The separation of specific enantiomers of anticancer reagent cytarabine using HPLC.

By
Shweta Ashok Kamble

A thesis submitted in partial fulfilment for the requirements for the degree of Masters of Science by Research at the University of Central Lancashire

January 2011

DECLARATION

I declare that while registered as a candidate for this degree I have not been registered as a candidate for any other award from an academic institution. The work present in this thesis, except where otherwise stated, is based on my own research and has not been submitted previously for any other award in this or any other University.

Signed

Shweta Ashok Kamble

ABSTRACT

During the research and development stage of any drug candidate, there is a growing need to investigate the properties of existing enantiomers, if the drug possesses chirality. It is becoming increasingly more of a requirement to separate specific enantiomers in order to optimize the potency and reduce the toxicity of certain drugs. Consequently, there is a huge interest in devising analytical methods for the analysis and separation of enantiomers, thus leading to improved treatments of marketed pharmaceutical products. Cytarabine is a nucleoside analogue used as a chemotherapeutic agent for the treatment of acute lymphoblastic and myelogenous leukaemia. The aim of this study was to separate the enantiomers of anticancer reagent cytarabine by HPLC and investigate the effects of cytarabine enantiomers on specific cancer cell lines in order to identify if the enantiomers possess any specificity. The present study carried out the attempted separation of cytarabine enantiomers on three polysaccharide based chiral stationary phases namely; Chiralcel OD-H, Chiralcel OJ-H and Chiralpak AD-H using HPLC system. The effect of the varying compositions and the flow rates of mobile phases resulted in varying degrees of elution and separation. The conditions that afforded the best separation involved the use of the Chiralpak AD-H column using mobile phase ratios consisting of *n*-hexane: 2-propanol (50:50, v/v), (60:40, v/v) (70:30, v/v) at a flow rate 1.5ml/min and *n*-hexane: 2-propanol: diethylamine (50:50:0.2, v/v/v) and (60:40:0.2, v/v/v) at flow rate 1ml/min with excessive overlap. The application of the Chiralcel OJ-H column using mobile phase composition of *n*-hexane: ethanol: diethylamine (60:40:0.2 v/v/v) at a flow rate of 0.5ml/min also afforded partial separation with excessive overlap. The application of Chiralcel OD-H with varying compositions proved to be less effective than

Chiralcel OJ-H and Chiralpak AD-H columns. Partial separation was achieved with the employment of a gradient elution using combinations of *n*-hexane with 2-propanol on the Chiralpak AD-H. However, the gradient method proved not to be as effective as the isocratic system. The investigation highlighted the difficulties that exist in separating effectively specific chiral compounds. The fact that there was variation in peak shape, even after analysing the same sample repeatedly was a significant challenge which clearly hampered resolving the peaks.

Dedicated To My Loving Family

Table of Contents

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

First and foremost I offer my sincerest gratitude to my supervisor, Dr. Leroy Shervington, who has supported me throughout my thesis with his patience and knowledge whilst allowing me the room to work in my own way. I attribute the level of my Masters degree to his encouragement and effort and without him this thesis, would not have been completed. One simply could not wish for a better or friendlier supervisor. I would also like to thank Prof. Jai Paul Singh for his support and encouragement throughout the work and Dr Tim Snape who kindly provided the use of his chiral columns.

My warm thanks are due to all those who have helped me with my work in the Department of Pharmacy and Biomedical Sciences. I would especially like to thank James Donnelly and Tamar Garcia for giving valuable knowledge about HPLC.

Many thanks go to my loving friends for all the support, care and happiness they have shared and influenced my life and this work. I will not forget the great times I spent with Nehala Parkar, Dr. Zarine Khan, Pooja Babar, Shantaram Muthaye, Mayur Gawde, Seema Jaiswal, Sukhmander Kaur, Okoh Adeyi, Shahid Chaudhary, and Bimee. Special thanks to Nehala Parkar, Zarine Khan and Mayur Gawde, for taking time to read my thesis and offer valuable suggestions.

Most especially I would like to extend my heartfelt gratitude to my parents, Ashok Kamble and Jayshree A Kamble and my brother Omkar A Kamble, to whom I dedicate my work. Through my childhood and educational years they have always encouraged me to follow my heart, for which my mere expression of thanks does not suffice. My eternal gratitude goes to my wonderful grandmother,

Mrs. Subhadra Tapase, for her immense love and pampering throughout. My loving thanks to my cousins, Shilpa, Sachin, Nikhil, Shivani and Sheetal.

I am ever grateful to God, the Creator and the Guardian, and to whom I owe my very existence.

LIST OF ABBREVIATIONS

ACN	Acetonitrile
Ara-C	Arabinosylcytosine
Ara-CMP	Ara-C Monophosphate
Ara-CTP	Ara-C Triphosphate
APaf-1	Apoptotic Protease Activating Factor
BCL	B-cell Lymphoma 2
BH3	Bcl-2 Homology Domain 3
CN-II	Cytosolic 5o-nucleotidase II
CSP	Chiral Stationary Phase
d	Dextrorotatory
dck	Deoxycytidine Kinase
DEA	Diethylamine
DNA	Deoxyribonucleic acid
DOPA	Dihydroxy-3,4 phenylalanine
DSC	Differential Scanning Calorimetry
ee	Enantiomeric Excess
EtOH	Ethanol
FDA	Food and Drug Administration
GC	Gas Chromatography
hENTI	Human Equilibrative Nucleoside Transporter
Hexane	*n*-hexane
HPLC	High Performance Liquid Chromatography
IPA	2-propanol
l	Levorotatory
min	Minute
MP	Mobile Phase
PMAIP1	Phorbol-12-Myristate-13-Acetate-Induced Protein 1
R enantiomer	Rectus Enantiomers
RNA	Ribonucleic acid

RNase H	Ribonuclease H
RSD	Relative Standard Deviation
S enantiomer	Sinister Enantiomer
SFC	Supercritical Fluid Chromatography
S-phase	Synthesis Phase
SMAC	Second Mitochondria-derived Activator of Caspases
TEA	Triethylamine
TLC	Thin Layer Chromatography
TRAIL	TNF Related Apoptosis Inducing Ligand
UV	Ultra Violet

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Chirality

Structural differences create a significant impact on the way molecules interact with the surrounding and other compounds. Even though the molecule can be identical in a number of ways for example molecular formula and connectivity, they can differ on their spatial properties that have a profound effect on the way they react with other compounds. Chirality is formally defined as the geometric property of a rigid object which is not superimposable with its mirror image and the existence of left and right position. It is applied to molecules, conformations as well as macroscopic objects such as crystals (Francotte and Lindner, 2006; Brown *et al.,* 2009). Chirality is an essential and ubiquitous property of living nature since many important molecules in nature are chiral including proteins, nucleic acids, enzymes, carbohydrates, alkaloids, and also all resulting macromolecules like deoxyribonucleic acid (DNA), ribonucleic acid (RNA) (Reddy and Mehvar, 2004). Thus, the phenomenon of chirality plays a key role in life of plants, animals, pharmaceutical, agricultural industry. The most fundamental development occurred when a tetrahedral carbon atom was proposed as a basis for molecular chirality by Dutch and French chemists Jacobus Henricus Van't Hoff and Joseph Achille LeBeli (Francotte and Lindner, 2006). When two chemically identical molecular species differ from each other as non-superimposable mirror images they are called as enantiomers for example S and R lactic acid (Beesley and Scott, 1998) (Fig 1.1).

S (+) lactic acid R (-) lactic acid

Figure1.1: The chemical structure of S and R lactic acid as enantiomers. (The chemical structure was drawn using the chemdraw ultra 12.0 software).

The simplest example of chirality is a sp^3-hybridized tetrahedral carbon atom to which four different substituents are attached, this molecule does not have a plane of symmetry and is called chiral for example the anti-depressant drug fluoxetine which has one chiral carbon and therefore exists as a pair of isomers called enantiomers (Fig 1.2), the S-enantiomers of which possesses a higher pharmacological activity (Shamsipur *et al.,* 2007).

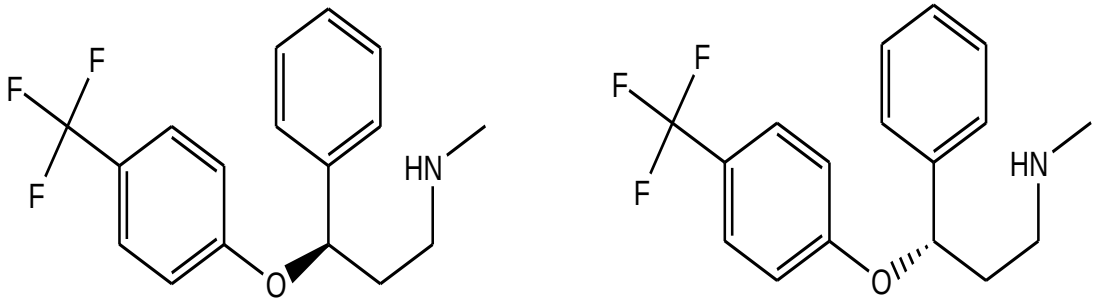


Figure 1.2: R and S enantiomers of fluoxetine with one chiral carbon and four different groups attached to it.

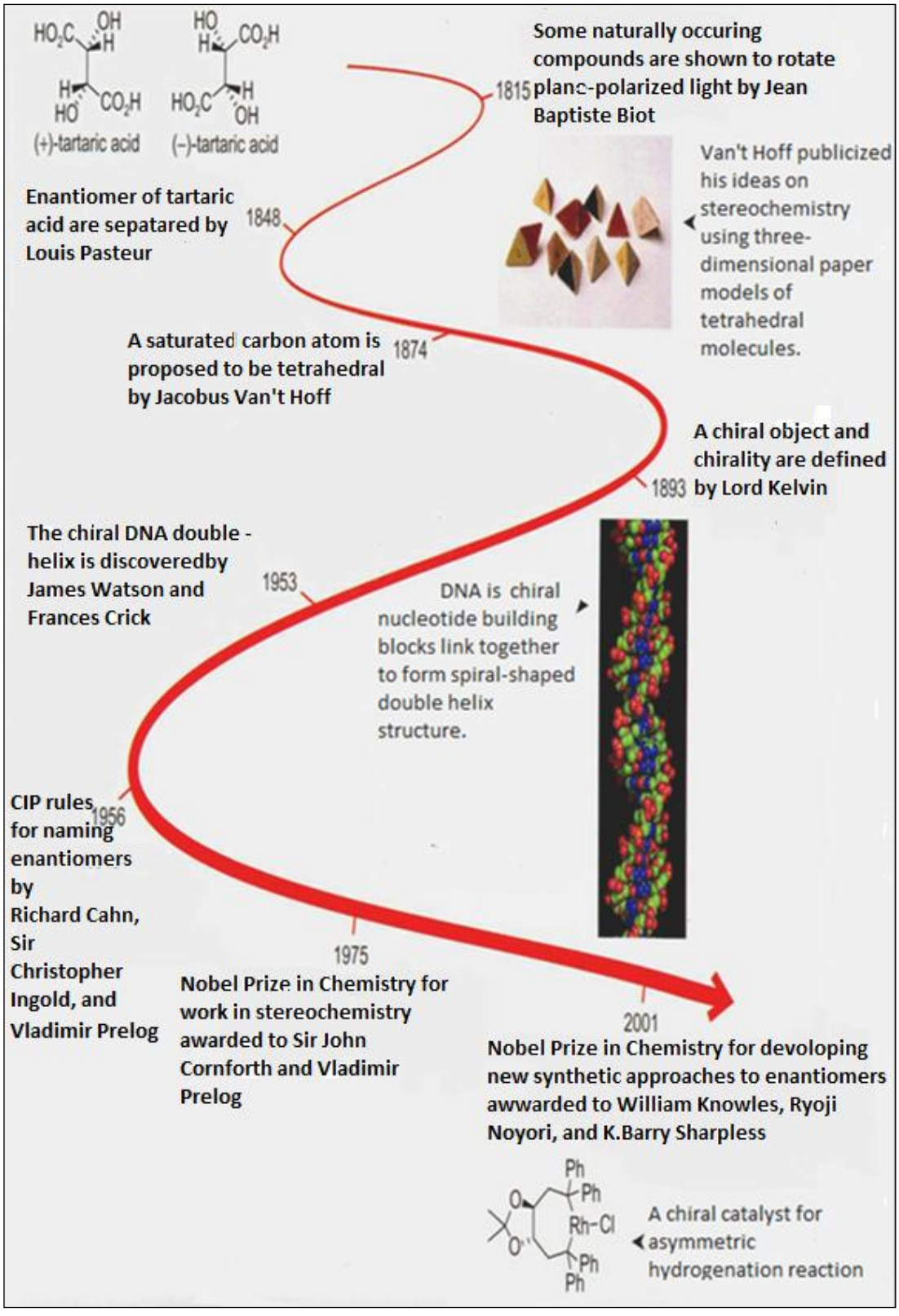


Figure 1.3: Some landmarks in chirality (Adapted from Burrows *et al*., 2009)**.**

1.2 Important terms of chiral chemistry

1.2.1 Achiral

If an object and its mirror image are superimposable then the original object is termed as achiral. An object can be called achiral when it has one or more elements of symmetry such as plane of symmetry and centre of symmetry for example cyclobutane (Fig 1.4), it has a centre of symmetry so is identical to its mirror image and is achiral (Brown *et al.,* 2009).

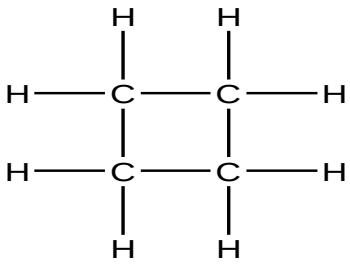
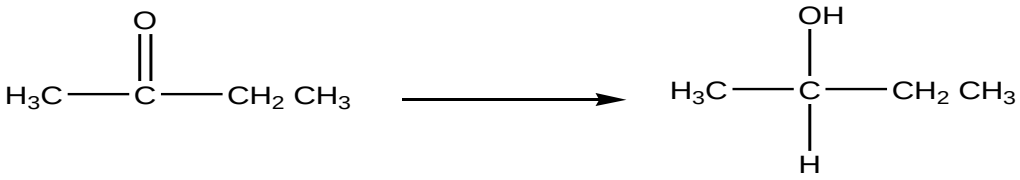


Figure 1.4: The chemical structure of achiral cyclobutane.

1.2.2 Prochiral

A molecule is considered prochiral if it can be converted into a chiral or achiral molecule in a single chemical step for example 2-butanone which gives 2-butanol on reduction (Fig 1.5) (McMurry and Begley, 2005).



2-Butanone (prochiral)

2-Butanol (chiral)

Figure 1.5: The chemical structure of prochiral 2-butanone which gets converted to 2-butanol, a chiral compound on reduction.

1.2.3 Isomers

These are compounds with same molecular formulae but different structural arrangements. The outcome of isomerism yields practically various compounds which possesses different properties from each other (Turker, 2002). The types of isomers are constitutional, stereoisomers, configurational isomers, conformational isomers, geometric isomers, optical isomers, diastereomers and enantiomers.

1.2.3.1 Diastereomers

The compounds with one chiral centre exist as enantiomeric pair, whereas compounds with two or more chiral centres can exist as diastereomers or diastereoisomers (Metzler and Metzler, 2003). They are stereoisomers with a different configuration and are not related as mirror images having different chemical and physical properties. Molecules possessing more than one chiral centre also exhibit diastereomerism because inverting one or more (but not all) of the centres leads to structures which do not have a mirror image relationship with the original. Inversion of a single chiral centre gives an epimer of the original structure. Inversion of all stereogenic centres gives the enantiomer. A molecule possessing `ń´ chiral centres has a maximum of $2^n$ stereoisomers, $2^n$ -1 pairs of enantiomers and `ń´ epimers (Burrows *et al*., 2009). Molecular symmetry within the molecule may result in a reduction of the numbers of different isomers due to internal compensation.

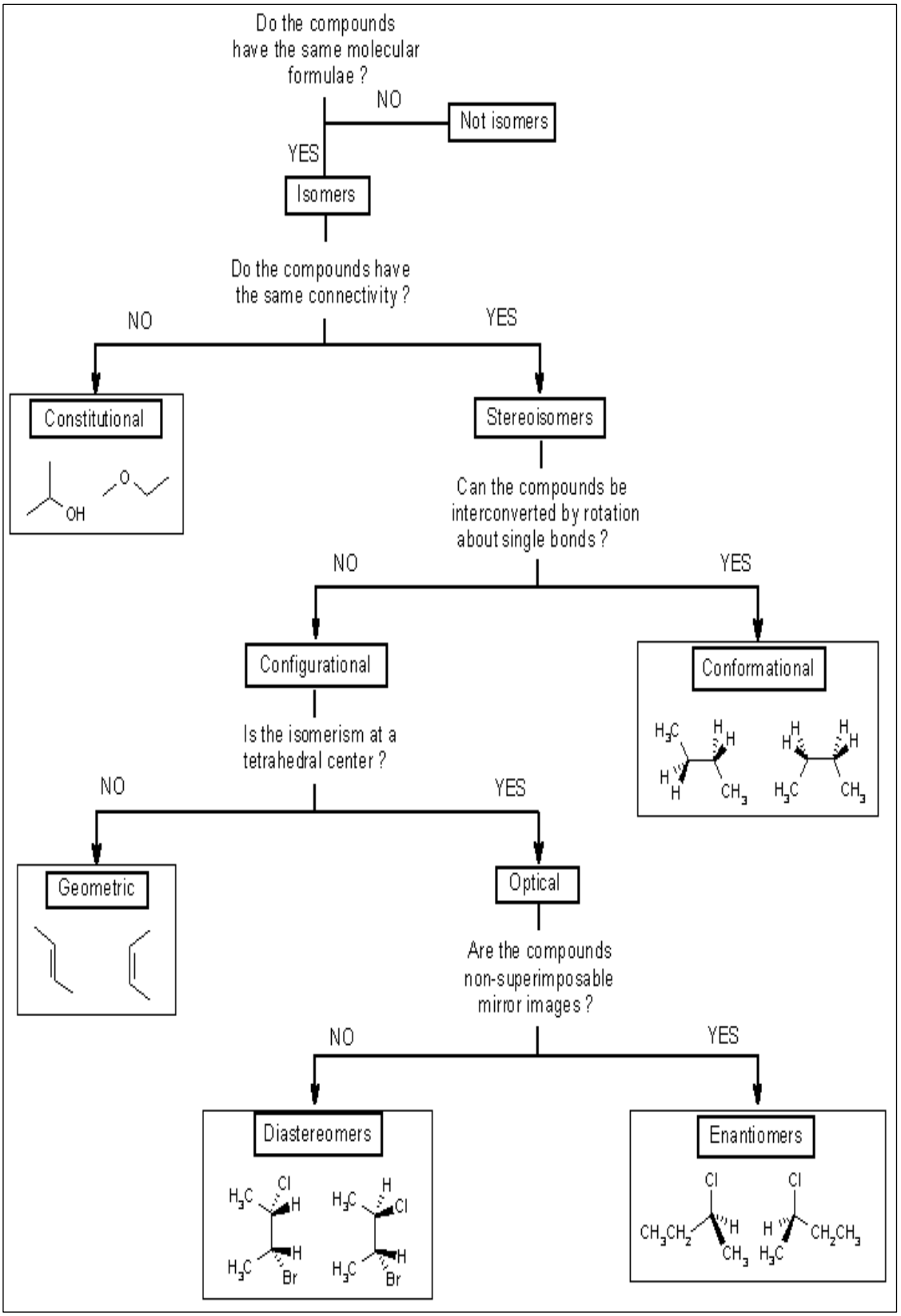
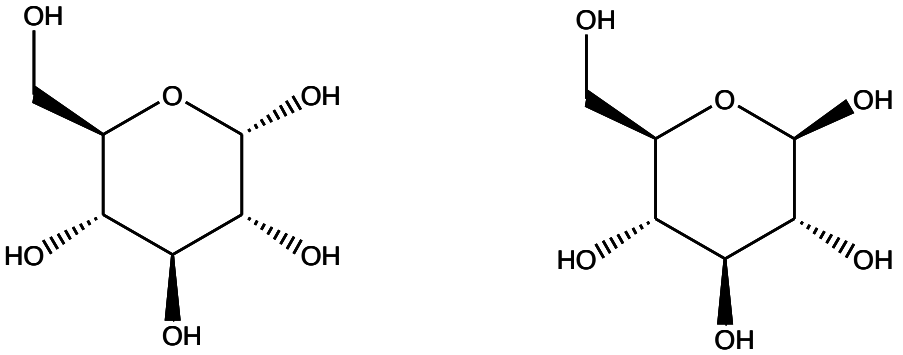


Figure 1.6: Relationship between isomers and their types (Adapted from http://www.chem.ucalgary.ca/courses).

1.2.4 Anomers

A pair of diastereomeric aldoses that differ only in configuration about the first chiral carbon are called anomers e.g. pair of aldoses α D(+) glucose and β D(+) glucose (Fig 1.7). They differ in configuration of H atom and the OH group about its first carbon atom and have the same configuration about the remaining carbon atoms (Beesley and Scott, 1998).



α D (+) glucose β D (+) glucose

Figure 1.7: The chemical structure of α D (+) glucose and β D (+) glucose.

1.2.5 Epimers

They are diastereomers that differ in configuration at only one position or stereogenic centre (Smith *et al.,* 2007). Pair of diastereomeric aldoses that differ only in configuration about second chiral carbon are known as epimers (Beesly and Scott, 1998). The structure of aldoses D glucose and D galactose only differ in configuration of the H atom and OH group in the second carbon atom but have the same configuration in the remaining carbon atoms (Fig 1.8). When a molecule contains more than one chiral centre and configurational inversion takes place at one chiral centre only, which is reversible, then the product formed is not an enantiomer of original compound but it is a diastereomer or more specifically an

epimer and this process is called as epimerisation (Nasipori, 1994). Thus the epimeric form of a chiral form can be converted to another using an enzymatic action and this is known as epimerisation.

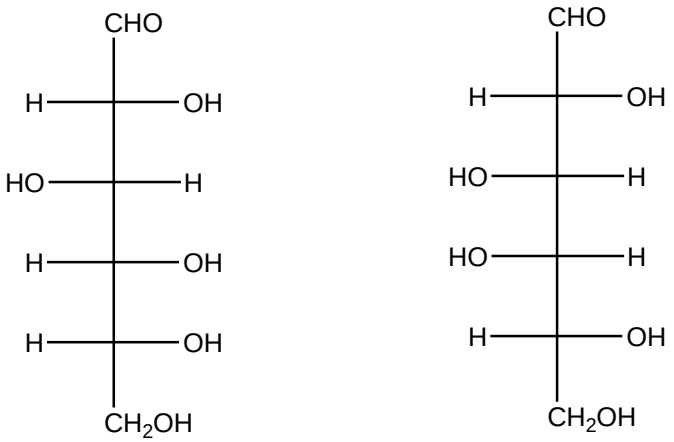
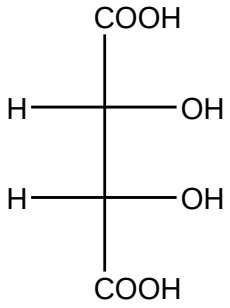


Figure 1.8: The chemical structure of D-glucose and D-galactose as epimers which differ in configuration of the H atom and OH group in the second carbon atom.

1.2.6 Meso compounds

Some compounds are superimposable mirror images of each other; compounds possessing such structures are not chiral. Their molecules have chiral centres but are not themselves chiral and are termed meso compounds. Chiral compounds have the property to rotate the plane of polarisation of plane polarised light but a meso compound is unable to do so, since they are not chiral they are not (-) levorotatory or (+) dextrorotatory (Smart and Gagan, 2002). When there are ``n´´ chiral centres, the isomeric configuration will be 2^n for example, ephedrine, pseudoephedrine. If the groups around the chiral centres are the same then the number of stereoisomers is less than 2^n. Therefore, when n=2 there are only three stereoisomers and not four for

example tartaric acid (Fig 1.9). These stereochemical principles apply to both acyclic and cyclic compounds (Dewick, 2006).



meso tartaric acid

Figure 1.9: The chemical structure of meso tartaric acid.

1.2.7 Optical purity (Enantiomeric excess)

While dealing with a pair of enantiomers it is very important to have a means of describing the composition of that mixture and the degree to which one enantiomer is in excess. The composition of mixture of enantiomers can be described by their optical purity. The optical purity is the specific rotation of a mixture of enantiomers divided by specific rotation of enantiomerically pure substance when they are in same concentration. Another way is to describe the composition of a mixture of enantiomers by its enantiomeric excess (ee). ee can be defined as the difference in the number of moles of each enantiomer in a mixture compared to the number of moles of both enantiomers (Brown *et al.,* 2009). Numerically optical purity and ee are the same. ee = % (R) - % (S) where ee = enantiomeric excess, R and S enantiomers % = percentage of enantiomers for example, if a mixture has 60% of R enantiomer and 40 % of S enantiomer, then the ee for R enantiomer will be 20%.

1.2.8 Nomenclature of enantiomers

1.2.8.1 Optical activity (+ or -)

Numerous substances have the property to rotate through a certain angle the plane of polarisation or plane of vibration of a polarised light which is passed through them, some of them rotate it to the left and others to right. Such substances can be called optically active or circular polarising and this phenomenon is called as optical activity (Burrows *et al.*, 2009). The enantiomer rotating the plane-polarized light in clockwise direction is called (+) enantiomer and the other enantiomer which rotates the plane-polarized light in anticlockwise direction is called (-) enantiomer (Burrows *et al.,* 2009).

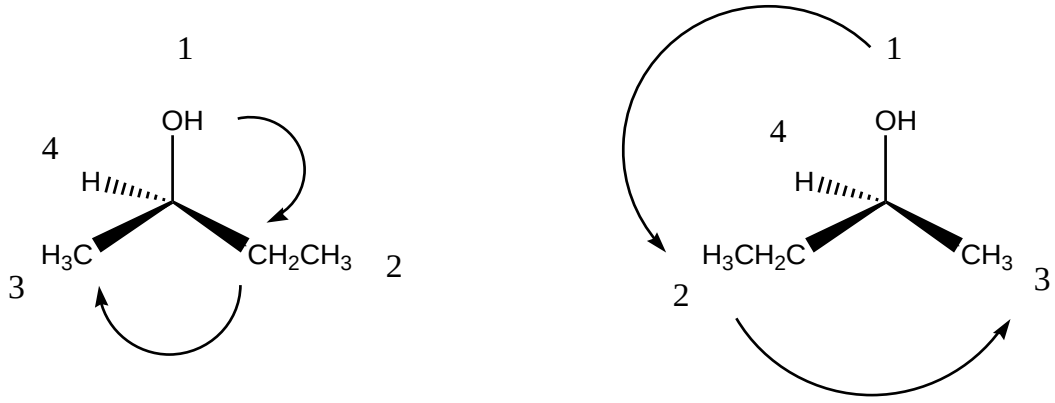
1.2.8.2 Absolute configuration (dextrorotatory and levorotatory)

The D/L labeling is unrelated to (+)/(−); it does not indicate which enantiomer is dextrorotatory and which is levorotatory. Rather, it says that the compound's stereochemistry is related to that of the dextrorotatory or levorotatory enantiomer of glyceraldehydes. If the OH group of the second carbon is on the right hand side than the enatiomer is named D and if the OH group of the second carbon is on the left hand side than the enatiomer is named as L (Burrows *et al.,* 2009).

1.2.8.3 R and S configuration

Cahn, Ingold and Prelog devised a system based on assigning sequence rules based on decreasing atomic number for projection formulas that allows absolute configuration assignments of R (rectus, Latin for right) and S (sinister Latin for left). This is the internationally accepted nomenclature for chiral molecules. The Cahn-Ingold-Prelog (CIP) rule is used for sp^3-hybridised carbon. The four groups are sorted on the basis of increasing mass of the atom attached to the asymmetric chiral centre and so on. If two atoms are identical e.g. 2-butanol, the next higher mass atom one bond away is

considered and so on. Next will be the molecule held by the lightest mass. Hence the three other substituents arranged in decreasing mass order define R-enantiomer. The mirror image of the R-2-Butanol is the S enantiomer (Fig 1.10). These rules can be used to find the absolute configuration of any chiral compound in nature (Crowe *et al*., 2006). The second way to designate these stereoisomers is to assign the configuration as R or S, based on stereostructure of enantiomers with one chiral centre. On the basis of their three dimensional structures R and S are relative configurations and it is not possible to predict if the R enantiomer will be dextrorotatory or levorotatory (Hoffman, 2004).



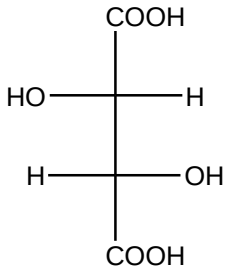
R- (2- butanol) S- (2-butanol)

Figure 1.10: R, S nomenclature for enantiomers of 2-butanol based on the increasing mass of the atom attached to the asymmetric chiral centre.

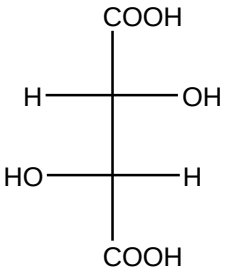
1.2.9 Racemate

Mixtures of substances that have dissymmetric molecular structures and are mirror images of each other in equimolar proportions are known as a racemic mixture. Each enantiomer rotates the plane of polarisation of plane polarised light in an angle but the racemic mixture is optically inactive due to the rotary effect of each enantiomer

cancels that of the other enantiomer, hence it cannot exhibit optical activity. This name is derived from racemic acid. For example racemic tartaric acid which was isolated from potassium tartrate in 1769 by the Swedish chemist Carl Wilhelm Scheele (Fig 1.11) (Burrows *et al*., 2009). Naturally occurring tartaric acid is chiral and it is used in the synthesis of other chiral materials. The natural form of tartaric acid is levotartaric acid (-)-tartaric acid 2, 3-dihydroxysuccinic acid. Pasteur was the first to produce a pure sample of levotartaric acid. The other enantiomer form is dextrotartaric or (+) tartaric acid. The mixture of these two levo and dextro forms is called racemic tartaric acid.



Levotartaric acid



Dextrotartaric acid

Figure 1.11: The Chemical structure of levotartaric acid and dextrotartaric acid, these when mixed together result in racemic tartaric acid.

1.2.10 Racemisation

When many optically active compounds are subjected to heat, in the presence of alkalis or acids they can go through optical inversion and an optically inactive compound is produced which contains equal quantities of two optical antipodes. The product is called a racemic product and this process is known as racemisation. It is a spontaneous post translation process (McCudden and Kraus, 2006).

1.2.11 Resolution

Enantiomers have identical physical properties and can be distinguished only in a chiral environment. The interaction between a mixture of enantiomers with single enantiomers of chiral compounds result in a mixture of diastereomers (Fig 1.12). Since diastereomers have different physical properties they can be separated. Once separation takes place the individual isomers can be obtained (Hoffman, 2004). Hence chiral resolution in stereochemistry is the separation of racemates into its enantiomers. It is also known as optical resolution or mechanical resolution.

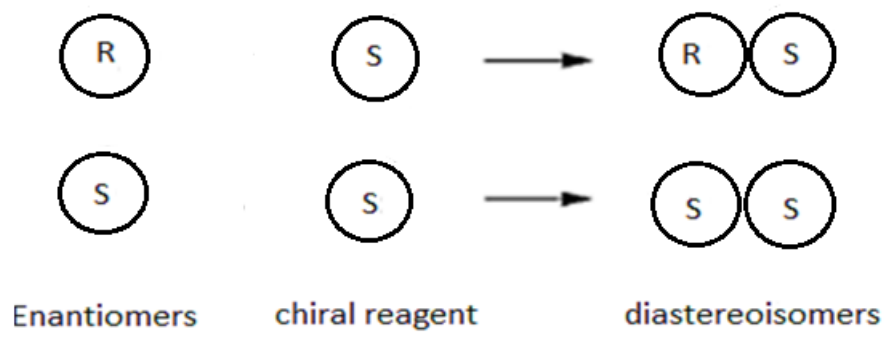


Figure 1.12: The formation of diastereomers by the interaction of mixture of enantiomers with single enantiomers (Adapted from Hoffman, 2004).

1.3 Polarimeter

The polarimeter measures the rotation of polarized light as it passes through an optically active fluid. It consists of a monochromatic light source, a polarizing filter, an analyzing filter and a sample tube (Fig 1.13). When the filled solvent tube is placed in the polarimeter, the analyzing filter must be adjusted so the field of view is dark. This position of the analyzing field is considered as 0^{o}. When the solution of optically compound is placed in the sample tube, some light passes through the filter. The optically active compound rotates the plane of polarized light from polarizing filter so

that it is no longer at an angle of 90° to analyzing filter. The analyzing filter is then rotated to restore darkness to the field. If the analyzing filter is turned to right (clockwise) then the compound is dextrorotatory and if it is turned to the left (counter clockwise) then it is called levorotatory (Brown *et al.,* 2009).

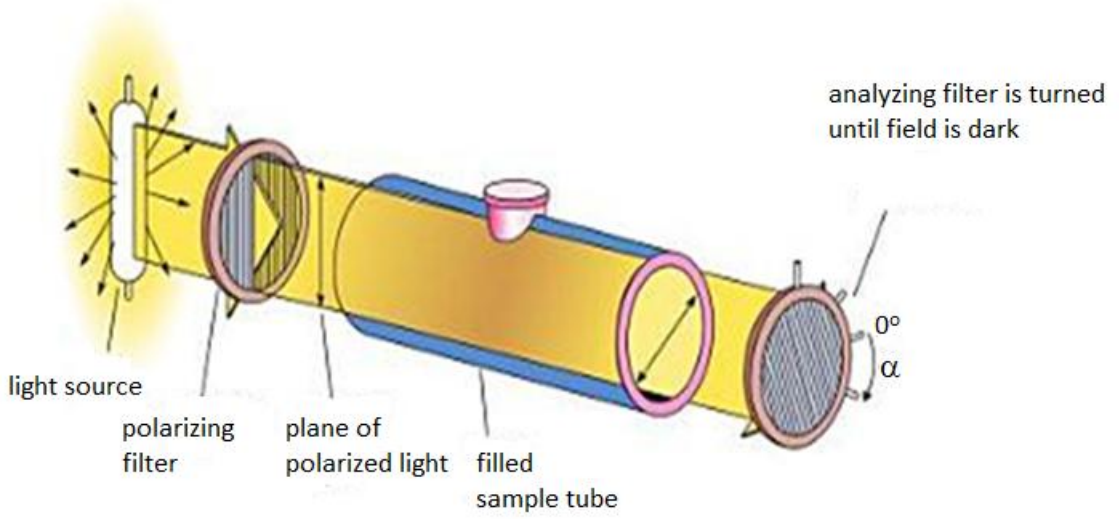


Figure1.13: Schematic diagram of a polarimeter. Showing the sample tube filled with the sample containing an optically active compound. With the analysing field turned clockwise to α degree to restore the dark field to the observer (Adapted from Brown *et al.,* 2009).

The angle through which the plane polarized light is rotated is called the optical rotation and is given symbol α. It is proportional to the number of optically active molecules present in the beam of polarized light so it depends on the concentration (c) of optically active compound and the path length (l). The standard for comparison of all molecules is called specific rotation $[\alpha]_D^{20}$ (where D is line of sodium lamp (589nm) and the superscript indicated the temperature in degrees Celsius).

$$[\alpha]_D^{20} = \frac{\text{observed rotation (degrees)} \times 100}{\text{path length (dm)} \times \text{ concentration (g per } 100\text{cm}^3)}$$

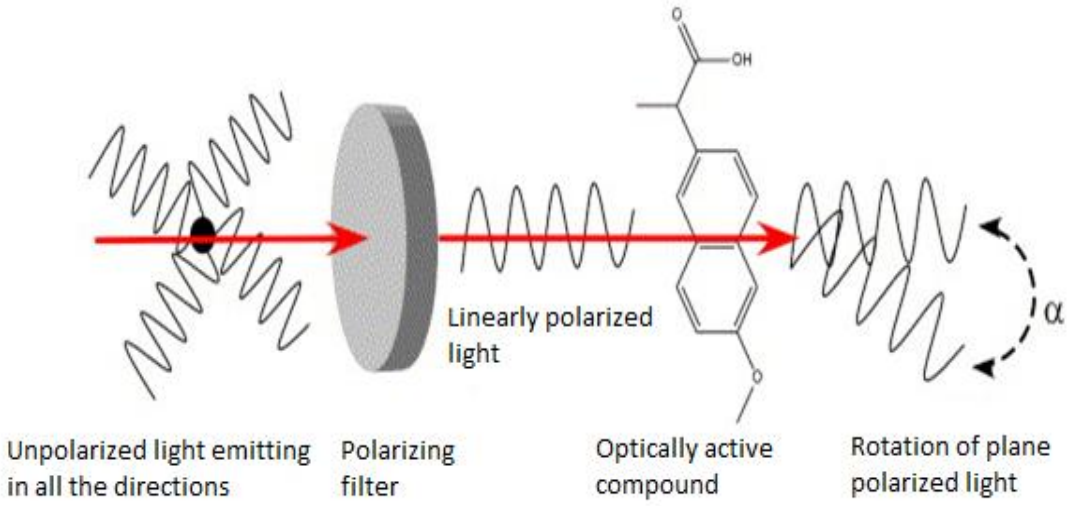


Figure1.14: Optical processes in the polarimeter (Adapted from Kott *et al*., 2006).

1.4 Importance of chirality

Except for inorganic salts and a few low molecular weight organic compounds, most of the molecules in living systems possess chirality (Brown *et al.,* 2009). Many of the most widely prescribed drugs today are chiral molecules. It is important to promote separation of enantiomers and their analysis for the requirement of stereo-pharmacokinetic studies and stereospecific drug assays (Burke and Henderson, 2002). Metabolic behaviour, bioavailability, distribution and excretion behaviour are crucial determinants of drug action. Very often one of the two enantiomers represents a more active isomer for a given action (eutomer), while the other enantiomer (distomer) may be active in a different way, leading to side effects, or acting as antagonist or displaying toxicity (Aboul-Enein and Wainer, 1997; Caldwell, 1995). US FDA (food and drug administration), European Committee for Proprietary Medicinal Products and other regulatory agencies have restricted the use racemic drugs. They have defined stricter requirements to patent new racemic drugs demanding full

documentation of pharmacological and pharmacokinetic profiles of individual enantiomers as well as their combinations (Ali *et al.,* 2009). Hence, some of the pharmaceutical companies are manufacturing pure enantiomeric drugs using enantiomeric separation techniques. Despite the fact that these differences of chiral enantiomers have been known for more than two decades, however, during the past decades tremendous advances have arisen in the development of enantiomerically pure compounds with the help of powerful analytical and preparative separation techniques (Maier *et al.,* 2001). The separation of optical isomers requires an asymmetric or chiral environment that allows diastereomeric interactions (Torok *et al*., 2005). Chiral chromatography is an important analytical tool for the determination and separation of inactive enantiomers in enantiomerically pure drugs (Nageswara *et al*., 2006b). It was only during start of this decade that the commercialisation of enantiomerically pure drugs was suggested as a desirable challenge with a number of practical limitations (Maier *et al.,* 2001). The drug has more complex pharmacological and analytical situation with the increasing number of chiral centres.

1.5 Toxicology of chiral drugs

The action of drugs is a result of pharmacological and pharmacokinetic processes by which it enters, interacts and leaves the body (Maier *et al.,* 2001). Large pharmacokinetic and pharmacodynamic differences are frequently observed between enantiomers. Hence enantiomers may result in stereoselective toxicity. The toxicological properties in a pair of enantiomers can be identical or entirely different. They can reside in the pharmacologically inactive enantiomer or the active one (Drayer, 1986). For example Dopa or dihydroxy-3,4 phenylalanine was used under racemic form d,l-dopa but due to the toxicity of d-isomer only levorotatory form

(L-dopa) is used in therapeutics. Another common example of a pair of enantiomers that proved to have devastating effects is Thalidomide (Thalomid ^{TM}) used to treat epilepsy (Melchert and List, 2007). During the last century, the thalidomide incident dramatically drew attention towards the importance of chiral activity and the need to use enantiomerically pure drug forms (Bosch *et al*., 2008). Due to the lack of sufficient efficacy as an anti-epileptic it was used as an antiemetic during pregnancy and a sleep aid (Randall, 1990). It exhibited irreversible neurotoxicity and teratological effects which led to the deformation of babies born. It was found that these effects were caused by the S-enantiomer and that the R-enantiomer had the required therapeutic activity (Fig 1.15). Prior to the introduction of thalidomide approved drugs could be racemic, but experience from that drug has now set new standards for purity assays (Webster and Kott, 2010). However, many chiral drugs are still marketed under mixed form because either their chiral separation is difficult or their pharmacological and toxical effects reside in the same enantiomer or due to high cost productions (Waldeck, 2003).

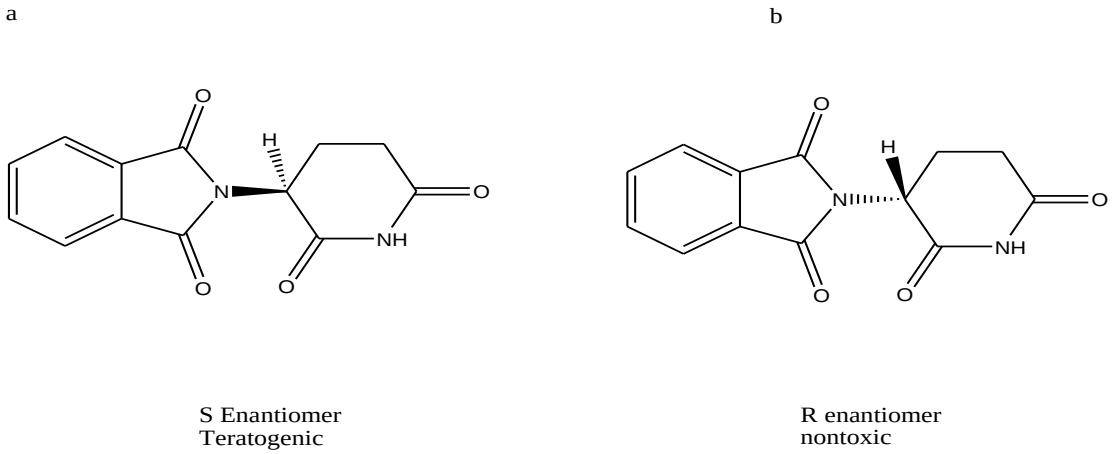


Figure 1.15: The structure of S and R enantiomer of thalidomide. The S-enantiomer had teratological effects while the R enantiomer had the required therapeutic activity.

1.6 Mechanism of biological activity

Biological processes that are mediated by or involve chiral molecules like enzymatically catalyzed biotransformation or receptor-ligand interactions can be stereoselective and this leads to implications of biologically active compounds interacting with them (Maier *et al.,* 2001). Certainly, biological systems can recognize the two enantiomers as different substances and therefore elicit different responses. The reason for the chiral recognition by drug receptors is a three-point interaction of the drug with the receptor site proposed by Easson and Stedman (Burke and Henderson, 2002) (Fig 1.16). The active enantiomer drug labelled A, B and C interacts with the corresponding binding sites (a, b, and c) while the inactive enantiomer cannot bind in the same way, consequently there is not an active response.

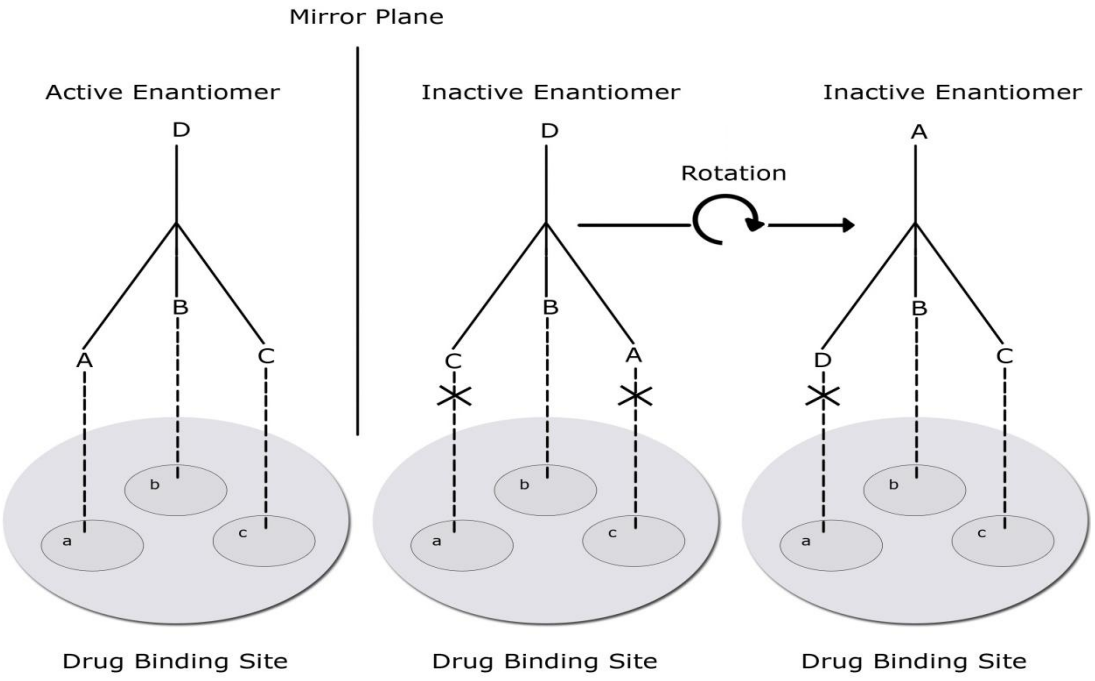


Figure 1.16: The three point interaction of the drug with its receptor site. The three substituent A, B, C of active enantiomer (left) can interact with the three binding sites by forming Aa, Bb, Cc whereas the inactive enantiomer cannot. (Adapted from McConalthy *et al*., 2003).

1.7 Cytarabine

Recently, nucleoside analogues have become the prime focus of many scientists for the treatment of cancer and various dreadful viral diseases. This class of drugs share the same pharmacodynamics and metabolism pathway with their purines and pyrimidines (Heidelberger *et al.*, 1983). The drugs when injected get incorporated into one of the DNA strands during DNA replication or DNA repair synthesis resulting in stalled DNA replication and chain termination. The cell triggers the activation of various checkpoint pathways which causes the arrest of the cell cycle during the S phase and also signals for DNA repair (Glick and Pasternak, 2007). If the survival mechanism fails to repair the damage DNA, the cell triggers another signalling pathway that initiate apoptotic processes (Cheson *et al.*, 1997). These nucleoside analogue shows diversity in their clinical activities in spite of sharing the same structural features and metabolic pathways. This study involves enantioseparation of a chiral drug by the name of cytarabine.

Nucleosides, which are frequently altered in the carbohydrate moiety, became the focus for the development of nucleic acid analogs. Most efforts were directed at the constituents and their configuration about the 2' - carbon, as this is a site at which DNA metabolizing enzymes are quite discriminating. One of the first pyrimidine nucleoside analogs to be developed containing alteration in carbohydrate moiety was cytarabine, an analog of deoxycytidine (ara-C, 1-β-D-Arabinofuranosylcytosine, Cytosar-U) (Sun *et al*., 2008). Cytarabine is an anticancer chemotherapeutic drug, and is among the most effective anticancer agent used for the treatment of both chronic and acute myeloblastic leukaemia (Stornio *et al*., 1999).

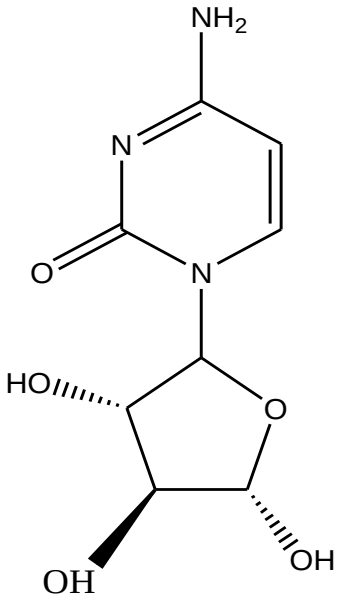


Figure 1.17: The chemical structure of cytarabine.

Synthesis of the lagging DNA strand at the replication fork occurs discontinuously and requires unwinding of the parental DNA strands, *de novo* synthesis of an RNA primer and elongation of the nascent DNA chain. Completion of the replication process requires excision of the RNA primer by ribonuclease H (RNase H) and ligation of the DNA chains by DNA ligase. Okazaki fragments occur as intermediates during replication of the lagging strand and contain the RNA primer and the nascently synthesized DNA associated with the lagging DNA strand through complementary base pairing (Russell, 2006) (Fig 1.18).

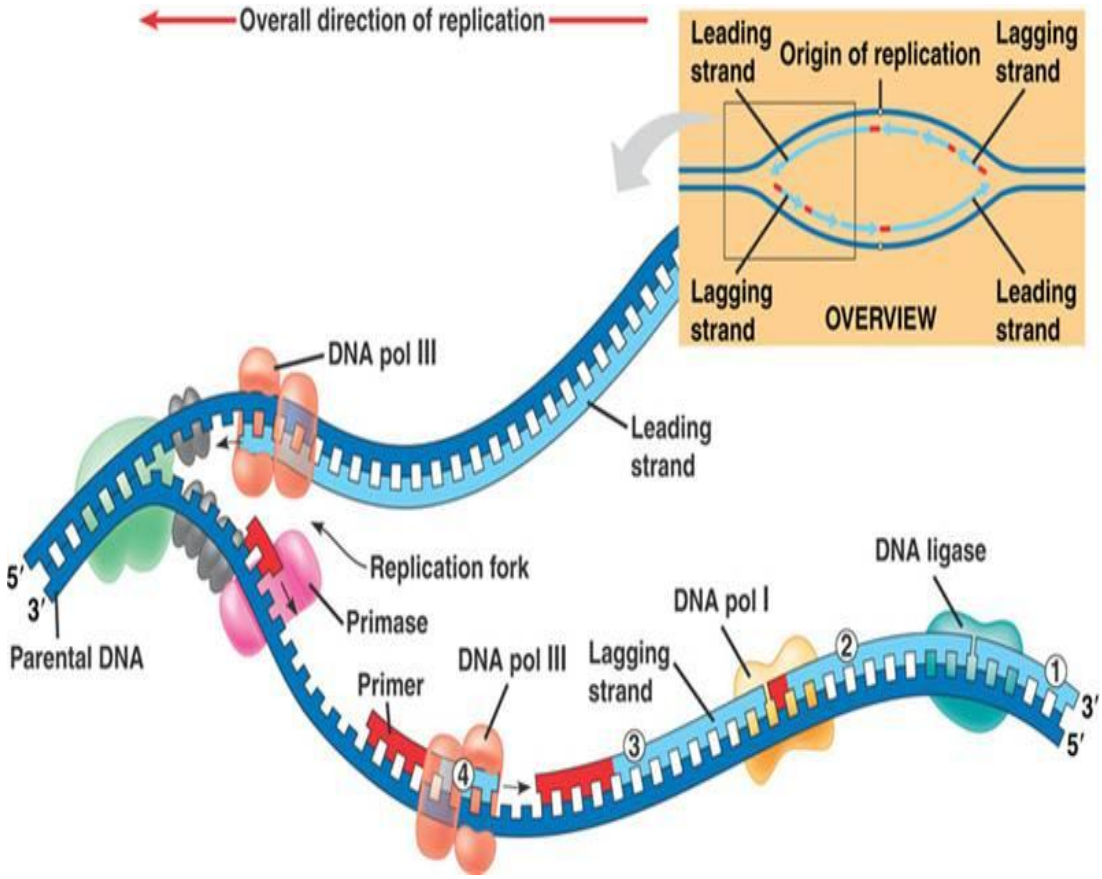


Figure 1.18: Depiction of Okazaki fragments that have occurred as intermediates during replication of DNA. Lagging strand synthesis took place in discontinuous manner. New DNA segments hydrogen bonded to the template strand have RNA primers at their 5‘ ends. RNA primers are removed by RNAse, DNA polymerase filling the resulting gaps and DNA ligase joins the adjacent gaps (Adapted from Gmeiner *et al.,* 1998).

One of the principal distinguishing characteristics between normal and malignant cells is the occurrence of nuclear DNA replication along with cell division (malignant cells lack G_0 phase). Cytarabine is cytotoxic to a variety of mammalian cells in culture. The exact mechanism of action of cytarabine has not been fully known, however it acts through DNA synthesis inhibition. It acts by blocking the utilisation of deoxycytidine triphosphate, thereby killing cells that are undergoing DNA synthesis (Yamauchi and Ueda, 2005). Its mechanism of action includes the transportation of Arabinosylcytosine (ara-C) into leukemic cells by membrane transporters such as

human equilibrative nucleoside transporter 1 (hENT1) (Wiley *et al.,* 1982). Upon entering the cell, an enzyme called deoxycitidine kinase (dCK) phosphorylates ara-C, and converts it into ara-C monophosphate. Ara-C monophosphate is then phosphorylated to ara-C diphosphate by deoxycytidine monophosphate kinase, and ultimately nucleoside diphosphate kinase phosphorylates it to ara-C triphosphate (ara-CTP) which is an active metabolite of ara-C (Garcia-Carbonero *et al*., 1996). Ara-C then incorporates into DNA strand in place of deoxycytidine triphosphate dCTP during the S-phase of the cell cycle, which results in the inhibition of DNA synthesis. Therefore the cytotoxicity property of cytarabine results from inhibition of DNA polymerase α and the incorporation of ara-CTP in place of dCTP (Yamauchi *et al*., 2009).

Intrinsic and extrinsic pathway leading to cell death mediated by cytarabine

Intrinsic pathway to cell death:

The intrinsic pathway of apoptosis triggered by DNA damage caused by nucleoside analogs results in mitochondrial-dependent activation of caspases, which executes the death program. Caspase activation causes the release of the *cytochrome c* from the mitochondria. Released *cytochrome c* triggers the formation of death complex apoptosome consisting of Apoptotic protease activating factor-1 (Apaf-1) and procaspase-9. Procaspase-9 activates the effectors caspase-3 and caspase-7 that leads to the characteristic apoptotic phenotype (Gunji *et al.*, 1991).

Ara-C mediated c-Jun N-terminal kinases (JNK) signalling directly participate in the intrinsic pathway leading to apoptosis by translocating to the mitochondria and interacting with the death proteins such as B-cell lymphoma 2 (Bcl) and second mitochondria-derived activator of caspases (SMAC). JNK regulates the

phosphorylation of p53 protein which regulates the mitochondrial apoptosome. The transcription factor p53 in turn causes induction of many pro-apoptotic genes such as Bcl-2–associated X protein (BAX), Bcl-2 Homology Domain 3 (BH3) and NOXA (Latin for damage) Noxa protein is also known as phorbol-12-myristate-13-acetate-induced protein 1(PMAIP1) (Fig 1.19) (Sampath *et al.,* 2003).

Extrinsic pathway to cell death:

The extrinsic pathway utilised by the nucleoside analogue to induce apoptosis require the participation of death receptors such as Fas receptor (FasR) that senses the death signal and translocated to the mitochondria. Ara-C once injected, causes upregulation of Fas and TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 without the requirement of p53 protein. Once induced, the death receptor causes activation of caspase-8 and caspase-3 that executes the cell death program (Sampath *et al.,* 2003) (Fig 1.19).

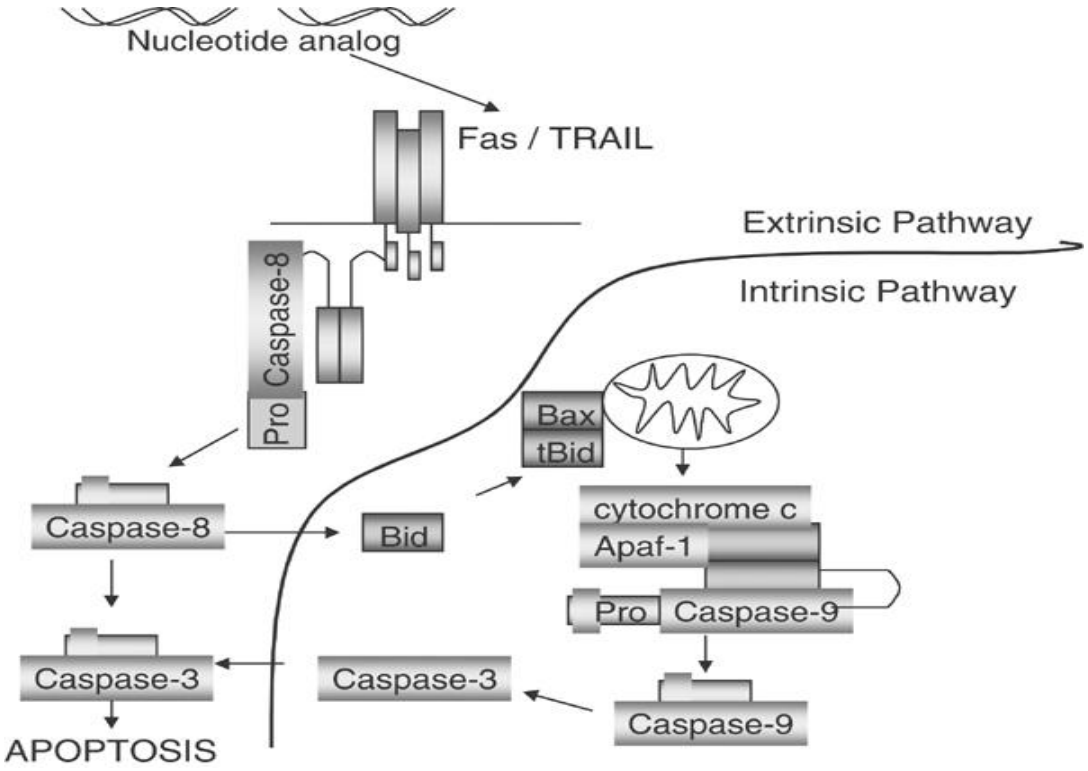


Figure 1.19: The nucleoside analogue induced extrinsic and intrinsic pathway cascades leading to cell death. (Adapted from Sampath *et al.,* 2003).

1.8 Enantiomer separation techniques

Chiral drugs require special analytical techniques that can differentiate between the various enantiomers at different levels (Wozniak *et al.,* 1991). There are different analytical techniques available depending on the type of analysis to be performed (Fig 1.20).

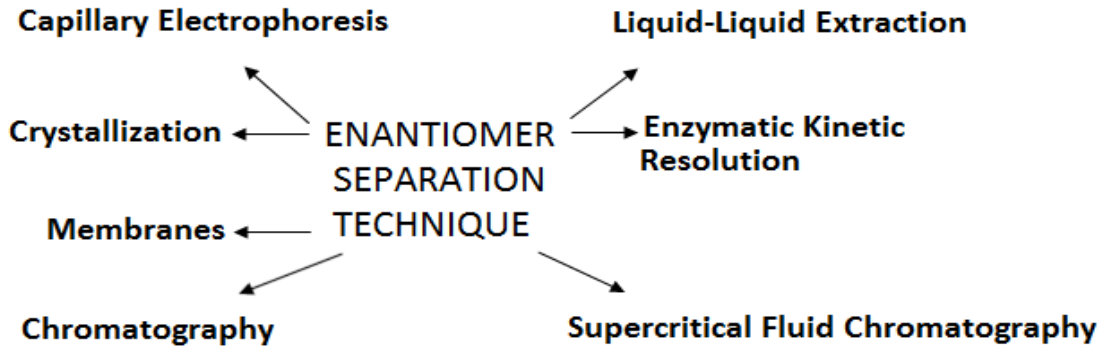


Figure 1.20: Some common enantiomer separation methods.

The conventional method of separating the optical isomers of racemic compounds involves the treatment of isomers with a chiral reagent to produce a pair of diastereomers (Sorrel, 2006). These diastereomeric intermediates can then be separated by differential crystallization, hydrolysis and purification (Lorenz *et al.*, 2007). Enzymatic Kinetic resolution uses enzymes like lipase, only one enantiomer of a chiral reactant fit to the active site properly and is able to undergo the reaction while the second enantiomer is left unreacted and in enantiomerically pure form. Enzymatic catalyzed kinetic resolution is the most common way to prepare enantiomerically pure compounds on an industrial scale (Martin-Matute and Backvall, 2007). Other techniques include differential scanning calorimetry (DSC), spectroscopy and chromatography. Infrared spectroscopy and differential scanning calorimetry can only discriminate between mixtures and individual enantiomers. The differential

scanning calorimetry uses the melting range of the analyte to distinguish between mixture of enantiomers and single enantiomer (Jacques *et al.,* 1981). Spectroscopic methods include infrared spectroscopy, nuclear magnetic resonance and optical rotation. Spectroscopic methods cannot trace low levels of enantiomeric impurities. It is furthermore difficult to differentiate between various isomers of compounds with one or more chiral centre. With optical rotation there is a requirement of a pure standard of one of the enantiomers. Chromatographic techniques are the most popular and most commonly used techniques for enantioselective analysis. It is a separation technique whereby the components of a mixture may be separated by allowing a sample (analyte) to be transported through a packed body of materials (stationary phase) by a fluid mobile phase (Heftmann, 2004). Chromatographic techniques can be divided into gas chromatography (GC), thin layer chromatography (TLC), supercritical fluid chromatography (SFC), capillary electrophoresis and high performance liquid chromatography (HPLC). In chiral liquid chromatography the most common detectors used are the ultra violet (UV), fluorescence and electrochemical detectors. Polarimetric detectors can be used in combination with chiral and non chiral liquid chromatography (Lloyd and Goodall, 1989; Salvadori *et al.,* 1991). The main advantage about chiral liquid chromatography is that it can be used for qualitative and quantitative work in combination with the polarimeter. It also provides reliable result accuracy compared to other techniques. These techniques except for infrared spectroscopy and differential scanning calorimetry can discriminate between individual enantiomers (+) from (-) and (-) or (+) from (+/-) and hence can be used for qualitative and quantitative analysis. This research utilizes HPLC for the separation anticancer reagent cytarabine.

1.9 High performance liquid chromatography (HPLC)

High performance liquid chromatography has been the fastest developing chromatographic technique over the past two decades (Fig 1.21). HPLC has very likely impacted the development of every drug candidate within the pharmaceutical industry. It is generally one of the most direct and efficient methods for the determination of enantiomeric purity (Karcher *et al*., 2005). The method is highly sensitive and accurate for quantitative determinations and is used extensively for substances that are of major interest to science, food industry, health care and forensics. HPLC can separate proteins, amino acids, hydrocarbons, pesticides, antibiotics, nucleic acids, steroids, metal and non metal organic substances (Krupadanam *et al*., 2001). It is comparatively fast as it forces the solvent to pass through the column under high pressure. Also it is highly automated and very sensitive. HPLC is more widely used compared to other chromatographic techniques, since the interaction between solute and chromatographic system takes place to a larger degree. This is due to the interactions favoured by lower operating temperatures. HPLC uses a broad choice of mobile phases and stationary phases. Furthermore, samples can be recovered without being destroyed. Its major advantage includes the reuse of columns.

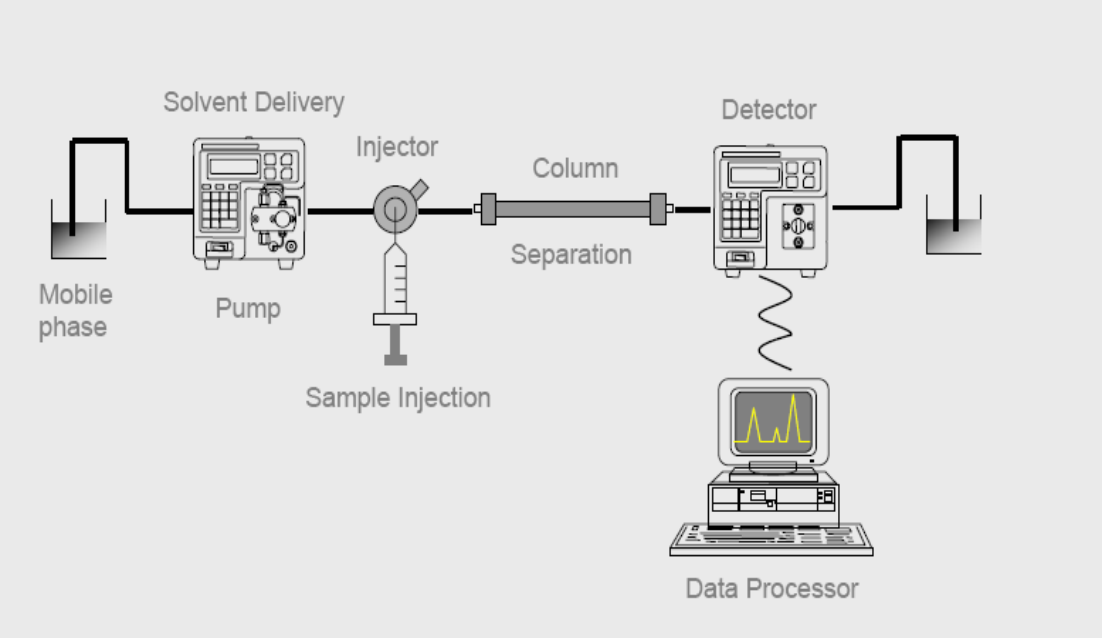


Figure 1.21: High Performance Liquid Chromatography system. (Adapted from http://www.jascofrance.fr/pdf/hplc.pdf).

1.9.1 HPLC components

A typical HPLC system includes the following components:

Mobile phase – it is the liquid flowing through the system which is chosen for the best separation of the components in an unknown mixture. This is chosen on the basis of polarity of the sample and the stationary phase.

Solvent reservoir – it is used for the storage of sufficient quantities of HPLC solvent for continuous operation of the HPLC system. It can include a degassing system and filters to keep the solvent isolated from the influence of environment.

Column – Based on the type of separation required an appropriate column is chosen. The column actually produces the separation of an analyte in the mixture. Different columns can produce different results due to varying composition of the packing material.

Column oven – usually a laboratory has an adequate climate control but temperature fluctuations can occur due to ventilation, heating or air conditioning. It is known that the column temperature plays an important role in HPLC retention and selectivity (Synder *et al*., 2009).

Pump – continuous, constant and pulse free flow of the mobile phase through the system is provided by the pump. Pumps are designed for either constant flow operation or constant pressure. As long as the resistance (column) remains constant, a constant pressure will produce a constant flow.

Injector - The analyte or analyte mixture is introduced into the stream of mobile phase before it enters the column through the injector. The maximum injection volume depends on the sample loop based on the injection valve.

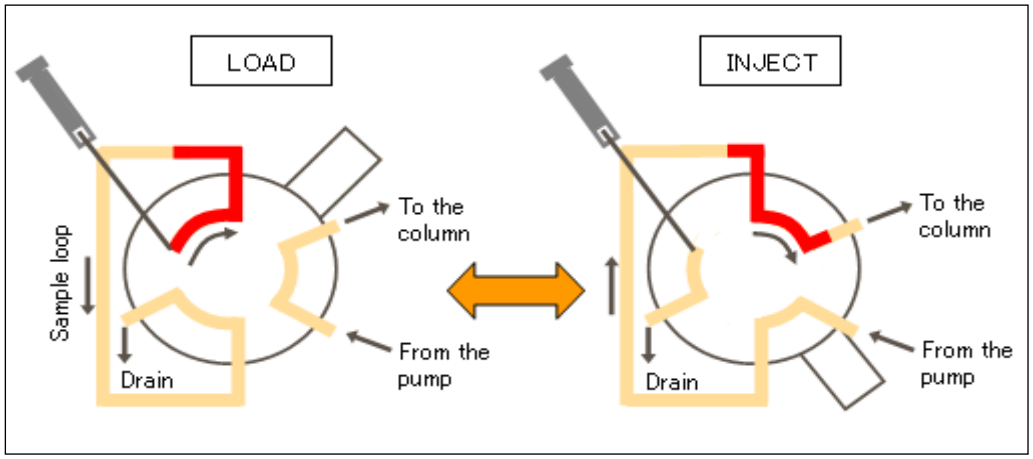


Figure 1.22: Diagram of a typical injector. (Adapted from http://www.hitachi-hitec.com/global/science/lc/img/lc_basic/lc_basic_3_3_e.gif).

For quantitative analysis it is always important to use a small loop and an overflow injection of the sample, so that the injector is fully saturated with the sample (Hanai, 1999).

Detector – this device allows continuous registration of specific physical (sometimes chemical) properties of column effluent. It is used for monitoring and continuous registration of UV absorbance over a span of wavelengths or a selected wavelength. Appearance of analyte in the detector causes the absorbance to change. A positive signal is obtained when the analyte absorbs greater than the mobile phase. They are widely used due to their relative low susceptibility to flow rate fluctuations.

Data acquisition and control system - these are computer based systems that control all parameters of HPLC instruments e.g. temperature, injection sequence. In addition, acquires data from the detectors and monitors system performance.

1.9.2 Working of HPLC

The technique involves separation of the components in a mixture pushed through the column usually at a constant flow rate using a liquid mobile phase. The solvent (mobile phase) is run through the high pressure pump which generates and measures the flow rate of the mobile phase in millilitres per minute (ml/min). The sample to be analysed is introduced in the injector from where it is introduced in the mobile phase flowing through the column consisting of chromatographic packing material. From the column the flow is directed to the detector. The components are then analyzed by the detector from where the chromatographic image is produced in the computer. The chromatogram produced displays the retention time and the peak area of the expected compound. The retention time is a characteristic of the sample for a particular set of conditions and helps in identification of the compound. Different compounds have different retention times based on the nature of the stationary phase, nature of mobile phase and the flow rate of mobile phase. The peak area of the

compound indicates its concentration (Lindsay and Barnes, 1992; Kazakevich and Lobrutto, 2007).

1.9.3 Direct and indirect mode of separation

The separation of enantiomers by chiral chromatography is divided into two modes indirect mode and direct mode. Both direct and indirect separation methods provide different options to achieve separation of enantiomers and their quantification. Methods are chosen on the basis of chemical structure of the solute. Indirect mode uses an off column conversion of the original enantiomers with an optically pure derivatizing agent by chemical reaction. It involves the formation of diastereomers before the separation. Diastereomers have different physical and chemical properties unlike enantiomers that have same physical and different chemical properties, thus enabling their separation in a non chiral environment. Indirect method requires a functional group in the molecule at which the derivatization can take place. The functional group must be located near the chiral centre. One of the advantages of the indirect mode of separation is that it can be performed on a traditional column such as C18. Its disadvantages are that the derivatization reaction requires lot of time and can result in unwanted products or racemisation. Furthermore optically pure derivatizing agents may not be available. Some derivatizing agents for indirect separation are amino acids, alcohols, carboxylic acid and amines.

The direct mode uses a chiral stationary phase for the chromatographic separation of enantiomers. In the chromatographic system it requires the creation of chiral environment. The direct mode involves different diastereomeric molecular association between the chiral non racemic stationary phase and the chiral analyte. For

enantiomers to be separated directly over chiral stationary phases (CSP), they must form short lived diastereomeric molecular complexes of non identical stability by interacting rapidly and reversibly (Francotte and Lindner, 2006). The present study involves the use of the direct mode of separation of the chiral anticancer reagent cytarabine.

1.10 Chiral stationary phases (CSP)

One of the most important and challenging aspects of chiral chromatography is to choose a good stationary phase. In chiral liquid chromatography, the separation of enantiomers provides a number of possibilities in experimental design and there are many stationary phases that can be used in combination with different mobile phase system to achieve resolution (Lammerhofer, 2010). Generally in chiral liquid chromatography, the column consists of small solid particles called sorbent and its purpose is to exhibit different affinities towards the two enantiomers in the column, and hence a difference is created between their retention times large enough to separate enantiomers. When any chiral compound passes through the chiral column it will be partitioned between the mobile phase and the sorbent used for elution and the affinity of each enantiomer will determine the time spent in the column during separation. While choosing a chiral stationary phase many important factors should be considered such as solubility of samples in mobile phase, structural characteristics of the analyte, cost of the column, mobile phase, waste treatment, analysis time, transferability or method robustness (Ahuja and Rasmussen, 2007).

The direct separation method used to separate chiral compounds using HPLC is extensively used in the pharmaceutical industry and in biochemistry

(Tachibana and Ohnishi, 2001). A large number of chiral stationary phases have been developed since the last 25 years such as ligand exchange, brush type, cyclodextrin, protein and polysaccharide types CSP (Lammerhofer, 2010).

1.10.1 Brush-type columns

Silica can be derivatized on almost any functional group; the resulting monomer structures are called brushes. It is assumed that silica based, brush-type stationary phases are characterized by a monomolecular organic layer on the silica surface, resulting from the attachment of a single chiral molecule to the silanol groups (Gasparrini *et al*., 2001). It was invented by William H. Pirkle, and is often referred to as pirkle phase. The chiral stationary phase in brush-type is composed of various selectors capable of ionic or covalent bonding. These types of chiral stationary phase preceded all the other types of chiral stationary phases historically. The structure of these stationary phases is based on a single strand of chiral selectors which posses either p-acceptor or p-donor aromatic fragments, together with dipole stacking inducing structure and hydrogen bonding agents. These are connected via amidic linkage onto aminopropyl silica.

1.10.2 Cyclodextrin columns

Cyclodextrins are produced by the partial degradation of starch and the enzymatic coupling of cleaved glucose unit into crystalline, homogenous toroidal structures of different molecular sizes. The α, β and γ are the most widely characterized containing 6, 7 and 8 glucose units respectively which are chiral (Fig 1.23) (Meyer, 2010). The cyclodextrin molecules are arranged to form a truncated cone. The cavity in

cyclodextrins is composed of glucoside oxygen and methylene hydrogen, giving it an apolar character. The external surface of the cavity is hydrophilic surrounded by hydroxyl groups. Normal phase type solvents can be used for cyclodextrin but most commonly aqueous solutions mixed with organic solvents are used. For chiral resolution to occur hydrogen bond region of the molecule must interact with mouth of cavity and a portion of molecule must enter hydrophobic cavity. It is important for the solute to have one aromatic ring if the separation is of reversed phase type because apolar molecule segments become attracted to apolar cavities. Hence cyclodextrin based CSP, due to their ability to separate enantiomers of many chiral compounds and especially neutral chiral molecules with aromatic units are widely used (Beesley and Scott, 1998).

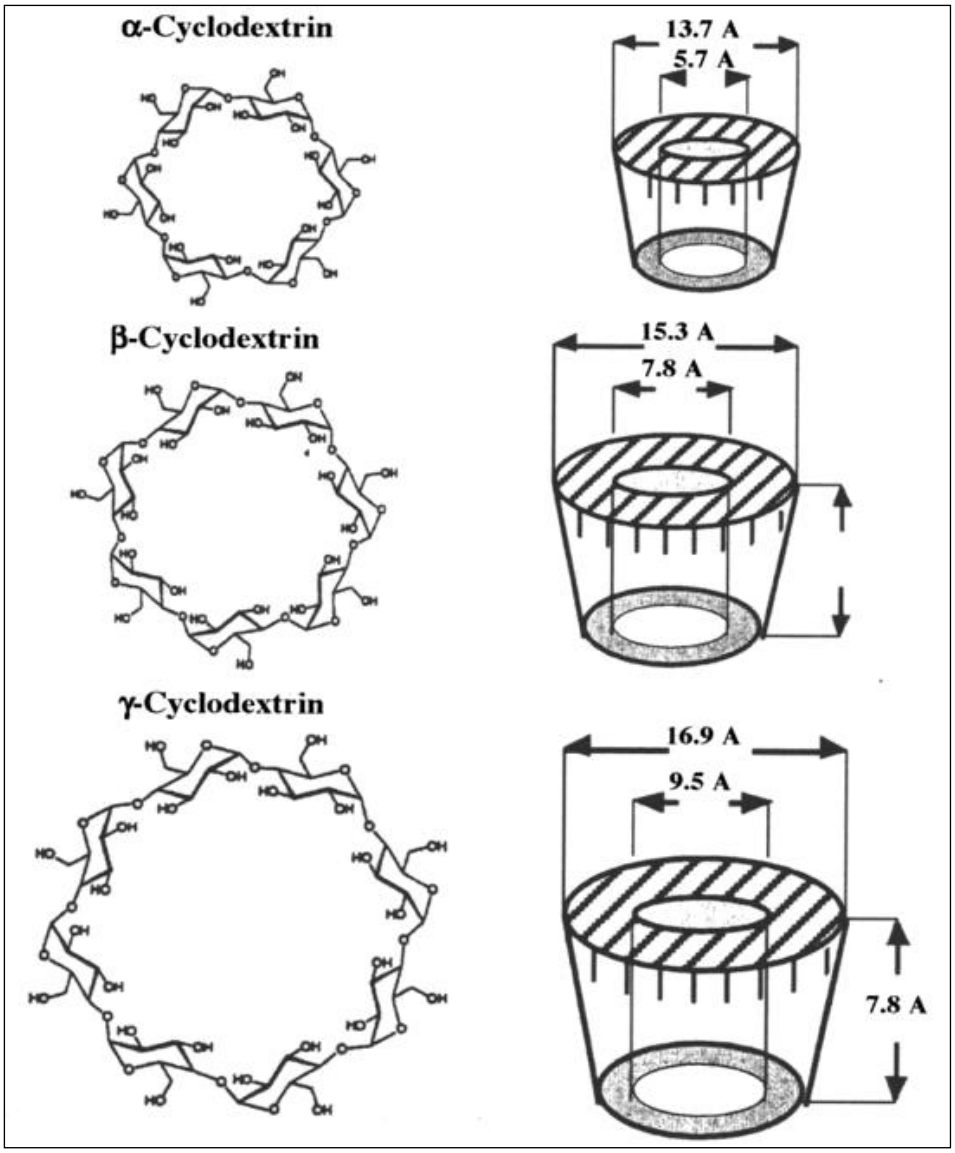


Figure 1.23: The molecular structure of α, β and γ cyclodextrins. (Adapted from http://www.chromatography-online.org/Chrial-GC/Contemporary-Chiral-Stationary-Phases/Cyclodextrin.html).

1.10.3 Ligand exchange phases

In ligand exchange chiral stationary phases, an amino acid such as L-proline is bonded to the silica gel support. This is a general approach to separate enantiomers that forms chelates with transition metal ions. Based on its high enantioselectivity chiral ligand exchange stationary phase was once the principle method to separate amino acid enantiomers (Poole, 2003). Factors such as temperature, ionic strength of mobile

phase pH affect the selectivity and efficiency of separation using ligand exchange phases. This method is mainly used for separation of α and β amino acids, hydroxy acids, amino alcohols and diamines (Gehrke and Wixom, 2010). It can separate such compounds because these compounds possess two polar functional groups and adequate spacing (Meyer, 2010). Ligand exchange columns are based on interactions between enantiomers to be separated during the formation of coordination bonds between complex forming metal ion present in solute and stationary phase. The differences in stability between complexes with R and S form of a solute result in separation of enantiomers. Enantioseparation can be carried out when a stationary phase contains chiral ligand metal complex. The metal complex used is mostly Cu^{2+} amino acid complex. For separation to be positive the solute must have two polar functional groups with correct spacing that can simultaneously act as ligand for copper ion.

1.10.4 Protein based chiral stationary phase

Proteins are complex, high molecular weight polymers composed of chiral amino acids. In protein based chiral stationary phases separation is based on combination of hydrophobic and polar interactions. Their enantioselective properties are based on a combination of the bio affinity which is hydrogen bonding, electrostatic interaction and hydrophobic interactions. It involves the use of protein immobilized on the surface of the silica gel. This protein can be either physically adsorbed or covalently bonded to silica gel (Ahuja and Rasmussan, 2007). These phases use stereoselective properties of proteins. The four types of proteins used in this kind of separation include bovine serum albumin, human serum albumin, ovamucoid proteins and human alpha acid

glycoprotein. Protein phases are expensive and delicate in handling and their loadabilities and performances are low (Meyer, 2010).

1.10.5 Polysaccharide based stationary phase

Polysaccharide based stationary phases are amongst the most used CSP's in organics, bio organics and pharmaceutical chemistry (Yashima, 2001). They are considered the leaders in the chiral separation science due to their remarkable recognition capabilities. The main polysaccharides are cellulose, amylose, chitosan, xylan, curdlan, dextrin and inulin (Yashima, 2001). Among these, cellulose and amylase approved to be the best polymers due to their good capabilities of chiral resolution (Fig 1.24) (Okamoto and Yashima, 1997). They consist of D-glucose units linked by 1-4 glucosidic bonds. Strands around chiral glucose can be formed by the derivatization of three hydroxyls on each glucose unit. The derivatized glucose unit can act as chiral site which can discriminate between enantiomers that act differently with strands. The derivatized polysaccharide chiral stationary phase supported on silica gel are made by the reaction of benzoyl chloride or a phenyl isocyanate with polysaccharide in homogenous conditions and by evaporation. These derivatives are then coated from a solution onto a macroporous γ-aminopropylsilica matrix (Franco *et al.*, 2001).

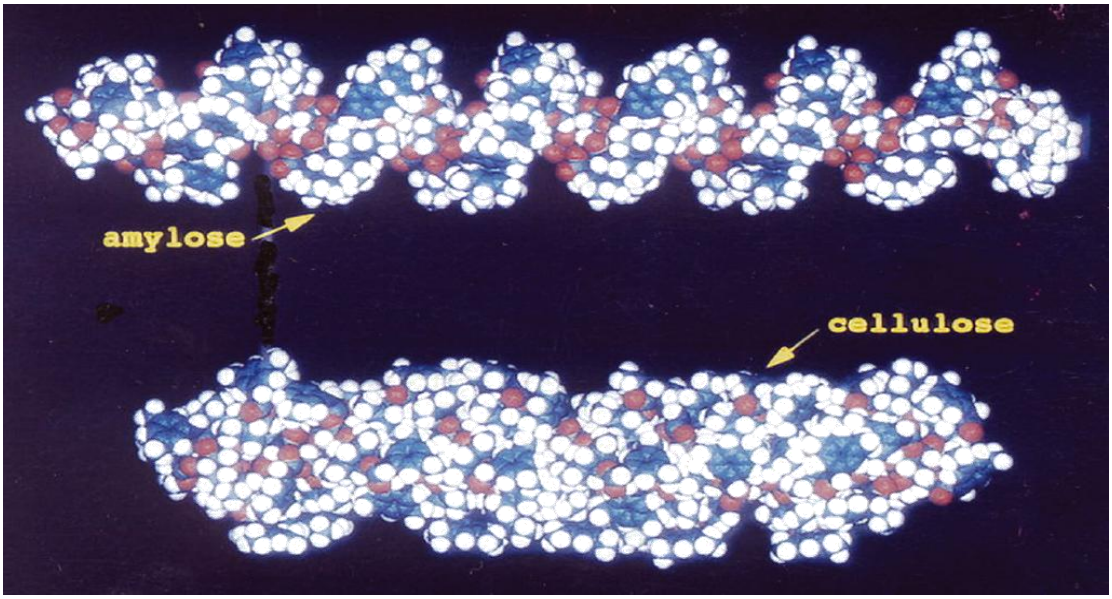


Figure 1.24: The three dimensional structure of amylose and cellulose (Adapted from-http://www.informaworld.com/smpp/section?content=a911179964&fulltext=713240928).

The derivatized polysaccharide coated on silica gel was relatively good because its chiral selectivity was not affected by dissolving the polysaccharide in organic solvents. Selectivity could be enhanced by using various kinds of substituent on the hydroxyl group of polysaccharide and by coating them on solid support such as silica support-aminopropylsilica. The interactions such as π-π interactions, hydrogen bonding, dipole-dipole stacking interactions are more effective under normal phase chromatography. The polysaccharide type chiral stationary phase can be used with normal phase eluents like hexane and ethanol or 2-propanol mixtures. The use of alkane: alcohol is possible since there is no ionic functional group in the polysaccharide chiral stationary phase and aqueous eluents can be used in reversed phase chromatography mode.

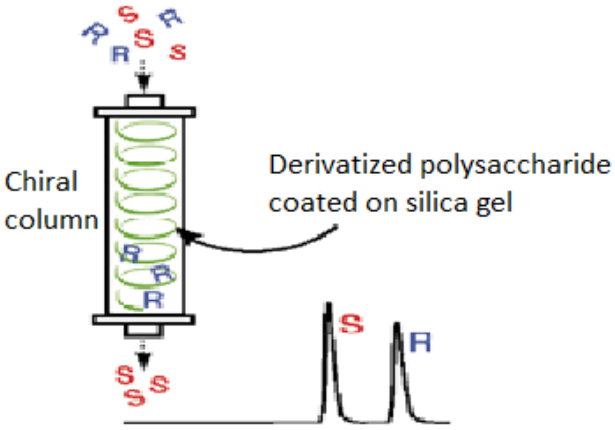


Figure 1.25: The separation of a mixture of enantiomers using polysaccharide based chiral stationary phase (Adapted from http://www.chemistry.or.jp/journals/bcsj/bc-cont/bc77-2.html).

Although polysaccharide coated on silica gel exhibit high chiral separation abilities they are compatible with only a limited number of solvents that can be used as mobile phase such as alkanes, alcohols, acetonitrile and their mixtures. Other solvents such as ethers, ketones, esters and chlorinated alkanes dissolve the polysaccharide and wash off the selector from the silica (Thunberg *et al.*, 2008). This limited use of solvents is a serious disadvantage of these chiral stationary phases, therefore suitable selection of solvents is very important for attaining chiral separation. Some of the commonly used stationary phases for primary normal phase screening, are Chiralpak AD-H, Chiralpak AS-H, Chiralcel OJ-H and Chiralcel OD-H (Ahuja, 2010). These columns can resolve a number of structurally different compounds and depict high loading capacities making them suitable for application in analytical as well as preparative scale resolution of enantiomers (Yamamoto and Okamoto, 2004; Francotte, 2005).

1.10.6 Columns used in this study

The three columns used in this study are commercially known as Chiralpak AD-H, Chiralcel OD-H and Chiralcel OJ-H columns. Several derivatives are available but these three have very complementary properties and numerous publications have demonstrated that they are able to achieve a chiral resolution of more than 80 % for drugs currently available on the market (Perrin *et al*., 2002).

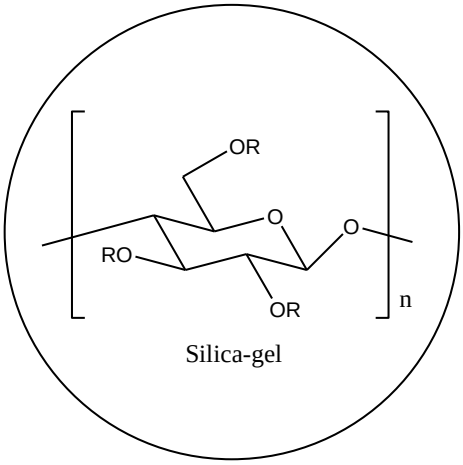


Figure 1.26: The chemical structure of silica gel used in preparation of Chiralcel OD-H and Chiralcel OJ-H columns.

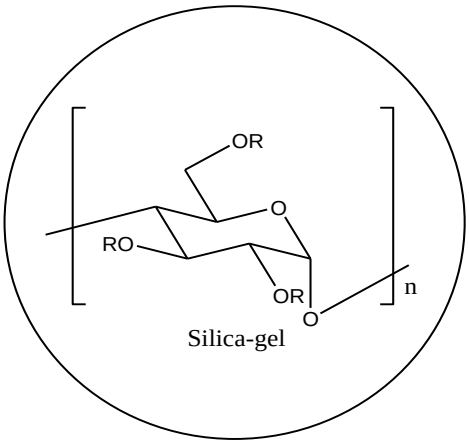


Figure 1.27: The chemical structure of silica gel used in preparation of Chiralpak AD-H.

1.10.6.1 Chiralpak AD-H

The packing composition of Chiralpak AD-H consist of amylose tris (3, 5-dimethylphenylcarbamate) coated on 5 µm silica gel.

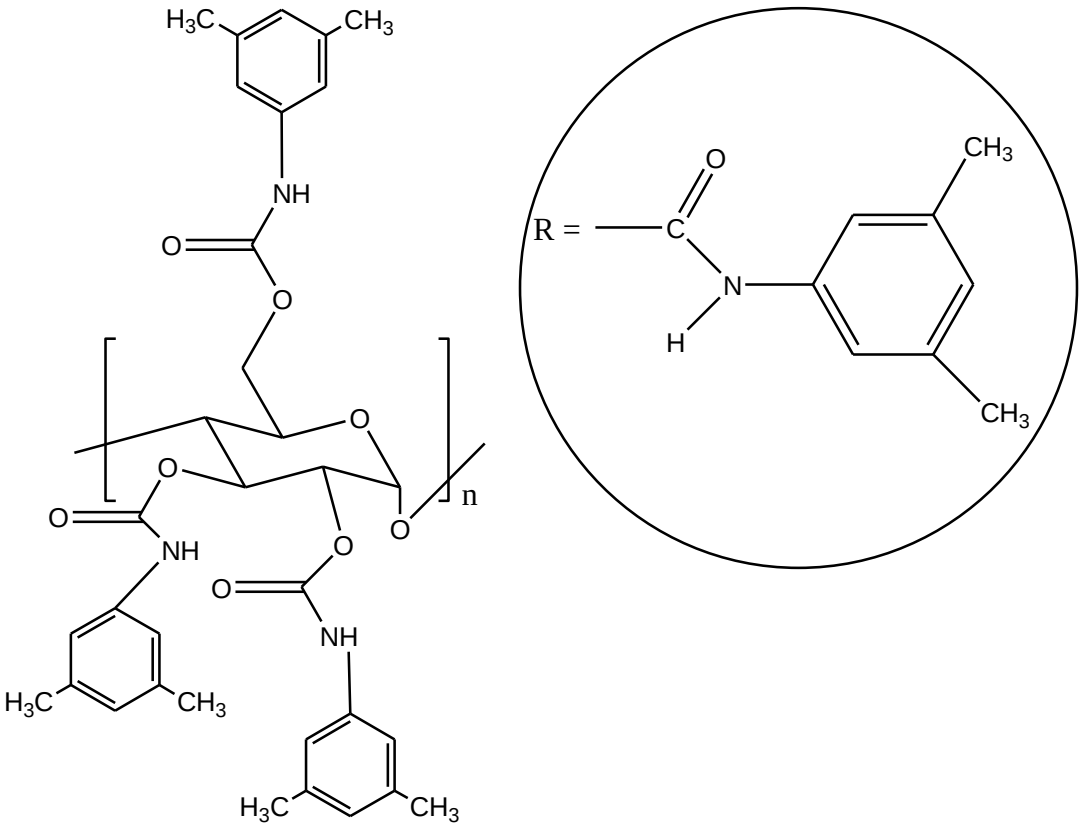


Figure 1.28: The chemical structure of amylose tris (3, 5-dimethylphenylcarbamate) coated on silica gel.

1.10.6.2 Chiralcel OD-H

The packing composition of Chiralcel OD-H consist of cellulose tris (3, 5-dimethylphenylcarbamate) coated on a 5µm silica gel.

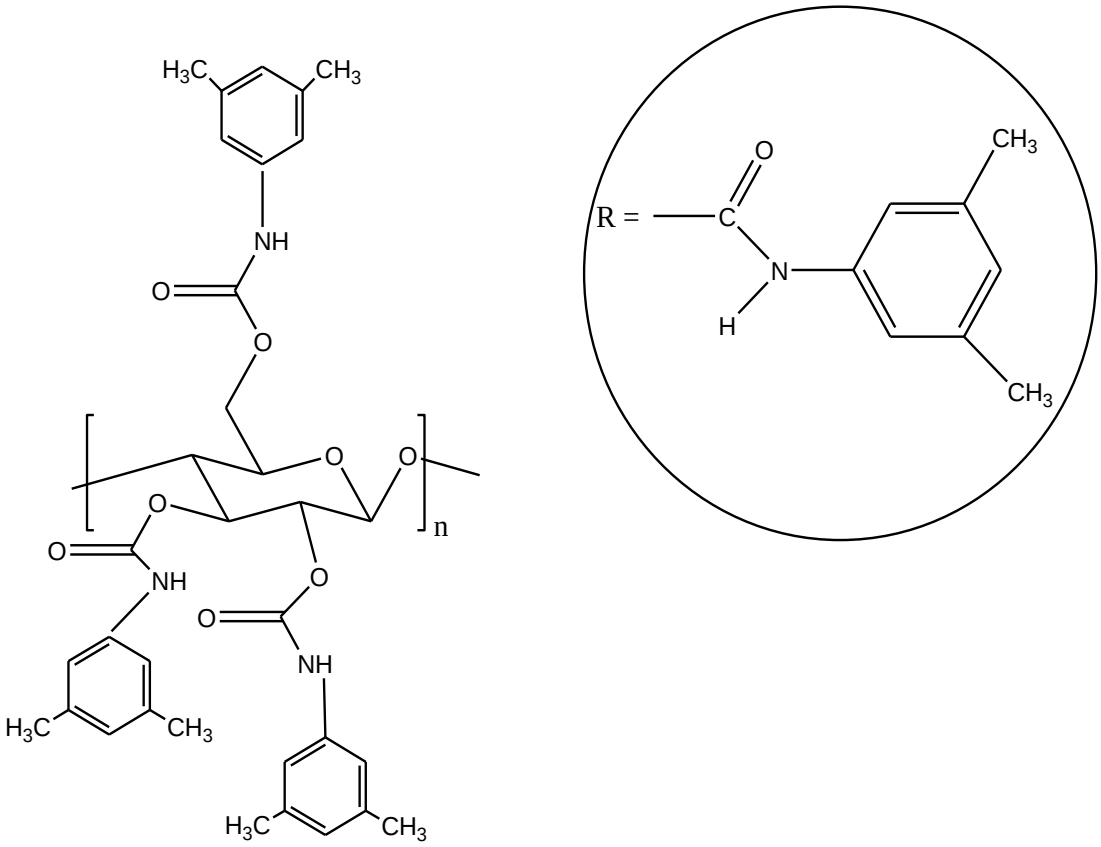


Figure 1.29 The chemical structure of cellulose tris (3, 5-dimethylphenylcarbamate) coated on silica gel**.**

1.10.6.3 Chiralcel OJ-H

The packing composition of Chiralcel OJ-H consist of cellulose tris (4-methylbenzoate) coated on a 5µm silica gel.

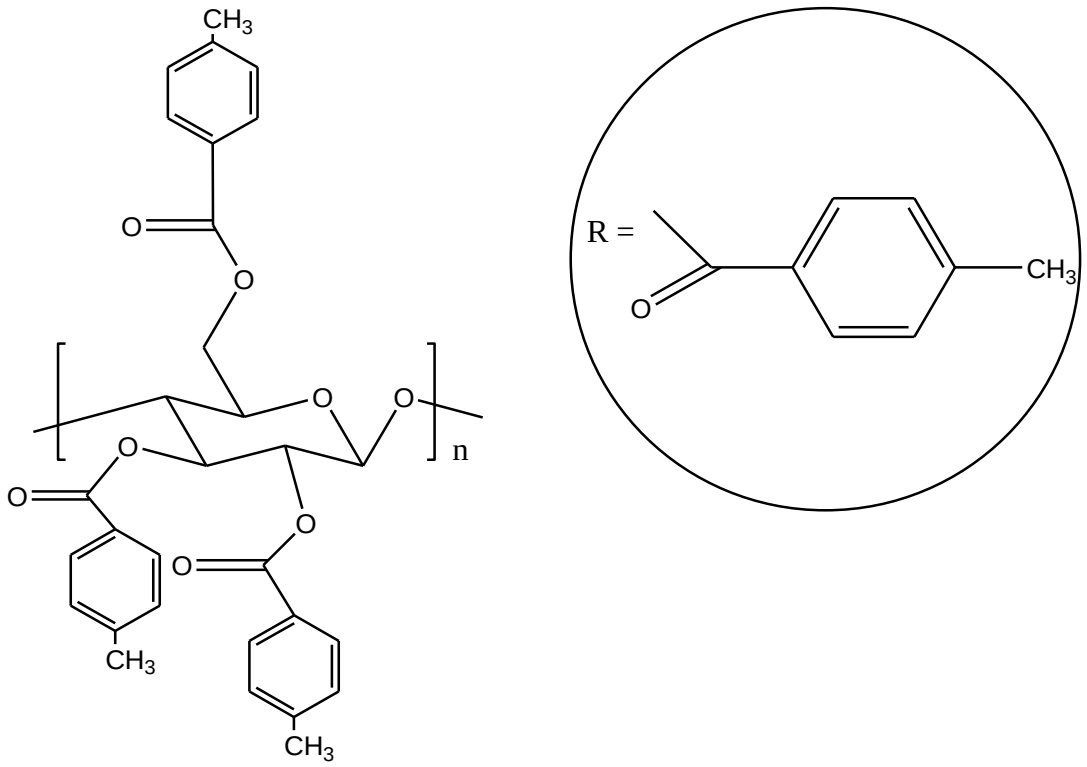


Figure 1.30: The chemical structure of cellulose tris (4-methylbenzoate) coated on silica gel.

1.11 Chiral additives

In the direct separation method chiral additives interact with the solute and form some kind of complex. This formation of additive solute complex results in pair of diastereomers which has different behaviour patterns in HPLC system. Diastereomers show different properties such as interaction with stationary phase and solubility in mobile phase. These differences help to separate the enantiomers of the solute. Additives are used in chiral HPLC employing polysaccharide based chiral stationary

phases under normal phase conditions (Ye *et al.*, 2004). A maximum of 1 % (v/v) acidic and basic additives are frequently incorporated into mobile phases to reduce tailing of polar analytes (Tang *et al.,* 1998). Compounds having amine groups require basic mobile phase additive such as triethylamine (TEA) and diethylamine (DEA) to avoid tailing. These are added to typical hexane: alcohol mixtures to obtain satisfactory chiral selectivity, whereas, for acidic compounds acidic additive such as acetic acid and trifluoroacetic acid could be used in the mobile phase for better efficiency and elution in normal phase mode (Ye *et al.*, 2004).

1.12 Types of liquid chromatography phases

The two common types of HPLC are normal phase liquid chromatography and reversed phase liquid chromatography. These two types can be distinguished on the basis of relative polarities of the mobile and stationary phase (Lindsay and Barnes, 1992).

1.12.1 Normal phase liquid chromatography

Normal phase liquid chromatography being the oldest chromatographic technique uses a highly polar stationary phase e.g. silica gel. The mobile phase is a non-polar or a low polarity solvent, such as hexane. These solvents are water immiscible and have low polarity. This technique separates the molecules based on their strength of interaction with the polar stationary phase. These solvents usually are used as a mixture. In normal phase liquid chromatography the non polar compounds elute faster than the polar compounds (Fig 1.31) (Krupadanam *et al.,* 2001).

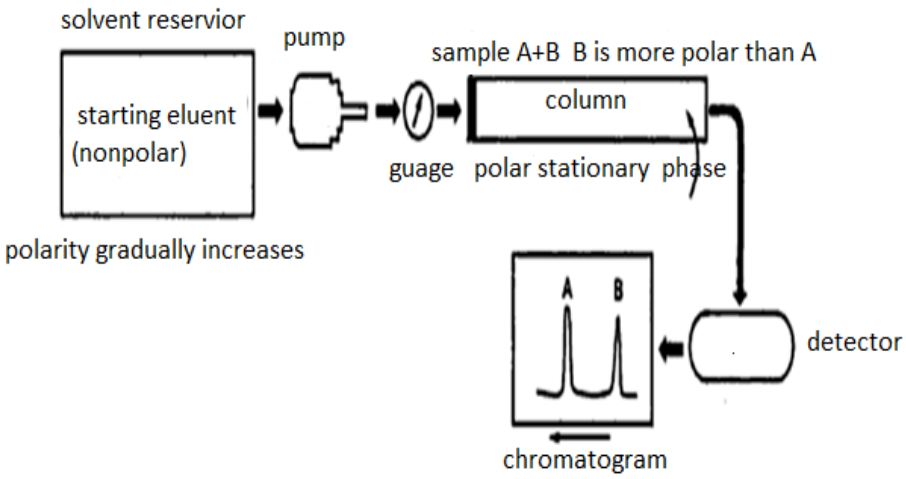


Figure1.31: The normal phase chromatographic run, the non polar compound elute faster than the polar compound (Adapted from Krupadanam *et al.,* 2001).

1.12.2 Reversed phase liquid chromatography

In reversed phase liquid chromatography, the stationary phase is non polar such as non-polar hydrocarbons, waxy liquids or bonded hydrocarbons such as C18, C8 or C4. While the mobile phase is a relatively polar solvent such as methanol, water, acetonitrile, or tetrahydrofuran (Skoog and Leary, 1992; Haghedooren *et al.,* 2007). There is a wide variety of presently available reversed phase packing system that differs by the way they are bonded and their ligand type (Claessen, *et al*., 1998). In reversed phase liquid chromatography the polar compounds elute faster than the non- polar compounds (Fig 1.32).

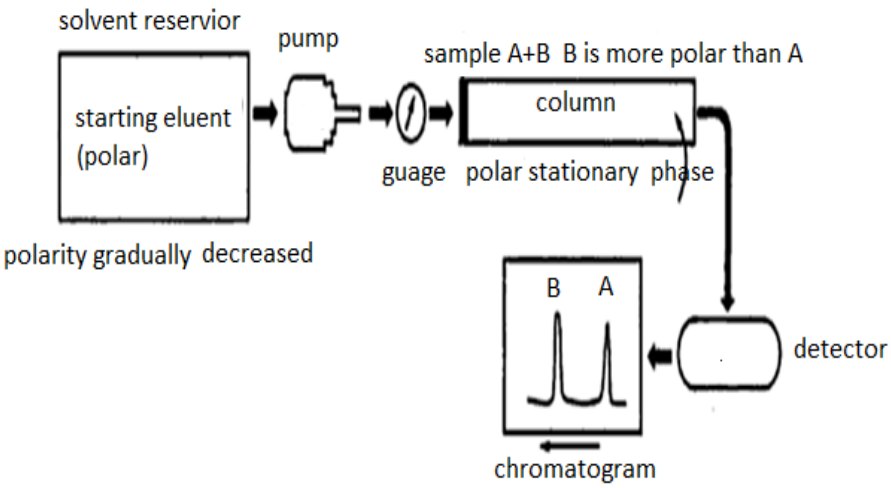


Figure1.32: The reversed phase chromatographic run, the polar compound elute faster than the non polar compound (Adapted from Krupadanam *et al*., 2001).

1.13 Elution mode in HPLC

After choosing an appropriate stationary phase, modifiers and additive, the next most important part in liquid chromatography system is to choose an eluent flow mode that can provide separations in suitable time scales. There are two modes of elution used namely, isocratic and gradient mode (Schellinger and Carr, 2006). These modes of separation can employ both normal and reversed phase systems. Isocratic elution is performed without changing the mobile phase composition; hence the mobile phase composition remains constant throughout the chromatographic run. In gradient mode, there are steady changes in the composition of mobile phase. In gradient mode of elution desired solvent polarity at specific times can be programmed and predetermined (Krupadanam *et al*., 2001).

1.13 The aims and hypothesis of the research

1.13.1 Working hypothesis

Is it possible to develop a chiral HPLC method in order to separate specific enantiomers of the anticancer reagent cytarabine using chiral HPLC.

1.13.2 Main aim

The main aim of the project was to utilize and possibly modify the separation of specific enantiomers of anticancer reagent cytarabine using chiral HPLC.

1.13.3 Specific aims of the research

1. To develop a HPLC stability-indicating method to separate enantiomers of the anticancer reagent cytarabine.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Chemicals and reagents used in the study are detailed in Table 2.1. All chemicals and reagents were of analytical grade and were handled and stored as recommended by the suppliers.

Table 2.1 Chemicals and reagents.

Name	Supplier
HPLC grade ethanol	Fisher scientific (UK)
HPLC grade *n*-hexane	Fisher scientific (UK)
HPLC grade 2-propanol	Fisher scientific (UK)
HPLC grade water	Fisher scientific (UK)
HPLC grade acetonitrile	Fisher scientific (UK)
Methylated spirit	Fisher scientific (UK)
Diethyl amine (DEA)	Sigma Aldrich (UK)
Sucrose	Sigma Aldrich (UK)

2.1.2 Drugs

Drugs used in this study are listed in Table 2.2.

Table 2.2 Drugs.

Name	Molecular weight	Supplier
Cytarabine	243.22	Sigma Aldrich (UK)
Trans-stilbene oxide	196.24	Sigma Aldrich (UK)

2.1.2.1 Trans-stilbene oxide

The standard used in this study to test the column efficiency was trans-stilbene oxide.

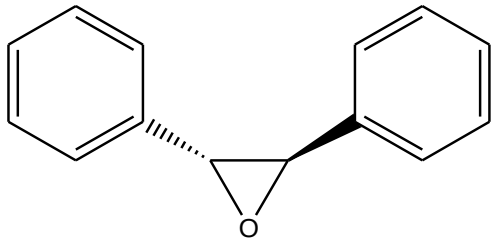


Figure 2.1: The chemical structure of trans-stilbene oxide.

2.1.3 Equipment

Equipments used in the study are listed in Table 2.3.

Table 2.3 Equipment**.**

Equipment	Type	Supplier
Analytical balance	Balance P/PI-114	Denver instruments (UK)
Sonicator	Ultra sonicator-ULT065	Ultrawave (UK)
HPLC Pump	Jasco PU-2028	Jasco (UK)
HPLC Pump	Jasco PU-1580	Jasco (UK)
HPLC Detector	Jasco UV-975	Jasco (UK)
HPLC Detector	UV-970M	Jasco (UK)
Quaternary Gradient Unit	LG 1580-04	Jasco (UK)
HPLC Printer	Hewlett Packard Desk Jet 890C	Hewlett Packard (UK)
pH Meter	pH meter 220	S & M Corning (UK)
HPLC columns	Chiralcel OD-H	Chiral technologies (Europe)
	Chiralcel OJ-H	Chiral technologies (Europe)
	Chiralpak AD-H	Chiral technologies (Europe)
Water bath	Nuve water bath *nb* 5	Biotech lab (Bulgaria)
Polarimeter	Jasco P2000	Jasco (UK)

2.2 Methods

2.2.1 Polarimeter

The polarimeter used for the measurement of optical rotation of cytarabine was a Jasco P2000 (Fig 2.2).

2.2.1.1 Sample preparation of sucrose for polarimeter

The sucrose sample was prepared by transferring 5 g of the compound into a 100 ml volumetric flask dissolving in water and then making up to the mark with the water to afford 5 g % solution of sucrose (0.05 g/ml).

2.2.1.2 Sample preparation of cytarabine for polarimeter

The cytarabine sample was prepared for polarimeter by transferring 100 mg of the compound into a 10 ml volumetric flask dissolving in water and then making up to the mark with the water to afford a concentration of 10 mg/ml.

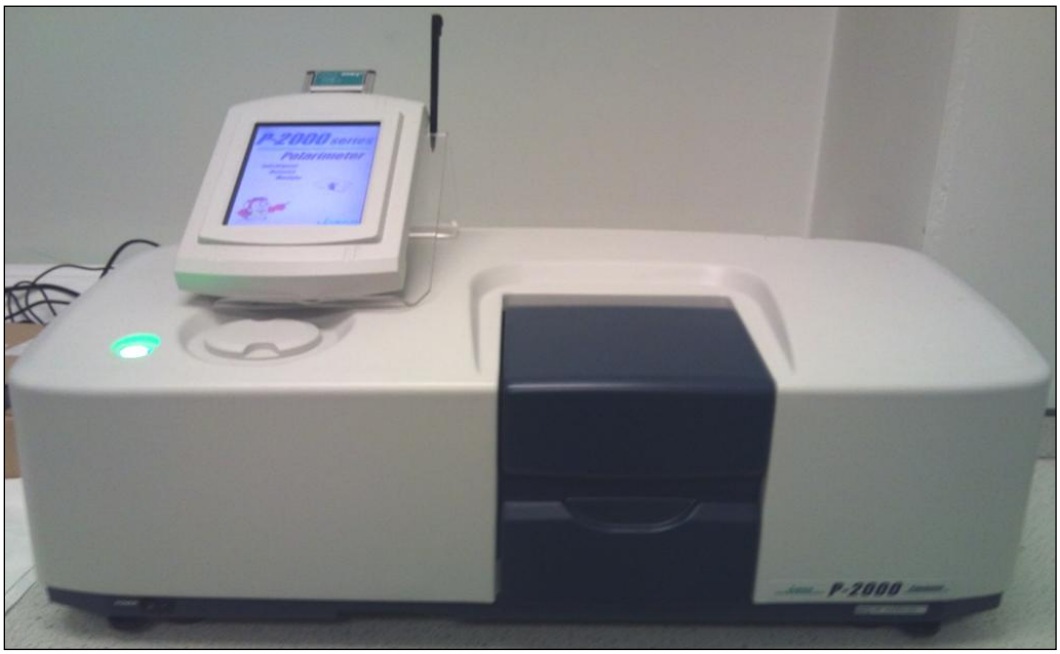


Figure 2.2: Jasco P2000 polarimeter used for measurement of optical rotation of cytarabine.

2.3 HPLC apparatus

2.3.1 Isocratic flow system

The HPLC system used in the study consisted of a Jasco PU-2028 Intelligent prep pump coupled to a UV-975 Intelligent UV/VIS Detector, DEGASYS- DG-3210 degasser, a Nokia 447ZA plus computer with an AZUR version 5.0.10.0 software package for the separation of cytarabine. The printer used was a Hewlett Packard Desk Jet 890C (Fig 2.3).

Figure 2.3: High Performance Liquid Chromatography system used for sample analysis using isocratic elution.

2.3.2 Gradient flow system

The HPLC system used in the study consisted of a Jasco PU-1580 pump attached to a Jasco UV-970M (4-λ intelligent), a Jasco LG 1580-04 Quaternary gradient unit and a Viglen Pentium-4 contender with an AZUR version 5.0.10.0 software package for separation of cytarabine (Fig. 2.4). The printer used was a Hewlett Packard DeskJet 890C.

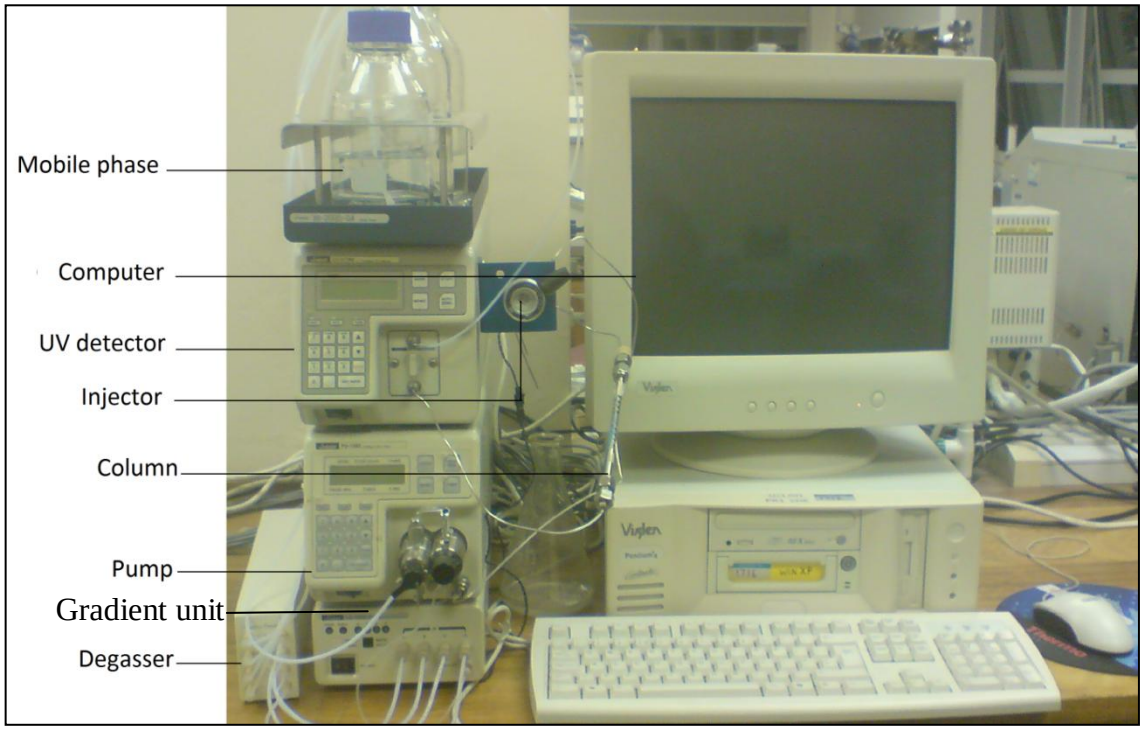


Figure 2.4 High Performance Liquid Chromatography used for sample analysis using gradient elution.

2.3.3 HPLC column

The present work was aimed at investigating the performance of the three chiral columns for the enantioseparation of cytarabine. The columns were Chiralcel OD-H, Chiralcel OJ-H, and Chiralpak AD-H. The dimensions of all three columns were 250 mm x 4.6 mm.

Table 2.4 Type of chiral columns.

Column	Type of adsorbent	Particle size (µm)
Chiralcel OD-H	Cellulose tris (3,5-dimethylphenylcarbamate)	5
Chiralcel OJ-H	Cellulose tris (4-methylbenzoate)	5
Chiralpak AD-H	Amylose tris (3,5-dimethylphenylcarbamate)	10

The analysis using these columns were carried out only when the column had been equilibrated (the time allowed to ensure that the column had equilibrated was approximately 30-60 min at a flow rate of 1ml/min). Manual injections were carried out using a Rheodyne model 7725i injector with a 20 µl loop. The detector was set to monitor at wavelengths 254 nm for all the chromatographic analysis.

2.4 Mobile phase used in the analysis of cytarabine

2.4.1 Mobile phases used for the isocratic flow system

a) Hexane: 2-propanol (v/v) in the ratio of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70.

b) Hexane: ethanol (v/v) in the ratio of 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70.

c) Hexane: 2-propanol: diethyl amine (v/v/v) in the ratio of 40: 60: 0.2, 50: 50: 0.2, 60: 40: 0.2, 70: 30: 0.2.

d) Hexane: ethanol: diethyl amine (v/v/v) in the ratio of 40: 60: 0.2, 50: 50: 0.2, 60: 40: 0.2, 70: 30: 0.2, 80: 20: 0.2.

e) Acetonitrile: 2-propanol: diethyl amine (v/v/v) in the ratio of 95: 5: 0.2 (pH - 12.7), 90: 10: 0.2 (pH - 12.2), 85: 15: 0.5 (pH - 12.5).

f) Acetonitrile: ethanol: diethyl amine (v/v/v) in the ratio of 95: 5: 0.2 (pH - 12.2), 90: 10: 0.2 (pH - 12.0), 85: 15: 0.5 (pH - 12.0).

The mobile phases were sonicated and filtered before use and each of the composition was run at flow rates of 0.5, 1.0 and 1.5 ml/min.

2.4.2 The mobile phase combinations used for the Gradient system using Chiralpak AD-H column

Varying combinations of hexane and alcohol (2-propanol or ethanol) were used.

a.

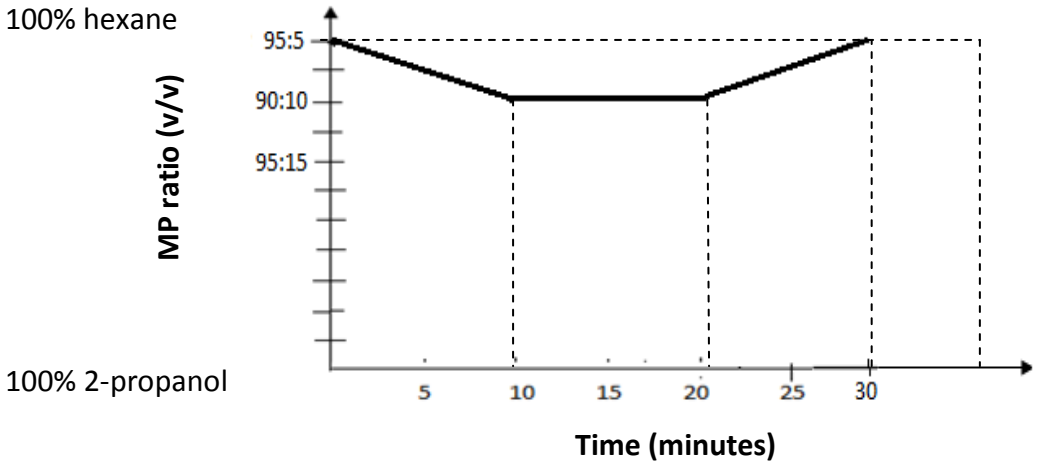


Figure 2.5: The gradient conditions ranging from 5 % to 10 % of 2-propanol in 30 min. MP ratio – hexane: 2-propanol.

b.

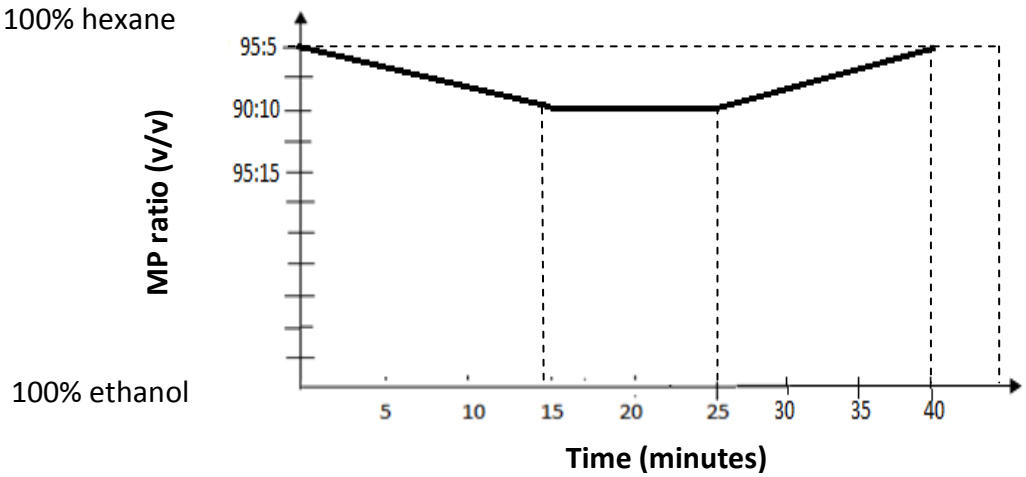


Figure 2.6: The gradient conditions ranging from 5 % to 10 % of ethanol in 40 min. MP ratio – hexane: ethanol.

c.

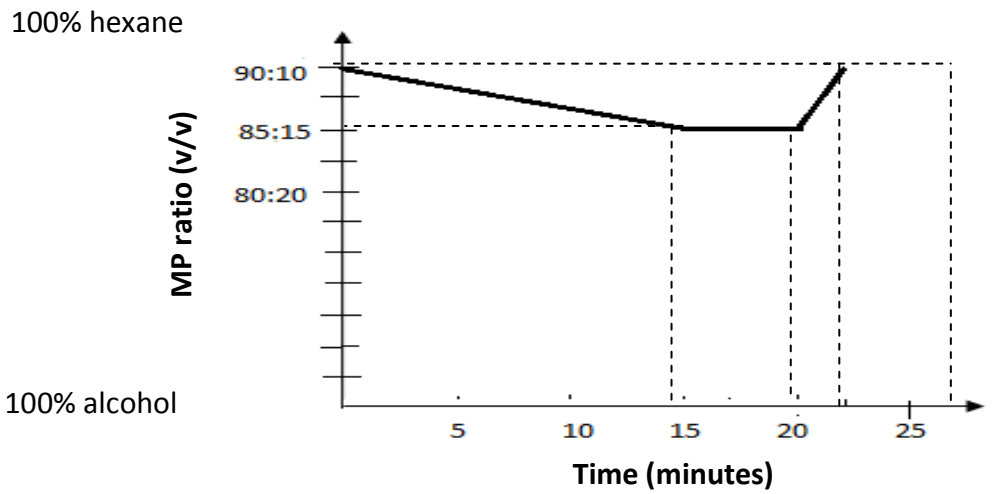


Figure 2.7: The gradient conditions from 10 % to 15 % of alcohol in 22 min. MP ratio – hexane: alcohol (2-propanol or ethanol).

d.

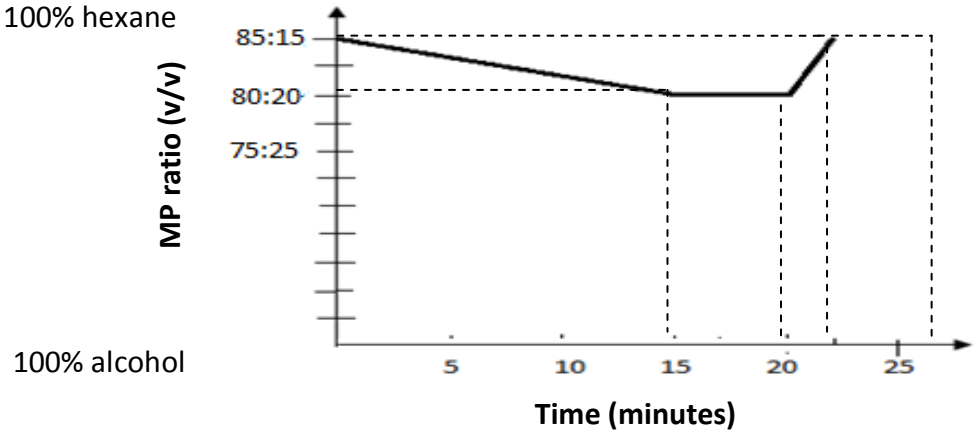


Figure 2.8: The gradient conditions for 15 % to 20 % of alcohol in 22 min. MP ratio – hexane: alcohol (2-propanol or ethanol).

e.

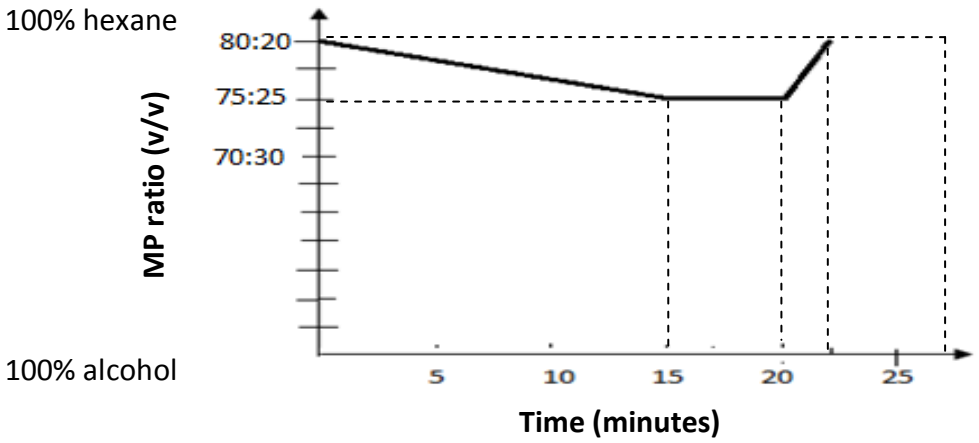


Figure 2.9: The gradient conditions for 20 % to 25 % of alcohol in 22 min. MP ratio – hexane: alcohol (2-propanol or ethanol).

f.

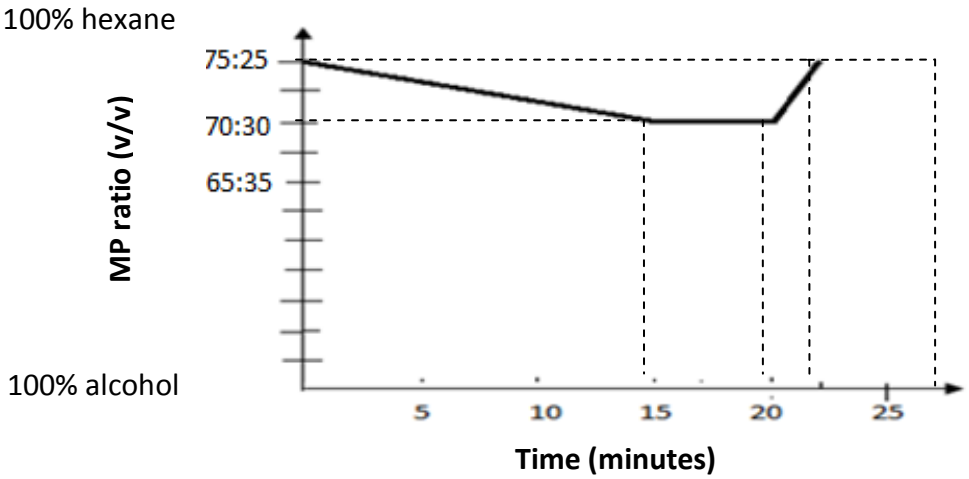


Figure 2.10: The gradient conditions from 25 % to 30 % of alcohol in 22 min. MP ratio – hexane: alcohol (2-propanol or ethanol).

g.

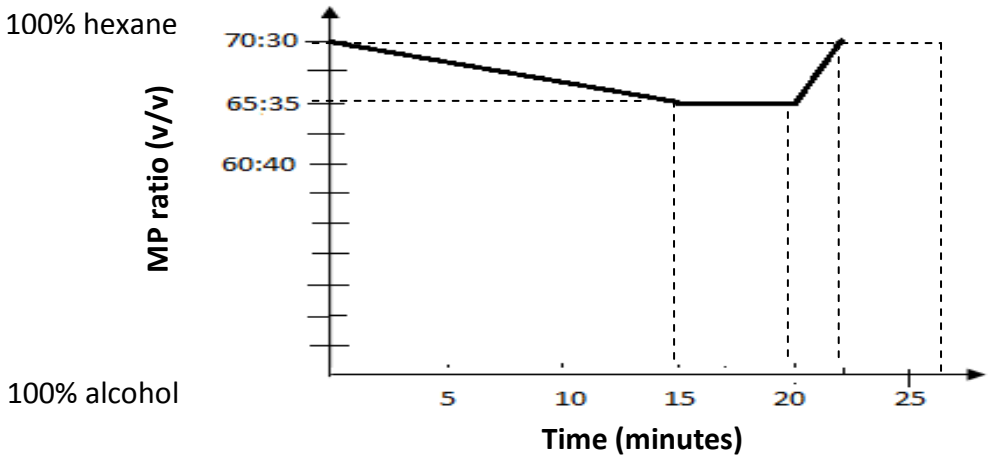


Figure 2.11: The gradient conditions from 30 % to 35 % of alcohol in 22 min. MP ratio – hexane: alcohol (2-propanol or ethanol).

h.

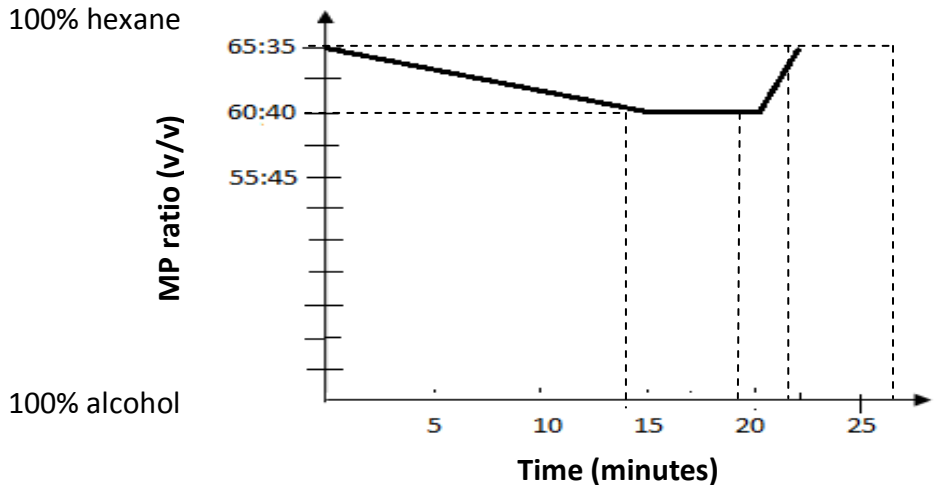


Figure 2.12: The gradient conditions for 35 % to 40 % of alcohol in 22 min. MP ratio – hexane: alcohol (2-propanol or ethanol).

i.

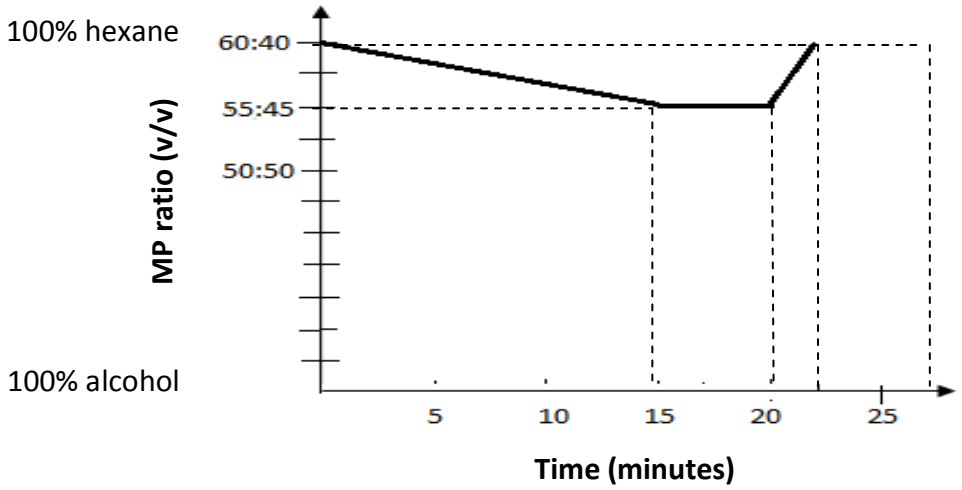


Figure 2.13: The gradient conditions for 40 % to 45 % of alcohol in 22 min. MP ratio – hexane: alcohol (2-propanol or ethanol).

j.

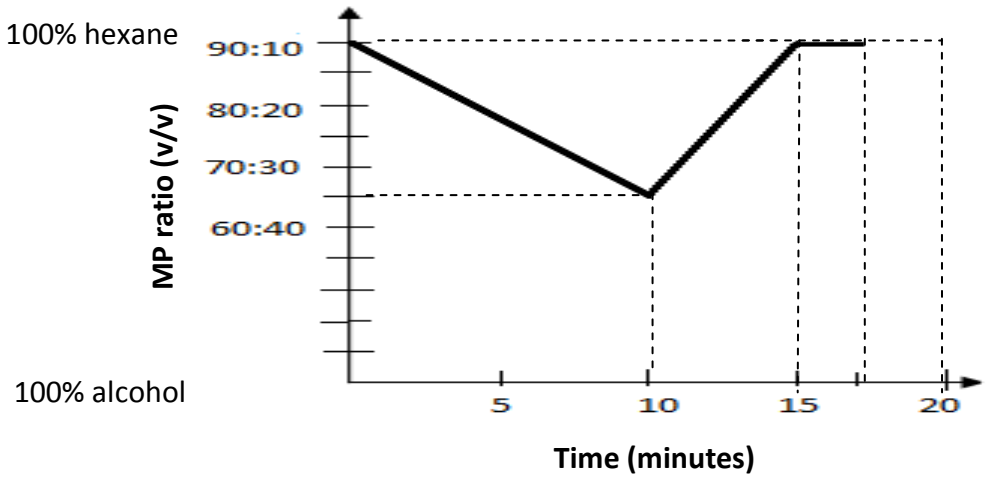


Figure 2.14: The gradient conditions for 10 % to 35 % of alcohol in 17 min. MP ratio – hexane: alcohol (2-propanol or ethanol).

The two alcohols used as organic modifier in hexane are 2-propanol and ethanol individually. The flow rate was held constant at 1.0 ml/min for all gradients, except for the mobile phase ratio ranging from (hexane: 2-propanol) 70:30 to 65:35 where two different flow rates of 0.5 ml/min and 1 ml/min were employed. All samples were injected after 30 min of isocratic equilibration. These mobile phases used in both isocratic and gradient conditions were sonicated, filtered & degassed before running it through the HPLC system.

2.5 Stock solution

The stock solution of cytarabine was prepared by transferring 10 mg of the compound into a 100 ml volumetric flask dissolving in a mixture of ethanol and 2-propanol (1:1) and then making up to the mark using the same composition of alcohol providing a final concentration of 10 mg % (0.1 mg/ml).

2.6 Sample preparation of trans-stilbene oxide

The trans-stilbene oxide sample was prepared by transferring 10 mg of the compound into a 10 ml volumetric flask dissolving in a mixture of hexane: 2-propanol (9:1) and then making up to the mark with the same composition of hexane and 2-propanol to afford a final concentration 1 mg/ml.

All the stock solutions were stored in the refrigerator at 4°C until further use.

2.7 Statistical analysis

For all the methods used for enantioseparation of anticancer reagent cytarabine, the experiments were performed in triplicate, and the results were expressed as the mean ±Standard Deviation (SD) from the three or four independent experiments, relative standard deviation (RSD) was calculated using where necessary. The data analysis was performed using SPSS version 18.0.3 and Microsoft Excel.

CHAPTER 3

RESULTS

3. RESULTS

3.1 Polarimeter results

The optical rotation for the anticancer reagent cytarabine was determined using polarimeter. Sucrose was used as a standard to check the performance of the polarimeter.

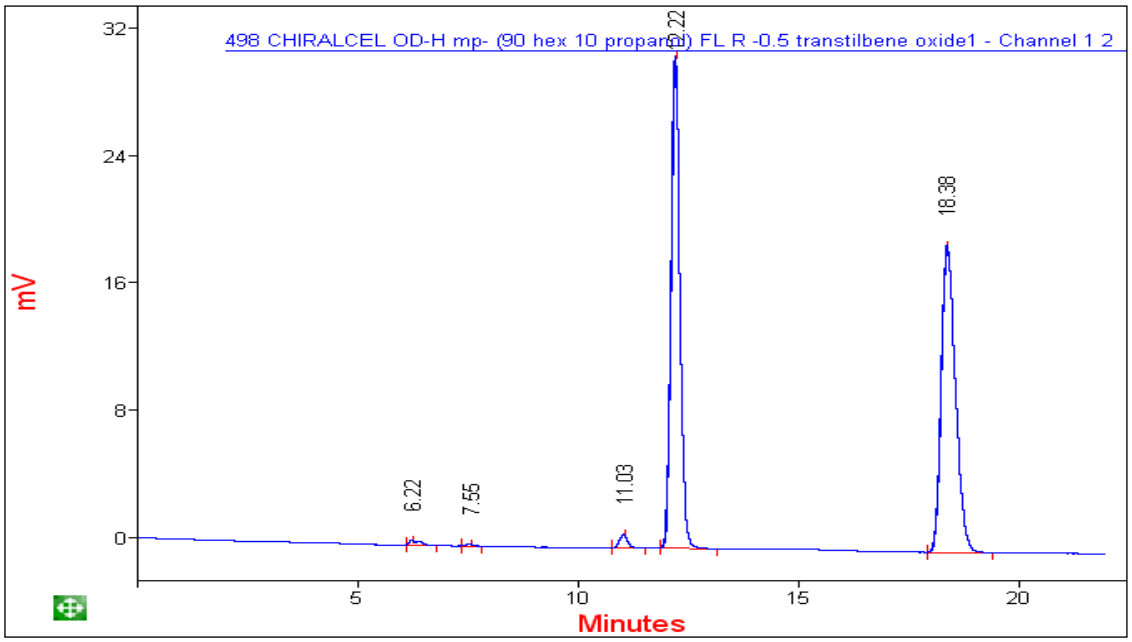
Table 3.1: Showing the optical and specific rotation of sucrose and cytarabine. Data values ± standard deviation.

Compound	Observed rotation n = 5	RSD	Specific rotation
Sucrose	3.2906 ± 0.0003	0.009	(+) 65.79
Cytarabine	1.5584 ± 0.0023	0.147	(+) 153.68

3.2 HPLC column performance

Column performance was regularly monitored for the three columns Chiralcel OD-H, OJ-H and Chiralpak AD-H by checking the resolution of trans-stilbene oxide as standard.

A.



B.

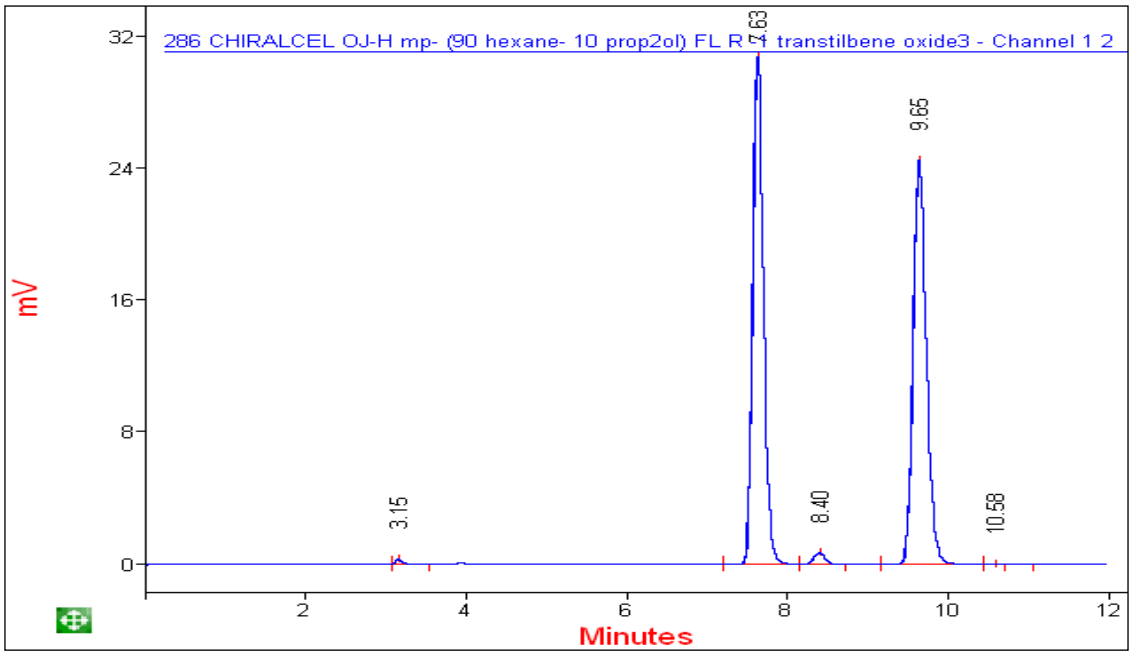


Figure 3.1: HPLC chromatogram showing the results for trans-stilbene oxide using 90% hexane and 10% 2-propanol (a) at a flow rate 0.5 ml/min on the Chiralcel OD-H column and (b) at a flow rate 1.0 ml/min on the Chiralcel OJ-H column.

C.

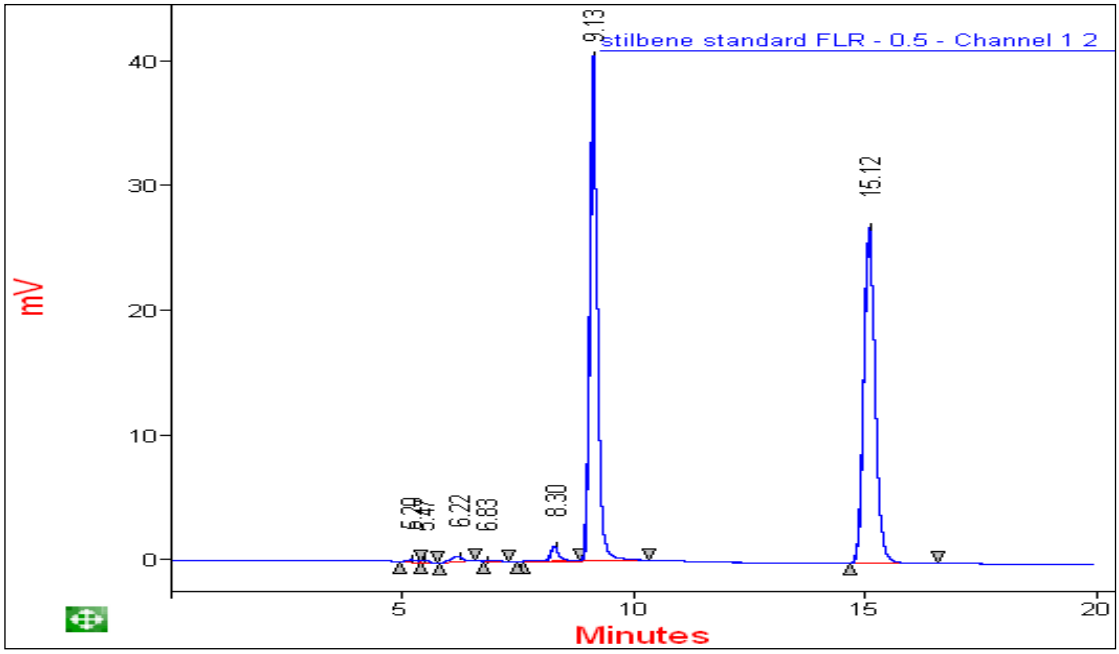


Figure 3.2: HPLC chromatogram showing the result for trans-stilbene oxide using 90% hexane and 10% 2-propanol at a flow rate 0.5 ml/min on the Chiralpak AD-H column.

3.3 The HPLC method using hexane: 2-propanol (v/v)

Chromatographic conditions- Chromatographic separation of cytarabine enantiomers was carried out using isocratic conditions at an ambient temperature, unless otherwise specified. Combinations of hexane: 2-propanol was used as the mobile phase. The flow rates used were 0.5, 1.0 and 1.5 ml/min and the cytarabine at a concentration of 10 mg % dissolved in a mixture of 2-propanol: ethanol (1:1) (v/v) was used. The injection volume was kept constant at 20 µl.

3.3.1 HPLC results for Chiralcel OD-H using hexane: 2-propanol (v/v)

The separation of enantiomers of cytarabine (10 mg %) was carried out using a mixture ranging from (90:10) to (30:70) (v/v) at flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralcel OD-H column. A significant decrease was observed in the retention time of cytarabine with increasing concentrations of 2-propanol.

Table 3.2: Effect of 2-propanol on selectivity and resolution of cytarabine enantiomers using the Chiralcel OD-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE HEXANE:IPA (v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
90:10	0.5	106.20	2182 ± 19.17
	1	54.60	1143 ± 11.43
	1.5	32.21	870 ± 22.11
80:20	0.5	19.80	6781 ± 13.16
	1	10.20	3034 ± 20.70
	1.5	6.22	2531 ± 36.05
70:30	0.5	11.02	6422 ± 88.33
	1	5.35	3862 ± 27.23
	1.5	3.48	2772 ± 18.82
60:40	0.5	8.08	7391 ± 22.07
	1	4.15	3972 ± 20.73
	1.5	2.65	2977 ± 20.32
50:50	0.5	7.28	7677 ± 17.28
	1	3.85	4045 ± 26.20
	1.5	2.40	3044 ± 12.24
40:60	0.5	6.90	7252 ± 4.45
	1	3.47	4067 ± 54.33
	1.5	2.27	2979 ± 36.81
30:70	0.5	6.45	6753 ± 47.11
	1	3.32	4078 ± 21.33
	1.5	2.20	2969 ± 20.43

3.3.2 HPLC results for Chiralcel OJ-H using hexane: 2-propanol (v/v)

The separation of enantiomers of cytarabine (10 mg %) was carried out using a mixture ranging from (90:10) to (30:70) (v/v) with flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralcel OJ-H column. A significant decrease was observed in the retention time of cytarabine with increasing concentration of 2-propanol. Partial separation was obtained using ratios 40:60 (Fig. 3.9), 50:50 (Fig. 3.6) and 70:30 (Fig. 3.4).

Table 3.3: Effect of 2-propanol on the selectivity and resolution of cytarabine enantiomers on the Chiralpak OJ-H column at three different flow rates. Data values ± standard deviation**.**

MOBILE PHASE HEXANE:IPA (v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
90:10	0.5	24.30	4596 ± 60.35
	1	11.47	3009 ± 126.21
	1.5	7.62	2197 ± 59.76
80:20	0.5	8.98	6718 ± 19.94
	1	4.47	3551 ± 166.40
	1.5	2.97	2378 ± 37.18
70:30	0.5	7.55	5301 ± 56.27
	1	3.57	1091 ± 28.11
		3.77	2312 ± 68.36
	1.5	2.38	2399 ± 19.42
60:40	0.5	7.05	5722 ± 22.98
	1	3.58	3554 ± 69.10
	1.5	2.28	2443 ± 32.45
50:50	0.5	6.58	2979 ± 51.37
		6.88	1756 ± 73.69
	1	3.28	3436 ± 192.27
	1.5	2.23	2358 ± 31.06
40:60	0.5	6.42	3503 ± 40.65
		6.77	1554 ± 5.42
	1	3.28	3171 ± 97.76
	1.5	2.17	2273 ± 110.01
30:70	0.5	6.02	3248 ± 65.43
	1	2.95	1668 ± 78.56
	1.5	2.04	1011 ± 37.36

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol (v/v) (70:30) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

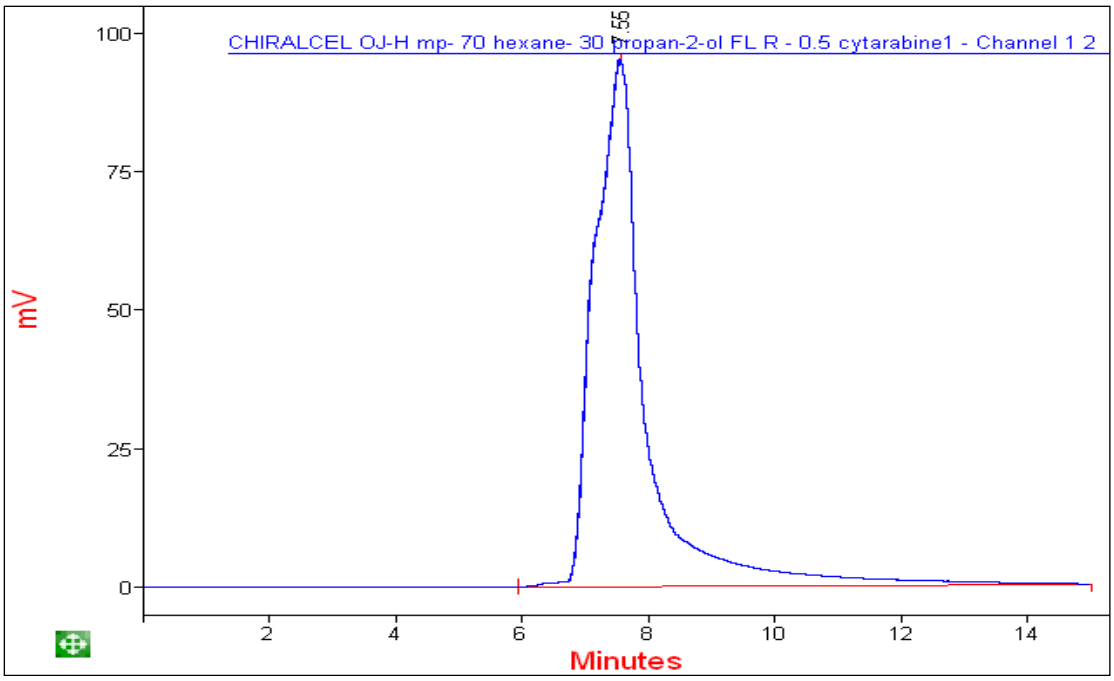


Figure 3.3: HPLC chromatogram showing the result for cytarabine using 70 % hexane and 30 % 2-propanol at a flow rate 0.5 ml/min on the Chiralcel OJ-H column.

B.

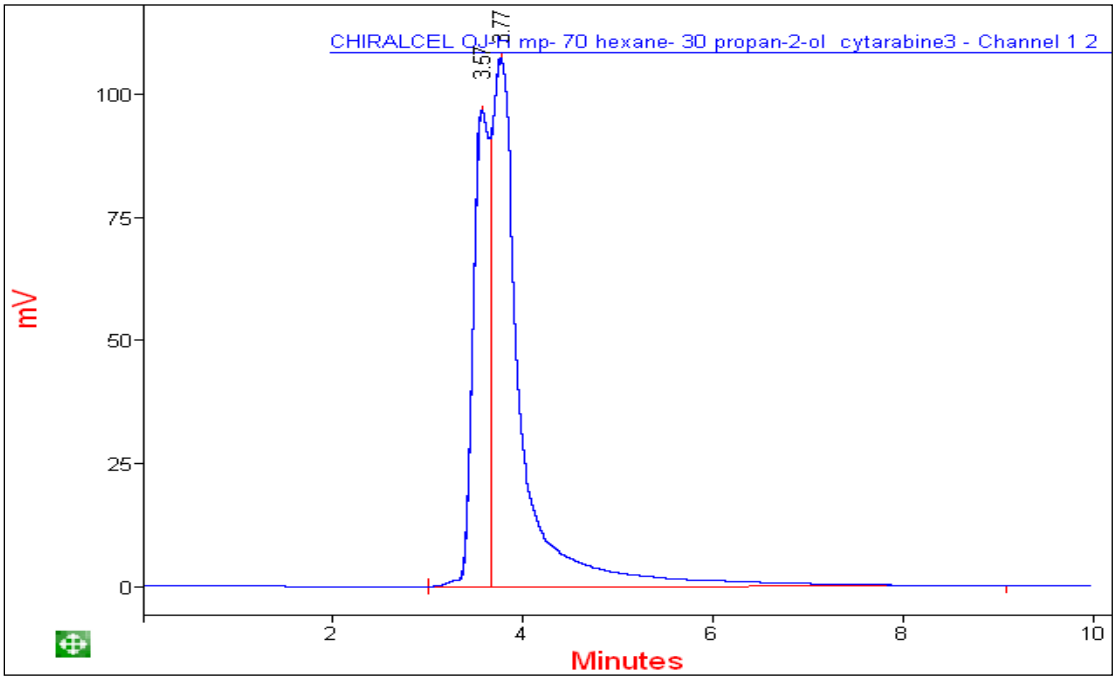


Figure 3.4: HPLC chromatogram showing the result for cytarabine using 70 % hexane and 30 % 2-propanol at a flow rate 1.0 ml/min on the Chiralcel OJ-H column.

C.

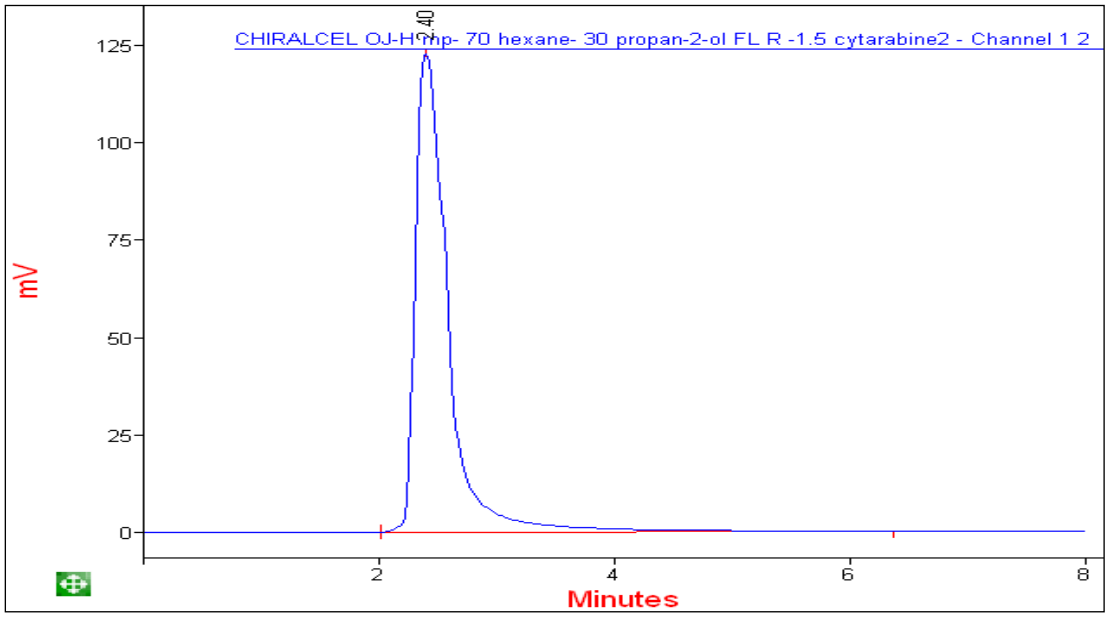


Figure 3.5: HPLC chromatogram showing the result for cytarabine using 70 % hexane and 30 % 2-propanol at a flow rate 1.5 ml/min on the Chiralcel OJ-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol (v/v) (50:50) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

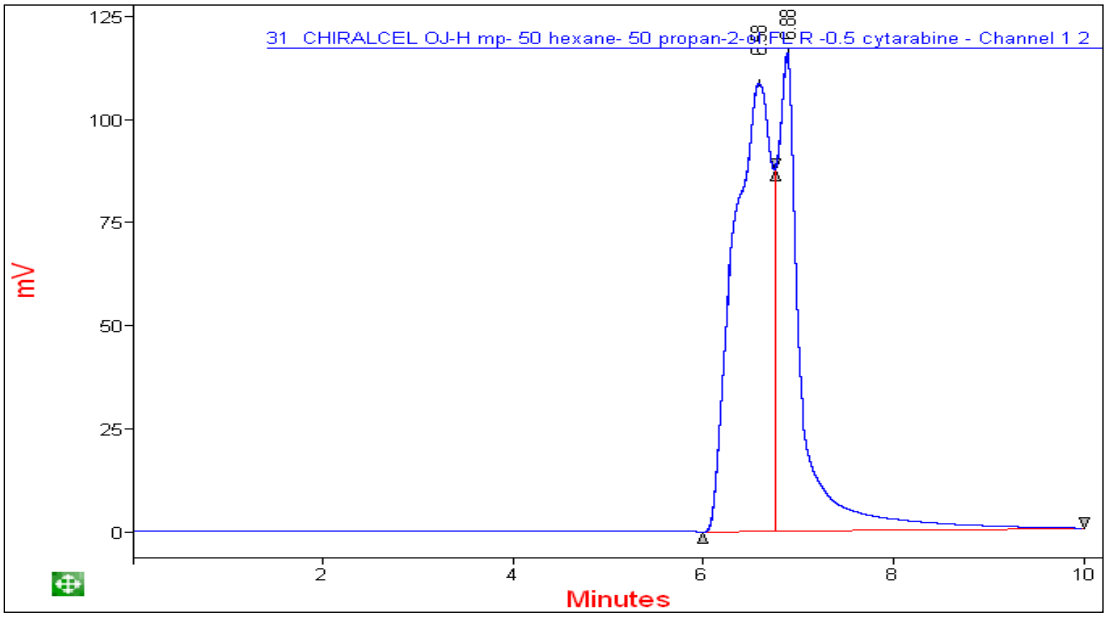


Figure 3.6: HPLC chromatogram showing the result for cytarabine using 50 % hexane and 50 % 2-propanol at a flow rate of 0.5 ml/min on the Chiralcel OJ-H column.

B.

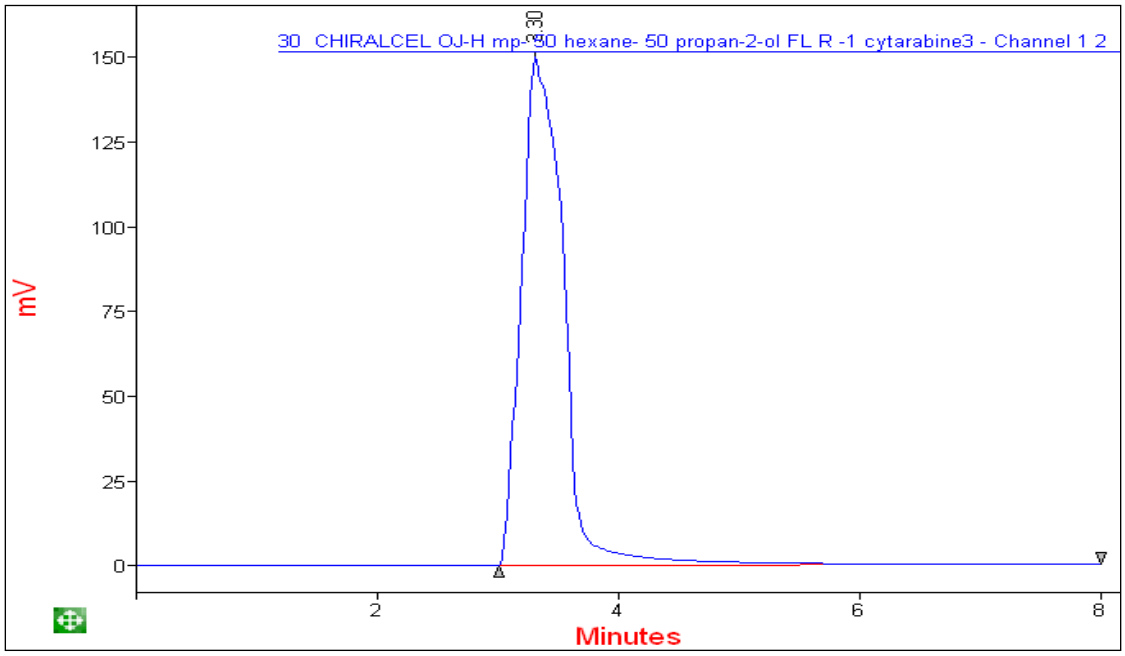


Figure 3.7: HPLC chromatogram showing the result for cytarabine using 50 % hexane and 50 % 2-propanol at a flow rate of 1.0 ml/min on the Chiralcel OJ-H column.

C.

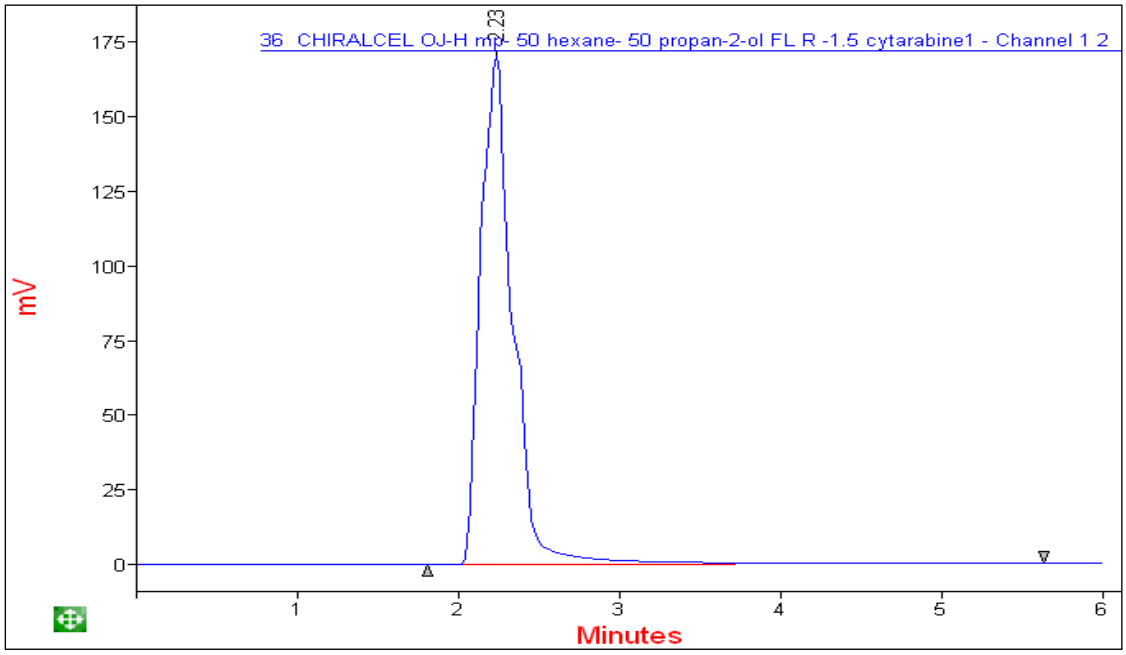


Figure 3.8: HPLC chromatogram showing the result for cytarabine using 50 % hexane and 50 % 2-propanol at a flow rate of 1.5 ml/min on the Chiralcel OJ-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol (v/v) (40:60) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

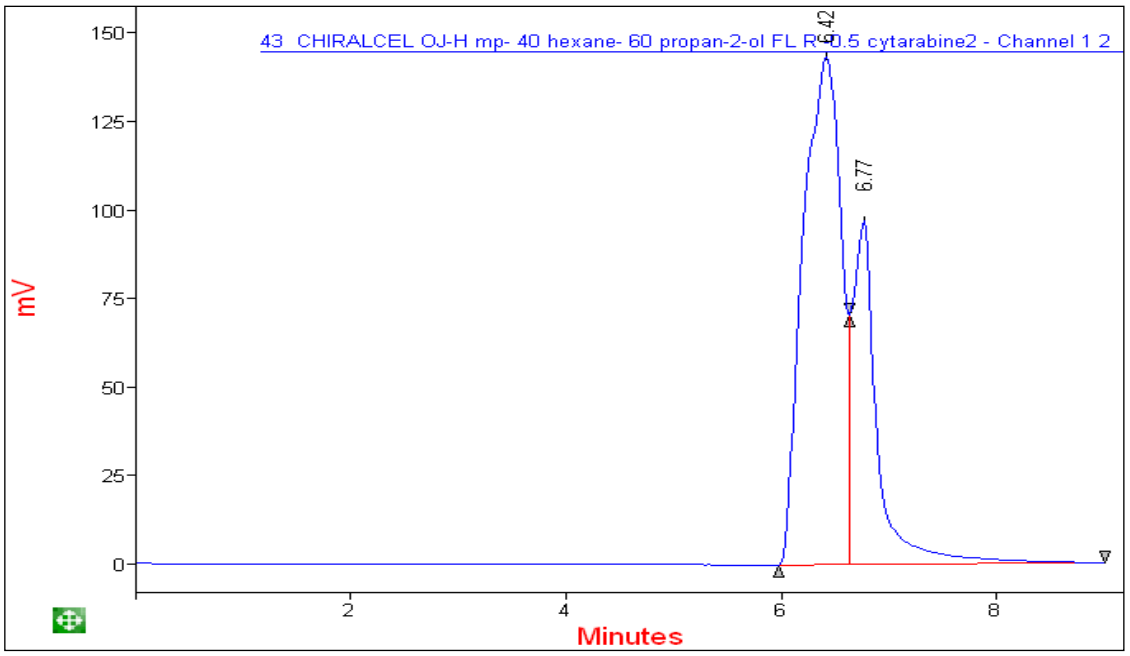


Figure 3.9: HPLC chromatogram showing the result for cytarabine using 40 % hexane and 60 % 2-propanol at a flow rate of 0.5 ml/min on the Chiralcel OJ-H column.

B.

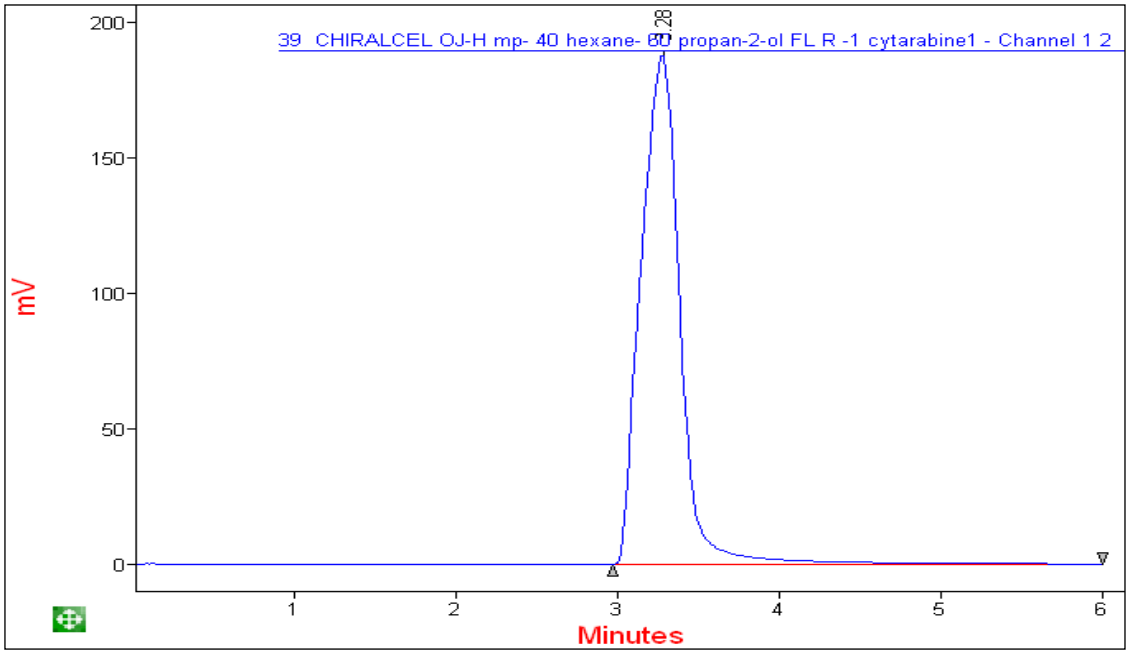


Figure 3.10: HPLC chromatogram showing the result for cytarabine using 40 % hexane and 60 % 2-propanol at a flow rate of 1.0 ml/min on the Chiralcel OJ-H column.

C.

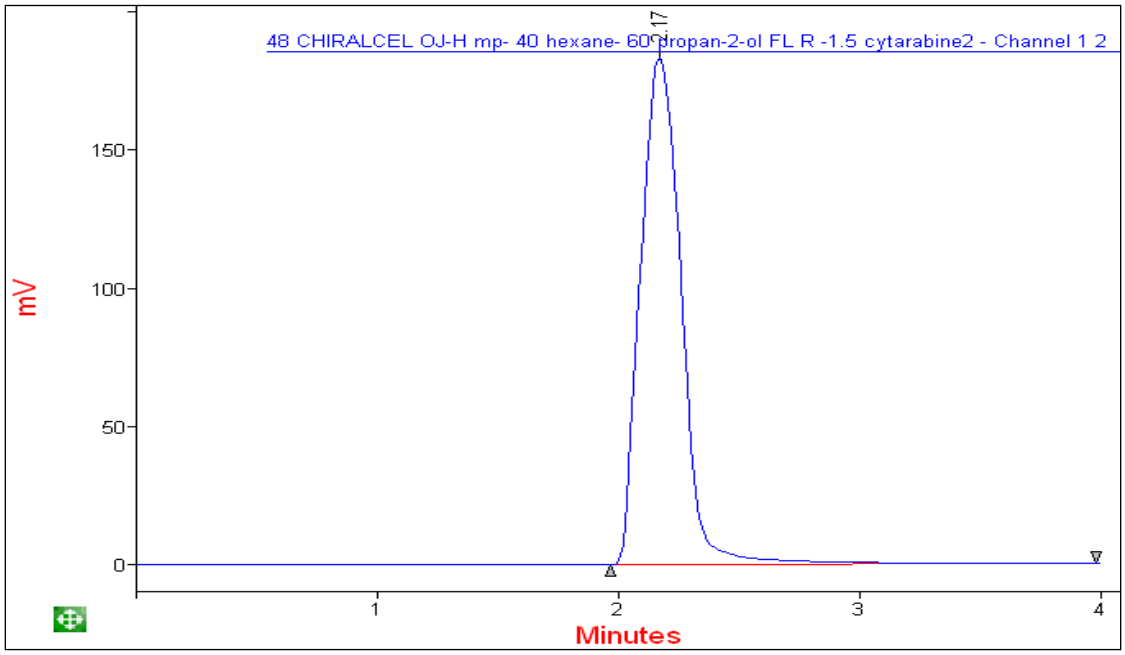


Figure 3.11: HPLC chromatogram showing the result for cytarabine using 40 % hexane and 60 % 2-propanol at a flow rate of 1.5 ml/min on the Chiralcel OJ-H column.

3.3.3 HPLC results for Chiralpak AD-H using hexane: 2-propanol (v/v)

The separation of enantiomers of cytarabine (10 mg %) was carried out using a mixture ranging from (90:10) to (30:70) (v/v) with flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralpak AD-H column. Partial separation was achieved using mobile phase ratios of 50:50 (Fig. 3.20), 60:40 (Fig. 3.17) and 70:30 (Fig. 3.14).

Table 3.4: Effect of 2-propanol on selectivity and resolution of cytarabine enantiomers on the Chiralpak AD-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE HEXANE:IPA (v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
90:10	0.5	41.88	4103 ± 66.66
	1	24.21	2715 ± 261.18
	1.5	15.23	1133 ± 38.42
80:20	0.5	10.75	5432 ± 163.03
	1	5.37	3681 ± 170.17
	1.5	3.69	2565 ± 98.87
70:30	0.5	7.28	1046 ± 37.72
		7.68	4090 ± 62.08
	1	3.57	840 ± 18.58
		3.82	2375 ± 115.11
	1.5	2.38	1103 ± 11.21
		2.57	1337 ± 183.90
60:40	0.5	6.63	1198 ± 50.29
		6.98	2886 ± 89.62
	1	3.25	1301 ± 230.77
		3.60	2569 ± 363.87
	1.5	2.18	1315 ± 20.11
		2.43	1093 ± 23.05
50:50	0.5	6.10	611 ± 15.29
		6.52	5629 ± 104.24
	1	3.13	3457 ± 74.62
	1.5	2.08	1230 ± 75.48
		2.30	1184 ± 57.33
40:60	0.5	6.37	6094 ± 57.25
	1	3.18	3382 ± 26.10
	1.5	2.02	2427 ± 30.45
30:70	0.5	5.97	5845 ± 15.43
	1	3.21	2968 ± 36.81
	1.5	2.01	1946 ± 11.08

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol (v/v) (70:30) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralpak AD-H column.

A.

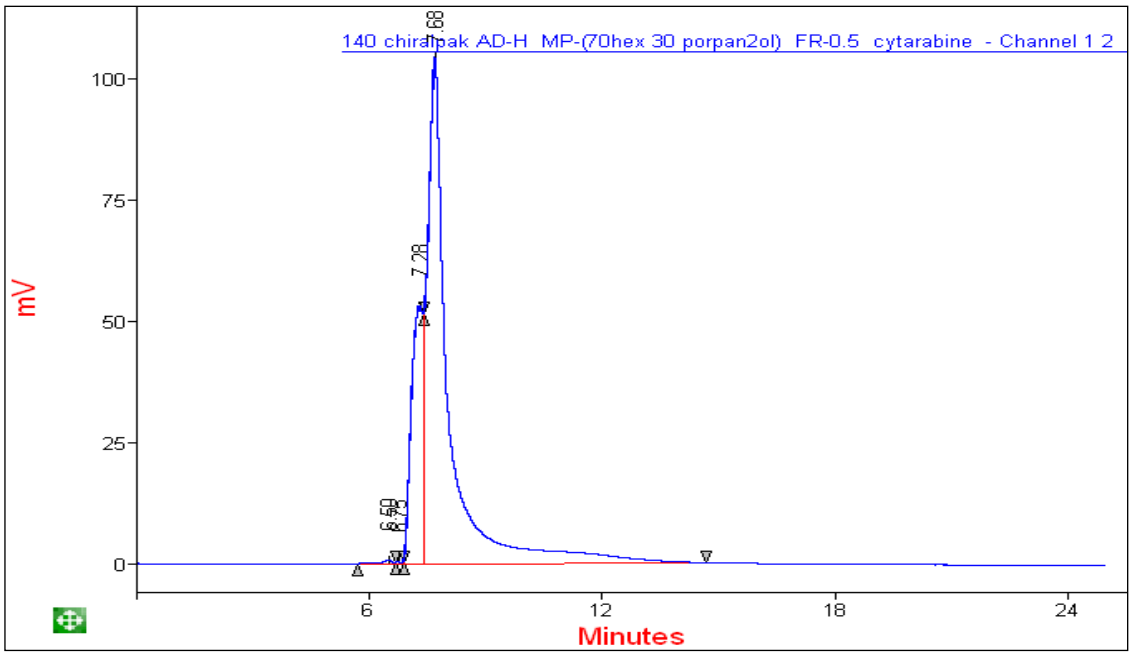


Figure 3.12: HPLC chromatogram showing the result for cytarabine using 70 % hexane and 30 % 2-propanol at a flow rate 0.5 ml/min on the Chiralpak AD-H column.

B.

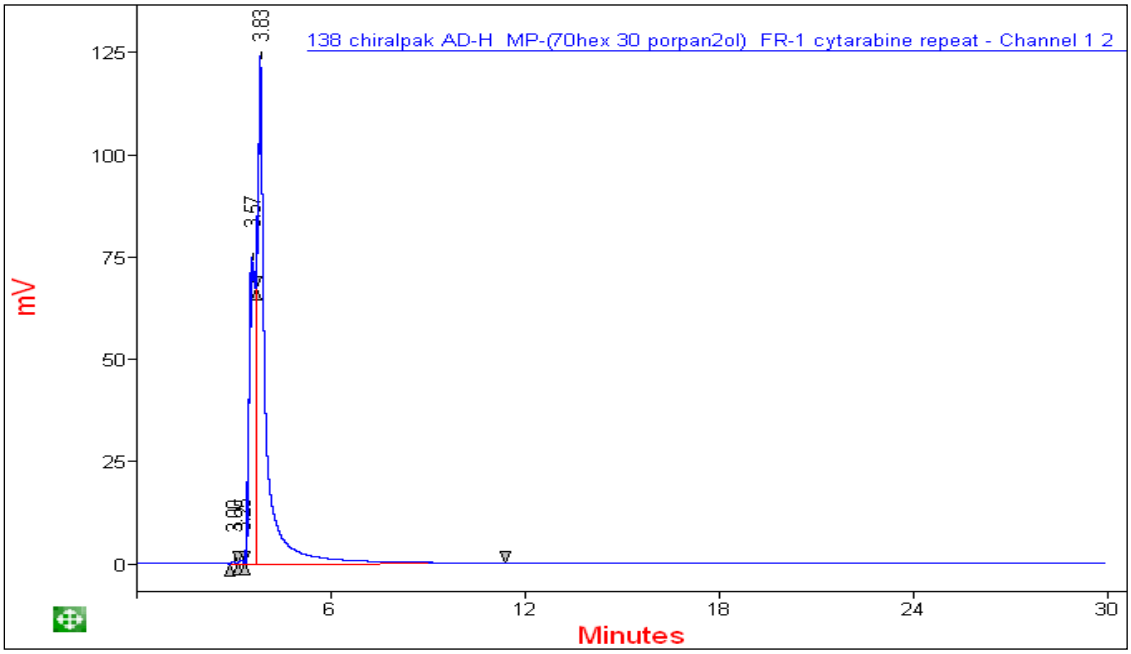


Figure 3.13: HPLC chromatogram showing the result for cytarabine using 70 % hexane and 30 % 2-propanol at a flow rate 1.0 ml/min on the Chiralpak AD-H column.

C.

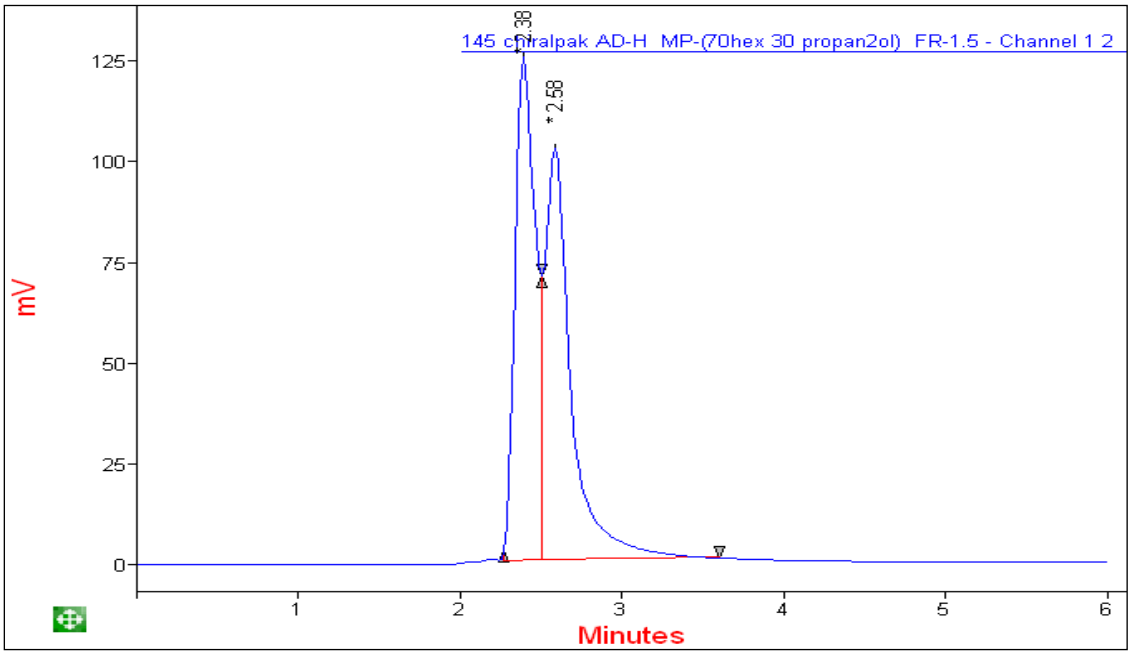


Figure 3.14: HPLC chromatogram showing the result for cytarabine using 70 % hexane and 30 % 2-propanol at a flow rate 1.5 ml/min on the Chiralpak AD-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol (v/v) (60:40) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralpak AD-H column.

A.

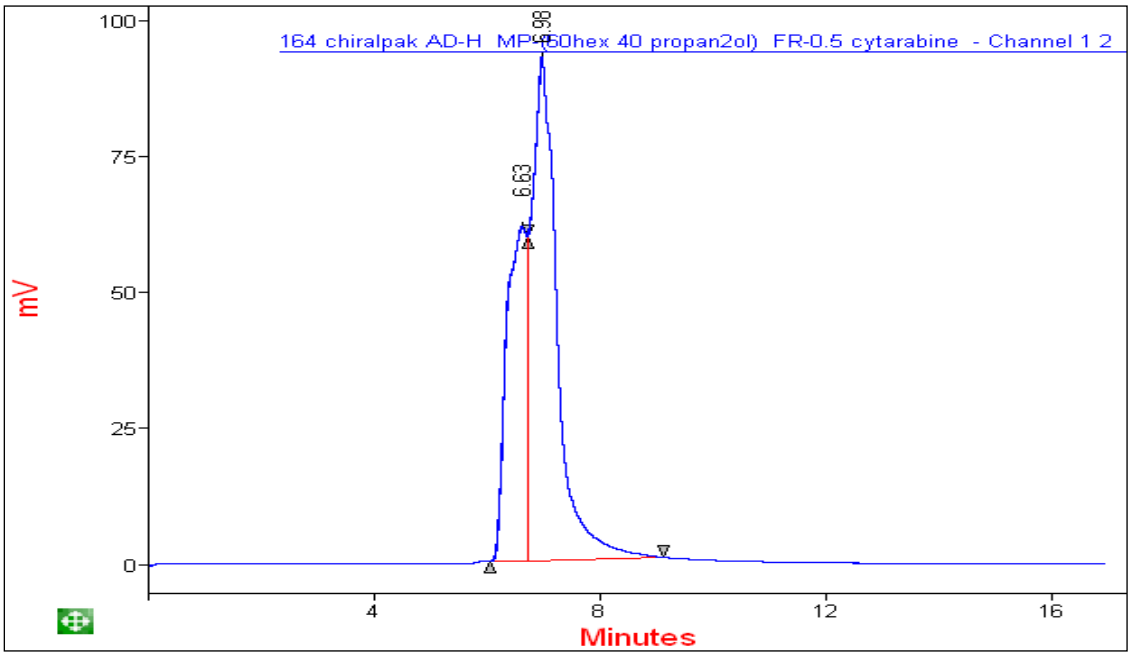


Figure 3.15: HPLC chromatogram showing the result for cytarabine using 60 % hexane and 40 % 2-propanol at a flow rate 0.5 ml/min on the Chiralpak AD-H column.

B.

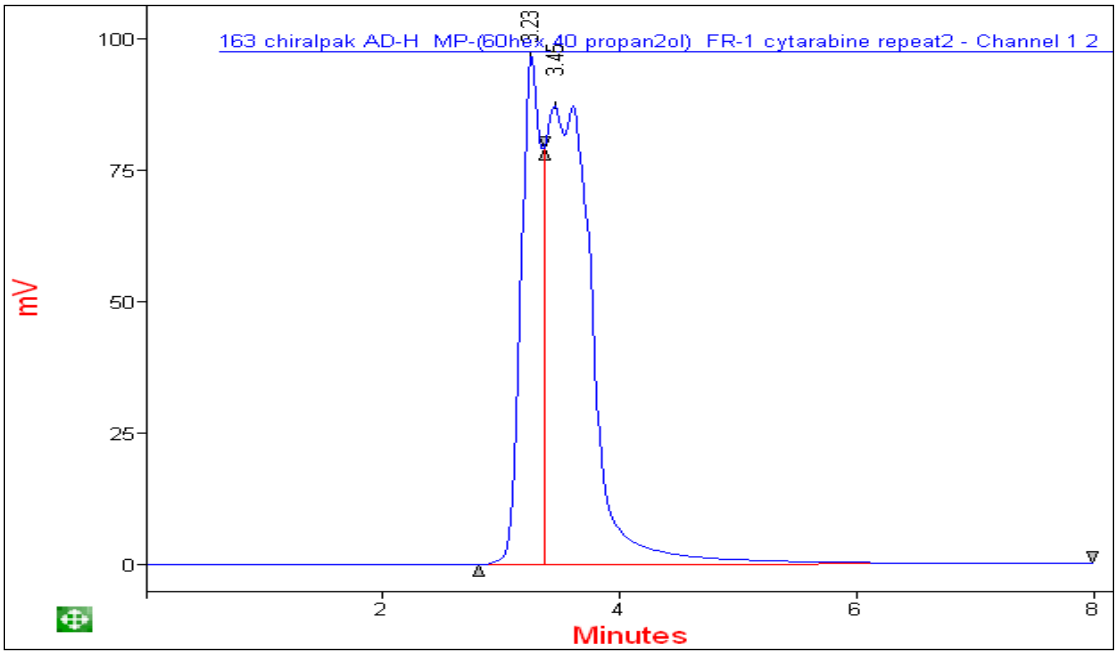


Figure 3.16: HPLC chromatogram showing the result for cytarabine using 60 % hexane and 40 % 2-propanol at a flow rate 1.0 ml/min on the Chiralpak AD-H column.

C.

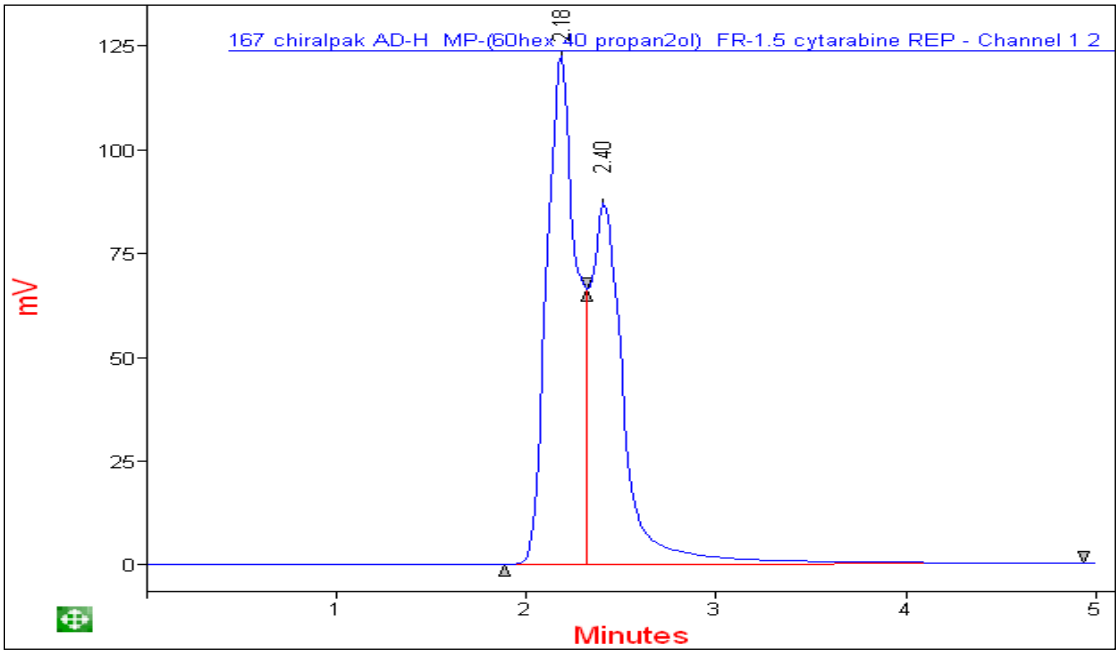


Figure 3.17: HPLC chromatogram showing the result for cytarabine using 60 % hexane and 40 % 2-propanol at a flow rate 1.5 ml/min on the Chiralpak AD-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol (v/v) (50:50) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralpak AD-H column.

A.

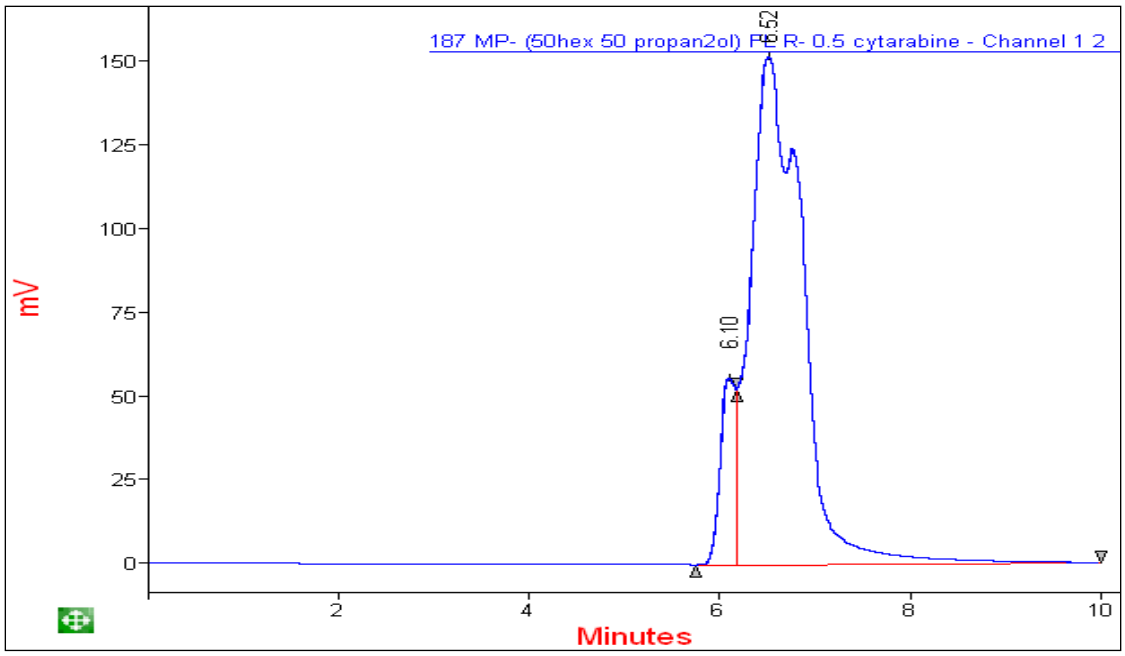


Figure 3.18: HPLC chromatogram showing the result for cytarabine using 50% hexane and 50% 2-propanol at a flow rate 0.5 ml/min on the Chiralpak AD-H column.

B.

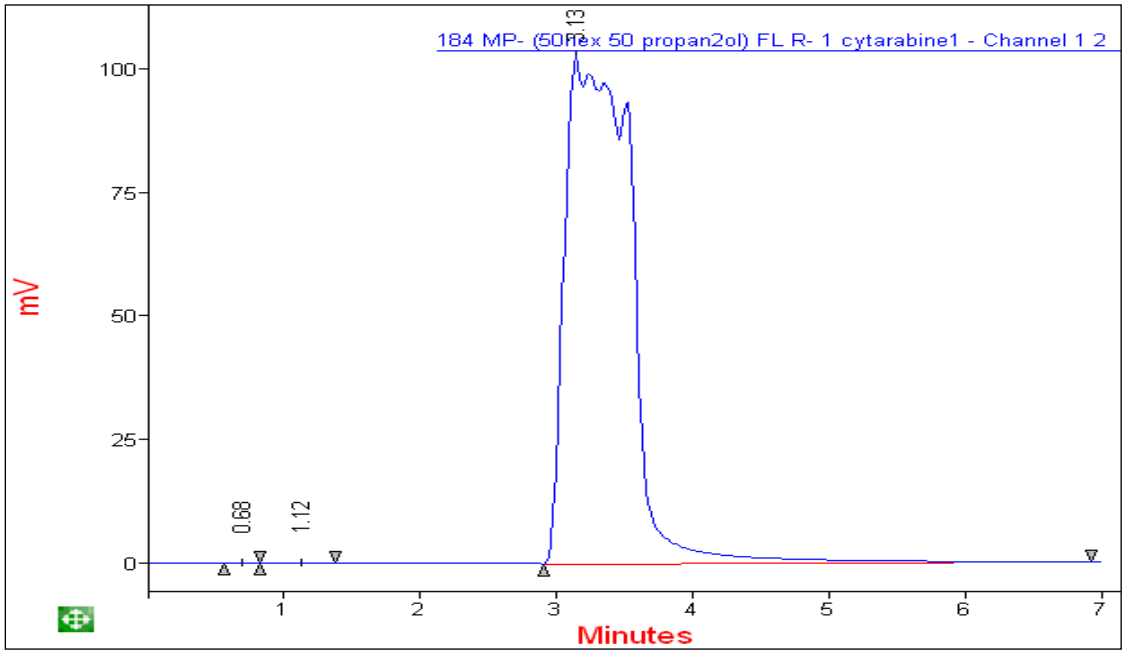


Figure 3.19: HPLC chromatogram showing the result for cytarabine using 50% hexane and 50% 2-propanol at a flow rate 1.0 ml/min on the Chiralpak AD-H column.

C.

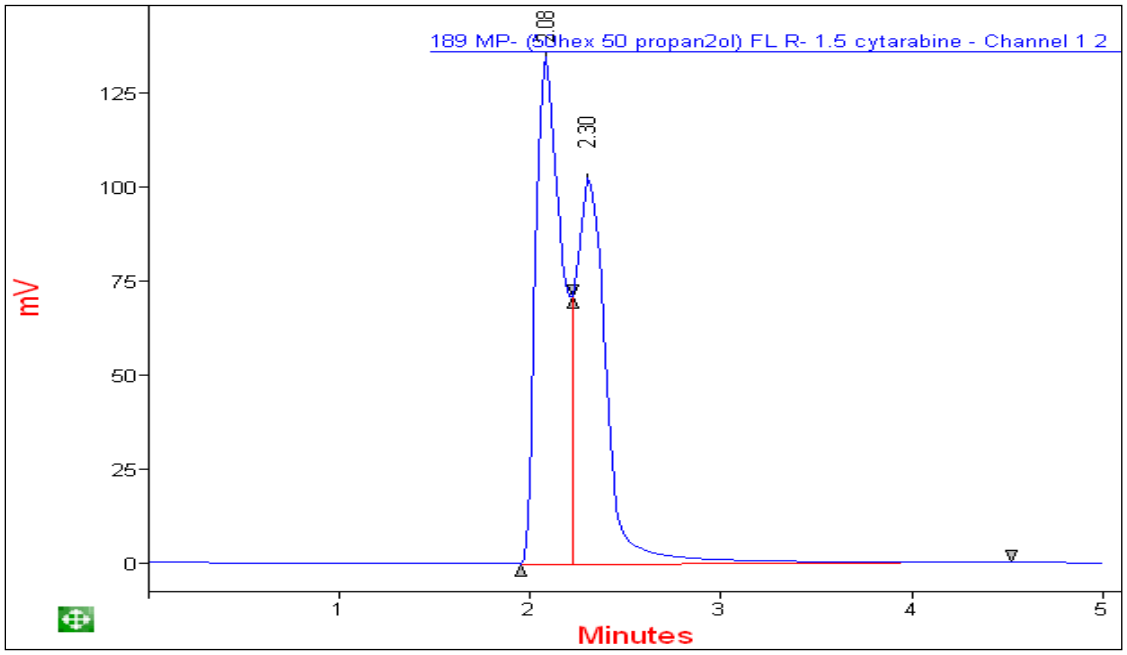


Figure 3.20: HPLC chromatogram showing the result for cytarabine using 50% hexane and 50% 2-propanol at a flow rate 1.5 ml/min on the Chiralpak AD-H column.

3.4 HPLC method using hexane and ethanol (v/v)

Chromatographic conditions - The chromatographic separation of cytarabine enantiomers was carried out using an isocratic system at ambient temperature, unless otherwise specified. A combination of hexane: ethanol ranging from (90:10) to (30:70) (v/v) was used as mobile phase with flow rates of 0.5, 1.0 and 1.5 ml/min. Cytarabine at a concentration of 10 mg % dissolved in a mixture of 2-propanol: ethanol (1:1) was used. The injection volume was kept constant at 20 µl.

3.4.1 HPLC results for Chiralcel OD-H using hexane: ethanol (v/v)

The separation of enantiomers of cytarabine (10 mg %) was carried out with flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralcel OD-H column. A significant decrease was observed in the retention time of cytarabine with increasing concentrations of ethanol.

Table 3.5: Effect of ethanol on the selectivity and resolution of cytarabine enantiomers using Chiralcel OD-H column at three different flow rates. Data values ± standard deviation**.**

MOBILE PHASE HEXANE: EtOH (v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
90:10	0.5	41.05	4568 ± 19.92
	1	20.58	2745 ± 38.06
	1.5	13.27	2135 ± 22.32
80:20	0.5	13.32	5818 ± 18.56
	1	6.43	3643 ± 18.15
	1.5	4.28	2413 ± 17.05
70:30	0.5	8.53	6788 ± 13.21
	1	4.22	3933 ± 17.27
	1.5	2.80	3038 ± 19.90
60:40	0.5	7.30	6353 ± 30.61
	1	3.68	4160 ± 36.88
	1.5	2.74	2759 ± 19.02
50:50	0.5	6.82	6974 ± 24.86
	1	3.53	4231 ± 17.97
	1.5	2.27	2953 ± 27.69
40:60	0.5	6.08	8306 ± 8.48
	1	3.15	4485 ± 27.29
	1.5	2.10	2918 ± 22.26
30:70	0.5	6.22	6315 ± 23.13
	1	3.12	4420 ± 37.56
	1.5	2.13	2955 ± 29.49

3.4.2 HPLC results for Chiralcel OJ-H using hexane: ethanol (v/v)

Attempted Separation of enantiomers of cytarabine (10 mg %) with flow rates of 0.5, 1.0 and 1.5 ml/min on Chiralcel OJ-H column. A significant decrease in the retention time of cytarabine was observed with increasing concentrations of ethanol. Partial separation was obtained using ratios 40:60 (Fig. 3.30), 50:50 (Fig. 3.27), 60:40 (Fig.3.24) and 70:30 (3.21).

Table 3.6: Effect of ethanol on selectivity and resolution of cytarabine enantiomers on the Chiralcel OJ-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE HEXANE:EtOH (v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
90:10	0.5	22.95	5940 ± 42.95
	1	11.40	3214 ± 117.12
	1.5	7.25	2354 ± 65.13
80:20	0.5	8.98	6046 ± 51.08
	1	4.47	3417 ± 127.28
	1.5	2.90	2373 ± 123.86
70:30	0.5	7.35	2087 ± 172.85
		7.65	2513 ± 113.90
	1	3.85	3240 ± 29.28
	1.5	2.52	2482 ± 147.91
60:40	0.5	6.96	3873 ± 26.15
		7.23	1813 ±16.58
	1	3.45	3464 ± 41.93
	1.5	2.32	2486 ± 197.69
50:50	0.5	6.57	3131 ± 97.61
		6.93	3028 ±76.89
	1	3.43	3343 ±138.46
	1.5	2.25	2378 ± 86.03
40:60	0.5	6.33	4525 ± 88.42
		6.83	2080 ± 69.20
	1	3.33	3550 ± 116.00
	1.5	2.15	2464 ± 39.27
30:70	0.5	6.13	4770 ±123.08
	1	3.10	3463 ± 133.64
	1.5	2.15	2313 ± 162.18

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: ethanol (v/v) (70:30) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

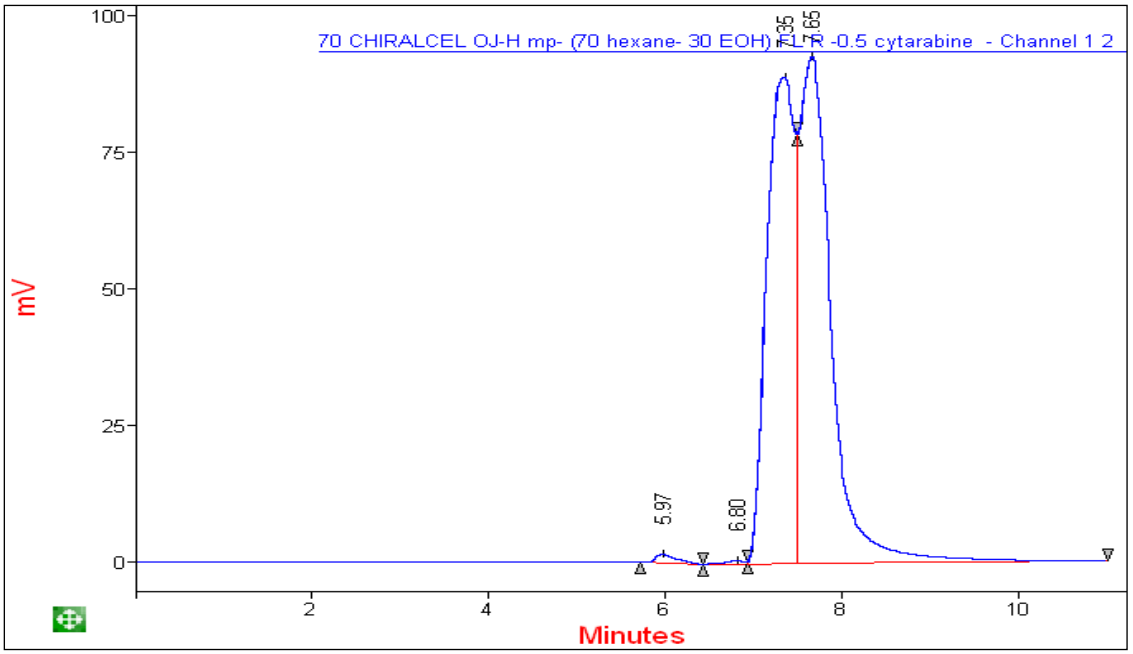


Figure 3.21: HPLC chromatogram showing the result for cytarabine with 70 % hexane and 30 % ethanol at a flow rate 0.5 ml/min on the Chiralcel OJ-H column.

B.

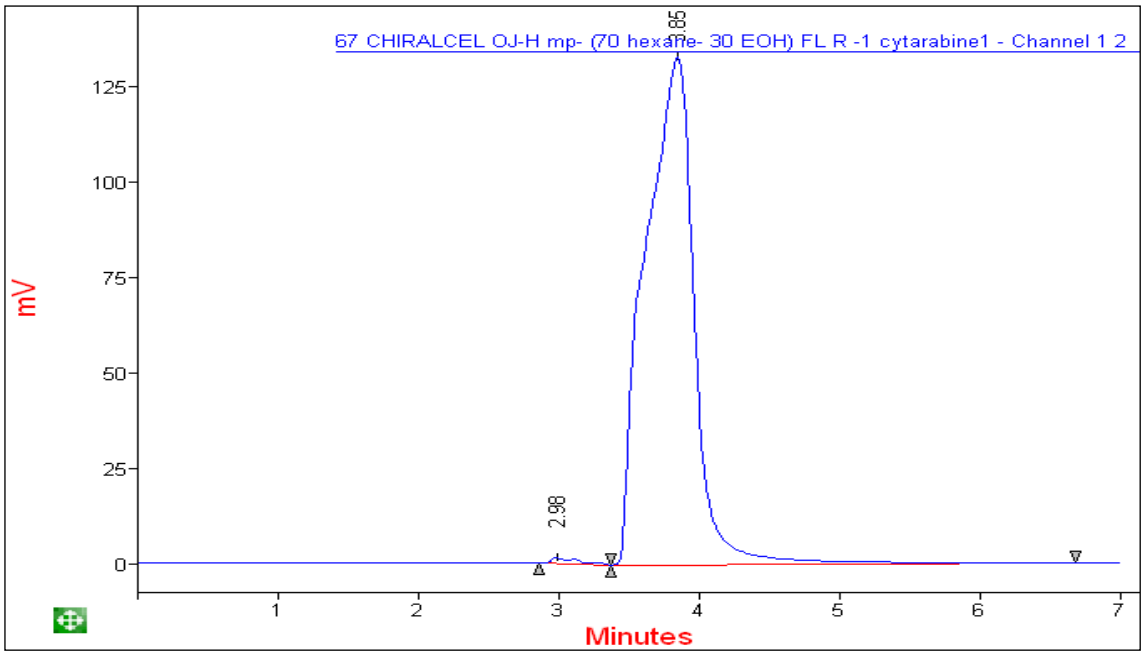


Figure 3.22: HPLC chromatogram showing the result for cytarabine with 70 % hexane and 30 % ethanol at a flow rate 1.0 ml/min on the Chiralcel OJ-H column.

C.

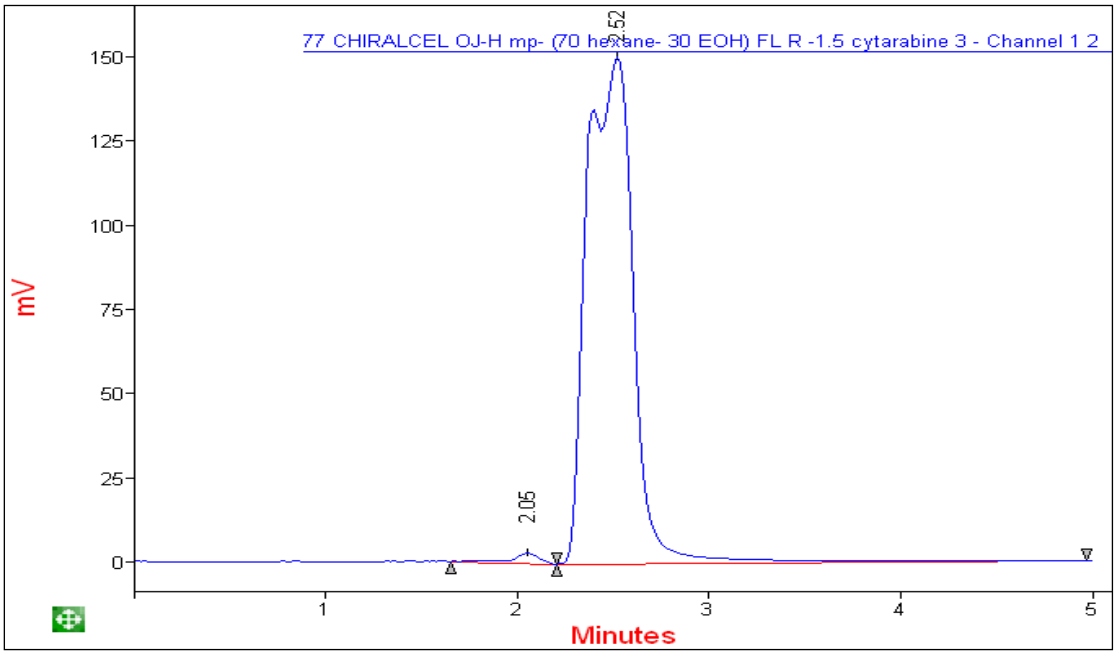


Figure 3.23: HPLC chromatogram showing the result for cytarabine with 70 % hexane and 30 % ethanol at a flow rate 1.5 ml/min on the Chiralcel OJ-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: ethanol (v/v) (60:40) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

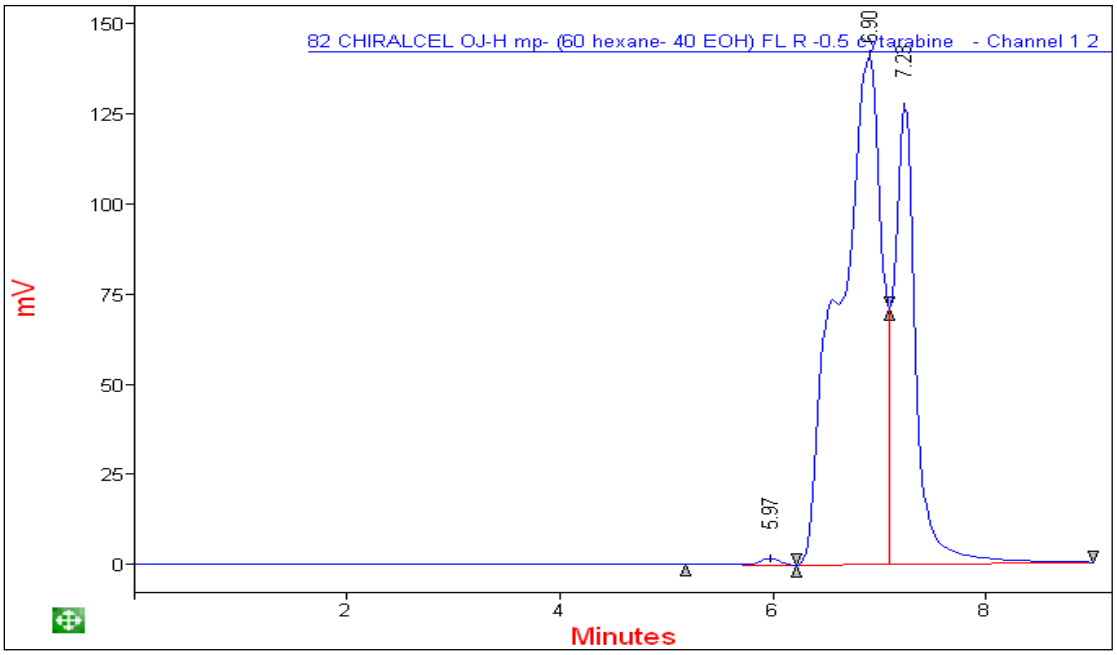


Figure 3.24: HPLC chromatogram showing the result for cytarabine with 60 % hexane and 40 % ethanol at a flow rate 0.5 ml/min on the Chiralcel OJ-H column.

B.

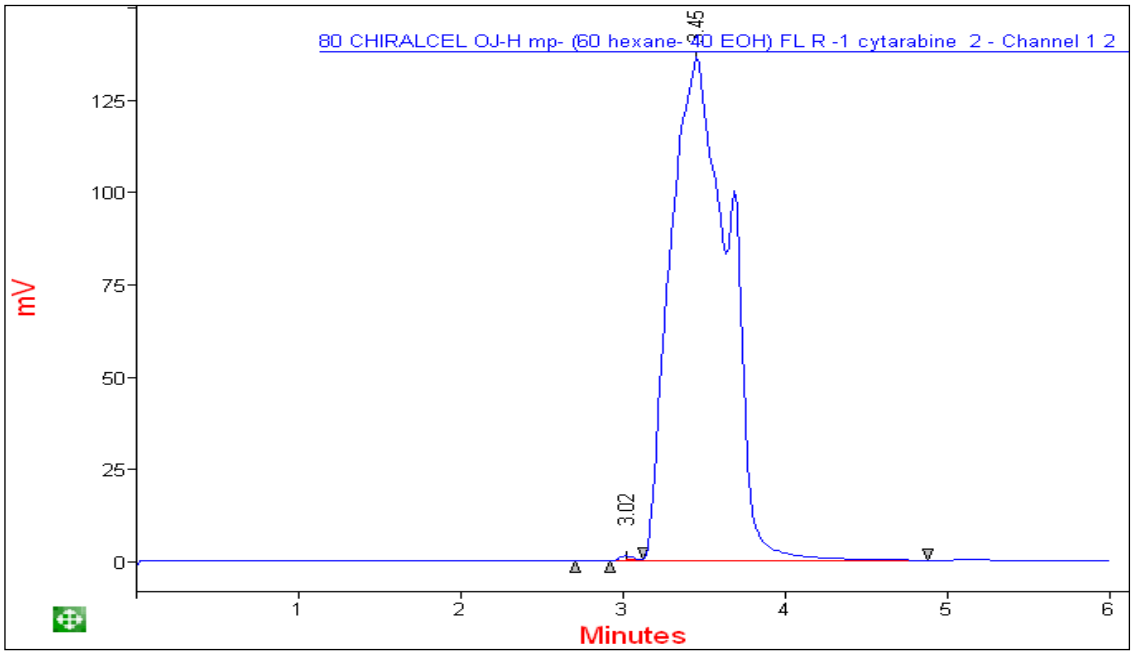


Figure 3.25: HPLC chromatogram showing the result for cytarabine with 60 % hexane and 40 % ethanol at a flow rate 1.0ml/min on the Chiralcel OJ-H column.

C.

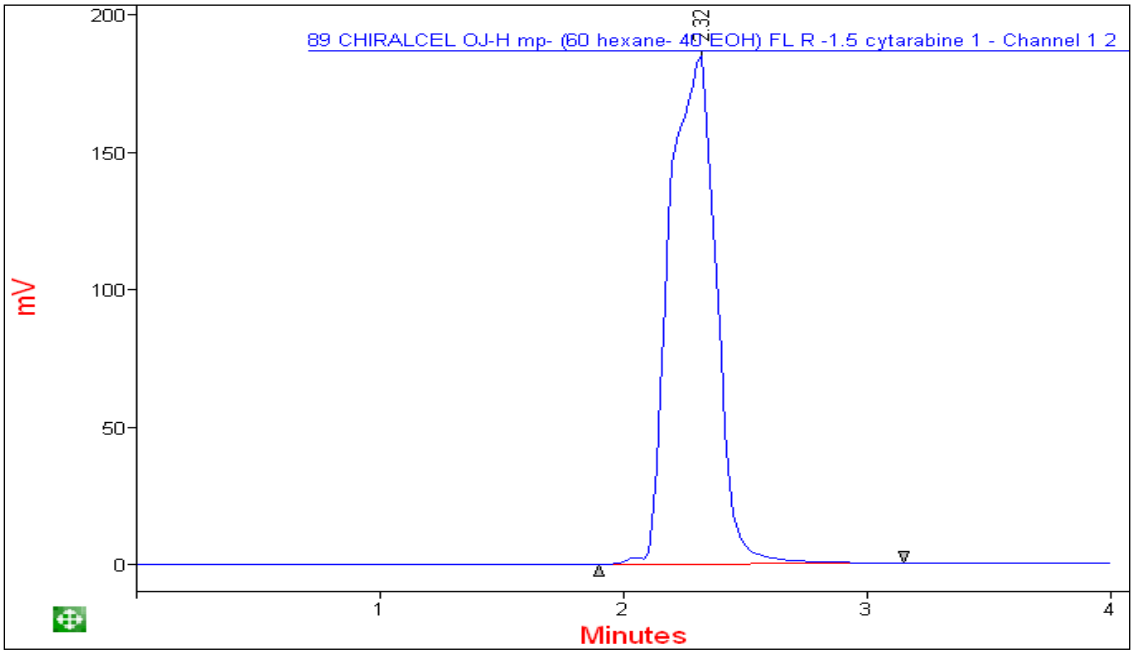


Figure 3.26: HPLC chromatogram showing the result for cytarabine with 60 % hexane and 40 % ethanol at a flow rate 1.5 ml/min on the Chiralcel OJ-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: ethanol (v/v) (50:50) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

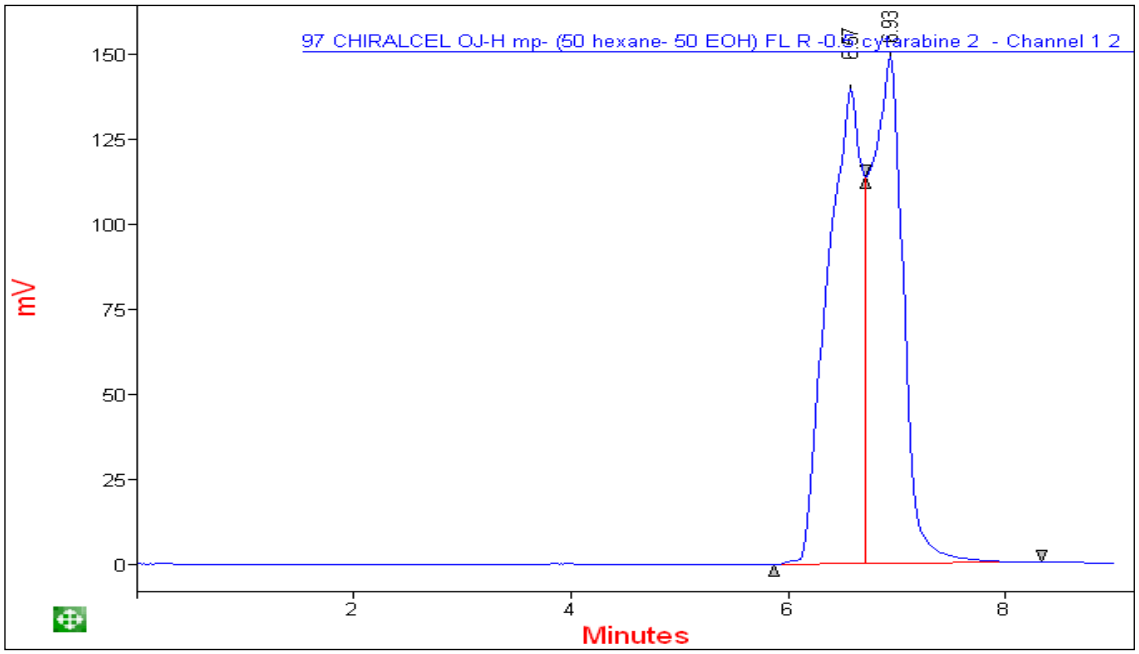


Figure 3.27: HPLC chromatogram showing the result for cytarabine with 50 % hexane and 50 % ethanol at a flow rate 0.5 ml/min on the Chiralcel OJ-H column.

B.

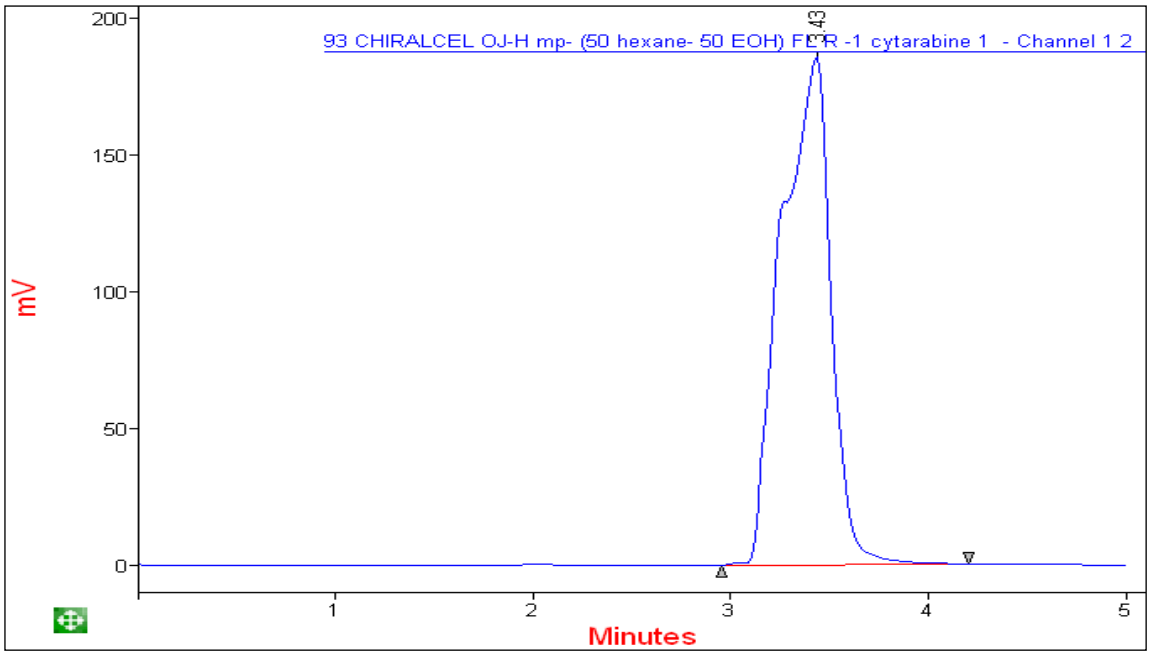


Figure 3.28: HPLC chromatogram showing the result for cytarabine with 50% hexane and 50% ethanol at a flow rate 0.5 ml/min on the Chiralcel OJ-H column.

C.

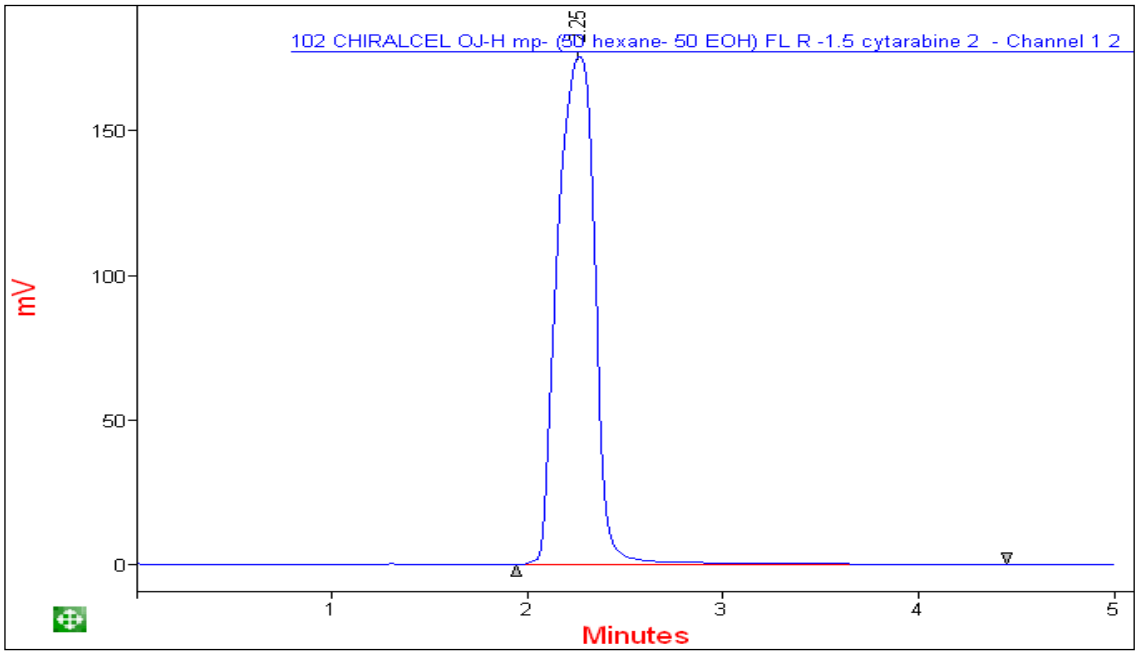


Figure 3.29: HPLC chromatogram showing the result for cytarabine with 50% hexane and 50% ethanol at a flow rate 0.5 ml/min on the Chiralcel OJ-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: ethanol (v/v) (40:60) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

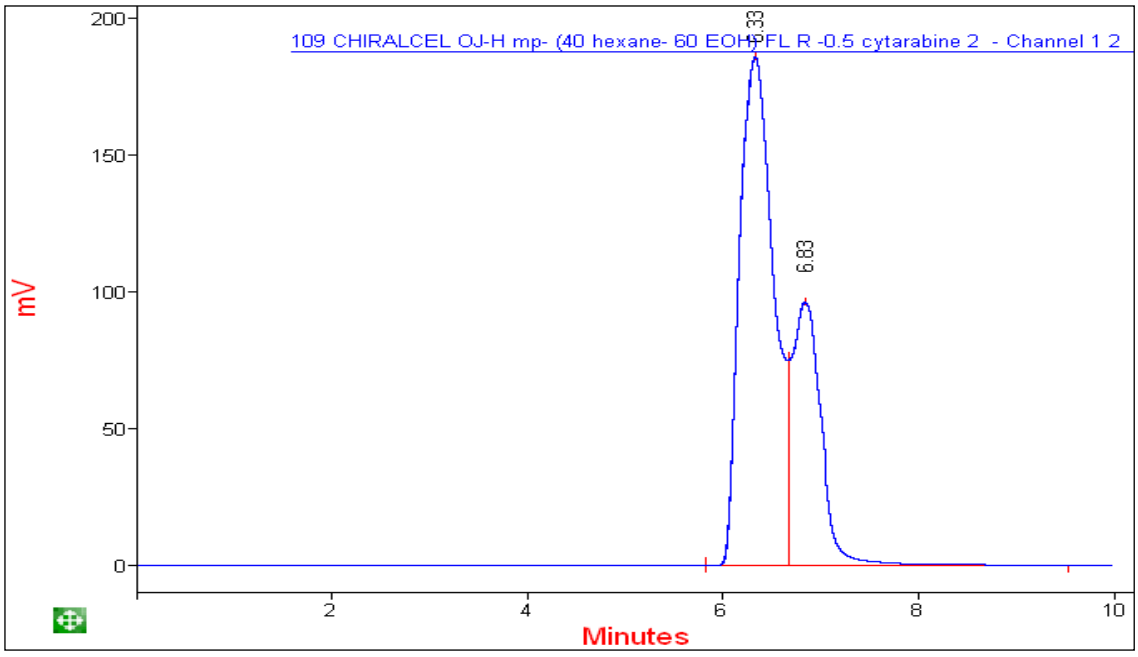


Figure 3.30: HPLC chromatogram showing the result for cytarabine with 40% hexane and 60% ethanol at a flow rate 0.5 ml/min on the Chiralcel OJ-H column.

B.

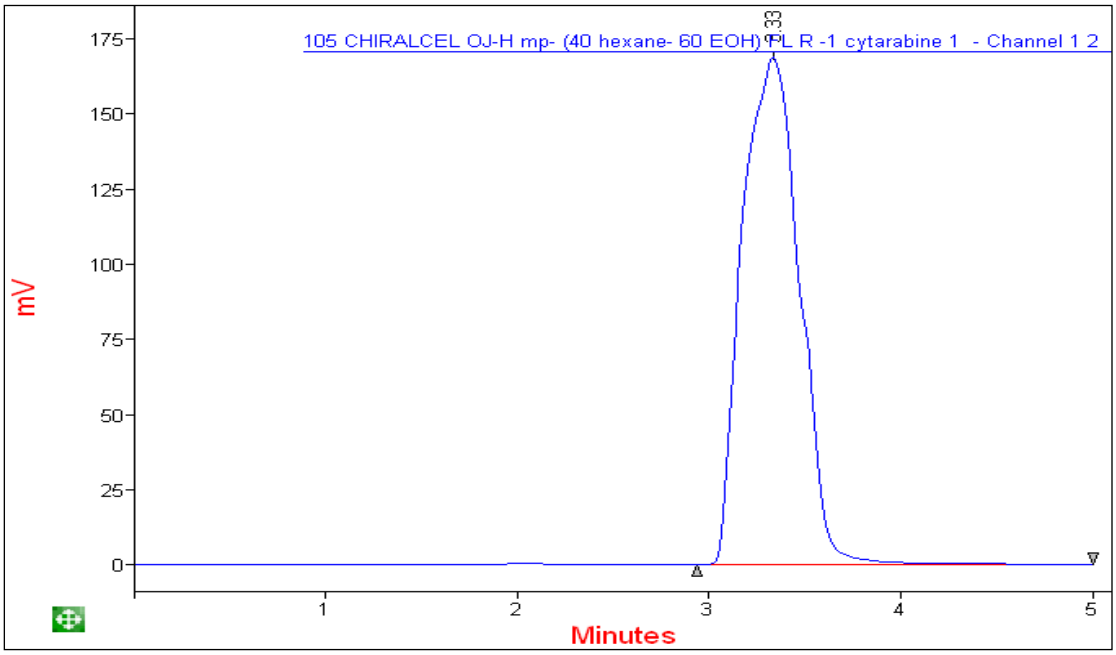


Figure 3.31: HPLC chromatogram showing the result for cytarabine with 40% hexane and 60% ethanol at a flow rate 1.0 ml/min on the Chiralcel OJ-H column.

C.

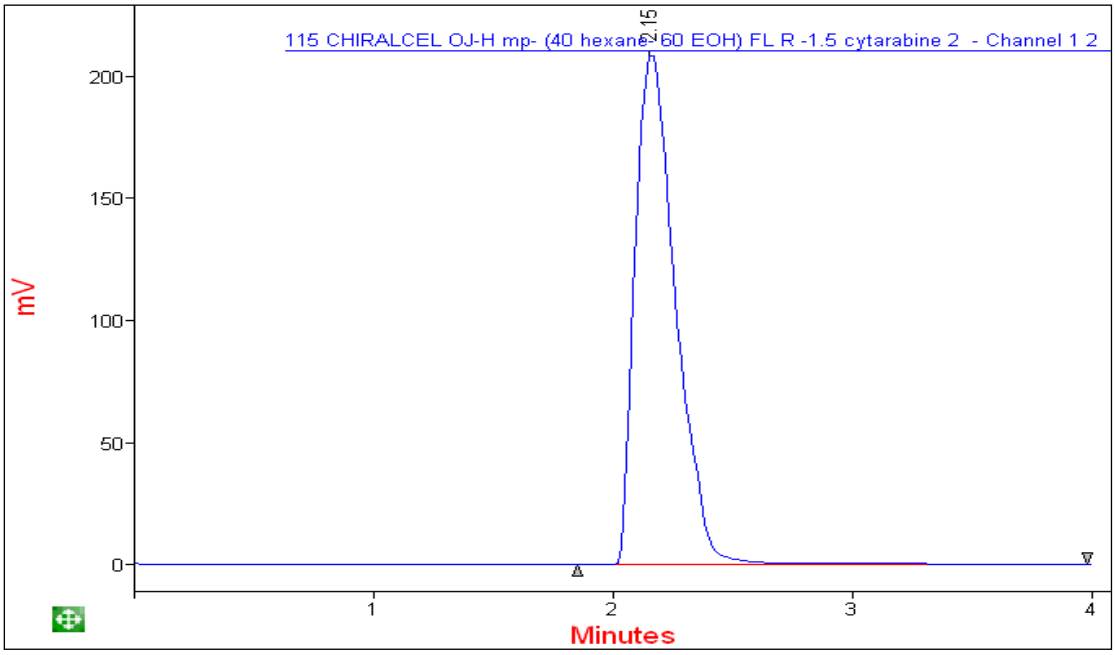


Figure 3.32: HPLC chromatogram showing the result for cytarabine with 40% hexane and 60% ethanol at a flow rate 1.5 ml/min on the Chiralcel OJ-H column.

3.4.3 HPLC results for Chiralpak AD-H using hexane: ethanol (v/v)

Attempted separation of enantiomers of cytarabine (10 mg %) with flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralpak AD-H column. Partial separation was achieved using a mobile phase ratios 50:50 (Fig. 3.39 & 3.40), 60:40 (Fig. 3.36, 3.37 & 3.38) and 80:20 (Fig. 3.33 & 3.34).

Table 3.7: Effect of ethanol on the selectivity and resolution of cytarabine enantiomers using the Chiralpak AD-H column at different flow rates. Data values ± standard deviation.

MOBILE PHASE HEXANE:EtOH (v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
90/10	0.5	8.21	6954 ± 33.03
	1	4.73	3298 ± 79.32
	1.5	2.43	1475 ± 19.74
80/20	0.5	13.07	2513 ± 109.67
		13.52	3203 ± 72.53
	1	6.38	1461 ± 66.42
		6.67	1844 ± 113.62
	1.5	4.38	2330 ± 56.68
70/30	0.5	8.22	5192 ± 60.64
	1	4.08	3550 ± 76.60
	1.5	2.70	2328 ± 50.63
60/40	0.5	6.70	1403 ± 65.05
		7.08	4995 ± 45.21
	1	3.48	4921 ± 65.86
		3.68	2247 ± 92.22
	1.5	2.30	1478 ± 128.79
		2.47	2469 ± 17.78
50/50	0.5	6.50	3521 ± 45.91
		6.82	2737 ± 67.54
		7.10	727 ± 7.74
	1	3.37	1936 ± 35.62
		3.57	1492 ± 38.41
	1.5	2.20	2407 ± 34.15
40/60	0.5	6.57	6196 ± 33.21
	1	3.23	3574 ± 172.12
	1.5	2.15	2443 ± 25.81
30/70	0.5	5.95	5535.80 ± 81.40
	1	3.10	2958.34 ± 20.29
	1.5	2.85	1544.74 ± 4.32

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: ethanol (v/v) (80:20) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralpak AD-H column.

A.

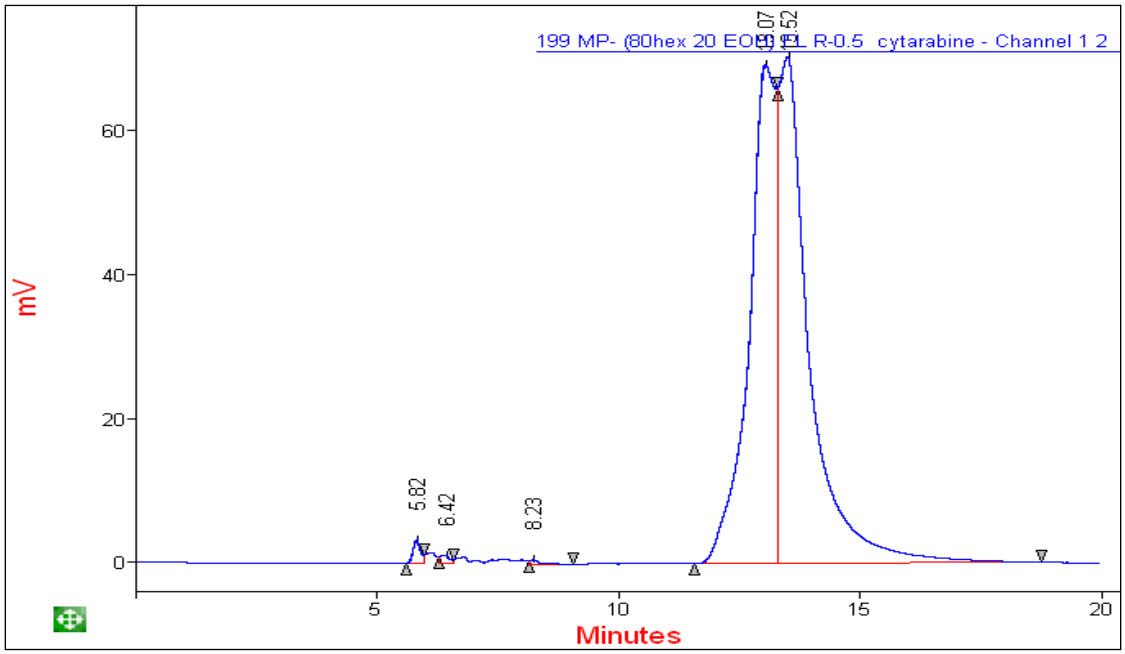


Figure 3.33: HPLC chromatogram showing the result for cytarabine with 80 % hexane and 20 % ethanol at flow rate 0.5 ml/min on the Chiralpak AD-H column.

B.

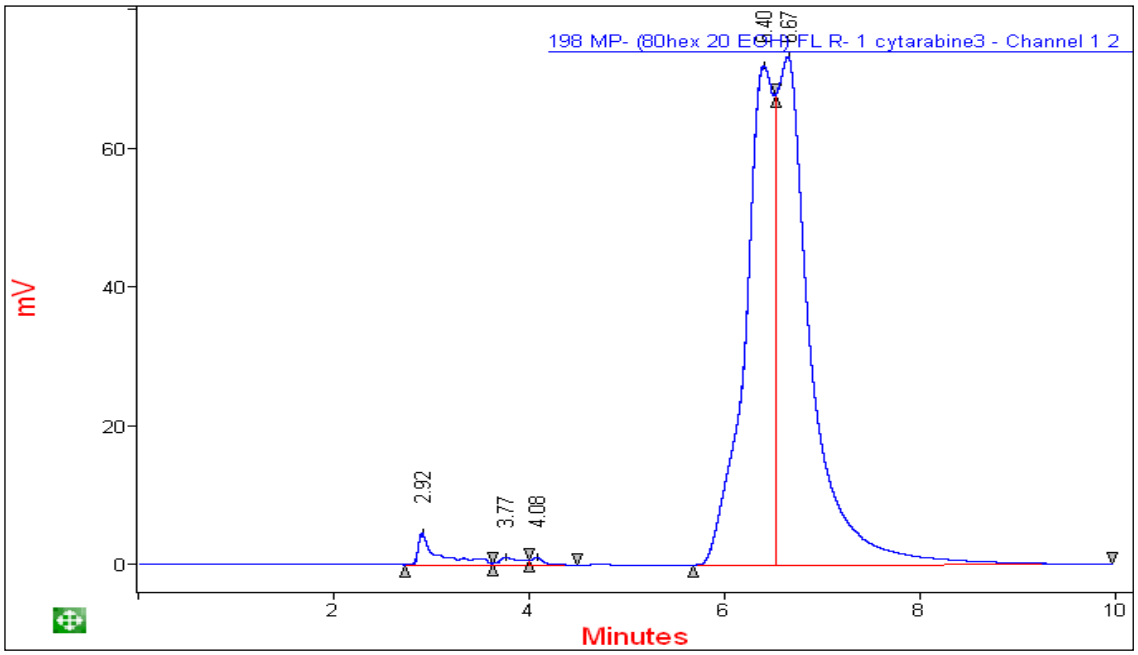


Figure 3.34: HPLC chromatogram showing the result for cytarabine with 80 % hexane and 20 % ethanol at flow rate 1.0 ml/min on the Chiralpak AD-H column.

C.

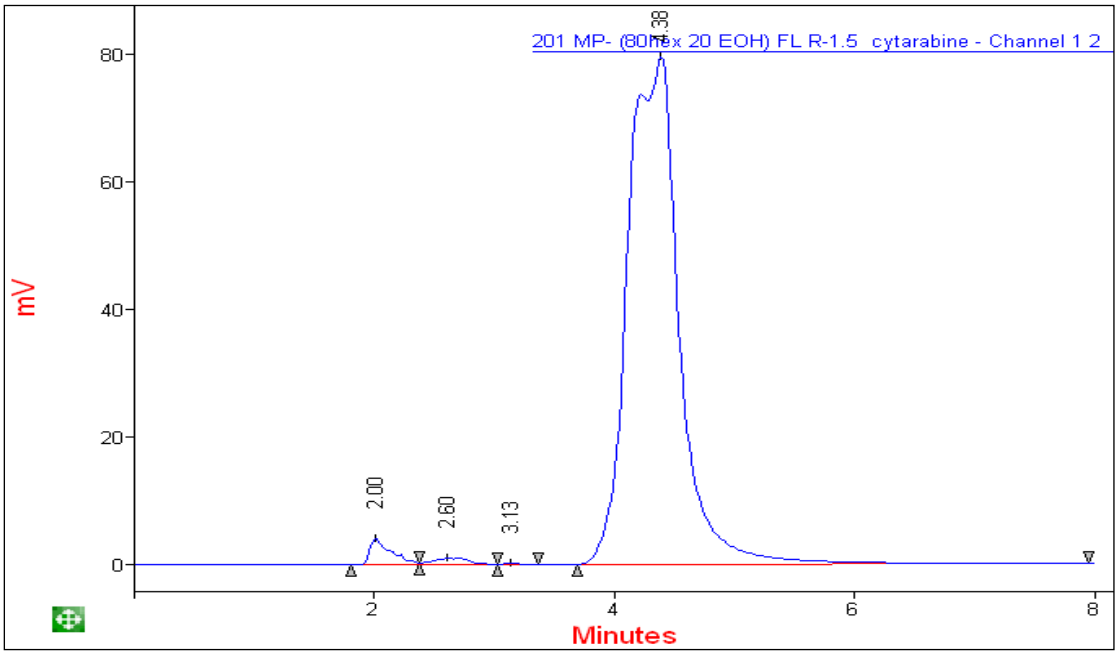


Figure 3.35: HPLC chromatogram showing the result for cytarabine with 80 % hexane and 20 % ethanol at flow rate 1.5 ml/min on the Chiralpak AD-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: ethanol (v/v) (60:40) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralpak AD-H column.

A.

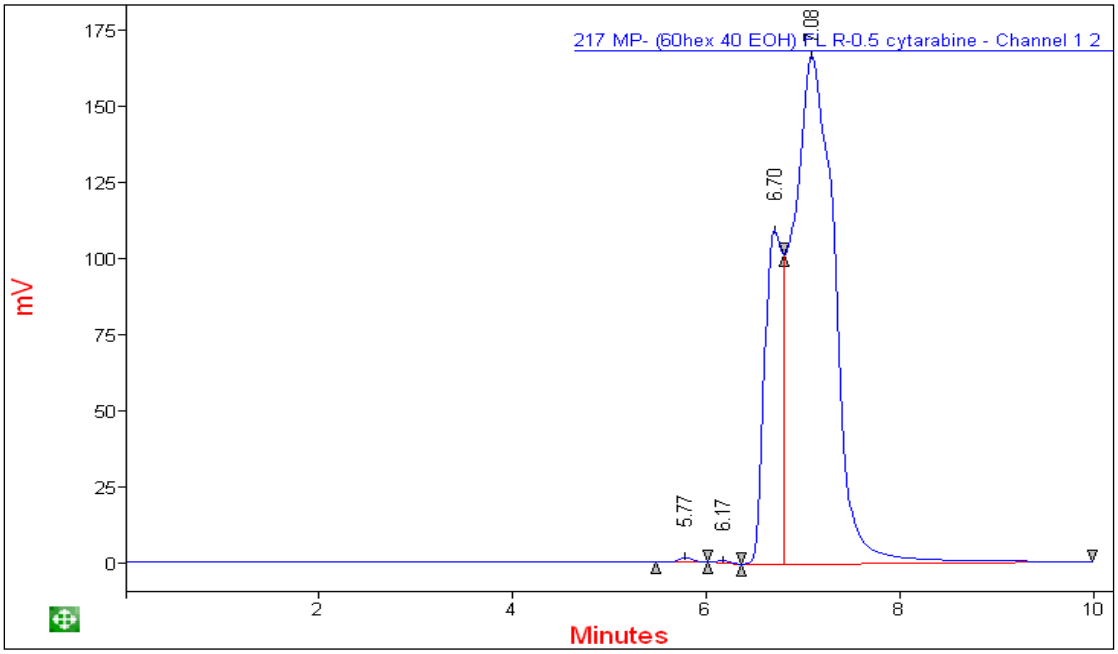


Figure 3.36: HPLC chromatogram showing the result for cytarabine with 60 % of hexane and 40 % ethanol at flow rate 0.5 ml/min on the Chiralpak AD-H column.

B.

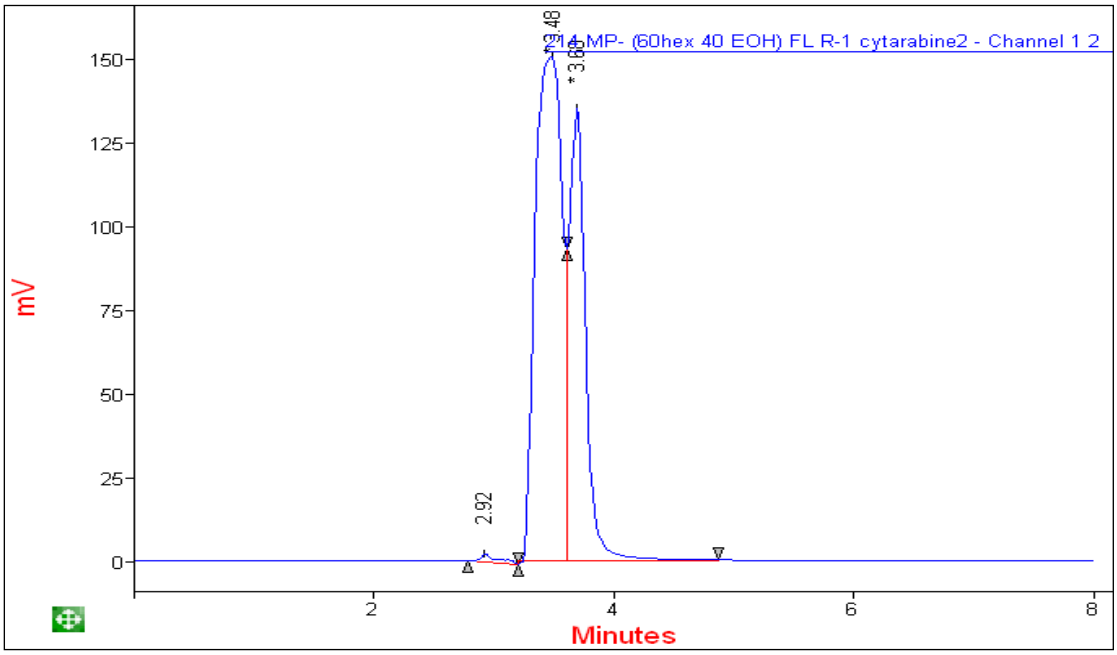


Figure 3.37: HPLC chromatogram showing the result for cytarabine with 60 % of hexane and 40 % ethanol at flow rate 1.0 ml/min on the Chiralpak AD-H column.

C.

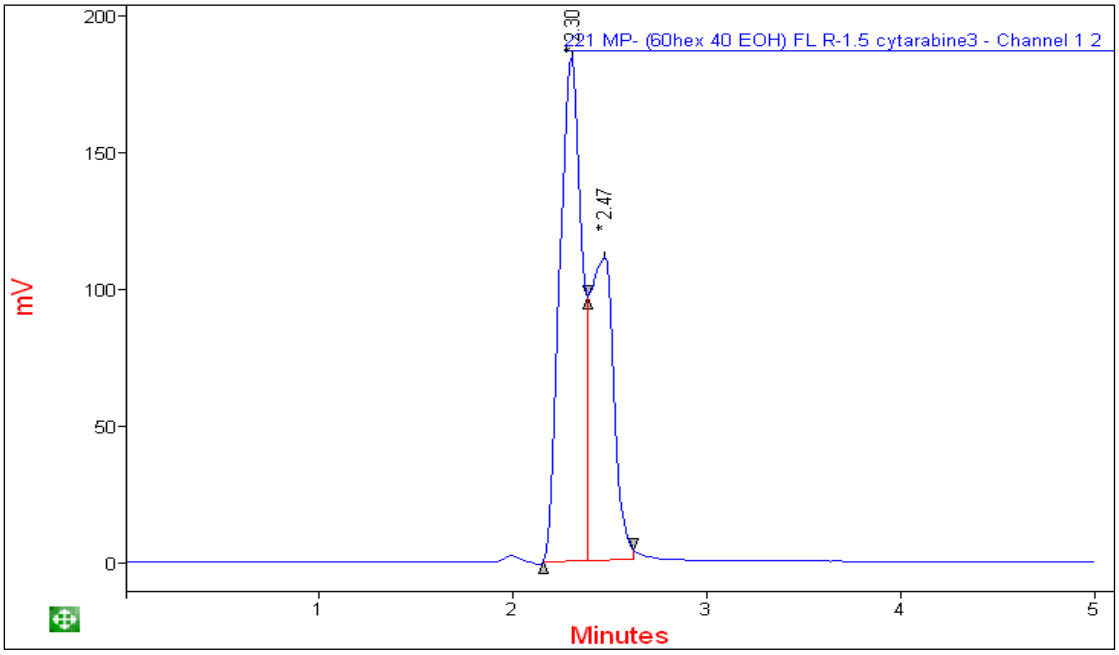


Figure 3.38: HPLC chromatogram showing the result for cytarabine with 60 % of hexane and 40 % ethanol at flow rate 1.5 ml/min on the Chiralpak AD-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: ethanol (v/v) (50:50) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralpak AD-H column.

A.

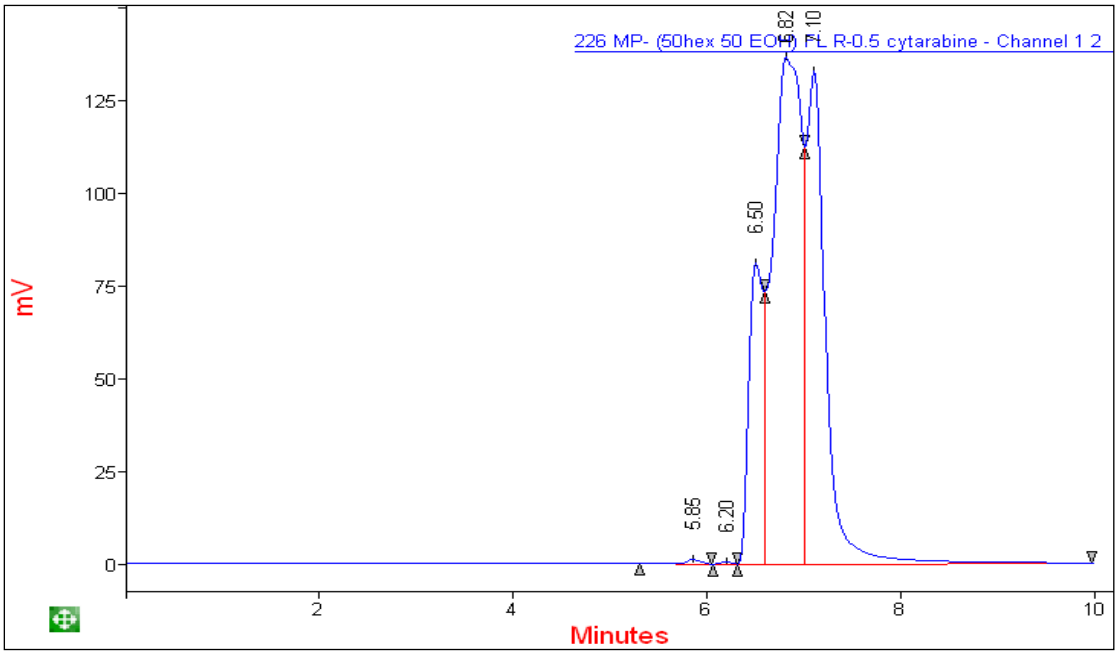


Figure 3.39: HPLC chromatogram showing the result for cytarabine 50 % of hexane and 50 % ethanol at flow rate 0.5 ml/min on the Chiralpak AD-H column.

B.

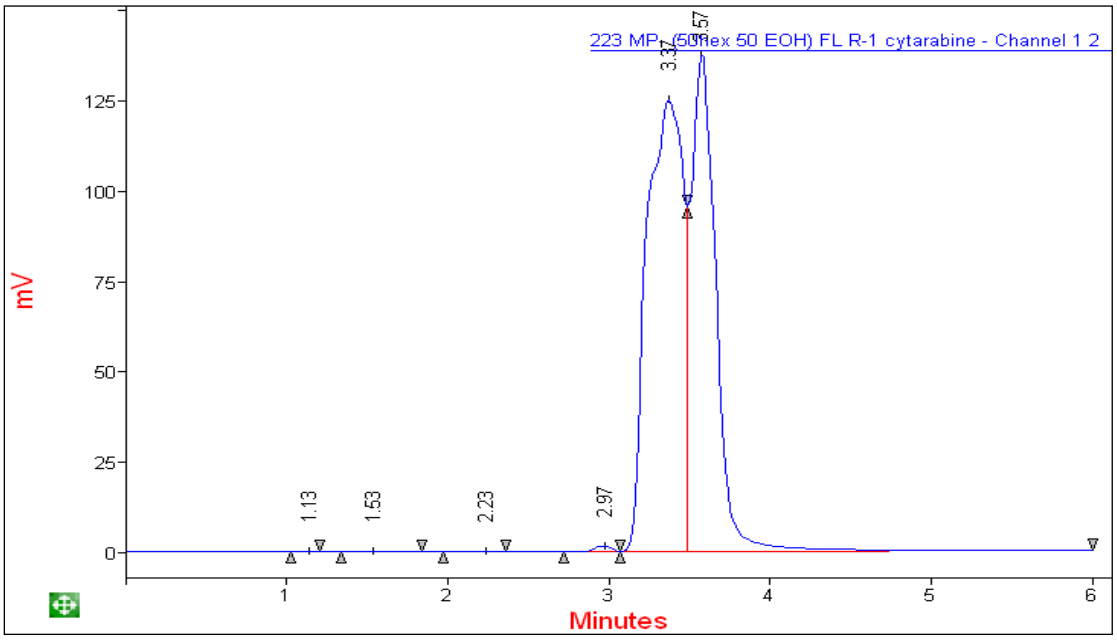


Figure 3.40: HPLC chromatogram showing the result for cytarabine 50 % of hexane and 50 % ethanol at flow rate 1.0 ml/min on the Chiralpak AD-H column.

C.

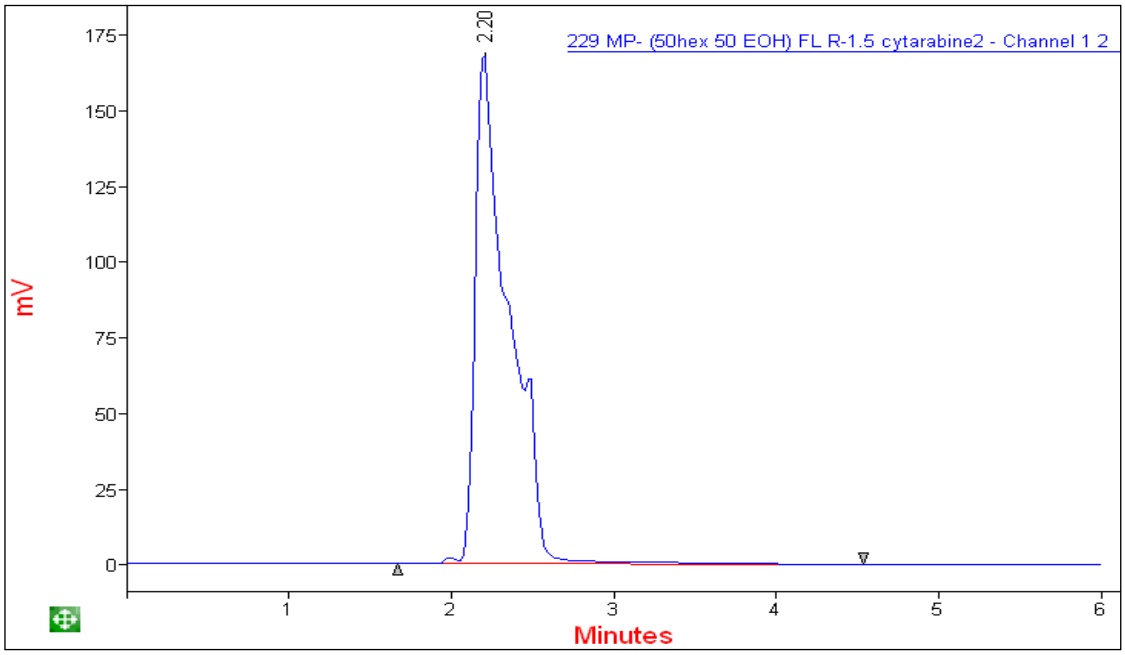


Figure 3.41: HPLC chromatogram showing the result for cytarabine 50 % of hexane and 50 % ethanol at flow rate 1.5 ml/min on the Chiralpak AD-H column.

3.5 HPLC method for selectivity and resolution of cytarabine enantiomers on Chiralcel OJ-H and Chiralpak AD-H columns using chiral modifier diethylamine (DEA)

The Chiralcel OJ-H and Chiralpak AD-H columns were used with different ratios of 2-propanol and ethanol as organic modifiers in hexane together with a basic modifier DEA. The selectivity of these two columns as well as the effect of the mobile phase characteristics on the retention and resolution of cytarabine enantiomers was investigated. The cytarabine was analysed at a wavelength of 254 nm.

Chromatographic conditions - Chromatographic separation of cytarabine enantiomers was carried out using an isocratic system at ambient temperature, unless otherwise specified. A combination of hexane: 2-propanol: DEA (0.2 %) (v/v/v) and hexane: ethanol: DEA (0.2 %) (v/v/v). DEA was added to various ratios which resulted in improved resolution using hexane: 2-propanol or ethanol with the three columns. Cytarabine was prepared at 10 mg % dissolved in a mixture of (1:1) 2-propanol: ethanol. The injection volume was kept constant at 20 µl.

3.5.1 HPLC results for Chiralcel OJ-H using hexane: 2-propanol: DEA (v/v/v)

From the results of using varying combinations of hexane: 2-propanol on the Chiralcel OJ-H column, the most promising conditions were chosen and repeated with the addition of DEA. Partial separation was achieved using the ratios 50:50:0.2 v/v/v (Fig 3.43), and 70:30:0.5 v/v/v (Fig 3.46 and 3.47).

Table 3.8: Effect of DEA on the selectivity and resolution of cytarabine enantiomers using three selected ratios of hexane and 2-propanol on the Chiralcel OJ-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE HEXANE:IPA:DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
40: 60: 0.2	0.5	6.30	6108 ± 162.93
	1	3.22	3776 ± 121.41
	1.5	2.15	2816 ± 56.61
50: 50: 0.2	0.5	6.40	2060 ±119.38
		6.78	4534 ± 82.61
	1	3.33	4230 ± 97.16
	1.5	2.22	2813 ±123.13
70: 30: 0.2	0.5	6.48	3275 ± 81.75
		6.92	2158 ± 106.54
	1	3.27	1817 ± 24.79
		3.47	2235 ± 71.26
	1.5	2.23	2782 ± 107.32

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol: DEA (v/v/v) (50:50:0.2) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

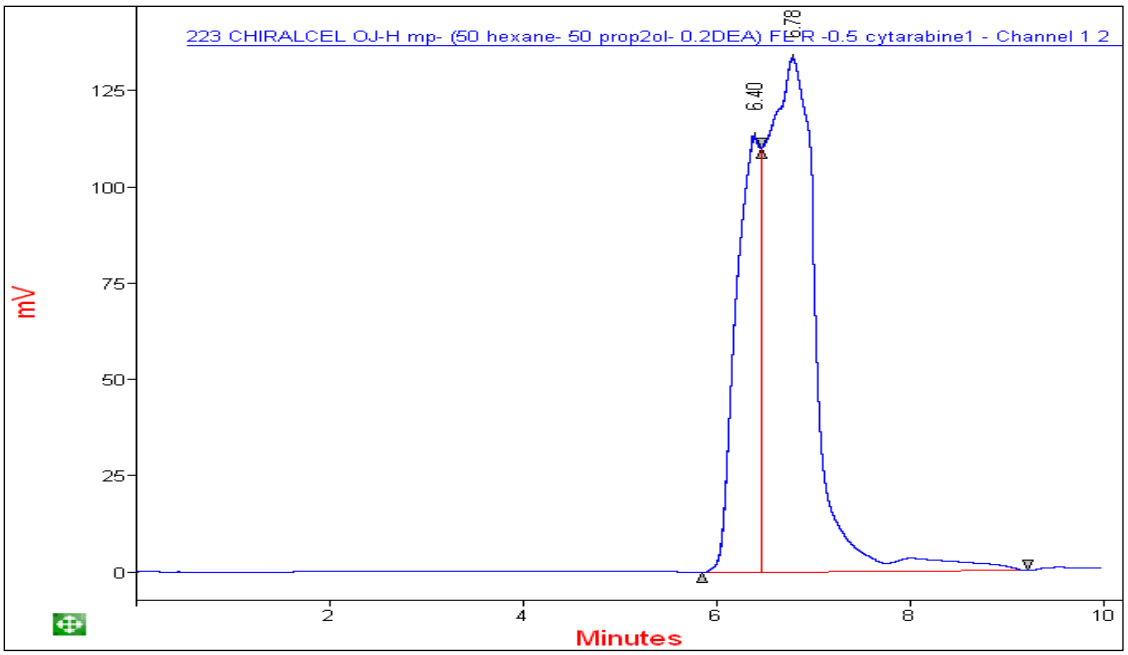


Figure 3.42: HPLC chromatogram showing the result for cytarabine using 50 % of hexane and 50 % 2-propanol and 0.2 % DEA at flow rate 0.5 ml/min on the Chiralcel OJ-H column.

B.

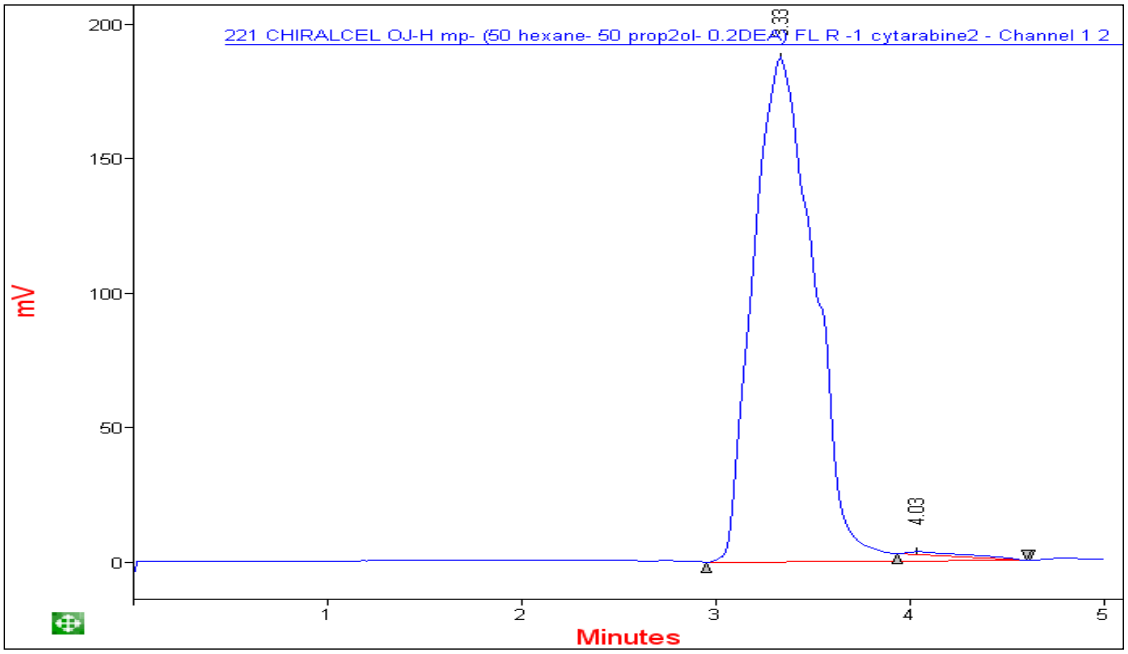


Figure 3.43: HPLC chromatogram showing the result for cytarabine using 50 % of hexane and 50 % 2-propanol and 0.2 % DEA at flow rate 1.0 ml/min on the Chiralcel OJ-H column.

C.

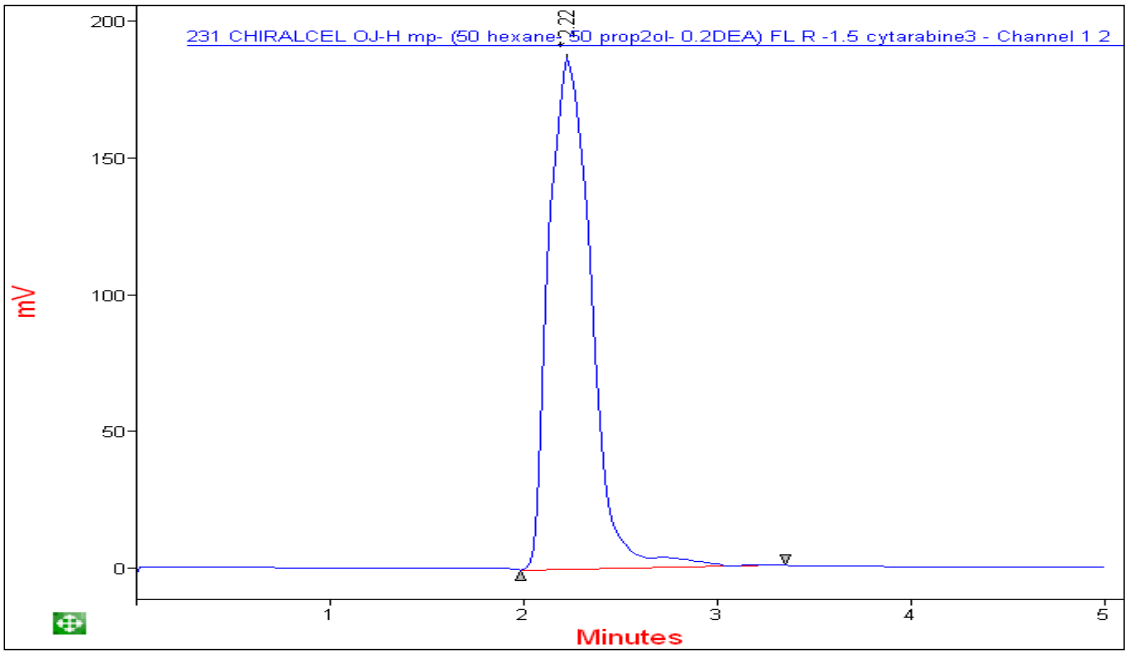


Figure 3.44: HPLC chromatogram showing the result for cytarabine using 50 % of hexane and 50 % 2-propanol and 0.2 % DEA at flow rate 1.5 ml/min on the Chiralcel OJ-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol: DEA (v/v/v) (70:30:0.2) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

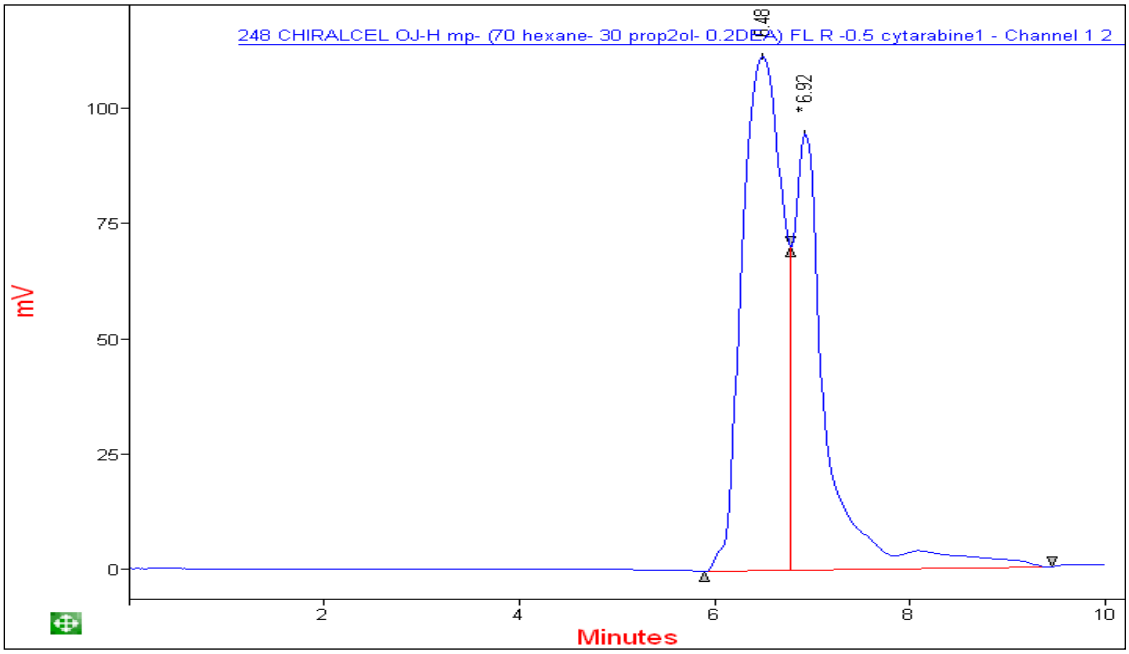


Figure 3.45: HPLC chromatogram showing the result for cytarabine using 70 % of hexane and 30 % 2-propanol and 0.2 % DEA at flow rate 0.5 ml/min on the Chiralcel OJ-H column.

B.

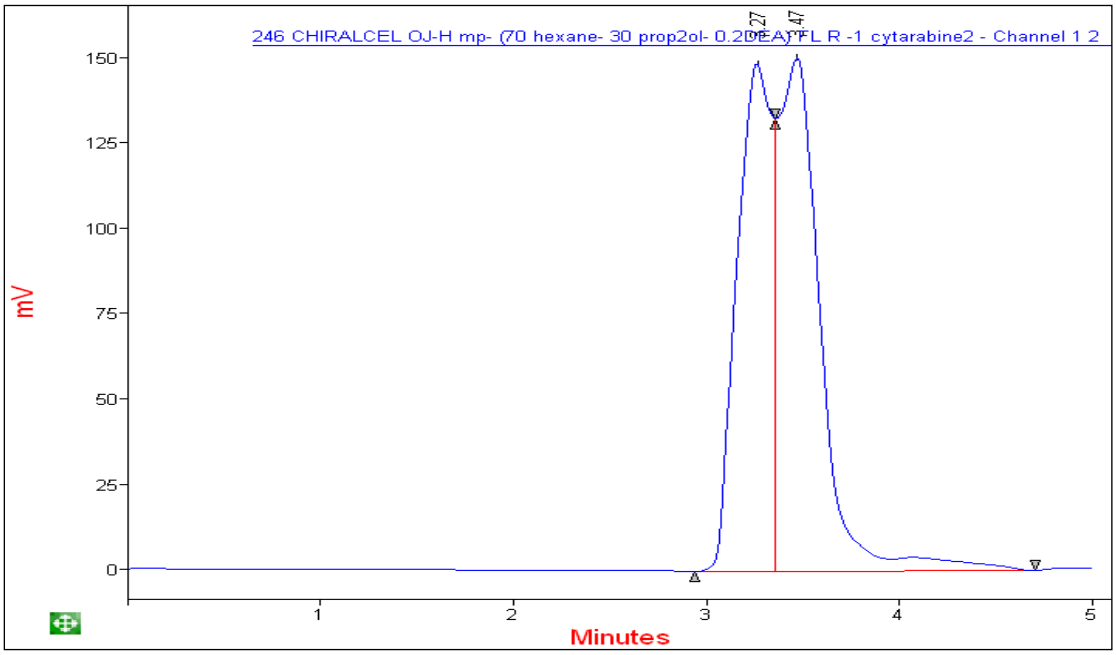


Figure 3.46: HPLC chromatogram showing the result for cytarabine using 70 % of hexane and 30 % 2-propanol and 0.2 % DEA at flow rate 1.0 ml/min on the Chiralcel OJ-H column.

C.

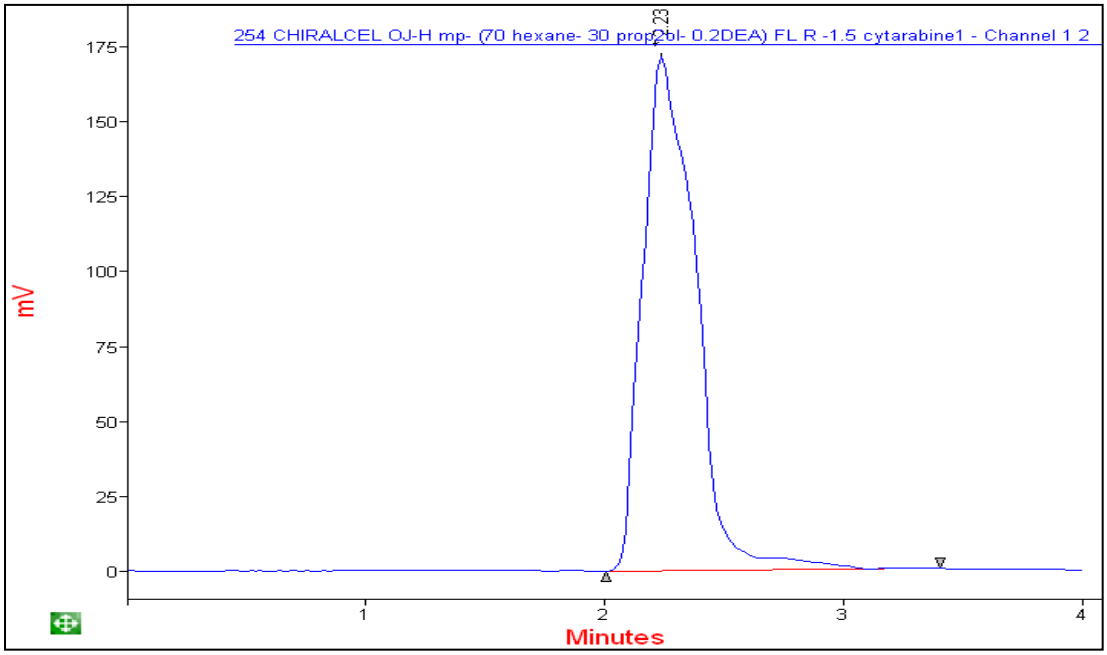


Figure 3.47: HPLC chromatogram showing the result for cytarabine using 70 % of hexane and 30 % 2-propanol and 0.2 % DEA at flow rate 1.5 ml/min on the Chiralcel OJ-H column.

3.5.2 HPLC results for the Chiralcel OJ-H using hexane: ethanol: DEA (v/v/v)

From the results using varying combinations of hexane: ethanol on the Chiralcel OJ-H column, the most promising conditions were chosen and repeated with the addition of DEA. Partial separation was achieved using ratios 50:50:0.2 v/v/v (Fig 3.49) and 60:40:0.5 v/v/v (Fig 3.52).

Table 3.9: Effect of ethanol and DEA on the selectivity and resolution of cytarabine enantiomers using selected ratios of hexane and ethanol on the Chiralcel OJ-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE HEXANE:EtOH:DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
50:50:0.2	0.5	6.78	5745 ± 191.92
		7.17	905 ± 5.53
	1	3.37	3702 ± 121.53
	1.5	2.22	2558 ± 150.06
60:40:0.2	0.5	6.65	2135 ± 110.57
		7.20	2326 ± 109.38
	1	3.53	3663 ± 141.41
	1.5	2.28	2363 ± 115.94
70:30:0.2	0.5	7.47	6115 ± 147.45
	1	3.80	3779 ± 140.35
	1.5	2.47	2593 ± 81.60

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: ethanol: DEA (v/v/v) (50:50:0.2) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

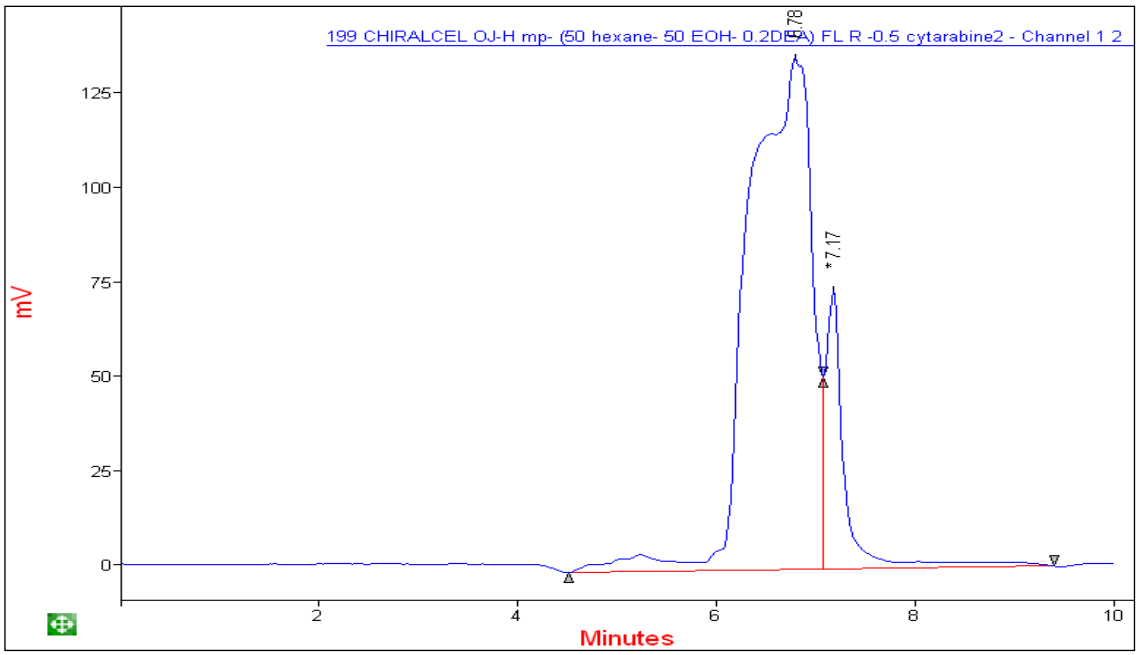


Figure 3.48: HPLC chromatogram showing the result for cytarabine using 50 % of hexane and 50 % ethanol and 0.2 % DEA at flow rate 0.5 ml/min on the Chiralcel OJ-H column.

B.

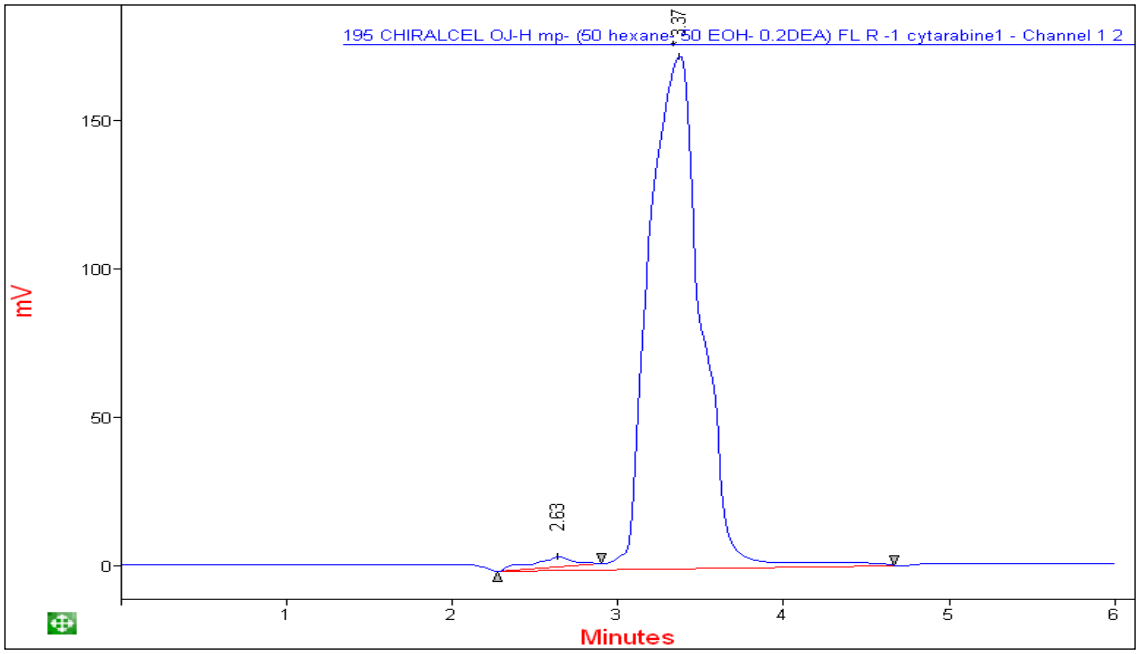


Figure 3.49: HPLC chromatogram showing the result for cytarabine using 50 % of hexane and 50 % ethanol and 0.2 % DEA at flow rate 1.0 ml/min on the Chiralcel OJ-H column.

C.

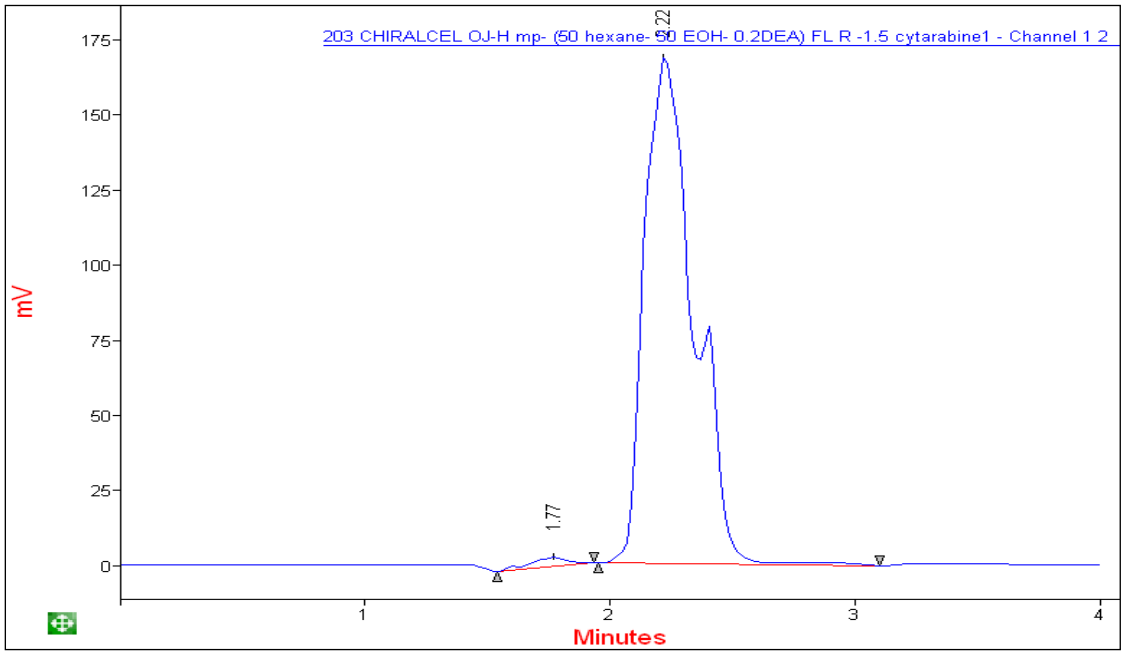


Figure 3.50: HPLC chromatogram showing the result for cytarabine using 50 % of hexane and 50 % ethanol and 0.2 % DEA at flow rate 1.5 ml/min on the Chiralcel OJ-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: ethanol: DEA (v/v/v) (60:40:0.2) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralcel OJ-H column.

A.

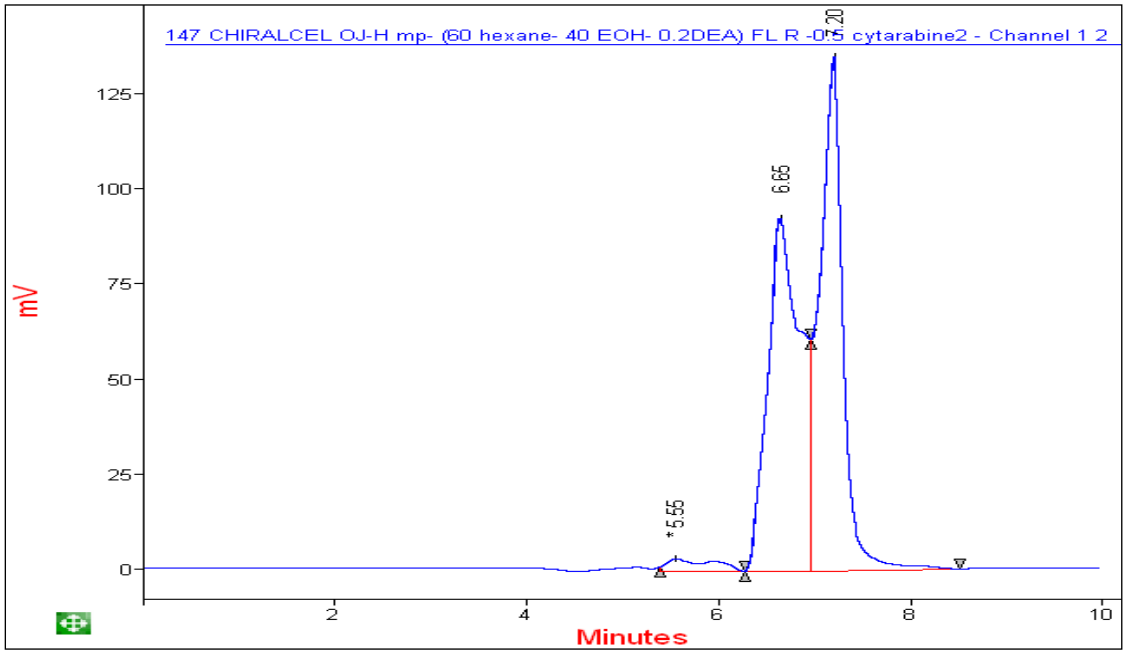


Figure 3.51: HPLC chromatogram showing the result for cytarabine using 60 % of hexane and 40 % ethanol and 0.2 % DEA at flow rate 0.5 ml/min on the Chiralcel OJ-H column.

B.

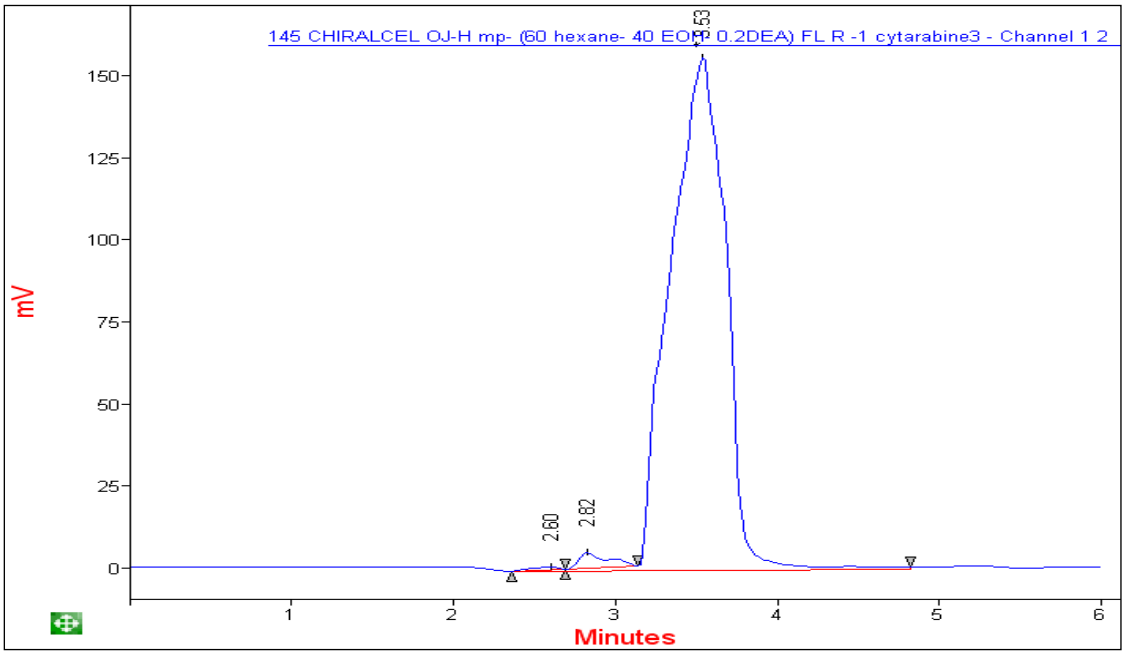


Figure 3.52: HPLC chromatogram showing the result for cytarabine using 60 % of hexane and 40 % ethanol and 0.2 % DEA at flow rate 1.0 ml/min on the Chiralcel OJ-H column.

C.

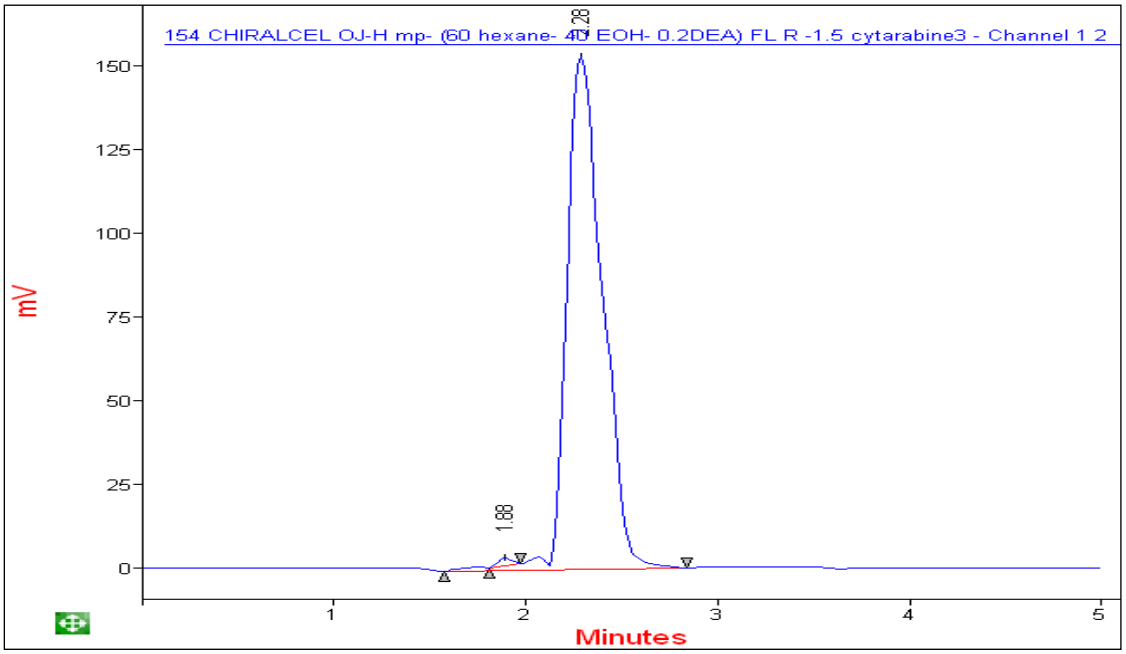


Figure 3.53: HPLC chromatogram showing the result for cytarabine using 60 % of hexane and 40 % ethanol and 0.2 % DEA at flow rate 1.5 ml/min on the Chiralcel OJ-H column.

3.5.3 HPLC results for Chiralpak AD-H using hexane: 2-propanol: DEA (v/v/v)

From the results using varying combinations of hexane: 2-propanol on the Chiralpak AD-H column, the most promising combinations were chosen and repeated with the addition of DEA. Partial separation was achieved using the ratios of 50:50:0.2 v/v/v (Fig 3.56) and 60:40:0.5 v/v/v (Fig 3.58, 3.59. 3.60).

Table 3.10: Effect of 2-propanol and DEA on the selectivity and resolution of cytarabine enantiomers on the Chiralpak AD-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE HEXANE: IPA: DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
50:50:0.2	0.5	6.10	1076 ± 51.87
		6.72	4939 ± 51.89
	1	3.10	1186 ± 60.59
		3.40	2254 ± 88.48
	1.5	2.07	2420 ± 45.89
60:40:0.2	0.5	6.28	854 ± 4.61
		6.65	1622 ± 22.25
		6.95	1506 ± 5.37
		7.20	1344 ± 33.83
	1	3.18	1154 ± 95.86
		3.57	2330 ± 99.72
	1.5	2.15	1501 ± 62.58
		2.42	870 ± 31.45

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol: DEA (v/v/v) (50:50:0.2) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralpak AD-H column.

A.

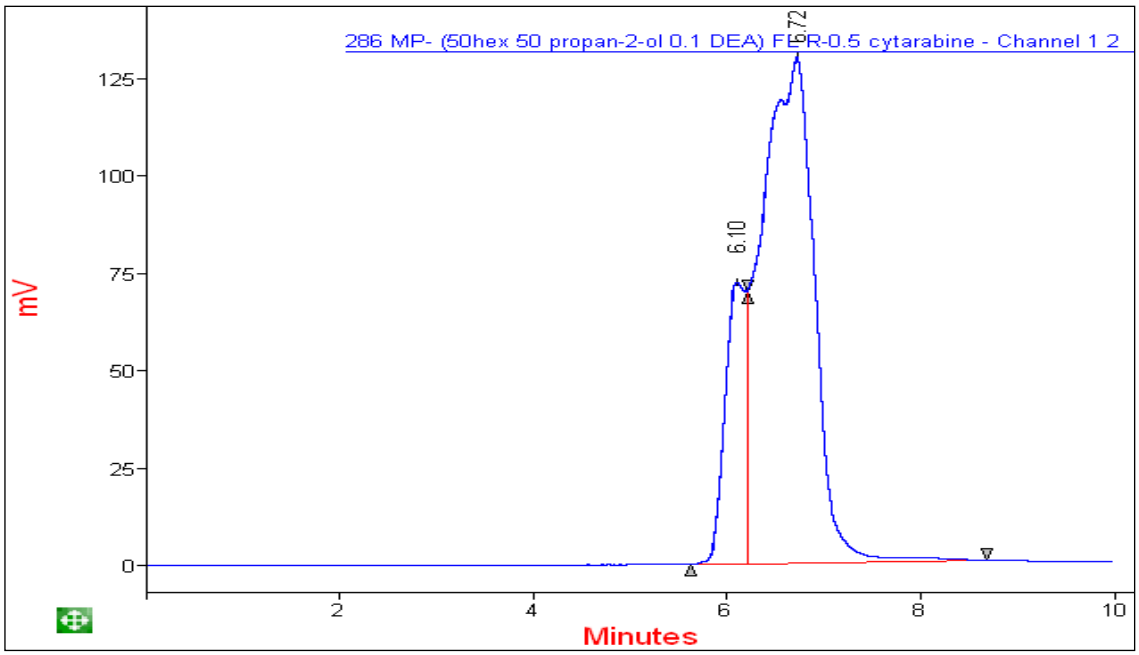


Figure 3.54: HPLC chromatogram showing the result for cytarabine using 50 % of hexane and 50 % 2-propanol and 0.2% DEA at flow rate 0.5 ml/min on the Chiralpak AD-H column.

B.

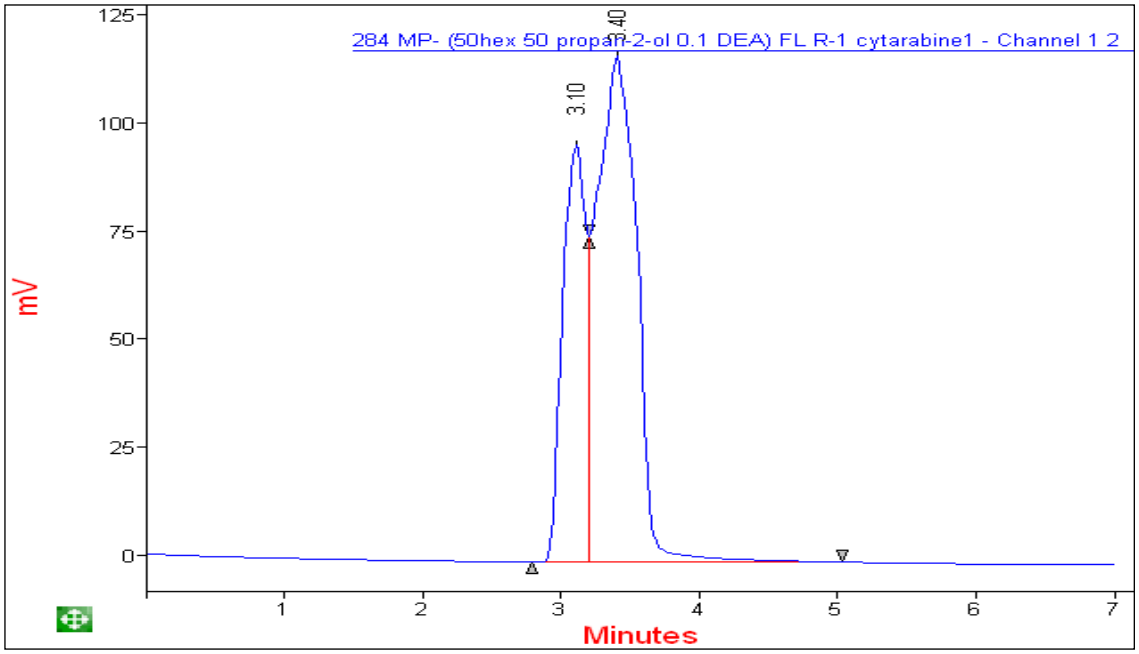


Figure 3.55: HPLC chromatogram showing the result for cytarabine using 50 % of hexane and 50 % 2-propanol and 0.2% DEA at flow rate 1.0 ml/min on the Chiralpak AD-H column.

C.

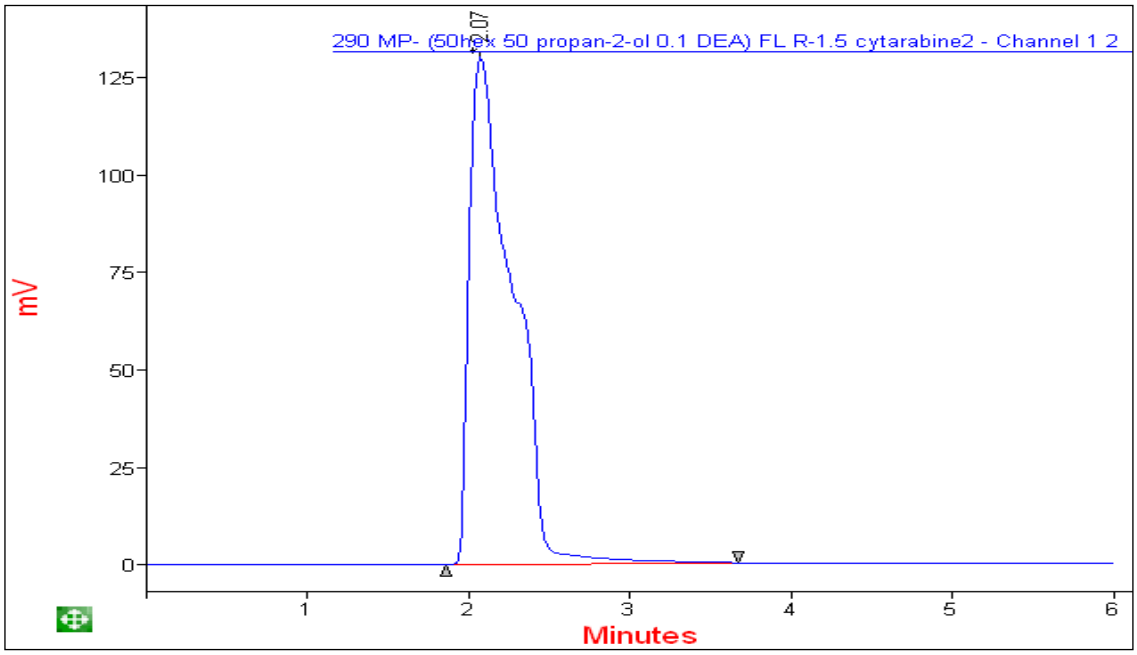


Figure 3.56: HPLC chromatogram showing the result for cytarabine using 50 % of hexane and 50 % 2-propanol and 0.2% DEA at flow rate 1.5 ml/min on the Chiralpak AD-H column.

Chromatograms showing partial resolution of cytarabine enantiomers using hexane: 2-propanol: DEA (v/v/v) (60:40:0.2) at flow rate 0.5 ml/min (A), 1 ml/min(B) and 1.5 ml/min (C) on the Chiralpak AD-H column.

A.

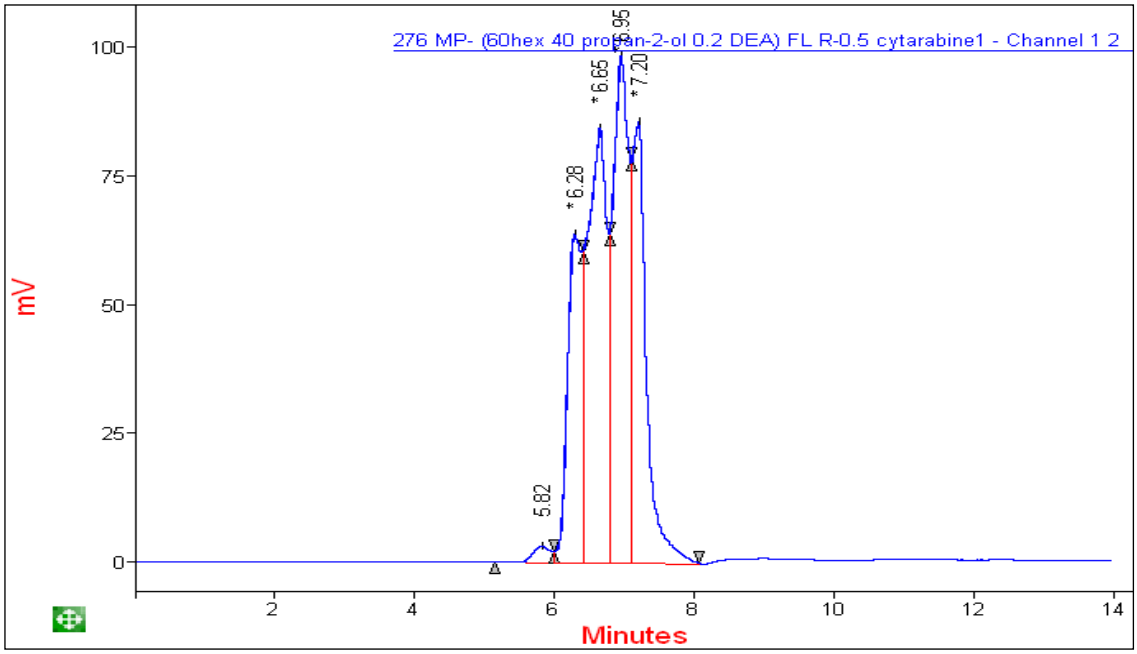


Figure 3.57: HPLC chromatogram showing the result for cytarabine using 60 % of hexane and 40 % 2-propanol and 0.2% DEA at flow rate 0.5 ml/min on the Chiralpak AD-H column.

B.

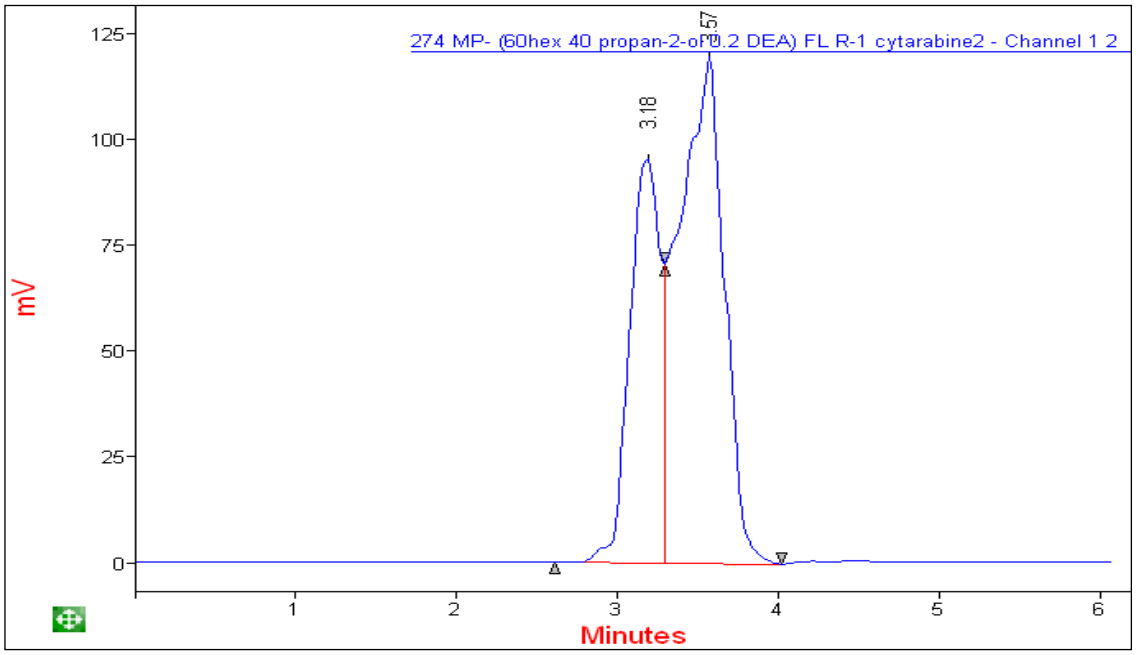


Figure 3.58: HPLC chromatogram showing the result for cytarabine using 60 % of hexane and 40 % 2-propanol and 0.2 % DEA at flow rate 1.0 ml/min on the Chiralpak AD-H column.

C.

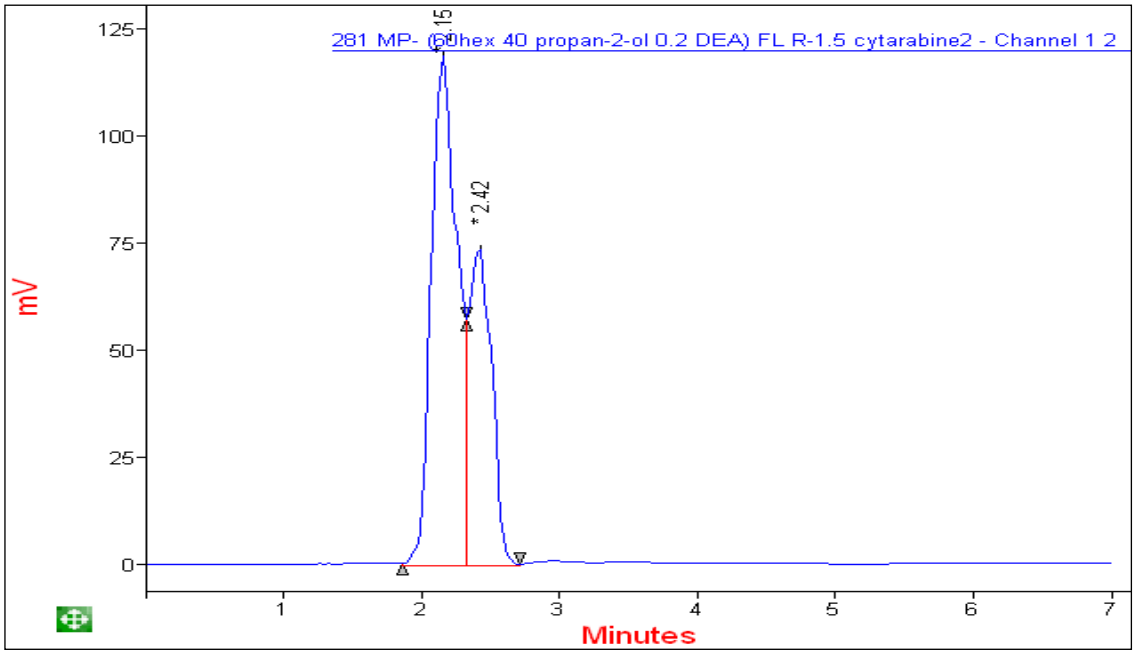


Figure 3.59: HPLC chromatogram showing the result for cytarabine using 60 % of hexane and 40 % 2-propanol and 0.2 % DEA at flow rate 1.5 ml/min on the Chiralpak AD-H column.

3.5.4 HPLC results for Chiralpak AD-H using hexane: ethanol: DEA (v/v/v)

From the results using varying combinations of hexane: ethanol on the Chiralpak AD-H column (Table 3), the most promising combinations were chosen and repeated with the addition of DEA. No significant signs of separation were observed.

Table 3.11: Effect of ethanol and DEA on the selectivity and resolution of cytarabine enantiomers using the Chiralpak AD-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE HEXANE:EtOH:DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
80:20:0.2	0.5	13.42	7882 ± 271.62
	1	6.62	5072 ± 162.11
	1.5	4.38	3451 ± 57.27
70:30:0.2	0.5	8.63	7980 ± 117.68
	1	4.30	4152 ± 51.78
	1.5	2.90	2742 ± 36.86
60:40:0.2	0.5	3.62	4295 ± 49.18
	1	2.97	3582 ± 32.00
	1.5	2.37	3042 ± 25.68
50:50:0.2	0.5	6.62	7183 ± 85.24
	1	3.35	4285 ± 43.80
	1.5	2.75	3681 ±29.11
40:60:0.2	0.5	6.42	8591 ± 65.91
	1	3.27	4354 ± 39.35
	1.5	2.18	3061 ± 27.03

3.6 HPLC method for the selectivity and resolution of cytarabine enantiomers on the Chiralcel OJ-H and Chiralpak AD-H using acetonitrile: 2-propanol: diethylamine and acetonitrile: ethanol: diethylamine

The Chiralcel OD-H, Chiralcel OJ-H and Chiralpak AD-H columns were used with different ratios of 2-propanol and ethanol as organic modifiers in acetonitrile together with DEA. The selectivity of these three columns as well as the effect of mobile phase characteristics on retention and resolution of cytarabine enantiomers was investigated.

Chromatographic conditions - Chromatographic separation of cytarabine enantiomers was carried out using an isocratic system at ambient temperature. Varying combinations of acetonitrile: 2-propanol: DEA (v/v/v) and acetonitrile: ethanol: DEA (v/v/v) ranging from (95:5:0.2) to (85:15:0.2) was used as mobile phase with flow rates of 0.5, 1.0 and 1.5 ml/min. Cytarabine at a concentration of 10 mg % in 2-propanol: ethanol (1:1). The injection volume was kept constant at 20 µl and cytarabine was analysed at a wavelength of 254 nm.

3.6.1 HPLC results for the Chiralcel OD-H using acetonitrile: 2-propanol: DEA (v/v/v)

Attempted separation of enantiomers of cytarabine using flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralcel OD-H column. A significant decrease in the retention time of cytarabine was observed with increasing concentrations of 2-propanol.

Table 3.12: Effect of 2-propanol on the selectivity and resolution of cytarabine enantiomers using the Chiralcel OD-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE ACN:IPA:DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
95:5:0.2	0.5	14.28	16130± 603.98
	1	6.97	8445 ±169.34
	1.5	5.12	4807 ± 205.34
90:10:0.2	0.5	9.87	11609 ±898.46
	1	5.53	5664 ± 165.41
	1.5	3.39	3345 ± 99.49
85:15:0.2	0.5	8.58	10364 ± 385.57
	1	4.22	3049 ± 61.90
	1.5	2.64	2513 ± 62.71

3.6.2 HPLC results for the Chiralcel OD-H using acetonitrile: ethanol: DEA (v/v/v)

Attempted separation of enantiomers of cytarabine using flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralcel OJ-H column. A significant decrease in the retention time of cytarabine was observed with increasing concentrations of ethanol.

Table 3.13: Effect of ethanol on the selectivity and resolution of cytarabine enantiomers using the Chiralpak OD-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE ACN:EtOH:DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA
85:15:0.2	0.5	6.00	12758 ± 656.21
	1	3.43	4882 ± 44.74
	1.5	2.57	1924 ± 58.26
90:10:0.2	0.5	9.23	12309 ± 479.99
	1	3.20	1708 ± 169.49
	1.5	2.21	831 ± 72.75
95:5:0.2	0.5	11.95	11744 ±503.23
	1	6.75	7656 ± 349.38
	1.5	5.87	3644 ±72.06

3.6.3 HPLC results for the Chiralcel OJ-H using acetonitrile: 2-propanol: DEA (v/v/v)

Attempted separation of cytarabine enantiomers using flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralcel OJ-H column.

Table 3.14: Effect of 2-propanol on the selectivity and resolution of cytarabine enantiomers using the Chiralpak OJ-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE ACN:IPA:DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
95:5:0.2	0.5	7.47	12042 ± 136.65
	1	3.50	7168 ± 216.13
	1.5	2.40	4505 ± 71.17
90:10:0.2	0.5	7.13	1209 ± 154.88
		7.93	3680 ± 228.46
	1	3.73	2558 ± 70.85
	1.5	3.15	2511 ± 165.16
85:15:0.2	0.5	5.85	11314 ± 876.77
	1	3.80	6054 ± 105.25
	1.5	2.58	3438 ± 131.23

3.6.4 HPLC results for the Chiralcel OJ-H using acetonitrile: ethanol: DEA (v/v/v)

Attempted separation of enantiomers of cytarabine using flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralcel OJ-H column. A significant decrease in the retention time of cytarabine was observed with increasing concentrations of ethanol.

Table 3.15: Effect of ethanol on the selectivity and resolution of cytarabine enantiomers using the Chiralpak OJ-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE ACN:EtOH:DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
95:5:0.2	0.5	9.87	14504 ± 695.41
	1	4.42	7005 ± 81.42
	1.5	2.95	6037 ± 91.33
90:10:0.2	0.5	9.82	9728 ± 67.58
	1	4.33	5748 ± 106.36
	1.5	2.91	4101 ± 77.41
85:15:0.2	0.5	8.30	8471 ± 245.40
	1	3.92	5107 ± 54.29
	1.5	2.55	3488 ± 144.72

3.6.5 HPLC results for the Chiralpak AD-H using acetonitrile: 2-propanol: DEA (v/v/v)

Attempted separation of enantiomers of cytarabine using flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralpak AD-H column. A significant decrease in the retention time of cytarabine was observed with increasing concentration of 2-propanol.

Table 3.16: Effect of 2-propanol on the selectivity and resolution of cytarabine enantiomers using the Chiralpak AD-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE ACN:IPA:DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
95:5:0.2	0.5	13.87	668 ± 229.08
	1	6.70	3716 ± 100.14
	1.5	4.48	2600 ± 71.67
90:10:0.2	0.5	11.73	6922 ± 31.13
	1	5.80	3906 ± 70.25
	1.5	3.78	2607 ± 52.72
85:15:0.2	0.5	10.63	5594 ±204.02
	1	5.17	3978 ± 119.66
	1.5	3.43	2738 ± 44.30

3.6.6 HPLC results for the Chiralpak AD-H using acetonitrile: ethanol: DEA (v/v/v)

Attempted separation of enantiomers of cytarabine using flow rates of 0.5, 1.0 and 1.5 ml/min on the Chiralpak AD-H column. A significant decrease in the retention time of cytarabine was observed, with increasing concentrations of ethanol.

Table 3.17: Effect of ethanol on the selectivity and resolution of cytarabine enantiomers on the Chiralpak AD-H column at three different flow rates. Data values ± standard deviation.

MOBILE PHASE ACN:EtOH:DEA (v/v/v)	FLOW RATE (ml/min)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 3
95:5:0.2	0.5	14.47	2262 ± 83.14
	1	7.02	3601 ± 167.31
	1.5	2.18	2949 ± 171.79
90:10:0.2	0.5	10.23	6976 ± 36.95
	1	4.98	4510 ± 69.26
	1.5	3.30	2787 ± 40.92
85:15:0.2	0.5	8.33	7970 ± 110.61
	1	4.22	4872 ± 123.43
	1.5	2.77	3188 ± 65.57

3.7 Gradient elution using the HPLC results for the Chiralpak AD-H column

Gradient HPLC analysis was performed using the Chiralpak AD-H column. The varying mobile phase ratios used, consisted of 2-propanol and ethanol as organic modifiers in hexane (Section 2.4.2). Cytarabine was analysed at 254 nm at room temperature.

Chromatographic conditions - The chromatographic separation of cytarabine enantiomers was carried out using a gradient system. Throughout the chromatographic separation a constant flow rate of 1 ml/min was maintained (unless otherwise specified). The injection volume was kept constant at 20 µl. After each run the column was allowed to equilibrate prior to the start of the next injection. Cytarabine of concentration 10 mg % was made up in 2-propanol: ethanol (1:1).

3.7.1 HPLC results for gradient system using hexane: 2-propanol (v/v) with a run time of 30 min

The gradient elution was carried out using 2-propanol as an organic modifier in hexane (Section 2.4.2 Fig 2.5) in 30 min at a flow rate of 1 ml/min.

Table 3.18: Effect of gradient elution using varying combinations of hexane: 2-propanol on selectivity and resolution of cytarabine enantiomers. Data values ± standard deviation.

MOBILE PHASE Hexane: IPA (v/v)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 2
95:5 - 90:10	24.10	4441 ± 81.12

3.7.2 HPLC results for the gradient system using hexane: 2-propanol (v/v) at flow rate 1.0 ml/min run time 22 min

The gradient elution was carried out using 2-propanol as an organic modifier in hexane (Section 2.4.2 Fig 2.7, Fig 2.8, Fig 2.9, Fig 2.10, Fig 2.11, Fig 2.12, and Fig 2.13). A significant decrease in the retention times of cytarabine was observed with increasing concentrations of the organic modifier. Mobile phase ratio of ranging from hexane: 2-propanol (70:30 - 65:35) showed partial separation.

Table 3.19: Effect of gradient elution using varying combinations of hexane: 2-propanol on selectivity and resolution of cytarabine enantiomers. Data values ± standard deviation.

MOBILE PHASE Hexane: IPA (v/v)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 2
90:10 - 85:15	12.03	7133 ± 58.68
85:15 - 80:20	6.93	6566 ± 22.12
90:10 - 85:15	5.30	6686 ± 6.56
75:25 - 70:30	4.17	6158 ± 14.59
70:30 - 65:35	3.45	1612 ± 16.82
	3.88	4774 ± 10.52
65:35 - 60:40	3.62	5523 ± 57.05
60:40 - 55:45	3.50	6416 ± 16.24

3.7.3 HPLC results for gradient system using hexane: 2-propanol (v/v) at flow rate – 0.5 ml/min in 22 min

From the attempted conditions given in Table 19, ratio consisting of 30 %-35 % of 2-propanol in hexane was chosen and repeated with a flow rate of 0.5 ml/min (Section 2.4.2, Fig 2.11).

Table 3.20: Effect of gradient elution using varying combinations of hexane: 2-propanol on selectivity and resolution of cytarabine enantiomers. Data values ± standard deviation.

MOBILE PHASE Hexane: IPA (v/v)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 2
70:30 - 65:35	7.45	3529 ± 1427.94
	8.08	6112 ± 1287.47

3.7.4 HPLC results for gradient system using hexane: ethanol (v/v) flow rate – 1.0 ml/min in 22 min

The gradient elution was carried out using ethanol as an organic modifier in hexane (Section 2.4.2, Fig 2.7, Fig 2.8, Fig 2.9, Fig 2.10, Fig 2.11, Fig 2.12, and Fig 2.13). A significant decrease in the retention times of cytarabine was observed with the increasing concentrations of the organic modifier.

Table 3.21: Effect of gradient elution using varying combinations of hexane: ethanol on selectivity and resolution of cytarabine enantiomers. Data values ± standard deviation.

MOBILE PHASE Hexane: EtOH (v/v)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 2
90:10 - 85:15	14.08	5895 ± 8.02
85:15- 80:20	9.28	6248 ± 43.91
70:30 - 65:35	5.02	6538± 50.74
65:35 - 40:40	3.90	5257 ± 60.01
60:40 - 55:45	3.82	6561 ± 28.51

3.7.5 HPLC results for gradient system using hexane: ethanol (v/v) flow rate – 1.0 ml/min in 40 min

The gradient elution was carried out using ethanol as an organic modifier in hexane (Section 2.4.2, Fig 2.6) in 40 min at a flow rate of 1ml/min**.**

Table 3.22: Effect of gradient flow of ethanol (5 % - 10 %) on selectivity and resolution of cytarabine enantiomers. Data values ± standard deviation.

MOBILE PHASE Hexane: EtOH (v/v)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 2
95:5 - 90:10	30.95	5171 ± 33.89

3.7.6 HPLC results for gradient system using hexane: 2-propanol (v/v)

The gradient elution was carried out using 2-propanol 10 % - 35 % as an organic modifier in hexane (Section 2.4.2, Fig 2.14) in 17 min at a flow rate of 1ml/min**.**

Table 3.23: Effect of gradient flow of 2-propanol (10% - 35%) in hexane on selectivity and resolution of cytarabine enantiomers. Data values ± standard deviation.

MOBILE PHASE Hexane: IPA (v/v)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 2
90:10 - 65:35	8.30	6526 ± 47.68

3.7.7 HPLC results for gradient system using hexane: ethanol (v/v)

The gradient elution was carried out using ethanol 10 % - 35 % as an organic modifier in hexane (Section 2.4.2, Fig 2.14) in 17 min at a flow rate of 1ml/min**.**

Table 3.24: Effect of gradient flow of ethanol (10 % - 35 %) in hexane on selectivity and resolution of cytarabine enantiomers. Data values ± standard deviation.

MOBILE PHASE Hexane: EtOH (v/v)	RETENTION TIME (min)	MEAN PEAK AREA *n* = 2
90:10 - 65:35	9.82	6264 ± 33.69

CHAPTER 4

DISCUSSION, CONCLUSION AND FUTURE PROSPECTS

4. DISCUSSION

Chiral molecules are at the forefront of strategies for more effective and safe drugs. The number of chiral pharmaceutical drugs in need of separation has been increasing steadily during recent years (De la Puente, 2004). The characteristic features which interests researchers in exploring chiral drugs is their ability to differ in their pharmacology and toxicology (Wang *et al*., 2003). Chiral chromatography is an important analytical technique used for the separation of enantiomers that could be inactive or harmful (Nageswara *et al*., 2006a). The separation of enantiomers requires a chiral or asymmetric environment that allows diastereomeric interactions, hence, HPLC is widely applied (Torok *et al*., 2005). In general practice chiral method development is usually accomplished via a trial and error approach, sometimes defined as a process of elimination (Blackwell *et al*., 1999). This study was an attempt to separate the enantiomers of cytarabine (Ara-C or 1-β-D-arabinofuranosylcytosine) using a polysaccharide based chiral stationary phase (CSP). Cytarabine is a well known nucleoside analogue used in chemotherapy for the treatment of acute lymphoblastic and myelogenous leukaemia (Foulon *et al*., 2009). The various factors identified in the literature affecting chiral resolution are the nature of chiral compound and chiral selectors, the mobile phase composition and condition, column efficiency and temperature (Peng *et al*., 2010). The effect of these factors on enantiomer recognition for cytarabine on three different columns namely, Chiralcel OD-H, Chiralcel OJ-H and Chiralpak AD-H were studied. Parameters such as the type of column, concentration of organic modifier (2-propanol or ethanol), effect of DEA, effect of acetonitrile in combination with the organic modifiers using normal isocratic elution and the effect of

gradient elution on Chiralpak AD-H on resolution of cytarabine enantiomers are discussed below.

4.1 Mechanism of Chiralcel OD-H, Chiralcel OJ-H and Chiralpak AD-H columns:

Polysaccharide based chiral stationary phases developed by Okamoto and co-workers were used since they showed the broadest applicability to date (Persson and Andersson, 2001). Three different polysaccharide based stationary phases were evaluated: Chiralcel OD-H cellulose tris (3, 5-dimethylphenylcarbamate), Chiralcel OJ-H cellulose tris (4-methylbenzoate) and Chiralpak AD-H amylose tris (3, 5-dimethylphenylcarbamate). The chiral recognition mechanism of these CSP's is due to the formation of solute chiral stationary phase complexes through inclusion of enantiomers into the chiral cavities in higher order structures of the CSP's. The carbamate structural unit has been found to be the most important adsorbing site on the phenylcarbamate derivatives of polysaccharides (Chiralcel OD-H and Chiralpak AD-H) which are used in chiral recognition. In carbamate derivative CSP's the binding of the solutes to the CSP's is through interaction between the solutes and the polar carbamate groups on the CSP's (Nageswara *et al*., 2006b). On one hand there are interactions through hydrogen bonding between the carbonyl group of the carbamate and the OH and NH groups of the solute. There are also interactions of the same type between the NH groups of the carbamate unit and the carbonyl groups of solute and through π-π interaction between solutes and phenyl groups of CSP (Kunath *et al*., 1996). The cellulose derivative has been reported to exist in a conformation of a left-handed threefold (3/2) helix, while the amylose derivative can be regarded as a left-handed fourfold (4/1) helix. Also the amylase derivative is more

helical in nature and has well defined cavities compared to cellulose analogues (Aboul-Enein and Ali *et al*., 2001). Therefore the resolving ability of amylose and cellulose derivatives with the same (3, 5-dimethylphenylcarbamate) moiety are very different (Nageswara *et al*., 2006a). In both chiral biopolymers a helical groove exists with polar carbamate residues and 3, 5-dimethylphenyl groups, the enantiomers enter the groove and interact as described with CSP's (Kunath *et al*., 1996).

It is generally thought that the chiral recognition mechanism of tris (4-methylbenzoate) of cellulose is similar to the other two polysaccharide derivatives of CSP's, based on the formation of transient enantiomer-CSP complexes through the insertion of enantiomer in the chiral cavities of the selector. The basic material of these phases consists of cellulose tris (4-methylbenzoate). Cellulose is a linear polysaccharide containing 1500 (+) glucose units per molecule, which are arranged in parallel bundles in cellulose fibres and are held together by numerous hydrogen bonds between various hydroxyl groups. Their helical structure makes it possible to interact stereospecifically with enantiomers. The main chiral adsorption site on the OJ-H column seems to be associated with polar carbonyl groups that are located along the polymer backbone and are capable of interacting with analyte via acceptor hydrogen bonding and dipole-dipole interactions. The aromatic groups that are positioned outside the polymer chain also provide sites for π-π and steric interaction with analytes (Cirilli *et al.,* 2009). These interactions are relatively weak and are considered more effective under normal-phase conditions (Tachibana and Ohnishi *et al*., 2001). These differences in the chiral recognition mechanism could be attributed to different configurations of the glucose residues (α and β linkages) and higher order structure of chiral stationary phase

(Nageswara *et al*., 2006b). Based on the chiral recognition model proposed for the Chiralcel OD-H and Chiralpak AD-H columns, it is assumed that the cytarabine enantiomers interact with the CSP's via (1) hydrogen bonding between the nitrogen atoms of cytarabine molecules and the amide proton of the carbamate moiety of the CSP's; (2) charge transfer (π-π) interactions between the pyridine moiety of cytarabine and the phenyl groups of the CSP's and (3) dipole-dipole interaction between the nitrogen atoms of the cytarabine and the carbonyl oxygen of the carbamate moiety of the Chiralcel OD-H and Chiralpak AD-H columns. The phenyl moiety can furthermore provide π-π interactions with the aromatic group of the compound (Ducret *et al*., 1998). For Chiralcel OJ-H (1) the hydrogen bonding between the nitrogen atoms of cytarabine molecule and the polar ester function via hydrogen bonding and dipole-dipole interactions. (2) Charge transfer (π-π) interactions between pyridine moiety of cytarabine and methyl groups of the CSP, however, a detailed separational mechanism for chiral solute has not yet been fully established.

It is well known that the performance of a specific column is affected by the composition and the flow rate of the mobile phase (Zhou *et al*., 2006). Experiments were carried out to find the optimal composition and flow rate for resolution of cytarabine enantiomers for each column. Two classes of mobile phases were attempted using the isocratic mode, normal phase hexane and polar phase acetonitrile. 2-propanol and ethanol were tested on both the phases as polar organic modifiers at three different flow rates of 0.5 ml/min, 1 ml/min and 1.5 ml/min. The most promising results were repeated with the addition of DEA.

4.2 Effect of 2-propanol and ethanol content of the mobile phase on Chiralcel OJ-H and Chiralpak AD-H upon enantioselectivity of cytarabine:

The effect of 2-propanol on peak characteristics of cytarabine was investigated by increasing the concentration of 2-propanol in mixed hexane: 2-propanol phase from 10 to 60 %, the results indicated partial resolution of cytarabine enantiomers with 2-propanol as a polar modifier in hexane, on Chiralcel OJ-H and Chiralpak AD-H columns shown in Tables 3.3 and Table 3.4 respectively. In comparison to Chiralcel OJ-H, Chiralpak AD-H showed better resolution of cytarabine enantiomers using 2-propanol at ratios of 30 % 40 % and 50 %. This could be due to the amylose backbone instead of cellulose in Chiralpak AD-H which exhibits rather high enantiomer resolving ability as compared to its cellulose analog (Chankvetadze *et al*., 2002). Comparatively, the separation was better at higher flow rates (1.5 ml/min) with the Chiralpak AD-column.

The effect of ethanol on peak characteristics of cytarabine was investigated by increasing the concentration of ethanol in hexane: ethanol combinations from 10 to 60 %. The results showed partial separation on Chiralcel OJ-H and Chiralpak AD-H columns shown in Table 3.6 and Table 3.7, respectively. Ethanol compared with 2-propanol, did not yield encouraging results, infact the broad nature of the peaks resulted in a loss in resolution. A combination of 60% hexane and 40% ethanol was found to be the best however, it did not resolve the enantiomers completely.

The retention and resolution of cytarabine enantiomers using 2-propanol in hexane differed from that of ethanol in hexane on the Chiralcel OJ-H and Chiralpak AD-H columns, the main reason for the differing separation property of the 2-propanol and ethanol may be due to a changed geometry and /or size of the chiral grooves of the

CSP's which is caused by the type of alcohol modifier used (Tatini *et al*., 2005). In this investigation it was also found that in the results showing partial resolution the peak shape varied with the use of two different modifiers, which could possibly be due to the steric hindrance of the alcohol content in the mobile phase. In the results indicating partial resolution, the possible change in the peak shape could be due to the change of the order of elution which can be associated with the use of different modifiers (2-propanol or ethanol) which may have caused alteration to the steric environment of the chiral cavities in the CSP's (Wang *et al*., 2003).

4.3 Effect of 2-propanol and ethanol content of the mobile phase on Chiralcel OD-H for resolution of cytarabine enantiomers:

The type and concentration of organic modifiers was found to influence the resolution and retention of cytarabine. The HPLC results for Chiralcel OD-H using 2-propanol Table 3.2 and ethanol Table 3.5 as organic modifiers in hexane clearly showed no signs of resolution of cytarabine enantiomers. A comparison of the results for the two organic modifiers indicated that ethanol provided improved selectivity for cytarabine while the retention time was less compared to 2-propanol. In the cavity of CSP's the lower alcohols could be inserted easily as compared to longer chain alcohols (Nageswara *et al*., 2006b). This phenomenon could have been attributed to the steric effects around the hydroxyl moiety of the 2-propanol and ethanol. The insertion of the polar mobile phase modifier into the cavities of CSP's could induce changes in the main chiral recognition mechanisms, leading to the formation of more stable diastereomeric complexes of enantiomers causing higher retention and better

selectivity. However, no further combinations of mobile phases were tried on Chiralcel OD-H due to time limitation.

4.4 Effect of concentration of alcohol modifier (2-propanol or ethanol) upon enantioselectivity of cytarabine:

The retention of the analyte is highly influenced by the amount of polar modifier in apolar mobile phases (Persson and Andersson, 2001). It was noted that the decrease in the polar component of the mobile phase resulted in reduction in the retention time of cytarabine. This phenomenon was observed in all three columns used, the decrease in retention time was due to the increased polarity of the MP with an increasing alcohol concentration (Torok *et al*., 2005). Since the solvent molecules compete with the solute molecules for the specific adsorption sites on the CSP, however, the solvation of the mobile phase increases with the increased modifier content.

4.5 Effect of diethylamine (DEA) as a residual silinol deactivator upon enantioselectivity of cytarabine using Chiralcel OJ-H and Chiralpak AD-H columns:

The influence of DEA on enantioseparation of cytarabine was assessed on Chiralcel OJ-H and Chiralpak AD-H columns by using 0.2 % in various mobile phases which had showed partial resolution consisting of mixtures of hexane: 2-propanol and hexane: ethanol (Table 3.8, Table 3.9, Table 3.10, and Table 3.11). It is known that basic mobile phase additives could be used in chiral separations to increase enantioselectivity, it minimizes peak broadening arising from the unwanted interactions between polar solutes and the stationary phase (Matthijs *et al*., 2006).

DEA was used to reduce peak tailing by masking the residual silanol of the CSP's (Sharma *et al.*, 1995). With the type of columns used, consisting of a free silanol groups on the silica surface, results in secondary interactions with the nitrogen atoms of cytarabine, leading to improved peak symmetry. DEA effectively masks the underivatized silanols, thus minimizing the adsorption effect and the resultant peak tailing (Tatini *et al.*, 2005). Although the use of DEA resulted in greater peak symmetry there was no apparent improvement observed in the retention or enantioselectivity of the cytarabine enantiomers. However, a ratio of hexane: ethanol: DEA (60:40:0.2) using OJ-H column showed some enantioselectivity compared to the other combinations.

4.6 Effect of Acetonitrile upon enantioselectivity of cytarabine:

The results of the effect of varying ratios of the organic modifier 2-propanol in acetonitrile together with DEA for Chiralcel OD-H, Chiralcel OJ-H and Chiralpak AD-H are mentioned in Table 3.12, Table 3.14 and Table 3.16, respectively. The varying compositions of ethanol in acetonitrile together with DEA had little or no effect on enantioselectivity of cytarabine shown in Table 3.13, Table 3.15 and Table 3.17 respectively. With the OD-H column the varying ratios of 2-propanol with acetonitrile provided higher retention times compared with ethanol. With the OJ-H column the ratios with 2-propanol resulted in the lowest retention time. With the AD-H column the varying ratios of 2-propanol as organic modifier resulted in a higher retention time compared to ethanol. Neither of these ratios proved to be useful in separating the enantiomers of cytarabine which could possibly be since the solvents (acetonitrile, ethanol and 2-propanol) used were polar.

4.7 Effect of gradient elution upon enantioselectivity of cytarabine on Chiralpak AD-H

A gradient elution of the cytarabine was carried out in order to see whether the enantiomers could be separated without having to make up various mobile phases and to see whether gradually changing the mobile phase composition to a more polar system would prove useful in separating the enantiomers. The gradient systems resulting in partial resolution are listed in Tables 3.19 and 3.20. However, the gradient method proved not to be as effective as the isocratic system. Increasing the polarity of the mobile phase decreased the retention time of the cytarabine as expected (Torok *et al*., 2005). The gradient elution was not explored on other two columns due to time limitation.

4.8 Limitations

1. There was considerable variation in the peak shape even after repeated injections of the cytarabine under the same conditions and was most likely attributed to the non-buffering mobile phase. It is worth noting that the presence of an aqueous environment would be detrimental to the integrity of the columns used.

2. Even with the analysis carried out using Chiralpak AD-H column at temperatures higher that the room temperature (30, 35 and 40) no significant improvement in resolving the peaks was achieved.

3. Due to time constraints gradient elution could not be explored using the Chiralcel OD-H and Chiralcel OJ-H columns.

4. The alcohol content (2-propanol or ethanol) more than 70 % in hexane was found unfavourable to the integrity of the HPLC system at flow rates of 0.5, 1.0 and 1.5

ml/min and hence could not be tested on Chiralcel OD-H, Chiralcel OJ-H and Chiralpak AD-H.

4.9 CONCLUSION

Separation of cytarabine enantiomers was attempted on three different polysaccharide-based chiral stationary phases namely the Chiralcel OD-H, Chiralcel OJ-H and Chiralpak AD-H column. The conditions that afforded the best separation involved the use of the Chiralcel OJ-H column using varying compositions of hexane, ethanol and diethylamine and use of the Chiralpak AD-H column using mobile phase ratios consisting of hexane: 2-propanol and hexane: 2-propanol: diethylamine. The application of the Chiralcel OD-H column with varying compositions proved to be less effective to separate cytarabine enantiomers. The application of gradient elution did not help much to resolve the enantiomers. It can be concluded that the Chiralcel OJ-H and Chiralpak AD-H columns can only give partial resolution using 2-propanol and ethanol as organic modifiers, while DEA did not help improve the resolving power, however it did reduce tailing. Using various combinations of organic modifiers (2-propanol or ethanol) in acetonitrile with the addition of DEA could not enhance separation. In conclusion, the aim of developing a chiral HPLC method in order to separate specific enantiomers of anticancer reagent cytarabine using chiral HPLC under specified conditions proved to be unsuccessful. Therefore the isolation and purification of individual enantiomers by semi-preparative HPLC could not be performed since the results varied considerably even after repeated injections under the same conditions which could have been attributed to non-buffering mobile phases.

4.10 SCOPE FOR FUTURE WORK

1. Although partial resolution of cytarabine enantiomers was achieved using ethanol and 2-propanol as organic modifier in hexane, the use of solvents such as methanol, 1-butanol, *tert*-butanol, 1-propanol, 1-pentanol and 1-hexanol could be investigated as organic modifiers to check their effect on enantioselectivity of cytarabine.
2. Further work could be carried out using varying column temperatures using the Chiralcel OD-H and Chiralcel OJ-H columns, to investigate their effect on enantioseparation of cytarabine.
3. Enantiomeric separations of cytarabine could be investigated by HPLC using macrocyclic glycopeptide-based chiral stationary phases.
4. Chiralpak AS-H column, that has a packing composition of amylose tris (S)-α-methylbenzylcarbamate can be investigated for its effect on separation of cytarabine enantiomers.
5. Reversed-phase liquid chromatographic separations can be investigated on polysaccharide based chiral stationary phases such as Chiralpak® AS-RH, Chiralpak® AD-RH, Chiralcel® OD-RH ,Chiralcel® OJ-RH, Chiral AGP column based on α1-acid glycoprotein (α1-AGP) and Chiral CBH based on Cellobiohydrolase (CBH) .
6. Due to time constraints the gradient elution of the Chiralcel OD-H, Chiralcel OJ-H columns was not carried out and therefore could be investigated.
7. Ligand-exchange based chiral stationary phase such as Chiralpak WH, Chiralpak WM and Chiralpak WE could be investigated.
8. Protein based chiral stationary phases such as Chiral AGP could be investigated for their effect on enantioseparation of cytarabine.

REFERENCES

5. REFERENCES

Aboul-Enein HY & Ali I (2001). Studies on the effect of alcohols on the chiral discrimination mechanisms of amylose stationary phase on the enantioseparation of nebivolol by HPLC. *J Biochem Biophys Methods* **48**, 175-188.

Aboul-Enein HY & Wainer IW (1997). *The Impact of Stereochemistry on Drug Development and Use 1st edition*. John Wiley & Sons , New York, USA, 45-263.

Ahuja S (2010). *Chiral separation methods for pharmaceutical and biotechnological products 1st edition.* John Wiley & Sons, Hoboken, New Jersey, 57-130.

Ahuja S & Rasmussen H (2007). *HPLC method devolopement for pharmaceuticals 1st edition.* Elvesier, North Carolina, USA, 85-105.

Ali I, Gaitonde VD, Aboul-Enein HY, & Hussain A (2009). Chiral separation of beta-adrenergic blockers on CelluCoat column by HPLC. *Talanta* **78**, 458-463.

Beesley TE & Scott RPW (1998). *Chiral chromatography 1st edition*. John Wiley & Sons, West Sussex, England, 1-368.

Blackwell JA, Stringham RW, Xiang D, & Waltermire RE (1999). Empirical relationship between chiral selectivity and mobile phase modifier properties. *J Chromatogr A* **852**, 383-394.

Bosch ME, Sanchez AJ, Rojas FS, & Ojeda CB (2008). Recent advances in analytical determination of thalidomide and its metabolites. *J Pharm Biomed Anal* **46**, 9-17.

Brown WH, Foote CS, Inverson BL & Anslyn EV (2009). Organic chemistry *5th edition*. Brooks/Cole cengage learning, Belmont, USA, 112-142.

Burke D & Henderson DJ (2002). Chirality: a blueprint for the future. *Br J Anaesth* **88**, 563-576.

Burrows A, Holman J, Parsons A, Pilling G & Price G (2009). *Chemictry^{3} 1st edition.* Oxford University press, New York, USA, 450-483.

Caldwell J (1995). Stereochemical determinants of nature and consequence of drug metabolism. *J Chromatogr A* **694**, 39-48.

Chankvetadze B **,** Kartozia I**,** Yamamoto C & Okamoto Y (2002). Comparative enantioseparation of selected chiral drugs on four different polysaccharide-type chiral stationary phases using polar organic mobile phases. *J Pharm Biomed Anal* **27**, 467-478.

Cheson BD, Keating MJ and Plunkett W. (1997). *Nucleoside Analogs in Cancer Therapy 1st edition*. Marcel Dekker, New York, USA, 1-47.

Cirilli R, Alcaro S, Fioravanti R, Secci D, Fiore S, La TF, & Ortuso F (2009). Unusually high enantioselectivity in high-performance liquid chromatography using cellulose tris(4-methylbenzoate) as a chiral stationary phase. *J Chromatogr A* **1216**, 4673-4678.

Claessen HA, Straten MAV, Cramers CA, Jeezierska M & Buszewski B (1998).Comparative study of test methods for reversed phase columns for high performance liquid chromatography. *J Chromatogr A* **826**, 135-156.

Crowe J, Bradshaw T & Monk P (2006). *Chemistry for the Biosciences 1st edition.* Oxford University press, Oxford, UK, 332-367.

De la Puente ML (2004). Highly sensitive and rapid normal-phase chiral screen using high-performance liquid chromatography-atmospheric pressure ionization tandem mass spectrometry (HPLC/MS). *J Chromatogr A* **1055**, 55-62.

Dewick PM (2006). *Essentials of organic chemistry 1st edition***.** John Wiley and Sons, West Sussex, England, 55-116.

Drayer DE (1986). Pharmacodynamic and pharmacokinetic differences between drug enantiomers in humans: an overview. *Clin Pharmacol Ther* **40**, 125-133.

Ducret A**,** Trani M**,** Pepin P & Lortie R (1998). Chiral high performance liquid chromotography resolution of ibuprofen esters. *J Pharm Biomed Anal* **16**, 1225-1231.

Foulon C, Tedou J, Peyrottes S, Perigaud C, Bonte JP, Vaccher C, & Goossens JF (2009). Separation of diastereoisomers of Ara-C phosphotriesters using solid phase extraction and HPLC for the study of their decomposition kinetic in cell extracts. *J Chromatogr B Analyt Technol Biomed Life Sci* **877**, 3475-3481.

Franco P, Senso A, Oliveros L, & Minguillon C (2001). Covalently bonded polysaccharide derivatives as chiral stationary phases in high-performance liquid chromatography. *J Chromatogr A* **906**, 155-170.

Francotte E (2005). *Preparative Enantioselective Chromatography 1st edition.* Blackwell Publishing, Oxford, UK, 48–77.

Francotte E & Lindner W (2006). *Chirality in Drug Research* (Volume-33). Wiley-VCH,Weinheim, Germany, 3-18.

Garcia-Carbonero R, Ryan DP & Chabner BA (1996). *Cytidine analogs 1st edition*. Lippincott- Raven Publishers, Philadelphia, 265–94.

Gasparrini F, Misiti D, & Villani C (2001). High-performance liquid chromatography chiral stationary phases based on low-molecular-mass selectors. *J Chromatogr A* **906**, 35-50.

Gehrke CW & Wixom RL (2010). *Chromatography a science of discovery 1st edition.* John Wiley & Sons, Hoboken, New Jersey, 109-198.

Glick BR & Pasternak, JJ. (2007). Regulation of mRNA Transcription In Eukaryotes. *Principles And Applications of Recombinant DNA.* American Society For Microbiology Press, Washington DC , 426-458.

Gmeiner WH, Skradis A, Pon RT, & Liu J (1998). Cytarabine-induced destabilization of a model Okazaki fragment. *Nucleic Acids Res* **26**, 2359-2365.

Gunji H, Kharbanda S & Kufe D. (1991). Induction of Internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-Arabinofuranosylcytosine. *Cancer Res* **51**, 741–743.

Haghedooren E, Diana J, Noszal B, Hoogmartens J, & Adams E (2007). Classification of reversed-phase columns based on their selectivity towards vancomycin compounds. *Talanta* **71**, 31-37.

Hanai T (1999). *HPLC: a practical guide 1st edition.* Royal society of Chemistry, Cambridge, UK, 11-30.

Heftmann E, (2004). *Chromatography 6th edition.* Elsevier BV, Amsterdam, Netherland, 1-131.

Heidelberger C, Danenberg PV and Moran RG (1983). Fluorinated pyrimidines and their nucleosides. *Adv. Enzymol. Relat. Areas Mol. Biol* **54**, 58–119.

Hoffman RV (2004). *Organic chemistry an intermediate text* 2nd edition. John Wiley & Sons, Hoboken, New Jersey, 124-183.

Jacques J, Collet A, Wilen SH (1981). *Enantiomers, Racemates and Resolution 1st edition*. John Wiley & Sons, New York, USA , 32-166.

Karcher BD, Davis ML, Dalaney EJ & Venil JJ (2005). A 21st century HPLC Workflow for Process R&D, *J Association for Laboratory Automation **10,** 381-393.*

Kazakevich Y, LoBrutto R (2007). *HPLC for pharmaceutical scientists.* John Wiley and Sons, Hoboken, New Jersey, 3-25.

Kott L, Holzheuer WB, Wong MM, & Webster GK (2007). An evaluation of four commercial HPLC chiral detectors: a comparison of three polarimeters and a circular dichroism detector. *J Pharm Biomed Anal* **43**, 57-65.

Krupadanam GLD, Prasad DV, Rao KV, Reddy KLN & Sudhakar C (2001). *Analytical chemistry* Universities Press, Hydrabad, India, 1-32.

Kunath A, Theil F & Jahnisch K (1996). Influence of kind of organic modifier on chiral separation on a Chiralpak AD column, *J Chromatogr A **728,** 249-257.*

Lammerhofer M (2010). Chiral recognition by enantioselective liquid chromatography: mechanisms and modern chiral stationary phases. *J Chromatogr A* **1217,** 814-856.

Lloyd DK & Goodall DM (1989). Polarimetric detection in high performance liquid chromatography. *Chirality* **1**, 251-264.

Lindsay S & Barnes J (1992). *High performance liquid chromatography 2nd edition*. John Wiley & Sons, West Sussex, UK 1-5.

Lorenz H, Capla F, Polenske D, Elsner MP, Seidel-Morgenstern A (2007). Crystallization based separation of enantiomers. *Journal of the University of Chemical ,Technology and Metallurgy* **42,** 5-16.

Maier NM, Franco P, & Lindner W (2001). Separation of enantiomers: needs, challenges, perspectives. *J Chromatogr A* **906**, 3-33.

Martin-Matute B & Backvall JE (2007). Dynamic kinetic resolution catalyzed by enzymes and metals. *Curr Opin Chem Biol* **11**, 226-232.

Matthijs N, Maftouh M, & Heyden YV (2006). Screening approach for chiral separation of pharmaceuticals IV. Polar organic solvent chromatography. *J Chromatogr A* **1111**, 48-61.

McCudden CR & Kraus VB (2006). Biochemistry of amino acids racemisation and clinical application to musculoskeletal disease. *Clin Biochem* **39,** 1112-1130.

McConathy J & Owens MJ (2003). Stereochemistry in Drug Action. *Prim Care Companion J Clin Psychiatry* **5**, 70-73.

McMurry J & Begley TP (2005). *Organic chemistry of biological pathways 1st edition*. Robert & Company publisher, Colorado, USA, 43-92.

Metzler DE & Metzler CM (2003). *Biochemistry the chemical reaction of living cells* (volume 2). Harcourt Academic press, California, USA, 39-94.

Meyer VR (2010). *Practical high performance liquid chromatography 5nd edition.* John Wiley & Sons, West Sussex, UK, 5-15.

Melchert M & List A (2007). The thalidomide saga. *Int J Biochem Cell Biol* **39**, 1489-1499.

Nageswara RR, Nagaraju D, & Narasa RA (2006a). Enantiomeric resolution of doxazosin mesylate and its process-related substances on polysaccharide chiral stationary phases. *J Pharm Biomed Anal* **41**, 766-773.

Nageswara RR, Narasa RA, & Nagaraju D (2006b). An improved and validated LC method for resolution of bicalutamide enantiomers using amylose tris-(3,5-dimethylphenylcarbamate) as a chiral stationary phase. *J Pharm Biomed Anal* **42**, 347-353.

Nasipori D (1994). *Stereochemistry of organic compounds 2nd edition.* New Age International (P) Limited Publishers, Daryaganj, New Delhi, 135-160.

Peng L, Jayapalan S, Chankvetadze B, & Farkas T (2010). Reversed-phase chiral HPLC and LC/MS analysis with tris(chloromethylphenylcarbamate) derivatives of cellulose and amylose as chiral stationary phases. *J Chromatogr A* **1217**, 6942-6955.

Perrin C, Matthijs N, Mangelings D, Granier-Loyaux C, Maftouh M, Massart DL, & Heyden YV (2002). Screening approach for chiral separation of pharmaceuticals part II. Reversed-phase liquid chromatography. *J Chromatogr A* **966**, 119-134.

Persson BA & Andersson S (2001). Unusual effects of separation conditions on chiral separations. *J Chromatogr A* **906**, 195-203.

Poole CF (2003). *The essence of chromatography 1st edition.* Elsevier BV, Amsterdam, Netherland, 793-839.

Randall T (1990). Thalidomide has 37-year history. *JAMA* **263**, 1474.

Reddy IK & Mehvar R (2004). *Chirality in drug design & devolopement 1st edition.* CRC press, New York, USA, 1-34.

Russell PJ. (2006). DNA Replication. *iGenetics: A Molecular Approach*. Cinnamon Hearst, San Francisco, 1-62.

Salvadori P, Bertucci C & Rosini C (1991). *Circular dichroism detection in HPLC*. *Chirality* **3**, 376-385.

Sampath S, Rao VA & Plunkett W. (2003). Mechanism of Apoptosis induction by nucleoside analogue. *Oncogene* **22,** 9063–9074

Shamsipur M, Dastjerdi LS, Haghgoo S, Armspach D, Matt D, & Aboul-Enein HY (2007). Chiral selectors for enantioresolution and quantitation of the antidepressant drug fluoxetine in pharmaceutical formulations by (19)F NMR spectroscopic method. *Anal Chim Acta* **601**, 130-138.

Schellinger AP, Carr PW (2006). Isocratic and gradient elution chromatography: a comparision in terms of speed, retention reproducibility and quantitation. *J Chromatogr A* **1109**, 253-266.

Sharma SC, Evans MB, & Evans SJ (1995). The enantiomeric separation of metipranolol and desacetylmetipranolol on a cellulose tris-3,5-dimethylphenyl-carbamate chiral stationary phase. *J Pharm Biomed Anal* **13**, 129-137.

Skoog DA and Leary JJ (1992). *Principles of instrumental analysis 4th edition*. Saunder college publishing, Florida, USA, 628-669.

Smart L & Gagan M (2002). *The molecular world the third dimension* (volume 1). The Open University Press, Milton Keynes, UK, 97-115.

Smith M, Smith MB & March J (2007). *March's Advanced Organic Chemistry: Reactions, Mechanisms and Structure 6th edition,* John Wiley & Sons*,* Hoboken, New Jersey, 136-233.

Sorrel TN (2006). *Organic chemistry 2nd edition.* University Science Books, Sausalito, California, 516- 530.

Storniolo AM, Enas NH, Brown CA, Voi M, Rothenberg ML and Schilsky R. (1999). An investigational new drug treatment program for patients with gemcitabine. *Cancer* **85**, 1261–126.

Sun Y, Sun J, Wen B, Shi S, Xu Y, Chen Y, Wang Y, Pan C, Zhang C, Zhang T, & He Z (2008). High-performance liquid chromatography/tandem mass spectrometry method for the simultaneous determination of cytarabine and its valyl prodrug valcytarabine in rat plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **870**, 121-125.

Synder LR, Kirkland JJ & Dolan JW (2009) *Introduction to modern liquid chromatography 3rd edition.* John Wiley & Sons, Hoboken, New Jersey, 104-143.

Tachibana K & Ohnishi A (2001). Reversed-phase liquid chromatographic separation of enantiomers on polysaccharide type chiral stationary phases. *J Chromatogr A* **906**, 127-154.

Tang Y, Zielinski WL, Bigott HM (1998). Separation of nicotine enantiomers via normal phase HPLC on derivatized cellulose chiral stationary phases. *chirality* **10,** 364-369.

Tatini R **,** Sadik O**,** Bernhard S & Abruna H (2005) Direct resolution of chiral 'pineno' fused terpyridyl ligands on amylose based chiral stationary phase using long chain alcohol modifiers. *Anal Chim Acta* **534**, 193-198.

Thunberg L, Hashemi J & Andersson S (2008). Comparative study of coated and immobilized polysaccharide- based chiral stationary phases and their applicability in resolution of enantiomers. *J Chromatogr B* **875**, 72–80.

Torok R, Bor A, Orosz G, Lukacs F, Armstrong DW, & Peter A (2005). High-performance liquid chromatographic enantioseparation of bicalutamide and its related compounds. *J Chromatogr A* **1098**, 75-81.

Turker L (2002). *Isomerism in the class of alternate hydrocarbons*. *J Molecular Structure Theochem* **584**, 183-187.

Waldeck B (2003). Three-dimensional pharmacology, a subject ranging from ignorance to overstatements. *Pharmacol Toxicol* **93**, 203-210.

Wang T, Robert M & Wenslow Jr. (2003). Effects of alcohol mobile-phase modifiers on the structure and chiral selectivity of amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase. *J Chromatogr A* **1015**, 99-110.

Webster GK & Kott L (2010). 7.Method devolopement for pharmaceutical chiral chromatography. *Separation Science and Technology* **10**, 251-282.

Wiley JS, Jones SP, Sawyer WH, Paterson AR (1982). Cytosine arabinoside influx and nucleoside transport sites in acute leukemia. *J Clin Invest* **69**, 479–89.

Wozniak TJ, Bopp RJ, & Jensen EC (1991). Chiral drugs: an industrial analytical perspective. *J Pharm Biomed Anal* **9**, 363-382.

Yamamoto C and Okamoto Y (2004). Optically active polymers for chiral separation. *Bulletin of chemical society of Japan* **77**, 227-257.

Yamauchi T , Negoro E, Kishi S, Takagi K, Yoshida A, Urasaki Y, Iwasaki H & Ueda T (2009). Intracellular cytarabine triphosphate production correlates to deoxycytidine kinase/cytosolic 50-nucleotidase II expression ratio in primary acute myeloid leukemia cells. *Biochem Pharmacol* **77,** 1780–1786.

Yamauchi T & Ueda T (2005). A sensitive new method for clinically monitoring cytarabine concentrations at the DNA level in leukemic cells. *Biochem Pharmacol* **69**, 1795-1803.

Yashima E (2001). Polysaccharide-based chiral stationary phases for high-performance liquid chromatographic enantioseparation. *J Chromatogr A* **906**, 105-125.

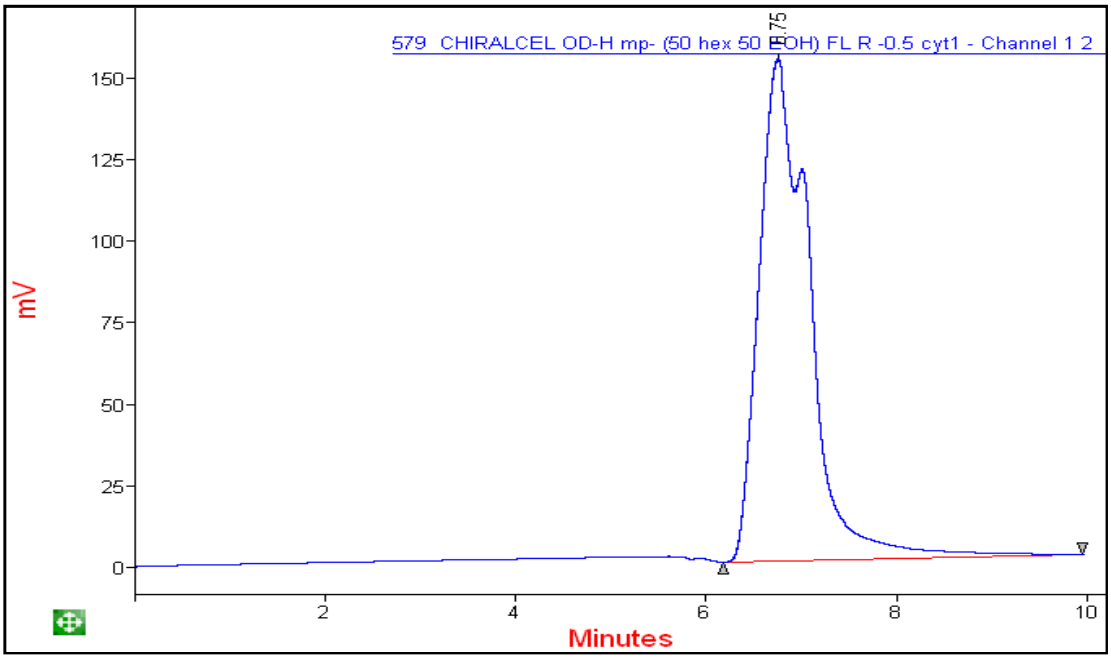
Ye YK, Stringham RW, & Wirth MJ (2004). Origin of enhanced chiral selectivity by acidic additives for a polysaccharide-based stationary phase. *J Chromatogr A* **1057**, 75-82.

Zhou J, Yang YW, Wei F, & Wu PD (2007). Comparison of the performance of chiral stationary phase for separation of fluoxetine enantiomers. *J Zhejiang Univ Sci B* **8**, 56-59.

6. APPENDIX

Appendix 1

A.



B.

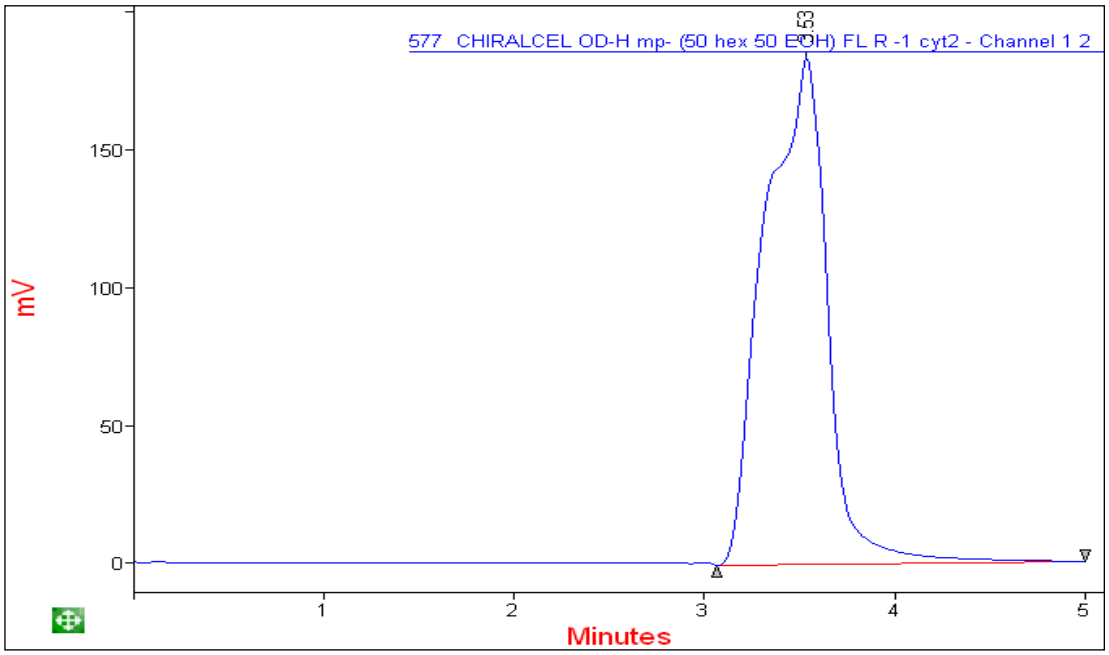


Figure 6.1: HPLC chromatogram showing results for cytarabine using 50% hexane and 50% ethanol (A) at flow rate 0.5 ml/min on the Chiralcel OD-H column and (B) at flow rate 1.0 ml/min on the Chiralcel OD-H column.

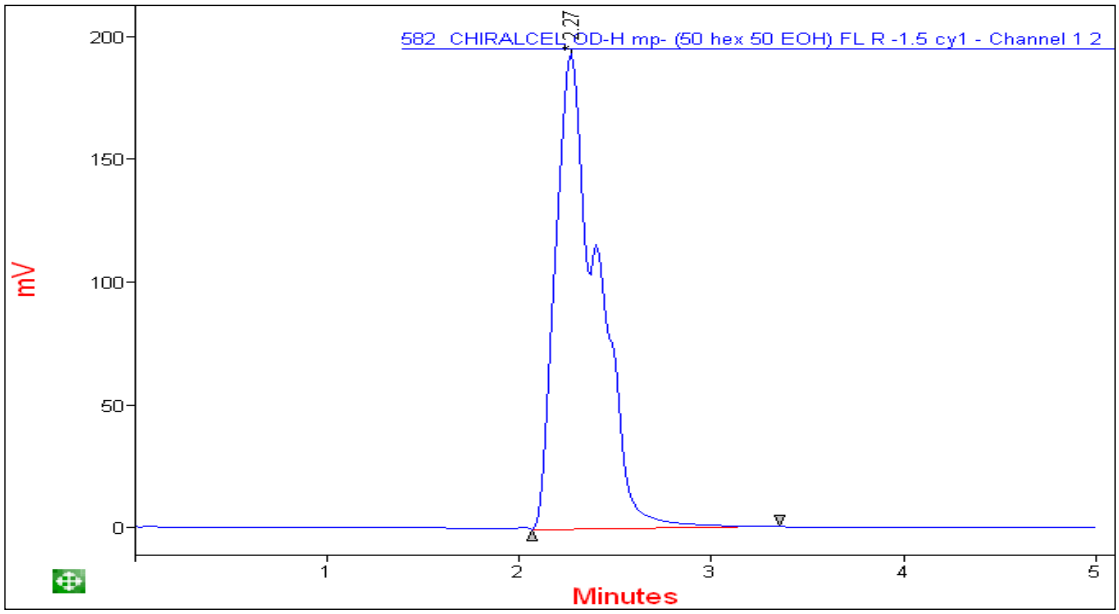
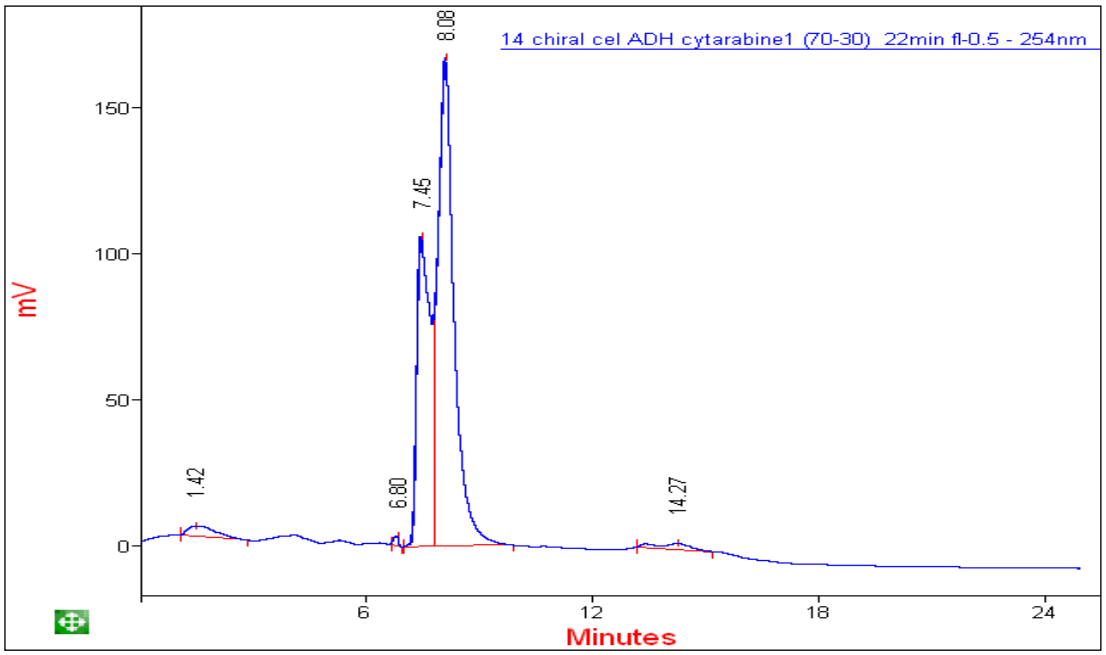


Figure 6.2: HPLC chromatogram showing results for cytarabine using 50% hexane and 50% ethanol at flow rate 1.5 ml/min on the Chiralcel OD-H column.

Appendix 2: HPLC Results for cytarabine using gradient elution on a Chiralpak AD-H column.

A.



B.

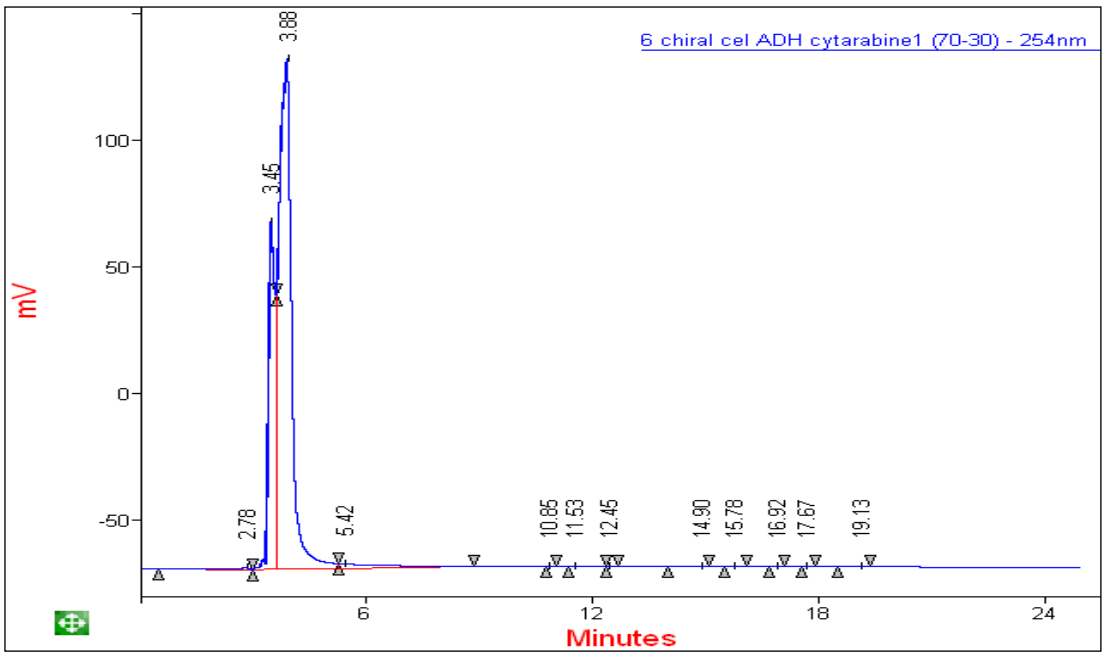


Figure 6.3: HPLC chromatogram showing results for cytarabine using gradient system with 2-propanol ranging from 30 to 35% in hexane in 22min (A) at flow rate 0.5 ml/min on the Chiralpak AD-H column and (B) at flow rate 1.0 ml/min on the Chiralpak AD-H column.

Appendix 3:

Calculation of specific rotation of sucrose:

$$[\alpha]_D^{20} = \frac{\text{observed rotation (degrees)} \times 100}{\text{path length (dm)} \times \text{concentration (g per } 100\text{cm}^3)}$$

$$[\alpha]_D^{20} = \frac{3.2906}{0.050010 \times 1}$$

$$= (+)\ 65.79$$

Calculation of specific rotation of cytarabine:

$$[\alpha]_D^{20} = \frac{1.5584}{0.01014 \times 1}$$

$$= (+)\ 153.68$$