanol, 85.5∶14.5, as mobile phase. The concentrations of *S*-OFL and *R*-OFL eluting from the column (retention times 7.5 and 8.7 min, respectively) were monitored by fluorescence detection with λex = 331 and λem = 488 nm. The detection and quantitation limits were 10 and 20 ng mL−1, respectively, for *S*-OFL and 11 and 21 ng mL^{-1} for *R*-OFL. Response was linearly related to concentration in the range 10 to 2500 ng mL^{-1}. Recovery was close to 93% for both compounds. The method was applied to determination of the enantiomers of OFL in plasma samples collected during pharmacokinetic studies.

Azzam *et al.* (2010) described a capillary electrophoretic method for the separation of the enantiomers of both ofloxacin and ornidazole. Several parameters affecting the separation were studied, including the type and concentration of chiral selector, buffer pH, voltage and temperature. Good chiral separation of the racemic mixtures was achieved in less than 16 min with resolution factors Rs=5.45 and 6.28 for ofloxacin and ornidazole enantiomers, respectively. Separation was conducted using a bare fused-silica capillary and a background electrolyte (BGE) of 50 mM H_3PO_4:1M tris solution; pH 1.85; containing 30 mg mL^{-1} of sulfated-beta-cyclodextrin. The separation was carried out in reversed polarity mode at 25 degrees C, 18 kV, detection wavelength at 230 nm and using hydrodynamic injection for 15 s. Acceptable validation criteria for selectivity, linearity, precision, and accuracy were studied. The limits of detection (LOD) and limits of quantitation (LOQ) of the enantiomers (ofloxacin enantiomer 1 (OF-E1), ofloxacin enantiomer 2 (OF-E2), ornidazole enantiomer 1 (OR-E1) and ornidazole enantiomer 2 (OR-E2)) were (0.52, 0.46, 0.54, 0.89) and (1.59, 1.40, 3.07, 2.70) microg mL^{-1}, respectively. The proposed method was successfully applied to the assay of enantiomers of both ofloxacin and ornidazole in pharmaceutical formulations. The computational calculations for the enantiomeric inclusion complexes rationalized the reasons for the different migration times between the ofloxacin and ornidazole enantiomers.

See *et al.* (2009) have developed a simple, rapid and validated capillary electrophoretic method for the separation and determination of ofloxacin and ornidazole in pharmaceutical formulations with detection at 230 nm. Optimal conditions for the quantitative separations were investigated. Analysis times shorter than 4 min were obtained using a background electrolyte solution

consisting of 25 mmol/L phosphoric acid adjusted with 1M Tris buffer to pH 8.5, with hydrodynamic injection of 5 s and 20 kV separation voltages. The validation criteria for accuracy, precision, linearity and limits of detection and quantitation were examined and discussed. An excellent linearity was obtained in concentration range 25-250 µg/mL. The detection limits for ofloxacin and ornidazole were 1.03 ± 0.11 and 1.80 ± 0.06 µg/mL, respectively. The proposed method has been applied for the analysis of ofloxacin and ornidazole both individually and in a combined dosage tablet formulation. The proposed validated method showed recoveries between 96.16 and 105.23% of the nominal contents.

1.17.7. Carvedilol

Lamprecht *et al.* (2002) have developed an HPLC column-switching method and validated for the enantioselective determination of (*R*)- and (*S*)-carvedilol in human plasma. Sample preparation was performed either off-line, by extraction with trichloromethane or back-extraction into 0.01M aqueous citric acid which was injected on to a LiChrosorb RP_8 column, or on-line, by injecting diluted (0.1M formic acid) plasma on to a LiChrosorb ADS column. In both instances separation was performed by gradient elution and on-line transfer of the fraction containing, the carvedilol on to an enantioselective Teicoplanin column. The enantiomers of carvedilol were separated isocratically by use of methanol-acetonitrile-triethylammonium acetate, 70:30:0.05 (v/v/w), as mobile phase. With fluorescence detection, the limits of quantitation were 0.30 ng mL^{-1} for (*R*)-carvedilol and 0.26 ng mL^{-1} for (*S*)-carvedilol; these were sufficient to enable investigation of the effect of exercise on plasma concentrations of (*R*)- and (*S*)-carvedilol after oral administration of either the racemate or the pure enantiomers. Although the operating conditions were optimized for sample preparation by on-line deproteination on a LiChrospher RP_{18} ADS column, the complete method was insufficiently rugged for analysis of large numbers of plasma samples—the enantioselectivity of the Teicoplanin column deteriorated too rapidly because of the transfer of enantioselectivity-poisoning interferences which could not be suppressed sufficiently. In contrast the liquid-liquid sample-extraction procedure combined with column switching resulted in an analytical method with long-term stability.

Poggi *et al.* (2012) have describer that their method involves plasma extraction with diisopropyl ether using metoprolol as internal standard and direct separation of the carvedilol enantiomers

on a Chirobiotic T1 (Teicoplanin) column. Protonated ions and their respective ion products were monitored at transitions of 407 > 100 for the carvedilol enantiomers and 268 > 116 for the internal standard. The quantification limit was 0.2 ng for both enantiomers in plasma. The method was applied to study enantioselectivity in the pharmacokinetics of carvedilol administered as a single dose of 25 mg to a hypertensive patient. The results showed a higher plasma concentration of (*R*)-carvedilol (AUC0–1 205.52 vs. 82.61 (ng h)), with an enantiomer ratio of 2.48.

Satto *et al.* (2006) presented a highly sensitive HPLC method for enantioselective determination of carvedilol in human whole blood and plasma. Carvedilol and *S*-carazolol as an internal standard extracted from whole blood or plasma were separated using an enantioselective separation column (Chiralpak AD column) without any chiral derivatizations. The mobile phase was hexane: isopropanol: diethylamine (78:22:1, v/v). The excitation and emission wavelengths were set at 284 and 343 nm, respectively. The limits of quantification for the *S*(-)- and *R*-(+)-carvedilol enantiomers in plasma and blood were both 0.5 ng/ml. Intra- and inter-day variations were less than 5.9%. As an application of the assay, concentrations of carvedilol enantiomer in plasma and blood samples from 15 patients treated with carvedilol for congestive heart failure were determined.

Peccinini *et al.* (2008) developed an enantioselective high-performance liquid chromatographic method for the analysis of carvedilol in plasma and urine and validated using (-)-menthyl chloroformate (MCF) as a derivatizing reagent. Chloroform was used for extraction, and analysis was performed by HPLC on a C_{18} column with a fluorescence detector. The quantitation limit was 0.25 ng/ml for *S*-(-)-carvedilol in plasma and 0.5 ng/ml for *R*- (+)-carvedilol in plasma and for both enantiomers in urine. The method was applied to the study of enantioselectivity in the pharmacokinetics of carvedilol administered in a multiple dose regimen (25 mg/12 h) to a hypertensive elderly female patient. The data obtained demonstrated highest plasma levels for the *R*-(+)-carvedilol (AUC(SS) (0-12) 75.64 vs 37.29 ng/ml). The enantiomeric ratio *R*-(+)/*S*-(-) was 2.03 for plasma and 1.49 for urine (Ae (0-12) 17.4 *vs* 11.7 µg).

Taraji *et al.* (2015) have developed a sensitive, selective and simple method for the simultaneous determination of carvedilol enantiomers in aqueous solution using stir bar sorptive extraction (SBSE) followed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. This method was based on the reaction of carvedilol enantiomers with (–)-menthyl chloroformate (MCF) after extraction by the SBSE method to produce diastereomeric derivatives. The separation was achieved by use of a C_{18} analytical column and the influence of mobile phase composition on the enantioseparation of carvedilol was studied. The applicability of two sorptive phases, poly (methyl methacrylate/ethyleneglycol dimethacrylate) (PA–EG) and polydimethylsiloxane, were tested for extraction of carvedilol enantiomers from aqueous samples. The obtained results showed excellent linear dynamic ranges and precisions for each of them. The least limit of detection for (*S*)- and (*R*)-carvedilol obtained 8 and 11 mg, respectively, using the PA–EG sorptive phase. Inter- and intra-mean recoveries were also satisfactory, ranging from 98 to 103%, with coefficient of variation in the range of 1–5% at three fortified levels using a PA–EG coated stir bar. The proposed SBSE (PA–EG)–MCF derivatization–HPLC–UV method was successfully applied to enantioselective analysis of carvedilol in water and pharmaceutical dosages, confirming the application of this method.

Swetha *et al.* (2015) developed a simple chiral HPLC method and validated for quantification of S-(-)-carvedilol in active pharmaceutical ingredient (API) and marketed tablet formulation of racemic carvedilol. Chiral resolution of enantiomers of carvedilol was achieved on Phenomenex Lux-cellulose–4 (250 mm × 4.6 mm; 5 µ particle size) chiral column by using a mobile phase, isopropanol and *n*-heptane (60:40 v/v), at a flow rate of 1.0 ml/min and by employing UV detection at 254 nm wavelength. The method was validated according to the ICH guidelines and was proved to be specific, linear, precise and accurate for the analysis of *S*-(-)-carvedilol.

Zakrzewski-Jakubiak *et al.* (2010) developed a simple, specific, sensitive, inexpensive and rapid HPLC method for enantioselective quantification of carvedilol in human plasma. *S*-(−)- and *R*-(+)-carvedilol and *R*-(+)-propranolol as the internal standard were extracted from human plasma by liquid–liquid extraction using methyl *tert*-butyl ether. Enantioseparation was performed on a reverse-phase C_{18} Phenomenex Luna 5 micron 150 mm × 2 mm column after chiral derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate. The mobile phase

was a mixture of water and acetonitrile. The peaks were detected using a fluorescence detector, where the excitation and emission wavelengths were set at 242 and 344 nm, respectively. The limits of quantification for the *S*-(−)- and *R*-(+)-carvedilol enantiomers were both 0.5 ng/ml. Combined intra- and inter-day variations for both enantiomers were less than 8.3%. The combined accuracy for both enantiomers ranged from 91.7 to 104.7%. This method was used to assay the carvedilol enantiomers in human plasma samples obtained from heavily medicated heart failure patients within the framework of a clinical trial.

1.17.8. Propranolol

Alanazi *et al.* (2014) developed a stereoselective high-performance liquid chromatographic (HPLC) method and validated to determine *S*-(−)- and *R*-(+)-propranolol in rat serum. Enantiomeric resolution was achieved on cellulose tris-(3,5-dimethylphenylcarbamate) immobilized onto spherical porous silica chiral stationary phase (CSP) known as Chiralpak IB. A simple analytical method was validated using a mobile phase consisted of n-hexane-ethanol-triethylamine (95:5:0.4%, v/v/v) at a flow rate of 0.6 mL/min and fluorescence detection set at excitation/emission wavelengths 290/375 nm. The calibration curves were linear over the range of 10-400 ng/mL ($R = 0.999$) for each enantiomer with a detection limit of 3 ng/ mL. The proposed method was validated in compliance with ICH guidelines in terms of linearity, accuracy, precision, limits of detection and quantitation, and other aspects of analytical validation. Actual quantification could be made for propranolol isomers in serum obtained from rats that had been intraperitoneally (i.p.) administered a single dose of the drug. The proposed method established in this study is simple and sensitive enough to be adopted in the fields of clinical and forensic toxicology. Molecular modeling studies including energy minimization and docking studies were first performed to illustrate the mechanism by which the active enantiomer binds to the β-adrenergic receptor and second to find a suitable interpretation of how both enantiomers are interacting with cellulose tris(3,5-dimethylphenylcarbamate) CSP during the process of resolution. The latter interaction was demonstrated by calculating the binding affinities and interaction distances between propranolol enantiomers and chiral selector.

Morante-Zarcero and Sierra (2012) compared the enantioselectivities of β-blockers (propranolol, metoprolol, atenolol and pindolol) on four different types of chiral stationary phases (CSPs): Chiralpak AD-H, Lux Cellulose-1, Chirobiotic T and Sumichiral OA-4900

using polar organic (PO) elution mode and normal phase (NP) elution mode. Method optimizations were demonstrated by modifying parameters such as organic modifier composition (ethanol, 2-propanol and acetonitrile) and basic mobile phase additives (triethylamine, diethylamine, ethanolamine, and buthylamine). In normal phase elution mode with Lux Cellulose-1, the four pairs of enantiomers can be separated in the same run in gradient elution mode. Additionally, a simple chiral HPLC–DAD method using a newly commercialized polysaccharide-based CSP by Phenomenex (Lux Cellulose-1) in NP elution mode for enantioselective determination of propranolol in water samples by highly selective molecularly imprinted polymers extraction was validated. The optimized conditions were a mobile phase composed by *n*-hexane/ethanol/DEA (70/30/0.3, v/v/v) at a flow rate of 1.0 mL min^{-1} and 25 °C. The method was selective, precise and accurate and was found to be linear in the range of 0.125–50 μg/mL (R^2 > 0.999) with a method detection limit (MLD) of 0.4 μg/mL for both enantiomers. Recoveries achieved with both enantiomers ranged from 97 to 109%.

Chen *et al.* (2008) describe the direct semipreparative resolution of racemic (rac)-propranolol hydrochloride by high-performance liquid chromatography using cellulose tris(3,5-dimethylphenylcarbamate) as chiral stationary phase and mobile phase systems containing petroleum ether and 2-propanol with the use of basic additives. At analytical scale, the retention factor of both enantiomers was less than 5 with the separation factor 1.95 and the resolution 2.4. Then, the analytical method was scaled up to semipreparative loading to obtain small amounts of both propranolol hydrochloride enantiomers. Petroleum ether, rather than *n*-hexane, was used to effectively reduce the production cost of (*R*)- and (*S*)-propranolol. To increase the throughput, overlapping injections were used, allowing an injection to be made every 6.5 min instead of every 12 min. At semipreparative scale, approximately 19 mg/h enantiomers were isolated. The first fraction [(*R*)-(+)- propranolol hydrochloride] was isolated with a purity of > 99.6% (e.e.) and > 97.0% yield, and the second [(*S*)-(–)-propranolol hydrochloride] was isolated with a purity of > 99.3% (e.e.) and > 95.0% yield. In addition, optical rotations of both propranolol hydrochloride enantiomers isolated were investigated.

Santoro *et al.* (2001) described a validated direct liquid chromatographic chiral methods for enantiomeric separation and quantitative determination of clinically significant β-blocking agent, propranolol. A liquid chromatographic method was validated and applied for enantiomeric determination of propranolol enantiomers in pharmaceutical formulations.

Separation were obtained in polar organic mode on a α-Burke 2® chiral stationary phase (250 x 4.6 mm, 5µm) with mobile phase composed of dichloromethane: methanol (90:10 v/v), along with 12 mM of ammonium acetate, at a flow rate of 0.9 mL/min. Detection was made by ultraviolet absorption at 280 nm. In all cases the run time was less than 10 min. The correlation coefficient for linear regression curves of *R*-propranolol and *S*-propranolol were 0.9995 and 0.9998 respectively. The intra-day precision, expressed as RSD was less than 2%. The accuracy determined by average recovery of *R*-propranolol and *S*-propranolol from sample matrices were 97.3% and 100.1% in commercial sample and 99.5% and 100.4% in simulated samples, respectively. Excellent levels of limit of detection (mean value = 1.34 ng) and limit of quantitation (mean value = 4.47 ng), along with rapid elution time of both enantiomers, made the method useful for routine enantiomeric quality control applications.

Bhushan and Nagar (2014) synthesized the diastereomers of three β-blockers (orciprenaline, betaxolol, and propranolol) using three new chiral derivatizing reagents that were prepared by substituting one fluorine atom in 1,5-difluoro-2,4-dinitrobenzene with two chirally pure amines and one L-amino acid. The reagents were characterized using ultraviolet-visible spectroscopy, infrared spectroscopy, elemental analysis, and proton nuclear magnetic resonance. Diastereomers were synthesized under microwave irradiation at 50 s at 80% of 800 W and also by stirring for 50 min at 45°C. The diastereomers were separated by reversed-phase high-performance liquid chromatography on a C_{18} column with detection at 340 nm using acetonitrile and aqueous trifluoroacetic acid as the mobile phase components. The conditions of derivatization and chromatographic separation were optimized. The method was validated for accuracy, precision, limit of detection, and limit of quantification.

Tamai *et al.* (1990) presented the chiral separation and determination of propranolol enantiomers in rat or mouse blood and tissue by column switching high performance liquid chromatography with ovomucoid bonded stationary phase. Resolution of propranolol (PL) enantiomers in biological samples was accomplished by column switching high performance liquid chromatography using a short precolumn and an analytical column of ovomucoid chiral phase. Plasma, whole blood or tissue homogenate sample was directly injected into the precolumn, and PL was adsorbed on Butyl Toyopearl 650-M. After column switching, the PL was backflushed and transferred to the analytical column (Ultron ES-OVM) by the eluant.

Fluorometric detection was carried out at λ_{ex} = 297 nm and λ_{em} = 340 nm with a detection limit of 0.5 pmol (signal to noise ratio = 2). The recovery (98.8–103%), reproducibility (coefficient of variance less than 3%) and enantiomer resolution (separation factor 1.15) were satisfactory using as eluant 50 mM sodium dihydrogenphosphate (pH 4.6) containing 12% ethanol. The time course of elimination of PL enantiomers in rat or mouse blood and tissues was also studied.

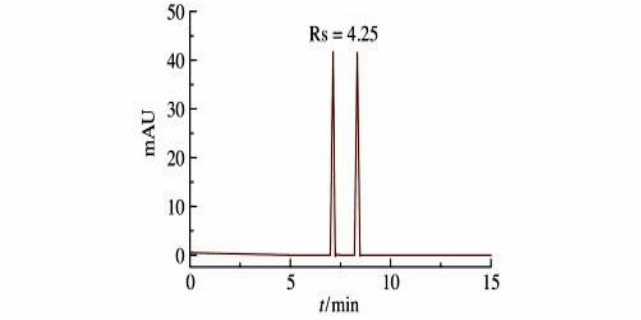
Xiao-yu *et al.* (2011) developed a normal phase-high performance liquid chromatography method for separation of the two enantiomers of propranolol. The synthesis of chiral stationary phase has been set up by means of cellulose derivatives. The mobile phase was the mixture of *n*-hexane-isopropanol-triethylamine (95: 5: 0.1%) with the flow rate of 0.5 mL/min, and the detection wavelength was set at 276 nm. The retention time of two enantiomers of propranolol was 8.67 mim and 14.36 min. Under the optimized conditions, enantiomers of propranolol were well separated from the baseline. The RSD of the inner-day and day to day precision was smaller than 3%. The recoveries were 95.5%-101.4% with RSD of 2.4%. The results showed that the method can enantioseparate the two enantiomers of propranolol by HPLC and the chiral stationary phase of big particle diameter cellulose derivative. This separation approach developed was simple, reliable and accurate, and and may be used for routine analysis of enantiomers of the tablet of propranolol.

Santoro *et al.* (2001) reported an application of chiral high-performance liquid chromatography (HPLC) in the separation and quantitative determination of propranolol isomers in tablets. The isomers were separated using a Chiralcel OD column (250 x 4.6 mm, 10 microm) with a mobile phase of hexane: ethanol (75:25 v/v) at a flow rate of 0.7 ml/min and ultraviolet detection at 280 nm. The coefficient of variation and average recovery of (*R*)-isomer for samples A, B, C, and D were 0.72% and 100.30%, 0.67% and 99.40%, 0.62% and 99.76%, and 0.70% and 99.68%, respectively. The coefficient of variation and average recovery of (*S*)-isomer for samples A, B, C, and D were 0.74% and 99.62%, 0.64% and 100.27%, 0.71% and 99.99%, and 0.70% and 99.72%, respectively.

1.17.9. Citalopram

Nozal *et al.* (2004) designed and synthesized a novel capillary for the high efficiency splitting of citalopram based on modified nanosize silica. The enantiomers of citalopram were separated within 10 min with a resolution (Rs) of 4.25.

Graphical abstract



Peng *et al.* (2016) have developed the enantiomeric separation of citalopram (CIT) using a reversed phase HPLC (RP-HPLC) with sulfobutylether-β-cyclodextrin (SBE-β-CD) as a chiral mobile phase additive. The effects of the pH value of aqueous buffer, concentration of chiral additive, composition of mobile phase, and column temperature on the enantioseparation of CIT were investigated on the Hedera ODS-2 C_{18} column (250 mm × 4.6 mm × 5.0 um). A satisfactory resolution was achieved at 25°C using a mobile phase consisting of a mixture of aqueous buffer (pH of 2.5, 5 mM sodium dihydrogen phosphate, and 12 mM SBE-β-CD), methanol, and acetonitrile with a volumetric ratio of 21: 3: 1 and flow rate of 1.0 mL/min. This analytical method was evaluated by examining the precision (lower than 3.0%), linearity (regression coefficients close to 1).

Mandrioli *et al.* (2003) developed a simple and fast capillary electrophoretic method for the enantioselective separation of citalopram and its main metabolites, namely *N*-desmethylcitalopram and *N*,*N*-didesmethylcitalopram, using beta-cyclodextrin (beta-CD) sulfate as the chiral selector. For method optimisation several parameters were investigated, such as CD and buffer concentration, buffer pH, and capillary temperature. Baseline enantioseparation of the racemic compounds was achieved in less than 6 min using a fused-silica capillary, filled with a background electrolyte consisting of a 35 mM phosphate buffer at pH 2.5 supplemented with 1% w/v beta-CD sulfate and 0.05% w/v beta-CD at 25 degrees C and applying a voltage of -20 kV. A fast separation method for citalopram was also optimized and applied to the analysis

of pharmaceutical formulations. Racemic citalopram was resolved in its enantiomers in less than 1.5 min using short-end injection (8.5 cm, effective length) running the experiments in a background electrolyte composed of a 25 mM citrate buffer at pH 5.5 and 0.04% w/v beta-CD sulfate at a temperature of 10 degrees C.

Dolzan *et al.* (2016) performed the enantiomeric separation of a novel series of twenty-eight racemic mixtures of citalopram analogues by high performance liquid chromatography (HPLC). Due to the effectiveness of citalopram as an antidepressant drug, the development of new compounds based on its chemical structure is interesting, and their enantiomeric separation is needed to allow further pharmacokinetic studies. Several bonded cyclodextrin (both native and derivatized) and macrocyclic glycopeptide based chiral stationary phases (CSPs) were evaluated for their ability to separate this set of compounds via HPLC. Polar ionic, polar organic and reversed phase modes were tested. Twenty-five of the racemic mixtures were separated with resolutions and enantiomeric selectivities up to 2.9 and 1.33, respectively. A total of eighteen baseline separations were achieved, while seven compounds were partially separated. Vancomycin based columns operated in the polar ionic mode resulted in the greatest number of separations. Lastly, the chromatographic behaviors of similar compounds were compared based on their chemical structure and also on the chiral selectors used.

Geryk *et al.* (2013) developed and validated HPLC method enabling chiral separation and determination of citalopram (CIT), a widely used antidepressant, and its synthetic precursor citadiol in one analysis. Moreover, supercritical fluid chromatography was also tested and was proved to be less effective for this separation purpose. The optimized HPLC system was composed of Chiralcel OD-H column and *n*-hexane/propane-2-ol/triethylamine 96/4/0.1 (v/v/v) as mobile phase, column temperature 25 °C, flow rate 1.0 mL min^{-1}, UV detection at 250 nm. The effects of amount of propane-2-ol, triethylamine addition, and temperature on enantioselectivity and resolution of the enantiomers were evaluated. The method was found to be suitable for determination of the enantiomeric purity of CIT in bulk drugs. Enantiomers of CIT were determined in two commercially available pharmaceuticals.

VK *et al.* (2011) described effective, selective and reproducible methods of chiral analysis of citalopram in human plasma. The sample preparation of human plasma was carried out by solid

phase extraction (SPE) C_{18} cartridges with elution via methanol having 0.1% acetic acid. The chiral analysis was achieved on AmyCoat column (150 x 46 mm) of the amylase type CSP, by using *n*-hexane-2-propanol-DEA (95:05:0.2, v/v/v) as mobile phase. Flow rate of mobile phase was 0.5 mL/min with detection at 240 nm. The values of k [*R*-(-)-], k [*S*-(+)-], α and Rs were 3.56, 4.00, 1.12 and 1.22, respectively. Linearity was in the concentration range of 100-500 µg/L with 10.0 ng as the detection limit. The percentage recovery by SPE was 98.00 and the validation parameters proved the precision of the method and its applicability for the determination of chiral citalopram in human plasma. *S*- (+)-Citalopram reacted more in human plasma than *R*-enantiomers.

Deshpande *et al.* (2015) developed a simple and cost effective chiral HPLC method for the separation of citalopram (CIT) enantiomers using chiral mobile phase additives (CMPAs). They synthesized sulfated beta cyclodextrin (S-β-CD) and evaluated as a CMPA. The parameters affecting the resolution were optimized. CIT enantiomers were resolved on an achiral Kromasil C8 column (150 mm × 4.6 mm, 5 µm) using methanol and 20 mM KH_2PO_4 (pH 3) containing 12 mM S-β-CD (35:65) as the mobile phase with a flow rate of 1 ml/min at 240 nm. Chiral resolution capacity of synthesized S-β-CD was compared to the marketed product. The method using synthesized S-β-CD as CMPA was validated and applied for the quantitative determination of CIT enantiomers in bulk drug and tablet formulation. Synthesized S-β-CD gave a better resolution than the marketed form. This method was validated as per ICH guidelines and was found to comply with the standard norms. A good linearity was observed in the concentration range of 1-30 µg/ml with R^2= 0.9993 for both enantiomers. The limit of detection and limit of quantification was 0.0272 and 0.0824 µg/ml for the R-enantiomer and 0.0303 and 0.0920 µg/ml for the S-enantiomer respectively. The method could be successfully applied for the quantitative determination of CIT enantiomers in bulk drug samples and pharmaceutical formulations.

1.18. Research objectives

In the 1960's, a drug called thalidomide was widely prescribed in the Western Europe to alleviate morning sickness in pregnant women. Many babies born to women who had taken thalidomide during pregnancy suffered from severe birth defects and made the children autistic.

Enantiomers exhibit same physical and chemical properties but may have different pharmacological activities, as well as different pharmacokinetic and pharmacodynamic effects (Maier *et al.*, 2001; Nguyen *et al.*, 2006). Thus, one isomer may produce the desired therapeutic activities, while the other may be inactive or, in worst cases, produce unwanted effects. Therefore, in order to eliminate the unwanted isomer from the preparation and to find an optimal treatment and a right therapeutic effect for the patient, it is important to promote the chiral separation and analysis of racemic drugs in pharmaceutical industry. According to the US Food and Drug Administration (FDA), a selective assay method should include for the separation and determination of enantiomeric and racemate drug substances to avoid the possible undesirable effects of a chiral drug. Therefore, it is important that chiral drugs are prepared and marketed as active single form. Hence, analytical methods of chiral drugs are highly needed for identification and separation (Santoro *et al.*, 2001; Nguyen *et al.*, 2006). According to the International Conference on Harmonization (ICH) guidelines, chiral identity, enantiomeric impurity and chiral assay tests may be needed in drug substance and product specifications. Therefore, analytical methods for separation and determination are most readily accomplished by means of chiral chromatography, and chiral HPLC is probably the most versatile and important tool employed today. However, the development of the methods for the quantitative analysis of chiral compounds and for the assessment of enantiomeric purity is extremely challenging, because the same physical and chemical properties of the two enantiomers make discriminating and separating them very difficulty. As many chiral drugs are marketed as racemates and single enantiomer in Bangladesh, the objective of the present investigation focuses on the quality assessment of some enantiomeric drugs available in Bangladesh. So in this study, enantiomeric identification, separation and quantity of enantiomers will be performed. Finally, purity for racemates and single enantiomer will be determined for the assessment of quality of some commonly used enantiomeric drugs in Bangladesh of different pharmaceutical companies. Therefore, the objective of the present investigation was to develop a rapid and easy chiral HPLC method for simultaneous quantification of chiral compounds with improved resolution, and to validate the method according to USP and ICH guidelines (*Requirements for registration of pharmaceuticals for human use. ICH harmonised tripartite guideline impurities : guideline for residual solvents.*).

Common diseases of Bangladeshi people are acidity, depression, inflammation, bacterial infections, asthma and heart diseases etc. So, we have chosen- omeprazole, esomeprazole, rabeprazole, pantoprazole, salbutamol, levosalbutamol, ibuprofen, dexibuprofen, ofloxacin, levofloxacin, citalopram, s-citalopram, carvedilol and propranolol.

In this study, chiral CD-PH, silica-based phenylcarbamated β-cyclodextrin; chiralpak IC, cellulose tris(3,5- dichlorophenylcarbamate); lux cellulose-3, cellulose tris(4-methylbenzoate) and chiral OD-H, Cellulose-tris-(3,5-dimethylphenylcarbamate) were used as stationary phases. Using chiral chromatography, identification of all enantiomers for respective chiral drugs were performed to ensure the presence of undesired compound and calculated the percentage content of enantiomers and compared their claimed content. For quality assessment the specific research objectives are:

1. to develop a fast, accurate, precise and easy enantioselective chiral HPLC method for identification of enantiomers present in drug samples.
2. to separate the enantiomers with improved resolution.
3. to validate the method according to the United States Pharmacopeia (USP) and ICH guidelines.
4. to determine the enantiomeric purity of currently marketed racemic drugs.
5. to determine the enantiomeric purity of currently marketed single enantiomeric drugs.
6. to evaluate the presence of distomer in single enantiomeric drugs, and finally
7. to apply the proposed methods for the routine analysis of respective chiral drugs in tablets, capsules and bulk drugs in pharmaceutical formulations available in Bangladesh.

Chapter 2: Method Development and Validation

Chapter 2 METHOD DEVELOPMENT AND VALIDATION

2.1 Chromatography and its classification

Chromatography is a technique by which the components in a sample, carried by the liquid or gaseous phase, are resolved by sorption-desorption steps on the stationary phase.

2.1.1. High performance liquid chromatography (HPLC)

HPL chromatographic separation is based on interaction and differential partition of the sample between the mobile liquid phase and the stationary phase. The commonly used chromatographic methods can be roughly devided into the following groups, not necessarily in order of importance:

- Chiral
- Ion--exchange
- Ion--pair/affinity
- Affinity chromatography
- Normal phase
- Reversed phase
- Size exclusion

2.1.1.1. Chiral chromatography

Separation of the enantiomers can be achieved on chiral stationary phases by formation of diastereomers via derivatizing agents or mobile phase additives on achiral stationary phases. When used as an impurity test method, the sensitivity is enhanced if the enantiomeric impurity elutes before the enantiomeric drug.

2.1.1.2. Ion-exchange chromatography

Separation is based on the charge-bearing functional groups, anion exchange for sample negative ion, or cation exchange for sample positive ion. Gradient elution by pH is common.

2.1.1.3 Ion-pair affinity chromatography

Separation is based on a chemical interaction specific to the target species. The more popular reversed phase mode uses a buffer and an added counter-ion of opposite charge to the sample

with separation being influenced by pH, ionic strength, temperature, concentration of and type of organic co-solvent(s).

2.1.1.4. Affinity chromatography

Common for macromolecules, employs a ligand (biologically active molecule bonded covalently to the solid matrix) which interacts with its homologous antigen (analyte) as a reversible complex that can be eluted by changing buffer conditions.

2.1.1.5. Normal phase chromatography

Normal phase chromatography is a chromatographic technique that uses organic solvents for the mobile phase and a polar stationary phase. Here, the less polar components elute faster than the more polar components.

2.1.1.6. Reversed phase chromatography

Reversed phase chromatography, a bonded phase chromatographic technique, uses water as the base solvent. Separation based on solvent strength and selectivity also may be affected by column temperature and pH. In general, the more polar components elute faster than the less polar components. UV detection can be used with all chromatographic techniques. The concern for this type of detector is the loss of sensitivity with lamp aging, and varying sensitivity at the low level depending on design and/or manufacturer. A point to note is that observations on the HPLC chromatograms, by UV detection in combination with reversed-phase HPLC, may not be a true indication of the facts for the following reasons:

- Compounds much more polar than the compound of interest may be masked (elute together) in the solvent.
- Compounds very less polar than the analyte may elute either late during the chromatographic run or are retained in the column.
- Compounds with lower UV extinction coefficients or different wavelength maxima may not be detectable at the low level relative to the visibility of the analyte since only one wavelength is normally monitored.

2.1.1.7. Size exclusion chromatography

Also known as gel permeation or filtration, separation is based on the molecular size or hydrodynamic volume of the components. Molecules that are too large for the pores of the

porous packing material on the column elute first, small molecules that enter the pores elute last, and the elution rates of the rest depend on their relative sizes.

2.1.2. Gas chromatography (GC)

Gas chromatography is based on the volatilized sample transported by the carrier gas as the moving phase through the stationary phase of the column where separation takes place by the sorption/desorption process. Samples for gas chromatographic analysis are normally low molecular weight compounds that are volatile and stable at high temperature. In this respect, residual solvents in drug substances and drug products are suitable for gas chromatographic analysis. Chemical derivatives can also be formed to achieve volatility and thermal stability.

Common detectors are flame ionization (FID) for carbon-containing compounds, electron capture (ECD) for halogenated compounds, flame photometric (FPD) for compounds containing sulphur or phosphorous and nitrogen-phosphorous (NPD) for compounds containing nitrogen or phosphorous. Chiral separation also can be achieved by gas chromatography. Separation by the packed column is rapidly being replaced by the capillary

column that provides improved resolution and analysis speed. The location of the analyte on the gas chromatogram is described by retention time (R) which is similar to HPLC.

2.1.3. Thin-layer chromatography (TLC)

Thin-layer chromatography is the simplest of the more common chromatographic techniques. Separation is based on migration of the sample spotted on a coated (stationary phase) plate with one edge dipped in a mixture of solvents (mobile phase). The whole system is contained in an enclosed tank. Detection techniques include fluorescence, UV and sprays (universal and

specific) for compounds that are not naturally colored. The location of the analyte on the TLC plate is described by the R, value which is the ratio of the migration distance of the compound of interest to the mobile phase front. Of the three techniques, gas, liquid and thin-layer, TLC is the most universal test method as all components are present on the plate and with appropriate detection techniques, all components can be observed. However, it normally is not as accurate or sensitive as HPLC. TLC has a higher analytical variation than HPLC, although one sees the "whole picture" when appropriate detection schemes are selected.

2.2. Analytical technique

Chromatographic methods are commonly used for the quantitative and qualitative analysis of raw materials, drug substances, drug products and compounds in biological fluids. The components monitored include chiral or achiral drug, process impurities, residual solvents, excipients such as preservatives, degradation products, extractables and leachables from container and closure or manufacturing process, pesticide in drug product from plant origin, and metabolites. The objective of a test method is to generate reliable and accurate data regardless of whether it is for acceptance, release, stability or pharmacokinetics study. Data are generated for the qualitative and quantitative testing during development and post approval of the drug products. The testing includes the acceptance of raw materials, release of the drug substances and products, in-process testing for quality assurance, and establishment of the expiration dating period. Separation of enantiomers has become very important in analytical chemistry, especially in the pharmaceutical and biological fields, because some stereoisomers of racemic drugs have quite different pharmacokinetic properties and different pharmacological or toxicological effects. This is one of the most vital reasons why the regulatory authorities insist more on stringent investigation for evaluating the safety and the effectiveness of drugs containing chiral centers. Most of the pharmaceutical industries are now concentrating towards the study of the therapeutic effect of pure enantiomers of the existing drug molecules. Enantiomeric separations have acquired importance in all the stages of drug development and the commercialization process. A control and accurate quantification of undesired enantiomers in active pharmaceutical ingredient is essential (Rao *et al.*, 2014), therefore, the development of new methods for efficient chiral separations mainly based on HPLC, capillary electrophoresis (CE) or gas chromatography (GC) is more than necessary. Among the chromatographic methods so far developed, HPLC methods based on chiral stationary phases are widely employed for the assays of drug isomers in pharmaceutical preparations and biological fluids.

For the bioanalysis of racemic drugs, two kinds of analytical methods have been developed: the physical methods and the enantioselective immunoassays.

2.2.1. Chiral analysis by physical methods

Chiral chromatography including high performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC) and capillary electrophoresis

(CE) are most readily accomplished for the enantiomer resolution. HPLC is the most widely used of the four methods. In industry, two HPLC techniques are used:

- indirect
- direct.

In contrast to industry, the indirect HPLC using chiral derivatization reagent with the formation of two diastereomers is frequently performed in bioanalysis because of its high sensitivity. However, this indirect technique requires a functional group in the analyte (drug) e.g. amine, hydroxyl, carboxyl, carbonyl and thiol.

A chiral derivating reagent (a pure single enantiomer) added in the sample will react with these functions to form two diastereomers that can be separated by a classical reversed-phase column (C_{18} or C_8) (Toyo'oka, 2002). For example, propranolols (ß-blocker), perhexiline (antianginal agent) have been determined by this indirect HPLC with fluorescent detector. (Pham-Huy *et al.*, 1994; Davies and Teng, 2003).

Because of the limitation of the indirect HPLC, direct chiral separations using chiral stationary phases CSPs are the most used because of its simplicity and its rapidity. Today, nearly a hundred CSPs have been developedand are recently marketed, but some types of chiral HPLC columns following are the most used in bioanalysis: cyclodextrine and its derivatives, carbohydrate (cellulose, amylose), Pirkle phases, chirobiotic phases. For example, many racemic drugs are well resolved with cyclodextrine and its derivative phases (Cyclobond I, Cyclobond I-2000 RSP, Cyclobond I-2000 SP) such as propranolol, (Robin *et al.*, 1995; Pham-Huy *et al.*, 1997; He *et al.*, 2004); methadone, (He *et al.*, 2004; Pham-Huy *et al.*, 1997); 3-hydroxybenzodiazepam (oxazepam, lorazepam, temazepam) (Pham-Huy *et al.*, 2002; He *et al.*, 2004). Cellulose and its derivatives such as Chiralcel OJ with pure ethanol as mobile phase can separate thalidomide enantiomers (Robin *et al.*, 1995), and tetrahydropalmatine enantiomers (synthetic alkaloid of carydalis used as antiarrhytmicand antihypertensive) (Hong *et al.*, 2005). Amylose and its derivatives such as Chiralcel OD is used to separate donepezil enantiomers (anti-acetylcholinesterase for the treatment of Alzheimer disease) (Radwan *et al.*, 2006). Chiralpak AD® to resolve metoprolol enantiomers (ß-blocker) (Boralli *et al.*, 2005) or other racemic drugs with polar organic solvent chromatography (Matthijs *et al.*, 2006). Antibiotics or chirobiotic phases such as vancomycin-CSP or eremomycin-CSP can also separate thalidomide and amino acids, respectively (Murphy-Poulton *et al.*, 2006; Staroverov *et al.*, 2006). Amide

derivative XAD-4CSP was used for chiral chromatographic separation of many ß-blockers (VK, Ali and Agarwal, 2011). Liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and capillary electrophoresis (CE) are other physical methods for the separation of numerous chiral pharmaceuticals (Martins *et al.*, 2005; Xie *et al.*, 2005; Wei *et al.*, 2006).

Unfortunately, until today, there is no single CSP that can resolve all classes of racemic compounds in bioanalysis, contrary to achiral reversed-phase C_{18} or C_8. The choice of a chiral column is in general examined on the interaction mechanism between CSP and chiral analyte (Bojarski *et al.*, 2005).

2.3. Method development

Method validation often evolves from method development. Method development can take a number of forms (*Method validation procedure*, no date a). At one extreme, it may involve adapting an existing method, making minor changes so that it is suitable for a new application (*Guidemethodvalidation.pdf*). It requires a lot of effort, and there is a degree of doubt initially to whether the method will be successful. It involves working on various ideas simultaneously and then finally picking one of those. Various steps involved in method development and validation are:

Step 1: selection of the HPLC method and initial system

Step 2: selection of initial conditions

Step 3: selectivity optimization

Step 4: system parameter optimization

Step 5: method validation.

2.3.1. Step 1: Selection of the HPLC method and initial system

The first step when developing an HPLC method is always to consult the literature to ascertain whether the separation has been previously performed and if so, under what conditions - this will save time doing unnecessary experimental work. Upon selecting an HPLC system, it must have a high probability of actually being able to analyze the sample; for example, if the sample includes polar analysts then reverse phase HPLC would offer both adequate retention and resolution, whereas normal phase HPLC would be much less feasible. Consideration must be given to the following:

Preparation of sample

Does the sample require dissolution, filtration, extraction, preconcentration or clean up? Is chemical derivatization required to assist detection sensitivity or selectivity?

Selection of chromatography

For the majority of samples reverse phase is preferred, but if acidic or basic analytes are present then reverse phase ion suppression (for weak acids or bases) or reverse phase ion pairing (for strong acids or bases) should be used. The stationary phase should be C_{18} bonded. For inorganic anion/cation analysis, ion exchange chromatography is best. Size exclusion chromatography would normally be considered for analyzing high molecular weight compounds.

For low/medium polarity analysts, normal phase HPLC is a potential candidate, particularly if the separation of isomers is required. Cyano-bonded phases are easier to work with than plain silica for normal phase separations.

Gradient HPLC

Gradient HPLC is an excellent method for initial sample analysis. Reverse phase gradient HPLC is commonly used in peptide and small protein analysis using an acetonitrile–water mobile phase containing 1% trifluoroethanoic acid.

Gradient HPLC will give greater sensitivity, particularly for analysts with longer retention times, because of the more constant peak width (for a given peak area, peakheight is inversely proportional to peak width).

This is only a requirement for complex samples with a large number of components (20–30) because the maximum number of peaks that can be resolved with a given resolution is much higher than in isocratic HPLC. This is a result of the constant peak width that is observed in gradient HPLC (in isocratic HPLC peak width increases in proportion to retention time). The method can also be used for samples containing analysts with a wide range of retentivities that would, under isocratic conditions, provide chromatograms with capacity factors outside of the normally acceptable range (0.5-1.5).

Detectors

Consideration must be given to the following:

- The analysts must have chromophores to enable UV detection.

- More selective/sensitive detection is needed.
- Necessity of detection limits.
- The samples require chemical derivatization to enhance detectability and/or improve the chromatography.
- Refractive index is preferred for preparative HPLC, because it can handle high concentrations without overloading the detector. Fluorescence or electrochemical detectors should be used for trace analysis.

Fluorescence wavelength

The excitation wavelength locates the excitation maximum; that is, the wavelength that gives the maximum emission intensity. The excitation is set to the maximum value then the emission is scanned to locate the emission intensity. Selection of the initial system could, therefore, be based on assessment of the nature of sample and analytes together with literature data, experience, expert system software and empirical approaches.

UV wavelength

For the greatest sensitivity λ_{max} should be used, which detects all sample components that contain chromophores. UV wavelengths below 200 nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.

A schematic flow diagram of HPLC is shown in figure 2.1.

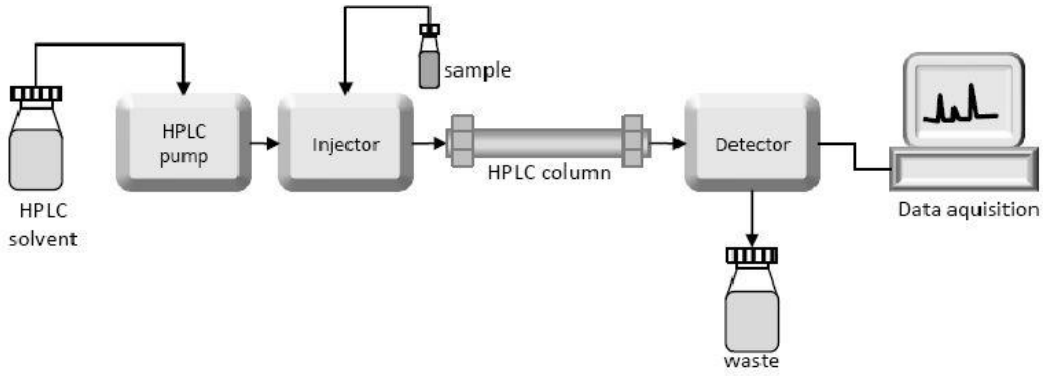


Figure 2.1: A flow scheme for HPLC.

2.3.2. Step 2: Selection of initial conditions

This step determines the optimum conditions to adequately retain all analysts; that is, ensures no analyst has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyst has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor delectability). Selection of the following is then required.

Initial conditions determination

The recommended method involves performing two gradient runs differing only in the run time. A binary system based on either acetonitrile/water (or aqueous buffer) or methanol/water (or aqueous buffer) should be used.

Gradient HPLC

With samples containing a large number of analysts (20–30) or with a wide range of analystretentivities, gradient elution will be necessary to avoid excessive retention.

Mobile phase solvent strength

The solvent strength is a measure of its ability to pull analysts from the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one. The aim is to find the correct concentration of the strong solvent. With many samples, there will be a range of solvent strengths that can be used within the aforementioned capacity limits. Other factors (such as pH and the presence of ion pairing reagents) may also affect the overall retention of analysts.

2.3.3. Step 3: Selectivity optimization

The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account. To minimize the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined. To select these, the nature of the analysts must be considered. For this, it is useful to categorize analysts into a few basic types. Once the analyst

types are identified, the relevant optimization parameters may be selected. Note that the optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization. Initially, gradient conditions should be optimized using a binary system based on either acetonitrile/water (or aqueous buffer) or methanol/water (or aqueous buffer). If there is a serious lack of selectivity, a different organic modifier should be considered.

2.3.4. Step 4: System parameter optimization

After satisfactory selectivity has been achieved system parameter optimization is used to find the desired balance between resolution and analysis time. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

2.3.5. Step 5: Method validation

It is important to isolate analytical method validation from the selection and development of the method. Method selection is the first step in establishing an analytical method and consideration must be given to what is to be measured, and with what accuracy and precision. Proper validation of analytical methods is important for pharmaceutical analysis when ensurance of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. The ability to control this quality is dependent upon the ability of the analytical methods, as applied under well-defined conditions and at an established level of sensitivity, to give a reliable demonstration of all deviation from target criteria. Method development and validation can be simultaneous, but they are two different processes, both downstream of method selection. Analytical methods used in quality control should ensure an acceptable degree of confidence that results of the analyses of raw materials, excipients, intermediates, bulk products or finished products are viable. Before a test procedure is validated, the criteria to be used must be determined.

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B) (*Guidance for Industry*; *Food and Drug Administration - USA*; *Method validation procedure*) and US Pharmacopoeia (USP) both refer to ICH guidelines. The most widely applied validation

characteristics are accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity, range, robustness and stability of analytical solutions. Method validation must have a written and approved protocol prior to use (*Food and Drug Administration - USA*).

2.4. HPLC method validation

A well-developed method is always to validate. While using any analytical technique for the estimation of the drug it needs a proper method to be developed. Let us take an example of a most complex analytical technique, i.e., high performance liquid chromatography (HPLC) which is complex in the sense that there are a wide variety of equipments, columns, eluents, and other parameters for operation which makes it so.

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

Analytical methods need to be validated or revalidated

- before their introduction into routine use;
- whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix); and
- whenever the method is changed and the change is outside the original scope of the method.

Method validation has received considerable attention in the literature and from industrial committees and regulatory agencies.

- The U.S. FDA CGMP request methods to be validated: The accuracy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented. These requirements include a statement of each method used in testing the sample to meet proper standards of accuracy and reliability, as applied to the tested product. The U.S. FDA has also proposed an industry guidance for Analytical Procedures and Methods Validation (*ISO/IEC 17025 - General requirements for the competence of testing and calibration laboratories*).

- ISO/IEC 17025 includes a chapter on the validation of methods with a list of nine validation parameters. The ICH has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics. ICH also developed guidance with detailed methodology.
- The U.S. EPA prepared guidance for method's development and validation for the Resource Conservation and Recovery Act (RCRA). The AOAC, the EPA and other scientific organizations provide methods that are validated through multi-laboratory studies.

The USP has published specific guidelines for method validation for compound evaluation (*Food and Drug Administration - USA*) USP defines eight steps for validation:

- Accuracy
- Precision
- Specificity
- Limit of detection
- Limit of quantitation
- Linearity and range
- Ruggedness
- Robustness

The FDA has also published a guidance for the validation of bioanalytical methods. The most comprehensive document is the conference report of the 1990 Washington conference: Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies, which was sponsored by, among others, the American Association of Pharmaceutical Scientists (AAPS), the AOAC and the U.S. FDA. The report presents guiding principles for validating studies of both human and animal subjects. The report has also been used as a basis for the FDA industry guidance document.

Representatives of the pharmaceutical and chemical industry have published papers on the validation of analytical methods. Patel (Patel *et al.*, 2011) applied the life cycle approach, developed for computerized systems, to the validation and revalidation of methods. Green (Green, 1996) gave a practical guide for analytical method validation, with a description of a set of minimum requirements for a method. Renger and his colleagues (Renger *et al.*, 1995) described the validation of a specific analytical procedure for the analysis of theophylline in a

tablet using high-performance thin layer chromatography (HPTLC). The validation procedure in this particular article is based on requirements for EU multistate registration.

Wegscheider (Wegscheider, 1996) has published procedures for method validation with a special focus on calibration, recovery experiments, method comparison and investigation of ruggedness. Seno (Seno *et al.*, 1997) have described how analytical methods are validated in a Japanese QC laboratory. The AOAC has developed a Peer-Verified Methods validation program with detailed guidelines on exactly which parameters should be validated. Winslow and Meyer (Meyer, 1997) recommend the definition and application of a master plan for validating analytical methods. J.Breaux and colleagues (Breaux *et al.*, 2003) have published a study on analytical methods development and validation. The key point is to develop methods for easy validation and revalidation. Koppenhoefer (Koppenhoefer *et al.*, 1994) published a guide for analytical method transfer, comparability, maintenance and acceptance criteria for the testing of biopharmaceuticals.

2.5. Parameters for method validation

The parameters for method validation have been defined in different working groups of national and international committees and are described in the literature. Unfortunately, some of the definitions vary between the different organizations. An attempt at harmonization was made for pharmaceutical applications through the ICH, where representatives from the industry and regulatory agencies from the United States, Europe and Japan defined parameters, requirements and, to some extent, methodology for analytical methods validation. The parameters, as defined by the ICH and by other organizations and authors, are summarized in table 2.1 and are described in brief in the following paragraphs.

- Specificity (1,2)
- Selectivity
- System Suitability
- Precision (1,2)
- repeatability (1)
- intermediate precision (1)
- reproducibility (3)

- Accuracy (1,2)
- Trueness
- Bias
- Linearity (1,2)
- Range (1,2)
- Limit of detection (1,2)
- Limit of quantitation (1,2)
- Robustness (2,3)
- Ruggedness (2)

(1) Included in ICH publications, (2) Included in USP (3) Terminology included in ICH publication but not part of required parameters.

2.5.1. Selectivity/specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). This definition has the following implications: Identification: to ensure the identity of an analyte. Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc. Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

2.5.2. System suitability

System suitability tests will be performed on both HPLC systems to determine the accuracy and precision of the system by injecting six injections of a solution containing analyte at 100% of test concentration. The following parameters will be determined: plate count, tailing factors, resolution, and reproducibility (percent RSD of retention time, peak area, and height for six injections). Print the chromatogram and record the data on the datasheet.

2.5.2.1. Retention factor (k)

The peak of interest should be well resolved from other peaks and the void volume; generally, k should be ≥2.0.

2.5.2.2. Resolution (Rs)

Rs should be ≥2 between the peak of interest and the closest eluted which is potentially interfering (impurity, excipient, and degradation product). Reproducibility: RSD for peak area, height, and retention time will be 1% for six injections.

2.5.2.3. Tailing factor (T)

T should be 2. Theoretical plates (N): ≥2000.

2.5.3. Precision and reproducibility

The precision of a method (table 2.2) is the extent to which the individual test results of multiple injections of a series of standards agree. The measured standard deviation can be subdivided into 3 categories: repeatability, intermediate precision and reproducibility. Repeatability is obtained when the analysis is carried out in a laboratory by an operator using a piece of equipment over a relatively short time span. At least 6 determinations of 3 different matrices at 2 or 3 different concentrations should be performed, and the RSD calculated. Schematic diagram for accuracy and precision is shown in figure 2.2.

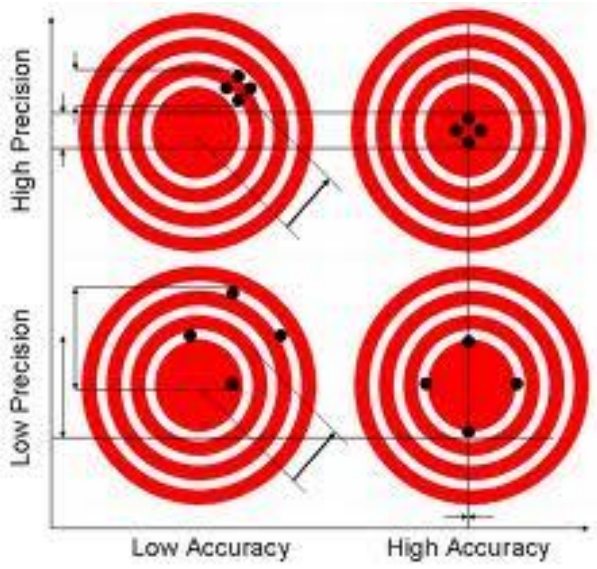


Figure 2.2: Accuracy and precision.

The ICH requires precision from at least 6 replications to be measured at 100 percent of the test target concentration or from at least 9 replications covering the complete specified range. For example, the results can be obtained at 3 concentrations with 3 injections at each concentration.

The acceptance criteria for precision depend very much on the type of analysis. Pharmaceutical QC precision of greater than 1 percent RSD is easily achieved for compound analysis, but the precision for biological samples is more like 15 percent at the concentration limits and 10 percent at other concentration levels. For environmental and food samples, precision is largely dependent on the sample matrix, the concentration of the analyte, the performance of the equipment and the analysis technique. It can vary between 2 percent and more than 20 percent.

The AOAC manual for the Peer-Verified Methods program includes a table with estimated precision data as a function of analyte concentration (table 2.2).

Intermediate precision is a term that has been defined by ICH as the long-term variability of the measurement process. It is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method's intermediate precision may reflect discrepancies in results obtained

- from different operators,
- from inconsistent working practice (thoroughness) of the same operator,
- from different instruments,
- with standards and reagents from different suppliers,
- with columns from different batches or
- a combination of these.

Table 2.1: Analytical concentration versus precision.

Analyte%	Analyte Ratio	Unit	RSD%
1	1	100%	1.3
10	10^{-1}	10%	2.8
1.0	10^{-2}	1%	2.7
0.1	10^{-3}	0.1%	3.7
0.01	10^{-4}	100 ppm	5.3
0.001	10^{-5}	10 ppm	7.3
0.0001	10^{-6}	1 ppm	11
0.00001	10^{-7}	100 ppb	15
0.000001	10^{-8}	10 ppb	21
0.0000001	10^{-9}	1 ppb	30

The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.

Reproducibility (table 2.2) as defined by the ICH represents the precision obtained between different laboratories. The objective is to verify that the method will provide the same results in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts, and by using operational and environmental conditions that may differ from, but are still within, the specified parameters of the method (interlaboratory tests). Validation of reproducibility is important if the method is to be used in different laboratories.

- Differences in room temperature and humidity
- Operators with different experience and thoroughness
- Equipment with different characteristics, e.g. delay volume of an HPLC system
- Variations in material and instrument conditions, e.g. in HPLC, mobile phases composition, pH, flow rate of mobile phase
- Variation in experimental details not specified by the method
- Equipment and consumables of different ages

- Columns from different suppliers or different batches
- Solvents, reagents and other material with varying quality

Table 2.2: Variables for measurements of precision, intermediate precision and reproducibility.

Parameters	**Precision**	**Intermediate Precision**	**Reproducibility**
Instrument	Same	Different	Different
Batches of accessories e.g. columns	Same	Different	Different
Operators	Different	Different	Different
Sample matrices	Different	Different	Different
Concentration	Different	Different	Different
Batches of material, e.g. reagents	Same	Different	Different
Environmental conditions, e.g., Temperature	Same	Different	Different
Laboratory	Same	Same	Different

2.5.4. Accuracy and recovery

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. Accuracy can also be described as the closeness of agreement between the value that is adopted, either as a conventional, true or accepted reference value, and the value found.

The true value for accuracy assessment can be obtained in several ways. One alternative is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known. Secondly, accuracy can be assessed by analyzing a sample with known concentrations (e.g., a control sample or certified reference material) and comparing the measured value with the true value as supplied with the material. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the

effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible. If validated correctly, the recovery factor determined for different concentrations can be used to correct the final results.

The concentration should cover the range of concern and should include concentrations close to the quantitation limit, one in the middle of the range and one at the high end of the calibration curve. Another approach is to use the critical decision value as the concentration point that must be the point of greatest accuracy.

Table 2.3: Analyte recovery at different concentrations.

Analyte%	Analyte Ratio	Unit	Mean Recovery(%)
1	1	100%	98-102
10	10^{-1}	10%	98-102
1.0	10^{-2}	1%	97-103
0.1	10^{-3}	0.1%	95-105
0.01	10^{-4}	100 ppm	90-107
0.001	10^{-5}	10 ppm	80-110
0.0001	10^{-6}	1 ppm	80-110
0.00001	10^{-7}	100 ppb	80-110
0.000001	10^{-8}	10 ppb	60-115
0.0000001	10^{-9}	1 ppb	40-120

The expected recovery (table 2.3) depends on the sample matrix, the sample processing procedure and the analyte concentration. The AOAC manual for the peer-verified methods program includes a table with estimated recovery data as a function analyte concentration.

The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations/three replicates each). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value, together with the confidence intervals.

2.5.5. Linearity and calibration curve

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analytes in samples within a given range or proportional by

means of well-defined mathematical transformations. Linearity may be demonstrated directly on the test substance (by dilution of a standard stock solution) and/or by using separate weighings of synthetic mixtures of the test product components, using the proposed procedure.

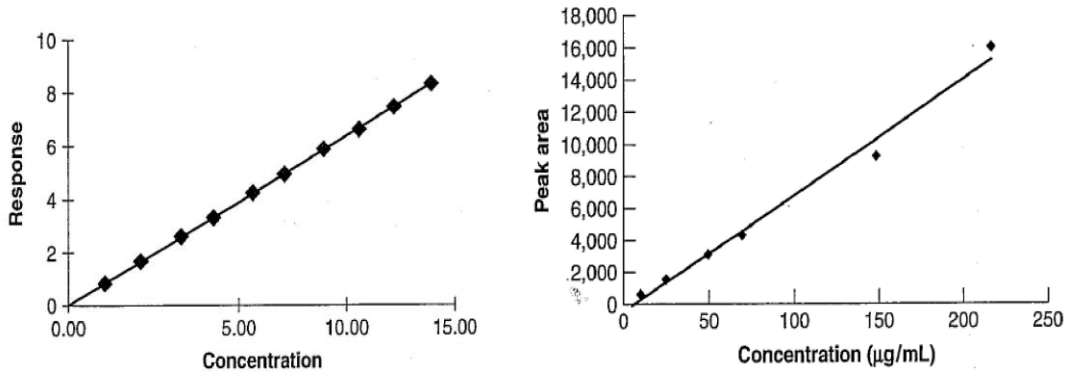
Linearity is determined by a series of 3 to 6 injections of 5 or more standards whose concentrations span 80–120 percent of the expected concentration range. The response should be directly proportional to the concentrations of the analytes or proportional by means of a well-defined mathematical calculation. A linear regression equation applied to the results should have an intercept not significantly different from 0. If a significant nonzero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method.

Frequently, the linearity is evaluated graphically, in addition to or as an alternative to mathematical evaluation. The evaluation is made by visually inspecting a plot of signal height or peak area as a function of analyte concentration. Because deviations from linearity are sometimes difficult to detect, two additional graphical procedures can be used. The first is to plot the deviations from the regression line versus the concentration or versus the logarithm of the concentration, if the concentration range covers several decades. For linear ranges, the deviations should be equally distributed between positive and negative values.

Another approach is to divide signal data by their respective concentrations, yielding the relative responses. A graph is plotted with the relative responses on the y-axis and the corresponding concentrations on the x-axis, on a log scale. The obtained line should be horizontal over the full linear range. At higher concentrations, there will typically be a negative deviation from linearity. Parallel horizontal lines are drawn on the graph corresponding to, for example, 95 percent and 105 percent of the horizontal line. The method is linear up to the point where the plotted relative response line intersects the 95 percent line. Figure 2.3 shows the linear curve and figure 2.4 shows a comparison of the two graphical evaluations on a sample of caffeine using HPLC.

The ICH recommends, for accuracy reporting, the linearity curve's correlation coefficient, y-intercept, slope of the regression line and residual sum of squares. A plot of the data should be included in the report. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an

analyte in a sample. In order to establish linearity, a minimum of five concentrations is recommended. Other approaches should be justified.



r > 0.997 **r < 0.997**

Figure 2.3: Usual acceptance criteria for a linear calibration curve – r>0.999; y-intercept a <0 to 5% of target concentration RSD (wrt calibration curve) < 1.5-2%.

Plotting the sensitivity (response/amount) gives clear indication of the linear range. Plotting the amount on a logarithmic scale has a significant advantage for wide linear ranges.

2.5.6. Range

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results (e.g., percentage, parts per million) obtained by the analytical method.

For assay tests, the ICH requires the minimum specified range to be 80 to 120 percent of the test concentration, and for the determination of an impurity, the range to extend from the limit of quantitation, or from 50 percent of the specification of each impurity, whichever is greater, to 120 percent of the specification.

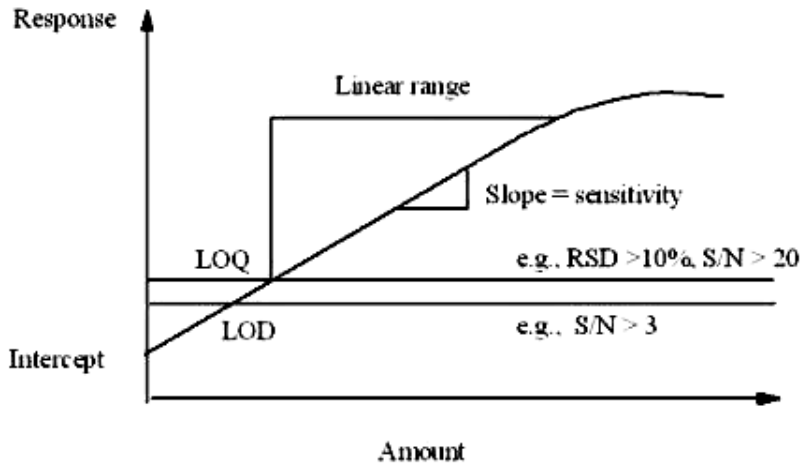


Figure 2.4: Definitions for linearity, range, LOQ, LOD.

2.5.7. Limit of detection

2.5.7.1. Detection limit

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

2.5.7.1.1. Based on visual evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

2.5.7.1.2. Based on signal-to-noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

2.5.7.1.3. Based on the standard deviation of the response and the slope

The detection limit (DL) may be expressed as: DL = 3.3 σ S; where, σ = the standard deviation of the response, S = the slope of the calibration curve, the slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example:

2.5.7.1.4. Based on the standard deviation of the blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

2.5.7.1.5. Based on the calibration curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended data

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification. Validation of Analytical Procedures: Methodology in cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

2.5.7.2. Quantitation limit

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

2.5.7.2.1. Based on visual evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The quantitation limit is generally determined by the analysis of samples

with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

2.5.7.2.2. Based on signal-to-noise approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1 which is shown in figure 2.5.

2.5.7.2.3. Based on the standard deviation of the response and the slope

The quantitation limit (QL) may be expressed as: $QL = 10\ \sigma\ S$;

where, σ = the standard deviation of the response, S = the slope of the calibration curve, the slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways for example:

2.5.7.2.4. Based on standard deviation of the blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

2.5.7.2.5. Based on the calibration curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

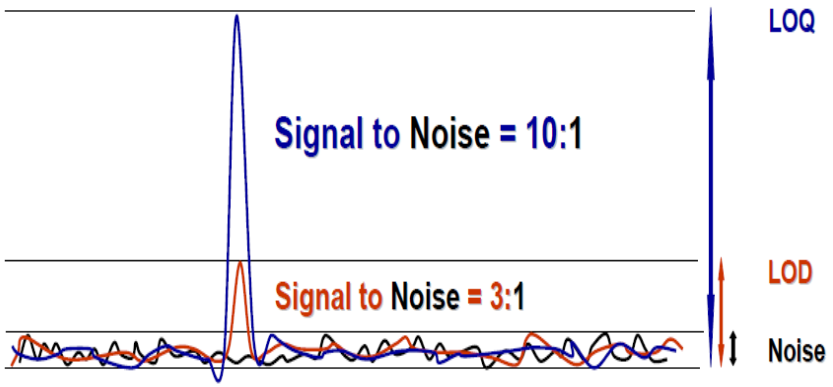


Figure 2.5: Limit of detection and limit of quantitation via signal to noise.

2.5.8. Ruggedness

Ruggedness is not addressed in the ICH documents. Its definition has been replaced by reproducibility, which has the same meaning as ruggedness, defined by the USP as the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.

2.5.9. Robustness

Robustness tests examine the effect that operational parameters have on the analysis results. For the determination of a method's robustness, a number of method parameters, for example, pH, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range.

Obtaining data on these effects helps to assess whether a method needs to be revalidated when one or more parameters are changed, for example, to compensate for column performance over time. In the ICH document, it is recommended to consider the evaluation of a method's robustness during the development phase, and any results that are critical for the method should be documented. This is not, however, required as part of a registration.

2.5.10. Stability

Many solutes readily decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method development should investigate the stability of the analytes and standards.

The term system stability has been defined as the stability of the samples being analyzed in a sample solution. It is a measure of the bias in assay results generated during a preselected time interval, for example, every hour up to 46 hours, using a single solution. System stability should be determined by replicate analysis of the sample solution. System stability is considered appropriate when the RSD, calculated on the assay results obtained at different time intervals, does not exceed more than 20 percent of the corresponding value of the system precision. If, on plotting the assay results as a function of time, the value is higher, the maximum duration of the usability of the sample solution can be calculated.

The effect of long-term storage and freeze-thaw cycles can be investigated by analyzing a spiked sample immediately after preparation and on subsequent days of the anticipated storage period. A minimum of two cycles at two concentrations should be studied in duplicate. If the integrity of the drug is affected by freezing and thawing, spiked samples should be stored in individual containers, and appropriate caution should be employed for the study of samples.

2.6. Data elements required for method validation

Compendial test requirements vary from highly exacting analytical determinations to subjective evaluation of attributes. This discussion covers only the most common catagories of tests for which validation data should be required. These categories are as follows:

Category 1: Analytical procedures for quantitation of major components in bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

Category 2: Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These procedures include quantitative assays and limit tests.

Category 3: Analytical procedures for determination of performance characteristics (e.g., dissolution, drug release, etc)

Category 4: Identification tests

Table 2.4: USP characteristics (*May be required, depending on the nature of specific test).

Analytical performance Characteristics	Category 1	Category 2		Category 3	Category 4
		Quantitative	Limit Tests		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	Yes
Detection Limit	No	No	Yes	*	No
Quantification Limit	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

Table 2.5: ICH characteristics (*may be required, depending on the nature of the specific test).

Analytical Task	Identification	Imputity		Assay
		Quantitative	Qualitative	Category 3
Accuracy	No	No	Yes	Yes
Precision	No	No	Yes	Yes
Repeatability	No	Yes	No	Yes
Interimediate precision	No	Yes	No	Yes
Reproducibility	No	No	No	No
Specificity	Yes	Yes	Yes	Yes
Limit of detection	No	No	Yes	Yes
Limit of quantification	No	Yes	No	No
Linearity	No	Yes	No	No
Range	No	Yes	No	Yes*

The validation parameters depend on the analytical task and the scope of the method. For example, both the USP and the ICH contain chapters on validation procedures for different analytical tasks, both of which are included to provide some ideas on what type of validations are required for different tasks (tables 2.4 and 2.5). For example, according to the ICH, accuracy, any type of precision and limits of detection and quantitation are not required if the

analytical task is identification. For assays in USP category 1, the major component or active ingredient to be measured is normally present at high concentrations; therefore, validation of limits of detection and quantitation is not necessary.

Because the type of analysis and the information that should be obtained from a sample have so much influence on the validation, the objective and scope of the method should always be defined as the first step of any method validation.

Chapter 3: Materials and Methods

Chapter 3 METHODS AND METERIALS

3.1. Materials for enantiomeric drugs

3.1.1. Propranolol

3.1.1.1. Materials

The reference standards of *S*-propranolol and propranolol (≥99 %) were gifted from Incepta pharmaceutical ltd. Commercial samples for all drugs were collected from different companies following good laboratory practice (GLP). Analytical grade ammonium acetate and HPLC grade methanol were purchased from Sigma- Aldrich (Spruce Street, St. Louis, Germany).

3.1.1.2. Equipment

A liquid chromatographic system, SIL 20 series Prominence HPLC (Shimadzu, Japan) consisted of an auto sampler (Model SIL-20 AC), dual pumps (Model 20 AD), column oven (Model CTO-20A), vacuum degasser (Model DGU-20A), UV-visible detector (Model SPD-20A), and LC solution software that runs on Windows was used. All solutions were prepared in ultrasonic bath (Ultrasons Medi-II, Spain). The column Chiral CD-Ph (phenylcarbamated β-cyclodextrin), coated on 5 μm silica gel particles (250×4.6 mm) (Chiral Technologies, Daicel Chemical Industries Ltd., Tokyo, Japan) was used.

3.1.1.3. Chromatographic conditions

Column: Chiral CD-PH (250x 4.6) mm, 5 μm
Flow rate: 0.6 mL/min
Wavelength: 254 nm
Column temperature: Room temperature
Injection volume: 20 μL
Run time: 20 minutes
Mobile phase: Ammonium acetate (pH 3.0) – methanol (10:90, v/v)
Diluent: Mobile phase
Elution: Isocratic

3.1.1.4. Standard solutions

Stock standard solutions of propranolol were prepared by dissolving 20 mg powder in 100 mL with diluents. Dilution of the stock solutions of propranolol with appropriate volumes of diluent was carried out to obtain solutions of concentrations of 40, 60, 80, 100, and 140 µg/mL. *S*-propranolol standard solution was prepared and also injected to confirm the enantiomers of propranolol. Before analysis, reagents were filtered using 0.45 µm membrane filter tips.

3.1.1.5. Sample solutions of propranolol tablets

Twenty tablets of six companies' propranolol samples were weighed and crushed with mortar and pestle. Samples powder equivalent to 5 mg of each brand was transferred to a 100 mL volumetric flask to make the concentration of 50 µg/mL. The content was dissolved with suitable volume of diluent, sonicated, make up the volume with diluent and inject immediately.

3.1.1.6. Application of the proposed method

This analytical method was applied to determine the % recovery of propranolol commercial samples. Then the enantiomeric purity of samples were calculated by using the formula provided (*Illustrated Glossary of Organic Chemistry* and *Optical Purity and all that....*).

enantiomeric purity = 50% - (% of major enantiomer) for racemate (1:1 mixture of enantiomers).

3.1.2. Carvedilol

3.1.2.1. Materials

The reference standards of carvedilol and *S* -carvedilol (≥99.98 %) were gifted from Incepta pharmaceutical ltd. Commercial samples for all drugs were collected from different companies following good laboratory practice (GLP). HPLC grade *n*-hexane, 2-propanol, acetic acid and diethyl amine were purchased from Sigma- Aldrich (Spruce Street, St. Louis, Germany).

3.1.2.2. Equipment

The equipment is common for all drugs except the column. The column Chiralpak IC column, 4.6x250 mm, cellulose tris-(3,5- dichlorophenylcarbamate) Chiral Technologies, Daicel group, Tokyo, Japan was used.

3.1.2.3. Chromatographic conditions

Column: Chiralpak IC column (4.6x250) mm, 5 µm

Flow rate: 1.0 mL/min

Wavelength: 220 nm

Column temperature: Room temparature

Injection volume: 20 µL

Run time: 20 minutes

Mobile phase: *n*-hexane: isopropyl alcohol: diethyl amine: acetic acid (40: 60: 0.7: 0.3, v/v)

Diluent: Mobile phase

Elution: Isocratic

3.1.2.4. Standard solutions

Dilution of the stock solutions of carvedilol with appropriate volumes of diluent was carried out to obtain solutions of concentrations of 30, 60, 80, 100, and 130 µg/mL. S-carvedilol standard solution was prepared and also injected to confirm the enantiomers of carvedilol.

3.1.2.5. Sample solutions of carvedilol

Twenty carvedilol tablets of each brand were weighed and crushed with mortar and pestle. Samples powder equivalent to 5 mg of each brand was transferred to a 100 mL volumetric flask to make the concentration of 50µg/mL. The content was dissolved with suitable volume of diluent, sonicated, make up the volume with diluent and inject immediately.

3.1.2.6. Application of the proposed method

This analytical method was applied to determine the % recovery of carvedilol commercial samples. Then the enantiomeric purity of samples were calculated by using the formula provided by (*Illustrated Glossary of Organic Chemistry* and *Optical Purity and all that....*):

enantiomeric purity = 50% - (% of major enantiomer) for racemate (1:1 mixture of enantiomers).

3.1.3. Citalopram

3.1.3.1. Materials

The reference standards of citalopram and *S*-citalopram, (≥99.99 %) were gifted from Incepta pharmaceutical ltd. Commercial samples for all drugs were collected from different companies following good laboratory practice (GLP). Analytical grade ammonium acetate and HPLC grade *n*-hexane, 2-propanol and methylene dichloride were purchased from Sigma-Aldrich (Spruce Street, St. Louis, Germany).

3.1.3.2. Equipment

The equipment is common for all drugs except the column. The column Chiral CD-Ph (phenylcarbamated β-cyclodextrin), coated on 5 μm silica gel particles (250×4.6 mm) (Chiral Technologies, Daicel Chemical Industries Ltd., Tokyo, Japan) was used.

3.1.3.3. Chromatographic condition

Column: Chiral CD-PH (250x 4.6) mm, 5 μm
Flow rate: 0.5 mL/min
Wavelength: 254 nm
Column temperature: Room temparature
Injection volume: 20 μL
Run time: 30 minutes
Mobile phase: ammonium acetate/ethanol/2-propanol/methylene dichloride (100:150:70:30, v/v)
Diluent: Mobile phase
Elution: Isocratic

3.1.3.4. Standard solutions

20 mg of citalopram standard was weighed and transferred into a 100 mL volumetric flask and dissolved with diluent (mixture of ethanol and 2-propanol) by applying sonication in an ultrasonic bath for 15 minutes. The solution was diluted with the solvent mixture up to the mark. The resulting solution had a concentration of 100 μg/mL. Dilution of this solution with

appropriate volumes of the solvent mixture was carried out to obtain solutions of concentrations of 40, 70, 90, 110, and 140 µg/mL. *S*-citalopram standard solution was also prepared and also injected to confirm the enantiomers of citalopram.

3.1.3.5. Sample solutions of citalopram and escitalopram

Twenty tablets of escitalopram samples were weighed and crushed with a mortar and pestle. Tablet powder equivalent to 5 mg of each brand was transferred to a 100 mL volumetric flask to make the concentration of 50 µg/mL. The content was dissolved with suitable volume of diluent and sonicated for 15 minutes. Then make up the volume with diluent and mix well and inject immediately.

3.1.3.6. Application of the proposed method

This analytical method was applied to determine the % recovery of escitalopram commercial samples. Then the enantiomeric purity of samples were calculated by using the formula provided by (*Illustrated Glossary of Organic Chemistry* and *Optical Purity and all that....*):

enantiomeric purity = (% of major enantiomer - % of minor enantiomer) (For single enantiomer)

3.1.4. Salbutamol

3.1.4.1. Materials

The reference standards of salbutamol and levosalbutamol (≥99.99%) were gifted from Incepta pharmaceutical ltd. Commercial samples for all drugs were collected from different companies following good laboratory practice (GLP). Analytical grade disodium hydrogen phosphate and HPLC grade methanol were purchased from Sigma- Aldrich (Spruce Street, St. Louis, Germany).

3.1.4.2. Equipment

The equipment is common for all drugs except the column. The column Chiral CD-Ph (phenylcarbamated β-cyclodextrin), coated on 5 µm silica gel particles (250×4.6 mm) (Chiral Technologies, Daicel Chemical Industries Ltd., Tokyo, Japan) was used.

3.1.4.3. Chromatographic conditions

Column: Chiral CD-PH (250x 4.6) mm, 5 µm

Flow rate: 0.5 mL/min

Wavelength: 225 nm

Column temperature: Room temparature

Injection volume: 10 µL

Run time: 20 minutes

Mobile phase: di-Sodium hydrogen phosphate (pH 6.01)/ methanol [50:50 (v/v)]

Diluent: Mobile phase

Elution: Isocratic

3.1.4.4. Standard solutions

Stock standard solution of salbutamol were prepared by dissolving 10 mg powder in 100 mL with diluents. Dilution of the stock solutions of salbutamol with appropriate volumes of diluent was carried out to obtain solutions of concentrations ranging from 5-100 µg/mL. Levo-salbutamol standard solution was prepared and also injected to confirm the enantiomers of salbutamol. Before analysis, reagents were filtered using 0.45 µm membrane filter tips.

3.1.4.5. Sample solutions of salbutamol and levosalbutamol (*R*- salbutamol)

Twenty tablets of salbutamol and levosalbutamol samples of each brand were weighed and crushed. Tablet powder and syrup samples were taken as equivalent to 1 mg of each brand and transferred to a 20 mL volumetric flask to make the concentration of 50 µg/mL with diluent for each brand, filtered through 0.45 µm membrane filter tips and then injected for analysis.

3.1.4.6. Application of the proposed method

This analytical method was applied to determine the % recovery of salbutamol and dexsalbutamol commercial samples. Then the enantiomeric purity of samples were calculated by using the formula provided by (*Illustrated Glossary of Organic Chemistry* and *Optical Purity and all that....*):

enantiomeric purity = (% of major enantiomer - % of minor enantiomer) (For single enantiomer) and

enantiomeric purity = 50% - (% of major enantiomer) for racemate (1:1 mixture of enantiomers).

3.1.5. Ibuprofen

3.1.5.1. Materials

The reference standards of ibuprofen and dexibuprofen (≥99.99 %) were gifted from Incepta pharmaceutical ltd. Commercial samples for all drugs were collected from different companies following good laboratory practice (GLP). HPLC grade acetic acid and acetonitrile were purchased from Sigma- Aldrich (Spruce Street, St. Louis, Germany).

3.1.5.2. Equipment

The equipment is common for all drugs except the column. The column lux cellulose-3, 4.6x250 mm, cellulose tris(4-methylbenzoate) (Chiral Technologies, Daicel Chemical Industries Ltd., Tokyo, Japan) was used.

3.1.5.3. Chromatographic conditions

Column: Lux cellulose-3 (250x 4.6) mm, 5 µm
Flow rate: 0.7 mL/min
Wavelength: 220 nm
Column temperature: Room temparature
Injection volume: 10 µL
Run time: 20 minutes
Mobile phase: Acetonitrile / 0.1% acetic acid at a ratio of 50: 50 (v/v)
Diluent: ethanol and 2-propanol (1:1)
Elution: Isocratic

3.1.5.4. Standard solution

Ibuprofen standard (20 mg) was weighed and transferred into a 100 mL volumetric flask and dissolved with diluent applying sonication in an ultrasonic bath for 15 minutes. The solution was diluted with the diluent up to the mark. The resulting solution had a concentration of 200 µg/mL. Dilution of this solution with appropriate volumes of the diluent was carried out to obtain solutions of concentrations of 40, 70, 90, 110, and 140 µg/mL. Dex-ibuprofen standard solution was also prepared and also injected to confirm the enantiomers of ibuprofen.

3.1.5.5. Sample solutions of ibuprofen and dex-ibuprofen

Twenty tablets of ibuprofen and dexibuprofen were collected, weighed and crushed with a mortar and pestle. Sample powder equivalent to 5 mg of each brand was transferred to a 100 mL volumetric flask to make the concentration of 50 µg/mL. The content was dissolved with suitable volume of diluent and sonicated for 15 minutes. Then make up the volume with diluent and mix well and inject immediately.

3.1.5.6. Application of the proposed method

This analytical method was applied to determine the % recovery of ibuprofen and dexibuprofen commercial samples. Then the enantiomeric purity of samples were calculated by using the formula provided by (*Illustrated Glossary of Organic Chemistry* and *Optical Purity and all that....*):

enantiomeric purity = (% of major enantiomer - % of minor enantiomer) (For single enantiomer) and

enantiomeric purity = 50% - (% of major enantiomer) for racemate (1:1 mixture of enantiomers).

3.1.6. Ofloxacin

3.1.6.1. Materials

The reference standards of ofloxacin and levofloxacin (≥99.98 %) were gifted from Incepta pharmaceutical ltd. Commercial samples for all drugs were collected from different companies following good laboratory practice (GLP). HPLC grade methyl tert butyl ether and ethanol were purchased from Sigma- Aldrich (Spruce Street, St. Louis, Germany).

3.1.6.2. Equipment

The equipment is common for all drugs except the column. The column Chiralpak IC column, 4.6x250 mm, cellulose tris(3,5- dichlorophenylcarbamate) Chiral Technologies, Daicel group, Tokyo, Japan) was used.

3.1.6.3. Chromatographic conditions

Column: Chiralpak IC column (250x 4.6) mm, 5 µm

Flow rate: 1.0 mL/min

Wavelength: 254 nm

Column temperature: Room temparature

Injection volume: 10 µL

Run time: 20 minutes

Mobile phase: ethanol: methyl tert butyl ether (50:50)

Diluent: Mobile phase

3.1.6.4. Standard solutions

Stock standard solutions of ofloxacin were prepared by dissolving twenty mg powder in 100 mL with diluent. Dilution of the stock solutions of ofloxacin and levofloxacin with appropriate volumes of diluent was carried out to obtain solutions of concentrations of 20, 50, 70, 110, and 130 µg/mL. Levofloxacin standard solution was prepared and also injected to confirm the enantiomers of ofloxacin.

3.1.6.5. Sample solutions of ofloxacin and levofloxacin

Twenty tablets of ofloxacin and levofloxacin samples were weighed and crushed with a mortar and pestle. Samples powder equivalent to 5 mg of each brand was transferred to a 100 mL volumetric flask to make the concentration of 50 µg/mL. The content was dissolved with suitable volume of diluents, sonicated, make up the volume with diluent and inject immediately.

3.1.6.6. Application of the proposed method

This analytical method was applied to determine the % recovery of ofloxacin and levofloxacin commercial samples. Then the enantiomeric purity of samples were calculated by using the formula provided by (*Illustrated Glossary of Organic Chemistry* and *Optical Purity and all that....*):

enantiomeric purity = (% of major enantiomer - % of minor enantiomer) (For single enantiomer) and

enantiomeric purity = 50% - (% of major enantiomer) for racemate (1:1 mixture of enantiomers).

3.1.7. Rabeprazole and pantoprazole

3.1.7.1. Materials

The reference standards of rabeprazole, *S*-rabeprazole, pantoprazole, *S*-pantoprazole (≥99.99 %) were gifted from Incepta pharmaceutical ltd. Commercial samples for all drugs were collected from different companies following good laboratory practice (GLP). HPLC grade n-hexane and ethanol were purchased from Sigma- Aldrich (Spruce Street, St. Louis, Germany).

3.1.7.2. Equipment

The equipment is common for all drugs except the column. The column Chiralpak IC column, 4.6x250 mm, cellulose tris-(3,5- dichlorophenylcarbamate) Chiral Technologies, Daicel group, Tokyo, Japan) was used.

3.1.7.3. Chromatographic conditions

Column: Chiralpak IC column (250x 4.6) mm, 5 µm
Flow rate: 0.7 mL/min
Wavelength: 230 nm
Column temperature: Room temparature
Injection volume: 20 µL
Run time: 20 minutes
Mobile phase: *n*-hexane/ Ethanol at a ratio of 50:50 (v/v)
Diluent: Mobile phase

3.1.7.4. Standard solutions

Stock standard solutions of rabeprazole and pantoprazole were prepared by dissolving twenty mg powder in 100 mL with diluent. Dilution of the stock solutions of rabeprazole and pantoprazole with appropriate volumes of diluent was carried out to obtain solutions of concentrations of 40, 70, 90, 110, and 140 µg/mL for each. *S*-rabeprazole and *S*-pantoprazole standard solutions were prepared and also injected to confirm the enantiomers of rabeprazole and pantoprazole.

3.1.7.5. Sample solutions of rabeprazole and pantoprazole

Twenty tablets of rabeprazole and pantoprazole samples were weighed and crushed with a mortar and pestle. Samples powder equivalent to 5 mg of each brand was transferred to a 100 mL volumetric flask to make the concentration of 50 µg/mL. The content was dissolved with suitable volume of diluents, sonicated, make up the volume with diluent and inject immediately.

3.1.7.6. Application of the proposed method

This analytical method was applied to determine the % recovery of rabeprazole and pantoprazole commercial samples. Then the enantiomeric purity of samples were calculated by using the formula provided by (*Illustrated Glossary of Organic Chemistry* and *Optical Purity and all that....*):

enantiomeric purity = 50% - (% of major enantiomer) for racemate (1:1 mixture of enantiomers).

3.1.8. Omeprazole

3.1.8.1. Materials

The reference standards of omeprazole and esomeprazole (≥99.99 %) were gifted from Incepta pharmaceutical ltd. Commercial samples for all drugs were collected from the companies following good laboratory practice (GLP). HPLC grade *n*-hexane, 2-propanol, acetic acid and triethylamine were purchased from Sigma- Aldrich (Spruce Street, St. Louis, Germany).

3.1.8.2. Equipment

The equipment is common for all drugs except the column. The column Chiralcel OD-H, 4.6x250 mm, Cellulose tris (3, 5-dimethylphenylcarbamate) was used.

3.1.8.3. Chromatographic conditions

Column: Chiralcel OD-H column (250x 4.6) mm, 5 µm

Flow rate: 1.2 mL/min

Wavelength: 300 nm

Column temperature: Room temparature

Injection volume: 20 µL

Run time: 20 minutes

Mobile phase: *n*-hexane/ 2-propanol/ acetic acid/ triethylamine at a ratio of 100:20:0.2:0.1 (v/v)

Diluent: mixture of *n*-hexane and 2-propanol (1:1)

3.1.8.4. Standard solutions

About 10 mg of racemic and (*S*)- omeprazole standard were accurately weighed, transferred into a 100 mL volumetric flask and dissolved in a mixture of n-hexane and 2-propanol (1:1) applying sonication in an ultrasonic bath for 15 minutes. The solution was diluted with the solvent mixture up to the mark. The resulting solution had a concentration of 100 µg/mL. Dilution of this solution with appropriate volumes of the solvent mixture was carried out to obtain solutions of concentrations of 50, 60, 70, 80, and 90 µg/mL.

3.1.8.5. Sample solutions of omeprazole and *S*-omeprazole

Twenty capsules of omeprazole and *S*-omeprazole samples were weighed and crushed with a mortar and pestle. Capsule powder equivalent of 5 mg of each brand was weighed accurately and transferred to a 100 mL volumetric flask. The content was dissolved with suitable volume of the mixture of *n*-hexane and 2-propanol (1:1) by sonication for 15 minutes and subsequently more solvent mixture was added into the volumetric flask up to the mark to make the concentration of 50 µg/mL. The solution was further diluted two times with the same solvent mixture to obtain the concentration of 50 µg/mL for each code, filtered through 0.45 µm membrane filter tips and then injected for analysis.

3.1.8.6. Application of the proposed method

This analytical method was applied to determine the % recovery of omeprazole and *S*-omeprazole commercial samples. Then the enantiomeric purity of samples were calculated by using the formula provided by (*Illustrated Glossary of Organic Chemistry* and *Optical Purity and all that....*):

enantiomeric purity = (% of major enantiomer - % of minor enantiomer) (For single enantiomer) and

enantiomeric purity = 50% - (% of major enantiomer) for racemate (1:1 mixture of enantiomers).

3.2. Method validation for enantiomeric drugs

3.2.1. Specificity

Specificity of the test method was determined by testing standard substances against potential interferences. The methods were found to be specific because of complete separation of all enantiomers were achieved without any interference.

3.2.2. System suitability

System suitability tests were carried out to verify whether the chromatographic system were suitable for the analysis. Retention factor (k), enantio- separation factor (α), and resolution (R) were calculated using the following equations:

$k = (t - t_0)/t_0$;

where, t and t_0 are the retention times of analyte and unretained solutes, respectively; $\alpha = k_R/k_S$, where k_S and k_R are the retention factors of (*S*)- and (*R*)-enantiomer, respectively;

$R = 2(t_R - t_S)/(w_R + w_S)$

Where t_S and t_R are retention times of the (*S*)- and (*R*)- enantiomer, respectively, and w_S and w_R are the baseline peak widths of the two enantiomers.

3.2.3. Linearity and calibration curve

The calibration curves were constructed by plotting the peak area of each enantiomer against the respective concentration, and the linearity was evaluated by the least-squares regression method, which was used to calculate the regression coefficient value (R), y-intercept and slope of the regression line.

3.2.4. Precision

For validation of the method intra-day precision was examined by determining the % RSD of a single solution of a particular concentration by injecting six times on the same day. Intermediate precision was examined by determining the %RSD for a solution of single concentration by injecting three times on three different days.

% RSD = (Standard deviation, SD/ Mean) × 100

3.2.5. Accuracy

Accuracy of the method was studied by recovery experiments which were performed by spiking solutions of known amount of the drug with pre- analyzed sample. The data of the

experiment were statistically analyzed to determine the recovery and the validity of the proposed method.

% Recovery = (Recovered conc. /Injected conc.) x 100

3.2.6. Detection limit

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were calculated using the following equations:

LOD= (SD / Slope) x 3.3

LOQ= (SD / Slope) x 10.

3.2.7. Robustness

The robustness of test method is demonstrated by carrying out method variations like mobile phase flow changes, mobile phase compositions, column oven temperature variations and change in wavelength and the % RSD should be reported. For the study, factors chosen were flow rate (± 0.2 mL/min) and wavelength (± 2 nm), where, n = 3.

3.2.8. Solution stability

The solution stability of standards and samples was established under normal bench top conditions, normal storage conditions, and sometimes in the instrument to determine where special storage conditions are necessary or not, for instance, refrigeration or protection from light.

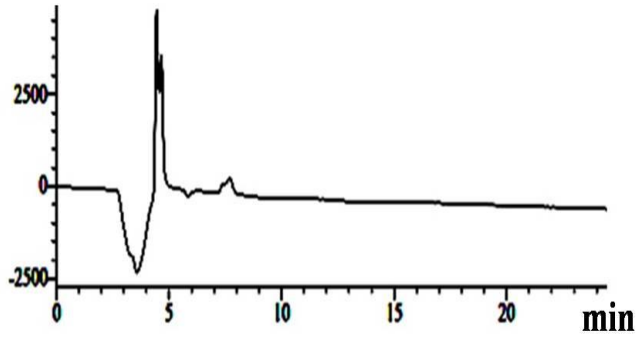
Chapter 4: Results and Discussion

4.1. Analysis of propranolol

4.1.1. Analytical HPLC

Our first effort was to develop a simple and easy method for determination of racemic mixture of propranolol hydrochloride. A number of mobile phases were initially tried through trial and error to elute propranolol and to achieve individual separation of propranolol enantiomers with good resulotion. For propranolol separation, CD-PH chiral columns were used which is applicable in both of the normal-phase and reversed-phase mode. Here, CD-PH was used as reverse-phase mode and normal-phase mode. Using different ratio of organic solvent or buffer as mobile phase, a suitable method was developed.

Some trial chiral methods with chromatogram are shown below with CD-PH on reverse phase mode:



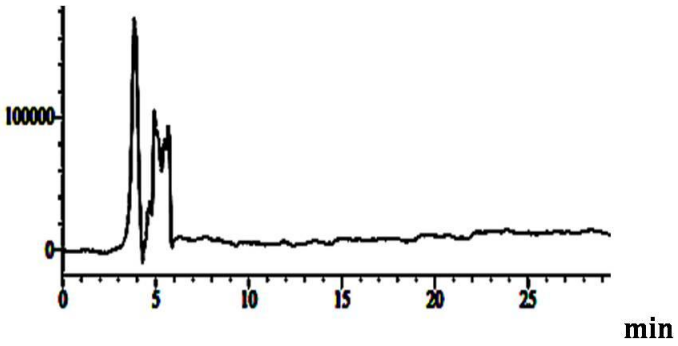
Mobile phase: MeOH: H_2O (50:50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: No peak observed

Figure 4.1.1: Trial-1.



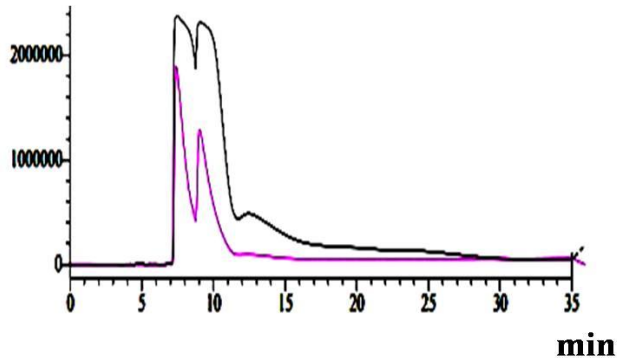
Mobile phase: ACN: H_2O (50: 50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: No peak observed

Figure 4.1.2: Trial-2.



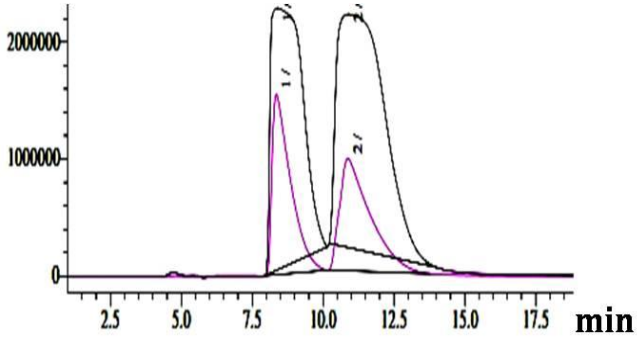
Mobile phase: NaOAc: MeOH (50: 50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.1.3: Trial-3.



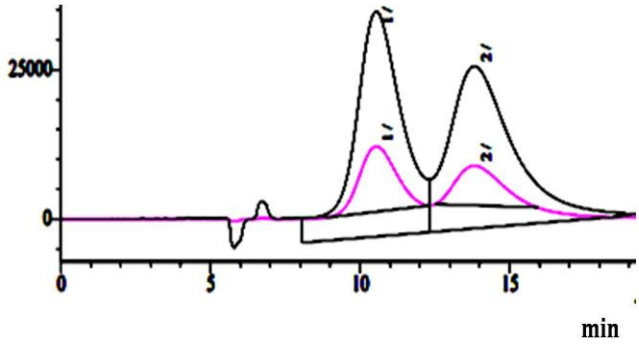
Mobile phase: NH_4OAc (pH 4.5): MeOH (50: 50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.1.4: Trial-4.



Mobile phase: NH_4OAc (pH 6.0): MeOH (50: 50)

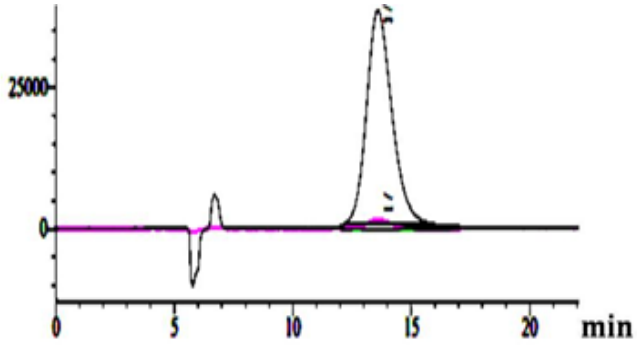
Flow rate: 1 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.1.5: Trial-5.

Some trial chiral methods with chromatogram have been shown below with CD-PH on polar phase mode:



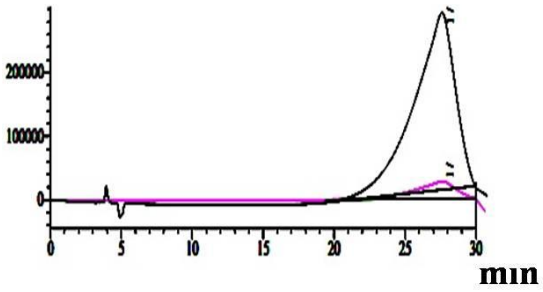
Mobile phase: IPA: EtOH (50: 50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: One peak observed

Figure 4.1.6: Trial-6.



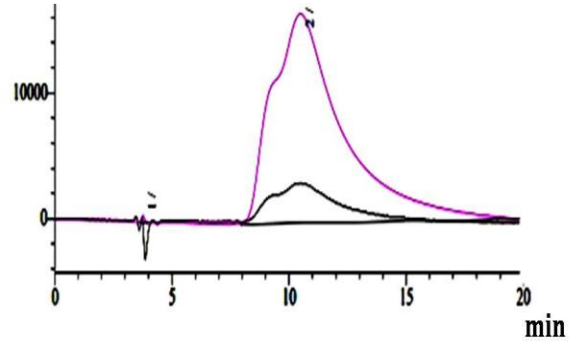
Mobile phase: IPA: NH_4OAc (50: 50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: One peak observed

Figure 4.1.7: Trial-7.



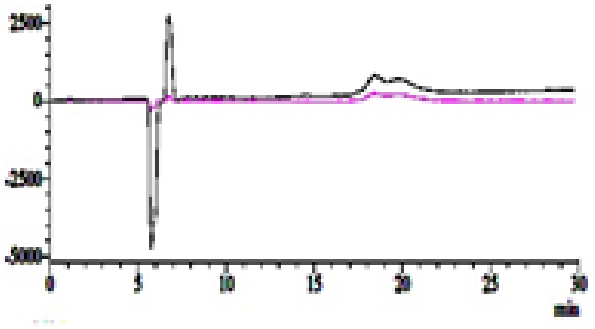
Mobile phase: Hx : IPA (50: 50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.1.8: Trial-8.



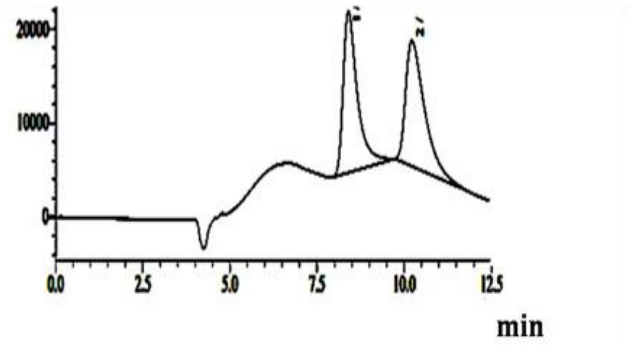
Mobile phase: Hx: IPA: NaOAc (40: 30: 30)

Flow rate: 1 mL/min

Detector: 254 nm

Result: Two peaks observe

Figure 4.1.9: Trial-9.



Mobile phase: IPA: NH_4OAc: MeOH (50: 30: 20)

Flow rate: 1 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.1.10: Trial-10.

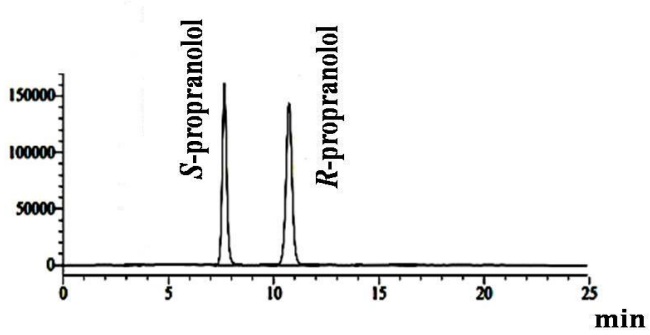
Summary of these trial chiral methods are shown in table 4.1.1.

Table 4.1.1: Trial chiral methods for propranolol.

Mobile Phase	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
Methanol: H_2O (50:50)	No peak observed	NA	NA	NA
Acetonitrile: H_2O (50: 50)	No peak observed	NA	NA	NA
Sodium acetate: Methanol (50: 50)	Two peaks observed	NA	NA	NG
Ammonium acetate (pH 4.5): Methanol (50: 50)	Two peaks observed	NA	NA	NG
Ammonium acetate (pH 6.0): Methanol (50: 50)	Two peaks observed	NA	NA	NG
IPA: Ethanol (50: 50)	One peak observed	NA	NA	NA
IPA: NH_4OAc (50: 50)	One peak observed	NA	NA	NA
Hx : IPA (50: 50)	Two peaks observed	NA	NA	NG
Hx : IPA: NaOAc (40: 30: 30)	Two peaks observed	NA	NA	NG
IPA: NH_4OAc: MeOH (50: 30: 20)	Two peaks observed	1332 1402	2.9 3.3	2.2

NA = Not applicable; NG = Not good

Finally, the best mobile phase composition was then found to be ammonium acetate (pH 3.0) and methanol in the ratio of (10: 90, v/v).



Mobile phase: Ammonium acetate (pH 3.0): methanol (10:90)
Flow rate: 0.6 mL/min
Detector: 254 nm
Result: Acceptable two peaks observed

Figure 4.1.11: Chromatogram with proposedmethod.

Then, this proposed chromatographic method was compared with the previously published methods and then validated according to the ICH guidelines.

4.1.2. Comparison of the present work

A comparison of present work with the other earlier reports on enantioresolution of propranolol is shown in table 4.1.2. It is clear that the present method is suitable in terms of resolution.

Table 4.1.2: Comparison of HPLC enantio-resolution of propranolol using different column.

Chiral column/ selector	Mobile phase	Rs	Reference
α-Burke-2	Dichloromethane: methanol (90: 10)	3.0	(Santoro *et al.* 2001)
Chiral CDMPC	Petroleum ether: isopropyl alcohol: diethylamine (85:15: 0.1)	2.4	(Chen *et al.*, 2008)
Chiralcel OD	*n*-Hexane: ethanol (75: 25)	Not given	(Santoro *et al.*, 2001)
Chiral CD-PH	Ammonium acetate (pH 3.75 3.0): methanol (10: 90)		Present work

Rs = Resolution

4.1.3. Validation of the proposed method

Finally, this present method was validated with respect to the following parameters.

4.1.3.1. Specificity

The specificity of the method was assessed from the chromatogram where complete separation of *R*-propranolol and *S*-propranolol was achieved without any interference. The peaks obtained were sharp, well separated at the baseline as shown in figure 4.12. Single enantiomer, *S*-propranolol was injected into HPLC for the detection of (*S*)- enantiomer in propranolol.

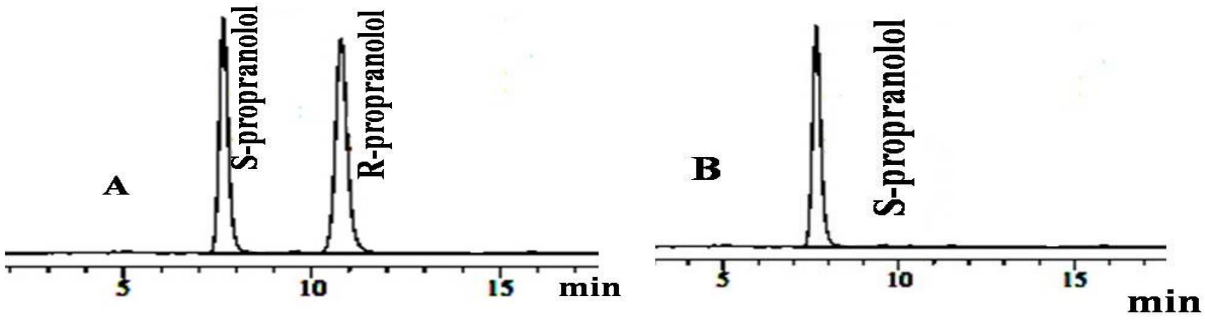


Figure 4.1.12: HPLC chromatograms of propranolol (A) and *S*-propranolol (B) for specificity testing.

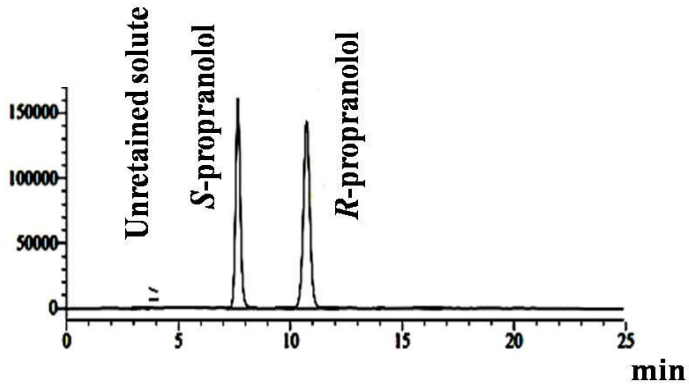
4.1.3.2. System suitability

System suitability parameters are reported in table 4.1.3. The chromatogram of system suitability is shown in figure 4.1.13.

Table 4.1.3: System suitability.

Parameters	***S*-propranolol**	***R*-propranolol**
Theoretical plates (≥2000) (n=5)	8074	7012
Tailing factor (≤2) (n=5)	1.14	1.04
Relative retention (k_S and k_R) (n=5), 0.5 < k < 10	1.43	2.39
Selectivity (α>1) (n=5)	1.67	
Resolution (≥2) (n=5)	3.75	

n = number of determinations



Where,

t_0 = 3.14 min,

t_S = 7.65 min,

t_R = 10.68 min

Figure 4.1.13: HPLC chromatogram of propranolol for system suitability testing.

4.1.3.3. Linearity and range

Linearity of the method was studied by injecting five concentrations of two enantiomers of propranolol prepared in the mobile phase in concentration range from 40 –140 µg/mL in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves. The linearity curves are shown in figure 4.1.14 and the parameters are given in table 4.1.4.

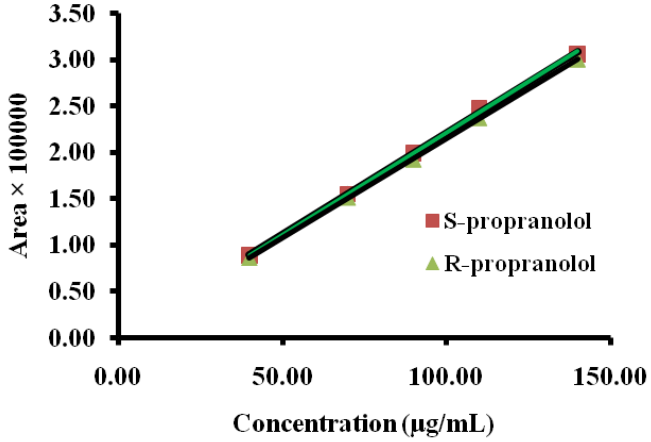


Figure 4.1.14: Linearity curve for (*S*)- and (*R*)-propranolol.

4.1.3.4. Precision

The precision of the method was verified by intra- and inter-day precision studies. Intra-day precision was performed by analysis of concentration for six times on the same day. The intermediate precision of the method was checked by studying on three different days. Results

are recorded in table 4.1.4. Bar diagram of precision (% RSD) for (*S*)- and (*R*)-propranolol has been shown in figure 4.1.15.

Table 4.1.4: Results of method validation parameters.

Parameters	***S*-propranolol**		***R*-propranolol**
Linear equation	y= 4394x+1655		y= 4289x+834.0
Coefficient of determination (r2>0.995)	0.999		0.999
Linearity range		40-140 µg/mL	
Precision (intra-day, n=6) (% RSD≤2)	0.09%		0.05%
Precision (inter-day, n=6) (% RSD≤2)	0.09%		0.05%

n = number of determinations

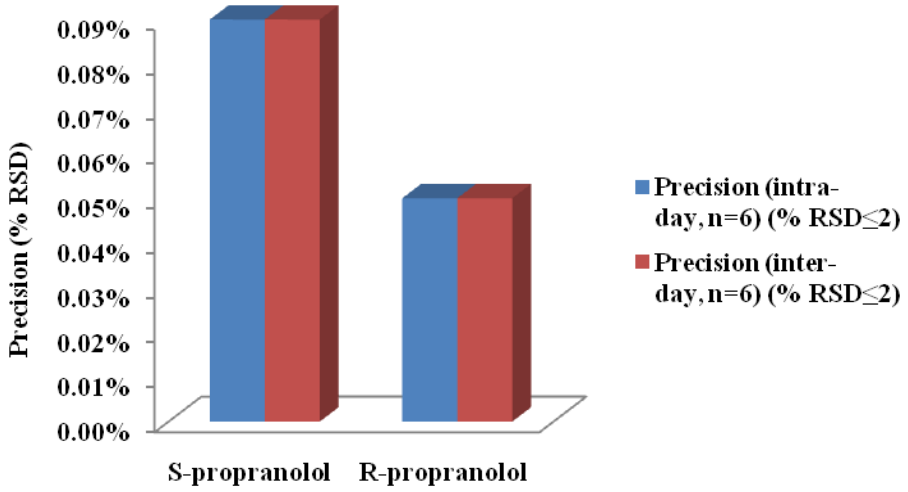


Figure 4.1.15: Bar diagram of precision (% RSD) for (*S*)- and (*R*)-propranolol.

4.1.3.5. Accuracy

Accuracy of the method was verified by studying recovery experiments which were performed by spiking solutions of known amount of the drug with pre-analyzed sample. To evaluate the accuracy of the method, successive analysis (n=3) of standard solutions of the drug was carried out and the results are given in table 4.1.5. Bar diagram of accuracy (% recovery) has been shown in figure 4.1.16.

Table 4.1.5: Results of accuracy for standard propranolol.

Parameters	*S*-propranolol	*R*-propranolol
Accuracy (n=3) (avg. % recovery)		
Standard+spike (µg/mL)		
(25+20)	99.95%	99.46%
(30+20)	100.28%	100.51%
(40+20)	100.06%	99.99%
LOD (µg/mL)	1.32	0.59
LOQ (µg/mL)	3.99	1.79

n = number of determinations

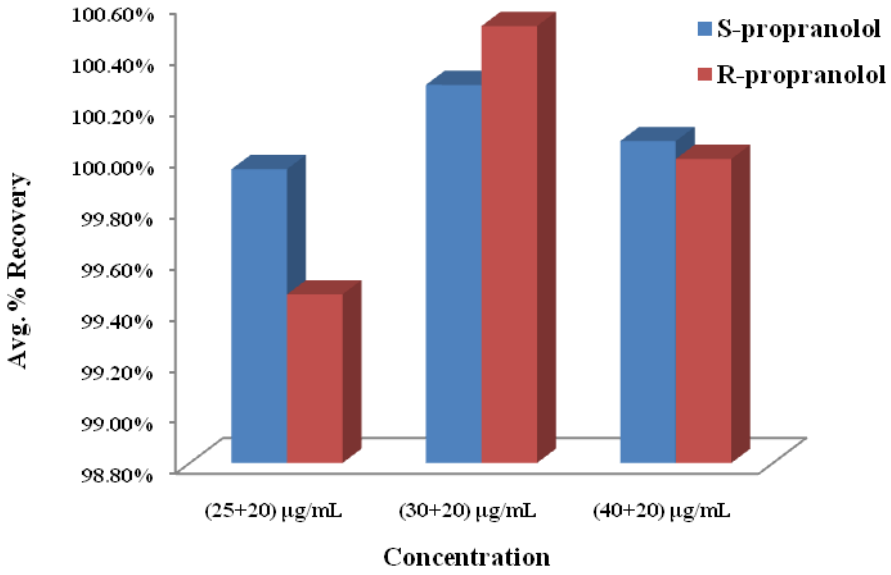


Figure 4.1.16: Bar diagram of accuracy (% recovery) for (*S*)- and (*R*)-propranolol.

4.1.3.6. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated using the following equations:

LOD= (SD / Slope) x 3.3

LOQ= (SD / Slope) x 10.

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of

regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). The results of LOD and LOQ obtained for studied drugs are recorded in table 4.1.5.

4.1.3.7. Solution stability testing

A standard solution of concentration of 60 µg/mL was kept in a tightly capped volumetric flask at room temperature (25 °C) on the laboratory bench and at 4 °C in a refrigerator for 3 days and its stability was tested. The results are given in table 4.6 and the bar diagram of solution stability (% RSD) for (*S*)- and (*R*)-propranolol has been shown in figure 4.1.17.

Table 4.1.6: Parameters for solution stability testing.

Day	At 25 °C		At 4 °C	
	***S*-propranolol (n=3) (% RSD)**	***R*-propranolol (n=3) (% RSD)**	***S*-propranolol (n=3) (% RSD)**	***R*-propranolol (n=3) (% RSD)**
Day 1	0.00%	0.03%	0.01%	0.01%
Day 2	0.01%	0.02%	0.03%	0.01%
Day 3	0.02%	0.01%	0.02%	0.00%
Avg.	0.01%	0.02%	0.02%	0.01%

n = number of determinations.

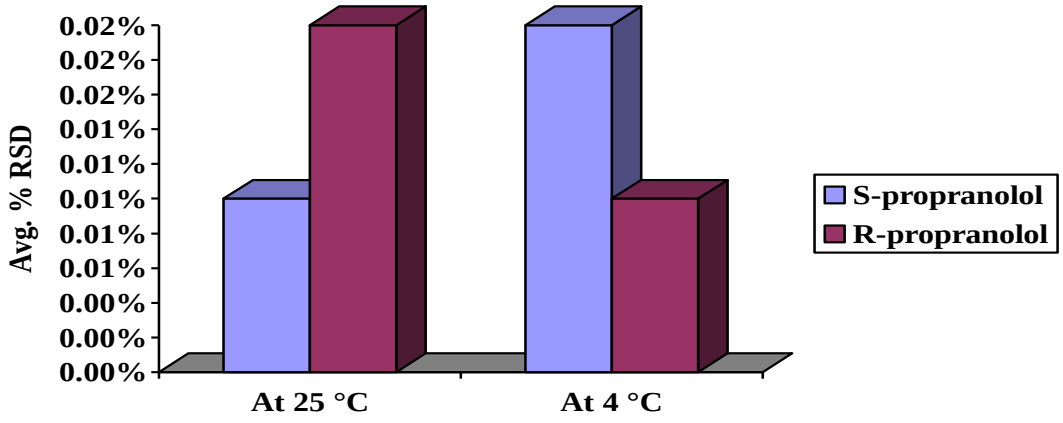


Figure 4.1.17: Bar diagram of solution stability testing (% RSD) for (*S*)- and (*R*)-propranolol.

4.1.3.8. Robustness of the method

To determine the robustness of this method, the experimental conditions were deliberately changed, like the flow rate and in the wavelength of detection and measuring the % RSD. For the present study, factors chosen were flow rate (0.6 ± 0.2mL/min) and wavelength (254 ± 2nm), and n = 3. % RSD has been reported in table 4.1.7. All analyte peaks are adequately resolved and elution orders remain unchanged. Bar diagram of robustness (% RSD) for (*S*)- and (*R*)-propranolol has been shown in figure 4.1.18.

Table 4.1.7: Robustness parameters.

Parameters (n=3)	***S*-propranolol (n=3) (% RSD)**	***R*-propranolol (n=3) (% RSD)**
Detection wavelength at 252nm	0.12%	0.12%
Detection wavelength at 256nm	0.12%	0.03%
Flow rate 0.4 mL/min	0.02%	0.03%
Flow rate 0.8 mL/min	0.05%	0.01%

n = number of determinations

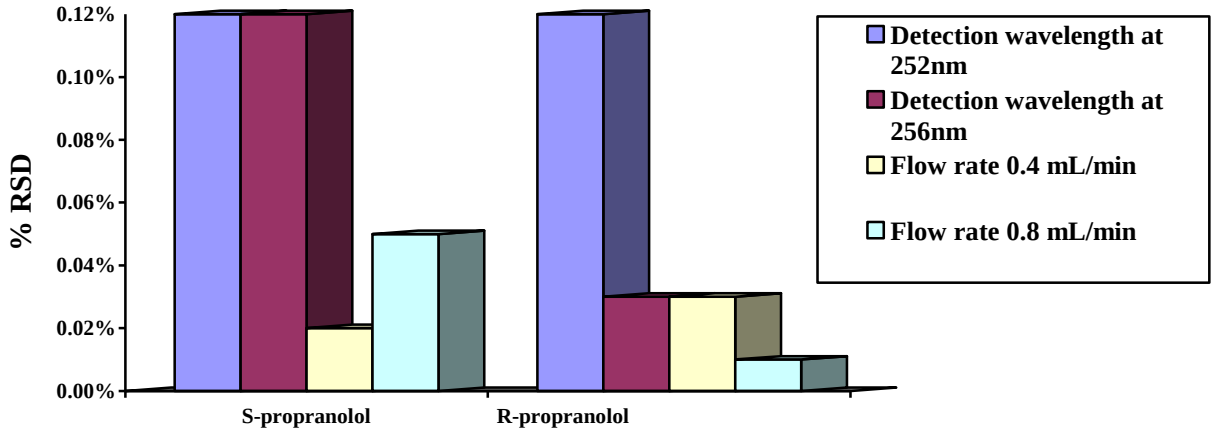


Figure 4.1.18: Bar diagram of robustness (% RSD) for (*S*)- and (*R*)-propranolol.

4.1.4. Applications of the method

This analytical method was applied to quantitate the content of *S*- and *R*- propranolol samples from six companies of Bangladesh and as well as to calculate the ratio of them. The average

content of *S*- propranolol was from 50.18% to 51.08% while the content of *R*-propranolol was from 48.91% to 49.82% in the formulations of racemic mixture of propranolol. The data and enantiomeric ratio of commercial samples are shown in table 4.1.8. According to USP, it states the limits for enantiomeric ratio between 45 to 55% of each isomer. Enantiomeric ratio of propranolol for all samples from different companies met the USP requirements. The chromatogram of one sample has been shown in figure 4.1.19. Bar diagram of enantiomeric ratio of racemic propranolol has been shown in figure 4.1.20.

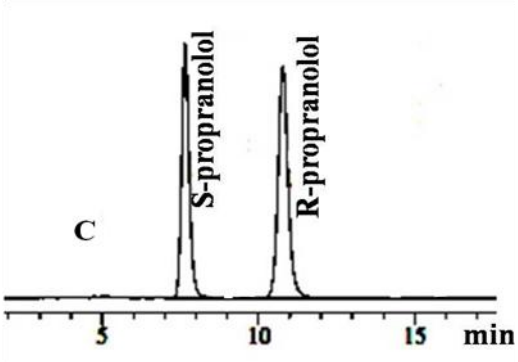


Figure 4.1.19: Chromatogram of one commercial sample (C).

Table 4.1.8: Enantiomeric ratio of racemic propranolol in commercial samples (n=10).

Identity of company	% content of *S*-propranolol	% EP= (50%-%enantiomer) of *S*-propranolol	% content of *R*-propranolol	% EP= (50%-%enantiomer) of *R*-propranolol	Enantiomeric ratio of propranolol
Company A	51.02%	-1.02%	48.97%	1.03%	51.02%/48.97%
Company B	50.36%	-0.36%	49.63%	0.37%	50.36%/49.63%
Company C	50.18%	-0.18%	49.82%	0.18%	50.18%/49.82%
Company D	51.08%	-1.08%	48.91%	1.09%	51.08%/48.91%
Company E	50.49%	-0.49%	49.51%	0.49%	50.49%/49.51%
Company F	50.72%	-0.72%	49.27%	0.73%	50.72%/49.27%

EP= enantiomeric purity, n = number of determinations

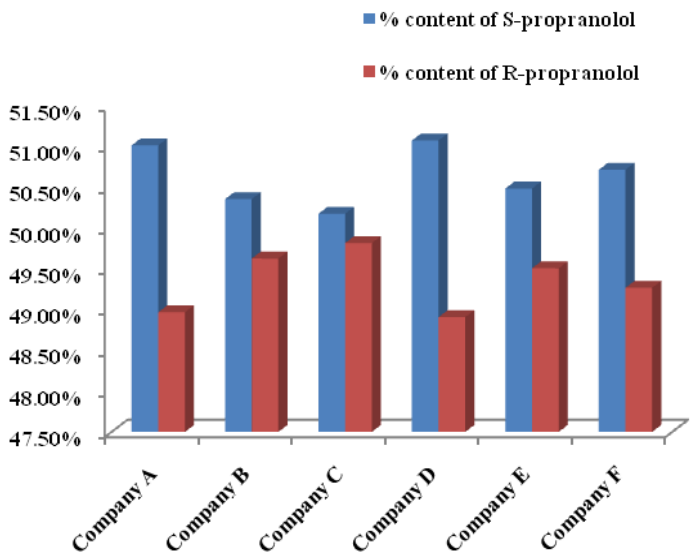


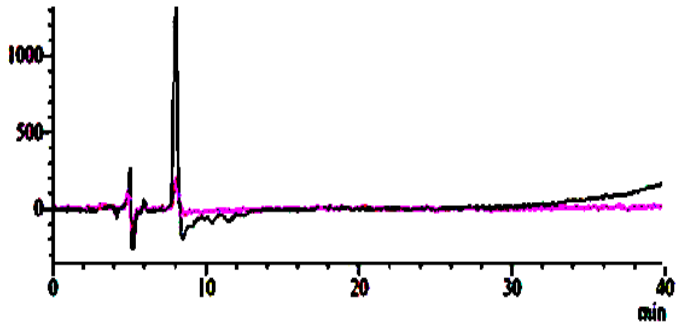
Figure 4.1.20: Bar diagram of enantiomeric ratio of racemic propranolol.

4.2. Analysis of carvedilol

4.2.1. Analytical HPLC

In order to develop a new and easy chiral HPLC method, a significant number of methods were tried for better separation of racemic mixture of carvedilol using a large number of polar and non-polar solvent mixtures as mobile phase with chirapak IC column. Chiralpak IC column is applicable in both of the normal-phase and reversed-phase mode. Here, it was used as reverse-phase mode and normal-phase mode. Using different organic solvent or buffer ratio as mobile phase, a suitable method was developed.

Some trial chiral methods with chromatograms are shown below with chiralpak IC on reverse phase mode:



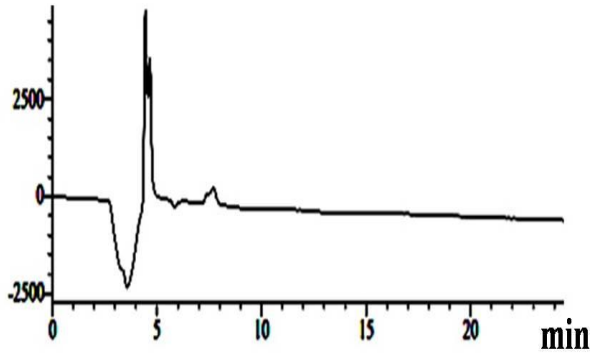
Mobile phase: MeOH: H_2O (50: 50)

Flow rate: 1 mL/min

Detector: 220 nm

Result: No peak observed

Figure 4.2.1: Trial-1.



Mobile phase:

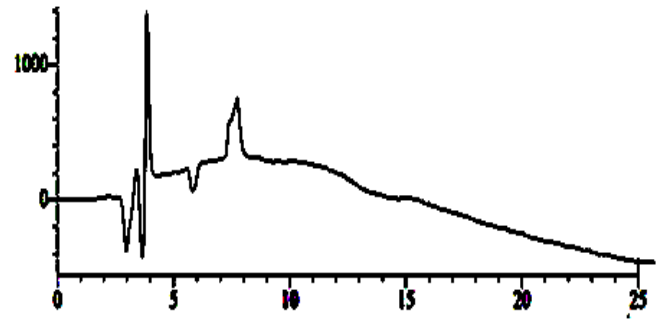
NH_4OAc: MeOH (50: 50)

Flow rate: 1 mL/min

Detector: 220 nm

Result: No peak observed

Figure 4.2.2: Trial-2.



Mobile phase:

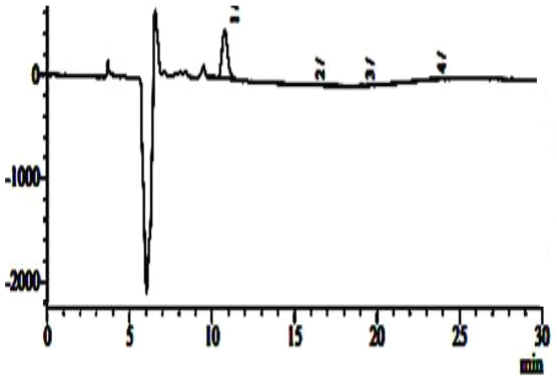
NH_4OAc: MeOH (20: 80)

Flow rate: 1 mL/min

Detector: 220 nm

Result: No peak observed

Figure 4.2.3: Trial-3.



Mobile phase:

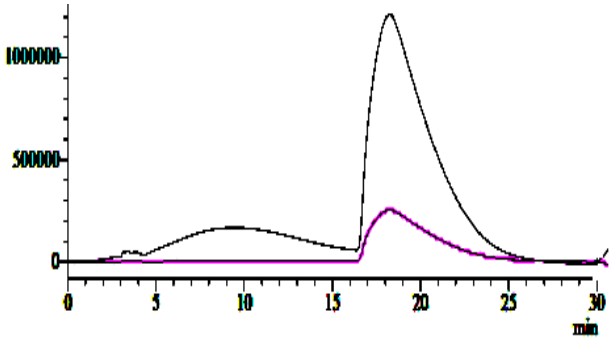
$NH_4H_2PO_4$: MeOH (50: 50)

Flow rate: 1 mL/min

Detector: 220 nm

Result: No identical peak observed

Figure 4.2.4: Trial-4.



Mobile phase:

$NH_4H_2PO_4$: MeOH (30: 70)

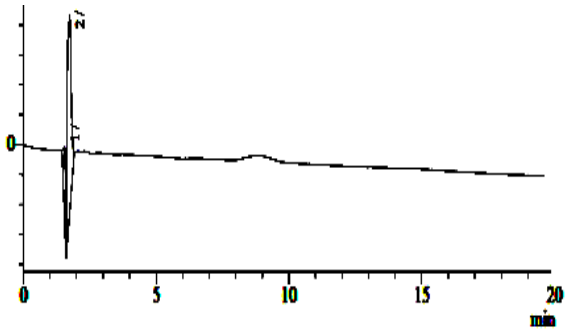
Flow rate: 1 mL/min

Detector: 220 nm

Result: No identical peak observed

Figure 4.2.5: Trial-5.

Some trial chiral methods with chromatograms are shown below with chiralpak IC on normal phase mode:



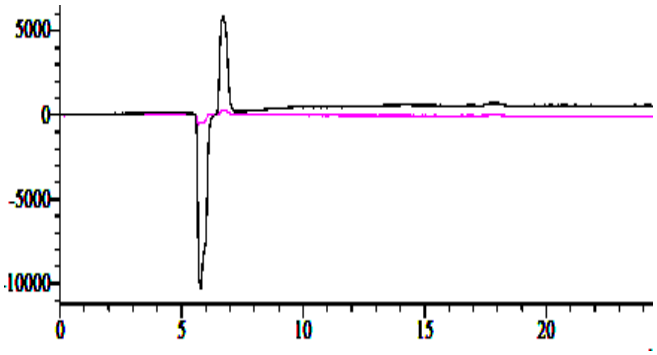
Mobile phase: Hx: IPA (30: 70)

Flow rate: 1 mL/min

Detector: 220 nm

Result: No peak observed

Figure 4.2.6: Trial-6.



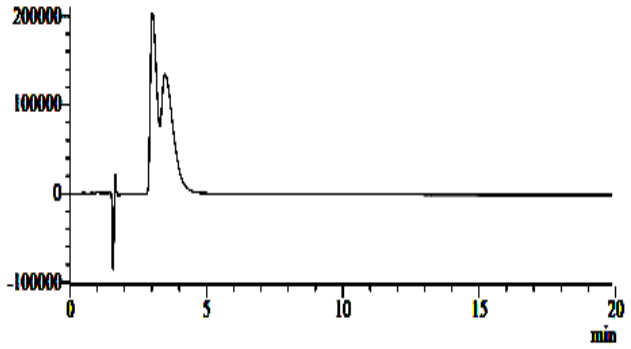
Mobile phase: Hx: IPA (70: 30)

Flow rate: 1 mL/min

Detector: 220 nm

Result: No identical peak observed

Figure 4.2.7: Trial-7.



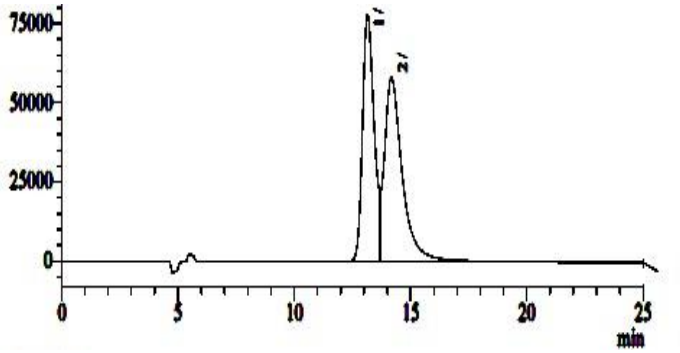
Mobile phase: Hx: IPA (50: 50)

Flow rate: 1 mL/min

Detector: 220 nm

Result: No identical peak observed

Figure 4.2.8: Trial-8.



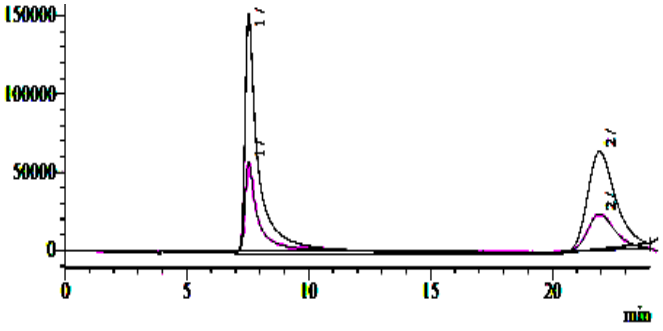
Mobile phase: Hx: IPA: DEA (50: 50: 0.1)

Flow rate: 1 mL/min

Detector: 220 nm

Result: No identical peak observed

Figure 4.2.9: Trial-9.



Mobile phase: Hx: IPA: DEA (50: 50: 0.1)

Flow rate: 1 mL/min

Detector: 220 nm

Result: No identical peak observed

Figure 4.2.10: Trial-10.

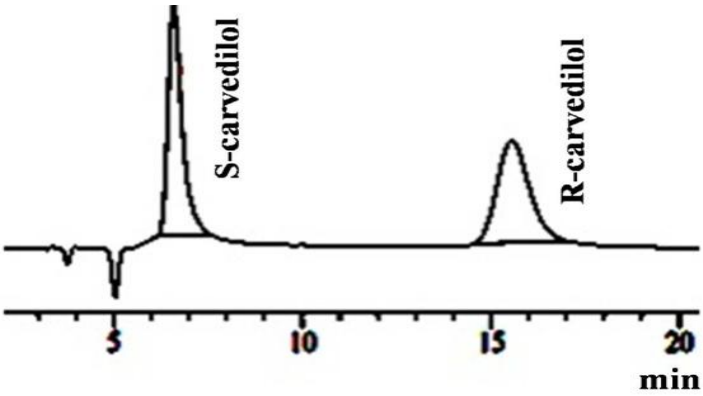
Summary of these trial chiral methods are shown in table 4.2.1.

Table 4.2.1: Trial chiral methods for carvedilol.

Polar Mobile Phase Buffer:Organic phase/Others	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
Methanol: H_2O (50:50)	No peak observed	NA	NA	NA
Ammonium acetate: Methanol (50:50)	No peak observed	NA	NA	NA
Ammonium acetate: Methanol (20:80)	No peak observed	NA	NA	NA
Ammonium dihydrogen phosphate: Methanol (50:50)	No peak observed	NA	NA	NA
Ammonium dihydrogen phosphate: Methanol (30:70)	No peak observed	NA	NA	NA
Non-polar Mobile phase	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
Hexane: Isopropylalcohol (50: 50)	One peak observed	NA	NA	NA
Hexane: Isopropylalcohol (90: 10)	One peak observed	NA	NA	NA
Hexane: Isopropylalcohol: Diethylamine (70: 30: 0.1)	Two peaks observed	NA	3.6 3.9	NA
Hexane: Isopropylalcohol: Acetic acid (40:50:0.1)	Two peaks observed	1010 998	1.5 1.3	1.07

NA = Not applicable

Finally, it has been found that good chromatographic condition achieved on chiralpak IC and n-hexane: isopropylalcohol: diethylamine: acetic acid (40: 60: 0.7: 0.3, v/v) as mobile phase because of adequate resolution, efficient theoretical plates number and symmetric peak shape.



Mobile phase: Hx: IPA: DEA: AA (40: 60: 0.7: 0.3)
Flow rate: 1.0 mL/min
Detector: 220 nm
Result: Acceptable two peaks observed

Figure 4.2.11: Chromatogram with IPA: DEA: AA (40: 60: 0.7: 0.3) using chiralpak IC.

Then, this proposed chromatographic method was compared with the previously published methods and then validated according to the ICH guidelines.

4.2.2. Comparison of the present work

A comparison of present work with the other earlier reports on enantioresolution of carvedilol using different chiral selectors is shown in table 4.2.2. It is noteworthy that resolution 7.9 was achieved using immobilized cellulose based CSP. It clearly establishes the novelty and superiority of the present report in terms of resolution.

Table 4.2.2: Comparison of HPLC enantioresolution of carvedilol using different column with the present work.

Chiral column/ selector	Mobile phase	Resolution (Rs)	Reference
Lux cellulose- 4	Isopropanol: *n*-Hexane (60:40)	1.9	(Swetha *et al*., 2015)
Chiral selector HP-β-CD	50 mM phosphate buffer (pH 4.0)	2.34	(Nguyen *et al*., 2006)
Chiral selector-β-CD	25 mM phosphoric acid (pH 2.5)	2.5	(Nguyen *et al*., 2006)
Chiralpak-IC	*n*-Hexane: isopropyl alcohol: diethylamine: acetic acid (40:60:0.7:0.3)	7.9	Present work

Finally, this present method has been extensively validated with respect to the following parameters.

4.2.3. Validation of the proposed method

4.2.3.1. Linearity and range

Linearity of the method was studied by injecting five concentrations of two enantiomers of carvedilol prepared in the mobile phase in concentration range from 30 –130 µg/mL in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves. The linearity curves are shown in figure 4.2.12 and the parameters are given in table 4.2.3.

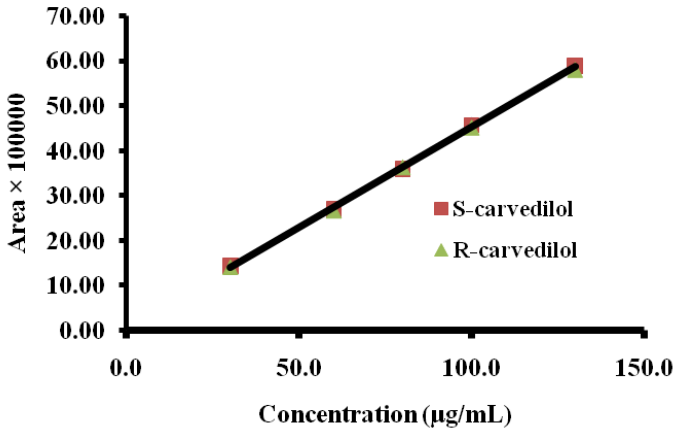


Figure 4.2.12: Linearity curve for (*S*)- and (*R*)-carvedilol.

4.2.3.2. Precision

The precision of the method was verified by intra- and inter-day precision studies. Intra-day precision was performed by analysis of on concentration for six times on the same day. The intermediate precision of the method was checked by studying on three different days. Results have been recorded in table 4.2.3. Bar diagram of precision (% RSD) for (*S*)- and (*R*)-carvedilol has been shown in figure 4.2.13.

Table 4.2.3: Results of method validation parameters.

Parameters	*S*-carvedilol	*R*-carvedilol
Linear equation	y= 89799x+49478	y= 88273x+86175
Coefficient of determination (r2>0.995)	0.999	0.999
Linearity range	30-130 µg/mL	
Precision (intra-day, n=6) (% RSD≤2)	0.02%	0.07%
Precision (inter-day, n=6) (% RSD≤2)	0.03%	0.07%

n = number of determinations

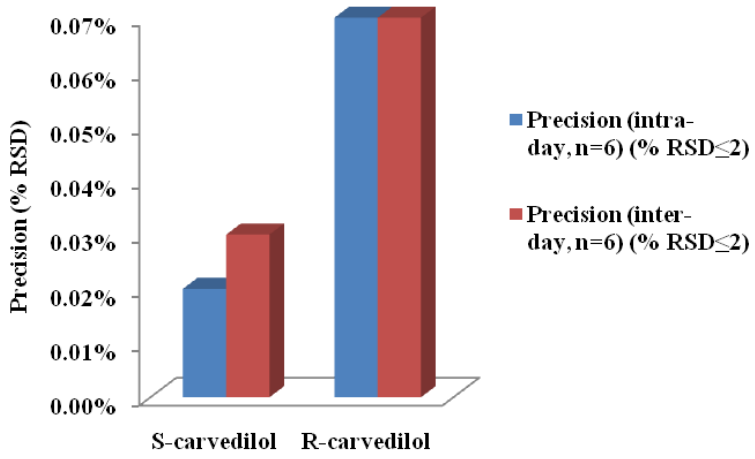


Figure 4.2.13: Bar diagram of precision (% RSD) for (*S*)- and (*R*)-carvedilol.

4.2.3.3. Accuracy

Accuracy of the method was verified by studying recovery experiments which were performed by spiking solutions of known amount of the drug with pre-analyzed sample. To evaluate the accuracy of the method, successive analysis (n=3) of standard solutions of the drug was carried out and the results are given in table 4.2.4. Bar diagram of accuracy (% Recovery) for (*S*)- and (*R*)-carvedilol has been shown in figure 4.2.14.

Table 4.2.4: Results of accuracy for standard carvedilol.

Parameters	*S*-carvedilol	*R*-carvedilol
Accuracy (n=3) (avg. % recovery)		
Standard+spike (µg/mL)		
(50+20)	99.59%	98.42%
(60+20)	100.83%	100.18%
(80+20)	99.79%	99.25%
LOD (µg/mL)	1.67	1.78
LOQ (µg/mL)	5.06	5.41

n = number of determinations

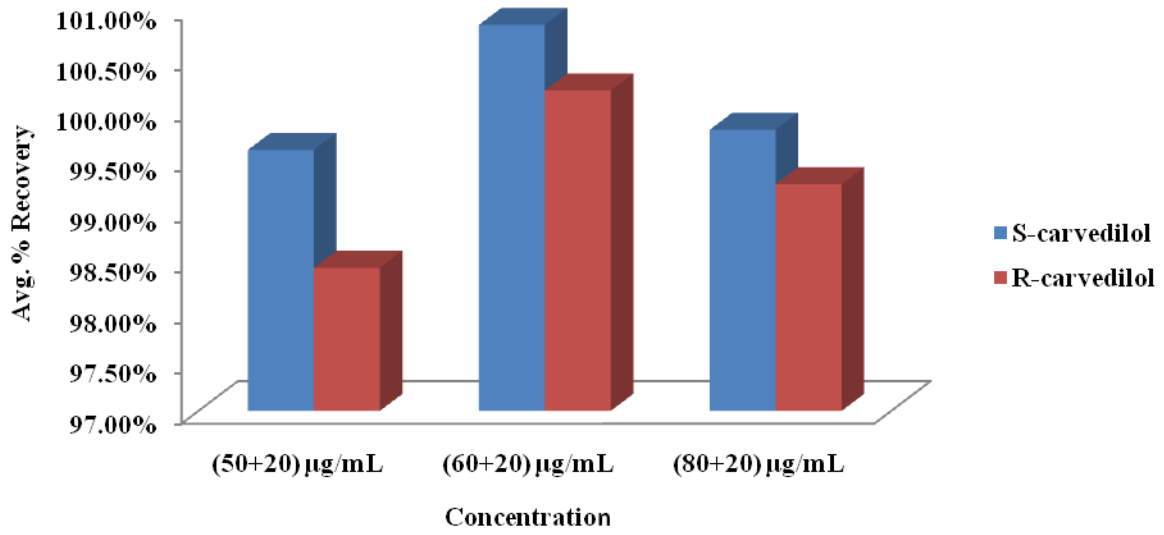


Figure 4.2.14: Bar diagram of accuracy (% recovery) for (*S*)- and (*R*)-carvedilol.

4.2.3.4. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated using the following equations:

LOD= (SD / slope) x 3.3

LOQ= (SD / slope) x 10.

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of

regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). The results of LOD and LOQ obtained for studied drugs are presented in table 4.2.4.

4.2.3.5. Specificity

The specificity of the method was assessed from the chromatogram where complete separation of *R*-carvedilol and *S*-carvedilol was achieved without any interference. The peaks obtained were sharp, well separated at the baseline as shown in figure 4.2.15. Single enantiomer, *S*-carvedilol was injected into HPLC for the detection of (*S*)- enantiomer in carvedilol.

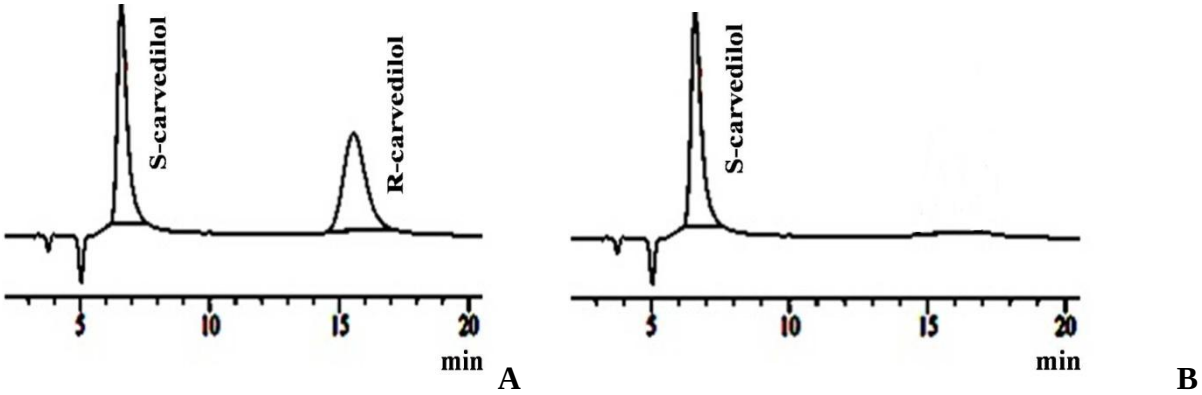


Figure 4.2.15: HPLC chromatograms of standard carvedilol (A) and *S*-carvedilol (B).

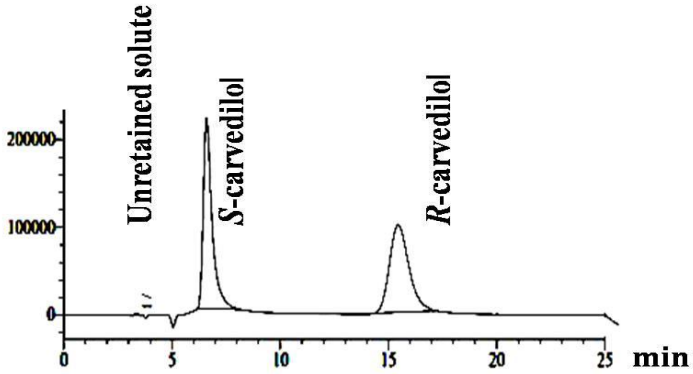
4.2.3.6. System suitability testing

System suitability parameters are reported in table 4.2.5. The chromatogram of system suitability is shown in figure 4.2.16.

Table 4.2.5: System suitability testing.

Parameters	***S*-carvedilol**	***R*-carvedilol**
Theoretical plates (≥2000) (n=5)	2198	2211
Tailing factor (≤2) (n=5)	1.23	1.62
Relative retention (k_S and k_R) (n=5), 0.5 < k < 10	0.96	3.59
Selectivity (α>1) (n=5)	3.73	
Resolution (≥2) (n=5)	7.9	

n = number of determinations



Where,

t_0 = 3.36 min,

t_S = 6.59 min,

t_R = 15.44 min

Figure 4.2.16: HPLC chromatogram of carvedilol for system suitability testing.

4.2.3.7. Solution stability testing

A standard solution of concentration of 60 µg/mL was kept in a tightly capped volumetric flask at room temperature (25 °C) on the laboratory bench and at 4 °C in a refrigerator for 3 days and its stability was tested. The results are given in Table 6. Bar diagram of solution stability testing (% RSD) for (*S*)- and (*R*)-carvedilol is shown in figure 4.2.17.

Table 4.2.6: Parameters for solution stability testing.

Day	**At 25 °C**		**At 4 °C**	
	***S*-carvedilol (n=3) (% RSD)**	***R*-carvedilol (n=3) (% RSD)**	***S*-carvedilol (n=3) (% RSD)**	***R*-carvedilol (n=3) (% RSD)**
Day 1	0.00%	0.03%	0.01%	0.01%
Day 2	0.01%	0.02%	0.03%	0.01%
Day 3	0.02%	0.01%	0.02%	0.00%
Avg.	0.01%	0.02%	0.02%	0.01%

n = number of determinations

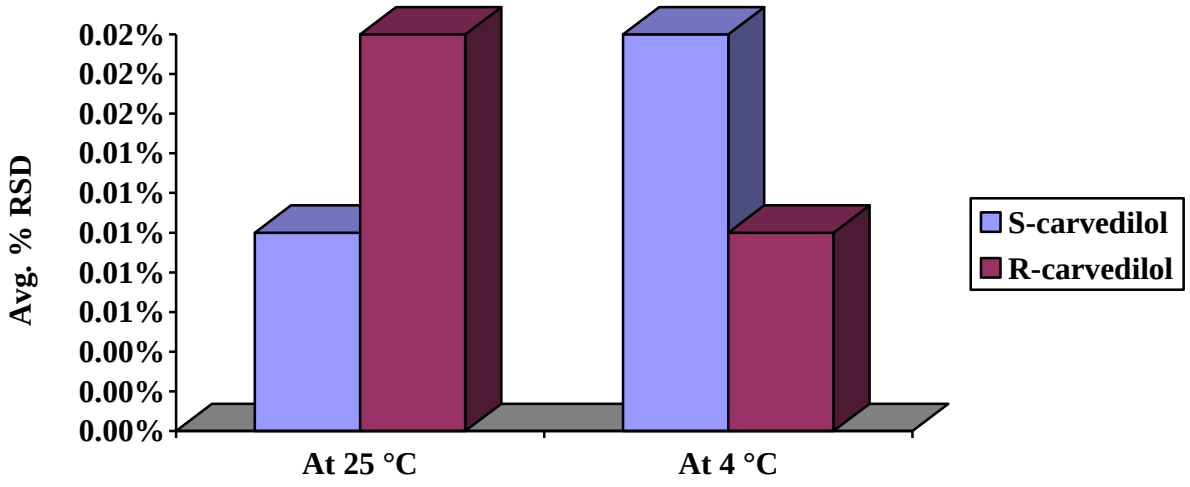


Figure 4.2.17: Bar diagram of solution stability testing (% RSD) for (*S*)- and (*R*) carvedilol.

4.2.3.8. Robustness study

To determine the robustness of this method, the experimental conditions were deliberately changed, like the flow rate and in the wavelength of detection and measuring the % RSD. For the present study, factors chosen were flow rate (1.0 ± 0.2 mL/min) and wavelength (220 ± 2 nm), and n = 3. The % RSD is reported in table 4.2.7. All analyte peaks were adequately resolved and elution orders remain unchanged. Bar diagram of robustness (% RSD) for (*S*)- and (*R*)-carvedilol is shown in figure 4.2.18.

Table 4.2.7: Parameters for robustness testing.

Change in flow rate (mL/min)	Average Rt of *S*-carvedilol ± SD	Average Rt of *R*-carvedilol ± SD	% RSD of *S*-carvedilol	% RSD of *R*-carvedilol
0.8	19.78 ± 0.01	8.31 ± 0.01	0.03%	0.07%
1.0	16.77 ± 0.01	6.63 ± 0.01	0.03%	0.09%
1.2	13.32 ± 0.01	5.56 ± 0.01	0.04%	0.10%
Change in wavelength (nm)	**Average area of *S*-carvedilol ± SD**	**Average area of *R*-carvedilol ± SD**	**% RSD of *S*-carvedilol**	**% RSD of *R*-carvedilol**
218	4432733 ± 5420.59	4227849 ± 5161.53	0.11%	0.12%
220	5002292 ± 1042.76	4851746 ± 1147.56	0.11%	0.02%
222	4847542 ± 1655.06	4636964 ± 5792.36	0.24%	0.12%

n=3; where, n = number of determinations

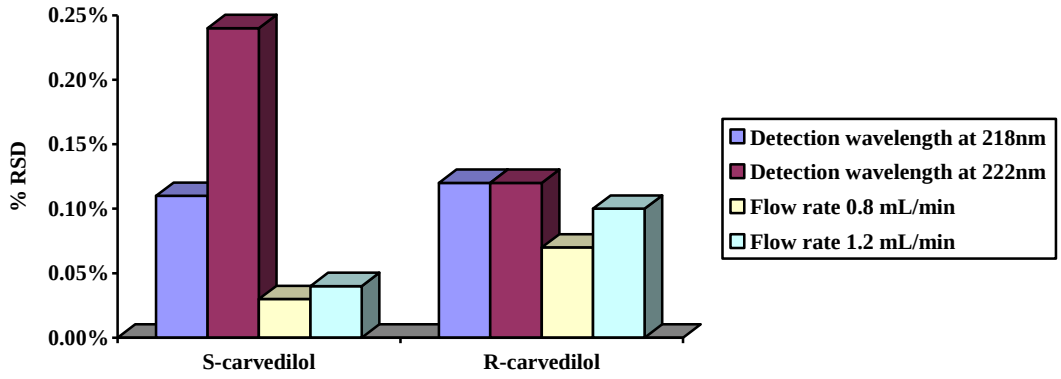


Figure 4.2.18: Bar diagram of robustness (% RSD) for (*S*)- and (*R*)-carvedilol.

4.2.4. Application of the developed method

This analytical method was applied to quantitate the content of *S*- and *R*- carvedilol in samples from five companies of Bangladesh and as well as to calculate ratio of them. The average

content of *S*- carvedilol was from 48.77% to 49.86% while the content of *R*-carvedilol was from 50.13% to 51.23% in the formulations of racemic mixture of carvedilol. Enantiomeric ratio of commercial samples is shown in table 4.2.9. Enantiomeric ratio of carvedilol for all companies' samples meet the USP requirements. The chromatogram of one sample is shown in figure 4.2.19. Bar diagram of enantiomeric ratio of racemic carvedilol is shown in figure 4.2.20.

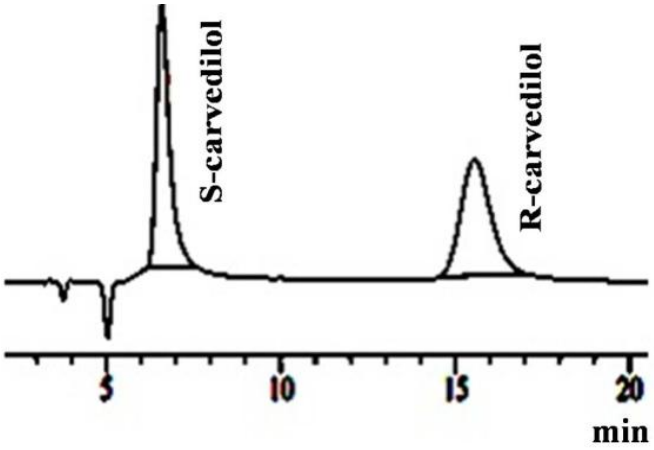


Figure 4.2.19: HPLC chromatogram of one commercial sample.

Table 4.2.8: Enantiomeric ratio of racemic carvedilol in commercial samples (n=10).

Identity of companies	**% content of *S*-carvedilol**	**%EP=(50%-% enantiomer) of *S*-carvedilol**	**% content of *R*-carvedilol**	**%EP=(50%-% enantiomer) of *R*-carvedilol**	**Enantiomeric ratio of carvedilol**
Company A	49.84%	0.16%	50.15%	-0.15%	49.84%/50.15%
Company B	49.45%	0.55%	50.54%	-0.54%	49.45%/50.54%
Company C	49.57%	0.43%	50.42%	-0.42%	49.57%/50.42%
Company D	48.77%	1.23%	51.23%	-1.23%	48.77%/51.23%
Company E	49.86%	0.14%	50.13%	-0.13%	49.86%/50.13%

EP= enantiomeric purity, n = number of determinations

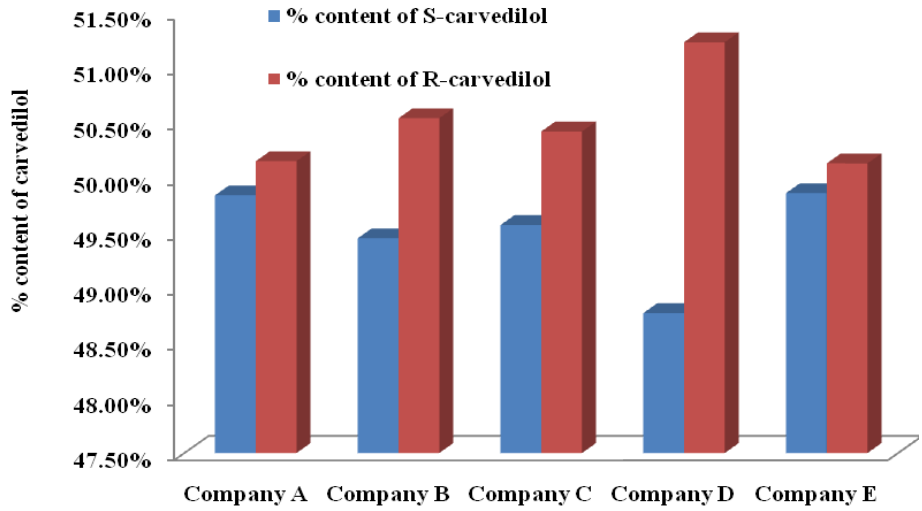


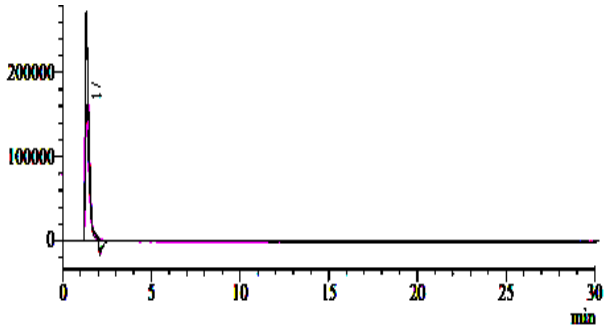
Figure 4.2.20: Bar diagram of enantiomeric ratio of racemic carvedilol.

4.3. Analysis of ofloxacin

4.3.1. Analytical HPLC

For developing a new and easy chiral HPLC method for ofloxacin, a significant number of methods were tried on trial and error basis using a large number of polar and non-polar mobile phase with chiralpak IC. Chiralpak IC column is applicable for both of the normal-phase and reversed-phase mode. Here, it was used as reverse-phase mode and normal-phase mode. Using different organic solvent or buffer ratio as mobile phase, a suitable method was developed.

Some trial chiral methods with chromatogram has been shown below with chiralpak IC on reverse phase mode:



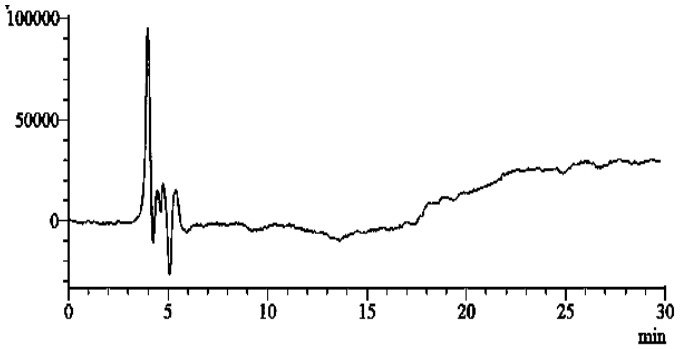
Mobile phase: NaOAc: MeOH (40:60)

Flow rate: 1 mL/min

Detector: 254 nm

Result: No peak observed

Figure 4.3.1: Trial-1.



Mobile phase:

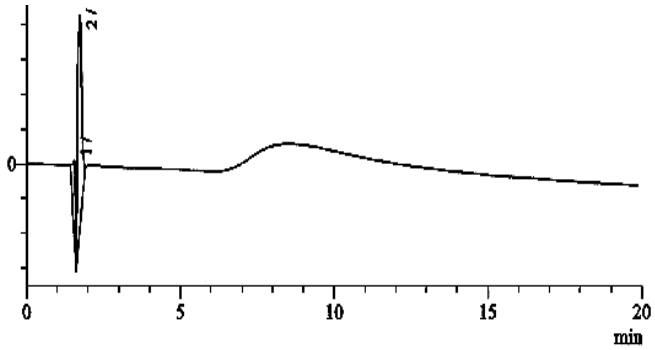
NH_4OAc: MeOH (50:50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: No peak observed

Figure 4.3.2: Trial-2.



Mobile phase:

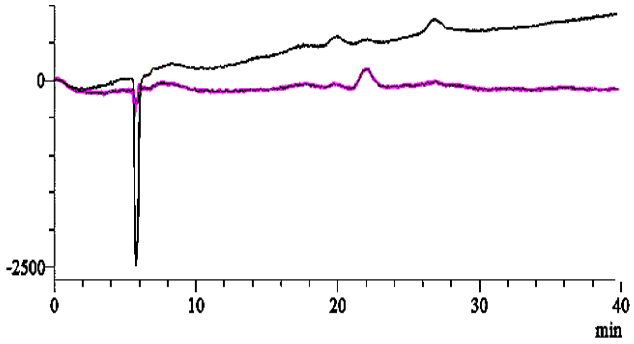
NH_4OAc: MeOH (10:90)

Flow rate: 1 mL/min

Detector: 254 nm

Result: No identical peak observed

Figure 4.3.3: Trial-3.



Mobile phase:

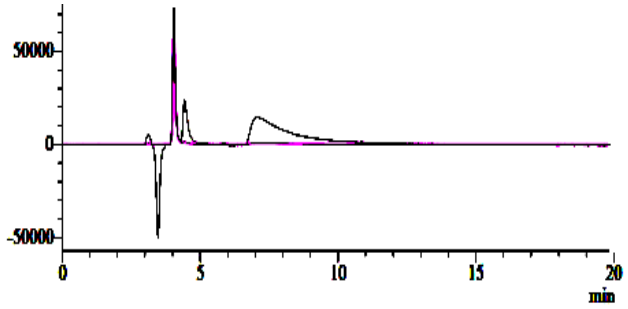
$NH_4H_2PO_4$: MeOH (40:60)

Flow rate: 1 mL/min

Detector: 254 nm

Result: No identical peak observed

Figure 4.3.4: Trial-4.



Mobile phase:

NaH_2PO_4: MeOH (30:70)

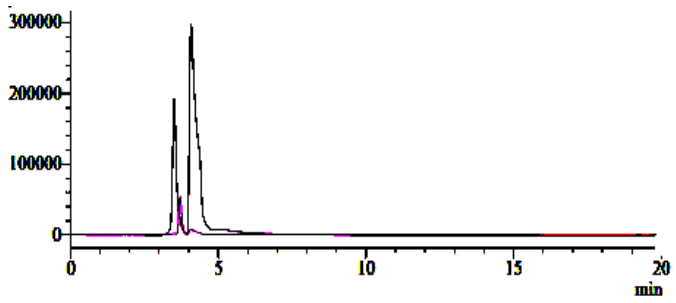
Flow rate: 1 mL/min

Detector: 254 nm

Result: No identical peak observed

Figure 4.3.5: Trial-5.

Some trial chiral methods with chromatogram are shown below with chiralpak IC on normal phase mode:



Mobile phase:

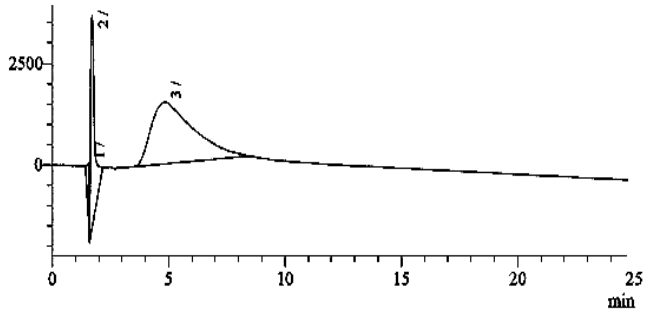
Hx: IPA (50: 50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: No identical peak observed

Figure 4.3.6: Trial-6.



Mobile phase:

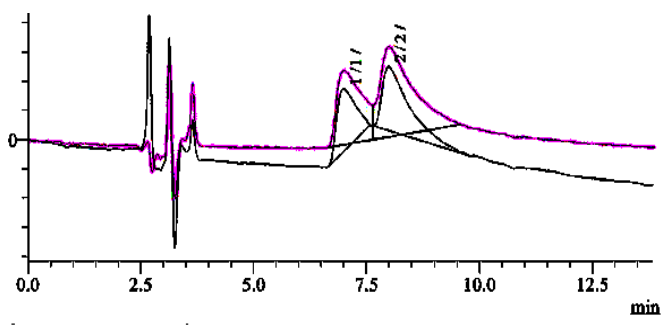
Hx: IPA: TEA (80: 20: 0.1)

Flow rate: 1 mL/min

Detector: 254 nm

Result: No identical peak observed

Figure 4.3.7: Trial-7.



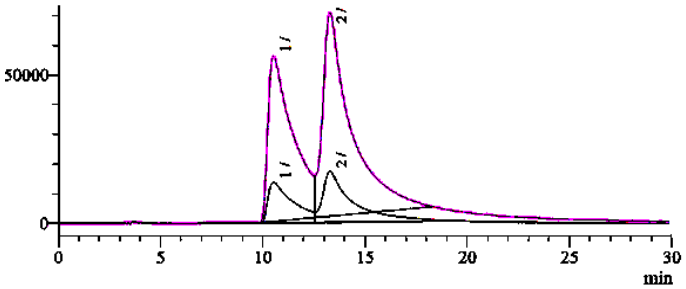
Mobile phase: Hp: MTBE: DEA (50: 50: 0.1)

Flow rate: 1 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.3.8: Trial-8.



Mobile phase:

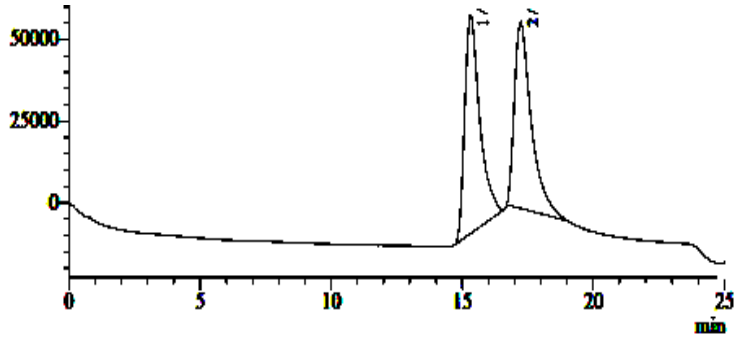
Heptane: MTBE (80: 20)

Flow rate: 1 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.3.9: Trial-9.



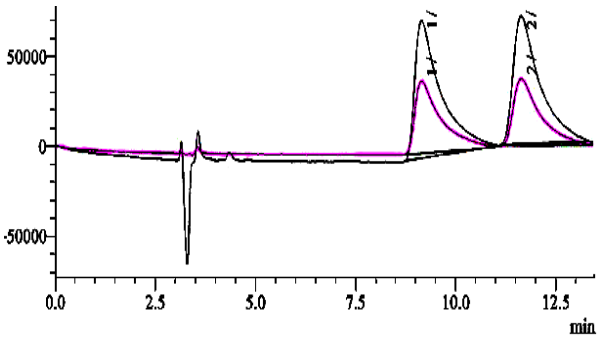
Mobile phase: Hx: EtOH: Dioxane (50:50:10)

Flow rate: 1 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.3.10: Trial-10.



Mobile phase: Hx: EtOH: Dioxane: DEA (40:50:10:0.1)
Flow rate: 1 mL/min
Detector: 254 nm
Result: Two peaks observed

Figure 4.3.11: Trial-11.

Summary of these trial chiral methods are shown in table 4.3.1.

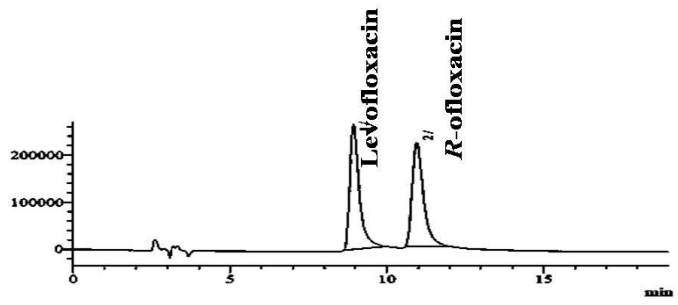
Table 4.3.1: Trial chiral methods for ofloxacin.

Polar Mobile Phase Buffer:Organic phase/Others	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
Sodium acetate: methanol (40:60)	No peak observed	NA	NA	NA
Ammonium acetate: methanol (50:50)	No peak observed	NA	NA	NA
Ammonium acetate: methanol (10:90)	No peak observed	NA	NA	NA
Ammonium dihydrogen phosphate: methanol (40:60)	No peak observed	NA	NA	NA
Sodium dihydrogen phosphate: methanol (30:70)	No peak observed	NA	NA	NA
Non-polar Mobile phase	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
Hexane: isopropyl alcohol (50: 50)	One peak observed	NA	NA	NA
Hexane: isopropylalcohol: triethylamine (80: 20: 0.1)	One peak observed	NA	NA	NA
Heptane: methyl tert -butyl ether: diethylamine (50: 50: 0.1)	Two peaks observed	510 1056	2.8 3.1	0.67

Heptane: methyl tert -butyl ether (80: 20)	Two peaks observed	311 1354	2.5 2.7	0.86
Hexane: ethanol: dioxane (50:50:10)	Two peaks observed	956 567	2.3 2.5	1.3
Hexane: ethanol: dioxane: diethylamine (40:50:10:0.1)	Two peaks observed	833 961	2.9 3.2	1.07

NA = Not applicable

But good chromatographic condition was achieved on chiralpak IC. The optimum mobile phase was found to be 50:50 % (v/v) ethanol: methyl *tert* butyl ether because of adequate resolution, efficient theoretical plates number and symmetric peak shape.



Mobile phase: EtOH: MTBE (50:50)
Flow rate: 0.7 mL/min
Detector: 254 nm
Result: Two acceptable peaks observed

Figure 4.3.12: Chromatogram with EtOH: MTBE (50:50) using chiralpak IC column.

Now, this proposed chromatographic method was compared with previous published methods and then validated according to the ICH guidelines.

4.3.2. Comparison of the present work

A comparison of present work with the other earlier reports on enantioresolution of ofloxacin using different chiral selectors is shown in table 4.3.2. It is noteworthy that resolution 3.42 was achieved using immobilized cellulose based CSP. It clearly establishes the novelty and superiority of the present report in terms of resolution.

Table 4.3.2: Comparison of HPLC enantioresolution of ofloxacin using different column with the present work.

Chiral column/ selector	Mobile phase	Resolution (Rs)	Reference
Zorbax-300SB	15% methanol in water containing 10mM phenylalanin and 5 mM cupper sulphate	1.56	Nguyen *et al*., 2015)
Chiral OD-H	*n*-hexane: ethanol: methanol: acetic acid: diethylamine (70:20:10:0.45: 0.05)	1.3	(Orlando and Bonato, 2003)
CI-Ph-β-CD	Acetonitrile: triethylammonium acetate (85: 15)	2.6	(Fang *et al.*, 2013)
Chiralpak-IC	Ethanol: methyl *tert*-butyl ether (50:50)	3.42	Present work

Finally, this present method has been extensively validated with respect to the following parameters.

4.3.3. Validation of the proposed method

4.3.3.1. Linearity and range

Linearity of the method was studied by injecting five concentrations of two enantiomers of ofloxacin prepared in the mobile phase in concentration range from 20 –130 µg/ml in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves. The linearity curves are shown in figure 4.3.13 and the parameters are given in table 4.3.3.

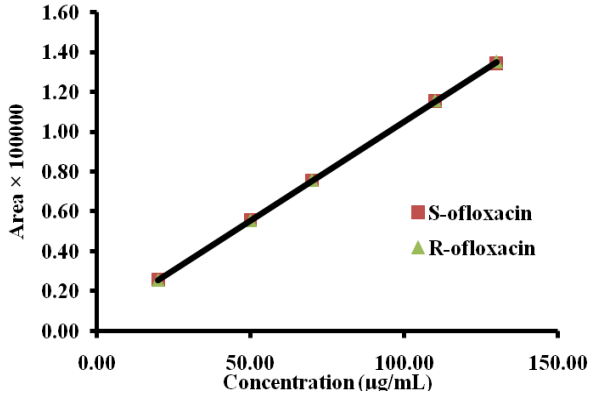


Figure 4.3.13: Linearity curve for (*S*)-ofloxacin and (*R*)-ofloxacin.

4.3.3.2. Precision

The precision of the method was verified by intra- and inter-day precision studies. Intra-day precision was performed by analysis of on concentration for six times on the same day. The intermediate precision of the method was checked by studying on three different days. Results are presented in table 4.3.3. Bar diagram of precision (% RSD) for ofloxacin is shown in figure 4.3.14.

Table 4.3.3: Results of method validation parameters.

Parameters	Levofloxacin	(*R*)- ofloxacin
Linear equation	y= 991.4x+5930	y= 1000x+5573
Coefficient of determination (r2>0.995)	1.0	1.0
Linearity range	20-130 µg/mL	
Precision (intra-day, n=6) (% RSD≤2)	0.19%	0.20%
Precision (inter-day, n=6) (% RSD≤2)	0.10%	0.10%

n = number of determinations

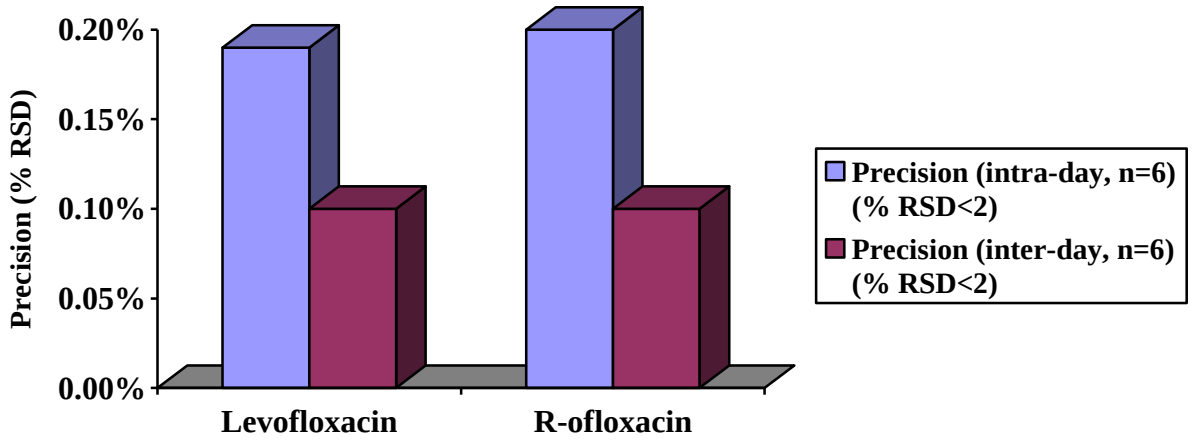


Figure 4.3.14: Bar diagram of precision (% RSD) for (*S*)- and (*R*)-ofloxacin.

4.3.3.3. Accuracy

Accuracy of the method was verified by studying recovery experiments which were performed by spiking solutions of known amount of the drug with pre-analyzed sample. To evaluate the

accuracy of the method, successive analysis (n=3) of standard solutions of the drug was carried out and the results are given in table 4.3.4.

Bar diagram of accuracy (% recovery) is shown in figure 4.3.15.

Table 4.3.4: Results of accuracy for standard ofloxacin.

Parameters	**Levofloxacin**	**(*R*)- ofloxacin**
Accuracy (n=3) (avg. % recovery)		
Standard+spike (µg/mL)		
(50+20) µg/mL	100.65%	100.29%
(70+20) µg/mL	100.63%	100.22%
(80+20) µg/mL	100.79%	100.72%
LOD (µg/mL)	1.42	0.79
LOQ (µg/mL)	4.30	2.40

n = number of determinations

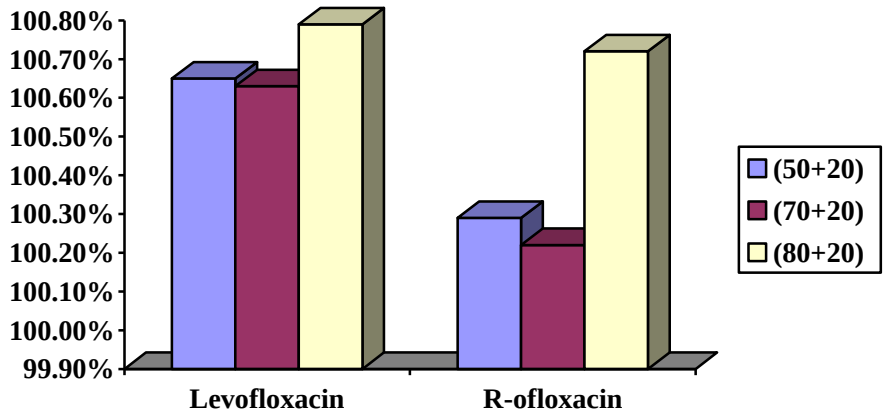


Figure 4.3.15: Bar diagram of accuracy (% recovery) for (*S*)- and (*R*)-ofloxacin

4.3.3.4. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated using the following equations:

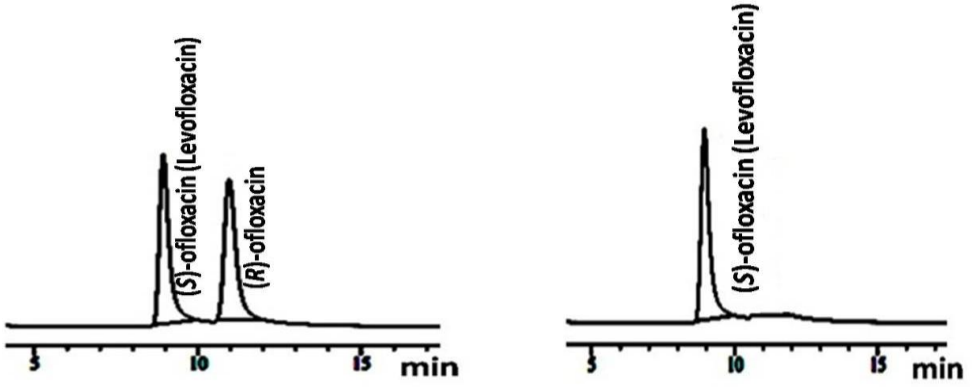
LOD= (SD / slope) x 3.3

LOQ= (SD / slope) x 10.

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). The results of LOD and LOQ obtained for studied drugs have been recorded in table 4.3.4.

4.3.3.5. Specificity

The specificity of the method was assessed from the chromatogram where complete separation of ofloxacin and levofloxacin was achieved without any interference. The peaks obtained were sharp, well separated at the baseline as shown in figure 4.3.16. Single levofloxacin was injected into HPLC for the detection of (*S*)- enantiomer (levofloxacin) in ofloxacin.



A **B**

Figure 4.3.16: HPLC chromatograms of ofloxacin (A) and levofloxacin (B).

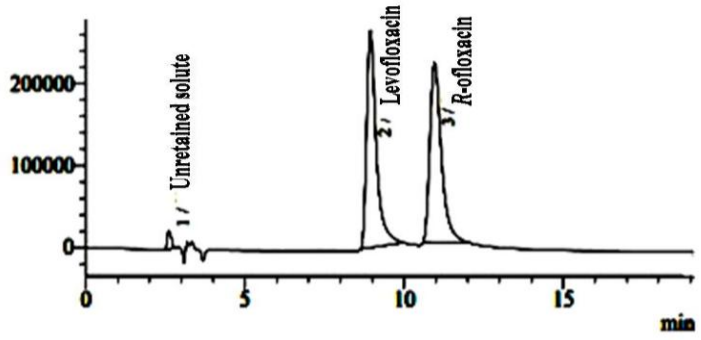
4.3.3.6. System suitability testing

System suitability parameters are reported in table 4.3.5. The chromatogram of system suitability is shown in figure 4.3.17.

Table 4.3.5: Parameters for system suitability testing.

Parameters	**Levofloxacin**	**(*R*)- ofloxacin**
Theoretical plates (≥2000) (n=5)	4534.75	4576.90
Tailing factor (≤2) (n=5)	1.79	1.60
Relative retention (k*S* and k*R*) (n=5), 0.5 < k < 10	2.43	3.21
Selectivity (α>1) (n=5)	1.32	
Resolution (≥2) (n=5)	3.42	

n = number of determinations



Where,

t_0 = 2.60 min,

t_S = 8.93 min,

t_R = 10.95 min

Figure 4.3.17: HPLC chromatogram of ofloxacin for system suitability testing.

4.3.3.7. Solution stability testing

A standard solution of concentration of 90 µg/mL was kept in a tightly capped volumetric flask at room temperature (25 °C) on the laboratory bench and at 4 °C in a refrigerator for 3 days and its stability was tested. The results are given in Table 6. Bar diagram of solution stability (% RSD) for (*S*)- and (*R*)-ofloxacin is shown in figure 4.3.18.

Table 4.3.6: Parameters for solution stability testing.

Day	**At 25 °C**		**At 4 °C**	
	Levofloxacin (n=3) (% RSD)	**(*R*)- ofloxacin (n=3) (% RSD)**	**Levofloxacin (n=3) (% RSD)**	**(*R*)- ofloxacin (n=3) (% RSD)**
Day 1	0.13%	0.20%	0.07%	0.08%
Day 2	0.12%	0.04%	0.10%	0.17%
Day 3	0.12%	0.07%	0.11%	0.11%
Avg.	0.12%	0.09%	0.10%	0.12%

n = number of determinations

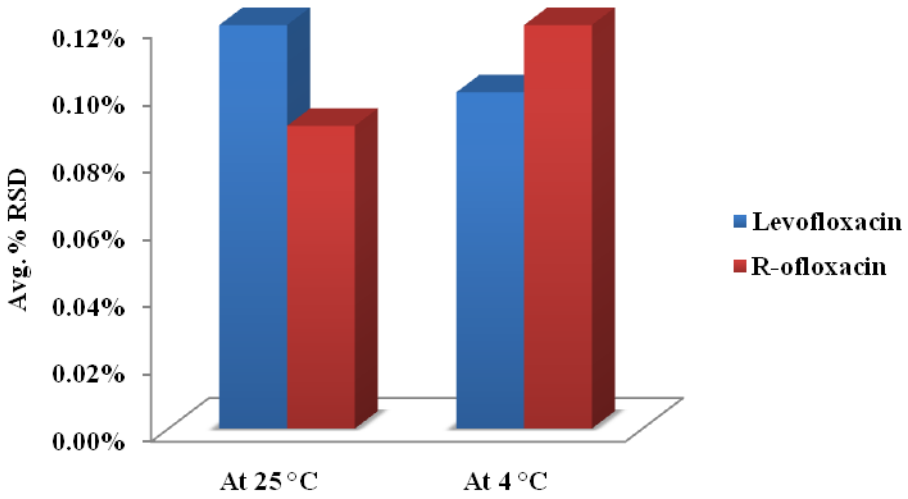


Figure 4.3.18: Bar diagram of solution stability testing (% RSD) for (*S*)- and (*R*)-ofloxacin.

4.3.3.8. Robustness of the method

To determine the robustness of the method, the experimental conditions were deliberately changed, like the flow rate and in the wavelength of detection and measuring the % RSD. For the present study, factors chosen were flow rate (0.7 ± 0.2mL/min) and wavelength (254 ± 2nm), and n = 3. The % RSD is reported in table 4.3.7. All analyte peaks were adequately resolved and elution orders remain unchanged. Bar diagram of robustness (% RSD) for (*S*)- and (*R*)-ofloxacin has been shown in figure 4.3.19.

Table 4.3.7: Robustness parameters.

Change in flow rate (mL/min)	**Average Rt of levofloxacin ± SD**	**Average Rt of (*R*)- ofloxacin ± SD**	**% RSD of levofloxacin**	**% RSD of (*R*)- ofloxacin**
0.5	13.11 ± 0.01	14.68 ± 0.01	0.04%	0.04%
0.7	8.93 ± 0.01	10.95 ± 0.01	0.06%	0.05%
0.9	6.95 ± 0.01	7.91 ± 0.01	0.01%	0.15%
Change in wavelength (nm)	**Average area of levofloxacin ± SD**	**Average area of (*R*)- ofloxacin ± SD**	**% RSD of levofloxacin**	**% RSD of (*R*)- ofloxacin**
256	93815 ± 94.54	94574 ± 74.90	0.11%	0.08%

254	45850 ± 45.18	45505 ± 55.77	0.11%	0.14%
252	68201 ± 151.04	68371 ± 78.10	0.24%	0.12%

n=3; where, n = number of determinations

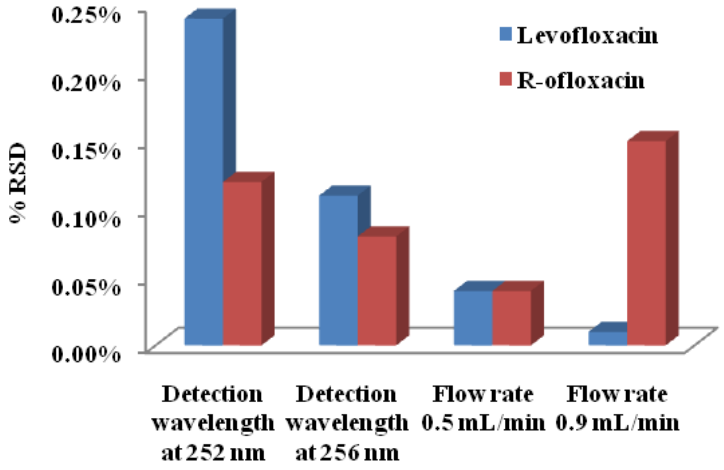


Figure 4.3.19: Bar diagram of robustness parameters (% RSD) for (*S*)- and (*R*)-ofloxacin.

4.3.4. Application of the developed method

This analytical method was applied to quantitate the content of levofloxacin and ofloxacin in commercial samples for ten and three companies of Bangladesh, respectively and as well as to calculate the % purity and ratio of them. The average content of levofloxacin was from 49.82% to 50.69% while the content of *R*-ofloxacin were from 49.31% to 50.18% in the formulations of racemic mixture of ofloxacin. For single enantiomeric samples, average content of levofloxacin was from 98.97% to 100.00% along with average content of *R*- ofloxacin were found as impurity from 0.00% to 1.03%. Enantiomeric purity (%EP) or the enantiomeric excess (ee%) of levofloxacin samples were found to be 97.94% to 100.00%. Enantiomeric ratio and purity of commercial samples are shown in table 4.3.8 and 4.3.9, respectively. Enantiomeric ratio of ofloxacin for all companies' samples met the USP requirements. HPLC chromatograms of one commercial sample for ofloxacin and levofloxacin are shown in figure 4.3.20. Bar diagram of enantiomeric ratio of racemic ofloxacin and bar diagram of % purity of levofloxacin are shown in figure 4.3.21 and figure 4.3.22, respectively.

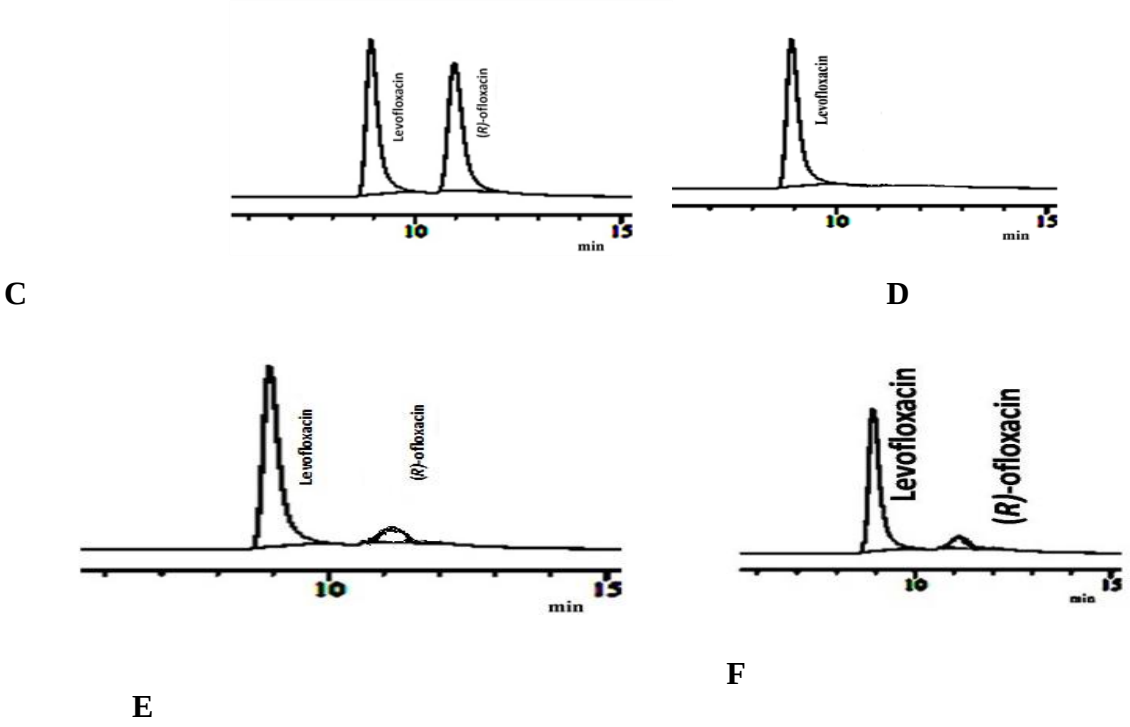


Figure 4.3.20: HPLC chromatograms of one commercial ofloxacin (C) and some commercial samples levofloxacin (D, E and F).

Table 4.3.8: Enantiomeric ratio of ofloxacin in commercial samples (n=10).

Identity of samples	% content of levofloxacin	% EP= (50%-%enantiomer) of levofloxacin	% content of *R*-ofloxacin	% EP= (50%-%enantiomer) of *R*-ofloxacin	Enantiomeric ratio of ofloxacin
Sample A	49.82%	0.18%	50.18%	-0.18%	49.82%/50.18%
Sample B	50.69%	-0.69%	49.31%	0.69%	50.69%/49.31%
Sample C	50.09%	-0.09%	49.91%	0.09%	50.09%/49.91%

EP= Enantiomeric purity, n = number of determinations

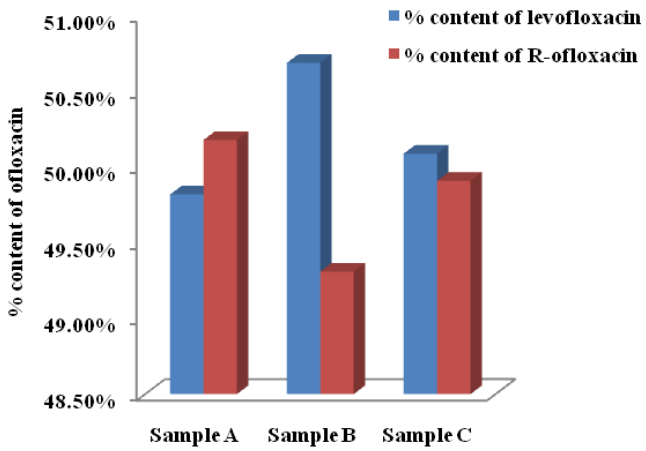


Figure 4.3.21: Bar diagram of enantiomeric ratio of racemic ofloxacin.

Table 4.3.9: Enantiomeric purity of levofloxacin in commercial samples (n=10).

Identity of company	% content of Levofloxacin	% content of *R*-ofloxacin in levofloxacin as impurity	%EP = (% of major enantiomer - % of minor enantiomer)
Company A1	100.00%	0.00%	100.00%
Company B1	100.00%	0.00%	100.00%
Company C1	100.00%	0.00%	100.00%
Company D1	100.00%	0.00%	100.00%
Company E1	100.00%	0.00%	100.00%
Company F1	99.71%	0.29%	99.42%
Company G1	99.89%	0.11%	99.78%
Company H1	99.99%	0.01%	99.98%
Company I1	99.82%	0.18%	99.64%
Company J1	98.97%	1.03%	97.94%

EP= Enantiomeric purity, n = number of determinations

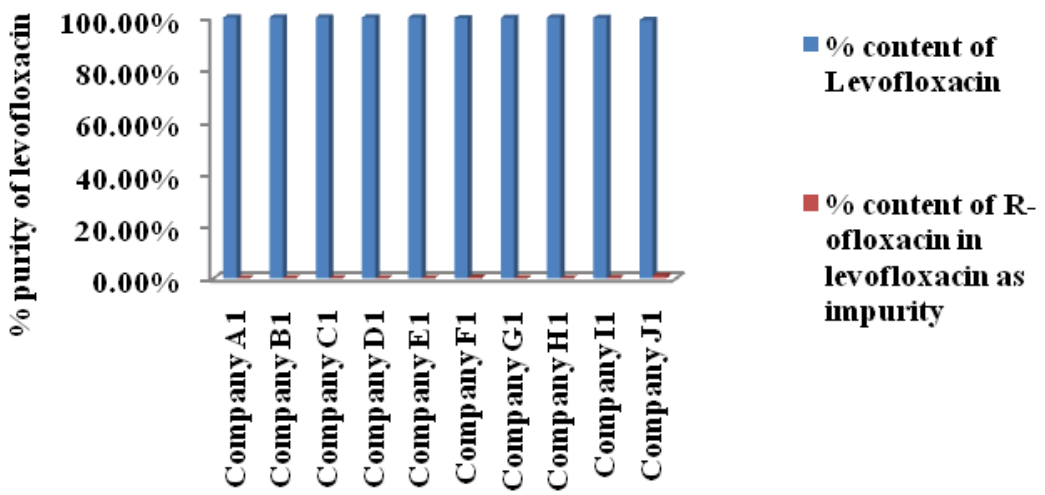


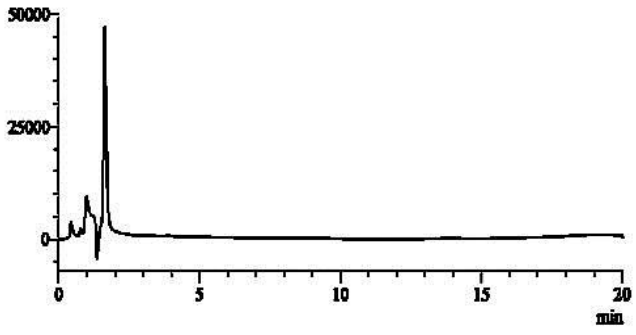
Figure 4.3.22: Bar diagram of % purity of levofloxacin.

4.4. Analysis of omeprazole

4.4.1. Analytical HPLC

For developing a new and easy chiral HPLC method for omeprazole, we tried a significant number of methods on trial and error basis using a large number of polar solvent mixtures as mobile phase with chiralcel OD-H column. This column is applicable for normal-phase mode. Using different organic solvent ratio as mobile phase, a suitable method was developed.

Some trial chiral methods with chromatogram are shown below with chiralcel OD-H:



Mobile phase: Heptane: IPA (50:50)

Flow rate: 1 mL/min

Detector: 300 nm

Result: No peak observed

Figure 4.4.1: Trial-1.

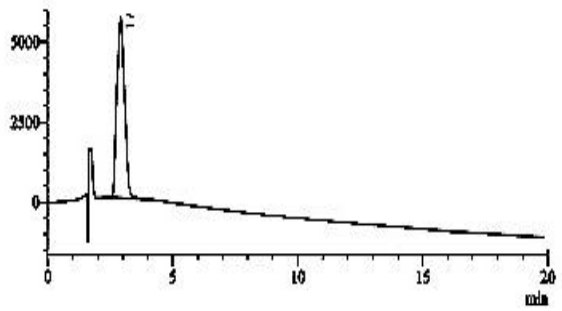


Figure 4.4.2: Trial-2.

Mobile phase: Heptane: IPA (70:30)
Flow rate: 1 mL/min
Detector: 300 nm
Result: No identical peak observed

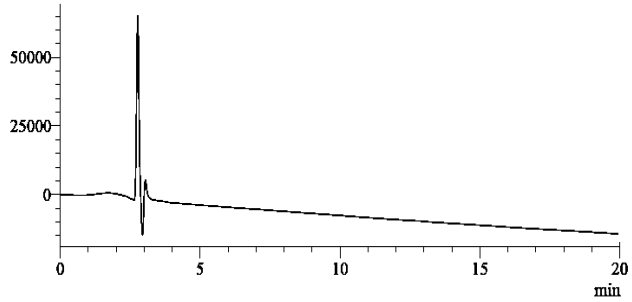


Figure 4.4.3: Trial-3.

Mobile phase: Heptane: IPA (30:70)
Flow rate: 1 mL/min
Detector: 300 nm
Result: No peak observed

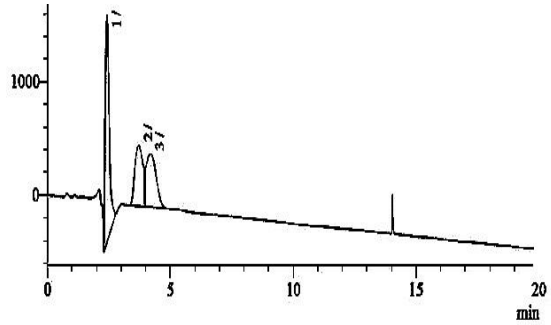
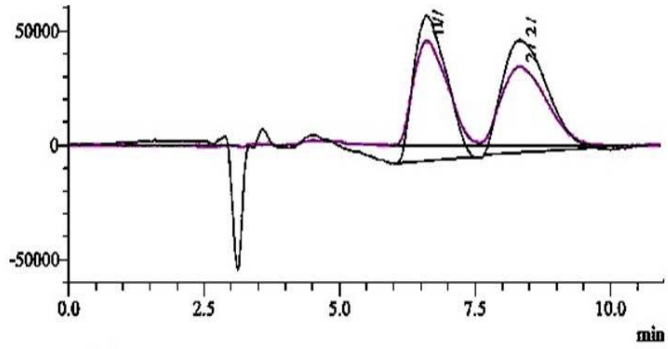


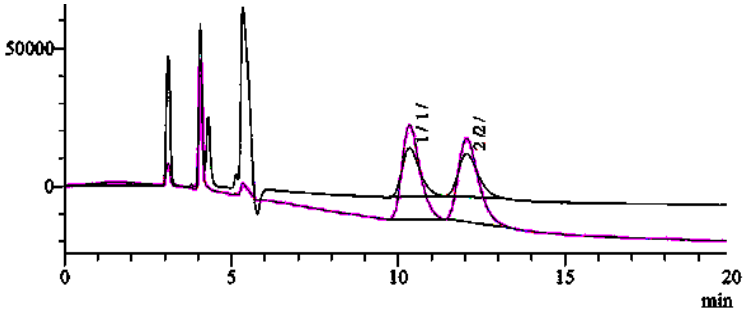
Figure 4.4.5: Trial-5.

Mobile phase: Hx: IPA (40:60)
Flow rate: 1 mL/min
Detector: 300 nm
Result: Two peaks observed



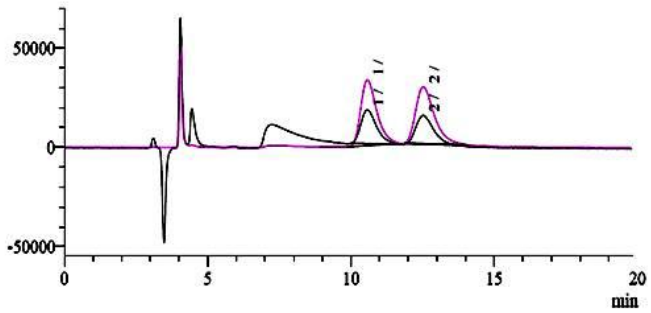
Mobile phase: Hx: IPA (70:30)
Flow rate: 1 mL/min
Detector: 300 nm
Result: Two peaks observed

Figure 4.4.6: Trial-6.



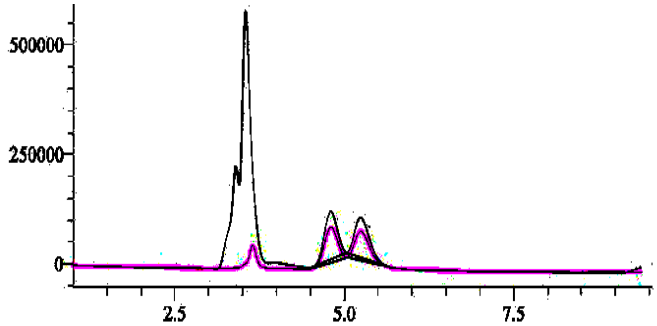
Mobile phase: Hx: IPA (80:20)
Flow rate: 1 mL/min
Detector: 300 nm
Result: Two peaks observed

Figure 4.4.7: Trial-7.



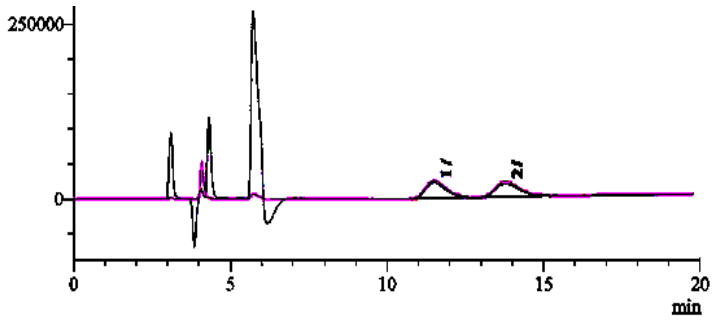
Mobile phaseHx: IPA: TEA (80:20: 0.2)
Flow rate: 1 mL/min
Detector: 300 nm
Result: Two peaks observed

Figure 4.4.8: Trial method-8.



Mobile phase: Hx: IPA: AA (20:80:0.2)
Flow rate: 1 mL/min
Detector: 300 nm
Result: Two peaks observed

Figure 4.4.9: Trial-9.



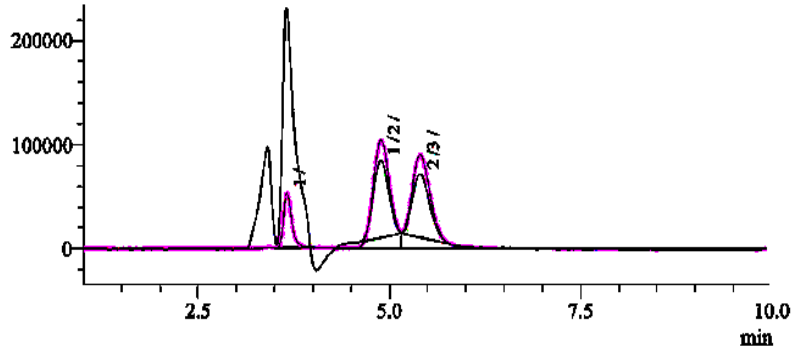
Mobile phase: Hx: IPA: AA (80:20:0.2)

Flow rate: 1 mL/min

Detector: 300 nm

Result: Two peaks observed

Figure 4.4.10: Trial-10.



Mobile phase: Hx: IPA: AA: TEA (20:80:0.2:0.2)

Flow rate: 1 mL/min

Detector: 300 nm

Result: Two peaks observed

Figure 4.4.11: Trial-11.

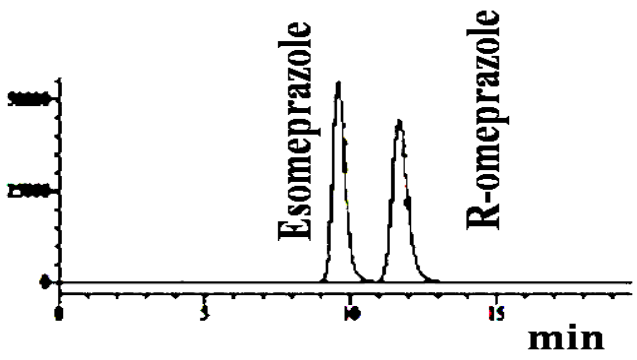
Summary of these trial chiral methods are shown in table 4.4.1.

Table 4.4.1: Trial chiral methods for omeprazole.

Non-polar Mobile phase	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
Hp: IPA(50:50)	No peak observed	NA	NA	NA
Hp: IPA: 70:30	No identical peak observed	NA	NA	NA
Hp: IPA (30:70)	No peak observed	NA	NA	NA
Hx: IPA (50:50)	Two peaks observed	NA	NA	1.25
Hx: IPA (40:60)	Two peaks observed	404.28 280.21	1.2 1.3	0.54
Hx: IPA (70:30)	Two peaks observed	468.23 463.27	1.3 1.4	1.24
Hx: IPA (80:20)	Two peaks	1787.52	1.35	1.45

	observed	1925.3	1.34	
Hx:IPA:TEA::80:20: 0.2	Two peaks observed	1863.78 1982.34	1.36 1.43	1.85
Hx:IPA:AA (20:80:0.2)	Two peaks observed	1889.34 1686.57	0.00 0.00	1.13
Hx:IPA:AA (80:20:0.2)	Two peaks observed	1323.16 1550.03	1.30 1.40	1.69
Hx:IPA:AA:TEA (20:80:0.2:0.2)	Two peaks observed	2468.17 2479.99	1.05 1.26	0.00 1.24
Hp: IPA(50:50)	No peak observed	NA	NA	NA

Table 4.4.1 demonstrates that all the results of all parameters are not suilable. So all the above methods are avoided. After a number of trials with chiralcel OD-H column with mobile phases of different composition, *n*-Hexane: isopropyl alcohol: acetic acid: triethyle amine (100:20:0.2:0.1, v/v) was selected as mobile phase because of adequate resolution, efficient theoretical plates number and symmetric peak shape.



Mobile phase: Hx: IPA: AA: TEA (100:20:0.2:0.1)

Flow rate: 1.2 mL/min

Detector: 300 nm

Result: Two acceptable peaks observed

Figure 4.4.12: Chromatogram with Hx: IPA: AA: TEA (100:20:0.2:0.1) using chiralcel OD-H column.

Now, this proposed chromatographic method was compared with the previously published methods and then validated according to the ICH guidelines.

4.4.2. Comparison of the present work

A comparison of present work with the other earlier reports on enantioresolution of omeprazole using different chiral selectors is shown in table 4.4.2. It is noteworthy to mention that resolution 2.36 was achieved. It clearly establishes the novelty and superiority of the present report in terms of resolution.

Table 4.4.2: Comparison of HPLC enantioresolution of omeprazole using different column with the present work.

Chiral column/ selector	**Mobile phase**	**Resolution (Rs>2)**	**Reference**
Chiralpak AS	*n*-hexane: ethanol (80:20)	2.0	(Orlando and Bonato, 2003)
Chiralpak OB-H	*n*-hexane: ethanol (93:07)	1.1	(Orlando and Bonato, 2003)
β-CD	Phosphate buffer (pH 2.5)	1.74	(Rosales-Conrado *et al.*, 2013)
HP-β-CD	Phosphate buffer (pH 2.5)	2.03	(Rosales-Conrado *et al.*, 2013)
Chiralpak AD-RH	Water:acetonitrile (50:50)	1.18	(Orlando and Bonato, 2003)
Chiralpak OD-H	Ethanol:hexane: isopropanol (6:91:3)	1.80	(Orlando and Bonato, 2003)
Chiralcel OD-H	n-hexane: isopropanol: acetic acid: triethylamine)100:20:0.2:0.1(	2.36	Present work

Finally, this present method has been extensively validated with respect to the following parameters.

4.4.3. Validation results of the proposed method

4.4.3.1. Specificity

Under the optimized chromatographic conditions, the overlaid chromatograms confirm the presence of (*S*)- and (*R*)- omeprazole in recemic omeprazole and (*S*)-omeprazole in single enantiomer at about 9.65 ± 0.01 min and 11.81 ± 0.01 min, respectively without any interference. It is said that the method was specific. Overlaid chromatograms of esomeprazole [(*S*)-omeprazole] and recemic omeprazole [mixture of (*S*)- and (*R*)- omeprazole] were shown in Figure 4.4.13.

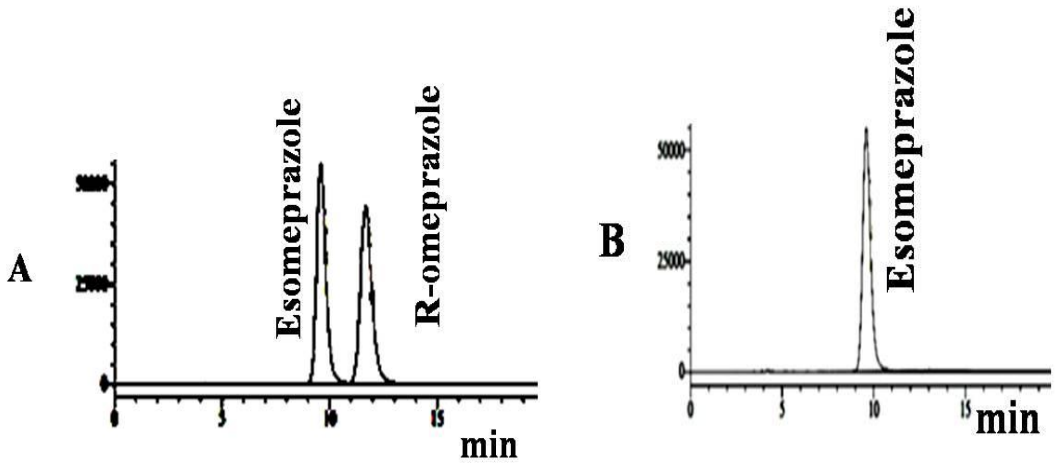
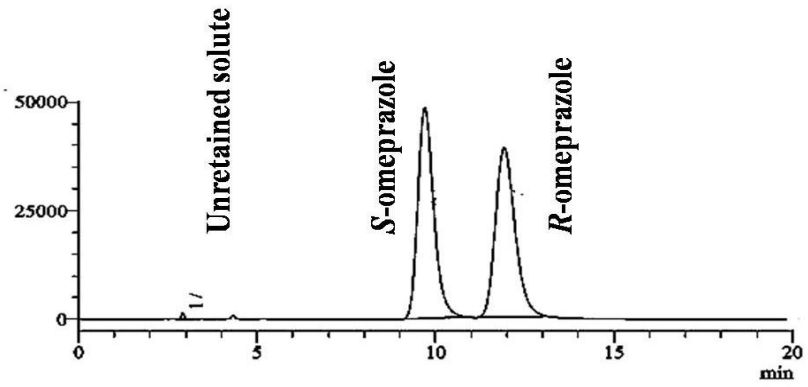


Figure 4.4.13: Chromatograms of esomeprazole [(*S*)-omeprazole] and recemic omeprazole [mixture of (*S*)- and (*R*)- omeprazole].

4.4.3.2. System suitabililty testing

This method also resulted in symmetric peak shape with tailing 1.24 and 1.23 and theoretical plates with 2345 and 2275 for (*S*)- and (*R*)- omeprazole, respectively. System suitability was carried out by injecting 3 replicate samples of 100% test concentration, where excellent resolution of 2.36 were obtained. The method showed insignificant deviation in the values of relative retention (k_S and k_R), selectivity (alpha), resolution (Rs) and number of theoretical plates (N). The chromatogram of system suitability are shown in table 4.4.3 and figure 4.4.14.



Where,

t_0 =2.90 min,

t_S = 9.65 min,

t_R = 11.81 min

Figure 4.4.14: HPLC chromatogram of omeprazole for system suitability testing.

4.4.3.3. Linearity and calibration curve

The calibration curve for (*S*)- and (*R*)- omeprazole constructed from the recemate-omeprazole solution over the concentration range of 50 µg/mL to 90 µg/mL showed linearity with coefficient of determination (r^2) values of 0.999 and 0.998, respectively which were found in the limit (r^2>0.995) indicating good linearity of calibration curve. The linearity curves are shown in figure 4.4.15. The parameters are given in table 4.4.3.

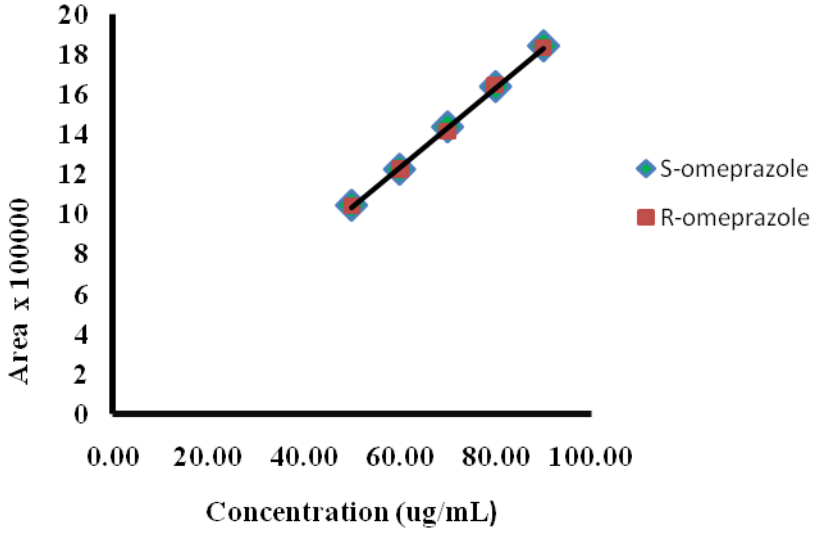


Figure 4.4.15: Linearity curves for *S*- omeprazole, and *R*-omeprazole.

4.4.3.4. Detection limit

The limit of detection (LOD) were found to be 0.71 µg/mL and 2.16 µg/mL for (*S*)- and (*R*)-omeprazole respectively. The limit of quantitation (LOQ) were 1.16 µg/mL and 3.51 µg/mL for (*S*)- and (*R*)- omeprazole, respectively.

4.4.3.5. Accuracy and precision

The average percentage of recovery was calculated and it was found to be 100.85% to 101.36% for (*S*)- and 99.81% to 101.62% for (*R*)- omeprazole against the concentration of 65, 75, and 85 µg/mL, respectively. The proposed method was found to be precise and reproducible with % RSD of 0.05% and 0.19% for (*S*)- and 0.03% and 0.13% for (*R*)- omeprazole, respectively. All the validation parameters are shown in table 4.4.3. Bar diagram of accuracy (% recovery) and precision (% RSD) for (*S*)- and (*R*)-omeprazole are shown in figure 4.4.16 and figure 4.4.17.

Table 4.4.3: Results of method validation parameters.

Parameters	(*S*)- omeprazole	(*R*)- omeprazole
Linear equation	y= 40252x+29041	y= 40010x+32889
Coefficient of determination (r^2>0.995)	0.999	0.998
Linearity range	50-90 µg/mL	50-90 µg/mL
Resolution (≥2) (*n*=3)	2.36	2.36
Theoretical plates (≥2000) (*n*=3)	2345	2275
Tailing factor (≤2) (*n*=3)	1.24	1.23
Relative retention (k_S and k_R, 0.5 < k < 10(	2.33	3.10
Selectivity (α>1)	1.33	
Precision (repeatability, *n*=6)	0.05%	0.03%
(% RSD ≤2)		
Intermediate precision (*n*=3) (% RSD≤2)		
Avg. of Day1, Day 2, Day 3	0.19%	0.13%
Accuracy (*n*=3) (average % Recovery)		
Standard + spike (µg/mL)		
(55 + 10)	101.07%	99.81%
(65 + 10)	101.36%	101.62%
(75 + 10)	100.85%	101.13%
LOD (µg/mL)	0.71 µg/mL	2.16 µg/mL
LOQ (µg/mL)	1.16 µg/mL	3.51 µg/mL

n = number of determinations

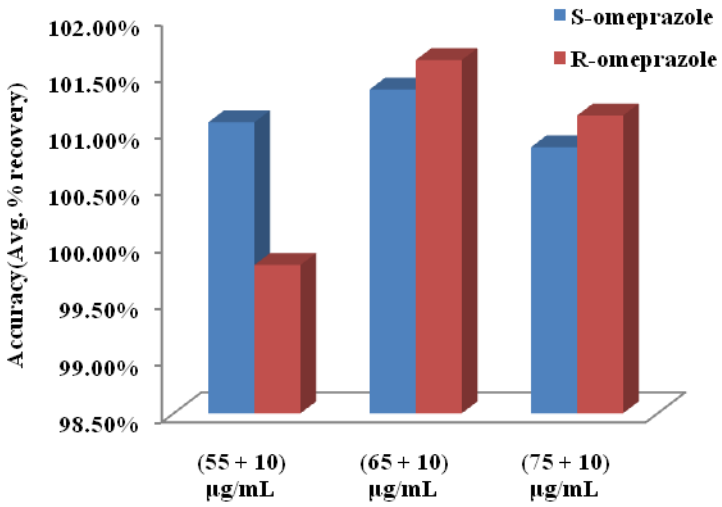


Figure 4.4.16. Bar diagram of accuracy (% recovery) for (*S*)- and (*R*)-omeprazole.

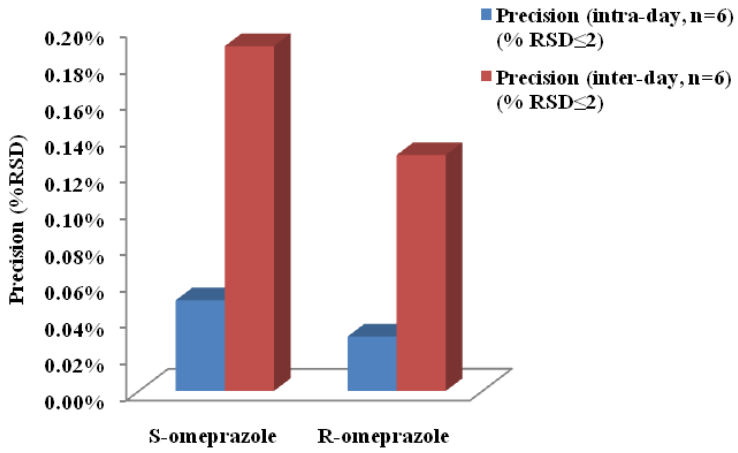


Figure 4.4.17: Bar diagram of precision (% RSD) for (*S*)- and (*R*)-omeprazole.

4.4.3.6. Solution stability testing

Solution stability study was carried out to calculate %RSD of area for three consecutive days at 25 ºC and 4 ºC. At 25 ºC the value of % RSD was found to be 0.06% and 0.09% for (*S*)- and (*R*)-omeprazole. At 4 ºC the value of % RSD was 0.10% and 0.15% for (*S*)- and (*R*)-omeprazole, respectively which demonstrated that the drug was fairly stable at normal temperature and 4ºC (table 4.4.4). Bar diagram of solution stability (% RSD) for (*S*)- and (*R*)-omeprazole are shown in figure 4.4.18.

Table 4.4.4: Results of solution stability testing.

Day	At 25 °C		At 4 °C	
	(*S*)-omeprazole (*n*= 3) (% RSD)	**(*R*)-omeprazole (*n*= 3) (% RSD)**	**(*S*)-omeprazole (*n*= 3) (% RSD)**	**(*R*)-omeprazole (*n*= 3) (% RSD)**
Day 1	0.05%	0.09%	0.08%	0.15%
Day 2	0.10%	0.06%	0.13%	0.18%
Day 3	0.04%	0.12%	0.10%	0.13%
Avg.	0.06%	0.09%	0.10%	0.15%

n = number of determinations

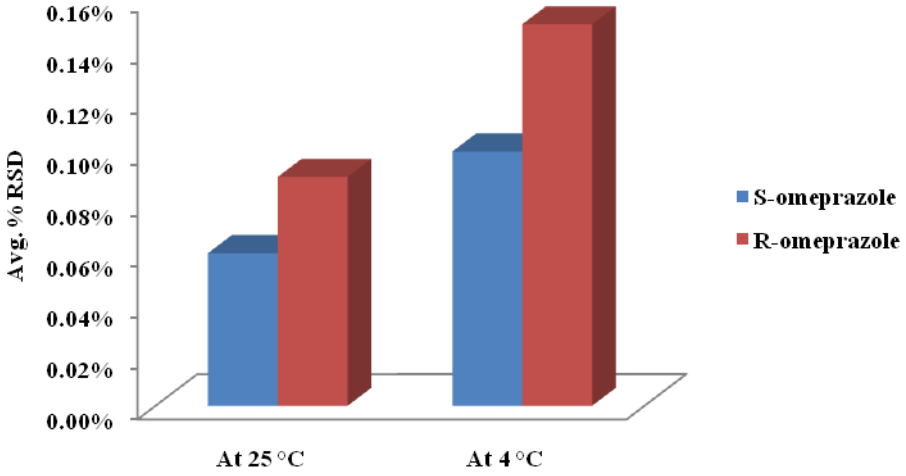


Figure 4.4.18: Bar diagram of solution stability testing(% RSD) for (*S*)- and (*R*)-omeprazole.

4.4.3.7. Robustness study

The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2mL/min). % RSD was calculated for each variation and reported. Values obtained are given in table 4.4.5 and bar diagram of robustness (% RSD) for (*S*)- and (*R*)-omeprazole has been shown in figure 4.4.19.

Table 4.4.5: Robustness study (n=3).

Change in flow rate (mL/min)	Average R_t of (*S*)-omeprazole ± SD	Average R_t of (*R*)-omeprazole ± SD	% RSD of (*S*)- omeprazole	% RSD of (*R*)-omeprazole
1.4	7.87 ± 0.05	9.76 ± 0.01	0.13%	0.06%
1.2	9.65 ± 0.01	11.81 ± 0.01	0.06%	0.05%
1.0	11.71 ± 0.01	13.91 ± 0.01	0.01%	0.04%
Change in wavelength (nm)	Mean area of (*S*)-omeprazole ± SD	Mean area of (*R*)-omeprazole ± SD	% RSD of (*S*)-omeprazole	% RSD of (*R*)-omeprazole
298	1144101 ± 343.42	1142985 ± 321.27	0.03%	0.03%
300	1043720 ± 381.24	1043849 ± 736.58	0.04%	0.07%
302	1243549 ± 598.34	1243268 ± 458.13	0.05%	0.04%

n = number of determinations

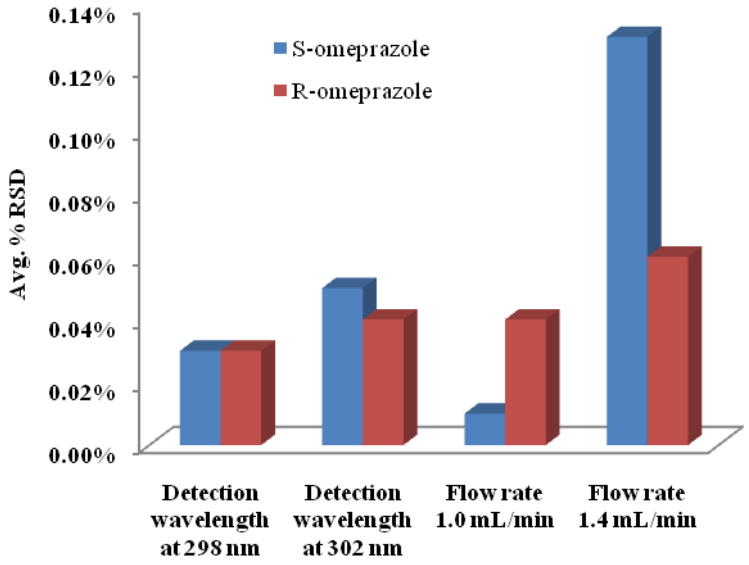
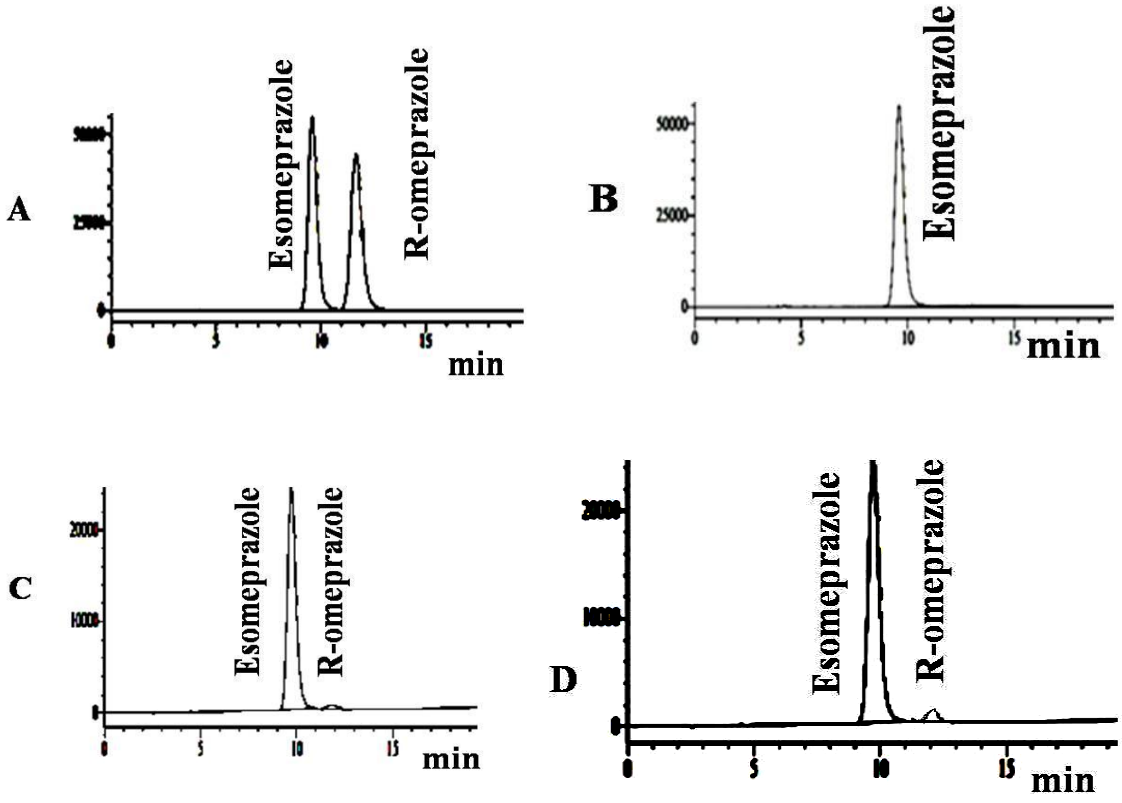


Figure 4.4.19: Bar diagram of robustness study (% RSD) for (*S*)- and (*R*)-omeprazole.

4.4.4. Application of the developed method

This analytical method was applied to quantitate the content of *S*- and *R*- omeprazole in commercial samples for fifty-two companies of Bangladesh and as well as to calculate the % purity and ratio of them. The average content of *S*- omeprazole was from 48.91% to 54.14% while the content of *R*-omeprazole was from 45.86% to 51.09% in the formulations of racemic

mixture of omeprazole. For single enantiomeric samples, average content of *S*- omeprazole was from 97.59% to 100.00% along with average content of *R*- omeprazole were found as impurity from 0.00% to 2.41%. Enantiomeric purity (%EP) or the enantiomeric excess (ee%) of esomeprazole samples were found to be 95.18% to 100.00%. Enantiomeric ratio and purity of commercial samples are shown in table 4.4.6 and 4.4.7, respectively. Enantiomeric ratio of omeprazole for all companies' samples met the USP requirements. HPLC chromatograms of some commercial samples for omeprazole and esomeprazole are shown in figure 4.4.20. Bar diagram of enantiomeric ratio of racemic omeprazole and % purity of esomeprazole are shown in figure 4.4.21 and figure 4.4.22, respectively.



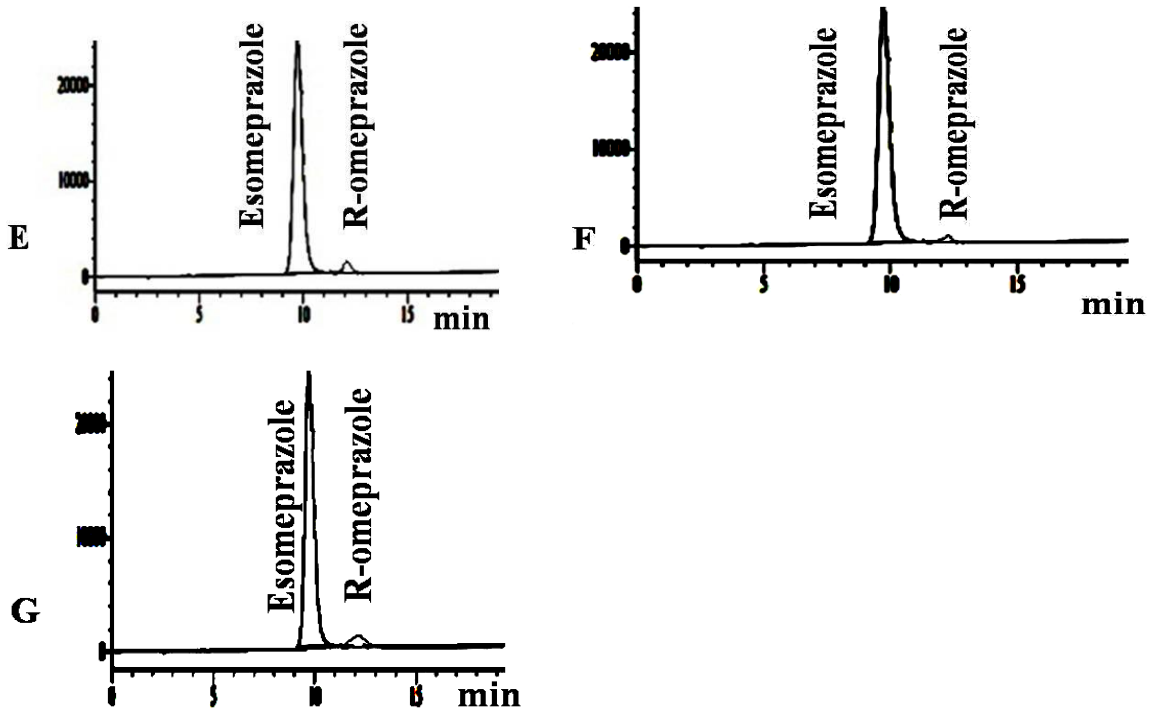


Figure 4.4.20: Typical chromatograms of both racemic (A) and esomeprazole samples (B-G) containing *R*-omeprazole.

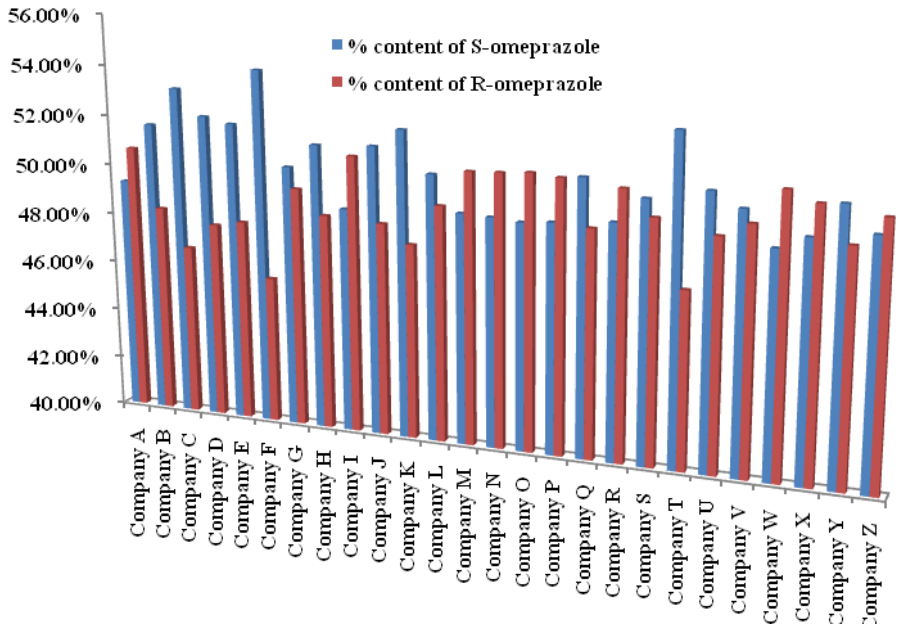


Figure 4.4.21: Bar diagram of enantiomeric ratio of racemic omeprazole.

Table 4.4.6: Enantiomeric ratio of racemic enantiomeric drugs (n=10).

Identity of company	% Content of *S*-omeprazole	% EP = (50% - % enantiomer) of *S*-omeprazole	% Content of *R*-omeprazole	% EP = (50% - % enantiomer) of *R*-omeprazole	Enantiomeric ratio of omeprazole
Company A	49.32%	0.68%	50.68%	-0.68%	49.32%/50.68%
Company B	51.69%	-1.69%	48.31%	1.69%	51.69%/48.31%
Company C	53.20%	-3.20%	46.80%	3.20%	53.20%/46.80%
Company D	52.17%	-2.17%	47.83%	2.17%	52.17%/47.83%
Company E	51.95%	-1.95%	48.05%	1.95%	51.95%/48.05%
Company F	54.14%	-4.14%	45.86%	4.14%	54.14%/45.86%
Company G	50.42%	-0.42%	49.58%	0.42%	50.42%/49.58%
Company H	51.38%	-1.38%	48.62%	1.38%	51.38%/48.62%
Company I	48.95%	1.05%	51.05%	-1.05%	48.95%/51.05%
Company J	51.50%	-1.50%	48.50%	1.50%	51.50%/48.50%
Company K	52.22%	-2.22%	47.78%	2.22%	52.22%/47.78%
Company L	50.60%	-0.60%	49.40%	0.60%	50.60%/49.40%
Company M	49.18%	0.82%	50.82%	-0.82%	49.18%/50.82%
Company N	49.13%	0.87%	50.87%	-0.87%	49.13%/50.87%
Company O	49.04%	0.96%	50.96%	-0.96%	49.04%/50.96%
Company P	49.14%	0.86%	50.86%	-0.86%	49.14%/50.86%
Company Q	50.95%	-0.95%	49.05%	0.95%	50.95%/49.05%
Company R	49.35%	0.65%	50.65%	-0.65%	49.35%/50.65%
Company S	50.34%	-0.34%	49.66%	0.34%	50.34%/49.66%
Company T	52.95%	-2.95%	47.05%	2.95%	52.95%/47.05%
Company U	50.82%	-0.82%	49.18%	0.82%	50.82%/49.18%
Company V	50.26%	-0.26%	49.74%	0.26%	50.26%/49.74%
Company W	48.91%	1.09%	51.09%	-1.09%	48.91%/51.09%
Company X	49.42%	0.58%	50.68%	-0.68%	49.42%/50.68%
Company Y	50.74%	-0.74%	49.26%	0.74%	50.74%/49.26%
Company Z	49.72%	0.28%	50.38%	-0.38%	49.72%/50.38%

EP= Enantiomeric purity, n = number of determinations

Table 4.4.7: Enantiomeric purity of esomeprazole (n=10).

Identity of company	**% Content of (*S*)-omeprazole**	**% Content of *R*-omeprazole in *S*-omeprazole as impurity**	**%EP = (% of major enantiomer - % of minor enantiomer)**
Company A1	99.94%	0.06%	99.88%
Company B1	100.00%	0.00%	100.00%
Company C1	100.00%	0.00%	100.00%
Company D1	99.77%	0.23%	99.54%
Company E1	100.00%	0.00%	100.00%
Company F1	98.70%	1.30%	97.40%
Company G1	99.96%	0.04%	99.92%
Company H1	100.00%	0.00%	100.00%
Company I1	99.82%	0.18%	99.64%
Company J1	100.00%	0.00%	100.00%
Company K1	99.90%	0.10%	99.80%
Company L1	100.00%	0.00%	100.00%
Company M1	99.24%	0.76%	98.48%
Company N1	100.00%	0.00%	100.00%
Company O1	100.00%	0.00%	100.00%
Company P1	100.00%	0.00%	100.00%
Company Q1	100.00%	0.00%	100.00%
Company R1	100.00%	0.00%	100.00%
Company S1	99.96%	0.04%	99.92%
Company T1	100.00%	0.00%	100.00%
Company U1	100.00%	0.00%	100.00%
Company V1	97.79%	2.21%	95.58%
Company W1	97.59%	2.41%	95.18%
Company X1	98.00%	2.00%	96.00%

Company Y1	100.00%	0.00%	100.00%
Company Z1	100.00%	0.00%	100.00%

EP=Enantiomeric purity, n = number of determinations

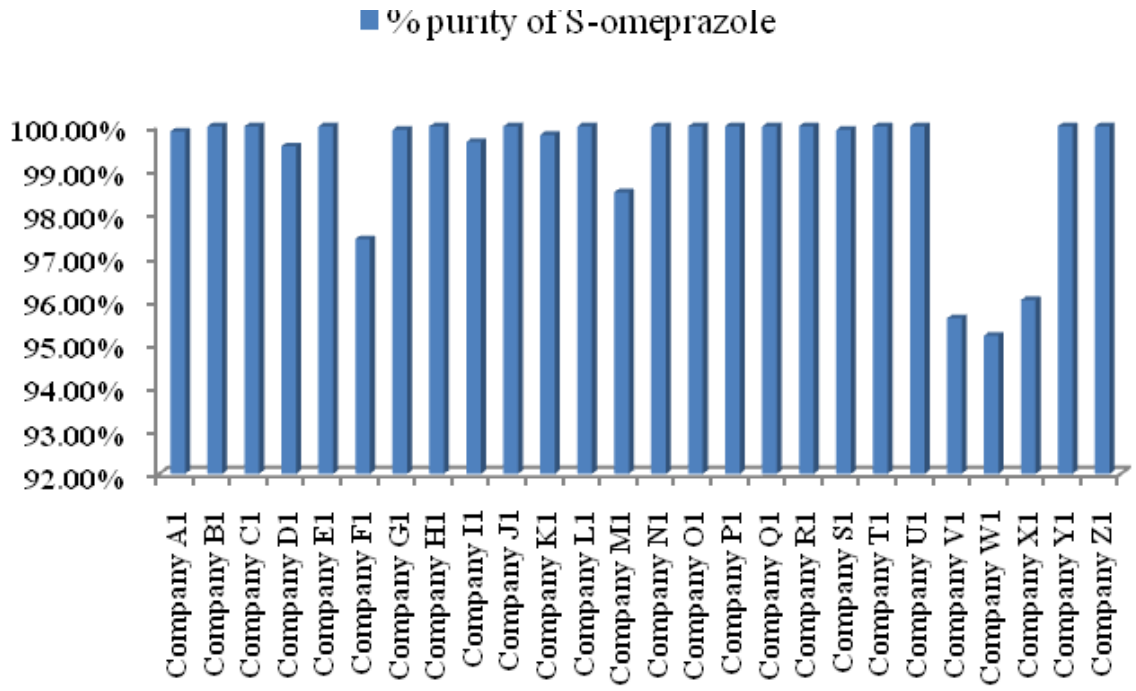


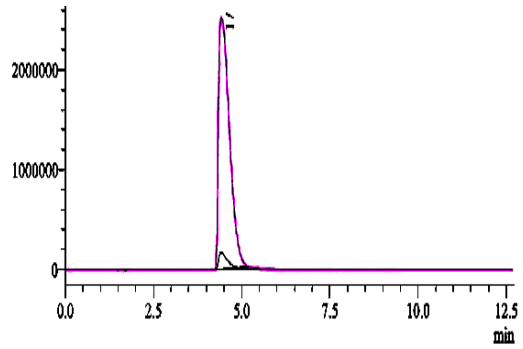
Figure 4.4.22: Bar diagram of % purity of omeprazole.

4.5. Analysis of salbutamol

4.5.1. Analytical HPLC

To develop a new and easy chiral HPLC method for salbutamol, we tried a significant number of methods on trial and error technique using a large number of solvent mixtures as mobile phase. For salbutamol separation, CD-PH chiral columns was used which column is applicable to both of the normal-phase and reversed-phase mode. Here, CD-PH was used as reverse-phase mode and normal-phase mode. Using different organic solvent or buffer ratio as mobile phase, a suitable method was developed.

Some trial chiral methods with chromatogram are shown below with CD-PH on reverse phase mode:



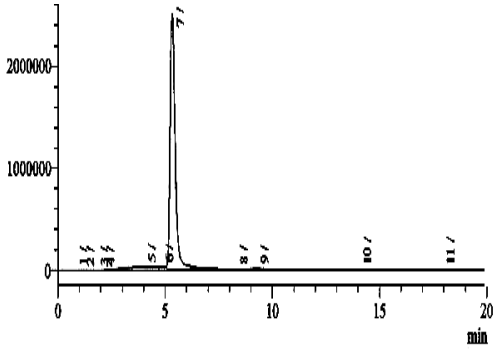
Mobile phase: ACN: H_2O: AA (50:50: 0.1)

Flow rate: 1 mL/min

Detector: 254 nm

Result: One peak observed

Figure 4.5.1: Trial-1.



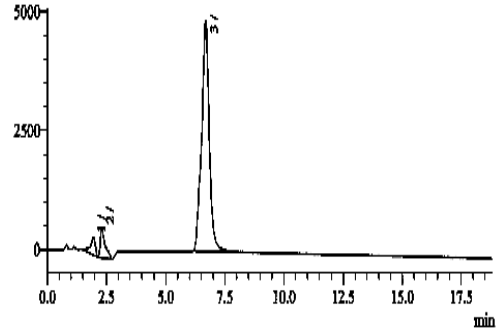
Mobile phase: ACN: NH_4OAc (50:50)

Flow rate: 1 mL/min

Detector: 254 nm

Result: One peak observed

Figure 4.5.2: Trial-2.



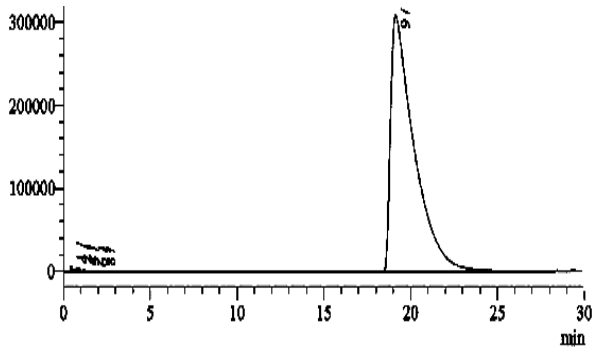
Mobile phase: ACN: NH_4OAc (20:80)

Flow rate: 1 mL/min

Detector: 254 nm

Result: One peak observed

Figure 4.5.3: Trial-3.



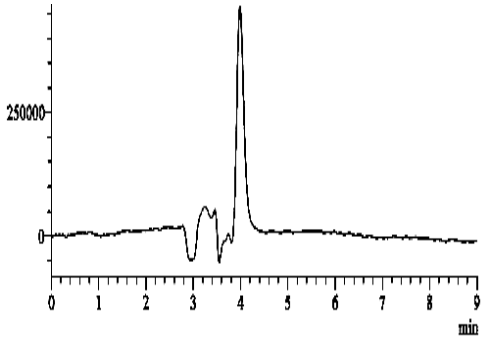
Mobile phase: ACN: $NH_4H_2PO_4$ (30:70)

Flow rate: 1 mL/min

Detector: 254 nm

Result: One peak observed

Figure 4.5.4: Trial-4.



Mobile phase: ACN: $NH_4H_2PO_4$ (05:95)

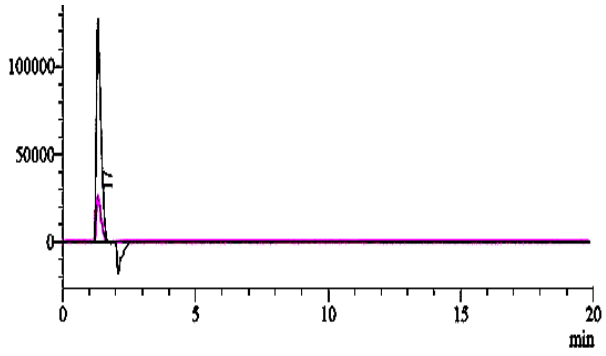
Flow rate: 1 mL/min

Detector: 254 nm

Result: One peak observed

Figure 4.5.5: Trial-5.

Some trial chiral methods with chromatogram have been shown below with CD-PH on normal phase mode:



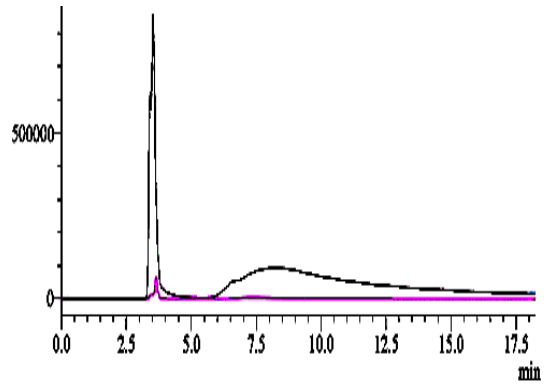
Mobile phase: Hx: IPA: TEA (50: 50: 0.1)

Flow rate: 1 mL/min

Detector: 254 nm

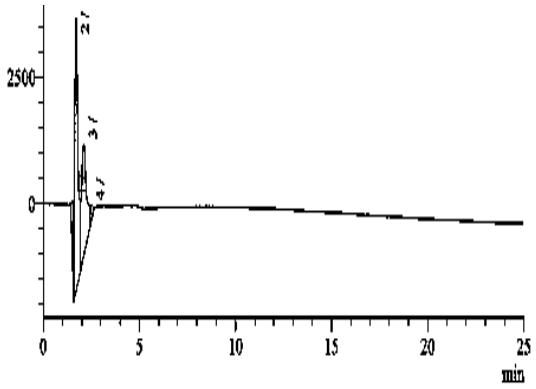
Result: No peak observed

Figure 4.5.6: Trial method-6.



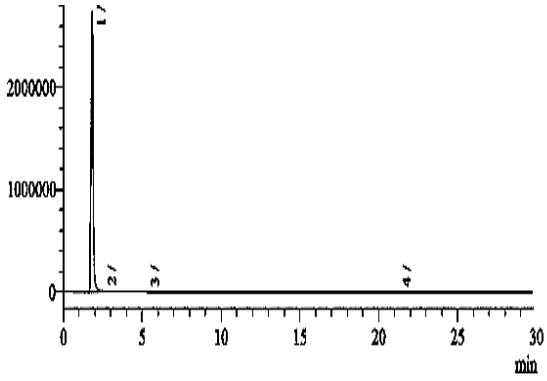
Mobile phase: Hx: IPA: TEA (60: 40: 0.1)
Flow rate: 1 mL/min
Detector: 254 nm
Result: No peak observed

Figure 4.5.7: Trial-7.



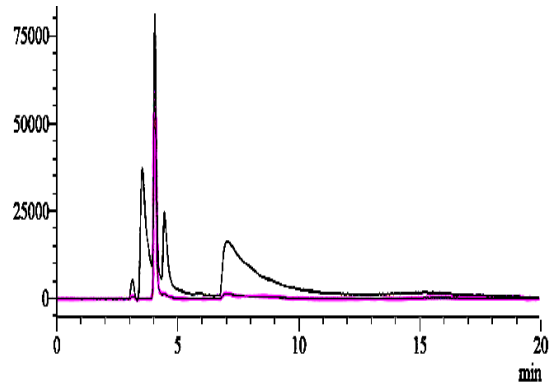
Mobile phase: Hx: IPA: AA (90: 10: 0.1)
Flow rate: 1 mL/min
Detector: 254 nm
Result: No peak observed

Figure 4.5.8: Trial-8.



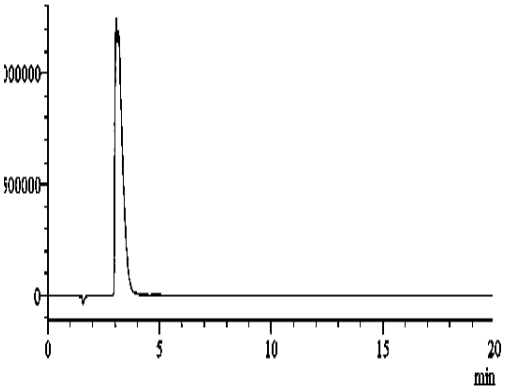
Mobile phase: Hx: IPA: AA (10: 90: 0.1)
Flow rate: 1 mL/min
Detector: 254 nm
Result: No peak observed

Figure 4.5.9: Trial-9.



Mobile phase: Hp: IPA: TEA (50: 50: 0.1)
Flow rate: 1 mL/min
Detector: 254 nm
Result: No identical peak observed

Figure 4.5.10: Trial-10.



Mobile phase: Hp: IPA: TEA (60: 40: 0.1)
Flow rate: 1 mL/min
Detector: 254 nm
Result: No identical peak observed

Figure 4.5.11: Trial-11.

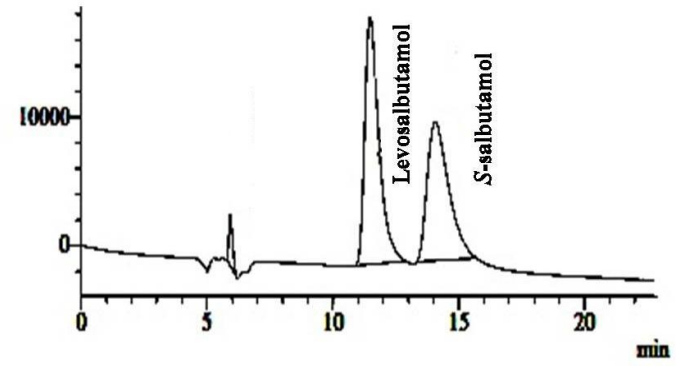
Summary of these trial chiral methods are showed in table 4.5.1.

Table 4.5.1: Trial and error experiment for separation of salbutamol.

PPolar Mobile Phase BBuffer:Organic phase/Others	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
ACN: H_2O: AA (50:50: 0.1)	One peak observed	NA	NA	NA
ACN: NH_4OAc (50:50)	One peak observed	NA	NA	NA
ACN: NH_4OAc (20:80)	One peak observed	NA	NA	NA
ACN: $NH_4H_2PO_4$ (30:70)	One peak observed	NA	NA	NA
ACN: $NH_4H_2PO_4$ (05:95)	One peak observed	NA	NA	NA
Non-polar Mobile phase	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
Hx: IPA: TEA (50: 50: 0.1)	No peak observed	NA	NA	NA
Hx: IPA: TEA (60: 40: 0.1)	No peak observed	NA	NA	NA
Hx: IPA: AA (90: 10: 0.1)	No peak observed	NA	NA	NA
Hx: IPA: AA (10: 90: 0.1)	No peak observed	NA	NA	NA
Hp: IPA: TEA (50: 50: 0.1)	No peak observed	NA	NA	NA
Hp: IPA: TEA (60: 40: 0.1)	No peak observed	NA	NA	NA

NA = Not applicable

Table 4.5.1 demonstrates that the results of all parameters are not suilable. So all the above methods are avoided. After a number of trials with CD-PH column with mobile phases of different composition, the best mobile phase composition was then found to be di-sodium hydrogen phosphate (pH 6.01)/ methanol (50:50, v/v), because of adequate resolution, efficient theoretical plates number and symmetric peak shape.



Mobile phase: $Na_2H_2PO_4$: MeOH (50:50)
Flow rate: 0.6 mL/min
Detector: 254 nm
Result: Two acceptable peaks observed

Figure 4.5.12: Chromatogram with $Na_2H_2PO_4$: MeOH (50:50) in CD- PH.

Now, this proposed chromatographic method has been compared with the previously published methods.

4.5.2. Comparison of the present work

A comparison of present work with the other earlier reports on enantio-resolution of salbutamol is shown in table 4.5.2. It is clear that the present method is suitable in terms of resolution.

Table 4.5.2: Comparison of HPLC enantioresolution of salbutamol with the present work.

Chiral column/ selector	Mobile phase	Resolution (Rs)	Reference
Chirobiotec T	Acetonitrile: methanol: acetic acid: triethylamine (60:40:0.3:0.2)	1.9	(Halabi *et al.*, 2004)
Teicoplanin based CSP	Methanol: acetonitrile: glacial acetic acid: diethylamine	1.8	(Rosales-Conrado *et al.*, 2013)
Teicoplanin Chirobiotic-T™	Methanol:20 mM ammonium acetate pH 4.5 (98:2, v/v)	1.6	(Rosales-Conrado *et al.*, 2013)
CD-PH	di-Sodium hydrogen phosphate: Methanol (50: 50)	2.08	Present work

Rs = Resolution

4.5.3. Validation of the proposed method

Finally, this present method was validated with respect to the following parameters.

4.5.3.1. Linearity and range

Linearity of the method was studied by injecting five concentrations of two enantiomers of salbutamol prepared in the mobile phase in concentration range from 5 –100 µg/mL in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves. The linearity curves are shown in figure 4.5.13 and the parameters are given in table 4.5.3.

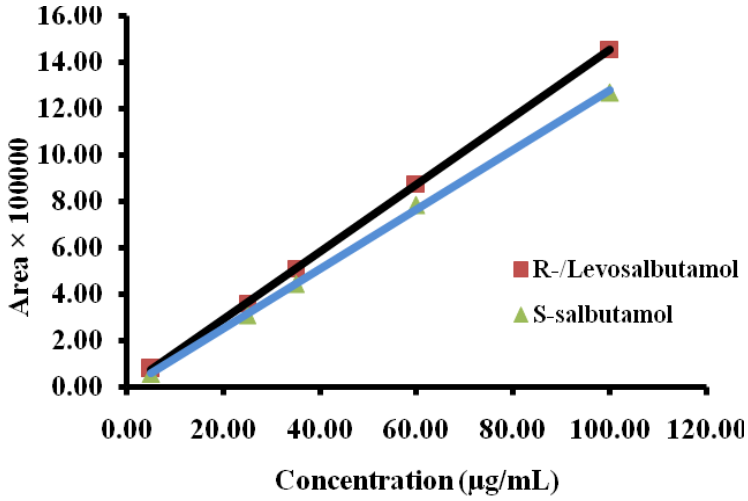


Figure 4.5.13: Linearity curve for (*S*)- and (*R*)-salbutamol.

4.5.3.2. Precision

The precision of the method was verified by intra- and inter-day precision studies. Intra-day precision was performed by analysis of on concentration for six times on the same day. The intermediate precision of the method was checked by studying on three different days. Results have been presented in table 4.5.3 and bar diagram of precision (% RSD) for (*S*)- and (*R*)-salbutamol is shown in figure 4.5.14.

Table 4.5.3: Results of method validation parameters for salbutamol.

Parameters	*S*-salbutamol	*R*-salbutamol
Linear equation	y= 4394x+1655	y= 4289x+834.0
Coefficient of determination (r2>0.995)	0.999	0.999
Linearity range	40-140 µg/mL	
Precision (intra-day, n=6) (% RSD≤2)	0.0.09%	0.05%
Precision (inter-day, n=6) (% RSD≤2)	0.09%	0.05%

n = number of determinations

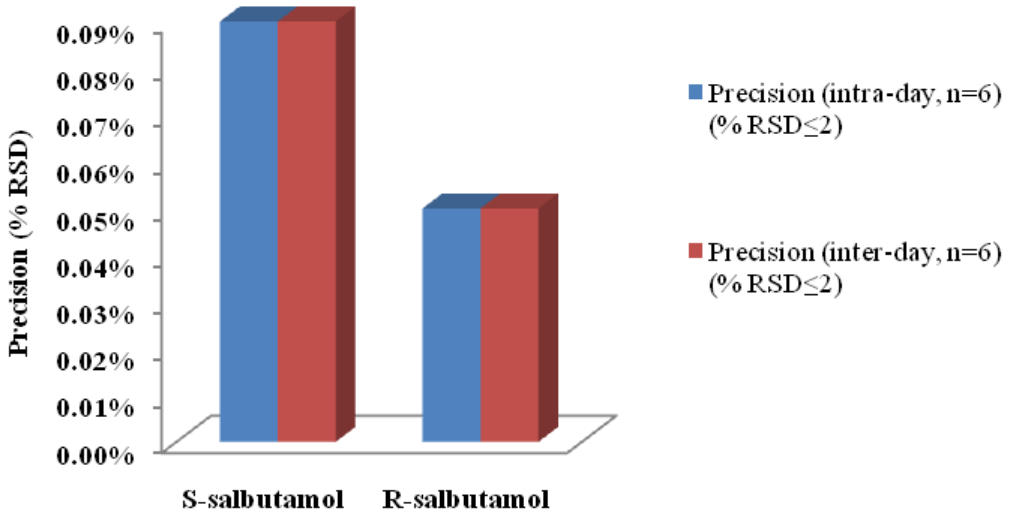


Figure 4.5.14: Bar diagram of precision (% RSD) for (*S*)- and (*R*)-salbutamol.

4.5.3.3. Accuracy

Accuracy of the method was verified by studying recovery experiments which were performed by spiking solutions of known amount of the drug with pre-analyzed sample. To evaluate the accuracy of the method, successive analysis (n=3) of standard solutions of the drug was carried out and the results are given in table 4.5.4 and bar diagram of accuracy (% Recovery) for (*S*)- and (*R*)-salbutamol has been shown in figure 4.5.15.

Table 4.5.4: Results of accuracy study for standard salbutamol.

Parameters	*S*-salbutamol	*R*-salbutamol
Accuracy (n=3) (avg. % recovery)		
Standard+spike (µg/mL)		
(25+20)	99.95%	99.46%
(30+20)	100.28%	100.51%
(40+20)	100.06%	99.99%
LOD (µg/mL)	1.32	0.59
LOQ (µg/mL)	3.99	1.79

n = number of determinations

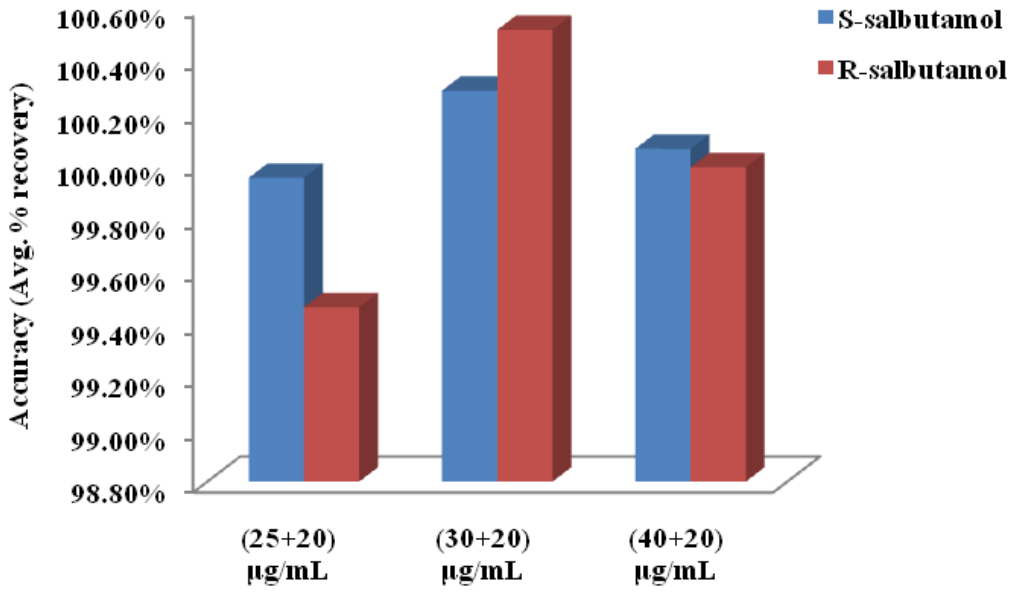


Figure 4.5.15: Bar diagram of accuracy (% recovery) for (*S*)- and (*R*)-salbutamol.

4.5.3.4. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated using the following equations:

LOD= (SD / slope) x 3.3

LOQ= (SD / slope) x 10.

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of

regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). The results of LOD and LOQ obtained for studied drugs have been recorded in table 4.3.4.

4.5.3.5. Specificity testing

The specificity of the method was assessed from the chromatogram where complete separation of salbutamol and levosalbutamol was achieved without any interference. The peaks obtained were sharp, well separated at the baseline as shown in figure 4.5.16. Single enantiomer, levosalbutamol was injected into HPLC for the detection of *R*- enantiomer in salbutamol.

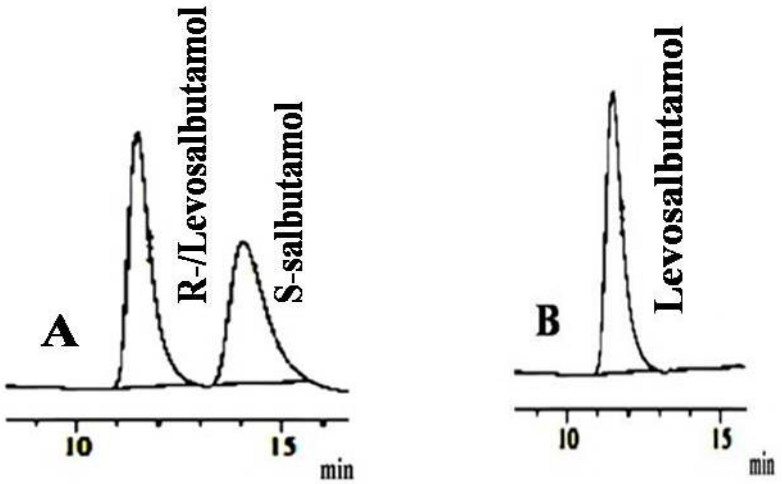


Figure 4.5.16: HPLC chromatograms of salbutamol (A) and levosalbutamol (B).

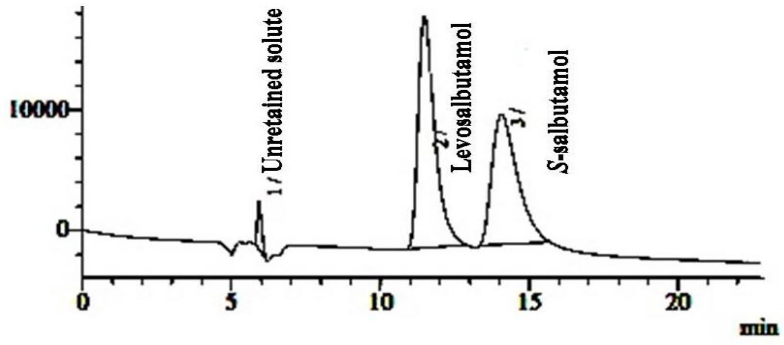
4.5.3.6. System suitability testing

System suitability parameters are reported in table 4.5.5 and chromatogram of salbutamol for system suitability is shown in figure 4.5.17.

Table 4.5.5: Parameters for system suitability testing.

Parameters	*S*-salbutamol	*R*-salbutamol
Theoretical plates (≥2000) (n=5)	8074	7012
Tailing factor (≤2) (n=5)	1.14	1.04
Relative retention (k*S* and k*R*) (n=5), 0.5 < k < 10	1.4	1.0
Selectivity (α>1) (n=5)	1.4	
Resolution (≥2) (n=5)	2.08	

n = number of determinations



Where,

t_0 =5.91 min,

t_S = 14.06 min,

t_R = 11.47 min

Figure 4.5.17: HPLC chromatogram of salbutamol for system suitability testing.

4.5.3.7. Solution stability testing

A standard solution of concentration of 60 µg/mL was kept in a tightly capped volumetric flask at room temperature (25 °C) on the laboratory bench and at 4 °C in a refrigerator for 3 days and its stability was tested. The results are given in table 4.5.6. Bar diagram of solution stability (% RSD) for (*S*)- and (*R*)-salbutamol is shown in figure 4.5.18.

Table 4.5.6: Parameters for solution stability testing.

Day	**At 25 °C**		**At 4 °C**	
	***S*-salbutamol (n=3) (% RSD)**	***R*-salbutamol (n=3) (% RSD)**	***S*-salbutamol (n=3) (% RSD)**	***R*-salbutamol (n=3) (% RSD)**
Day 1	0.00%	0.03%	0.01%	0.01%
Day 2	0.01%	0.02%	0.03%	0.01%
Day 3	0.02%	0.01%	0.02%	0.00%
Avg.	0.01%	0.02%	0.02%	0.01%

n = number of determinations

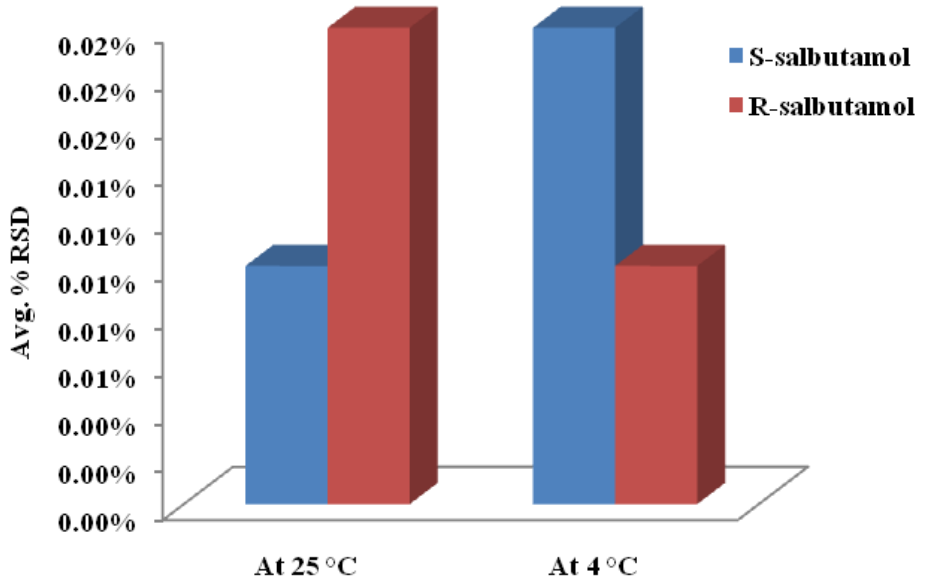


Figure 4.5.18: Bar diagram of solution stability testing (% RSD) for (*S*)- and (*R*)-salbutamol.

4.5.3.8. Robustness of the method

To determine the robustness of this method, the experimental conditions were deliberately changed, like the flow rate and in the wavelength of detection and measuring the % RSD. For the present study, factors chosen were flow rate (0.6 ± 0.2mL/min) and wavelength (254 ± 2nm), and n = 3. The % RSD is reported in table 4.5.7. All analyte peaks were adequately resolved and elution orders remain unchanged. Bar diagram of robustness study (% RSD) for (*S*)- and (*R*)- salbutamol is shown in figure 4.5.19.

Table 4.5.7: Robustness parameters.

Parameters (n=3)	***S*-salbutamol (n=3) (% RSD)**	***R*-salbutamol (n=3) (% RSD)**
Detection wavelength at 252 nm	0.11%	0.12%
Detection wavelength at 256 nm	0.24%	0.12%
Flow rate 0.4 mL/min	0.03%	0.07%
Flow rate 0.8 mL/min	0.04%	0.10%

n = number of determinations

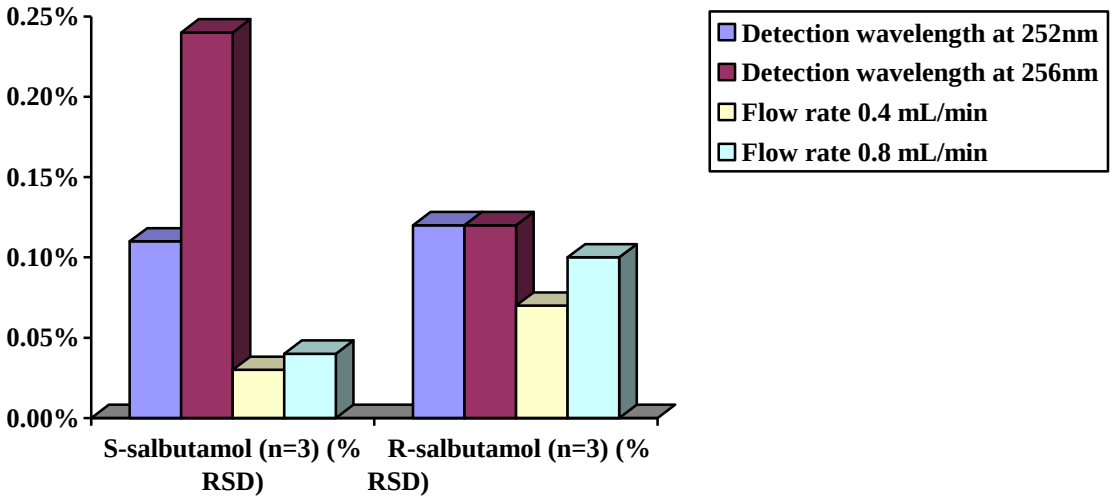


Figure 4.5.19: Bar diagram of robustness testing (% RSD) for (*S*)- and (*R*)- salbutamol.

4.5.4. Application of the developed method

This analytical method was applied to quantitate the content of levosalbutamol and salbutamol in twelve and ten companies of Bangladesh, respectively and as well as to calculate the % purity and ratio of them. The average content of levosalbutamol were from 47.46% to 52.17% while the content of *S*-salbutamol was from 47.83% to 52.54% in the formulations of racemic mixture of salbutamol. For single enantiomeric samples, average content of levosalbutamol were from 98.45% to 100.00% along with average content of *S*- salbutamol were found as impurity from 0.00% to 1.55%. Enantiomeric purity (%EP) or the enantiomeric excess (ee%) of levosalbutamol samples were found to be 98.45% to 100.00%. Enantiomeric ratio and purity of commercial samples are shown in table 4.5.8 and 4.5.9, respectively. Enantiomeric ratio of salbutamol for all companies' samples met the USP requirements. HPLC chromatograms of one commercial sample for salbutamol and levosalbutamol are shown in figure 4.5.20. Bar diagram of enantiomeric ratio of racemic salbutamol and % purity of levosalbutamol are shown in figure 4.5.21 and figure 4.5.22, respectively.

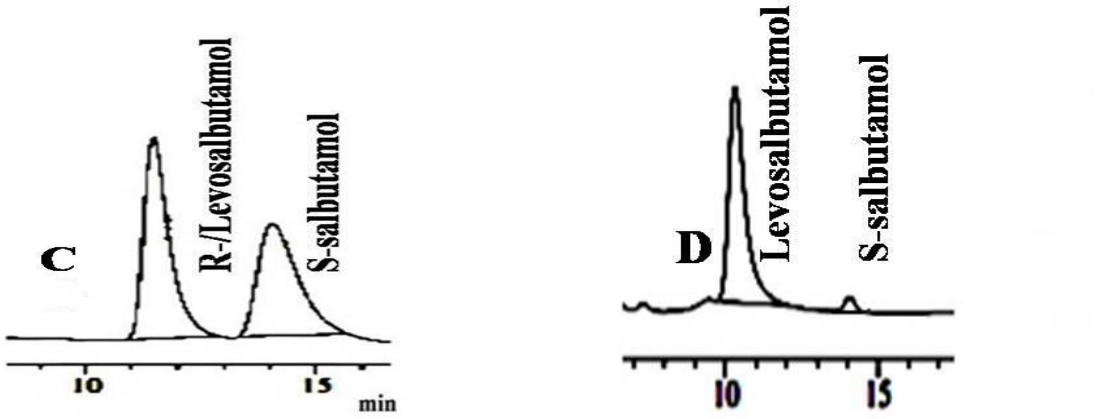


Figure 4.5.20: Chromatogram of one commercial sample (C) salbutamol and (D) levosalbutamol.

Table 4.5.8: Enantiomeric ratio of salbutamol in commercial samples (n=10).

Identity of company	% Content of levosalbutamol	% EP = (50% - % enantiomer) of levosalbutamol	% Content of dexsalbutamol	% EP = (50% - % enantiomer) of dexsalbutamol	Enantiomeric ratio of salbutamol
Company A	47.46%	2.54%	52.54%	-2.54%	47.46%/52.54%
Company B	50.67%	-0.67%	49.33%	0.67%	50.67%/49.33%
Company C	51.20%	-1.20%	48.80%	1.20%	51.20%/48.80%
Company D	52.17%	-2.17%	47.83%	2.17%	52.17%/47.83%
Company E	50.93%	-0.93%	49.07%	0.93%	50.93%/49.07%
Company F	49.58%	0.42%	50.42%	-0.42%	49.58%/50.42%
Company G	48.59%	1.41%	51.41%	-1.41%	48.59%/51.41%
Company H	47.70%	2.30%	52.30%	-2.30%	47.70%/52.30%
Company I	48.75%	1.25%	51.25%	-1.25%	48.75%/51.25%
Company J	51.30%	-1.30%	48.70%	1.30%	51.30%/48.70%

EP= Enantiomeric purity, n = number of determinations

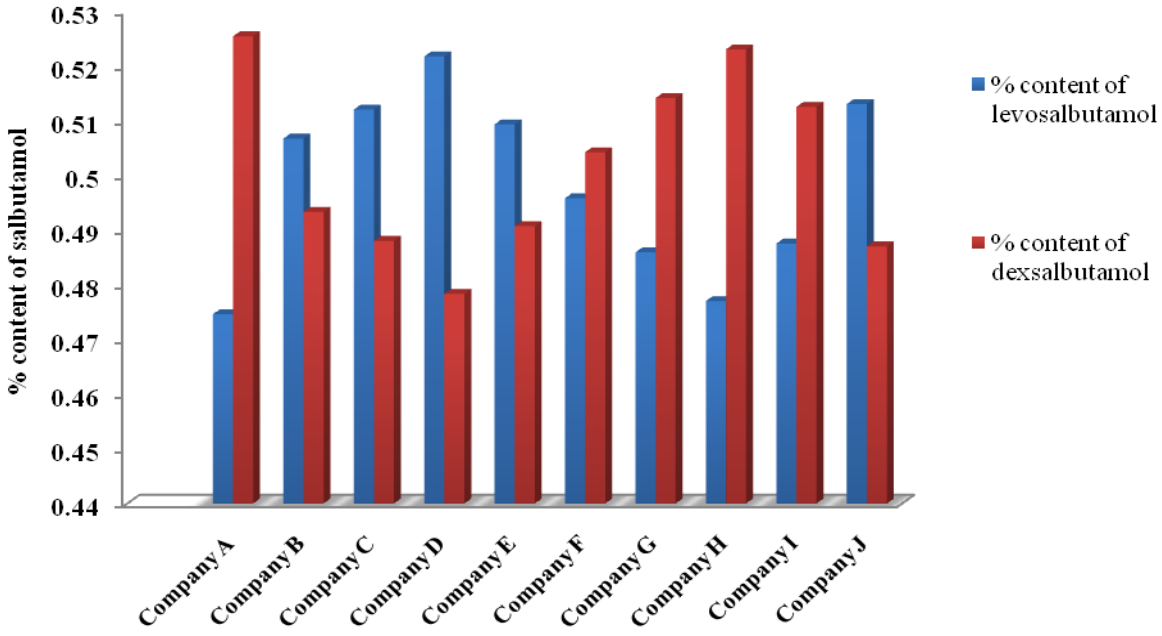


Figure 4.5.21: Bar diagram of enantiomeric ratio of racemic salbutamol.

Table 4.5.9: Enantiomeric purity of levosalbutamol in commercial samples (n=10).

Identity of company	% Content of levosalbutamol	%Content of dexsalbutamol in levosalbutamol as impurity	%EP = (% of major enantiomer - % of minor enantiomer)
Company A1	100.00%	0.00%	100.00%
Company B1	100.00%	0.00%	100.00%
Company C1	100.00%	0.00%	100.00%
Company D1	100.00%	0.00%	100.00%
Company E1	100.00%	0.00%	100.00%
Company F1	99.70%	0.30%	99.40%
Company G1	98.86%	1.14%	97.72%
Company H1	99.88%	0.12%	99.76%
Company I1	99.99%	0.01%	99.98%
Company J1	98.45%	1.55%	96.90%
Company K1	99.39%	0.61%	98.78%
Company L1	99.45%	0.55%	98.90%

EP=Enantiomeric purity, n = number of determinations

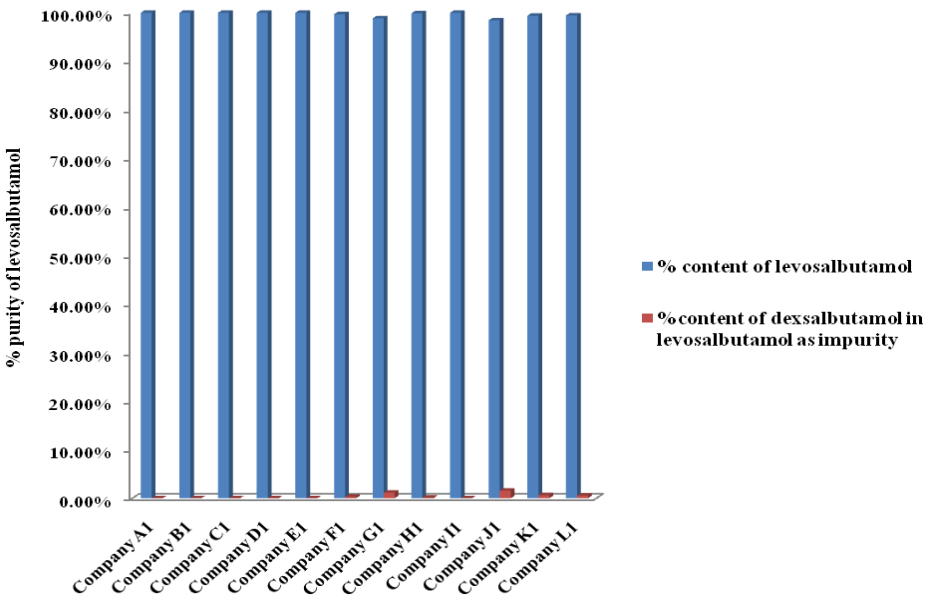


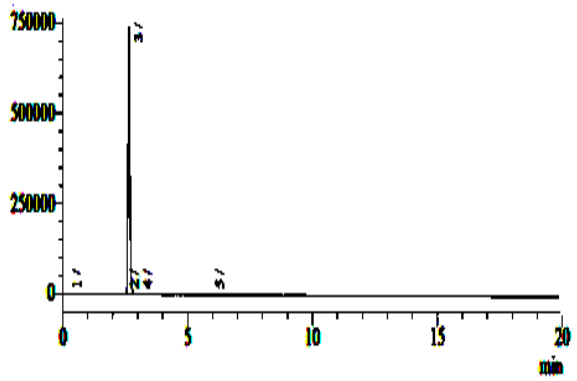
Figure 4.5.22: Bar diagram of % purity of levosalbutamol.

4.6. Analysis of escitalopram

4.6.1. Analytical HPLC

To develop a new and easy chiral HPLC method for citalopram, we tried a significant number of methods on trial and error basis using a large number of solvent mixtures as mobile phase. For citalopram separation, CD-PH chiral columns was used which column is applicable in both of the normal-phase and reversed-phase mode. Here, CD-PH was used as reverse-phase mode and normal-phase mode. Using different organic solvent or buffer ratio as mobile phase, a suitable method was developed.

Some trial chiral methods with chromatogram are shown below with CD-PH column:



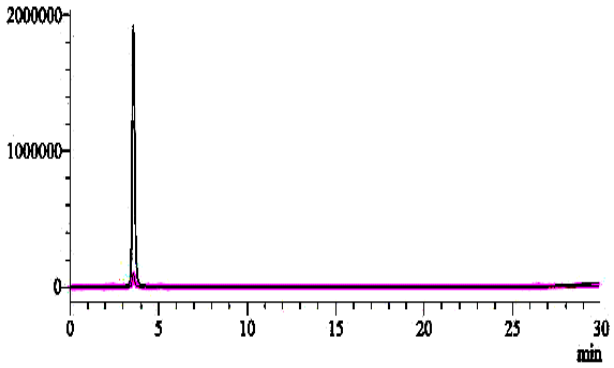
Mobile phase: MeOH: H_2O (50:50)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: No peak observed

Figure 4.6.1: Trial-1.



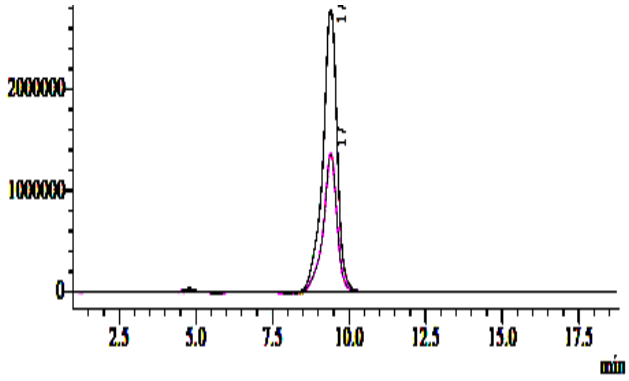
Mobile phase: IPA: EtOH (50:50)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: No peak observed

Figure 4.6.2: Trial-2.



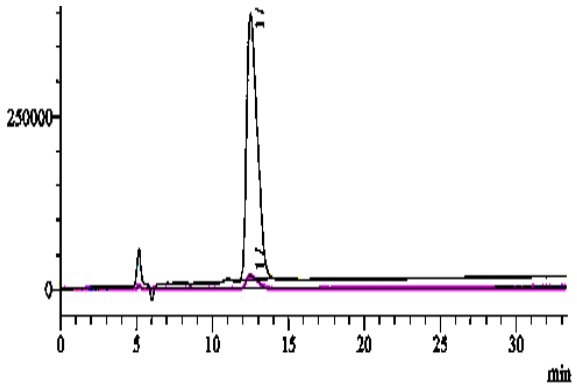
Mobile phase: Hx: IPA: MeOH (40:40:20)

Flow rate: 1.0 mL/min

Detector: 254 nm

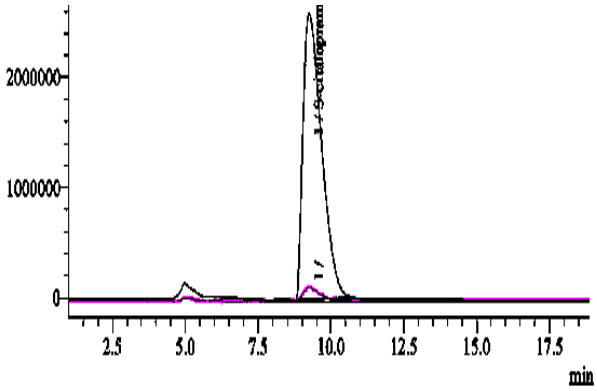
Result: One peak observed

Figure 4.6.3: Trial-3.



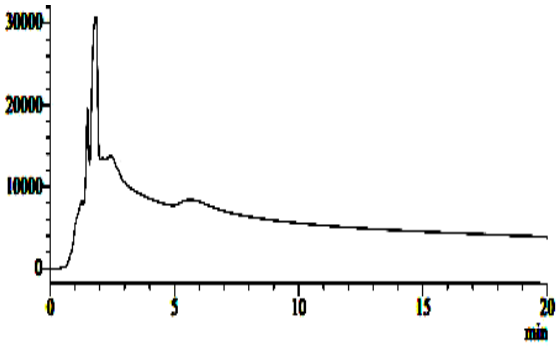
Mobile phase: Hx: IPA: EtOH (60:40:10)
Flow rate: 1.0 mL/min
Detector: 254 nm
Result: One peak observed

Figure 4.6.4: Trial-4.



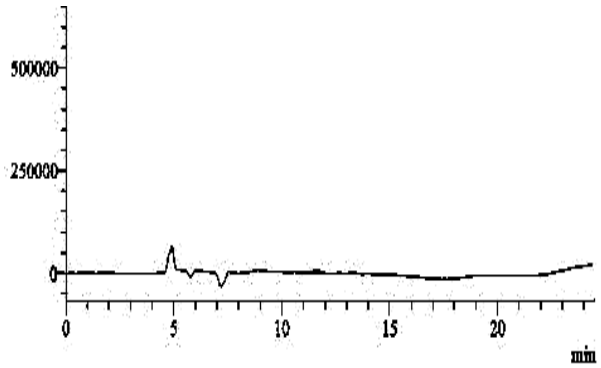
Mobile phase: Hx: IPA: NH_4OAc (30: 70: 60)
Flow rate: 1.0 mL/min
Detector: 254 nm
Result: One peak observed

Figure 4.6.5: Trial-5.



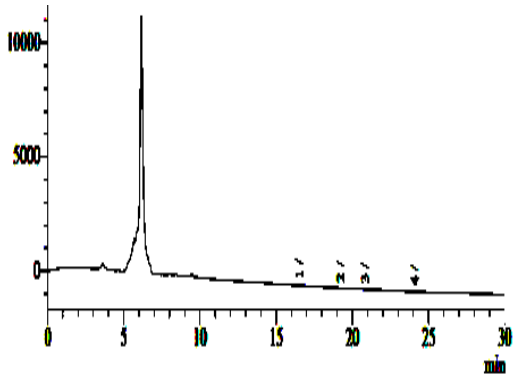
Mobile phase: Hx: MeOH: NH_4OAc (60: 50: 40)
Flow rate: 1.0 mL/min
Detector: 254 nm
Result: One peak observed

Figure 4.6.6: Trial-6.



Mobile phase: Hx: IPA: TEA (60: 40: 0.1)
Flow rate: 1.0 mL/min
Detector: 254 nm
Result: No peak observed

Figure 4.6.7: Trial-7.



Mobile phase: Hx: IPA: TEA (50: 50: 0.1)
Flow rate: 1.0 mL/min
Detector: 254 nm
Result: No peak observed

Figure 4.6.8: Trial-8.

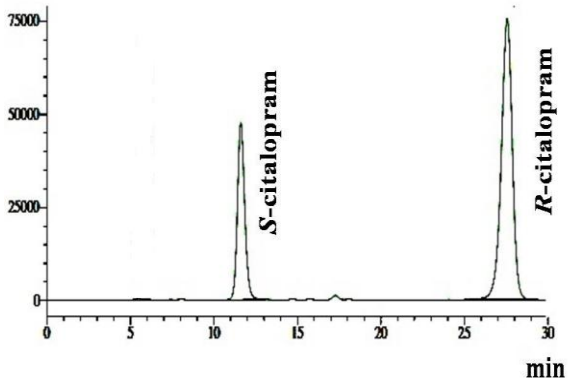
Summary of these trial chiral methods are shown in table 4.6.1.

Table 4.6.1: Trial and error experiment for separation of citalopram.

Mobile Phase	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
MeOH : H_2O (50:50)	No peak observed	NA	NA	NA
IPA: EtOH (50:50)	No peak observed	NA	NA	NA
Hx:IPA: MeOH (40:40:20)	One peak observed	NA	NA	NA
Hx:IPA:EtOH (60:40:10)	One peak observed	NA	NA	NA
Hx: IPA: NH_4OAc (30: 70: 60)	One peak observed	NA	NA	NA
Hx: MeOH: NH_4OAc (60: 50: 40)	One peak observed	NA	NA	NA
Hx: IPA: TEA (60: 40: 0.1)	No peak observed	NA	NA	NA
Hx: IPA: TEA (50: 50: 0.1)	No peak observed	NA	NA	NA

NA = Not applicable

Table 4.6.1 demonstrates that all the results of all parameters are not suitable. So all the above methods were avoided. After a number of trials with CD-PH column with mobile phases of different composition, the best mobile phase composition was then found to be ammonium acetate (pH 3.0: ethanol: isopropanol: methylene chloride (100:150:70:30, v/v), because of adequate resolution, efficient theoretical plates number and symmetric peak shape.



Mobile phase: NH_4OAc (pH 3.0): EtOH: IPA: CH_2Cl_2 (100:150:70:30, v/v)
Flow rate: 0.6 mL/min
Detector: 254 nm
Result: Two acceptable peaks observed

Figure 4.6.9: Chromatogram with NH_4OAc (pH 3.0): EtOH: IPA: CH_2Cl_2 (100:150:70:30, v/v) in CD-PH column.

Now, this proposed chromatographic method has been compared with the previously published methods.

4.6.2. Comparison of the present work

A comparison of present work with the other earlier reports on enantio-resolution of citalopram is shown in table 4.6.2.

Table 4.6.2: Comparison of HPLC enantioresolution of citalopram with the present work.

Chiral column/ selector	Mobile phase	Resolution (Rs>2)	Reference
Luna C_{18}	50 mM copper sulphate and 100 mM L-histidine in 50 mM potassium phosphate (pH 5): methanol: acetonitrile: tetrahydrofuran (70:20:10:0.6)	3.67	(Salama *et al.*, 2014)
AmyCoat (amylose CSP)	n-Hexane: isopropanol: diethylamine (95:05:0.2)	1.22	(VK *et al.*, 2011)
Hedera ODS-2 C_{18}	5 mM sodium dihydrogen phosphate and 12 mM SBE-β-CD(pH 2.5):methanol:acetonitrile (21:03:01)	2.5	(Peng *et al.*, 2016)
CD-PH	Ammonium acetate (pH 3.0): ethanol: isopropanol: methylene chloride (100:150:70:30)	15.63	Present work

It is clear that the present method is very suitable in terms of resolution.

Finally, this present method has been extensively validated with respect to the following parameters.

4.6.3. Results and discussion of validation parameters

4.6.3.1. Specificity

Specificity of the test method was determined by testing standard substances against potential interferences. The method was found to be specific because of absence of interference to the test solutions. Under the optimized conditions, the chromatograms (figure 4.6.1) confirm the presence of (***S***)- and (***r)***- enantiomers in citalopram standard solution at about 11.74 ± 0.01 min and 28.32 ± 0.01 min, respectively without any interference. System suitability was examined by injecting five replicates of standard citalopram solution into the system. Excellent resolution of 15.63 was obtained.

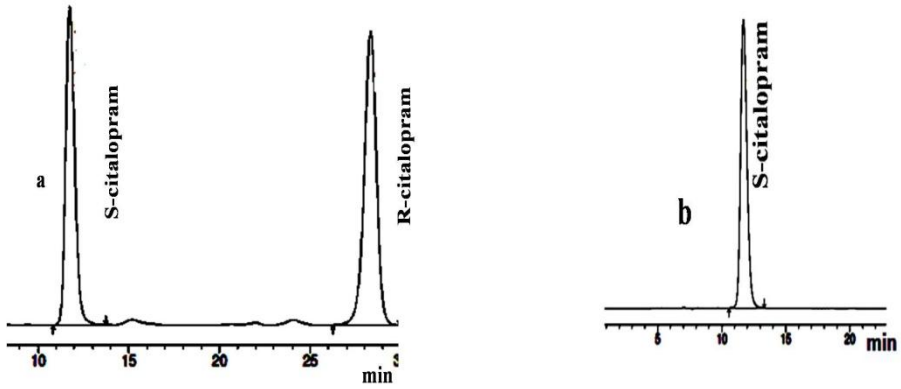


Figure 4.6.10: Chromatograms of (a) standard citalopram, and (b) standard *S*-citalopram.

4.6.3.2. System suitability testing

Capacity factor (k)

In the present work, k values for two enantiomers were 1.15 and 4.19, respectively.

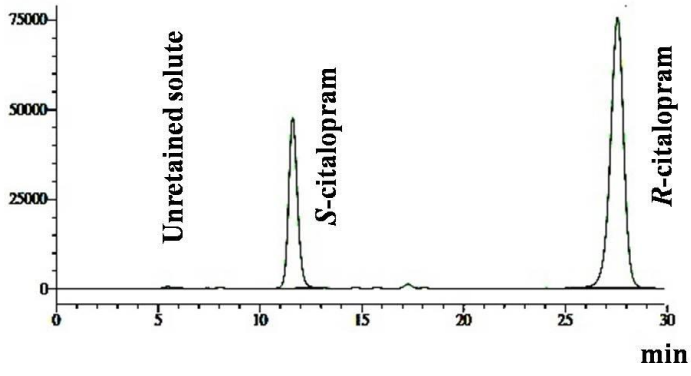
Selectivity factor (α)

In the present study the selectivity parameter for separation of citalopram was found to be 3.60.

Resolution (R)

In this work the resolution value for separation of citalopram was 15.63.

This method also resulted in symmetric peak shape with tailing 1.16 and 0.91 and good no. of theoretical plates with 23097 and 58185 for (*S*)- and (*R*)- citalopram, respectively. System suitability parameters are reported in table 4.6.2 and chromatogram are shown in figure 4.6.11.



Where,

t_0 =5.45 min,

t_S = 11.74 min,

t_R = 28.32 min

Figure 4.6.11: HPLC chromatogram of citalopram for system suitability testing.

4.6.3.3. Linearity and range

The calibration curve for escitalopram shows good linearity with coefficient of determination (r^2) values of 0.998 in the concentration range from 20-70 µg/mL. The linearity curves are shown in figure 4.6.12 and the parameters are given in table 4.6.3.

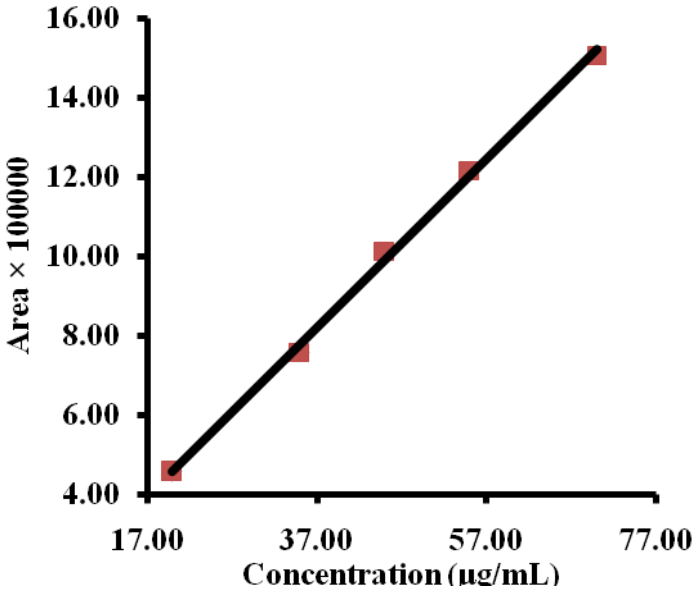


Figure 4.6.12: Linearity curve for *S*-citalopram.

4.6.3.4. Precision

The precision of the method was verified by intra- and inter-day precision studies. Intra-day precision was performed by analysis of concentration for six times on the same day. The intermediate precision of the method was checked by studying on three different days. The value of %RSD for intra-/inter- day precisions were less than 2.00% for escitalopram. The results are presented in table 4.6.5 and bar diagram of precision (% RSD) is shown in figure 4.6.13.

4.6.3.5. Accuracy

Accuracy of the method was verified by studying recovery experiments which were performed by spiking solutions of known amount of the drug with pre-analyzed sample. To evaluate the accuracy of the method, successive analysis (n=3) of standard solutions of the drug was carried out. The average percentage of recovery was calculated and it was found to be 100.28% to

102.86% for (*S*)- citalopram against the concentration of 45, 60, and 65 µg/mL. All data are shown in table 4.6.6 and bar diagram of accuracy (% recovery) is shown in figure 4.6.14.

4.6.3.6. Detection limit

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated using the following equations:

LOD= (SD / slope) x 3.3

LOQ= (SD / slope) x 10.

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). The results of LOD and LOQ obtained for studied drugs have been shown in table 4.6.3.

4.6.3.7. Parameters for solution stability testing

Escitalopram standard solution with concentration of 30 µg/mL was kept in a tightly capped volumetric flask at room temperature (25 $^{\circ}$C) on the laboratory bench and at 4 $^{\circ}$C in a refrigerator for 3 days and its stability was tested. Solution stability study was carried out to calculate % RSD of area for three consecutive days at 25 $^{\circ}$C and 4 $^{\circ}$C. At 25 $^{\circ}$C the value of % RSD was found to be 0.08%. At 4 $^{\circ}$C the value of % RSD was 0.05% which demonstrated that the drug was fairly stable at normal and freezing temperatures. Values obtained are given in table 4.6.7 and bar diagram of solution stability (% RSD) for (*S*)- citalopram is shown in figure 4.6.15.

4.6.3.8. Robustness testing

The robustness of the test method was carried out changing the flow rate ± 10%, i.e. 0.6 mL to 0.8 mL/min) and wavelength ± 2 nm, i.e. 254 nm to 256 nm), and n = 3. The method was found to be robust. The % RSD was calculated for each variation. Values obtained are given in table 4.6.8 and bar diagram of robustness (% RSD) for (*S*)- citalopram is shown in figure 4.6.16.

Table 4.6.3: Results of method validation parameters

Parameters	**(*S*)- citalopram**
Linear equation	y= 21269x+31568
Coefficient of determination (r2>0.995)	0.998
Linear range	20-70 µg/mL
LOD (µg/mL)	2.54 µg/mL
LOQ (µg/mL)	7.68 µg/mL

Table 4.6.4: Results of system suitability testing parameters.

Parameters	**(*S*)- citalopram**	**(*R*)- citalopram**
Theoretical plates (≥2000) (n=5)	23097	58185
Tailing factor (≤2) (n=5)	1.16	0.91
Relative retention (k*S* and k*R*) (n=5), 0.5 < k < 10	1.15	4.19
Selectivity (α>1) (n=5)	3.64	
Resolution (≥2) (n=5)	15.63	

n = number of determinations

Table 4.6.5: Results of precision intra/inter-day validation of the method.

Precision (%RSD≤2)	**(*S*)- citalopram**
Intra-day, n=6	0.16%
Inter-day, n=6	0.07%

n = number of determinations

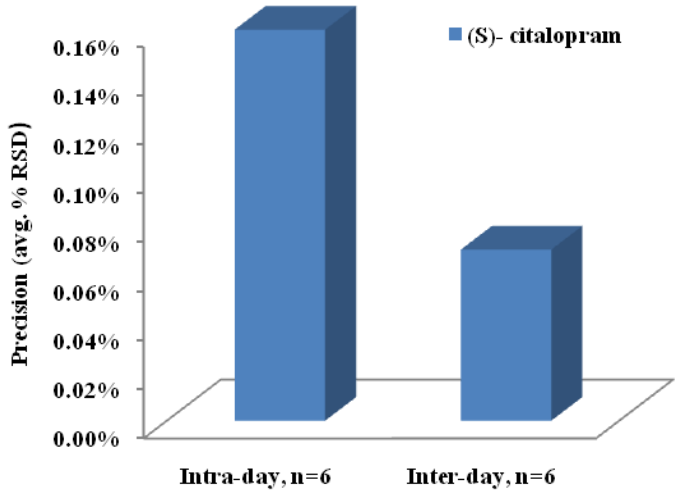


Figure 4.6.13: Bar diagram of precision (% RSD) for (*S*)- citalopram.

Table 4.6.6: Accuracy of *S*-citalopram.

Accuracy (n=3) (avg. % recovery)		
Standard+spike (µg/mL)		
Added concentration (µg/mL)	Recovered concentration (µg/mL)	% Recovery
45.00	46.29	102.86
60.00	60.53	100.89
65.00	65.18	100.28

n = number of determinations

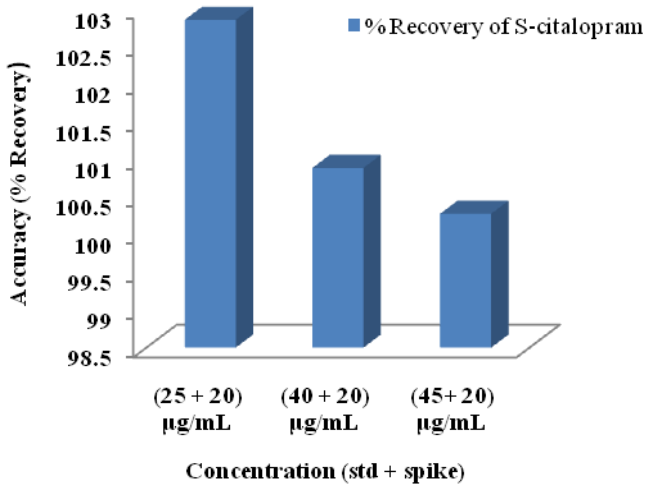


Figure 4.6.14: Bar diagram of accuracy (% recovery) for (*S*)- citalopram.

Table 4.6.7: Parameters for solution stability testing.

Day	% RSD at 25 °C	% RSD at 4 °C
	***S*-citalopram (n=3) (% RSD)**	
Day 1	0.07%	0.06%
Day 2	0.08%	0.03%
Day 3	0.09%	0.05%
Avg.	0.08%	0.05%

n = number of determinations

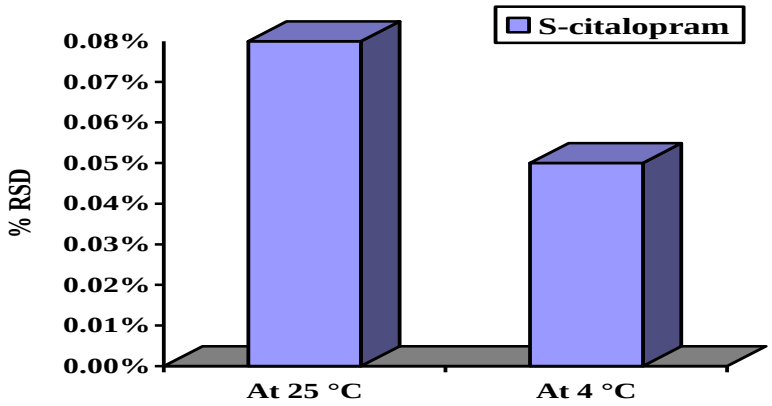


Figure 4.6.15: Bar diagram of solution stability testing (% RSD) for (*S*)- citalopram.

Table 4.6.8: Robustness study of *S*-citalopram (n=3).

Change in flow rate (mL/min)	Average Rt of (*S*)- citalopram ± SD	% RSD
0.8	8.03±0.01	0.12
0.6	11.74±0.01	0.09
0.4	18.63±0.01	0.03
Change in wavelength (nm	**Average area of (*S*)- citalopram ± SD**	**% RSD**
256	3107622±6624.57	0.22
254	1123455±1894.97	0.17
252	2658804±5563.27	0.21

n = number of determinations

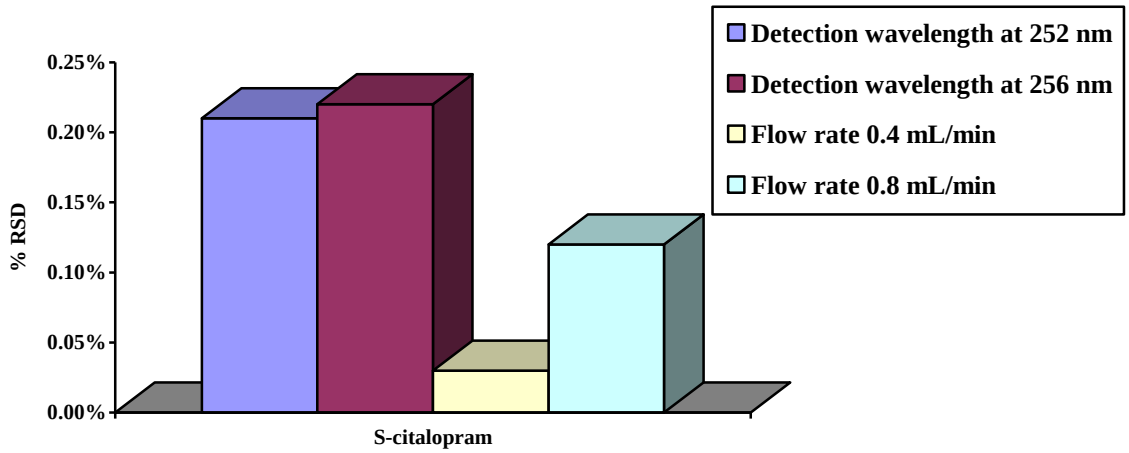


Figure 4.6.16: Bar diagram of robustness (% RSD) for (*S*)- citalopram.

4.6.4. Application of the developed method

This analytical method was applied to quantitate the content of (*S*)- citalopram in samples from seven companies of Bangladesh and as well as to calculate the % purity. The average content of (*S*)- citalopram was found to vary from 99.10% to 100.00% in the formulations while the average content of (*R*)- citalopram found as impurity varied from 0.02% to 0.45%. The enantiomeric purity (% EP) or enantiomeric excess (% ee) of sample was determined. The results are recorded in table 4.6.9 and all the chromatogram of samples are shown in figure 4.6.17. Bar diagram of % purity of *S*-citalopram is shown in figure 4.6.18.

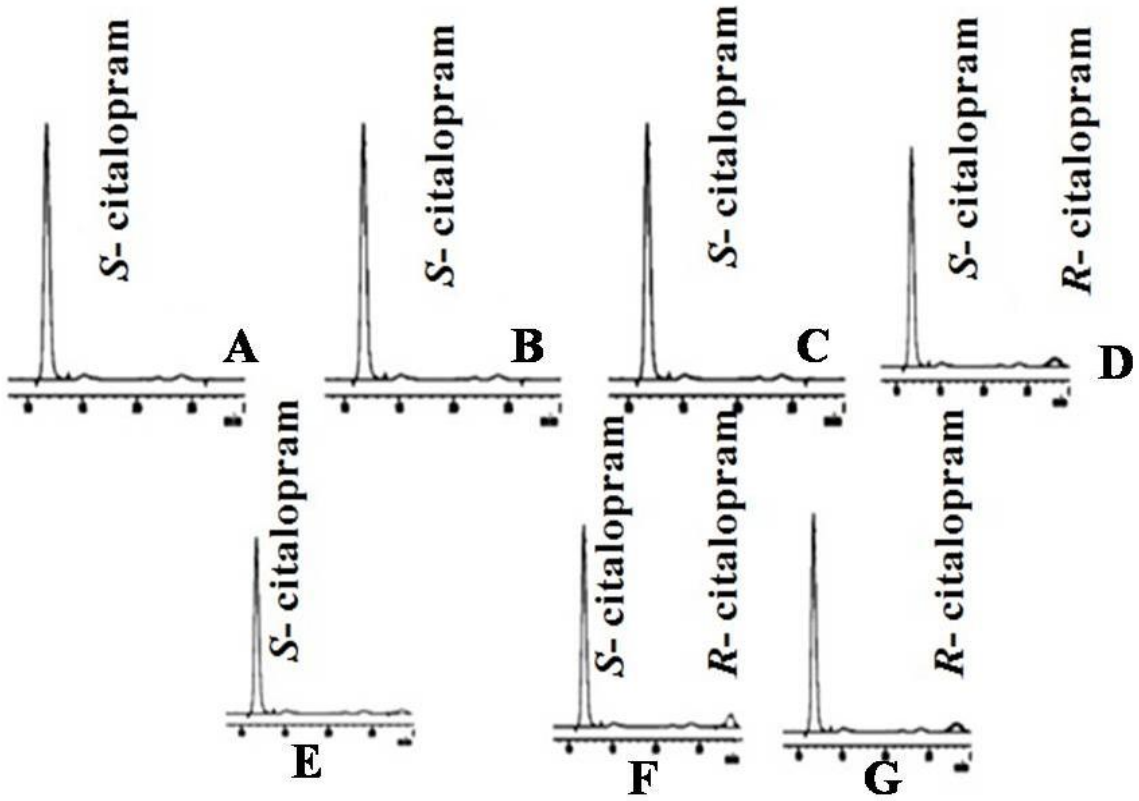


Figure 4.6.17: Chromatograms of escitalopram market preparations (A-G) used for testing.

Table 4.6.9: Enantiomeric purity of escitalopram market samples (n=10).

Identity of company	% Content of *S*-citalopram	% Content of *R*-citalopram in *S*-citalopram as impurity	%EP= (% of major enantiomer - % of minor enantiomer)
Company A	100.00%	0.00%	100.00%
Company B	100.00%	0.00%	100.00%
Company C	100.00%	0.00%	100.00%
Company D	99.70%	0.30%	99.40%
Company E	99.98%	0.02%	99.96%
Company F	99.55%	0.45%	99.10%
Company G	99.72%	0.28%	99.44%

EP= Enantiomeric purity, n = number of determinations

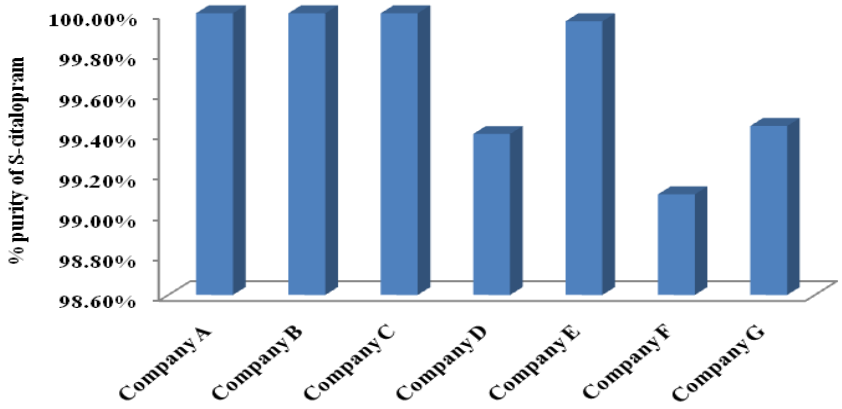


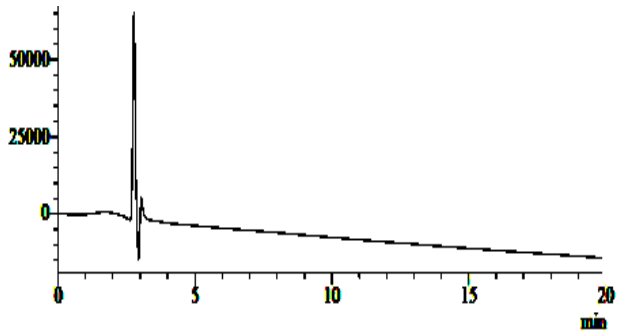
Figure 4.6.18: Bar diagram of % purity of *S*-citalopram.

4.7. Analysis of rabeprazole and pantoprazole

4.7.1. Analytical HPLC

Our first effort was to develop a simple and easy method for determination of racemic mixture of rabeprazole and pantoprazole. A number of mobile phases were initially tried through trial and error basis to elute rabeprazole and pantoprazole and to achieve individual separation of rabeprazole and pantoprazole enantiomers with good resulotion. For these separation, chiralpak IC column was used which is known to be applicable in both normal-phase and reversed-phase mode. Using different organic solvent or buffer ratio as mobile phase, a suitable method was developed.

Some trial chiral methods with chromatogram has been shown below with chiralpak IC:



Mobile phase:

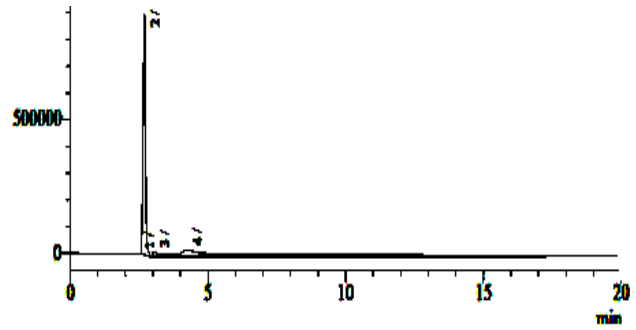
ACN: H2O (50: 50)

Flow rate: 0.7 mL/min

Detector: 230 nm

Result: No peak observed

Figure 4.7.1: Trial-1.



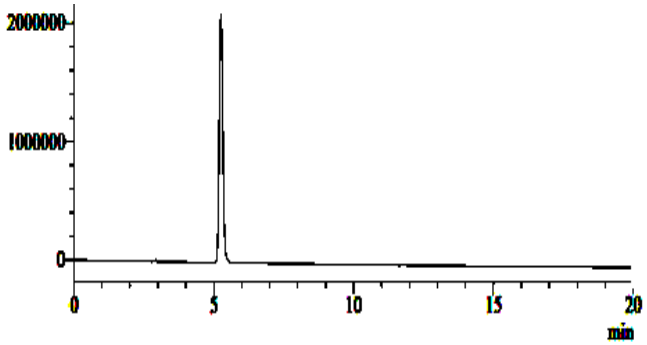
Mobile phase: MeOH: NH_4OAc (50: 50)

Flow rate: 0.7 mL/min

Detector: 230 nm

Result: No peak observed

Figure 4.7.2: Trial-2.



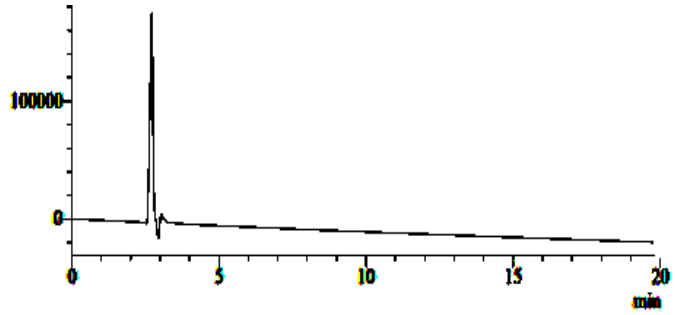
Mobile phase: MeOH: NH_4OAc (30: 70)

Flow rate: 0.7 mL/min

Detector: 230 nm

Result: No peak observed

Figure 4.7.3: Trial-3.



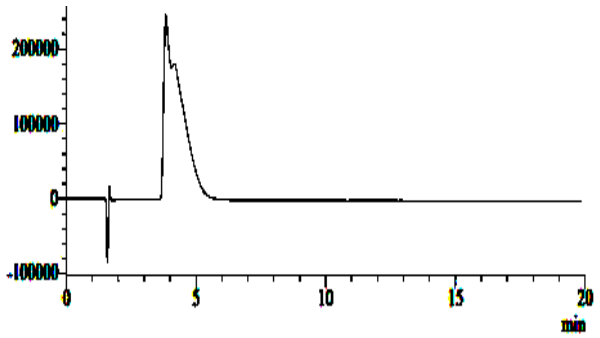
Mobile phase: MeOH:KH_2PO_4 (50: 50)

Flow rate: 0.7 mL/min

Detector: 230 nm

Result: No peak observed

Figure 4.7.4: Trial-4.



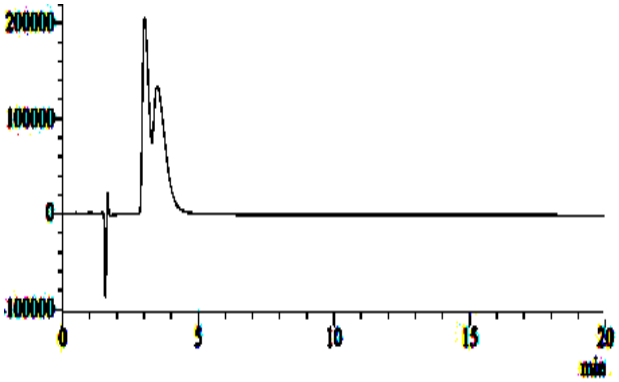
Mobile phase: ACN:KH_2PO_4 (60: 40)

Flow rate: 0.7 mL/min

Detector: 230 nm

Result: No identical peak observed

Figure 4.7.5: Trial-5.



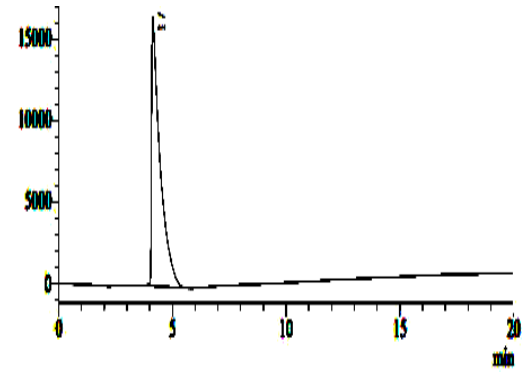
Mobile phase: ACN: NaH_2PO_4 (20: 80)

Flow rate: 0.7 mL/min

Detector: 230 nm

Result: No identical peak observed

Figure 4.7.6: Trial-6.



Mobile phase: ACN: NaOAc (50: 50)

Flow rate: 0.7 mL/min

Detector: 230 nm

Result: One peak observed

Figure 4.7.7: Trial-7.

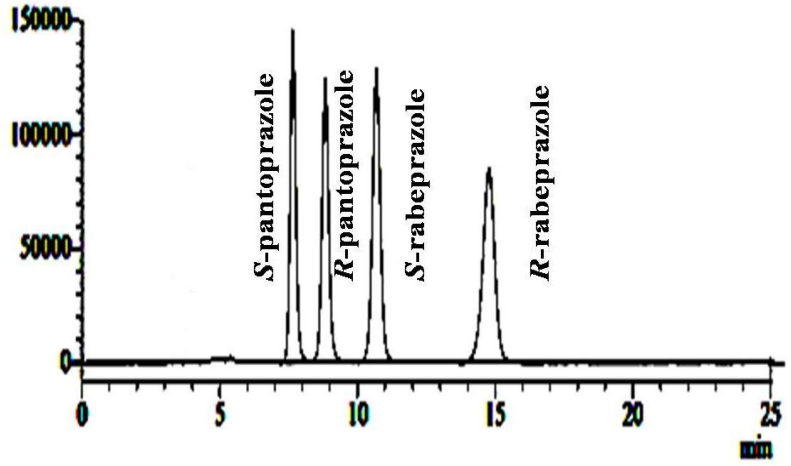
Summary of these trial chiral methods are shown in table 4.7.1.

Table 4.7.1: Trial chiral methods for rabeprazole and pantoprazole.

Mobile Phase	Retention time	Theoretical plates (≥2000)	Tailing factor (≤2)	Resolution (≥2)
ACN:H_2O (50: 50)	No peak observed	NA	NA	NA
MeOH:NH_4OAc (50: 50)	No peak observed	NA	NA	NA
MeOH:NH_4OAc (30: 70)	No peak observed	NA	NA	NA
MeOH:KH_2PO_4 (50: 50)	No peak observed	NA	NA	NA
ACN:KH_2PO_4 (60: 40)	No identical peak observed	NA	NA	NA
ACN:NaH_2PO_4 (20: 80)	No identical peak observed	NA	NA	NA
ACN:NaOAc (50: 50)	One peak observed	NA	NA	NA

NA = Not applicable

Table 4.7.1 demonstrates that the results of all parameters are not suilable. So all the above methods are avoided. After a number of trials with chiralpak IC column with mobile phases of different composition, the best mobile phase composition was then found to be *n*-Hexane/ ethanol at a ratio of 50:50 (v/v) for simultaneous determination of rabeprazole and pantoprazole, because of adequate resolution, efficient theoretical plates number and symmetric peak shape.



Mobile phase: Hx: EtOH (50:50)
Flow rate: 0.7 mL/min
Detector: 230 nm
Result: Four acceptable peaks observed

Figure 4.7.8: Chromatogram with proposed method.

Now, this proposed chromatographic method was compared with the previously published methods which was discussed in 4.7.2.

4.7.2. Comparison of the present work

A comparison of present work with the other earlier reports on enantioresolution of rabeprazole and pantoprazole are shown in table 4.7.2 and table 4.7.3. It is clear that the present method is suitable in terms of resolution.

Table 4.7.2: Comparison of HPLC enantio-resolution of rabeprazole using different column with the present work.

Chiral column/ selector	Mobile phase	Resolution (Rs>2)	Reference
EDABV	*n*-Hexane: ethanol (80:20)	4.29	(Lourenco *et al.*, 2010)
Chiralpak AS	Heptane: isopropanol: triethylamine (60:40:0.1)	2.70	(Andersson *et al.*, 2007)
Chiralpak AD-H	*n*-Hexane: ethanol: isopropyl alcohol (75: 15: 10)	3.24	(Nageswara *et al.*, 2006)
Chiralpak IC	*n*-Hexane: ethanol: diethylamine (50:50:0.1)	Not given	(Chennuru *et al.*, 2013)
Chiralpak IC	*n*-Hexane: ethanol: diethylamine (30:70:0.05)	6.08	(Kim *et al.*, 2017)
Chiralpak IC	*n*-Hexane: ethanol (50:50)	5.91	Present work

Table 4.7.3: Comparison of HPLC enantio-resolution of pantoprazole using different column with the present work.

Chiral column/ selector	Mobile phase	Resolution (R>2)	Reference
Chiral AGP	10 mM ammonium acetate (pH 5.5): acetonitrile (93: 07)	1.77	(Zhiyong *et al*., 2005)
SBE-β-CD	Phosphate buffer	2.10	(Hancu *et al*., 2015)
β-CD	Phosphate buffer (pH 7.0)	2.50	(Hancu *et al*., 2015)
Chiralpak AD-H	*n*-Hexane: ethanol: trifluoroacetic acid (80:20: 0.1)	3.0	(Jadhav, 2015)
Chiralpak IC	*n*-Hexane: ethanol (50:50)	2.57	Present work

4.7.3. Validation of the proposed method

Finally, this present method has been validated with respect to the following parameters.

4.7.3.1. Linearity and range

Linearity of the method was studied by injecting five concentrations of two enantiomers of rabeprazole and pantoprazole prepared in the mobile phase in concentration ranging from 40 – 140 µg/mL in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves. The linearity curves are shown in figure 4.7.9 and figure 4.7.10 and the parameters are given in table 4.7.4 and table 4.7.5, respectively.

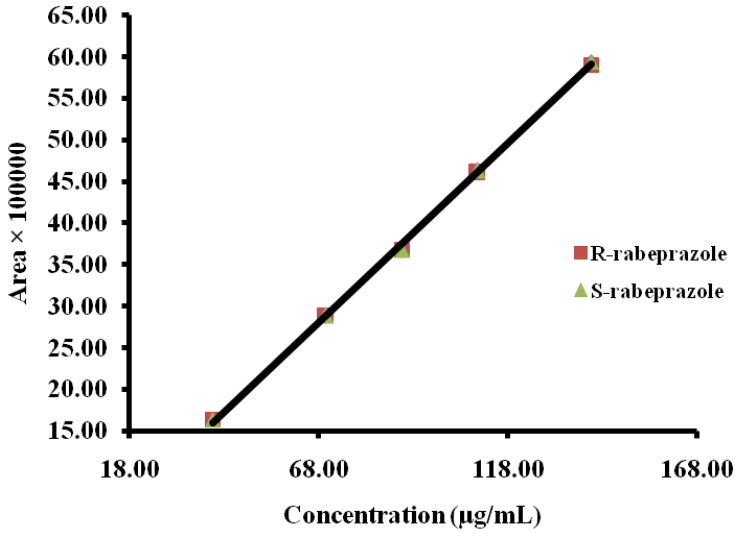


Figure 4.7.9: Linearity curve for (*S*)- and (*R*)-rabeprazole.

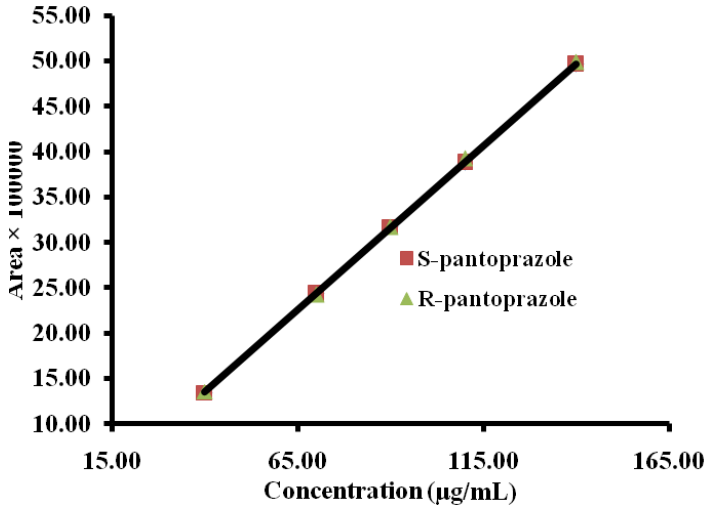


Figure 4.7.10: Linearity curve for (*S*)- and (*R*)-pantoprazole.

4.7.3.2. Precision

The precision of the method was verified by intra- and inter-day precision studies. Intra-day precision was performed by analysis of concentration for six times on the same day. The intermediate precision of the method was checked by studying on three different days. Results are recorded in table 4.7.4. Bar diagram of precision (% RSD) for (*S*)- and (*R*)-rabeprazole and (*S*)- and (*R*)-pantoprazole are shown in figure 4.7.11 and figure 4.7.12, respectively.

Table 4.7.4: Results of method validation parameters for rabeprazole.

Parameters	***S*-rabeprazole**	***R*-rabeprazole**
Linear equation	y= 86129x-12180	y= 85337x-93650
Coefficient of determination (r2>0.995)	0.999	0.999
Linearity range	40-140 µg/mL	
Precision (intra-day, n=6) (% RSD≤2)	0.0.05%	0.14%
Precision (inter-day, n=6) (% RSD≤2)	0.03%	0.06%

n = number of determinations

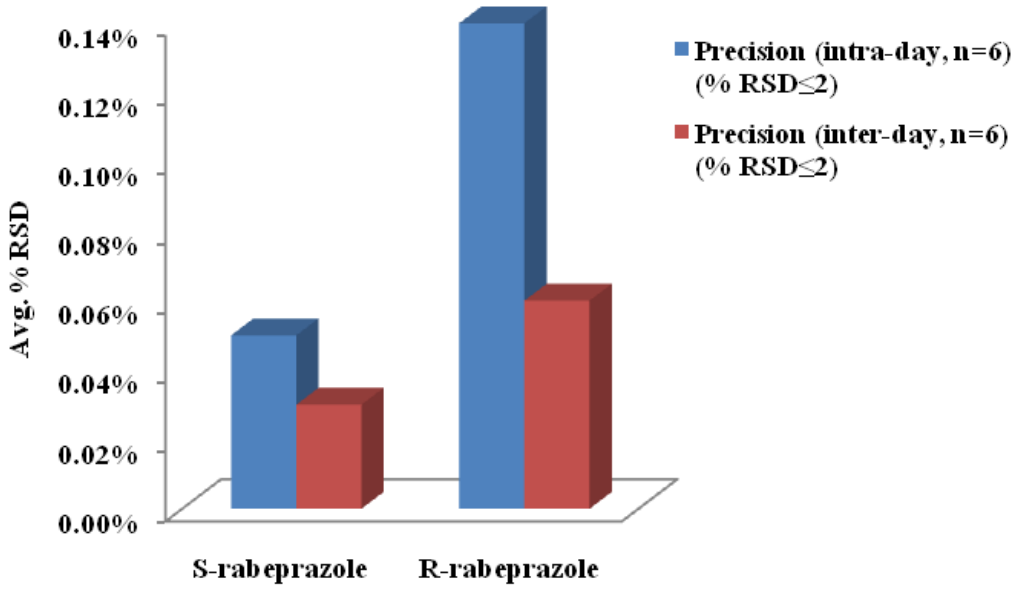


Figure 4.7.11: Bar diagram of precision (% RSD) for (*S*)- and (*R*)-rabeprazole.

Table 4.7.5: Results of method validation parameters for pantoprazole.

Parameters	*S*-pantoprazole	*R*-pantoprazole
Linear equation	y= 72260x-94800	y=72913x-10968
Coefficient of determination (r^2>0.995)	0.999	0.999
Linearity range	40-140 µg/mL	
Precision (intra-day, n=6) (% RSD≤2)	0.24%	0.19%
Precision (inter-day, n=6) (% RSD≤2)	0.31%	0.21%

n = number of determinations

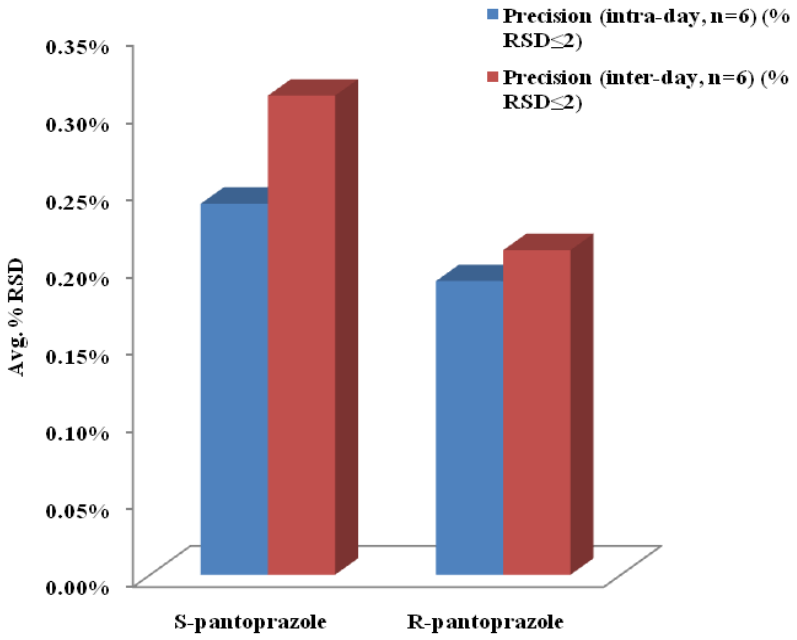


Figure 4.7.12: Bar diagram of precision (% RSD) for (*S*)- and (*R*)-pantoprazole

4.7.3.3. Accuracy

Accuracy of the method was verified by studying recovery experiments which were performed by spiking solutions of known amount of the drug with pre-analyzed sample. To evaluate the accuracy of the method, successive analysis (n=3) of standard solutions of the drug was carried out and the results are given in table 4.7.6 and table 4.7.7. Bar diagram of accuracy (% recovery) for (*S*)- and (*R*)-rabeprazole and (*S*)- and (*R*)-pantoprazole are shown in figure 4.7.13 and figure 4.7.14, respectively.

Table 4.7.6: Results of accuracy study for standard rabeprazole.

Parameters	*S*-rabeprazole	*R*-rabeprazole
Accuracy (n=3) (avg. % recovery)		
Standard+spike (µg/mL)		
(20+10)	100.25%	101.85%
(30+10)	100.81%	100.05%
(40+10)	100.07%	101.72%
LOD (µg/mL)	1.83	1.79
LOQ (µg/mL)	5.54	5.43

n = number of determinations

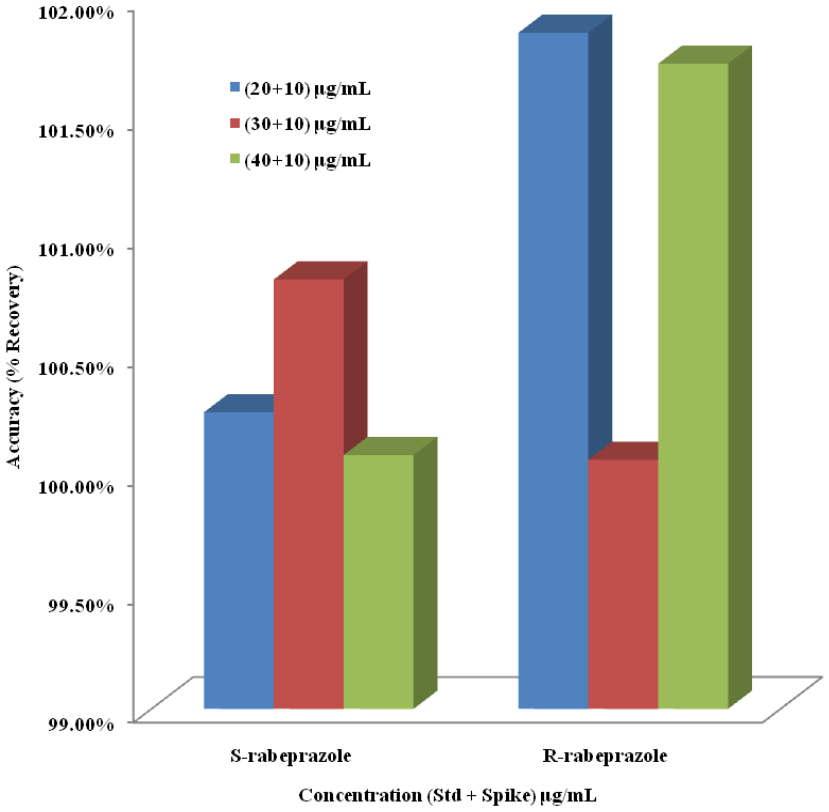


Figure 4.7.13: Bar diagram of accuracy (% recovery) for (*S*)- and (*R*)-rabeprazole.

Table 4.7.7: Results of accuracy study for standard pantoprazole.

Parameters	*S*-pantoprazole	*R*-pantoprazole
Accuracy (n=3) (avg. % recovery)		
Standard+spike (µg/mL)		
(20+10)	101.59%	101.75%
(30+10)	98.86%	100.10%
(40+10)	100.14%	99.95%
LOD (µg/mL)	1.29	1.06
LOQ (µg/mL)	3.92	3.20

n = number of determinations

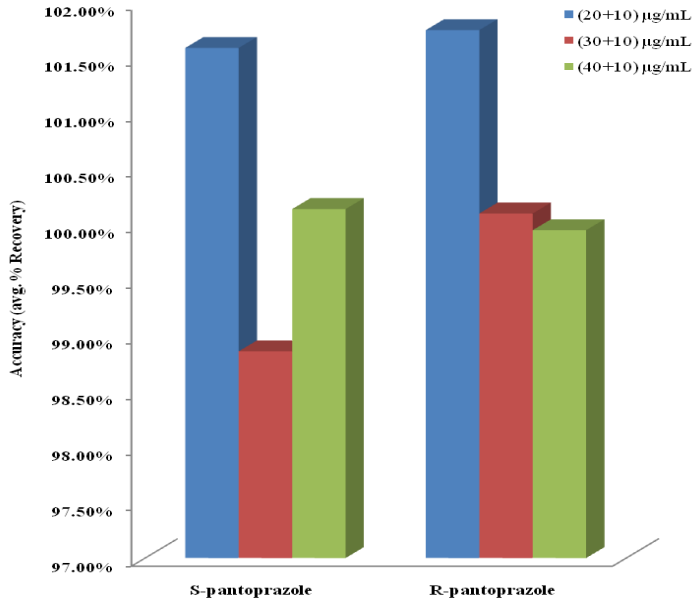


Figure 4.7.14: Bar diagram of accuracy (% recovery) for (*S*)- and (*R*)-pantoprazole.

4.7.3.4. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated using the following equations:

LOD= (SD / slope) x 3.3

LOQ= (SD / slope) x 10.

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of

regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). The results of LOD and LOQ obtained for studied drugs have been recorded in table 4.7.7.

4.7.3.5. Specificity

The specificity of the method was assessed from the chromatogram where complete separation of rabeprazole, pantoprazole and their single enantiomers *S*-rabeprazole and *S*-pantoprazole were achieved without any interference. The peaks obtained were sharp, well separated at the baseline as shown in figure 4.7.15 and figure 4.7.16, respectively. Single enantiomers, *S*-rabeprazole and *S*-pantoprazole were injected into HPLC for the detection of (*S*)- enantiomer in rabeprazole and pantoprazole. Simultaneous determination of pantoprazole and rabeprazole has been shown in figure 4.7.17.

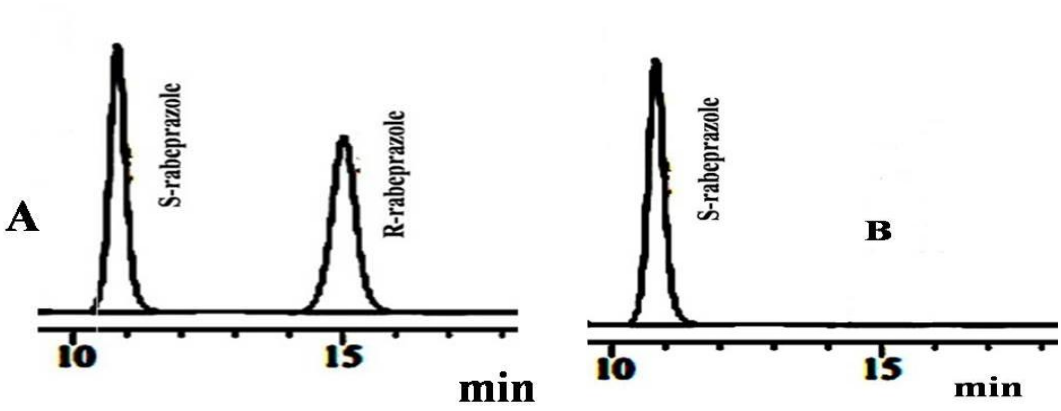


Figure 4.7.15: HPLC chromatograms of rabeprazole (A) and *S*-rabeprazole (B)

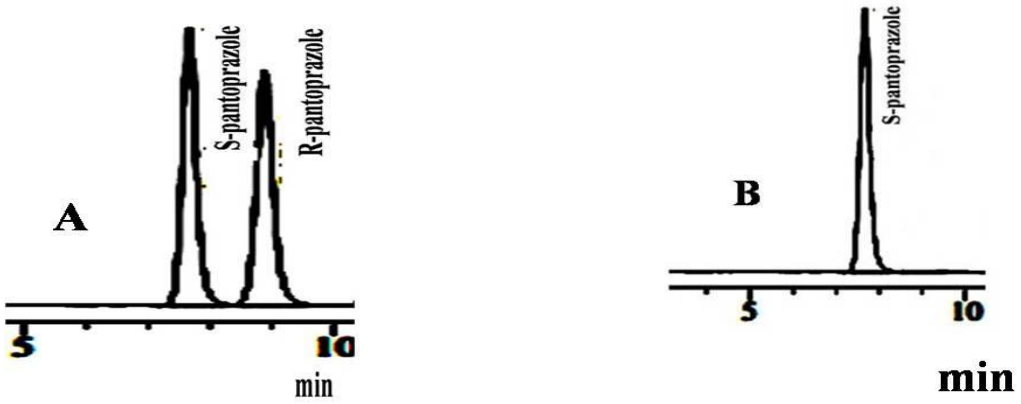


Figure 4.7.16: HPLC chromatograms of pantoprazole (A) and *S*-pantoprazole (B).

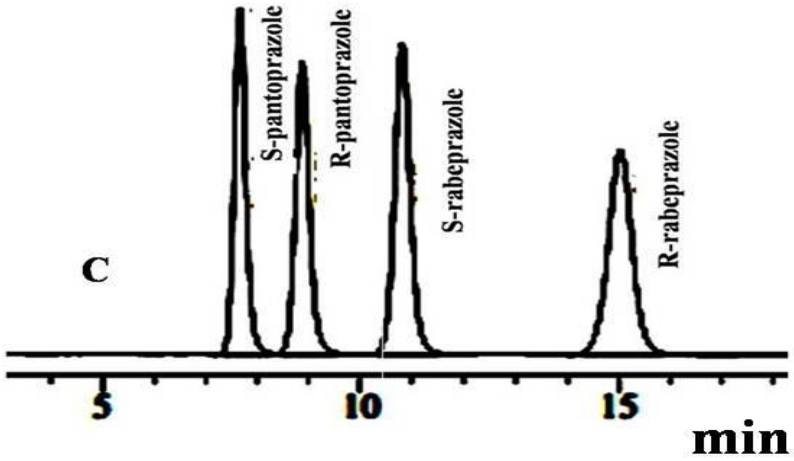


Figure 4.7.17: Simultaneous determination of pantoprazole and rabeprazole (C).

4.7.3.6. Parameters for system suitability testing

System suitability parameters are reported in table 4.7.8 and table 4.7.9. The chromatogram of system suitability is shown in figure 4.7.18.

Table 4.7.8: Parameters for system suitability testing for rabeprazole.

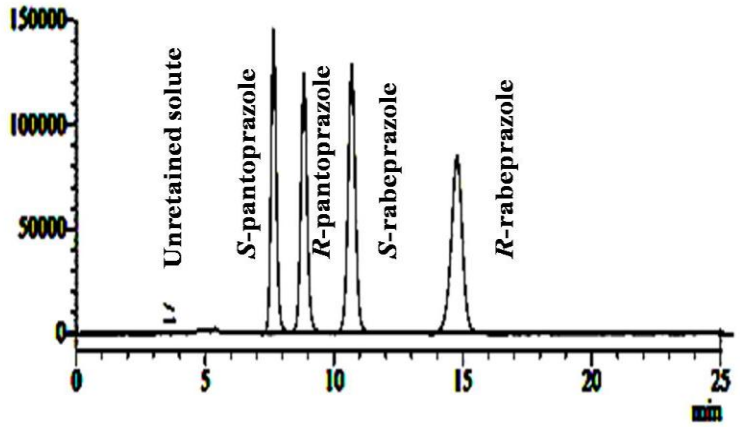
Parameters	*S*-rabeprazole	*R*-rabeprazole
Theoretical plates (≥2000) (n=5)	5707	4911
Tailing factor (≤2) (n=5)	1.11	1.02
Relative retention (k_S and k_R) (n=5), 0.5 < k < 10	2.39	3.69
Selectivity (α>1) (n=5)	1.54	
Resolution (≥2) (n=5)	5.91	

n = number of determinations

Table 4.7.9: Parameters for system suitability testing for pantoprazole.

Parameters	*S*-pantoprazole	*R*-pantoprazole
Theoretical plates (≥2000) (n=5)	4986	4890
Tailing factor (≤2) (n=5)	1.18	1.14
Relative retention (k*S* and k*R*) (n=5), 0.5 < k < 10	1.43	1.80
Selectivity (α>1) (n=5)	1.25	
Resolution (≥2) (n=5)	2.57	

n = number of determinations



For pantoprazole,

t_0 =3.14 min,

t_S = 7.65 min,

t_R = 8.83 min.

For rabeprazole,

t_0 = 3.14 min,

t_S = 10.68 min,

t_R = 14.77 min.

Figure 4.7.18: HPLC chromatograms of pantoprazole and rabeprazole for system suitability.

4.7.3.7. Parameters for solution stability testing

A standard solution of concentration of 60 µg/mL was kept in a tightly capped volumetric flask at room temperature (25 °C) on the laboratory bench and at 4 °C in a refrigerator for 3 days and its stability was tested. The results are given in table 4.7.10 and table 4.7.11. Bar diagram of solution stability (% RSD) for (*S*)- and (*R*)-rabeprazole and (*S*)- and (*R*)-pantoprazole are shown in figure 4.7.19 and figure 4.7.20, respectively.

Table 4.7.10: Solution stability parameters for rabeprazole.

Day	At 25 °C		At 4 °C	
	***S*-rabeprazole (n=3) (% RSD)**	***R*-rabeprazole (n=3) (% RSD)**	***S*-rabeprazole (n=3) (% RSD)**	***R*-rabeprazole (n=3) (% RSD)**
Day 1	0.01%	0.01%	0.01%	0.01%
Day 2	0.00%	0.01%	0.01%	0.01%
Day 3	0.01%	0.03%	0.02%	0.00%
Avg.	0.01%	0.02%	0.01%	0.01%

n = number of determinations

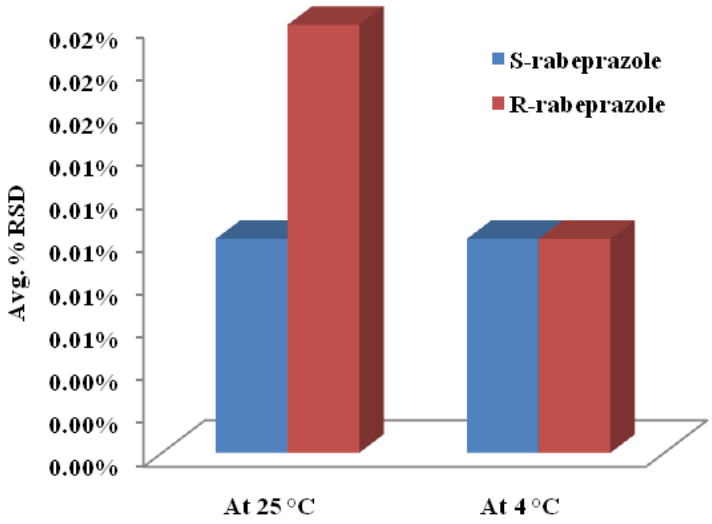


Figure 4.7.19: Bar diagram of solution stability testing (% RSD) for (*S*)- and (*R*)-rabeprazole.

Table 4.7.11: Solution stability parameters for pantoprazole.

Day	At 25 °C		At 4 °C	
	***S*-pantoprazole (n=3) (% RSD)**	***R*-pantoprazole (n=3) (% RSD)**	***S*-pantoprazole (n=3) (% RSD)**	***R*-pantoprazole (n=3) (% RSD)**
Day 1	0.07%	0.13%	0.15%	0.14%
Day 2	0.04%	0.14%	0.10%	0.14%
Day 3	0.09%	0.13%	0.06%	0.15%
Avg.	0.07%	0.13%	0.10%	0.14%

n = number of determinations

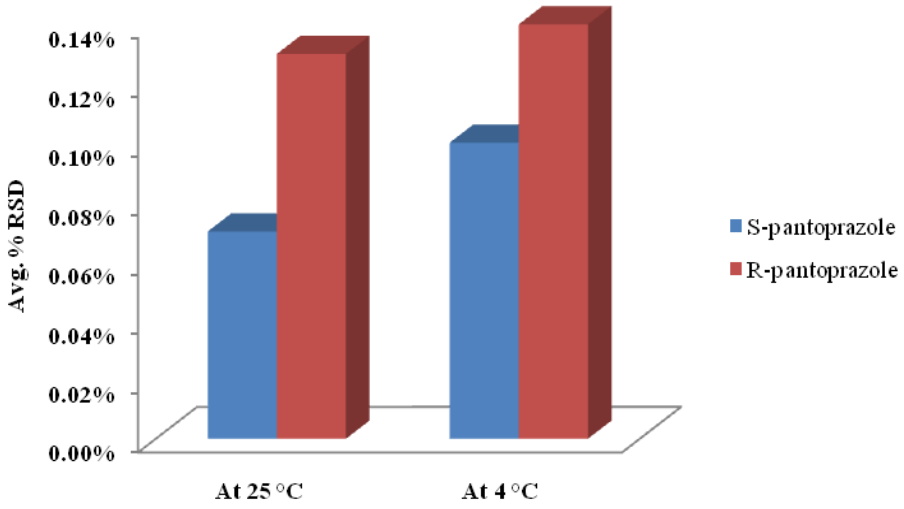


Figure 4.7.20: Bar diagram of solution stability (% RSD) for (*S*)- and (*R*)-pantoprazole.

4.7.3.8. Robustness testing of the method

To determine the robustness of this method, the experimental conditions were deliberately changed, like the flow rate and in the wavelength of detection and measuring the % RSD. For rabeprazole and pantoprazole study, factors chosen were flow rate (0.7 ± 0.2mL/min) and wavelength (230 ± 2nm), and n = 3. % RSD is reported in table 4.7.12 and table 4.7.13. All analyte peaks were adequately resolved and elution orders remain unchanged.

Bar diagram of robustness (% RSD) for (*S*)- and (*R*)-rabeprazole and (*S*)- and (*R*)-pantoprazole are shown in figure 4.7.21 and figure 4.7.22, respectively.

Table 4.7.12: Robustness parameters for rabeprazole.

Parameters (n=3)	***S*-rabeprazole (n=3) (% RSD)**	***R*-rabeprazole (n=3) (% RSD)**
Detection wavelength at 232nm	0.08%	0.06%
Detection wavelength at 228nm	0.10%	0.09%
Flow rate 0.5 mL/min	0.04%	0.03%
Flow rate 0.9 mL/min	0.07%	0.05%

n = number of determinations

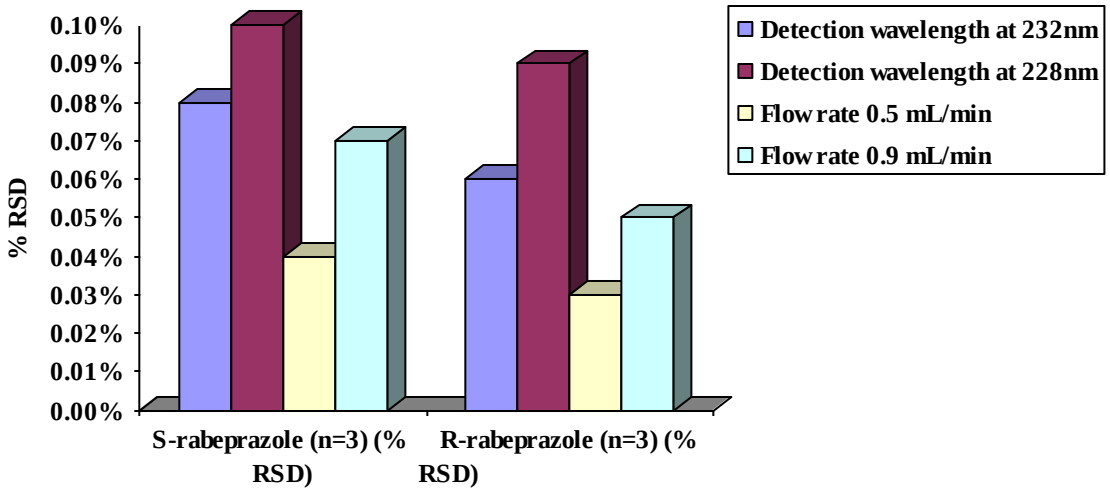


Figure 4.7.21: Bar diagram of robustness (% RSD) for (*S*)- and (*R*)-rabeprazole.

Table 4.7.13: Robustness parameters for pantoprazole.

Parameters (n=3)	*S*-pantoprazole (n=3) (% RSD)	*R*-pantoprazole (n=3) (% RSD)
Detection wavelength at 232 nm	0.02%	0.15%
Detection wavelength at 228 nm	0.06%	0.13%
Flow rate 0.5 mL/min	0.05%	0.05%
Flow rate 0.9 mL/min	0.10%	0.08%

n = number of determinations

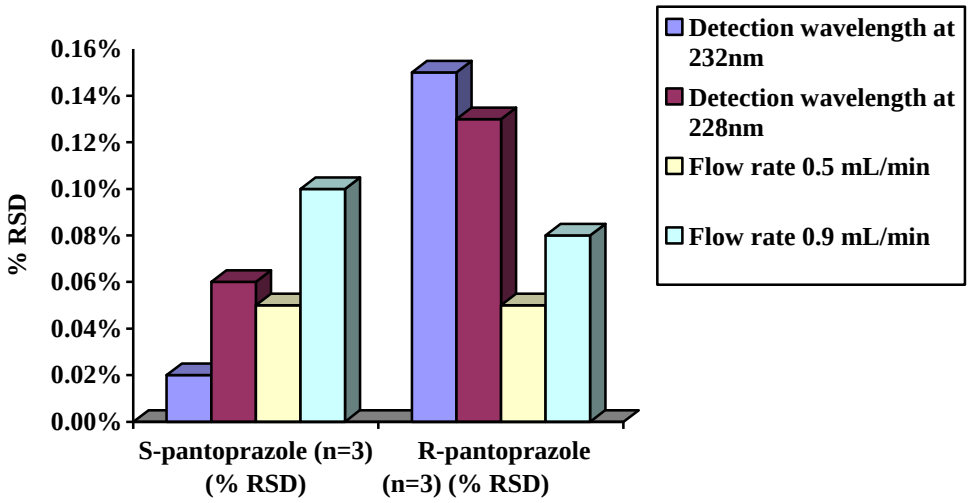


Figure 4.7.22: Bar diagram of robustness (% RSD) for (*S*)- and (*R*)-pantoprazole.

4.7.4. Application of the developed method

4.7.4.1. Rabeprazole

This analytical method was applied to quantitate the content of *S*- and *R*-rabeprazole samples from sixteen companies of Bangladesh and as well as to calculate the ratio of them. The average content of *S*-rabeprazole were from 49.13% to 53.11% while the content of *R*-rabeprazole were from 48.00% to 50.87% in the formulations of racemic mixture of rabeprazole. Enantiomeric ratio of commercial samples is shown in table 4.7.14. Enantiomeric ratio of rabeprazole for all

samples from different companies' met the USP requirements. The chromatogram of one sample and bar diagram of enantiomeric ratio of racemic rabeprazole are shown in figure 4.7.23 and 4.7.24, respectively.

4.7.4.2. Pantoprazole

This analytical method was also applied to quantitate the content of *S*- and *R*- pantoprazole samples from twenty companies of Bangladesh and as well as to calculate the ratio of them. The average content of *S*- pantoprazole was from 49.80% to 51.37% while the content of *R*-pantoprazole was from 48.62% to 50.42% in the formulations of racemic mixture of pantoprazole. Enantiomeric ratio of commercial samples showed in table 4.7.15. Enantiomeric ratio of pantoprazole for all samples from different companies met the USP requirements. The chromatogram of one sample and bar diagram of enantiomeric ratio of racemic pantoprazole are shown in figure 4.7.23 and 4.7.25, respectively.

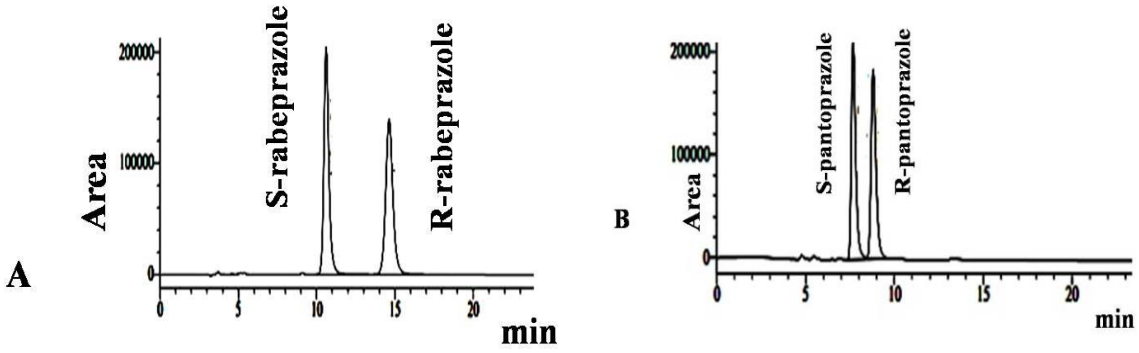


Figure 4.7.23: Chromatograms of one commercial sample of rabeprazole (A) and pantoprazole (B).

Table 4.7.14: Enantiomeric ratio of racemic rabeprazole in commercial samples (n=10).

Identity of company	% of S-rabeprazole	% EP = (50% - % enantiomer) of *S*-rabeprazole	% of R-rabeprazole	% EP = (50% - % enantiomer) of *R*-rabeprazole	Enantiomeric ratio of rabeprazole
Company A	50.20%	-0.20%	49.79%	0.21%	50.20%/49.79%
Company B	50.31%	-0.31%	49.68%	0.32%	50.31%/49.68%
Company C	50.31%	-0.31%	49.69%	0.31%	50.31%/49.69%
Company D	50.44%	-0.44%	49.55%	0.45%	50.44%/49.55%
Company E	50.42%	-0.42%	49.57%	0.43%	50.42%/49.57%
Company F	50.18%	-0.18%	49.81%	0.19%	50.18%/49.81%
Company G	50.17%	-0.17%	49.82%	0.18%	50.17%/49.82%
Company H	50.70%	-0.70%	49.29%	0.71%	50.70%/49.29%
Company I	50.70%	-0.70%	49.29%	0.71%	50.70%/49.29%
Company J	50.70%	-0.70%	49.29%	0.71%	50.70%/49.29%
Company K	49.58%	0.42%	50.41%	-0.41%	49.58%/50.41%
Company L	52.10%	-2.10%	48.00%	2.00%	52.10%/48.00%
Company M	51.99%	-1.99%	48.11%	1.89%	51.99%/48.11%
Company N	50.88%	-0.88%	49.12%	0.88%	50.88%/49.12%
Company O	53.11%	-3.11%	46.89%	3.11%	53.11%/46.89%
Company P	49.13%	0.86%	50.87%	-0.86%	49.13%/50.87%

EP= Enantiomeric purity, n = number of determinations

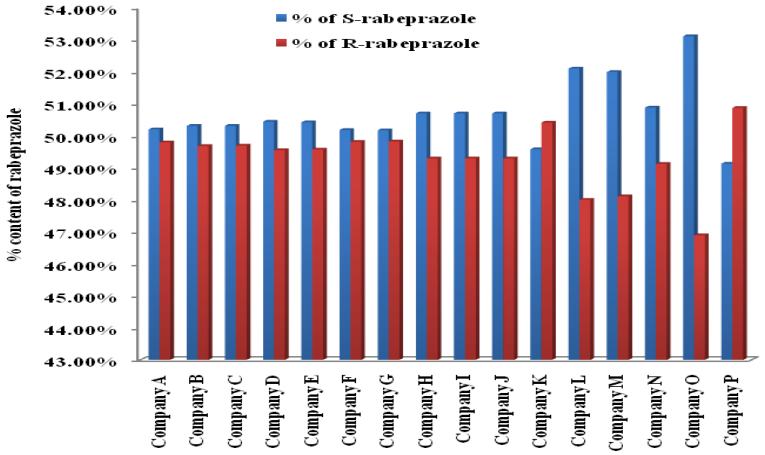


Figure 4.7.24: Bar diagram of enantiomeric ratio of racemic rabeprazole.

Table 4.7.15: Enantiomeric ratio of racemic pantoprazole in commercial samples (n=10).

Identity of company	% of S-pantoprazole	% EP = (50% - % enantiomer) of *S*-pantoprazole	% of R-pantoprazole	% EP = (50% - % enantiomer) of *R*-pantoprazole	Enantiomeric ratio of pantoprazole
Company A	51.37%	-1.37%	48.62%	1.38%	51.37%/48.62%
Company B	50.13%	-0.13%	49.86%	0.14%	50.13%/49.86%
Company C	50.38%	-0.38%	49.61%	0.39%	50.38%/49.61%
Company D	50.11%	-0.11%	49.89%	0.11%	50.11%/49.89%
Company E	49.80%	0.20%	50.19%	-0.19%	49.80%/50.19%
Company F	49.86%	0.14%	50.14%	-0.14%	49.86%/50.14%
Company G	50.26%	-0.26%	49.74%	0.26%	50.26%/49.74%
Company H	51.16%	-1.16%	48.83%	1.17%	51.16%/48.83%
Company I	49.86%	0.14%	50.13%	-0.13%	49.86%/50.13%
Company J	50.37%	-0.37%	49.62%	0.38%	50.37%/49.62%
Company K	50.98%	-0.98%	49.01%	0.99%	50.98%/49.01%
Company L	51.04%	-1.04%	48.95%	1.05%	51.04%/48.95%
Company M	51.17%	-1.17%	48.82%	1.18%	51.17%/48.82%
Company N	50.29%	-0.29%	49.70%	0.30%	50.29%/49.70%
Company O	50.37%	-0.37%	49.63%	0.37%	50.37%/49.63%
Company P	50.30%	0.86%	49.69%	-0.86%	50.30%/49.69%
Company Q	50.19%	-0.19%	49.80%	0.20%	50.19%/49.80%
Company R	49.57%	0.43%	50.42%	-0.42%	49.57%/50.42%
Company S	50.36%	-0.36%	49.63%	0.37%	50.36%/49.63%
Company T	50.57%	-0.57%	49.42%	0.58%	50.57%/49.42%

EP= Enantiomeric purity, n = number of determinations

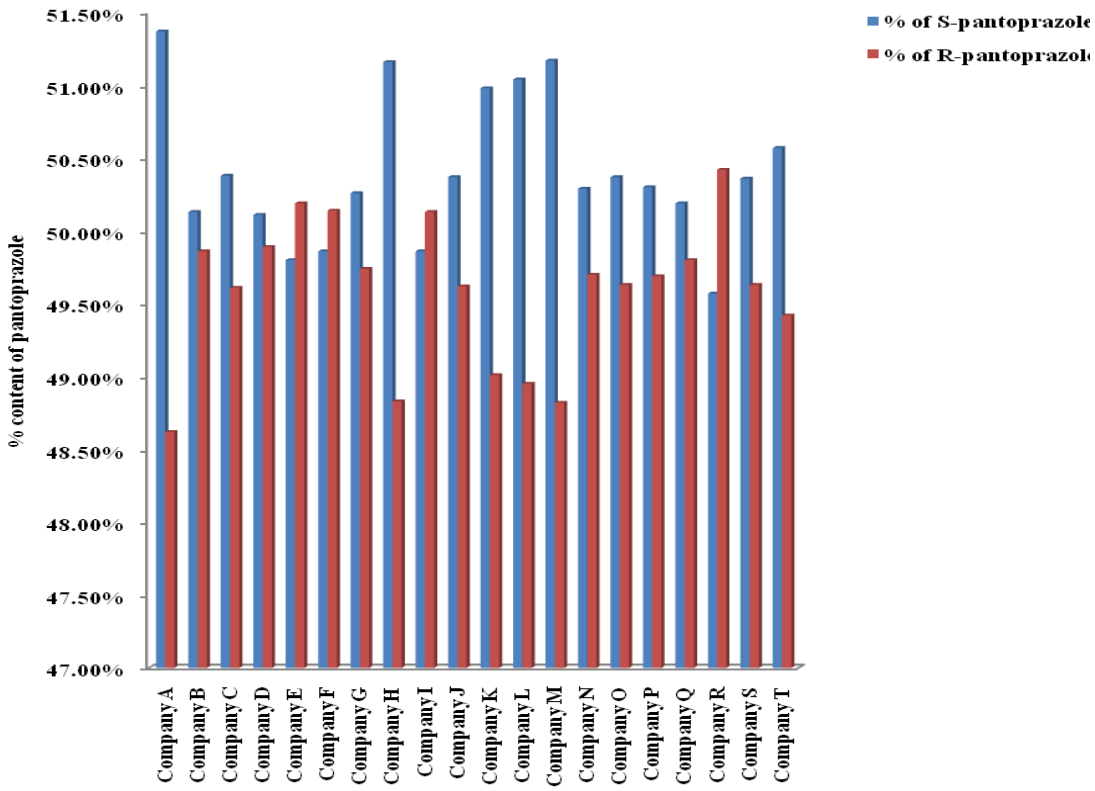


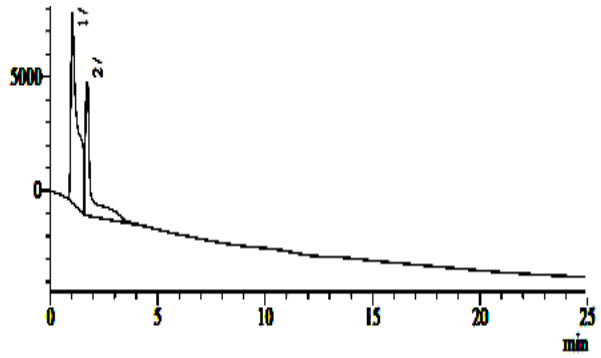
Figure 4.7.25: Bar diagram of enantiomeric ratio of racemic pantoprazole.

4.8. Analysis of ibuprofen

4.8.1. Analytical HPLC

For developing a new and easy chiral HPLC method we tried a significant number of methods on trial and error basis using a large number of polar and non-polar solvent mixtures as mobile phase with chiral column. For ibuprofen separation, lux-cellulose-3 is generally used which is applicable in both of the normal-phase and reversed-phase mode. Here, it was used both in reverse-phase mode and normal-phase mode. Using different organic solvent or buffer ratio as mobile phase, a suitable method was developed.

Some trial chiral methods with chromatograms are shown below with lux-cellulose-3 on reverse phase mode:



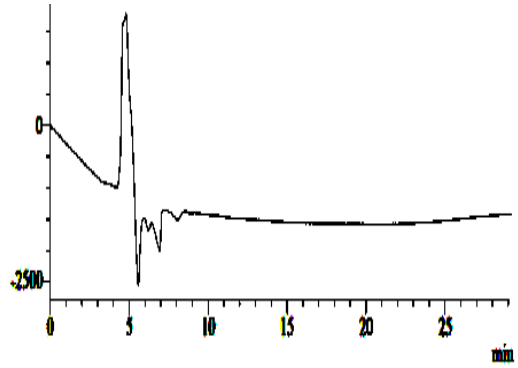
Mobile phase: Acetonitrile: H_2O (50:50)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: No peak observed

Figure 4.8.1: Trial-1.



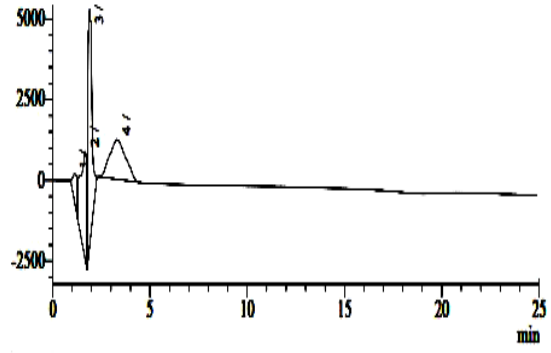
Mobile phase: ACN: NH_4OAc (50:50)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: No peak observed

Figure 4.8.2: Trial-2.



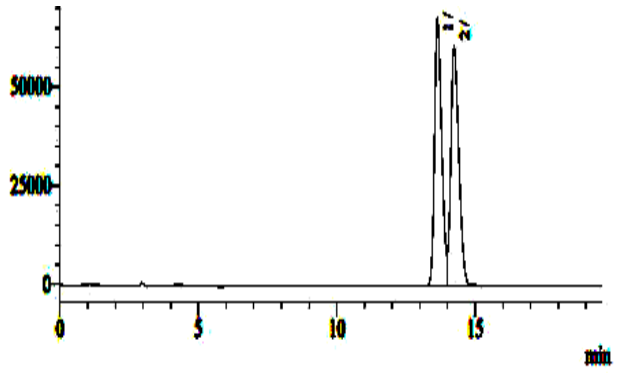
Mobile phase: ACN: NH_4OAc (20:80)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: No identical peak observed

Figure 4.8.3: Trial-3.



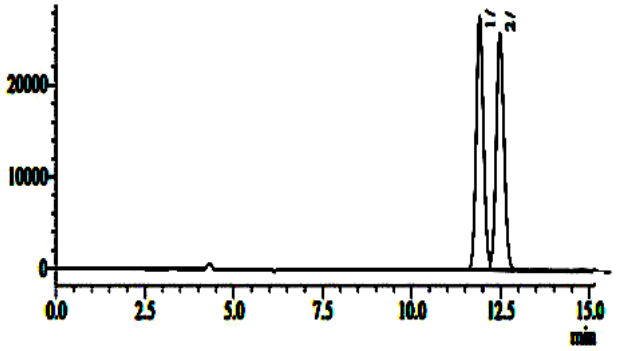
Mobile phase: ACN: $NH_4H_2PO_4$ (40:60)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.8.4: Trial-4.



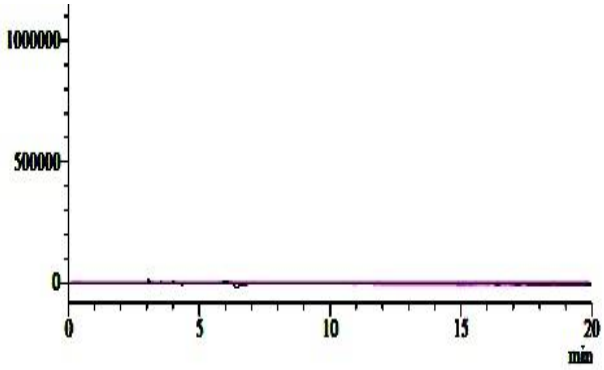
Mobile phase: ACN: $NH_4H_2PO_4$ (70:30)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.8.5: Trial-5.



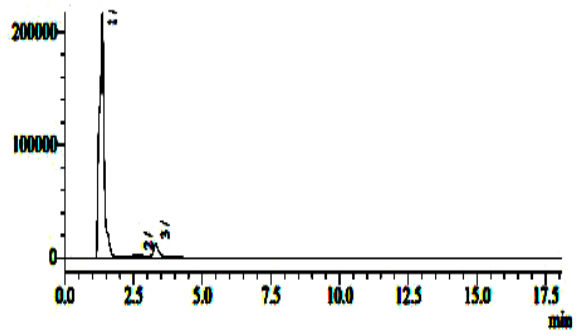
Mobile phase: ACN: $NH_4H_2PO_4$ (30:70)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: No peak observed

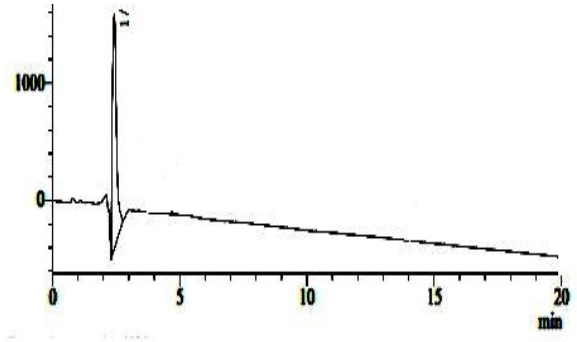
Figure 4.8.6: Trial-6.



Mobile phase: ACN: NaOAc (50:50)
Flow rate:1.0 mL/min
Detector: 254 nm
Result: No identical peak observed

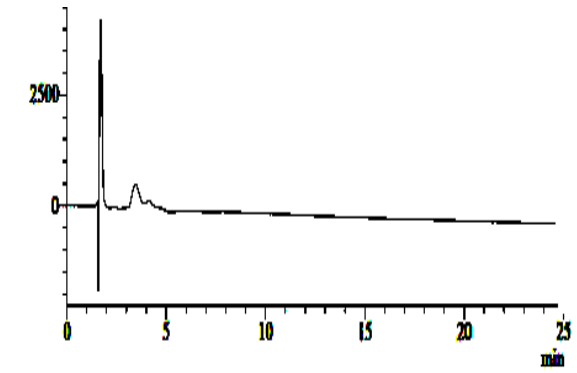
Figure 4.8.7: Trial-7.

Some trial chiral methods with chromatogram are shown below with lux-cellulose-3 on normal phase mode:



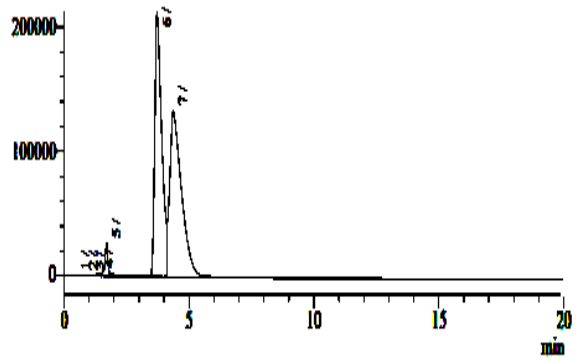
Mobile phase: Hx: IPA (50: 50)
Flow rate: 1.0 mL/min
Detector: 254 nm
Result: No peak observed

Figure 4.8.8: Trial-8.



Mobile phase: Hx: IPA: TEA: AA (50: 50: 0.1: 0.1)
Flow rate: 1.0 mL/min
Detector: 254 nm
Result: No peak observed

Figure 4.8.9: Trial-9



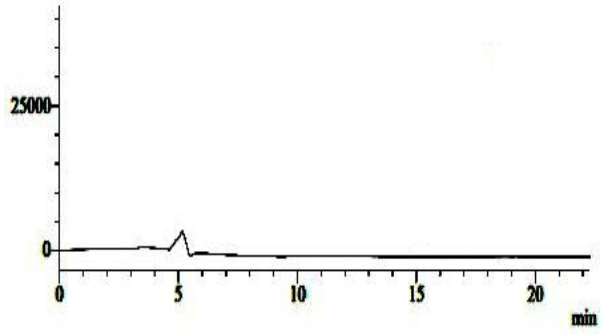
Mobile phase: Hx: IPA: AA (80: 20: 0.1)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: Two peaks observed

Figure 4.8.10: Trial-10.



Mobile phase: Hx: IPA: TEA (50: 50: 0.1)

Flow rate: 1.0 mL/min

Detector: 254 nm

Result: No peak observed

Figure 4.8.11: Trial-11

Summary of these trial chiral methods are shown in table 4.8.1.

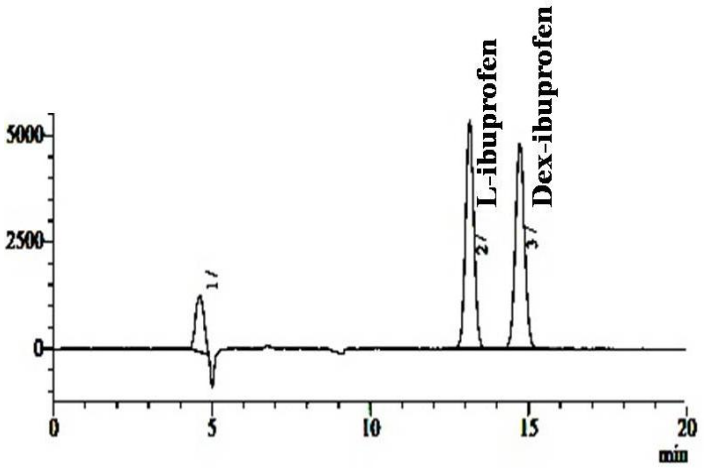
Table 4.8.1: Trial chiral methods for ibuprofen.

Polar Mobile Phase Buffer:Organic phase/Others	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
ACN: H_2O (50:50)	No peak observed	NA	NA	NA
ACN: NH_4OAc (50:50)	No peak observed	NA	NA	NA
ACN: NH_4OAc (20:80)	No identical peak observed	NA	NA	NA
ACN: $NH_4H_2PO_4$ (40:60)	Two peaks observed	NA	NA	1.13
ACN: $NH_4H_2PO_4$ (70:30)	Two peaks observed	NA	NA	1.38

ACN: $NH_4H_2PO_4$ (30:70)	No peak observed	NA	NA	NA
ACN: NaOAc (50:50)	No peak observed	NA	NA	NA
Non-polar Mobile phase	**Retention time**	**Theoretical plates (≥2000)**	**Tailing factor (≤2)**	**Resolution (≥2)**
Hx: IPA (50: 50)	No peak observed	NA	NA	NA
Hx: IPA: TEA: AA (50: 50: 0.1: 0.1)	No peak observed	NA	NA	NA
Hx: IPA: AA (80: 20: 0.1)	Two peaks observed	748 364	NA	0.89
Hx: IPA: TEA (50: 50: 0.1)	No peak observed	NA	NA	NA

NA= Not applicable

Table 4.8.1 demonstrates that all the results of all parameters are not suilable. So all the above methods were avoided. After a number of trials with lux Cellulose-3 column with mobile phases of different composition, the best mobile phase composition was then found to be acetonitrile / 0.1% acetic acid at a ratio of 50:50 (v/v), because of adequate resolution, efficient theoretical plates number and symmetric peak shape.



Mobile phase: ACN:0.1% AA (50:50)
Flow rate: 0.7 mL/min
Detector: 254 nm
Result: Two acceptable peaks observed

Figure 4.8.12: Chromatogram with acetonitrile: 0.1% AA (50:50) using lux Cellulose-3

Now, this proposed chromatographic method was compared with the previously published methods which is discussed in 4.8.2.

4.8.2. Comparison of the present work

A comparison of present work with the other earlier reports on enantioresolution of ibuprofen using different chiral selectors is shown in table 4.8.2. It is noteworthy that resolution 3.03 was achieved using cellulose based CSP. It clearly establishes the novelty and superiority of the present report in terms of resolution.

Table 4.8.2: Comparison of HPLC enantioresolution of ibuprofen using different column with the present work.

Chiral column/ selector	Mobile phase	Resolution (Rs>2)	Reference
Chiralcel OJ-H	n-hexane: 2-propanol: trifluoroacetic acid (98:02:0.1)	1.7	(Nagamalleswari *et al.*, 2015)
Ultron ES-OVM	di-Potassium phosphate: methanol: ethanol (85:10:05)	1.9	(Awad *et al*., 2012)
(R-R)- Whelk-02	Ethanol:10mM ammonium acetate (30: 40)	2.6	(El-Fatatry *et al.*, 2016)
Chiralcel OD-H	Hexane	2.17	(Ameur *et al.*, 2017)
Chiralpak ® IA	Hexane	1.11	(Ameur *et al.*, 2017)
Chiralpak AD	Hexane: 2-propanol (50: 50)	1.73	(Ameur *et al.*, 2017)
Lux cellolose-3	Acetonitrile: 0.1% acetic acid (50:50)	3.03	Present work

Finally, this present method has been extensively validated with respect to the following parameters.

4.8.3. Validation of the proposed method

4.8.3.1. Linearity and range

Linearity of the method was studied by injecting five concentrations of two enantiomers of ibuprofen prepared in the mobile phase in concentration range from 20 –140 µg/ml in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves. The linearity curves are shown in figure 4.8.13 and the parameters are given in table 4.8.3.

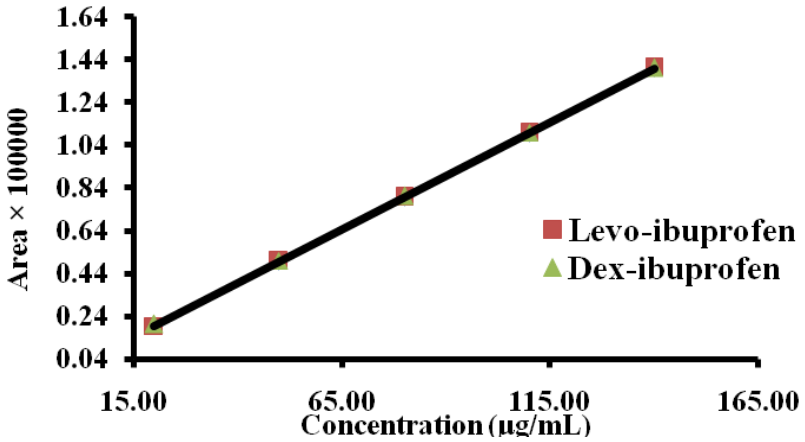


Figure 4.8.13: Linearity curve for *L*-ibuprofen and dexibuprofen.

4.8.3.2. Precision

The precision of the method was verified by intra- and inter-day precision studies. Intra-day precision was performed by analysis of on concentration for six times on the same day. The intermediate precision of the method was checked by studying on three different days. Results are recorded in table 4.8.3. Bar diagram of precision (% RSD) for *L*- and Dex-ibuprofen is shown in figure 4.8.14.

Table 4.8.3: Results of method validation parameters.

Parameters	***L*-ibuprofen**	**Dexibuprofen**
Linear equation	y= 1004x-629.7	y= 998.7x+186.0
Coefficient of determination (r2>0.995)	1.0	1.0
Linearity range	20-140 µg/mL	
Precision (intra-day, n=6) (% RSD≤2)	0.20%	0.21%
Precision (inter-day, n=6) (% RSD≤2)	0.10%	0.09%

n = number of determinations

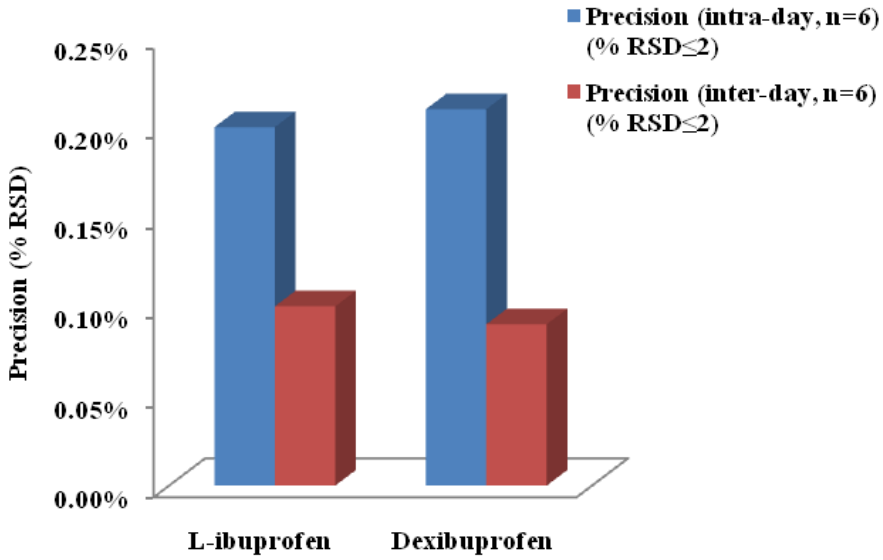


Figure 4.8.14: Bar diagram of precision (% RSD) for ibuprofen.

4.8.3.3. Accuracy

Accuracy of the method was verified by studying recovery experiments which were performed by spiking solutions of known amount of the drug with pre-analyzed sample. To evaluate the accuracy of the method, successive analysis (n=3) of standard solutions of the drug was carried out and the results are given in table 4.8.4. Bar diagram of accuracy (% recovery) is shown in figure 4.8.15.

Table 4.8.4: Results of accuracy study.

Parameters	***L*-ibuprofen**	**Dexibuprofen**
Accuracy (n=3) (avg. % recovery)		
Standard+spike (µg/mL)		
(50+20)	100.65%	99.93%
(70+20)	100.36%	100.04%
(80+20)	100.70%	101.58%
LOD (µg/mL)	0.84	0.21
LOQ (µg/mL)	2.55	0.62

n = number of determinations

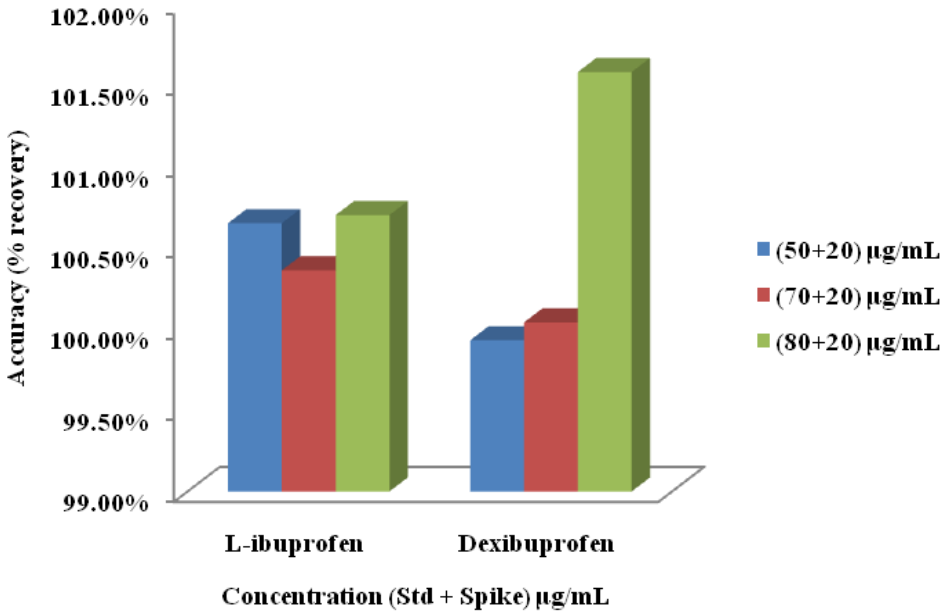


Figure 4.8.15: Bar diagram of accuracy (% recovery) for ibuprofen.

4.8.3.4. Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated using the following equations:

LOD= (SD / slope) x 3.3

LOQ= (SD / slope) x 10.

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). The results of LOD and LOQ obtained for studied drugs have been recorded in table 4.8.4.

4.8.3.5. Specificity

The specificity of the method was assessed from the chromatogram where complete separation of ibuprofen and dexibuprofen were achieved without any interference. The peaks obtained were sharp, well separated at the baseline as shown in figure 4.8.16. Single dexibuprofen was injected into HPLC for the detection of (*S*)- enantiomer (dexibuprofen) in ibuprofen.

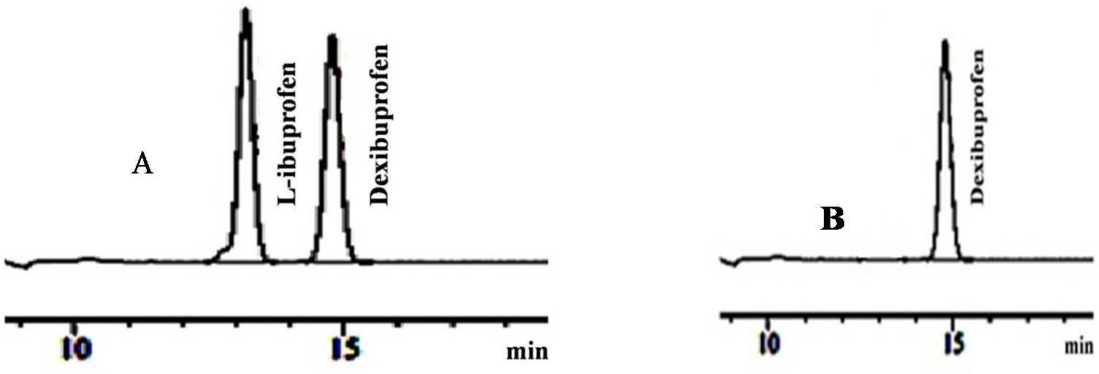


Figure 4.8.16: HPLC chromatograms of ibuprofen (A) and dexibuprofen (B).

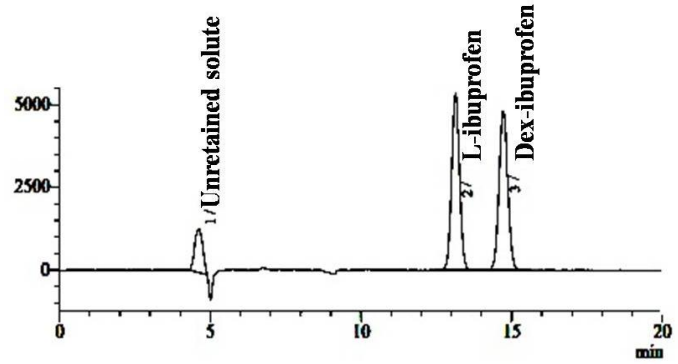
4.8.3.6. System suitability testing

System suitability parameters are reported in table 4.8.5. The chromatogram of system suitability is shown in figure 4.8.17.

Table 4.8.5: Parameters for system suitability testing.

Parameters	***L*-ibuprofen**	**Dexibuprofen**
Theoretical plates (≥2000) (n=5)	11379.09	11698.47
Tailing factor (≤2) (n=5)	1.06	1.08
Relative retention (k_S and k_R) (n=5), 0.5 < k < 10	1.85	2.19
Selectivity (α>1) (n=5)	1.18	
Resolution (≥2) (n=5)	3.03	

n = number of determinations



Where,

t_0=4.61 min,

t_L= 13.13 min,

t_D= 14.72 min.

Figure 4.8.17: HPLC chromatogram of ibuprofen for systemsuitability testing.

4.8.3.7. Solution stability testing

A standard solution of concentration of 90 µg/mL was kept in a tightly capped volumetric flask at room temperature (25 °C) on the laboratory bench and at 4 °C in a refrigerator for 3 days and its stability was tested. The results are given in table 4.8.6. Bar diagram of solution stability (% RSD) for *L*- and Dex-ibuprofen is shown in figure 4.8.18.

Table 4.8.6: Parameters for solution stability testing.

Day	At 25 °C		At 4 °C	
	***L*-ibuprofen (n=3) (% RSD)**	**Dexibuprofen (n=3) (% RSD)**	***L*-ibuprofen (n=3) (% RSD)**	**Dexibuprofen (n=3) (% RSD)**
Day 1	0.13%	0.08%	0.08%	0.06%
Day 2	0.11%	0.06%	0.10%	0.05%
Day 3	0.12%	0.04%	0.12%	0.12%
Avg.	0.12%	0.06%	0.10%	0.08%

n = number of determinations

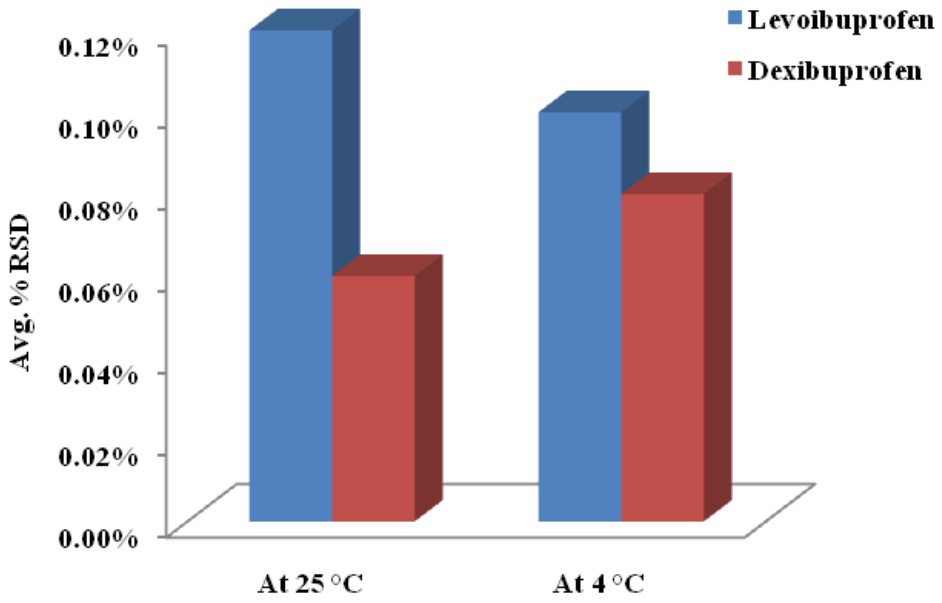


Figure 4.8.18: Bar diagram of solution stability testing (% RSD) for ibuprofen.

4.8.3.8. Robustness of the method

To determine the robustness of this method, the experimental conditions were deliberately changed, like the flow rate and in the wavelength of detection and measuring the % RSD. For the present study, factors chosen were flow rate (0.7 ± 0.2mL/min) and wavelength (254 ± 2nm), and n = 3. The % RSD is reported in table 4.8.7. All analyte peaks were adequately resolved and elution orders remain unchanged. Bar diagram of robustness (% RSD) for *L*- and Dex-ibuprofen is shown in figure 4.8.19.

Table 4.8.7: Robustness parameters.

Parameters (n=3)	***L*-ibuprofen (n=3) (% RSD)**	**Dexibuprofen (n=3) (% RSD)**
Detection wavelength at 252nm	0.27%	0.15%
Detection wavelength at 256nm	0.23%	0.19%
Flow rate 0.5 mL/min	0.03%	0.02%
Flow rate 0.9 mL/min	0.01%	0.05%

n = number of determinations

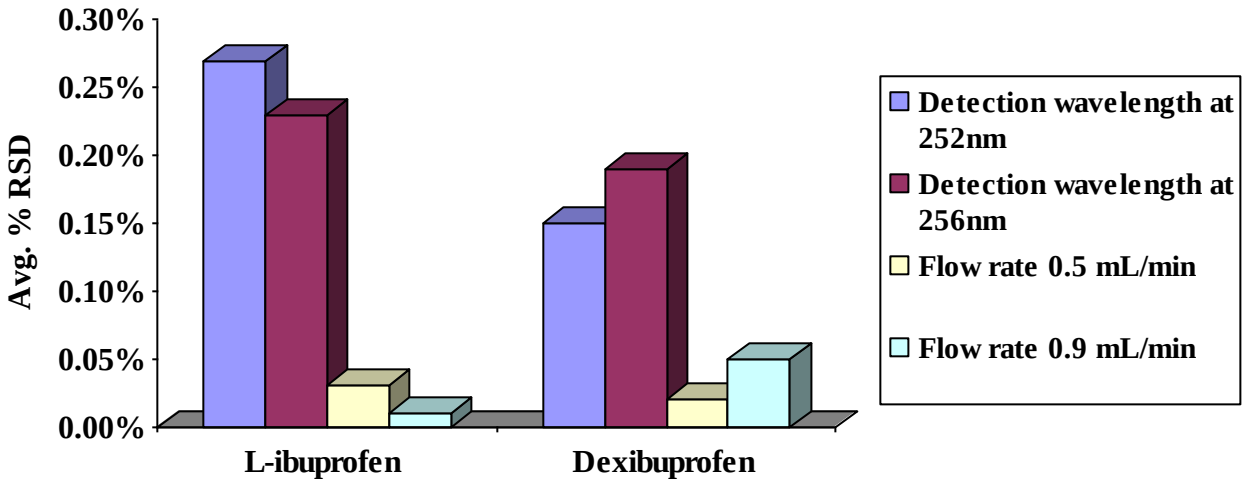


Figure 4.8.19: Bar diagram of robustness study (% RSD) for ibuprofen.

4.8.4. Application of the developed method

This analytical method was applied to quantitate the content of *L*-ibuprofen and dexibuprofen in commercial samples for three companies of Bangladesh for each, respectively and as well as to calculate the % purity and ratio of them. The average content of *L*-ibuprofen was from 49.89% to 50.02% while the content of dexibuprofen were from 49.98% to 50.10% in the formulations of racemic mixture of ibuprofen. For single enantiomeric samples, average content of dexibuprofen found the value of 100.00%. Enantiomeric purity (%EP) or the enantiomeric excess (ee%) of dexibuprofen samples were found to be 100.00%. Enantiomeric ratio and purity of commercial samples are shown in table 4.8.8 and 4.8.9, respectively. Enantiomeric ratio of ibuprofen samples for all different companies met the USP requirements. HPLC chromatograms of one commercial sample for ibuprofen and dexibuprofen are shown in figure 4.8.20. Bar diagram of enantiomeric ratio of racemic ibuprofen and % purity of levofloxacin are shown in figure 4.8.21 and 4.8.22, respectively.

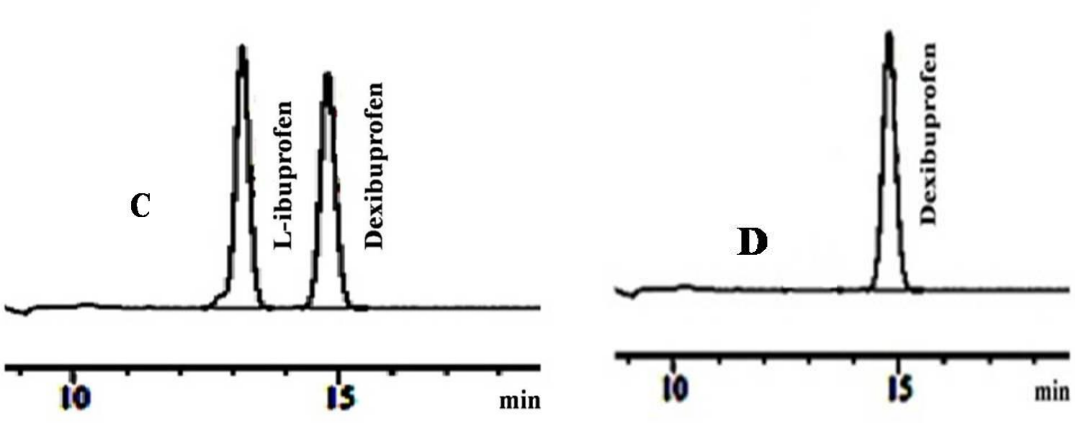


Figure 4.8.20: HPLC chromatograms of one commercial ibuprofen (C) and dexibuprofen (D).

Table 4.8.8: Enantiomeric ratio of racemic ibuprofen in commercial samples (n=10)

Identity of company	% Content of dexibuprofen	% EP = (50% - % enantiomer) of dexibuprofen	% Content of levoibuprofen	% EP = (50% - % enantiomer) of levoibuprofen	Enantiomeric ratio of ibuprofen
Company A	50.10%	-0.10%	49.89%	0.11%	50.10%/49.89%
Company B	49.98%	0.02%	50.02%	-0.02%	49.98%/50.02%
Company C	50.03%	-0.03%	49.99%	0.01%	50.03%/49.99%

EP= Enantiomeric purity, n = number of determinations

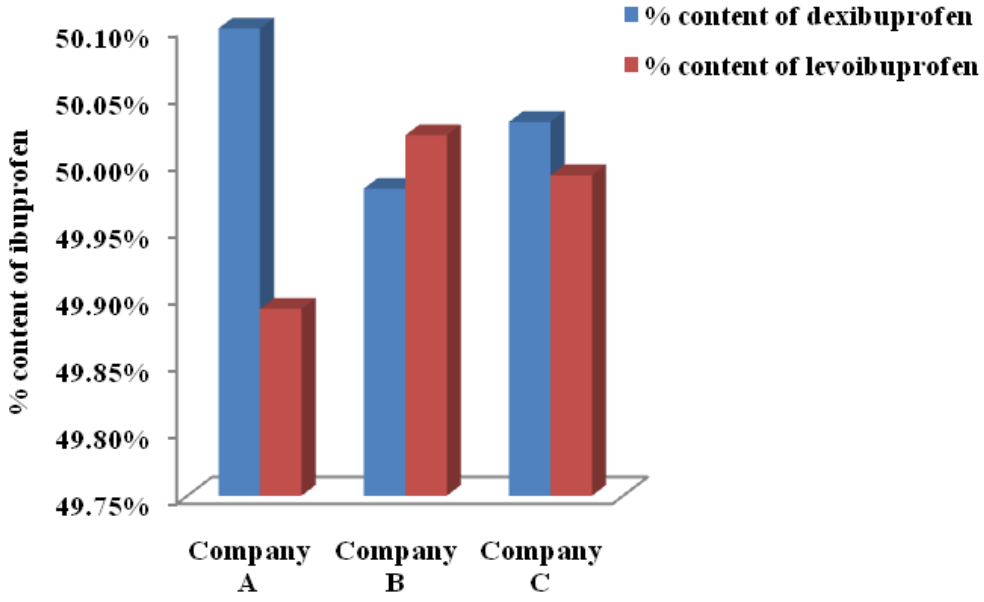


Figure 4.8.21: Bar diagram of enantiomeric ratio of racemic ibuprofen.

Table 4.8.9: Enantiomeric purity of dexibuprofen in commercial samples (n=10).

Identity of company	% Content of dexibuprofen	%Content of levoibuprofen in dexibuprofen as impurity	% EP= (% of major enantiomer - % of minor enantiomer)
Company A1	100.00%	0.00%	100.00%
Company B1	100.00%	0.00%	100.00%
Company C1	100.00%	0.00%	100.00%

EP= Enantiomeric purity, n = number of determinations

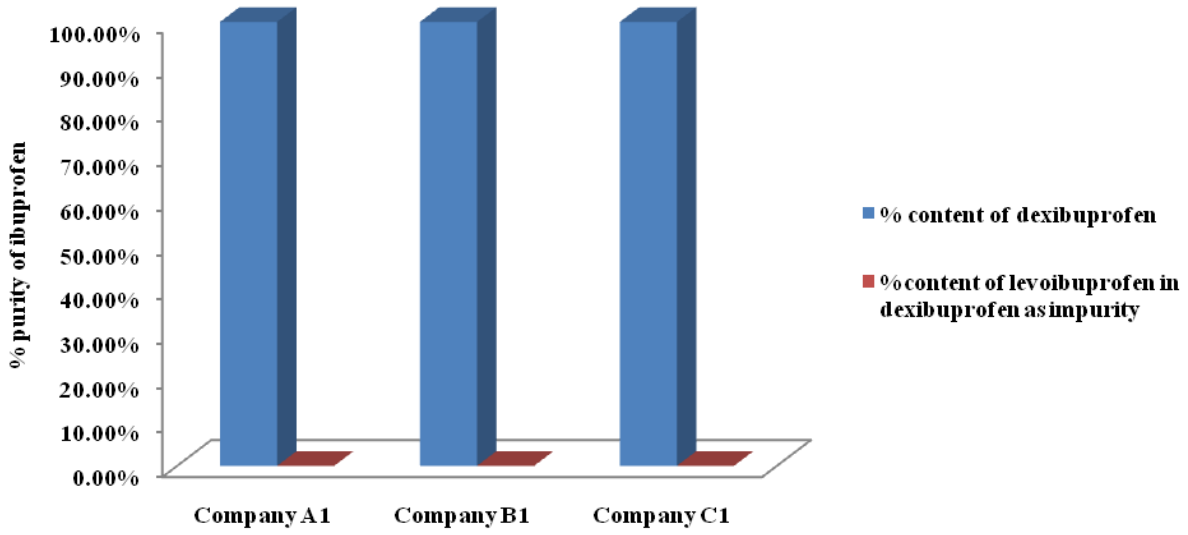


Figure 4.8.22: Bar diagram of % purity of ibuprofen.

All the developed chiral methods are validated according to ICH and USP guidelines. Results of all parameters-specificity, system suitability, linearity, solution stability, accuracy, precision, detection limit and robustness meet with the acceptance limit. In this chapter, enantiomeric purity and enantiomeric ratio are determined by applying all the validated methods which meet the USP requirements.

Chapter 5: Conclusion

CONCLUSION

At present, separation of racemic and its single enantiomeic drugs with chiral chromatography is very widely employed technique in pharmaceutical industry as well as in clinical therapeutics. After separation, determination of enantiomeric purity for the assessment of quality of chiral drugs for clinical purpose is very vital issue. Therefore, the development of new chiral separation techniques is and will be a topic subject in academic research as well as in industrial advance. In this work, very simple but very significant methods were developed by chiral chromatography and validated to get the actual therapeutic efficacy of commonly used chiral drugs which are very available and regularly administered in Bangladesh.

A sensitive, simple, and accurate method for enantioseparation of propranolol enantiomers was developed by chiral CD-Ph. The method provided good enantioseparation (α = 1.67) and resolution (Rs = 3.75) with the mobile phase of comprising of ammonium acetate (pH 3.0): methanol (10: 90, v/v). In this method it is very significant that a single chromatographic run allowed the identification of the (*S*)- and (*R*)- enantiomers in a short time for both (within 15 min).

The enantioseparation of carvedilol was developed by chiralpak IC column. The method provided good enantioseparation (α = 3.26) and resolution (Rs = 7.9) with *n*-hexane: isopropyl alcohol: diethyl amine: acetic acid (40: 60: 0.7: 0.3, v/v). The main advantage of this method was that a single chromatographic run allowed the identification of the (*S*)- and (*R*)- enantiomers in a short time for both (within 17 min).

A sensitive method for enantioseparation of ofloxacin enantiomers was developed by chiralpak IC column using immobilized cellulose tris-(3,5- dichlorophenylcarbamate) chiral selector. The method provided good enantioseparation factor (α = 1.32) and resolution (Rs = 3.42) with the mobile phase of ethanol: methyl *tert* butyl ether (50: 50, v/v). Less time consuming is significant of this method that's why a single chromatographic run allowed the identification of the (*S*)- and (*R*)- enantiomers in a short time for both (within 12 min).

For omeprazole, the developed method was found to be simple, accurate, precise, and less time consuming with improved resolution. This method was developed by chiralcel OD-H with the mobile phase of *n*-hexane: 2-propanol: acetic acid: triethylamine at a ratio of 100:20:0.2:0.1 (v/v). The method showed good linearity over the concentration range with coefficients of determinations of 0.999 and 0.998 for *S*- and *R*- omeprazole, respectively; high sensitivity with detection limit (LOD) of 0.71 and 1.16 µg/mL and quantitation limit (LOQ) of 2.16 and 3.51 µg/mL for *S*- and *R*- omeprazole, respectively. The average percentage of recovery and average percentage of relative standard deviation (% RSD) for intra- and inter- day precision were found to be within the range.

For salbutamol, the mobile phase composition was found to be di-Sodium hydrogen phosphate (pH 6.01): methanol (50:50, v/v) which good linearity over the concentration range with coefficients of determinations of 0.999 for both *S*- and *R*- salbutamol. The method provided good enantioseparation factor (α = 1.67) and resolution (R = 3.75). It also showed high sensitivity with detection limit (LOD) of 1.32 and 0.59 µg/mL and quantitation limit (LOQ) of 3.99 and 1.79 µg/mL for *S*- and *R*- salbutamol, respectively. The average percentage of recovery and average percentage of relative standard deviation (% RSD) for intra- and inter- day precision were found to be within the range.

This study presented an improved method for the enantioseparation of citalopram. This method was developed by chiral CD-PH with the mobile phase of ammonium acetate (pH 3.0: ethanol: isopropanol: methylene chloride (100:150:70:30, v/v). It showed good linearity over the concentration range with coefficients of determinations of 0.998. The method also provided good enantioseparation factor (α = 3.60) and resolution (Rs = 15.63). It was found to be simple, accurate, and precise with improved resolution compared to reported methods.

This study showed an excellent method by which simultaneous determination of rabeprazole and pantoprazole occurred. This method was developed by chiralpak IC with the mobile phase of n-hexane: ethanol at a ratio of 50:50 (v/v). Separation occurs for four enantiomers within very short time approximately 20 minutes. This was the main advantage of this method. This method also showed good linearity over the concentration range with coefficients of determinations of 0.999 for both *S*- and *R*- rabeprazole and also for both *S*- and *R*- pantoprazole.

Relative retention factor for *S*- and *R*- rabeprazole were found 2.39 and 3.69 and 1.43 and 1.80 for *S*- and *R*- pantoprazole, respectively. This method also showed good resolution of 5.91 and 2.57 for rabeprazole and pantoprazole, respectively. So this method found very simple easy and less time consuming.

The method for ibuprofen established was achieved on Lux Cellulose-3 with mobile phase consisting of acetonitrile: 0.1% acetic acid at a ratio of 50:50 (v/v) showed excellent linearity of 1.0 for both *L*- and dexibuprofen. The average percentage of recovery and average percentage of relative standard deviation (% RSD) for intra- and inter- day precision were found to be within the range. Separation occur within very short time, 16 minutes. Relative retention factor for *L* and dexibuprofen were found to be 1.85 and 2.19, respectively and resolution was 3.03.

From this study, it was found that all the chiral methods are very simple, accurate, precise, and less time consuming with improved resolution compared to previously reported methods. So, the established method can be successfully applied for the routine analysis of all respective enantiomers in pharmaceutical companies. All the proposed methods for enantiomeric separation and quantitative determination of enantiomers in tablets, capsules and bulk drugs were found to efficient and sensitive. This study showed that during the analysis the excipients of the commercial samples did not interfere with the reference solution, which proved the enantio-specificity of the methods. The main advantage is that a single chromatographic run allows the identification of the *S*- and *R*- enantiomers in short time for both and permits the analyst to analyze large number of samples within short period of time. Applying these methods, it was possible to determine the enantiomeric purity as well as enantiomeric ratio. So, these chiral methods can be applied in pharmaceutical companies of Bangladesh.

Chapter 6: References

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Chapter 7: List of Publications

PUBLICATIONS

Rahman, A., Haque, M.R., Sultan, M.Z., Rahman, M.M., and Rashid, M.A. (2017). Enantiomeric determination of omeprazole and esomeprazole by a developed and validated chiral HPLC method and stability studies by microthermal analysis. *Dhaka University Journal* of *Pharmaceutical Sciences*, 16(2), 221-233.

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