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Optical Method Developement for Enantiomeric Separation and Chromatographic Purification of a Pharmaceutical Ingridient

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This research work was conducted in Orion Corporations Chemical Analytics Laboratory situated in Espoo, Finland. The laboratory is specialized in analyzing samples such as synthesized pharmaceutical ingredients.

When a medicine is being developed containing isomers, part of the development process is to monitor the compounds individually in order to determine their specific properties and functions. Isomers are different compounds with the same molecular formula but with different chemical structures. There are various types of isomers and one of them is the enantiomer. They are molecules which are non-superimposable mirror images of each other. In some cases enantiomers might behave differently according to their structures. For this it is necessary to separate the enantiomers.

The purpose of this thesis was to develop a specific method for separating enantiomers from a target pharmaceutical ingredient molecule. The method was applicable on a certain Agilent's HPLC device. This created method was then used for preparative chromatography to actually separate the enantiomers. Prior to the method development process, several in-house screening runs were made in order to find the suitable atmosphere and parameters for the enantiomer separation. The end products were also analyzed chromatographically by HPLC technique. The reaction process and progress of a composition is followed also by using UPLC-MS technique.

As a result of this research, a successful method was developed for separating specific enantiomers. The method was saved into the database for later use and can be used for separating similar type of samples.

Keywords	optical isomerism, enantiomeric separation, chromatography, HPLC, method development

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Tämä tutkimus toteutettiin yhteistyössä Orionin analyyttisen kemian laboratoriossa Espoossa, Suomessa. Kyseinen laboratorio on erikoistunut näytteiden, kuten syntetisoitujen farmaseuttisten ainesosien analysointiin.

Kun lääkekehityksessä on lääkeaineita jotka sisältävät isomeerejä, kehityksen olennaisena osana on tutkia niitä aineita erikseen. Isomeerit ovat yhdisteitä, joilla on sama molekyylikaava,mutta erilainen molekyylirakenne. On olemassa erilaisia isomeereja ja enantiomeerit ovat yksi niistä. Enantiomeerit ovat aineita, joiden molekyylit ovat toistensa peilikuvia, mutta muuten niillä ei ole muuta rakenteellista eroa. Joskus enantiomeerit käyttäytyvät eri tavalla riippuen niiden muodosta. Tästä johtujen enantiomeerien erottaminen on tarpeellista, jotta niiden ominaisuudet voitaisiin analysoida paremmin.

Tämän tutkielman tavoite oli kehittää spesifinen optinen menetelmä erottaaksemme enantiomeerejä tietyn farmaseuttisen ainesosan molekyylistä. Kyseinen metodi olisi käytettävissä Agilentin HPLC laitteisiin. Metodia joka oli luoto testattiin käytänössä ja lääkeaine puhdistettiin käyttämällä preparatiivista kromatografia. Lopputuote analysoitiin myös kromatografisesti HPLC-tekniikalla. Ennen metodin kehittämistä useita Orioinin talon sisäisiä skriinausajoja toteutettiin optimaalisen ympäristön sekä sopivien parametrien löytämiseksi. Reaktion prosessia ja etenemistä seurattiin myös LC-MS tekniikkaa hyödyntäen.

Tutkimuksen tuloksena löytyi metodi enantiomeerien erottamiseen. Menetelmä on tallennettu Orionin analyyttisen laboratorion tietokantaan myöhempää käyttöä varten ja sitä voidaan jatkossa hyödyntää vastaaviin näytteisiin.

Avainsanat	optinen isomeria, enantiomeerien erotus, kromatografia, HPLC, menetelmän kehitys

Contents

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I am very grateful to Sauli Huopalahti for giving me the opportunity to work in Orion Corporation. That itself was a life changing experience. I am also very thankful to senior researcher Tarja Hiissa and lecturer Timo Laitinen for supervising my thesis and giving a direction to my work.

Writing this thesis and graduating with this degree would not have been possible without the support of my family and friends.

Especially I am grateful to my parents for being the best parents ever and always being by my side. You have always made my life easy and smooth.

I would like to thank my husband and baby daughter for their endless love, support and encouragement. You have made my world a better place.

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I have a lot to learn from you.

Also I am grateful to my aunt Dr. Rezwana Ashraf for being such an inspiration and guiding me towards the right path.

Abbreviations

API	Active Pharmaceutical Agents
DAD	Diode Array Detection
ELS	Evaporative Light Scattering
ESI	Electrospray Ionization
FIH	First In Human (trial)
GLP	Good Laboratory practice
HPLC	High performance Liquid Chromatograph
IND	Investigational New Drug Application
LC-MS	Liquid Chromatography–Mass Spectrometry
NCE	New Chemical Entity
RT	Retention Time
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet
VIS	visible light

1 Introduction

The research for this thesis took place in the chemical analytics laboratory of Orion Corporation situated in Espoo. The aim of this study was to process a certain pharmaceutical ingredient and develop a suitable optical method for separating an isomeric compound with HPLC device. After that the molecule was also purified chromatographically. The pharmaceutical agent was being analysed for an on-going first stage drug development project.

Developing a new drug is a very expensive and extensive process. Starting from an idea or necessity of a new kind of medicine and actually being able to bring that drug to the consumers is a multi-stage procedure. Each step is linked to each other and consists of scientific research as well as co-operation and approval from the correct authorities.

In the pharmaceutical industry often enantiomers are used as ingredients for medicine. Enantiomers belong to the group of isomers. They are molecules which are non-superimposable mirror images of each other. Sometimes enantiomers behave differently according to their structures. Because of this, quite much research is done on isomeric molecules and on the separation of their enantiomers. When separating enantiomers often HPLC separation is used and each separation might require precise HPLC method, and as mentioned before that was the specific target of this experiment.

This thesis consists of several chapters and sections. The theory chapter will explore the necessary literature in order to comprehend the conducted research. It will give a better understanding of the research process and its background. The techniques and equipment used will also be introduced in order to give better understanding on how the research was made possible. It will also provide the necessary information of the environment in a pharmaceutical laboratory facility. A section is also dedicated to explain the complex drug discovery and development process, especially because the research was done in the biggest pharmaceutical company in Finland.

At the end there will be discussion on the result analysis as well as conclusion and summary parts.

2 Orion Corporation

Orion Corporation was originally established in 1917. Quite soon after the start of the company, Orion began to manufacture drugs, which was in the early 1920s. By the end of 1930s Orion was the biggest pharmaceutical company in Finland.

Orion develops, manufactures and markets human and veterinary products, active pharmaceutical agents as well as diagnostic tests. Orion pharmaceutical R&D concentrates on central nervous system, oncology and respiratory systems researches.

Orion is the leading supplier of pharmaceutical products in Finland. As much as every third drug sold in the pharmacy comes from Orion pharmaceuticals. Besides this the company has marketing partners in over hundred countries. Orion has gained share listings in NASDAQ, Helsinki.

The company has manufacturing facilities in Espoo, Turku, Salo and Kuopio. Orion's daughter company Orion Diagnostics is specialized in making diagnostic tests and is situated in Espoo. [1] Also the company Fermion is fully owned by Orion and has facilities in Espoo, Hanko and Oulu. Fermion is specialized in manufacturing active pharmaceutical ingredients (API). [2]

2.1 Orion's R&D

Orion prefers to create its pharmaceutical innovations within its own R&D organization. The company gives great value to research and aims to spend on pharmaceutical research and product development purposes about 10-15% of their net sales. Orion's pharmaceutical research concentrates on early-phase drug development, pre-clinical studies and Phase I and Phase II clinical trials. The phase III clinical trials are often done in collaboration with partners selected for further development and marketing. The company also collaborates extensively with other pharmaceutical companies in early-phase research and targets to increase the number of innovative and safe new medicinal treatments. There are about 500 professionals working for the research and development in Orion.

Besides the facilities in Finland, Orion has operational units in Nottingham, England and Mumbai, India. [3]

Research areas

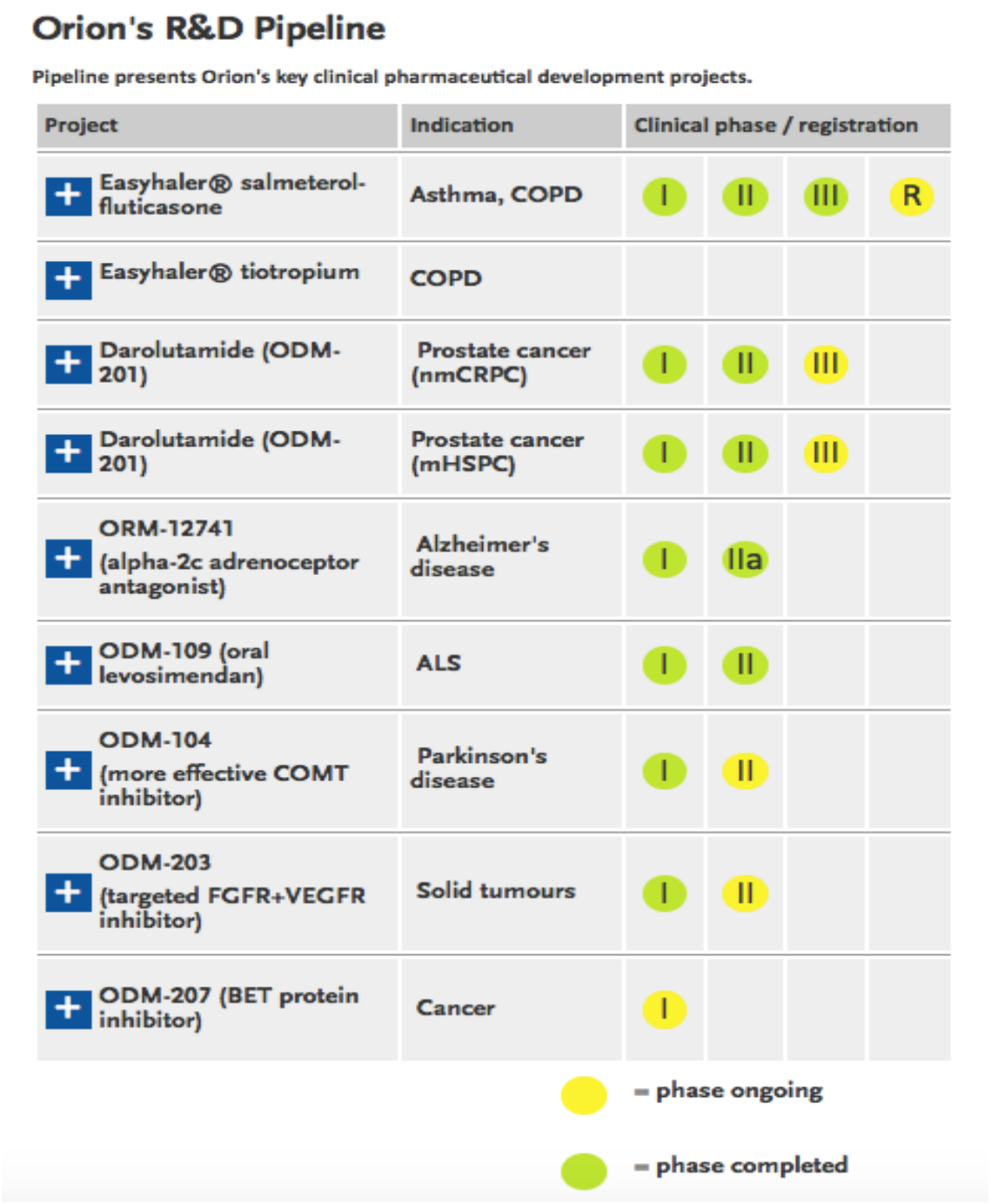
The R&D is focuses on 5 different areas at the moment (Table 1).

Table 1 Research areas in Orion's R&D.

Central nervous system	This therapy area includes drugs for the treatment of neurological and psychiatric diseases as well as pain medicines. Orion has been doing research for finding a treatment for Parkinson's disease for a long time. The outcome of Orion's most successful proprietary drugs Comtess and Stalevo are from these research projects.
Oncology and critical care	Orion develops novel proprietary drugs for the treatment of cancer. The company focuses on hormone-dependent cancer research and with partnering the company has expanded its research activities to include other types of cancer and supportive treatments for cancer patients.
Inhalable Drugs	Orion's own patented product, Easyhaler® is an environmentally friendly and easy-to-use dry powder inhaler.
Veterinary Medicine	Orion's veterinary product development utilizes expertise in its own therapy areas and formulation technology, and its human drug and formulation innovations when new therapies and treatment methods are being developed for animals.
Generic Drugs	Generic drugs are medicines which contain the same active pharmaceutical ingredient as a matching proprietary product with an expired patent. Orion develops and co-develops several new generic medicinal substances with its partners. The development of generic drugs is easier and economical than the development of proprietary drugs since the necessary studies on the active ingredient have already been carried out. The development of generics usually takes two to three years.

[4]

Orion's clinical pharmaceutical development projects are stated bellow (Picture 1).



Orion's R&D Pipeline

Pipeline presents Orion's key clinical pharmaceutical development projects.

Project	Indication	Clinical phase / registration			
Easyhaler® salmeterol-fluticasone	Asthma, COPD	I	II	III	R
Easyhaler® tiotropium	COPD				
Darolutamide (ODM-201)	Prostate cancer (nmCRPC)	I	II	III	
Darolutamide (ODM-201)	Prostate cancer (mHSPC)	I	II	III	
ORM-12741 (alpha-2c adrenoceptor antagonist)	Alzheimer's disease	I	IIa		
ODM-109 (oral levosimendan)	ALS	I	II		
ODM-104 (more effective COMT inhibitor)	Parkinson's disease	I	II		
ODM-203 (targeted FGFR+VEGFR inhibitor)	Solid tumours	I	II		
ODM-207 (BET protein inhibitor)	Cancer	I			

= phase ongoing

= phase completed

Picture 1. Orion's research and development projects [5]

3 Theory

3.1 Medicinal development

Drug development to discovery, leading to a successful outcome is a long and expensive procedure. The process might require 10-15 years and huge amount of investment. Due to its lengthy time line, high cost and low rate in accepting new drug, the chances of a new chemical entity (NCE) making its way to become a successful new therapeutic is extremely low. Only one in 5000-10,000 NCEs are authority approved in both United States and Europe. From there in average one in nine compounds enter clinical development end up as approved product. Success rates can also vary depending on the therapeutic area. The NCEs which enter the first in human trials (FIH) and from there make it to being marketed drugs have the following percentage according to therapeutic area. Drugs for cardiovascular disease have a 20 % success rate for becoming marketed products, arthritis and pain linked drugs have a 16 % success rate, and finally oncology and central nervous system related medicines have 5 to 8 percent success rate. The percentage rate according to therapeutic areas is presented in chart 1.

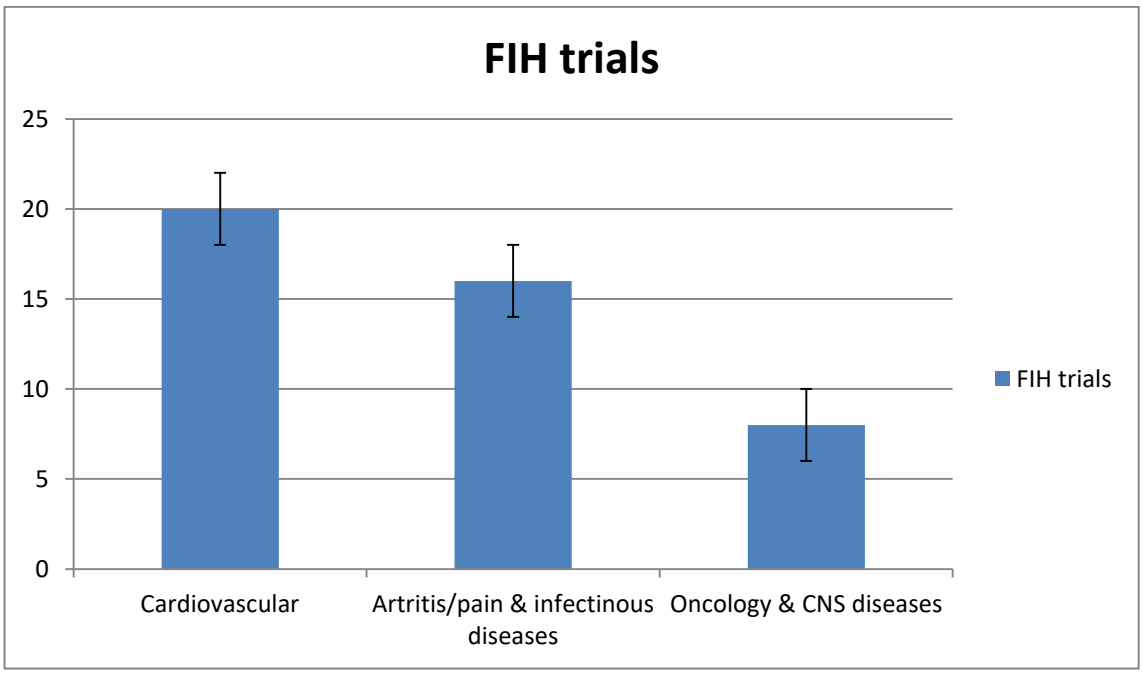


Chart 1. Medicinal development success rate according to therapeutic areas. [6]

Thus how are new drugs actually developed and marketed so that they reach into the hands of consumers. Like many other things it all starts with an idea.
The medicinal development process mainly follows three stages, which are drug discovery or research, drug development and manufacturing for the consumers. [7]

3.1.1 Drug discovery

The objective of drug discovery is to find a compound, which would inhibit the target protein in our body and change the characteristics of the disease. The first step is to test the hypothesis with artificial molecules. If desired results are obtained, then the next step is to move to the main research. [8]

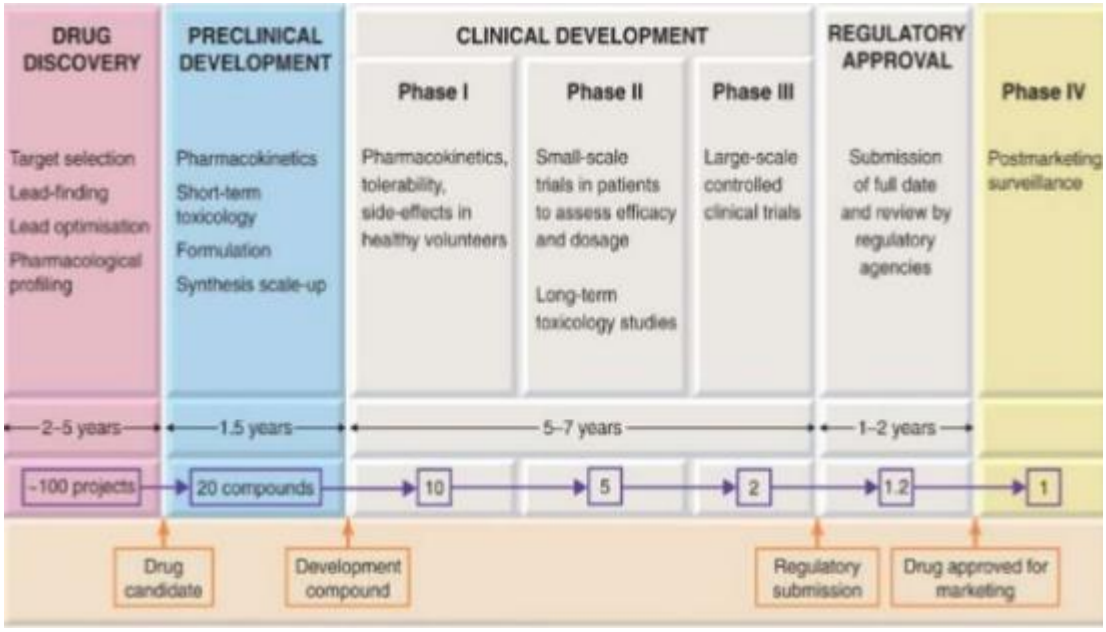
The first step of discovery is target identification, where a pathogenesis such as an enzyme or molecule associated with the disease is identified. When identifying the target, certain criteria are followed and specific characteristics evaluated. The ADME (absorption, distribution, metabolism, excretion) properties are an important guideline when choosing the target.

Also the Lipinski rule of five is followed during drug discovery. The factors predict that poor absorption or permeation is more likely to occur if two parameters are out of range. The five rules are stated below. [9]

- No more than five hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms)
- No more than 10 hydrogen bond acceptor (nitrogen or oxygen atoms)
- Molecular weight below 500 Da
- Calculated octanol - water partition coefficient (c log P0 bellow 5)

Following the target identification whether they are actual or virtual, computer based models are built in order to research their ability to bind with the target protein. This is the start of Hit-to-Lead identification where the small molecule hits from a high throughput screen (HTS) are evaluated and screened in order to find perspective lead compounds. These lead compounds are again screened in order to reach the lead's optimization, which is the last phase of drug discovery. The phases of drug development must be done within the parameters of Good Laboratory Practice (GLP). [10]

Already in the drug discovery phase the screening process starting off with thousands of compounds is narrowed down to only a few compounds for the pre-clinical testing (Pictures 2-3).



Picture 2. Drug discovery to development time line. [11]

3.1.2 Drug development

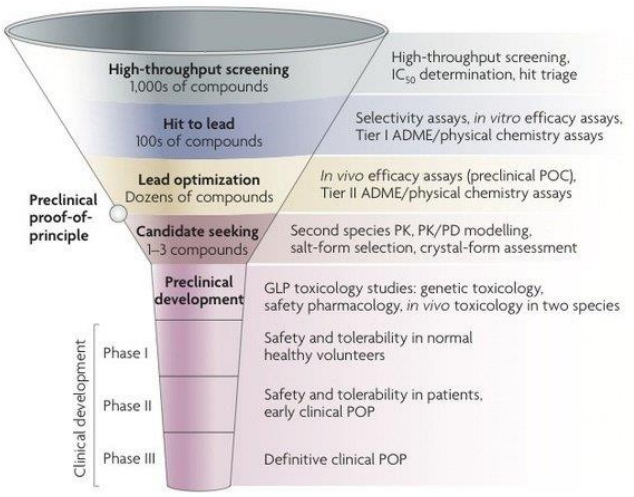
The development stage contains preclinical and clinical tests. The preclinical tests are done *in vitro* so with living organisms and *in vivo* with lab animals. If the results are successful an application called the Investigational New Drug Application (IND) is filed to assess the safety of the drug. With the approval of IND the clinical testing can take place.

Clinical testing consists of three stages called Phase I, Phase II and Phase III. These phases must proceed in this particular order and one phase must be completed successfully in order to go to the next phase. [12] Phase IV also exists and takes place after the drug has been marketed and consumed. It is a real life study of how the patients have reacted to the drug and the reasons behind it. Orion's drug development path is presented in Picture 5. [13]

In Phase I the trials are done with 20-30 healthy people. The safety, dosage and side effects of the new drug are observed. In Phase II the effectiveness is assessed on 100-300 patients. In Phase III the effects of long term drug use are examined on 1000-6000

clinical patients as the new drug is compared to existing therapies and drug combinations. This is the longest and most expensive phase of drug development.

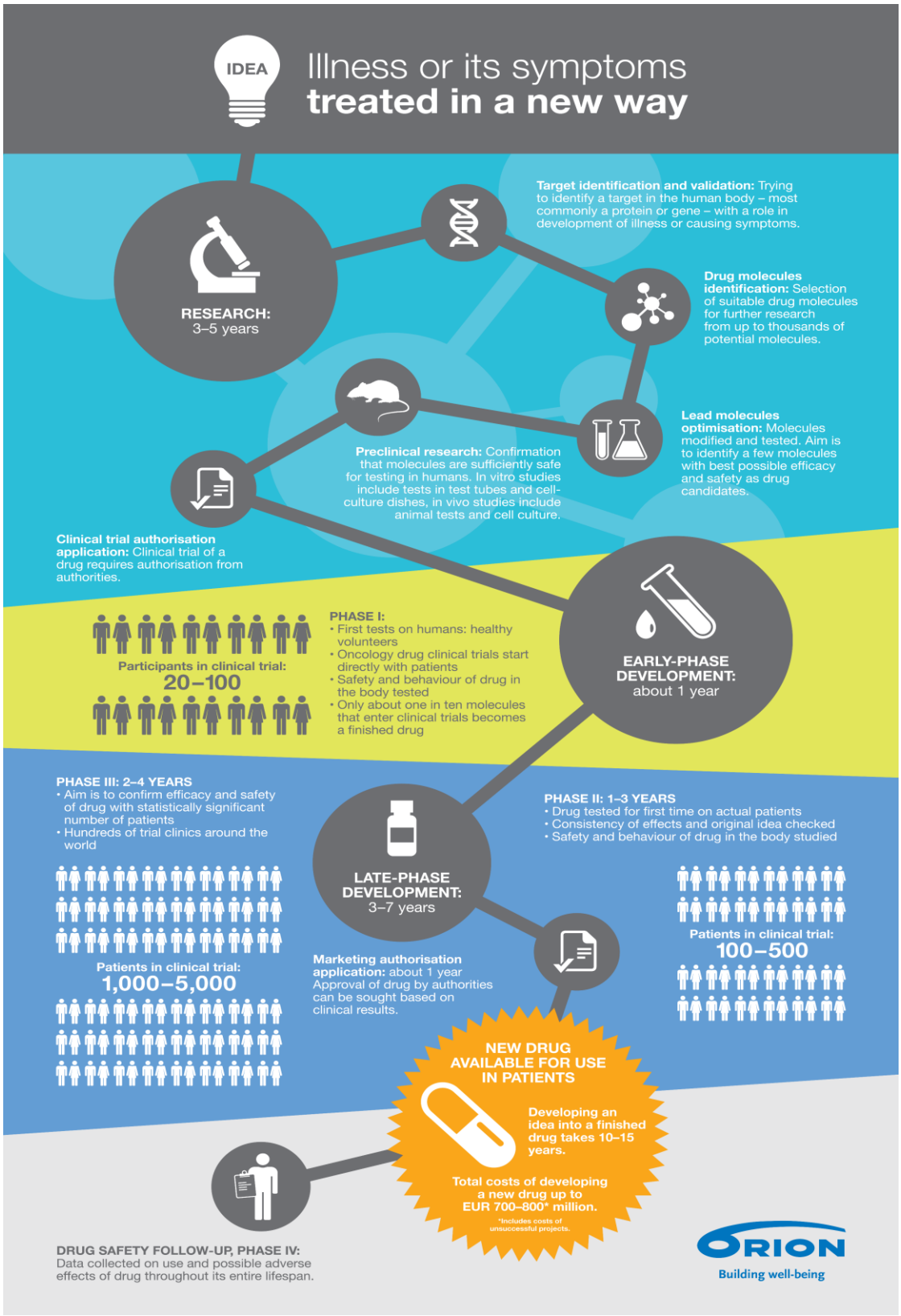
If the entire clinical test shows positive results a new NDA is filed for marketing approval. [14]



Picture 3. Drug development timeline in detail. [15]

Regarding drug development in Orion pharmaceutical, the company concentrates on early stage drug development and research. The company has delivered several proprietary products onto the market in various therapy areas.

During the early and final stages of research projects Orion like many other pharmaceutical companies works together with partners, such as universities and other organizations in the field. Orion also does its clinical trials in-house according to the high-level ethical principles as well as international and national regulatory requirements (Picture 4).



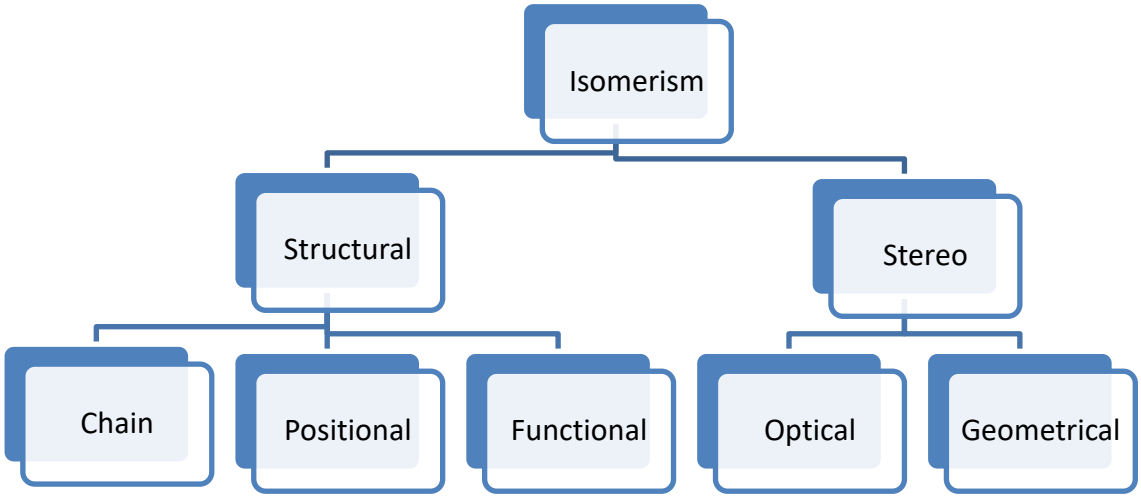
Picture 4. Orion's drug development and discovery. [16]

3.3 Isomerism

3.3.1 General concepts

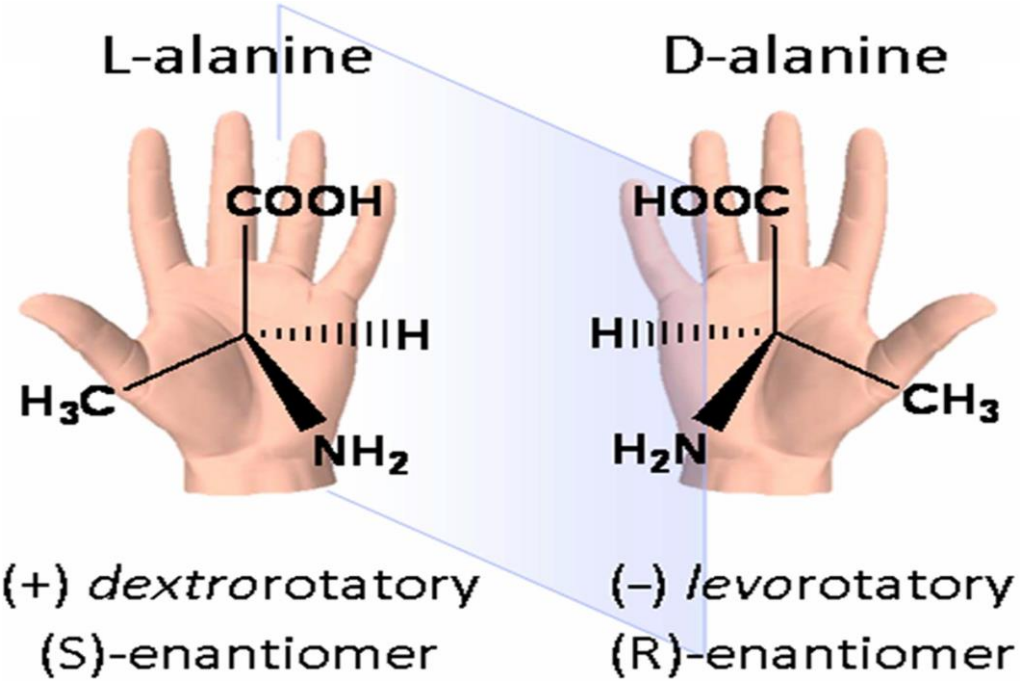
Molecules with the same molecular formula, but different arrangements of atoms are known as isomers. Many different types of isomers occur depending on their shape and positioning. Isomerism has two main forms, which are structural isomerism and stereoisomerism (Picture 5).

Structural isomerism, also known as constitutional isomerism, is a form in which molecules have the same molecular formula but differ in the bonding structure. Three categories of constitutional isomers are chain-, positional-, and functional isomers.



Picture 5. Forms of isomerism [17]

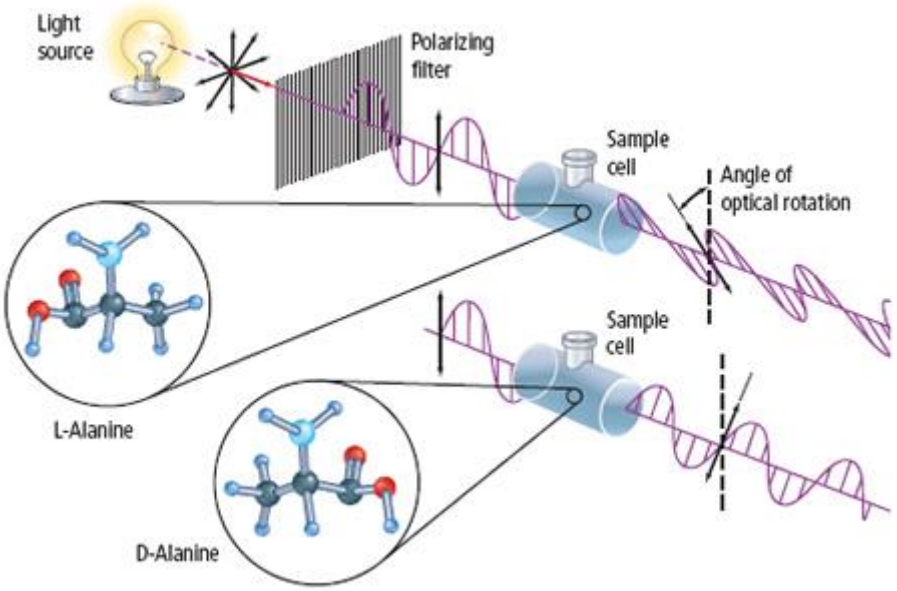
In stereoisomers the molecular structure remains the same, but differs in the spatial arrangement. The optical isomers belong to this group type. Optical isomers come in pairs, which contain the same number and kinds of atoms and bonds but have different spatial arrangements of the atoms. This class includes enantiomers, which are non-superimposable mirror-images of each other (Picture 6). On the other hand there are diastereomers, which do not match as mirror images. Enantiomers always contain chiral centres. Diastereomers usually do not have chiral centres. In some cases there are particular diastereomers which are neither chiral nor have chiral centers.



Picture 6. Mirror image of enantiomers [18]

Geometrical isomers have the same structural formulas but differ in the arrangement of groups at a single atom, at double bonds, or in rings. *Cis*- and *trans*-platin are examples of geometric isomers based on the different arrangement of groups at a single atom. [19]

Enantiomers are able to rotate the plane of polarized light that is passed through a solution of the compound. This ability makes the compounds optically active. It is a physical property of the compound that is measured using a polarimeter and is expressed in terms of degrees of rotation. Two compounds that are enantiomers have equal magnitude when come to optical rotations but in opposite sign. When two enantiomers are present in equal amounts, the mixture is optically inactive because the rotations cancel out. The same amount of two enantiomers in a mixture is called a racemic mixture (Picture 7). [20]

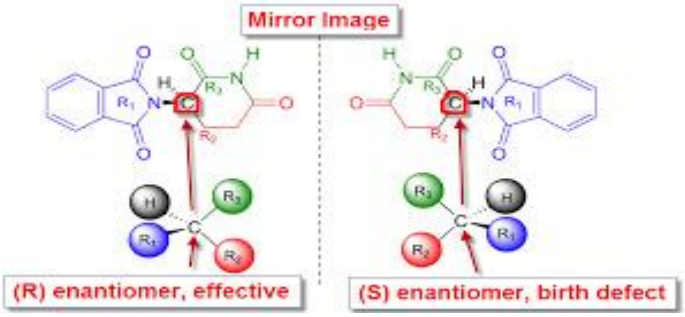


Picture 7. Polarized light effect on optical isomers. [21]

3.3.2 Impact of enantiomers in the pharmaceutical industry

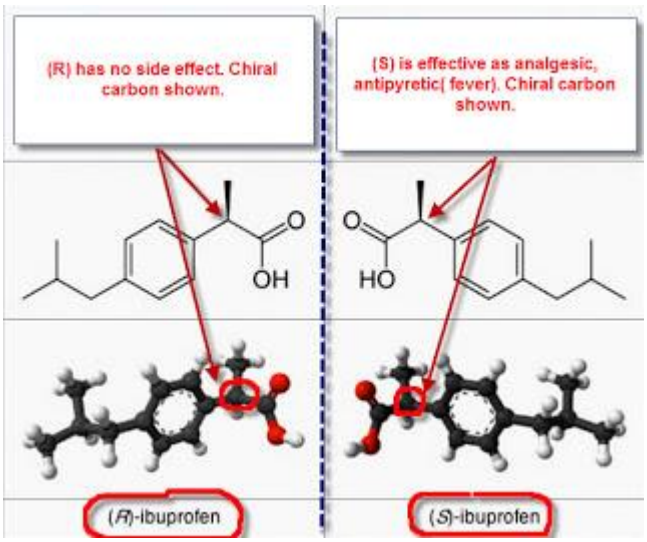
From the medicines currently in use, 56% of them are chiral products and 88% of them are marketed as racemates consisting of an equimolar mixture of two enantiomers. [22] Now a day's separation of enantiomers and dealing with their different form is common in the pharmaceutical industry. In the 1960's a well-known drug called Talidomade was marketed as an anti-nausea medicine for early pregnancy. Later researches has proven that the drug had caused uncountable miscarries, early infant mortality and disabled babies.

The medicine Thalidomide is a racemic glutamic acid mixture of R- and S- enantiomers. In a physiological environment the S- and R-enantiomers are able to change to each other due to the acidic hydrogen in the chiral center as shown in picture 8. The effect of the medicine was based on the R-enantiomer where in the other hand S form cause severe cell mutation leading to teratogenicity (Picture 8). [23]



Picture 8. Mirror image of enantiomers in Talidomide

Many drugs are made from racemic enantiomers with equal R- and S form. It is often cheaper to synthesize racemic mixtures. The very familiar drug ibuprofen is racemic compound where the S-enantiomer reduces fever and pain but the R-enantiomer is inactive with no side effects. The formula is explained in Picture 9.



Picture 9. Mirror image of ibuprofen [24]

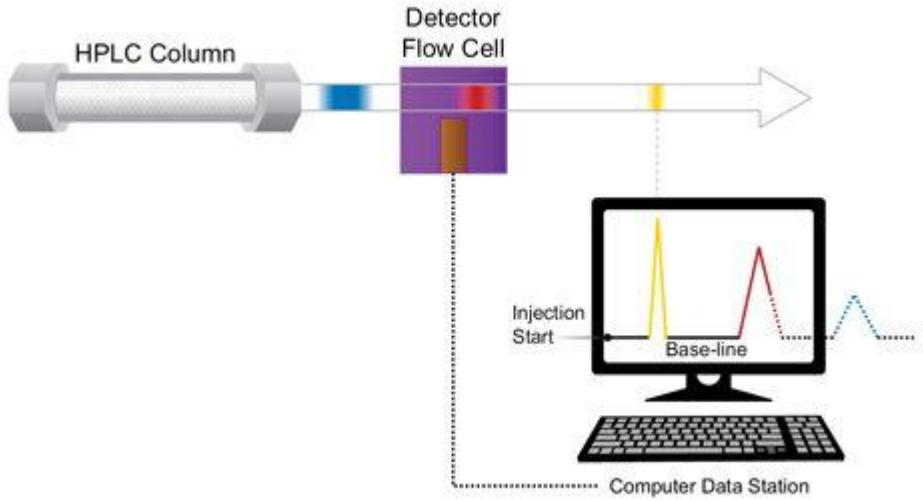
3.4 Chromatography

Chromatography is essentially a physical method of separating components in two phases. One of the phases is the stationary phase, and the other one is the mobile phase, which moves in a different direction. [25]

3.4.1 Column chromatography

Column chromatography operates so that different phases move through a tubular column packed with solid particles and filled with solvent (Picture 10). To explain the process better a solution containing two solutes are named A and B. As the machine takes in the mixture, it flows down into the column. Fresh solvent is applied into the column, which washes down the column by continuous solvent flow. If the solid particles absorb solute A more strongly than solute B, then the solute A spends a smaller fraction of the time free in the solution. This makes the solute A move down the column slower than solute B. This way the two solutes have been separated in the mixture. [26]

The separated mixture then enters the detector where the band formation takes place. The detector identifies each separated compound band against a background of mobile phase. A suitable detector like UV, evaporative light scattering (ELS) or fluorescence, has the ability to sense the presence of a compound and send its matching electrical signal to a computer data station where it is recorded as a peak. The detector responds to the concentration changes of the specific analyte molecules within the band, where the center of the band is interpreted by the detector as the top of the peak as the picture bellow clearly states. The base line appears as a straight line in the bottom of the screen as soon as the sample is injected. That is when the chromatogram begins and the baseline represents pure mobile phase passing through the flow cell over time.

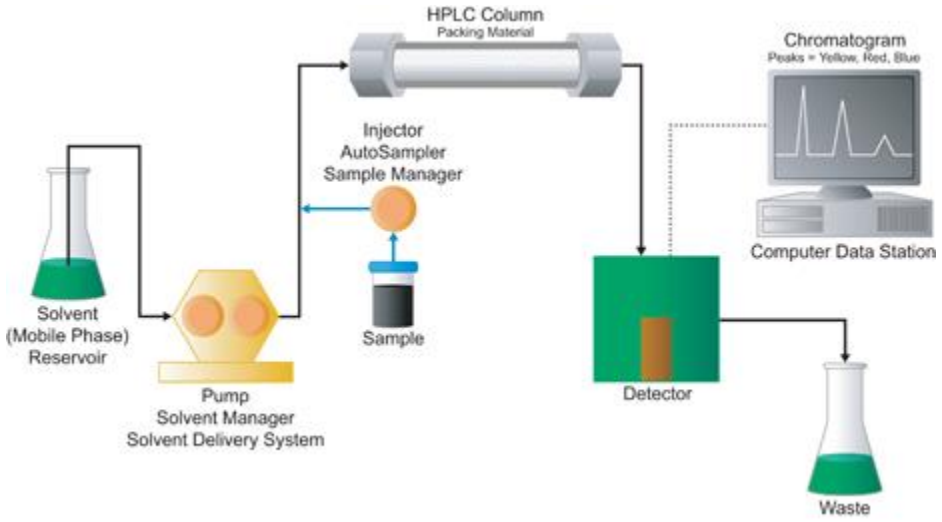


Picture 10. The process of peaks being created into the chromatogram [27]

The chromatography process and the basic materials are illustrated in Picture 11. Five chromatographic methods that use columns are listed below:

- gas chromatography (GC)
- liquid chromatography (LC)
- Ion exchange chromatography (IEC)
- size exclusion chromatography (SEC)
- chiral chromatography

The basic principles of chromatography can be applied to all five methods. [28]



Picture 11. Chromatography system [29]

3.4.2 Concept of columns

In chromatography, sometimes columns are used for separating the components. There are three basic types of liquid chromatographic columns

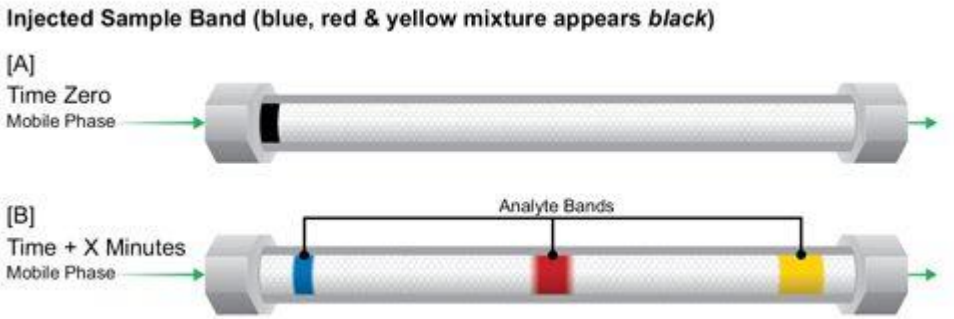
- liquid-liquid
- liquid-solid
- Ion-exchange

Liquid-liquid chromatographic columns contain the liquid stationary phase bonded or absorbed to the surface of the column, or packed material. Liquid-liquid chromatographic columns are not used as frequently because of their instability and inconvenience compared to other ones. Partitioning occurs between the two different liquids of the mobile and stationary phases.

In liquid-solid chromatographic columns, the stationary phase is a solid and the analyte absorbs onto the stationary phase, which separates the components of the mixture.

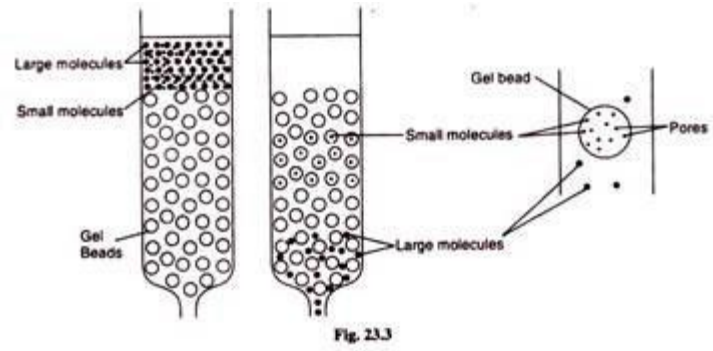
In ion-exchange chromatographic columns, the stationary phase is an ion-exchange resin and the separation occurs through ion exchanges, which occurs between the analyte and stationary phase.

The basic column separation starts few minutes within the sample injection, during which mobile phase flows continuously and steadily through the packing material particles. Picture 12 shows a separation of a mixture containing three colours, blue, red and yellow. At the beginning of the separation process the individual colours have moved in separate bands at different speeds. This is because there is a competition between the mobile phase and the stationary phase for attracting each of the colours or analytes. In the example picture the yellow colour band moves the fastest and is about to exit the column. This shows that the yellow colour has a greater affinity for the mobile phase compared to the other colours. The blue dye band is the most retained compound in the sample mixture and has a greater affinity for the packing material rather than the mobile phase. Its stronger attraction to the particles causes it to move considerably more slowly. The red colour has an intermediate attraction for the mobile phase and therefore moves at a medium speed through the column. As each component moves at a different speed, it allows separating the mixture chromatographically.



Picture 12. Concept of HPLC columns [30]

Usually HPLC has a guard column is placed before the analytical column to protect and extend the life of the analytical column. The guard column removes any unwanted matter, contaminants, and molecules that might bind irreversibly to the column. The stationary phase in the guard column is similar to that of the analytical column being used.

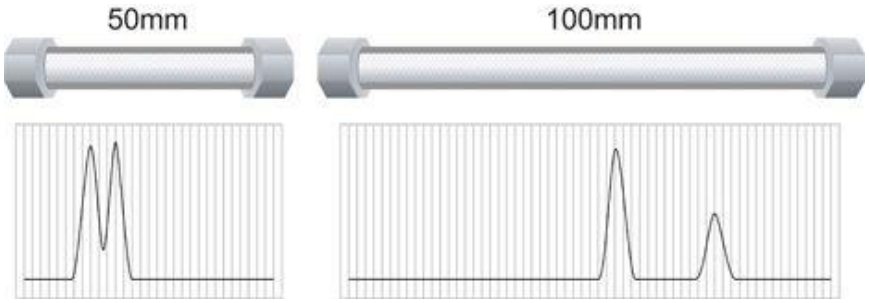


Picture 13. Internal material of a column [31]

Typical HPLC analytical columns are from 3 to 25 cm long and have a diameter of 1 to 5 mm. The columns are often straight unlike the GC columns. Particles that are used as packing material for the columns have a typical diameter between 3 to 5 µm as seen in picture 15. Liquid chromatographic columns will increase in efficiency when the diameter of the packed particles inside the column decreases (Picture 13). [32]

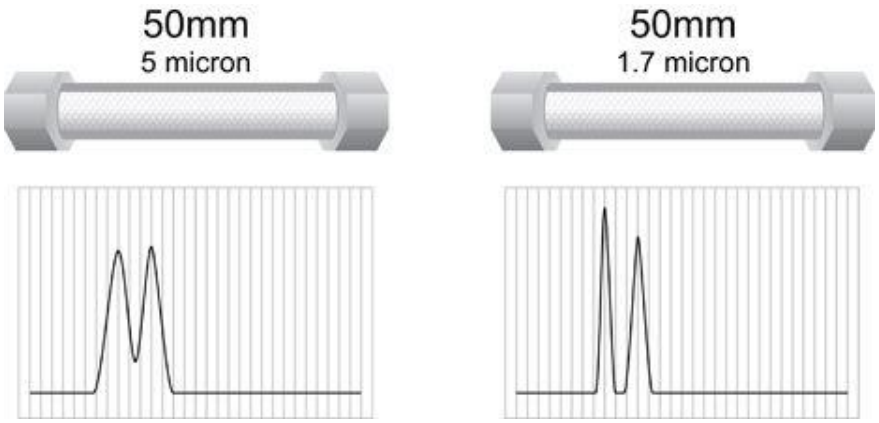
If a column bed is stable and uniformly packed, then the separation efficiency is determined by the column length and the particle size. Smaller-particle chromatographic beds have higher efficiency and higher backpressure. Better separation results are obtained by increasing column length. However longer column length means it takes longer for the product to come out. This creates longer chromatographic run times, greater solvent consumption, and higher backpressure. Shorter column lengths mini-

mize all these factors, but the results of the separation are usually weaker as shown in Picture 14.



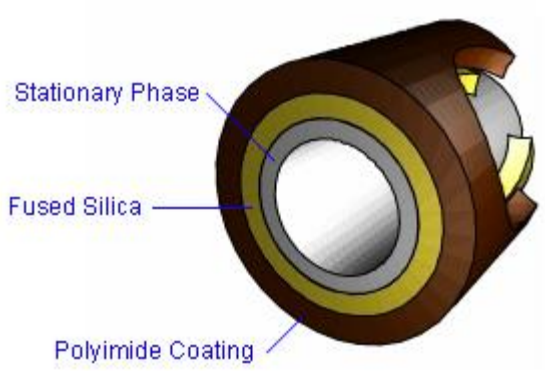
Picture 14. Quality of enantiomer separation due to column length

Particle sizes in a column also play a significant role in the quality of separation. As shown in Picture 15 below, two column of the same length, the one with smaller particle size will deliver a better peak separation. In this case, the backpressure needs to be relatively high.



Picture 15. Quality of enantiomer separation due to column particle size [33]

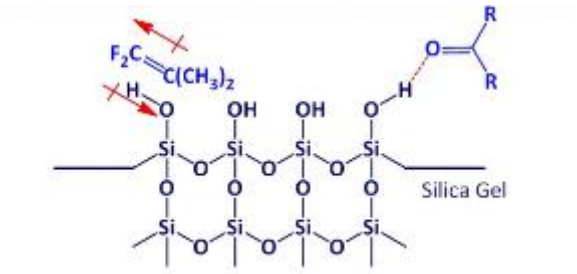
HPLC columns are often made from stainless steel, which is authentic chromium-nickel-molybedum steel. This allows the column to be able to resist the HPLC pressure. It is also important that the column be inert to able to avoid corrosion. They can be also made out of thick glass, polymers such as polyetherethelketone, a combination of stainless steel and glass, or a combination of stainless steel and polymers.



Picture 16. Inner surface material of basic silica column [34]

Silica gel is often used for coating chromatography columns because of its polar adsorbent feature and slightly acidic nature. It has the capacity to absorb basic contents that might be present in the material that needs separation or purification and does not interact with any of the substances that are passed through it. Silica maintains its own stable structure throughout the chromatography process (Picture 16). [35]

Silica gel (SiO_2) has hydroxyl groups at the surface of the particle which makes it highly polar. The polar functionality binds through hydrogen bonds or dipole-dipole interactions. In silica columns more polar compound will have greater interaction with the stationary phase and as a result, they will move slower creating separation from the mobile phase (Picture 17).

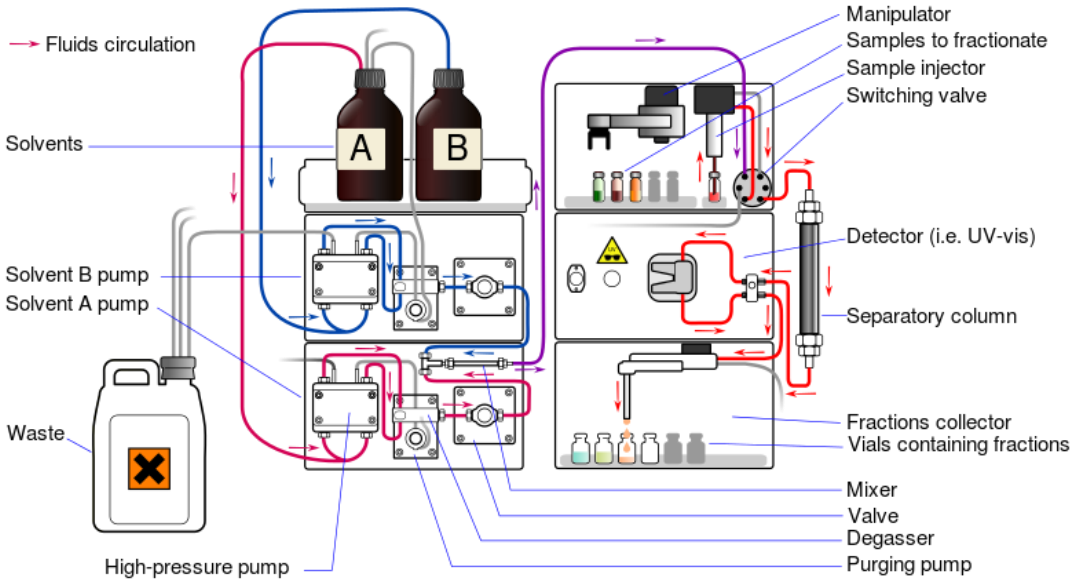


Picture 17. Example of silica binding method. [36]

3.4.3 Comparison of preparative and analytical chromatography

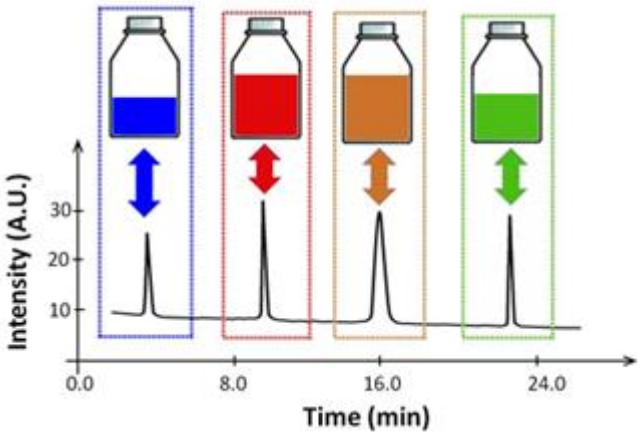
Preparative chromatography is a kind of scaling up chromatography because it is done in larger volume and using a larger column keeping all the other parameters constant. The preparative HPLC device is often equipped with a fraction collector. For the collection if the products, commonly set of vials or small bottles are used. After the sample is run in the preparative HPLC, the isolated compound is collected to an infraction collector for further use (Picture 18).

When a method is being developed for preparative separation, one way is to focus on finding the right parameters through a screening process. Once an analytical method is established, it can be scaled up to a preparative separation. An optimal method would show as clearly as possible the peak of interest and the one or two adjacent peaks. Any possible other peaks would be directed to waste and separated from the actual target compound by collecting them into individual tubes. Compared to analytical HPLC, which aims towards the quantification or identification of compounds with the sample going from the detector to waste, the preparative chromatography aims at the isolation of compounds with the sample going to the fraction collector. When applying the analytical separation method into preparative scaling up environment the performance features, selectivity behavior, and recyclability of the stationary phase material should be identical in both separations. The only exception is in the particle size. [37]



Picture 18. Preparative HPLC system [38]

In preparative separation each peak can be collected into individual vials as shown in Picture 19.



Picture 19. Specific compounds being collected into vials in preparative separation [39]

When applying the selected method into preparative columns instead of analytical HPLC column, the measurements are usually made according to Table 2.

Table 2. Parameters in preparative LC and analytical LC

Column type	Sample Quantity range	Column diameter (mm)	Column length	Flow rate range
Preparative LC	mg - g	>4	15 - 250	5 - 20
Analytical LC	µg - mg	2 - 4	15 - 250	0.2 - 1

[40]

3.5 Equipment description

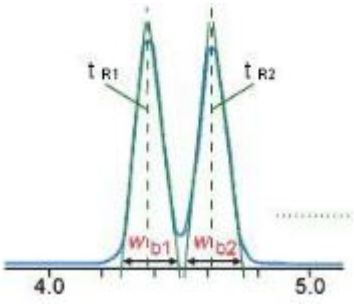
3.5.1 HPLC

High performance liquid chromatography (HPLC) is a type of liquid chromatography. HPLC uses a liquid mobile phase. The same basic principles from gas chromatography are applied to liquid chromatography. This is an extremely fast and accurate way to analyze compounds. It also allows the usage of very small particle size for the column packing material, which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This way much better separation of the components in the mixture can take place.

Different equations are applied when using the HPLC. Usually these equations are fed to the apparatus, which then is able to calculate the results almost instantly. A commonly used equation is presented in order to understand a factor which plays a vital role in the separation process. One way to determine the efficiency of HPLC separation is by measuring the resolution R_s. When reading a HPLC run result the resolution states the separation of two neighboring peaks following the equation bellow.

$$R_s = \frac{2 \cdot (t_{r2} - t_{r1})}{(w_{b1} + w_{b2})} \quad (1)$$

Where R_s is resolution time, t_{r1} is retention time of first peak, t_{r2} is retention time of second peak, w_{b1} is first peaks width in the base, and w_{b2} is second peaks width in the base (Picture 20).



Picture 20. Measuring the separation of neighboring peaks [41]

Main types of HPLC and their separation principles are presented below (Table 3).

Table 3. Different types of HPLC separation methods.

Separation mechanism	**Types of HPLC**
Hydrophobic forces	Reversed phase
	Ion pair
	HIC
	Non-aqueous phase (NARP)
Difference in polarity	HILIC
	Normal phase
	Aqueous normal phase
Ion interaction	Cation exchange
	Anion exchange
	Ion exchange on amphoteric and zwitterionic phase
	Ion exclusion
	Ligand exchange
	Immobilized metal affinity
	Ion moderated
Size exclusion	Gel filtration
	Gel permeation
Displacement	Displacement
Bioaffinity	Bioaffinity not always HPLC
Chiral	Chiral stationary phase
	Chiral mobile phase
Various principles together	Multi-enode

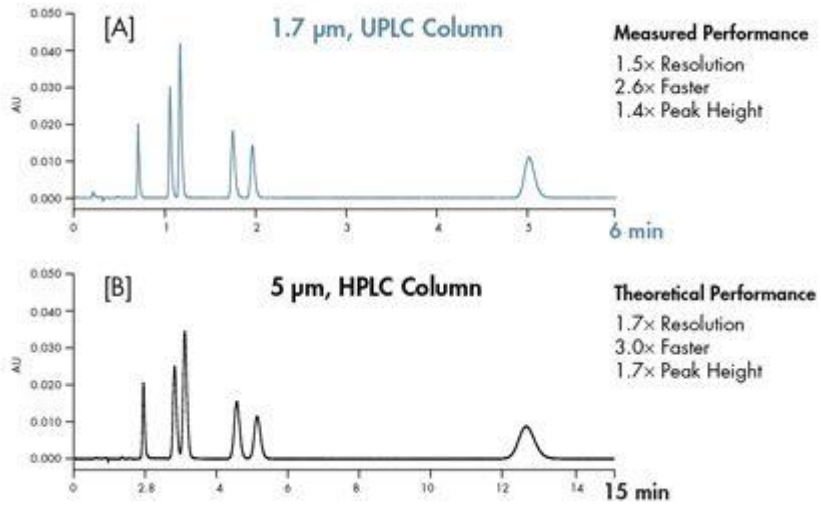
[42]

A basic HPLC apparatus is presented in the Picture 21

Picture 21. HPLC apparatus [43]

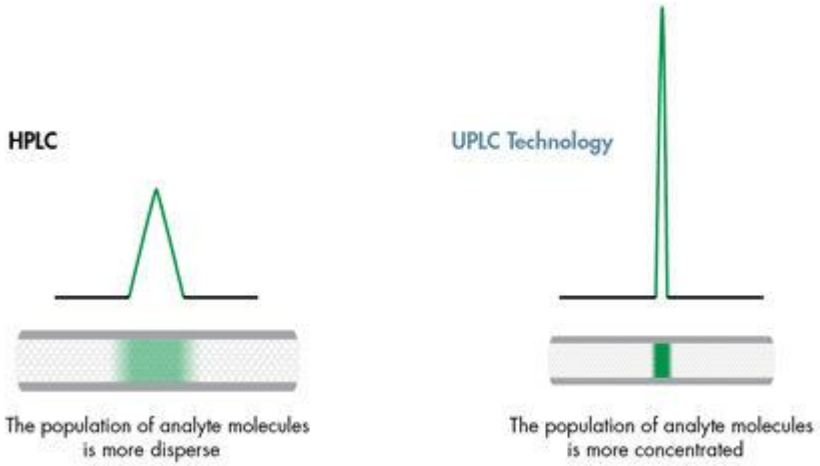
3.5.2 UPLC

UPLC (ultra performance liquid chromatography) systems were first introduced in 2004 by Waters and the name is their own trademark. The principles of UPLC are basically same as HPLC, the elementary difference is in designer of the column material particle size which less than 2-µm. This technique allows operating in high pressure (to 15,000 psi). It gives more rapid flow rates, speed and better resolution compared to HPLC (Picture 22).



Picture 22. Effectives of UPLC column compared to HPLC column

One of the features of modern UPLC is the ability to minimize band spreading in peaks. Narrower and sharper chromatographic bands are obtained, resulting in higher efficiency. Taller, narrower chromatographic peaks are easier for the detector to see, allowing for higher sensitivity and resolution due to a more concentrated analyte band. UPLC is thought to be an improvement on total productivity (Picture 23).

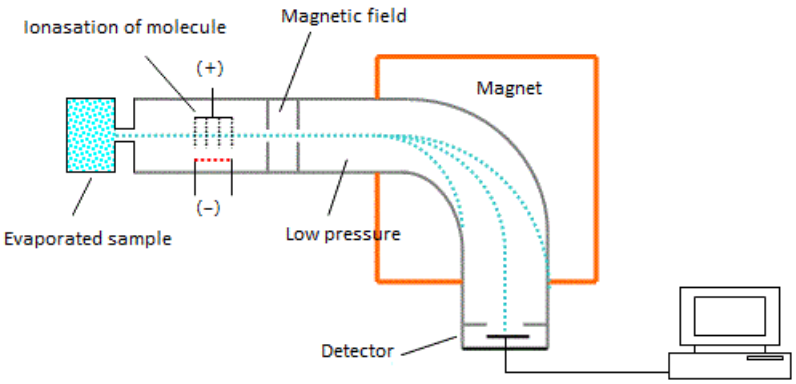


Picture 23. Peak formation in HPLC and UPLC technology [44]

3.5.3 LC-MS

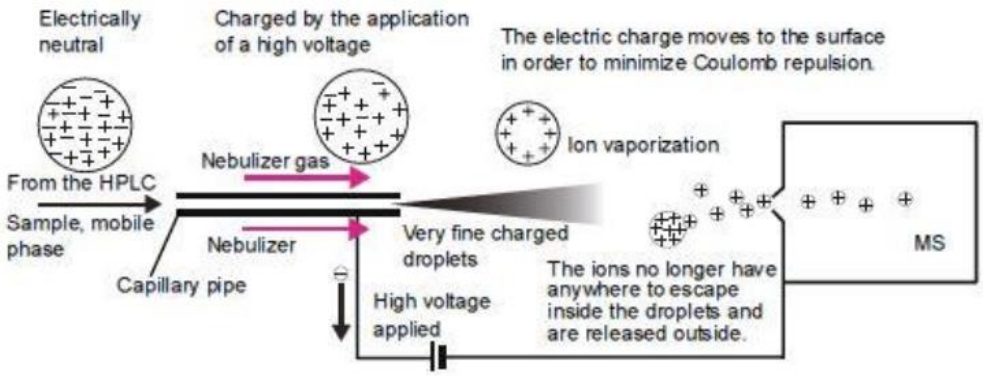
Mass spectrophotometry is commonly used in the pharmaceutical industry because of its ability to determine molecular weight and its structure in a very short period of time. First the, molecular sample is separated and the brought to the mass spectrometer for mass determination. Also the MS feature enables the detection of charged ions. The use of LS-MS has modernized the pharmaceutical industry in a tremendous manner.

The LC-MS device has different sections which treat the sample in order to determine the molecular weight. Depending on the device, it is able to measure up to 150,000 daltons. [45] The sample must be in charged ion form and gas phase in order to be analysed. For the apparatus it is necessary to have an ionization section, analyser and detector (Picture 24).

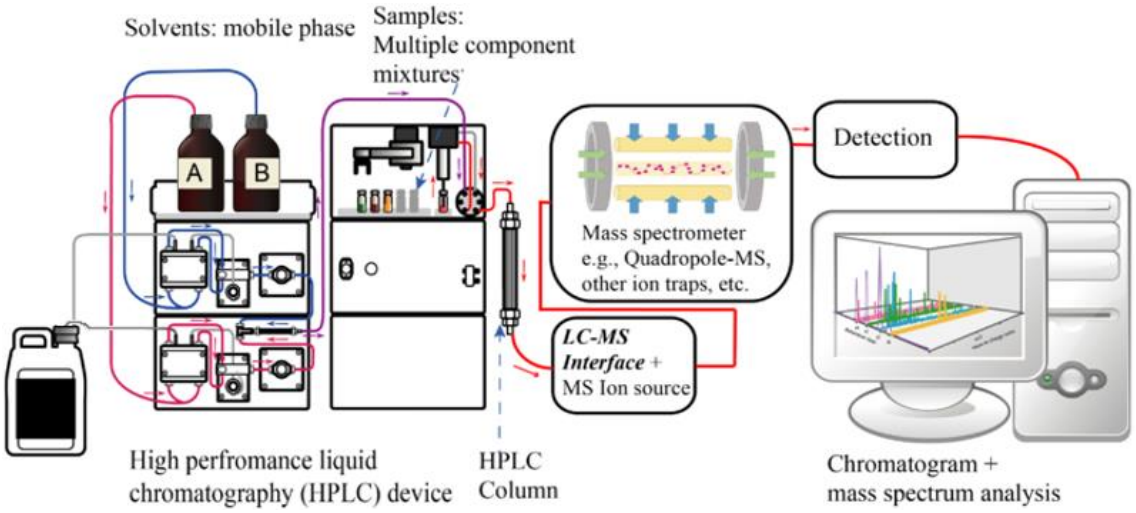


Picture 24. Mass spectrometry [46]

One way to ionize the sample is through electrospray ionisation (ESI). This is an ionization technique for small amounts of large molecules. ESI operates at atmospheric pressure. A sample solution is sprayed from a small tube into a strong electric field in the presence of a flow of warm nitrogen to assist dissolving. A very evident feature of an ESI spectrum is that the ions carry multiple charges, which reduces their mass-to-charge ratio compared to a singly charged species (Picture 25).



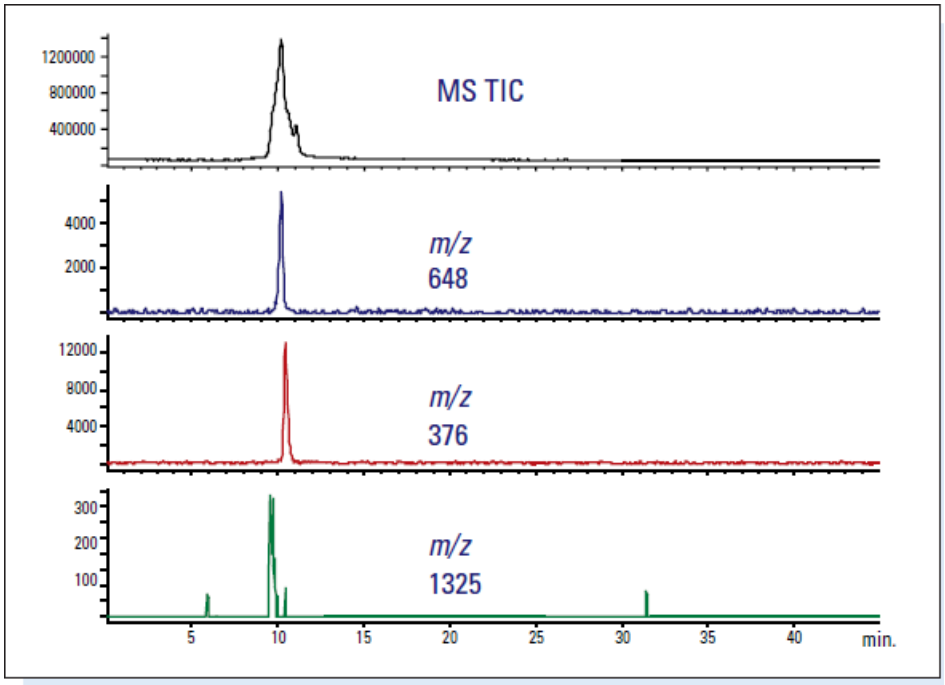
Picture 25. Electrospray ionization [47]



Picture 26. Principles of LC-MS [48]

The MS is easy to combine with LC detectors like Diode Array detection (DAD) for acquiring data on specific ultra violet (UV) and visible light (VIS) wavelengths and spectra. Actually it is more effective to use UV detector and mass selective detector rather than using them alone. The reason for this is because certain compounds specially which have low molecular weight might give very similar UV spectrums. This leads to difficulties in detecting impurities. Sometimes impurities share the same mass. However, they rarely share the same mass as well as same UV-Vis spectra (Picture 26).

An example is presented in Picture 27, where a polymer isomerism is separated using LC-MS. Many pharmaceutical companies choose to have an up to date LC-MS for these features, which a conventional LC-MS does not offer. This is also a very economical option as analysis because of the short analysis time which may be as less as three minutes.



Picture 27. Separation of isomers. [49]

4 Experimental procedures

The method development research process is presented in this chapter in the order it was conducted in the laboratory.

Solvents used in the research

All the solvents used in the research are presented in Table 4.

Table 4. The solvent details used in the research.

Solvent	Formula	Manufacturer	Grade
Acetonitrile	C_2H_3N	Merck, Darmstadt, Germany	Liquid chromatography
Ethanol	C_2H_6O	Merck, Darmstadt, Germany	Aa
Methyl tert-butyl ether (MTBE)	$C_5H_{12}O$	Merck, Darmstadt, Germany	Liquid chromatography
N-hexane	C_6H_{14}	Merck, Darmstadt, Germany	Liquid chromatography
Isopropyl alcohol (IPA)	C_3H_8O	Merck, Darmstadt, Germany	Liquid chromatography
Diethanolamine (DEA)	$HN(CH_2CH_2OH)_2$	Merck, Darmstadt, Germany	Liquid chromatography
Methanol	CH_3OH	J.T. Baker, Nether-lands	Liquid chromatography
Water	H_2O	Water center, Orion, Finland	Milli-Q ultrapure

Sample processing

The pharmaceutical ingredient sample was originally in powder form. 4 mg of the sample was measured and diluted in ethanol to a concentration of 4 mg/ml. The complete dissolving of the compound into the solvent was followed visually. The sample vial was placed into the HPLC to start the screening process.

Screening process

The aim of this screening process was to find the suitable kind of column, solvent combination, temperature, pH, pressure among other parameters for the separation. The use of a modifier additive was also important to determine. The choice of additive can have a significant impact on the retention and resolution, so various additives were taken into consideration.

The screening process set-up was done according to Orion's chemical analytics laboratory's own screening format. This specific screening method is also Orion's in-house system for finding out the appropriate method for analyzing and separating their samples.

Altogether 13 runs with different solvents and 2 column combinations were made in order to choose the perfect option for the final separation. The target was to find a method which would be able give a clear separation for the two peaks in a relatively short retention time (RT) and appropriate resolution. Retention time is the measure of time the sample is inside the column after being injected. Basically, it is the pass through time of the solute inside a column from injection to detection. Different compounds have unique retention times depending on their chemical composition, so each compound elutes from the column in different time. This can also be used as a recognition method for different compounds. The unit for RT is often seconds or minutes. When choosing a method, it is important not to choose a method with a very small retention time even though the target is to develop a method which does not consume much time. The reason for not choosing a very short method is that when the method is applied to preparative environment and the run is set for very short period of time, the product might not have enough time to elute from the preparative HPLC column. Sometimes the product might take a little longer time to elute from preparative column compared to analytical columns mostly because of the scale-up. This factor has to be considered when applying an analytical method into preparative environment. Agilent's 1200 series HPLC were used for the screening process.

The two different columns used for the screening were Diacels Chiralpal IA and IC, which are commonly used in the laboratory for similar separations. The column Chiralpak IA is a polysaccharide-derived chiral chromatographic column coated on a silica matrix. For better solvent versatility, an amylose derivative is immobilized onto the silica gel. Chiralpal IC is a similar polysaccharide-derived silica based column with a cellulose tris polymer immobilization. This column is also supposed to have high solvent versatility and high selectivity in the resolution of enantiomers.

The solvent selection amongst other parameters applied in the method was determined from the routine screening chart. The solvents used were ethanol, MTBE, N-hexane and IPA in different combinations. A 0.2 % DEA was added with the solvents hoping it

would improve the shape on the peaks. The flow rate for solvents was set up to run at 1 ml/min. injection volume of sample was 5 µl. The screening runs were done in three different wavelengths, which were 235 nm, 250 nm and 300 nm.

Chiral purification with preparative chromatography

The preparative separation method was based on the analytical method chosen from the screen run (Table 5, p.34). Slight modifications were made and the runtime was shortened to 30 minutes instead of the original 45 minutes. The reason for this was because in the screening process the peaks retention time was in 19 minutes and 25 minutes. The amount of starting material was approximately 180 mg. The sample concentration was 30 mg/ml in ethanol and injection volume for sample was 100 µl. The whole run consisted of 24 injections. The injection volume was 250 µl of sample per injection. The wavelength for the method will be 300 nm according to the UV-maximum (λmax), which is the wavelength of the peak's highest point (picture 29) on page 36. Also the screen run chromatogram proved the compound to have good absorbance in that wavelength.

After the preparative separation both peaks were analyzed separately with optical purification in order to insure their quality and separation rate. The mentioned separations and purification were done by Agilent's 1200 series HPLC. The column used for preparative separation was Chiralpak IC length 20 x 250 mm with particle size of 5 µm. The optical purification column was the same Chiralpak IC with shorter length of 4.6 x 250 mm.

HPLC purification

HPLC purity was done with Waters UPLC. The method used for the determination was a routine chromatographic method taken from the database of Orion's analytical laboratory. It is a five minute run in basic pH 2.2 environment. Both peaks were analyzed separately. The mobile phase solvent used for the run was 15 mM potassium dihydrogen phosphate buffer in pH 2.2 and ultra-purified water. The concentration of the sample was 2 mg/ml in ethanol. Aqcuity BEH C18 2.1 x 50 mm column was used for the run.

Pure ethanol was used for needle washes in between sample injections as well as for cleaning the column in between the actual sample run. This was to ensure that any residue from the prior sample was not present in the column when a new sample was injected. Also the samples were diluted in ethanol. The peaks appearing from ethanol run is deleted from the actual sample run. The chromatogram from the ethanol run is in Appendix 1.

Mass spectrophotometry determination

The mass spectrophotometry determination was supposed to ensure that the required compound had been isolated properly and that the chromatogram does not show any other compound or impurities. If the compound had broken, this analysis would be able to detect that in a short 3 minute runtime. Also for this analysis an existing method was used with a run of as short as 3 minutes. Both peaks were analyzed separately. Sample concentration was diluted in 4 mg/ml ethanol and the sample injection was 1 µl. The analysis was done with Waters mass spectrophotometry.

5 Result analysis

Screening results

The best method for separating the enantiomers was chosen from the screening chart below (Table 5) with the following parameters.

Column: Chiralpak IC

Solvent 1: MTBE + 0.2 % DEA

Solvent 2: IPA + 0.2% DEA

Solvent flow rate: 1 ml/min

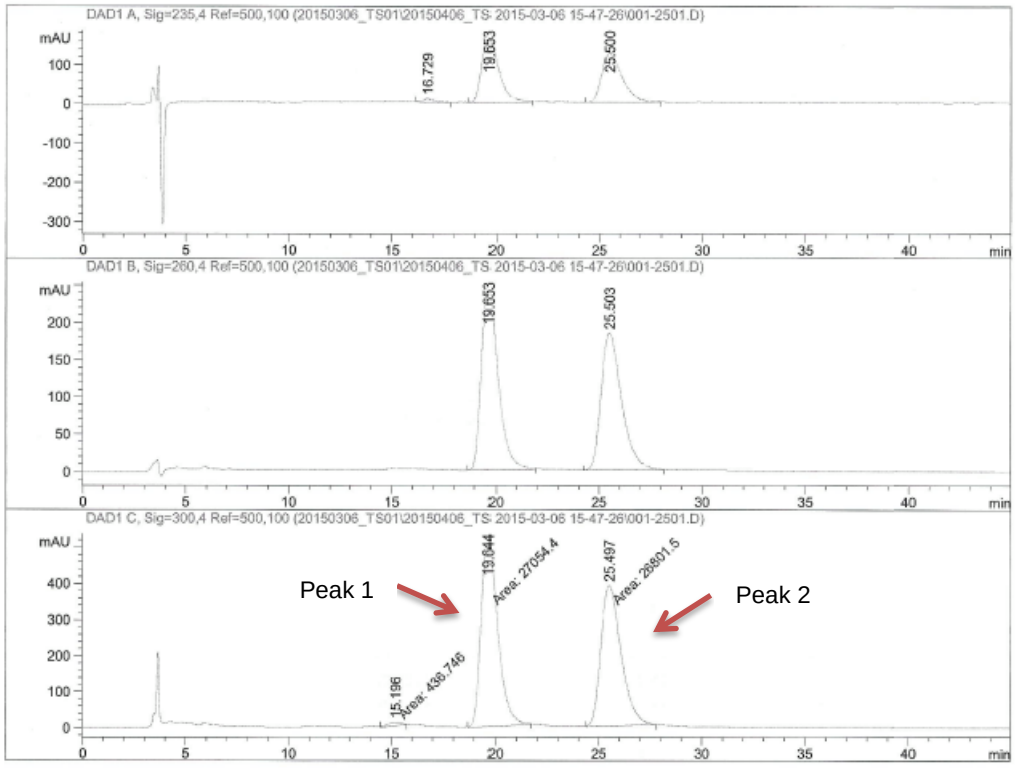
Wavelength: 300 nm

Runtime: 30 min

Table 5. Screening Chart

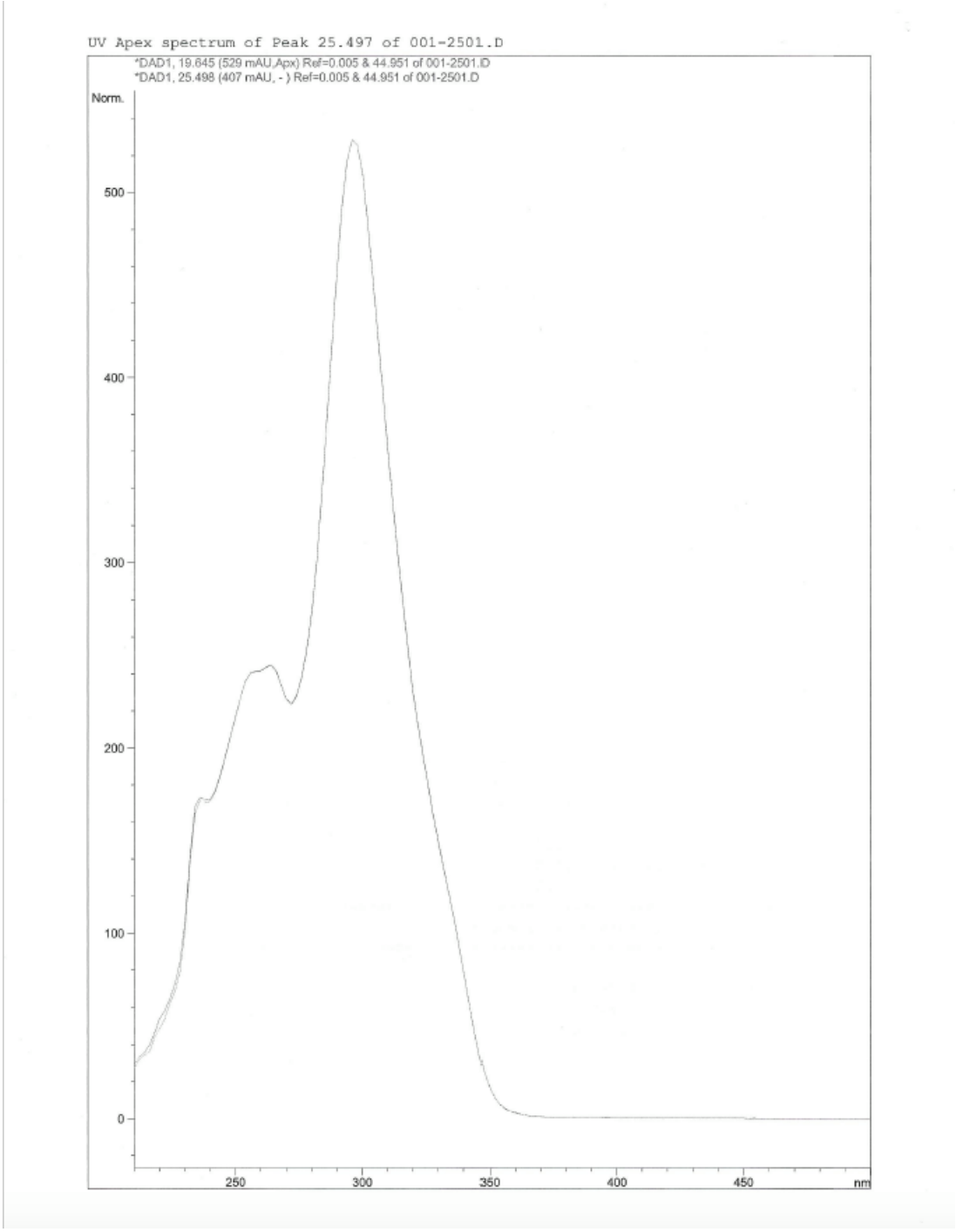
Column	Eluent mixture	Solvent flow rate	Max λ nm	Result	Duration
Chiralpak IA	n-hexane:EtOH 80%:20%	1 ml/min	*	2 peaks, no baseline separation, RT 10 min & 12 min	45 min
	n-hexane:EtOH 80%:20%	1 ml/min	*	2 peaks, separated, wide peaks	45 min
	n-hexane:EtOH 80%:20%	1 ml/min	*	Not visible	45 min
	n-hexane:EtOH 80%:20%	1 ml/min	*	2 peaks, Wide peaks, RT 13 min & 19 min	45 min
	n-hexane:EtOH 80%:20%	1 ml/min	*	1 peak	45 min
	n-hexane:EtOH 80%:20%	1 ml/min	*	Not separated	45 min
	n-hexane:EtOH 80%:20%	1 ml/min	*	1 peak	45 min
	+ 0.2 % DEA			RT = Retention time (amount of time the sample is inside the column)	
	* 235 nm, 250 nm, 300 nm				
Chiralpak IC	n-hexane:EtOH 90%:10%	1 ml/min	*	2 peaks, separated, RT 10 min & 12 min	45 min
	n-hexane:EtOH 95%:5%	1 ml/min	*	2 peaks, separated, RT 8 min & 11 min	45 min
	MTBE:EtOH 90%:10%	1 ml/min	*	2 peaks, separated but joint baseline, RT 10 min & 11 min	45 min
	MTBE:EtOH 95%:5%	1 ml/min	*	2 wide peaks, separated, RT 23 min & 29 min	45 min
	MTBE:IPA 80%:20%	1 ml/min	*	2 peaks, separated, RT 19 min & 25 min	45 min
	MTBE:IPA 80%:20%	1 ml/min	*	Not separated	45 min
	+ 0.2 % DEA				

This method option gave the best separation in a short retention time and good resolution. Both peaks eluted from the column with sharp separated peaks as shown in the results chromatogram (Picture 28). The red arrows indicate the peaks of interest.



Picture 28. Chromatogram of the best screen run.

The UV spectrums of both separated enantiomer peaks are put together on top of each other to check their similarity. It is clearly visible that both spectrums are a perfect match of each other (Picture 29). The same UV spectrums here prove that both of the enantiomers have the same absorbency as they overlap one another.

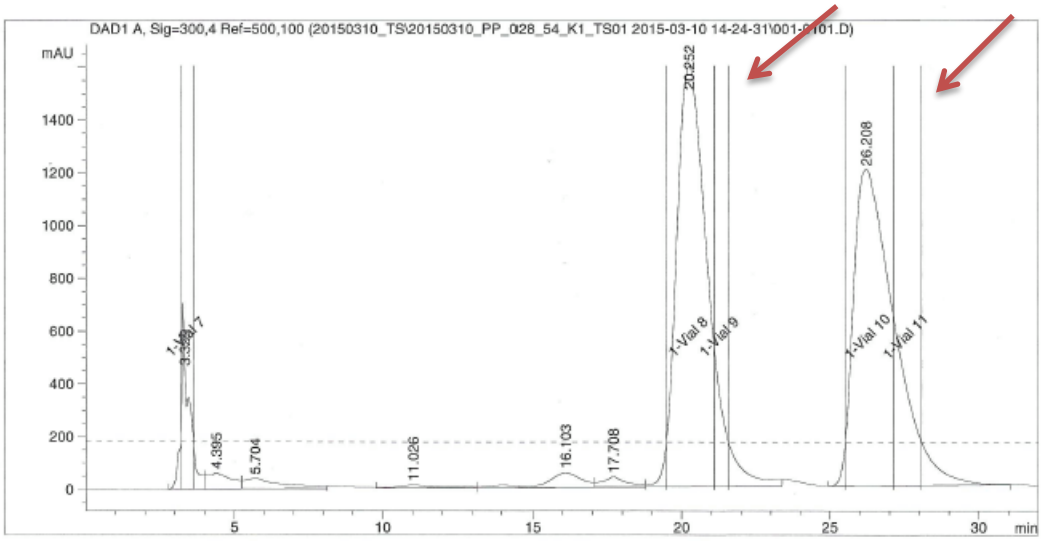


Picture 29. UV spectrum of the peaks

Preparative chromatography purification results

The enantiomers separated with the first peaks retention time of 20 minutes and second peaks retention time of 26 minutes as seen in picture 34. The compounds were collected into vials according to the vials shown in the chromatogram for further

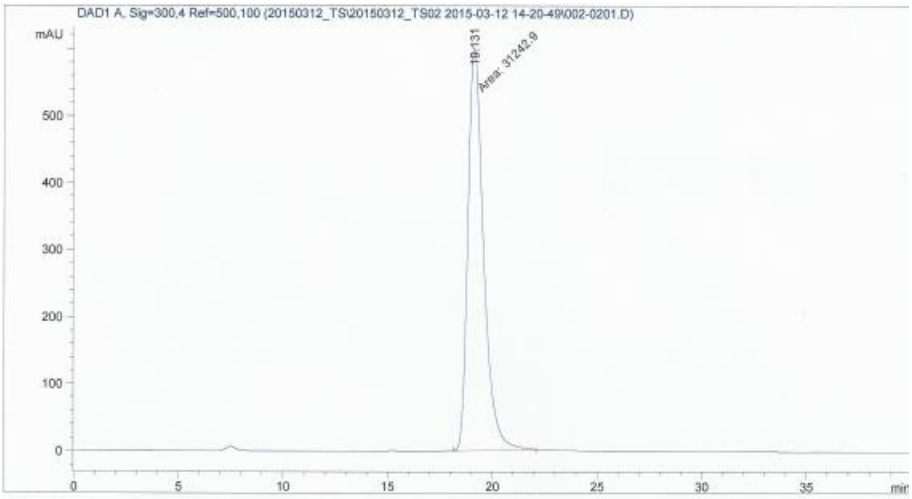
research. Approximately 1.2 litres on sample was collected for peak one and 1.4 litres of sample was collected for peak two. The red arrows point at the peaks of interest (Picture 30).



Picture 30. Collection of separated compounds into vials.

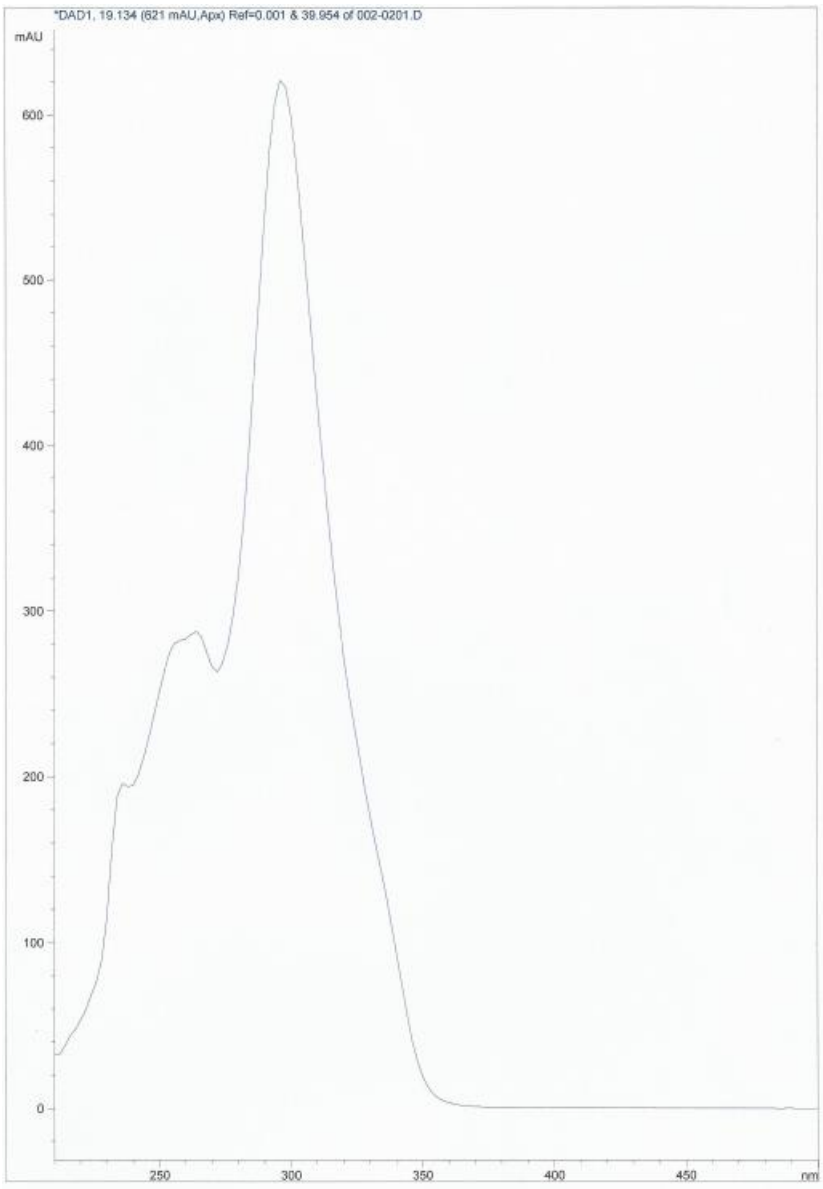
Optical purity

Optical purity analysis shows that peak one eluates neatly in retention time of 19 minutes (Picture 31). There are no indications of any other peaks or major impurities in the chromatogram.



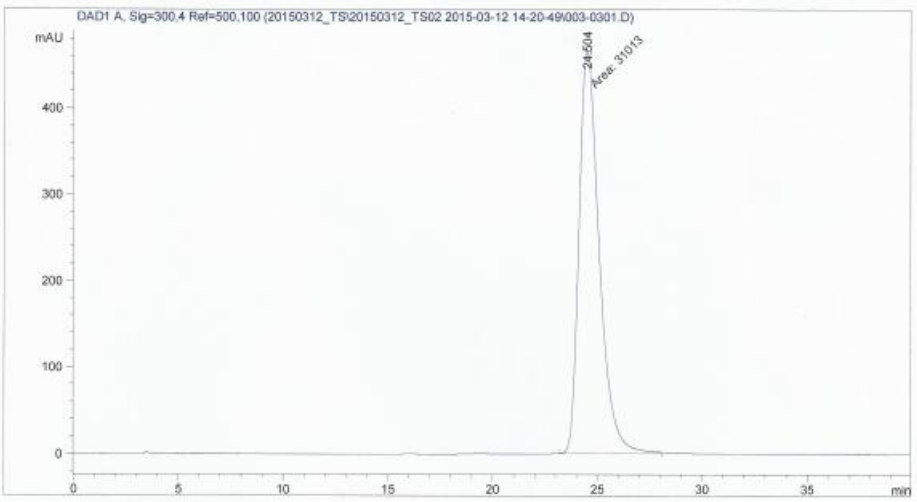
Picture 31. Chromatogram of peak one

The UV spectrum of the peak one shows the expected absorbancy and shape as from the preparative purification run (Picture 32).



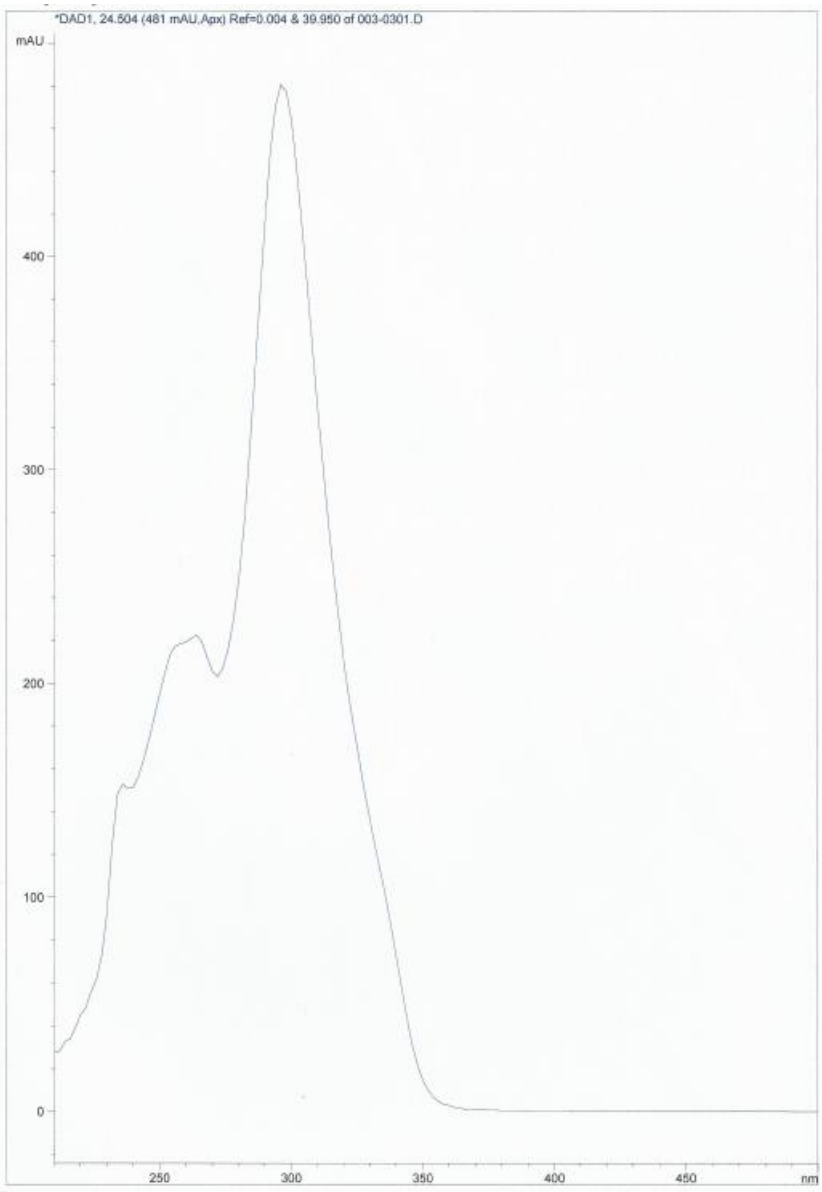
Picture 32. UV-spectrum of peak one

The chromatogram of peak two shows no indication of any remains from peak one or any other peaks (Picture 33). The chromatogram clearly shows a clean eluated peak at 24 minutes. No indication of remains from peak one is visible around the retention time of 20 minutes.



Picture 33. Chromatogram of peak two

The UV spectrum of the peak two shows the expected absorbancy and shape as from the preparative purification run (Picture 34).



Picture 34. UV-spectrum of peak two

The optical purity results indicate that the purity level for both peaks is 100% and has good resulation. Both of the enantiomers were collected and the samples were sent for further research which was not anymore a part of this particular experiment. The amounts of end products are mentioned here to give an overview of the final results. The amount of 44.9 mg of final product was obtained after evaporating 1.2 litres of sample collected as peak one. Also 41.4 mg of peak two was obtained from 1.4 litres of sample. Usually approximately two third of the material is obtained from a work similar to this one. The rest is lost thought evaporation and other procedures.

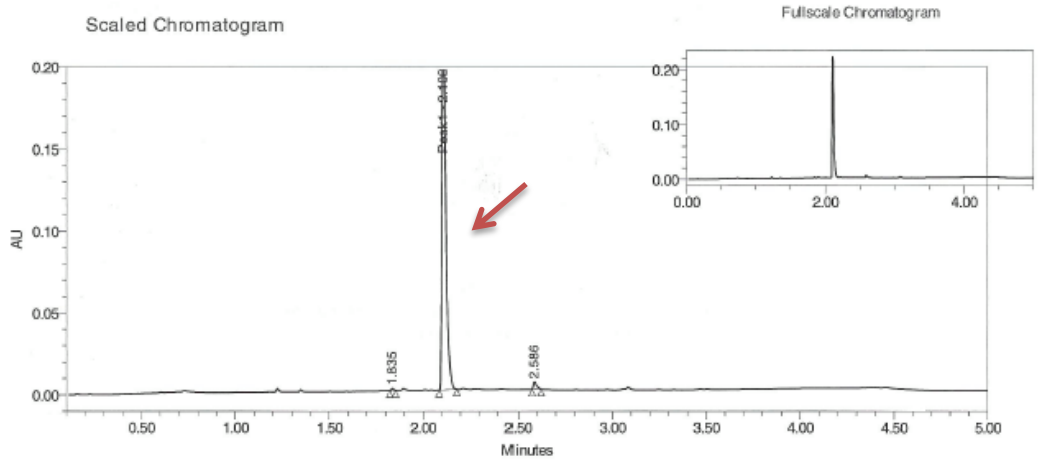
HPLC purity results

The result from the chromatographic purity shows that the separated peak one is 98% pure. This is because the chromatogram shows very few minor unknown impurity peaks, which can be ignored at this stage. One is able to disregard the other two peaks present in the chromatogram because the end result of the target peak covers 98 % of the chromatograms area (Picture 35).

The PDA spectrum plot presents the UV-spectrum of the compound showing one clear spectrum with the wavelength of 304 nm. The purity plot is used in the HPLC application to check whether there any impurities hidden beneath the target peak, which in this case is not the matter (Picture 36)

Peak two is 100 % pure and shows no other peak in the chromatogram except the target peak (picture 35). The spectrum plot and purity plot shows the same as in peak one (Picture 36).

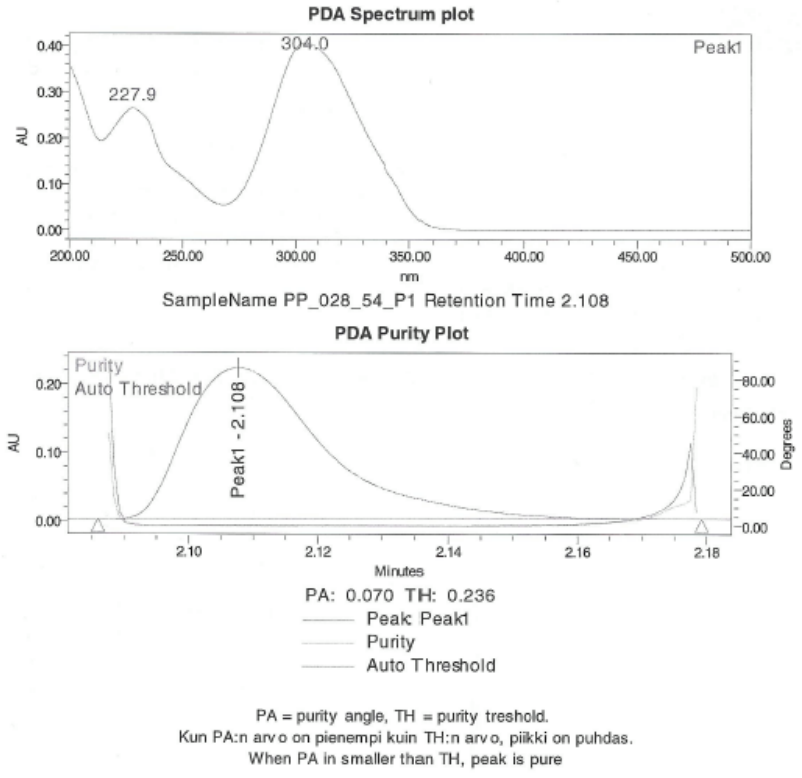
In Orion the in-house target is that the purity percentage of the analysed compound has to be around 90-95 % in order for it to be considered for further analysis. In this case the target was met.



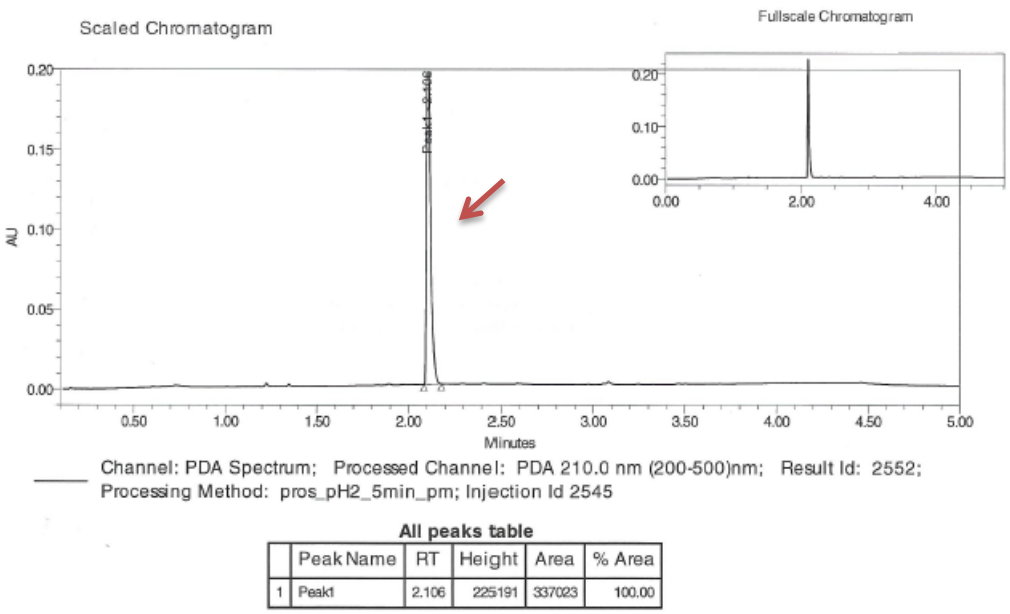
All peaks table

	Peak Name	RT	Height	Area	% Area
1		1.835	1294	111	0.34
2	Peak1	2.108	220214	326541	98.04
3		2.586	4537	5398	1.62

Picture 35. Purity of peak one



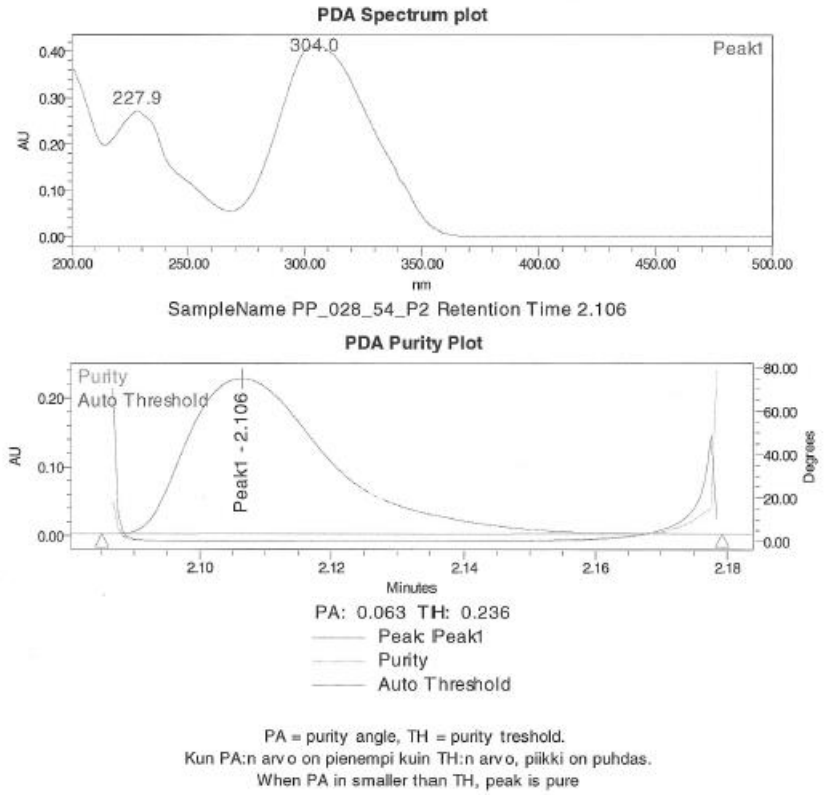
Picture 36. Purity plot of peak one



All peaks table

	Peak Name	RT	Height	Area	% Area
1	Peak1	2.106	225191	337023	100.00

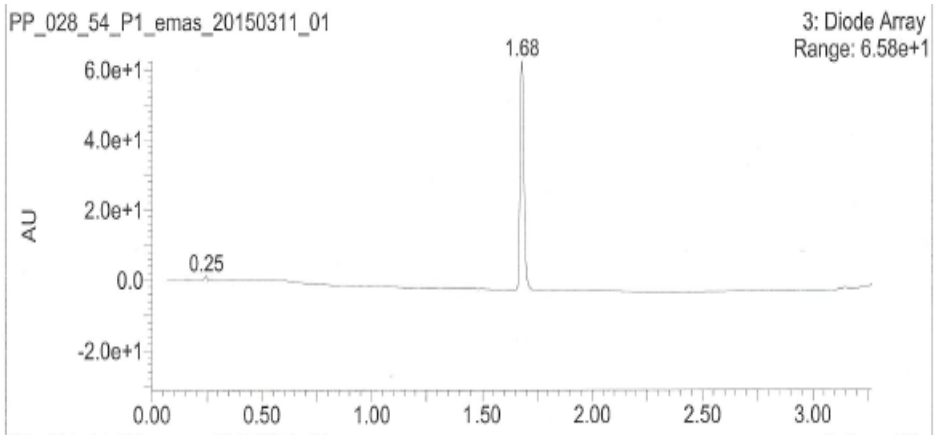
Picture 37. Purity of peak two



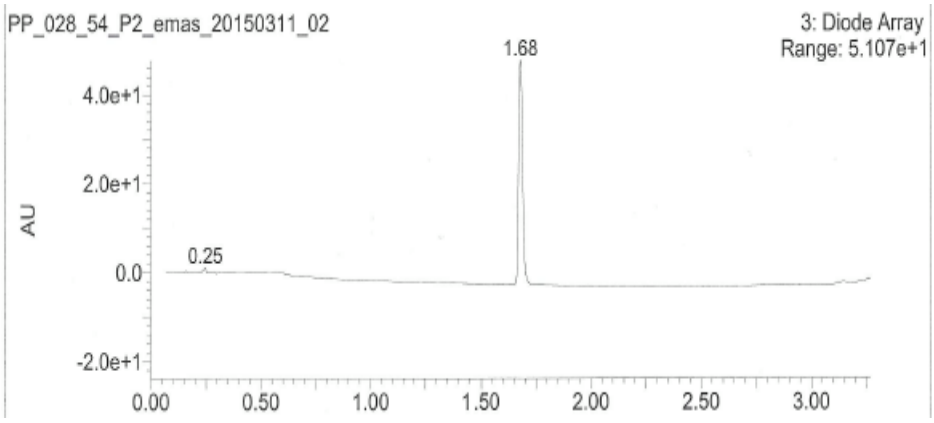
Picture 38. Peak purity plot of peak two

Mass spectrophotometry results

The mass spectrophotometry chromatogram for both separated enantiomers did not identify any other compounds confirming that the separation was successful (Pictures 39 and 40). Only one clear and visible peak is present in the diode array wavelength detector pictures for both of the peaks, and they also have the same retention time at approximately 1.68 minutes.



Picture 39. LC-MS chromatogram of peak one



Picture 40. LC-MS chromatogram of peak two

6 Summary and Conclusion

The aim of this research was to find a suitable HPLC purification method, which would separate an enantiomeric compound being tested for a certain drug development process. The research consisted of several parts, which were related to each other. While writing this thesis, it was a challenge to compile the entire process as one whole, so that it would be understandable to the readers. Part of the process was done according to Orion's in-house rules and regulations and might vary according to different companies.

All in all the end result was successful. The separation of enantiomers happened as expected according to the method development. Both of the peaks eluted in retention time of approximately 20 minutes for the first peak and 26 minutes for the second peak. The compounds were collected into vials for further research.

The efficiency of the method was tested by purifying the compound chromatography with a result over 98 % and 100 % purity for both peaks. The qualities of the optical purification for both peaks were 100% according to Orion's in-house standards.

It would have been more effective to start a design of experiment set up prior to the screening process. That could have given the method developing a more scientific framework. It might have also been more economical and less time consuming with fewer HPLC runs. Something to consider as a step towards green chemistry, as Orion's laboratory is aiming to reduce chemical waste.

Also it would have been interesting to expand the screening with more than just two columns. This way it might have been possible to determine whether separation of these enantiomers is tied to a specific type of column. Since there was a time limitation, and the focus was not on column determination, we limited our screening process by screening with two routine used columns in the laboratory.

The research to find a suitable method for separating a medicinal molecule was successful. The method is archived in Orion's medicinal chemistry laboratory and is used to this day for similar kind of molecules. The separated compound reached the pre-candidate phase.

References

[1] https://www.orion.fi/en/Orion-group/about-orion/ Read 1.2.2018

[2] https://www.fermion.fi/about-us/ Read 14.3.2018

[3] https://www.orion.fi/en/rd/orion-rd/ Read 19.2.2018

[4] https://www.orion.fi/en/rd/orion-rd/research-areas/ Read 13.3.2018

[5] https://www.orion.fi/en/rd/orion-rd/pipeline/ Read 13.03.2018

[6] Early drug development: strategies and routes to first – in –human trials, edited by

[7] Mitchell N. Cayen. Copyright 2010 John Wiley & Sons, Inc p. 3-5

[8] http://sic.fimea.fi/4_2014/laakekehitys_eilen_tanaan_ja_huomenna Read 6.3.2018

[9] Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Christopher A. Lipinski, Franco Lombardo, Beryl W. Dominy, Paul J. Feeney, Elsevier, 1996

[10] Early drug development: strategies and routes to first – in –human trials, edited by Mitchell N. Cayen. Copyright 2010 John Wiley & Sons, Inc page 8-10

[11]https://www.slideshare.net/karunkumar/drug-discovery-and-development-41609017 Read 1.4.2018

[12] Innovation and Entrepreneurship in Biotechnology, an International Perspective: Concepts, Theories and Cases, Damian Hine & John Kapeleris, Edward Elgar Publishing, Cheltenham, UK 2006, page 38, 180, 135, 191, 193

[13] http://sic.fimea.fi/4_2014/laakekehitys_eilen_tanaan_ja_huomenna Read 6.3.2018

[14] Innovation and Entrepreneurship in Biotechnology, an International Perspective: Concepts, Theories and Cases, Damian Hine & John Kapeleris, Edward Elgar Publishing, Cheltenham, UK 2006, page 38, 180, 135, 193

[15]https://www.quora.com/In-the-drug-development-process-what-is-a-lead-compound

[16] https://www.orion.fi/tutkimus/laakekehitys/ Read 12.6.2016

[17] Tamanna Sarapat, Drawing for thesis, 2018

[18] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3153011/ Read 3.3.2018

[19] Fundamentals of organic chemistry, 4TH edition, John Mcmurry, Brooks Cole, 1997

[20] Organic Chemistry Concepts and Applications for Medicinal Chemistry , Josef E. Rice, 2014 Elsevier, page 69.

[21]http://glencoe.mheducation.com/sites/007874637x/student_view0/chapter21/standardized_test_practice.html Read 8.4.2018

[22] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3614593/ Read 7.4.2018

[23]http://sic.fimea.fi/arkisto/2015/2_2015/laakehaittojen-hallinta/talidomidi_eilen_ja_tanaan Read 12.3.2018

[24]http://lawrencekok.blogspot.fi/2011/04/medicine-and-drugs_07.html Read 10.3.2018

[25]The essence of chromatography, Colin F. Poole, Elsevier, 2003

[26]Quantitative Chemical Analysis, Daniel C. Harris, Freeman, 1999

[27]http://www.waters.com/waters/en_FI/Chromatographic-Bands%2C-Peaks-and-Band-Spreading/nav.htm?cid=134803614&locale=en_FI Read 20.3.2018

[28]http://chemwiki.ucdavis.edu/Analytical_Chemistry/Instrumental_Analysis/Chromatography/Chromatographic_Columns) Read 14.01.2014

[29]http://www.waters.com/waters/en_US/How-Does-High-Performance-Liquid-Chromatography-Work%3F/nav.htm?cid=10049055

[30]http://www.waters.com/waters/en_FI/Chromatographic-Bands%2C-Peaks-and-Band-Spreading/nav.htm?cid=134803614&locale=en_FI Read 22.3.2018

[31]http://www.biologydiscussion.com/biochemistry/chromatography-techniques/3-main-types-of-chromatography-techniques-with-diagram/16583 Read 8.3.2018

[32]https://www.crawfordscientific.com/technical/gc-column-selection-guides/agilent-j-w-selection-principles Read 8.3.2018

[333]http://www.waters.com/waters/en_FI/HPLC-Column-Hardware-/nav.htm?cid=10049068&locale=en_FI Read 31.3.2018

[34]https://www.crawfordscientific.com/technical/gc-column-selection-guides/agilent-j-w-selection-principles Read 8.3.2018

[35]http://column-chromatography.com/the-benefits-of-silica-gel-chromatography/ Read 9.4.2018

[36] http://slideplayer.com/slide/6823479/ Read 8.3.2018

[37]https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/preparative-separation Read 6.3.2018

[38]http://www.cmtlaboratory.com/learn/potency/ Read 27.3.2018

[39]https://www.chromacademy.com/Preparative_HPLC_Essential_Guide.html Read 28.3.1

[40] Bioanalytics: Analytical Methods and Concepts in Biochemistry and Molecular biology. Edited by Friedrich Lottspeich, Joachim Engels, Wiley-VCH 2018 page 23

[41] https://core.ac.uk/download/pdf/39979900.pdf Read 1.4.2018

[42] Essentials in modern HPLC separation, Cerban c. Moldoveanu, Elsevier 2013 page 2, 14

[43] http://www.wikiwand.com/fi/Korkean_erotuskyvyn_nestekromatografia Read 21.3.2018

[44]http://www.waters.com/waters/en_FI/The-Promise-of-Small-Particles/nav.htm?cid=134804750&locale=en_FI Read 21.3.2018

[45] https://www.agilent.com/cs/library/support/documents/a05296.pdf page 5

[46]http://www03.edu.fi/oppimateriaalit/laboratorio/analyysimenetelmat_5-5_massaspektrometria.html Read 6.3.2018 (Edited)

[47]https://www.theseus.fi/bitstream/handle/10024/86536/Opinnaytetyo%2019%2012%202014.pdf?sequence=1 page 5 Read 10.3.2018

[48]https://en.wikipedia.org/wiki/Liquid_chromatography%E2%80%93mass_spectrometry Read 22.3.2018

[49]https://www.agilent.com/cs/library/support/documents/a05296.pdf page 3 Read 9.3.2018

Appendix 1. HPLC chromatogram of ethanol base run

Empower2

Liuotinajo_5min_pH2

SAMPLE INFORMATION

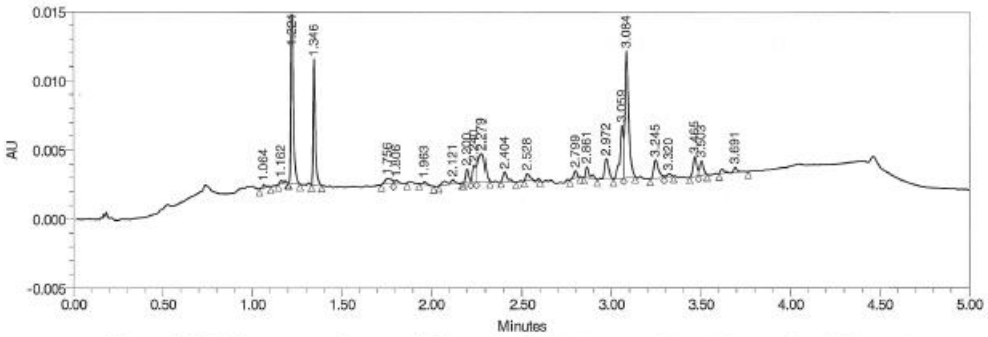
Sample Name:	EtOH	System Name:	Ruffe
Sample Type:	Unknown	Sample Set	20150312_pH2_Col1_TN02
Vial:	1:F,1	Acq Method Set	A1B1_Col1_B05_85_5min_ms
Injection #:	1	Processing Metho	pros_pH2_5min_pm
Injection Volume:	0.20 ul	Channel Name:	210 nm
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	PDA 210.0 nm (200-500)nm
Date Acquired:	3/12/2015 11:37:55 AM EET		
Date Processed:	3/12/2015 12:05:50 PM EET		

Buffer: 15mM KH2PO4 pH2,2

Column Name Aqcuity BEH C18 1,7um 2,1x50mm

Sample_info 2 mg/ml EtOH

Scaled Chromatogram

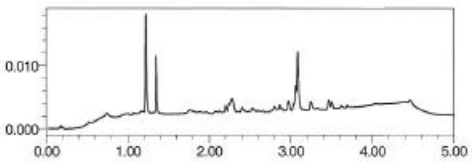


Channel: PDA Spectrum; Processed Channel: PDA 210.0 nm (200-500)nm; Result Id: 2547;
Processing Method: pros_pH2_5min_pm; Injection Id 2539

All peaks table

	RT	Height	Area	% Area
1	1.064	256	314	0.49
2	1.162	243	374	0.58
3	1.221	15608	13143	20.49
4	1.346	9102	7424	11.57
5	1.756	417	1075	1.68
6	1.806	242	413	0.64
7	1.963	218	280	0.44
8	2.121	294	1084	1.69

Fullscale Chromatogram



Reported by User: MC-analytics Espoo (MC_Espoo) ne: MC-Espoo-Ruffe_2015\MC-Espoo_Maaliskuu_2015
Report Method: Liuotinajo_5min_pH2 Date Printed:
Report Method ID 1336 3/12/2015
Page: 1 of 2 12:06:21 PM Europe/Helsinki

All peaks table

	RT	Height	Area	% Area
9	2.200	1004	1186	1.85
10	2.240	1238	1628	2.54
11	2.279	2007	5136	8.01
12	2.404	651	1089	1.70
13	2.528	485	897	1.40
14	2.799	499	633	0.99
15	2.861	786	1187	1.85
16	2.972	1492	2406	3.75
17	3.059	3805	5255	8.19
18	3.084	9152	13191	20.57
19	3.245	1331	2441	3.81
20	3.320	220	354	0.55
21	3.465	1420	2115	3.30
22	3.503	1039	1473	2.30
23	3.691	376	1039	1.62

Match Plot Group group contains no data.

PA = purity angle, TH = purity treshold.
Kun PA:n arvo on pienempi kuin TH:n arvo, piikki on puhdas.
When PA in smaller than TH, peak is pure

Reported by User: MC-analytics Espoo (MC_Espoo) ne: MC-Espoo-Ruffe_2015\MC-Espoo_Maaliskuu_2015
Report Method: Liuotinajo_5min_pH2 Date Printed:
Report Method II 1336 3/12/2015
Page: 2 of 2 12:06:21 PM Europe/Helsinki