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Faculty of Biological Sciences

Institute of Pharmacy

Pharmaceutical/Medical Chemistry

HPLC method for separation of chiral impurities of dolutegravir

DIPLOMA THESIS

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Supervisors: Assoc. Prof. Petra Štěrbová, Ph.D., Prof. Dr. Gerhard Scriba

Jena, Germany 2019

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Place, date: Hradec Králové, 30/08/2019

…………………………………

Júlia Lengvarská

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ABSTRACT

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Title of Diploma thesis**:**

HPLC method for separation of chiral impurities of dolutegravir

Following ICH Guidelines Q3A(R2) and Q3B(R2), every new drug substance and every new drug product must be checked for pharmaceutical quality based on Good Manufacturing Practice (GMP).

In this Diploma thesis three batches of a new drug substance and one batch of a new drug product (tablets) are tested by validated analytical method using chiral stationary phase Lux Cellulose-4. This HPLC-UV method is able to separate a main substance which is an antiviral drug - dolutegravir and its stereoisomeric impurities as well as some other related substances.

The main aim is to establish this method as a future monograph method in The International Pharmacopoeia for impurities testing of dolutegravir Sodium.

Impurities in pharmaceutical products have potential carcinogenic, mutagenic, or teratogenic effects. Herein even low chiral contamination of enantiomer and also diastereomer developed through the synthesis of the substance can be detected and separated. Therefore, due to this analytical procedure is possible to insure chiral purity, safety and quality of drug dolutegravir.

ABSTRAKT

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Názov diplomovej práce:

HPLC separačná metóda chirálnych nečistôt dolutegravíru

Dodržiavaním ICH predpisov Q3A(R2) a Q3B(R2), každé nové liečivo či každá nový liečivý prípravok musí byť otestovaný na kvalitu, čo je súčasťou Správnej výrobnej praxe (SVP).

V tejto Diplomovej práci boli testované tri šarže nového liečiva a jedna šarža nového liečivého prípravku, a to validovanou analytickou metódou pomocou chirálnej stacionárnej fázy Lux Cellulose-4. Táto HPLC-UV metóda je schopná oddeliť hlavnú látku, teda antivirotikum – dolutegravir, ďalej jeho stereoizomerické nečistoty, ale aj iné príbuzné látky.

Hlavným cieľom je zaviesť túto metódu pre budúce testovanie nečistôt v monografe Dolutegravir Sodium v Medzinárodnom liekopise.

Nečistoty vo farmaceutických produktoch majú potenciálny karcinogénny, mutagénny či teratogénny efekt. Aj malá kontaminácia enantiomérom a tiež diastereomérom získaná syntézou látky dokáže byť detekovaná v tejto DP. Takže vďaka tejto analytickej metódy je možné zabezpečiť chirálnu čistotu, bezpečnosť a kvalitu lieku dolutegravir.

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1 INTRODUCTION

Dolutegravir is one of the latest antiviral drugs with an activity of integrase inhibitor. Even though it is widely used and sold, it still does not have any pharmacopoeia monograph. (1) (2)

A monograph in pharmacopoeia usually consists of many parts as basic chemical information, substance description and specific instructions for identification, tests for impurities, assays and others. To prepare the monograph three following parties are involved like experts from academia, from industry and it all has its regulatory bodies. (3)

First effort for monograph has come from World Health Organization to provide quality control of dolutegravir worldwide. Therefore, on demand of World Health Organization, which is a regulatory body for International Pharmacopoeia, Institute of Pharmacy at Friedrich Schiller University in Jena participated in monograph development as academia and pharmaceutical companies Aurobindo and Cipla provided samples for testing.

During my Erasmus stay at Friedrich Schiller University in Jena I had a chance to be a part of this process, especially to work on HPLC method development for separation of chiral impurities of dolutegravir which became the main aim of this Diploma Thesis.

In earlier studies, there was one chiral method performed by HPLC and carried on column Lux® Cellulose-4 (250 x 4.6 mm, 5 μm) from company Phenomenex®. The first aim of my work was to adjust these chromatographic conditions for similar column but with different length and particles size, Lux® Cellulose-4 (150 x 4.6 mm, 3 μm). After finding the appropriate flow rate and composition of mobile phase, the final method was set. Next step was to prove desired properties of the method by required tasks from regulatory body as system suitability and recovery test. Once it was all done, a few batches of dolutegravir API (active pharmaceutical ingredient), racemic mixture and tablets could be analyzed and the HPLC method could be written done in a form of pharmacopoeia monograph for achieving future accomplishment of drug quality, efficacy and safe.

2 THEORETICAL PART

2.1 STEREOCHEMISTRY

2.1.1 Chirality

One of the simplest most concerning asymmetrical molecules in chemistry would be an atom of tetrahedral carbon with four different attached groups to it (Figure 1). This molecule has a dissymmetric center called a center of chirality or a stereocenter - a chiral tetravalent carbon atom which can be denoted by an asterisk. Molecules like this one with one stereocenter are always chiral. (4) (5)

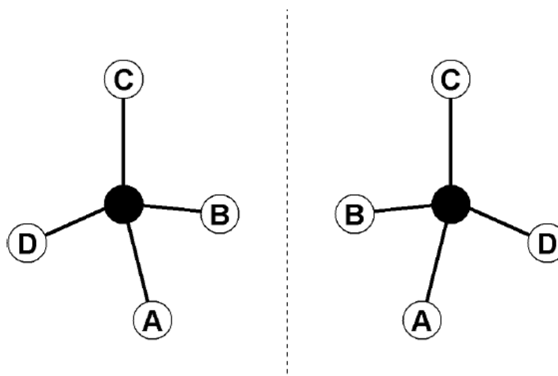


Figure 1: Asymmetrical mirror images of tetrahedral carbon atom. (4)

Not only carbon with tetrahedral hybridization can be a chiral atom, some other elements which can act as an asymmetric center are phosphorus, sulfur and nitrogen.

Thus, the mirror image of the molecule is not superimposable due to the presence of four nonidentical ligands. This molecule becomes **chiral** (cheir, Greek, hand/handedness). Each isomer of this nonidentical, nonsuperimposable pair of mirror images is called **an enantiomer** (enantios, Greek, opposite).

When at least two of the ligands are identical, the molecule becomes **achiral** and its mirror image is superimposable. See Figure 2. (4) (5)

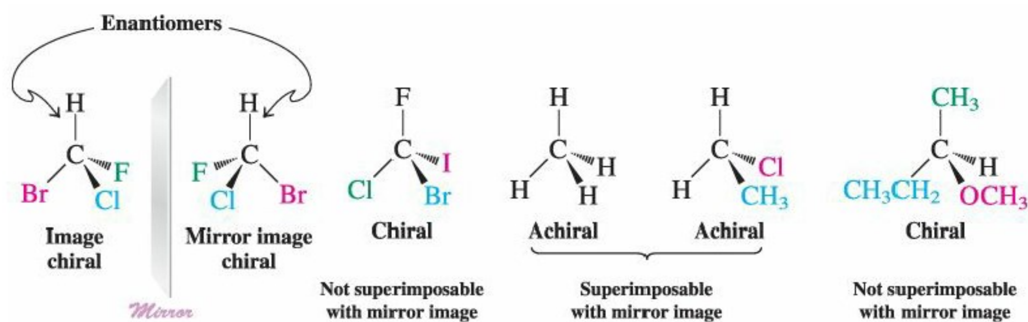


Figure 2: Examples of chiral and achiral molecules (5)

Many chiral molecules or objects do not have centers of chirality. To determine if a molecule is chiral or not, is not always easy. The nonsuperimposable character of mirror images is the only criterion for chirality though. A simple method is to look for the presence or absence of a plane of symmetry in the molecule. Chiral molecules cannot present a plane of symmetry. In nature, not constantly both enantiomers exist, many chiral organic compounds exhibit as a single enantiomer. (5)

2.1.2 Arrangements of molecules

Here a few definitions of molecule arrangements, which will be frequently used in the text, are listed:

- constitution refers to the order of connection of atoms and bonds building up the molecule (connectedness) but disregarding any spatial arrangement,
- conformation is related to a steric 3D arrangement of the atoms of a molecule which is energetically permissible under the given conditions (generally as normal conditions - room temperature and atmospheric pressure). This arrangement produces stereoisomers that distinct from each other formally by rotation(s) around single bonds,
- configuration is an arrangement of a well-defined and under the given conditions. Its parts of the molecule can only be characterized by spatial features (stereodescriptors). (6)

2.1.3 Isomerism

Isomers are molecular entities which share identical molecular formula (the same number of atoms of the same kind – the same atomic composition) but differ each other in structures. Not only line and stereochemical formulas but also their chemical and physical properties can be dissimilar. (6)

The word itself stands for "equal parts" from the Greek words "isos" and "meros" which underline its meaning in having the same parts but being not the same indeed. (7)

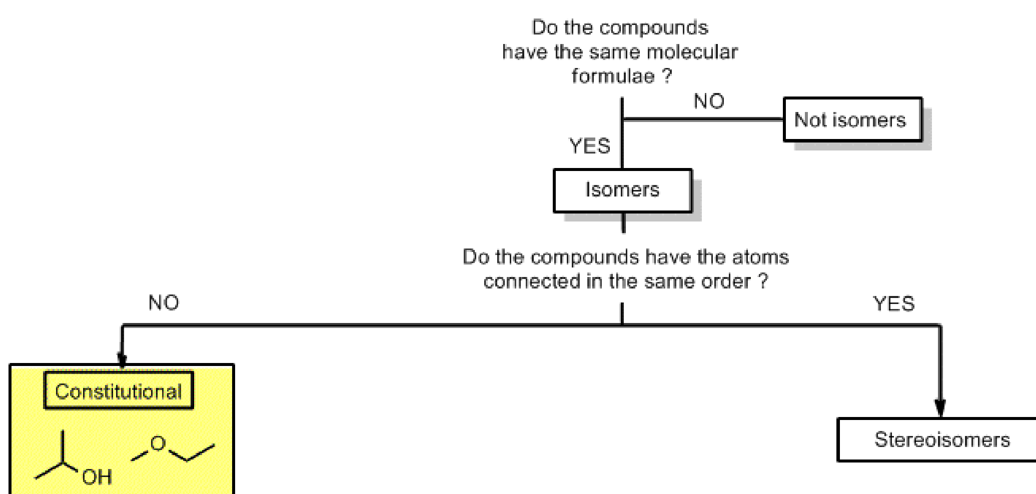


Figure 3: Isomers, either constitutional, or stereoisomers (8)

There are two main forms of isomerism (Figure 3):

1) Constitutional (structural) isomers are isomers which have different constitutions and present dissimilar linear formulas (e. g: CH_3OCH_3 and CH_3CH_2OH as in Figure 4). Their atoms are of different connectivity or bond order of isomers. (6)

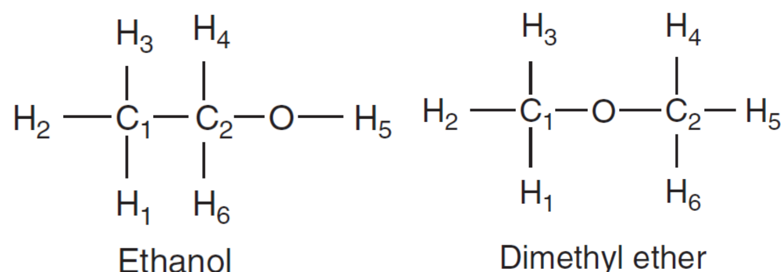


Figure 4: Structural chemical formula of two constitutional isomers. (6)

Constitutional isomers (Figure 5) are therefore different compounds with different chemical structures. (9)

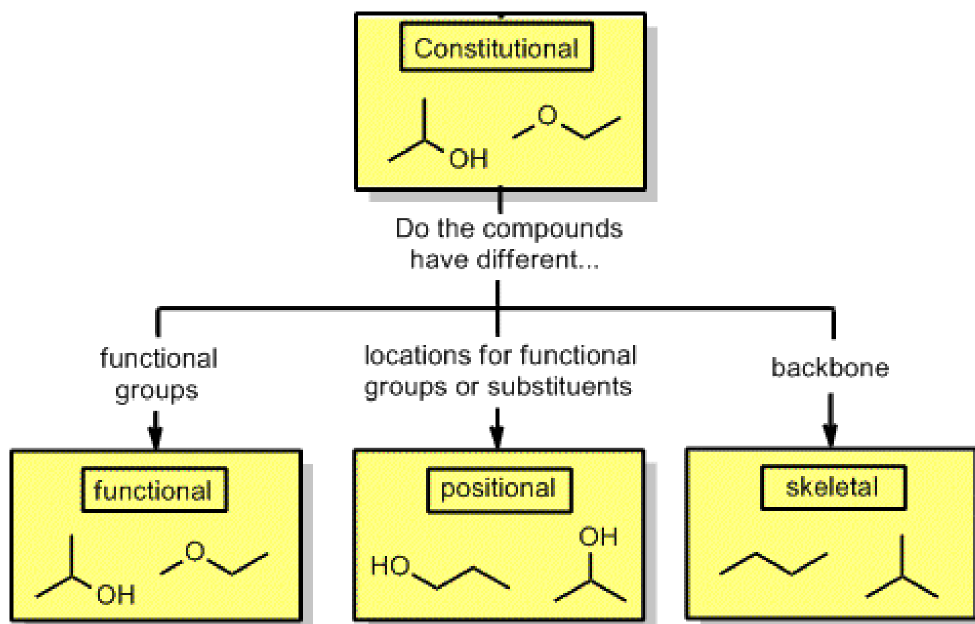


Figure 5: Constitutional isomers (8)

2) Stereoisomers are isomers which do have the same constitution. Their atoms are of the different spatial arrangement, but without the distinct in connectivity or bond order of isomers. Figure 6 shows the difference between two types of stereoisomers. (4) (6)

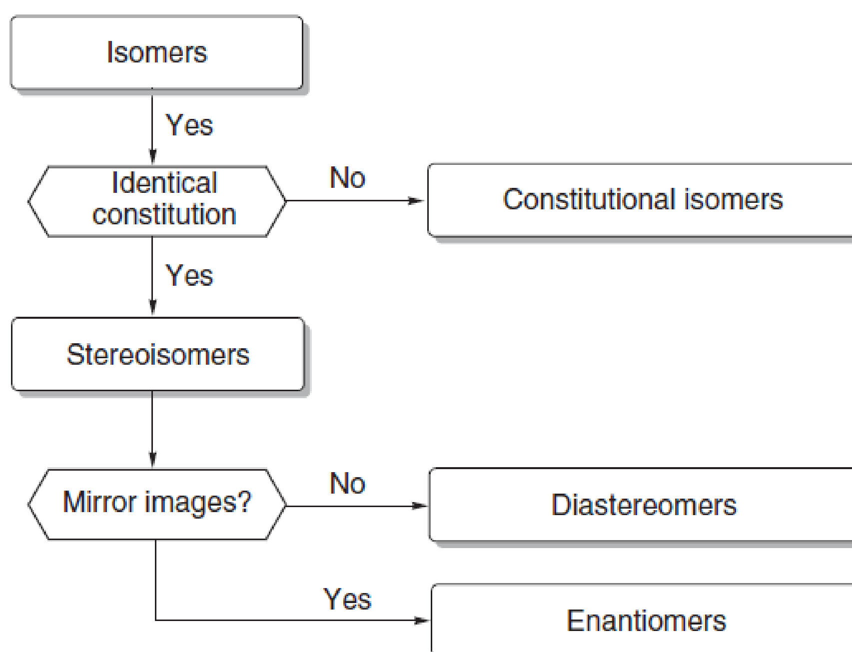


Figure 6: Isomers' and stereoisomers' forms(6)

According to the mirror images there exist two basic kinds of stereoisomers (Figure 7):

a) Diastereomers, molecular entities which are not related as mirror images. They are different in all their physical properties and in their chemical behavior (melting and boiling points, refractive indices, solubilities, chemical reactivities, optical rotations). Diastereomers can be either chiral or achiral. (4) (6)

b) Enantiomers are those stereoisomers which are nonsuperimposable and able to form a pair of mirror images. They must be chiral. Their physical properties are identical but they distinct in vectorial physical properties, such as optical rotation. Their chemical behavior may differ in chiral environment too. (6)

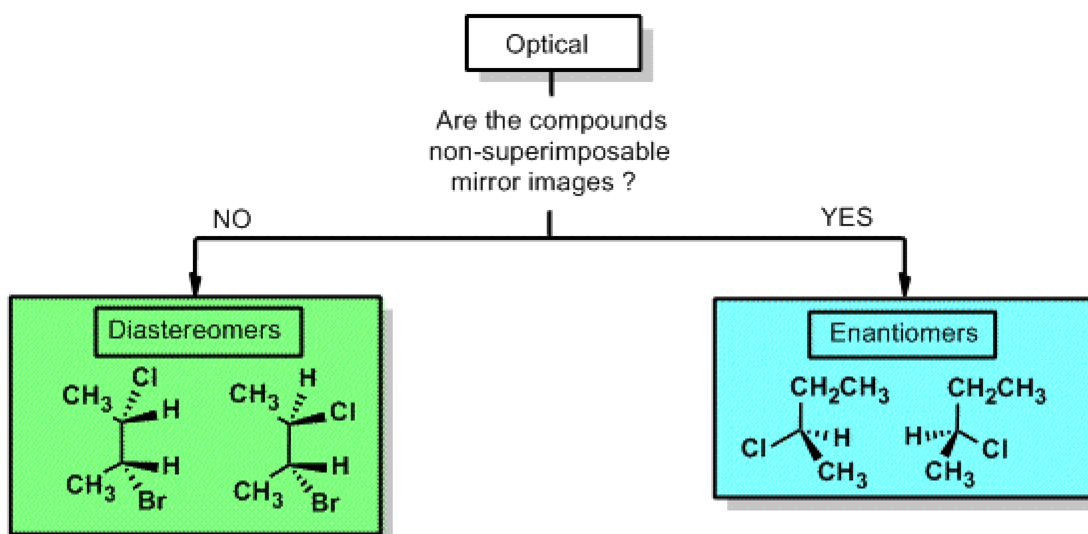


Figure 7: Optical stereoisomers(8)

Previous figures showed many different types of isomers nicely. Not all of them will be further explained but for the concept of isomerism was good to mention all of it and so to be able to see the distinct between them.

2.1.4 Optical activity

A pair of enantiomers share most of their basic physical properties (such a density, boiling point, melting point, refractive index, etc.) that is caused by their identical bonds and so their energy extents. They distinguish in the spatial orientation of their atoms and as chiral molecules are able to rotate plane-polarized light. It was remarked in 1815 for the first time by Biot. Back then he noticed the difference in rotation of plane-polarized light passing through the forms of tartaric acid. Molecules that rotates the plane-polarized light are called to be optically active. (4) (5)

One of the enantiomers can rotate the plane of polarization of incoming light in one direction (either clockwise, or counterclockwise). While another of the enantiomers rotates the plane of polarization by exact amount but in the opposite direction which is due to their related character as mirror images. As it is in Figure 8.

An instrument called a polarimeter is used for measuring these optical rotations of enantiomers. (4) (5)

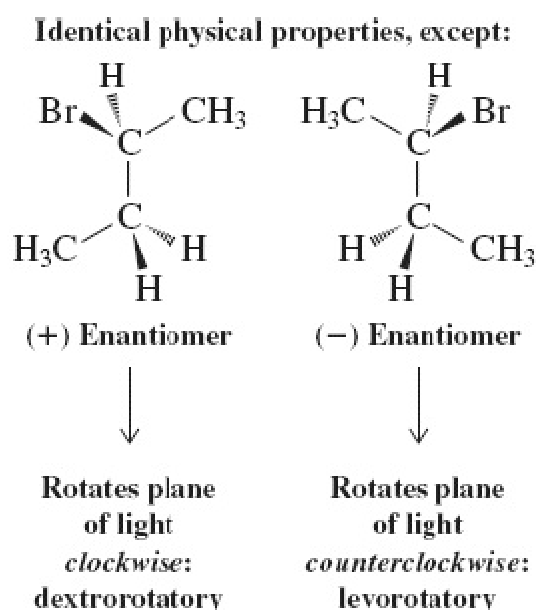


Figure 8: Optical activity of a pair of the enantiomers (5)

Dextrorotatory (D, dexter, Latin, right) enantiomer rotates the plane of light in a clockwise direction and is noted as the (+) enantiomer. Consequently, the other - levorotatory (L, laevus, Latin, left) enantiomer rotates the plane-polarized light in a counterclockwise sense, and is referred to the (−) enantiomer. (4) (5)

2.1.5 R, S rule

Three chemists R. S. Cahn, C. Ingold, and V. Prelog came up with an idea to develop clear system for naming the handedness in the molecule, called also the C.I.P. convention. (4) (5)

1) At the beginning all four substituents attached to the chiral center must be labeled in order from the largest atomic number to the smallest. In Figure 9, as an example of ranking, letters a, b, c and d in sequence of decreasing priority are used. (4) (5)

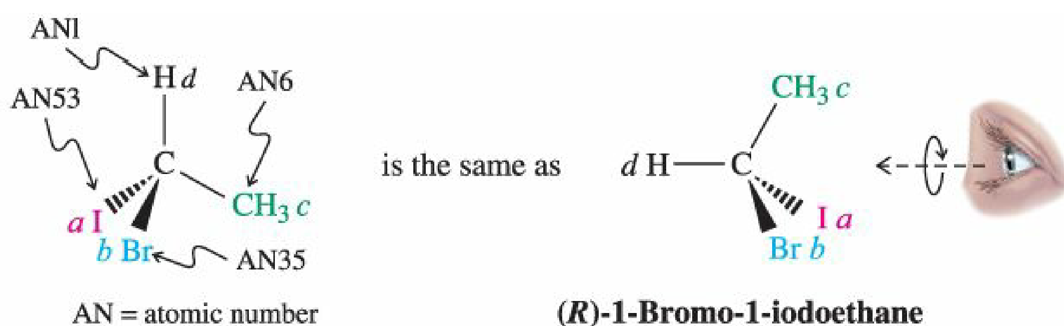


Figure 9: Ranking of substituents(5)

2) Next step is to orient the molecule the way that the lowest priority substituent is directed in the back, away from the viewer´s eye. Like in Figure 10. (4) (5)

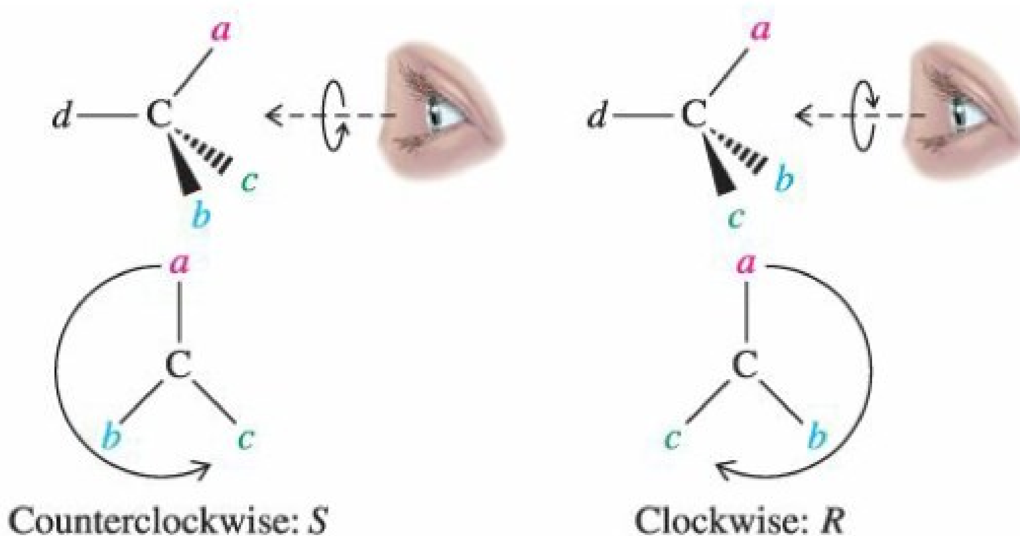


Figure 10: R, S rule in practice(5)

3) Now, there are two possibilities of arrangements. Either the move in order a – b – c becomes counterclockwise orientated which is called as **S** (sinister, Latin, left) configuration at the center of chirality, or the move designs clockwise rotation that is named as **R** (rectus, Latin, right). (4) (5)

These symbols R and S are often written in brackets in front of the name of chiral molecules, such enantiomers. Also, the mark of the rotation of the plane of polarized light might be appended ahead of the compound name next to the R/S sign of stereocenter configuration. A brief example: (S)-(+) -2-bromobutane and (R)-(−) -2-bromobutane. It is important to keep in mind that the sign of α (+/−) does not always correlate with R, S rule. (4) (5)

2.1.6 Racemic mixture

As mentioned earlier, a single enantiomer rotates a beam of the plane-polarized light in given direction, another of the pair does in opposite direction. The optical activity hits its maximum when there is only one enantiomer present in the sample and so it is optically pure.

When a sample within a 1:1 mixture of (+) and (−) enantiomers is measured by polarimeter no rotation is shown and thus the mixture is optically inactive, it is at zero. It can happen only if both enantiomers are present in the mixture in equal quantity. This mixture is named as **racemic mixture**, **racemate** and it is developed from the process of racemization. There is a certain prefix used in the chemical name to differ the racemic mixture from a single enantiomer. It is either (±)-, (D, L)-, (R, S)-, or rac-. Not only the optical activity of this kind of mixture is changed. In comparison to a single enantiomer also some other physicochemical properties might distinct significantly. (4) (5) (6)

Firstly, a pair of enantiomers had similar physiological activity, or there could be one of them which was inactive or had less potent for a certain action. It is called **distomer**, now. And on the contrary, the enantiomer with higher potent in particular

action (being able to cure, being biologically active) is named **eutomer**. Due to this fact of their common activities, the enantioselective development of drugs was unnecessary.

Secondly, racemic resolution was, and it still is expensive and has extra costs of drug development.

Later, more than a few cases showed that the activity of each enantiomer does not have be so similar as it was thought. New properties of racemate in a meaning of either opposite, or harmful activity were found. For example, one of the enantiomers can behave as a blocker of the receptor and so diminishes the positive effect of the other enantiomer or expected drug at all. In worse case, a chiral molecule can offer a toxic enantiomer to human body. (4) (5) (6)

Some examples of different character of enantiomers are displayed below.

- The plant asparagus and its juice were used for the first amino acid isolation. The isolated amino acid, asparagine, occurs in two enantiomer forms. Enantiomer with S-configuration at chiral center of molecule, L-asparagine, has bitter flavor, whereas its *R*-enantiomer tastes sweet (Figure 11). (4) (5) (6)

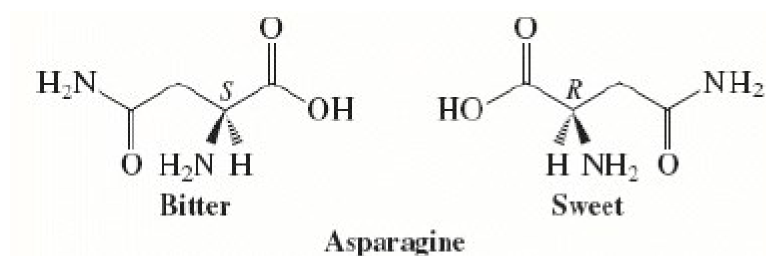


Figure 11: Asparagine´s forms (5)

- Albuterol, also called as salbutamol, and its mirror image are significantly different. The R-enantiomer widens bronchial airways, while the other (S-enantiomer) does not provide this activity, it blocks the positive effect and moreover, it is being considered as inflammatory agent (Figure 12). (4) (5) (6)

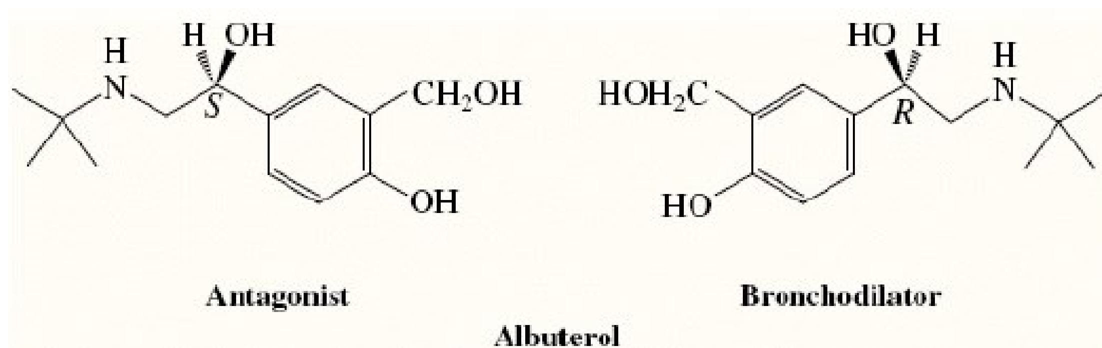


Figure 12: Albuterol´s forms (5)

Regarding the previous discoveries, the U.S. Food and Drug Administration (FDA) reconditioned its guidelines for chiral drugs and determined to produce pure distomer of medicinal products. It resulted in higher biological efficiency of medical products, it enriched knowledge of racemic resolution, and multiplied research of new enantioselective synthesis (especially enantioselective catalysis of 3 chemists was awarded with the Nobel prize in 2001). The importance of paying attention on the enantioselectivity becomes obvious by following sentence. Nowadays, more than 80% of drugs of small molecule which have FDA approvals are chiral. (4) (5) (6)

2.2 CHROMATOGRAPHY

Chromatography stands for a group of separation methods where attention is given to migration time while the sample is carried by a mobile phase and passed through a stationary phase. The mobile phase could be either a gas, a liquid, or a supercritical fluid. As the stationary phase, a solid, a liquid, or even a gel can be used. (9)

Nowadays, it is very important and the most frequently used technique in pharmaceutical analysis. (9)

2.2.1 Basics

Chromatography composes of two phases. One phase moves through the system, called the mobile phase (MP). The other one is immobile, so called the stationary phase (SP) which in case of solid particles remains in a tube – a column. Thank to pumps the mobile phase is pumped through the column at particular speed. (9)

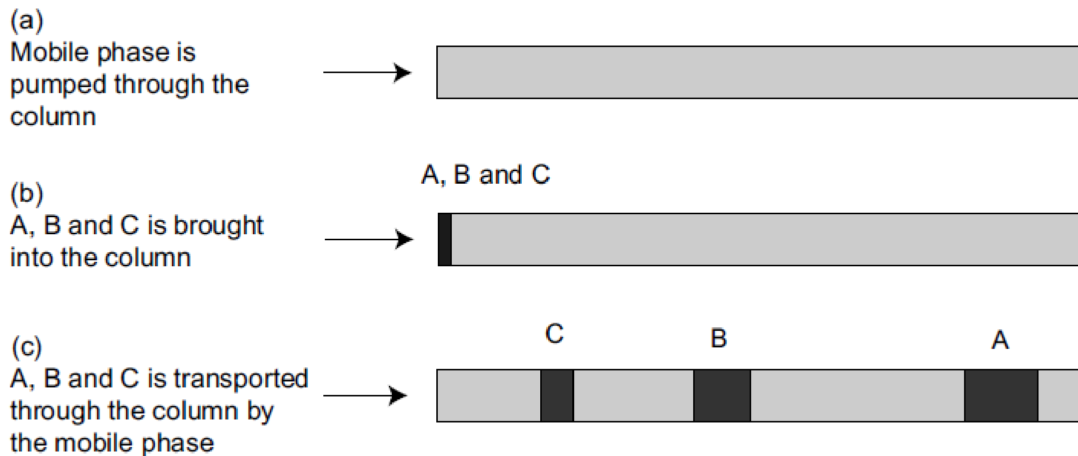


Figure 13: The separation of compounds A, B, C (9)

The mixture of components A, B, C is injected on the mobile phase and brought into the column (Figure 13). There the sample interacts with the stationary phase. Due to the structures of components, some of them will retard in the column and will migrate slower than the actual speed of the mobile phase is. Then the components A, B and C will be transported with different speeds through the column. The component A moves with the mobile phase while B and C interact more with the stationary phase and thus migrate at lower speed. (9)

The sample is injected to the column by an injector. The injector is situated in front of the column. A detector is an equipment that detects the compound – after the elution from the column.

Chromatogram is a visual outcome of separation. It is an outcome of Gaussian peaks on a baseline. (9)

2.2.2 The most frequently used chromatographic techniques

Chromatography can be classified according to the character of MP. Using gas as MP it becomes gas chromatography and similarly using liquid as MP then it is liquid chromatography. Another classification is according to the nature of stationary phase. In gas chromatography, it could be either liquid or solid. In liquid chromatography, there is thin layer chromatography (TLC) which has SP attached on a plate and high-performance liquid chromatography (HPLC) with SP packed in a column. (9)

To briefly compare gas and liquid chromatography:

Gas chromatography (GC)

This method is the choice of testing volatile samples. From tens to thousands of assays can be carried by carrier gas helium, hydrogen, nitrogen, argon or other gases. Compounds are vaporized at about 400 degrees Fahrenheit before separation usually on a long capillary column of 10 – 45 meters length. It has limited applicability and variability though. Drawbacks are also higher possibility of racemization in experimental temperature, flammability of carrier gas or the need of very high temperature stability. (9) (10) The system is shown in Figure 14.

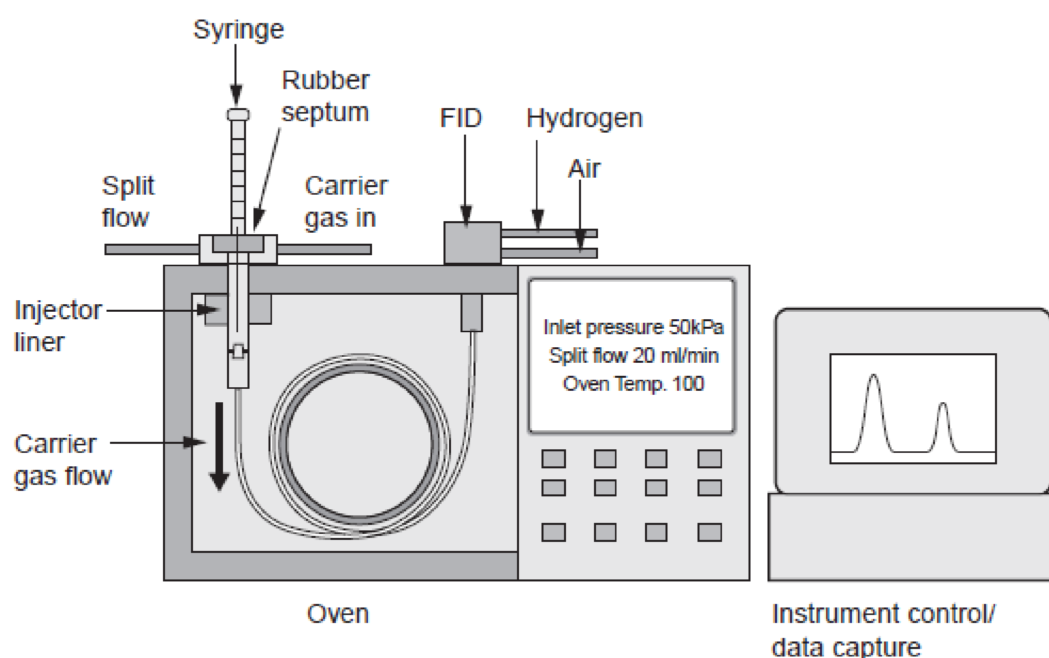


Figure 14: Composition of GS system (11)

High-performance liquid chromatography (HPLC)

HPLC is nowadays quick, automated method and provides high accuracy, efficiency and repeatability. Basic schema of HPLC system is in Figure 15:

It is capable of analyzing samples containing both compounds of small and large molecules. The analyte can also be unstable at higher temperature (due to lower temperature operation) in comparison with GC. It is widely used in medical, forensic, environmental and manufacturing laboratories and is extremely precise when it comes to identification and quantification of samples. The composition of solvents can play a role in large-scale variability. It is more expensive tough. (4) (5) (10) (12)

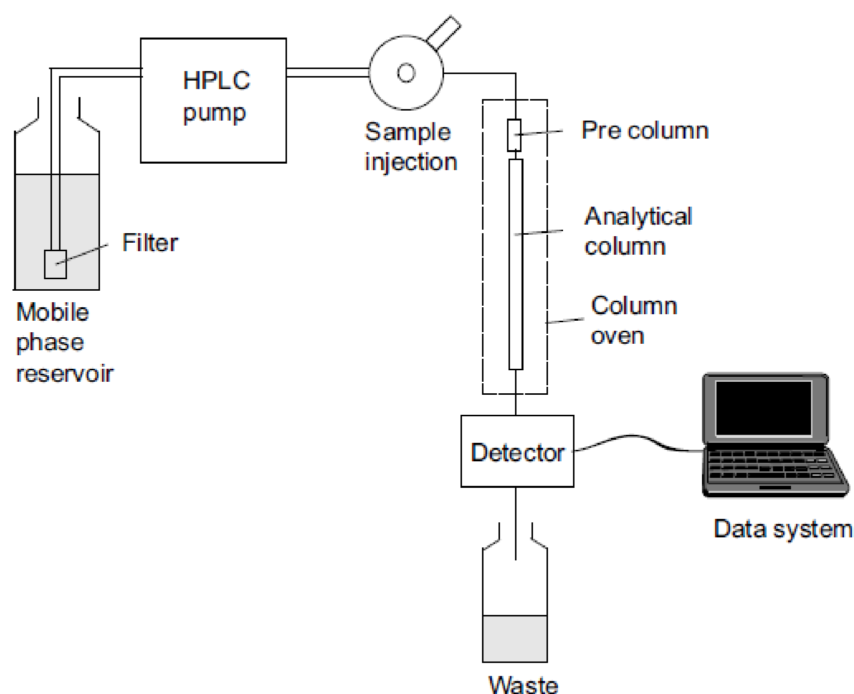


Figure 15: Schema of HPLC system (9)

2.2.3 Selected chromatographic parameters

2.2.3.1 Retention

The compound retention can be characterized by following parameters.

Retention time (t_R) is the time in which the compound travels through the system, from the injection to maximum response of the compound in a **related volume (V_R)** of mobile phase (the second peak at Figure 16).

Hold-up time (t_M) is the retention time of an unretained compound (the first peak at Figure 16) and it is the time which is needed for the mobile phase to elute from the column, so the compound migrates with the same speed as the mobile phase.

Hold-up volume (V_M) is therefore the volume of MP which was released at hold-up time from the column. (9)

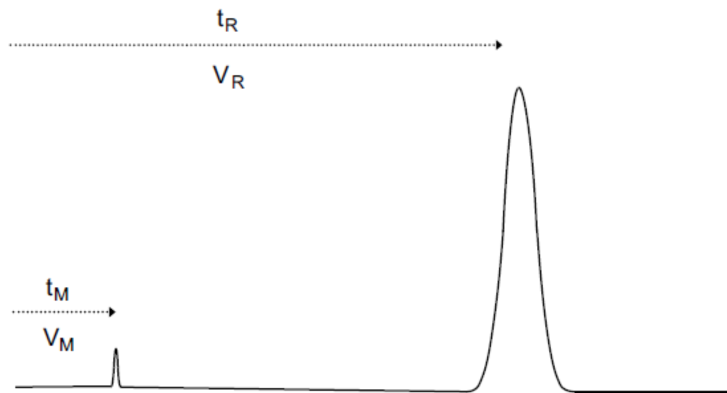


Figure 16: Retention time and retention volume (9)

The following equations can be used to calculate V_M and t_M (9):

$$V_M = t_M \times F \tag{1}$$

$$V_R = t_R \times F \tag{2}$$

V_M – the volume of mobile phase needed for an unretained compound

V_R – the retention volume of mobile phase needed for a retained compound

F – the volumetric flowrate of mobile phase passing through the column (9)

The retention of a compound can be explained by the following relationship between the V_R and its **distribution constant K_C** (9):

$$V_R = V_M + K_C \times V_S \tag{3}$$

$K_C V_S$ is the additional volume of MP needed for elution of the compound. K_C of an unretained compound is equal 0. A greater value of K_C means a higher affinity of the compound to the SP and a stronger retention of the compound.

Retention times are often used to determine a compound′s retention. A few factors, as the speed of the MP and a length of the column, influence the retention time.

Retention factor (k) is not influenced by these factors. It is defined as the ratio of amounts of compound in the SP and in the MP, which shows the distribution of the drug compound between those phases. It depends on the K_C, **V_s** – volume of the stationary phase, **V_m** – volume of the mobile phase and can be expressed as (9):

$$k = K_C \times \frac{V_s}{V_m} \tag{4}$$

Using the chromatogram, k can be determined as follows (9):

$$k = \frac{t_R - t_M}{t_M} \tag{5}$$

2.2.3.2 Column Efficiency

The width of a peak expresses how efficient is the migration of a compound through the column. The narrower the peaks are, the more efficient the separation of this column is. Parameter **N – plate number** or **number of theoretical plates** expresses the peak broadening through the column and can be used to express column efficiency. (9)

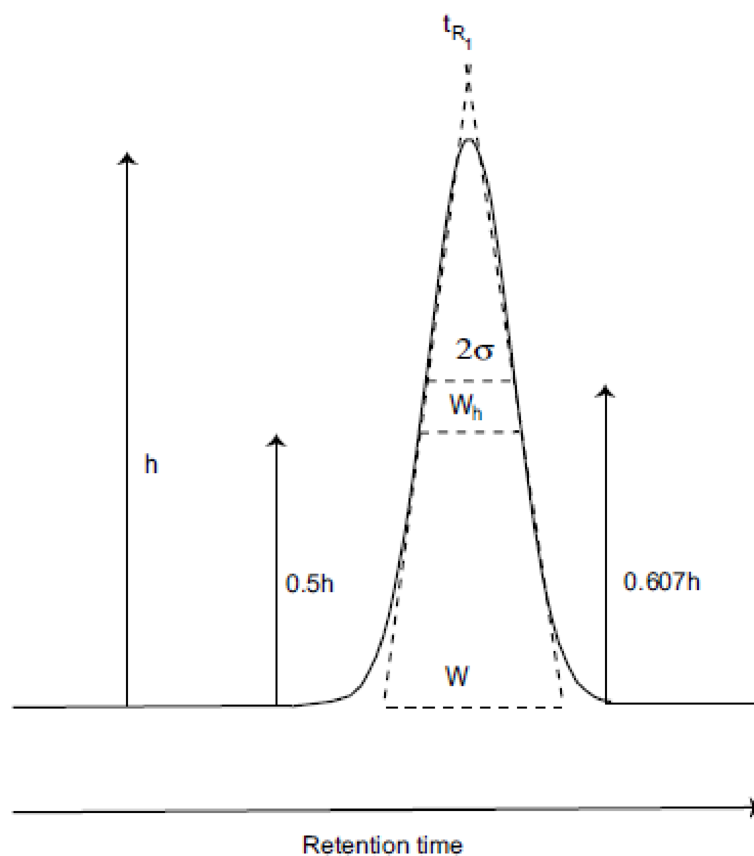


Figure 17: Peak widths. (9)

A picture (Figure 17) and equations (Equation 6 – 13) show different ways how to express peak width and calculate column efficiency.

The number of theoretical plates (N) is defined as follows, where $\mathbf{t_R}$ is retention time of the peak and **σ** refers to standard deviation (9):

$$N = \left(\frac{t_R}{\sigma}\right)^2 \qquad (6)$$

The peak width is shown as 2σ, that is at 0.607 height. In European Pharmacopeia, **the width $\mathbf{W_h}$** is measured at 0.5h (**half-height**) of the peak because it is difficult to take accurate height at 0.607 in practice and can be calculated using Equation 7. (9)

$$W_h = 2.354\sigma \qquad (7)$$

Therefore, N then can be calculated according to following equation (9):

$$N = 5.54\left(\frac{t_R}{W_h}\right)^2 \qquad (8)$$

Another way how to calculate N is to use **W – width of the peak at the baseline**, which is:

$$W = 4\sigma \quad (9)$$

and N will be then (9):

$$N = 16\left(\frac{t_R}{W}\right)^2 \quad (10)$$

For example, a peak which elutes at 6 min and has the W_h of 0.5 or 0.2 min, will have the number of theoretical plates equal (9):

$$N = 5.54\left(\frac{6}{0.5}\right)^2 = 798 \quad (11) \qquad N = 5.54\left(\frac{6}{0.2}\right)^2 = 4986 \quad (12)$$

To compare the efficiency of columns with different lengths, **H – the height of a single theoretical plate** and **L – the column length** can be used. Efficient columns and their high plate number N results in small H numbers (9):

$$H = \frac{L}{N} \quad (13)$$

2.2.3.3 Selectivity

The separation factor (α) is used to describe selectivity of a chromatographic system. Relative retentions of two near-by peaks in chromatogram are used:

$$\alpha = \frac{k_2}{k_1} \quad (14)$$

k_2 – is the retention factor of later eluting peak of a pair

k_1 – is the retention factor of first eluting peak of a pair (9)

It is independent of column length or type. When the value of α is equal 1, the peaks are not separated. The larger α, the better separation. (9)

2.2.3.4 Peak Symmetry

The symmetry can be determined by following equation:

$$A_S = \frac{w_{0.05}}{2d} \quad (15)$$

A_S – the peak symmetry

$w_{0.05}$ – the peak width at 1/20 of its height

d – the distance between a vertical line from the peak maximum and the edge of the peak at its $w_{0.05}$ (9)

The value $A_S = 1$ refers to symmetry of the peak. Values greater than 1 appear as tailing of the peak (Figure 18) and values smaller than 1 show as fronting of the peak. (9)

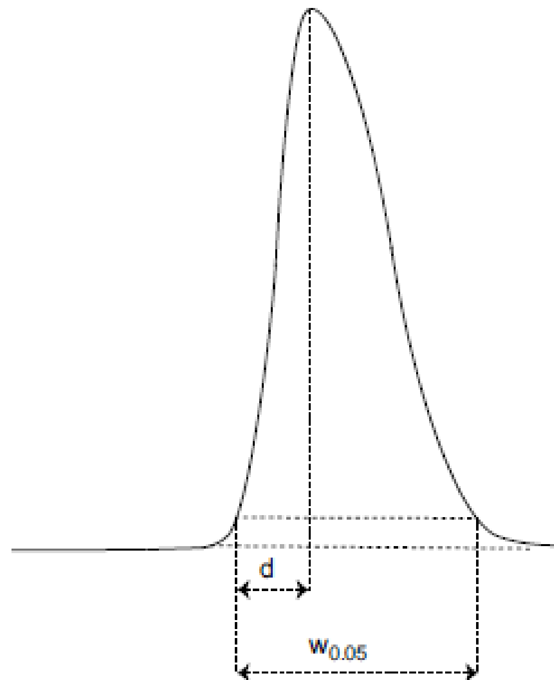


Figure 18: Peak asymmetry - a tailing peak (9)

2.2.3.5 Resolution

Resolution (R_S) expresses how well the peaks are separated. It can be calculated by following Equation 16 (Figure 19). Also, a value of 2 can be used instead of 1.18. W_h is the peak width at half-height of the first peak (W_{h1}) and of the second peak (W_{h2}). (9)

$$R_S = 1.18\,\frac{t_{R2} - t_{R1}}{w_{h1} + w_{h2}} \qquad (16)$$

If R_S is equal 1.0, the separation is only 94% and R_S value of 1.5 is considered to be baseline separation. The value R_S greater than 1.5 is when peaks are completely separated. (9)

When the substances are completely separated the response of detector must reach a baseline between the chromatographic peaks. The example of non-base line and base line separation is demonstrated in Figure 20. (9)

The value of resolution for base line separation is 1.5 or greater. (13)

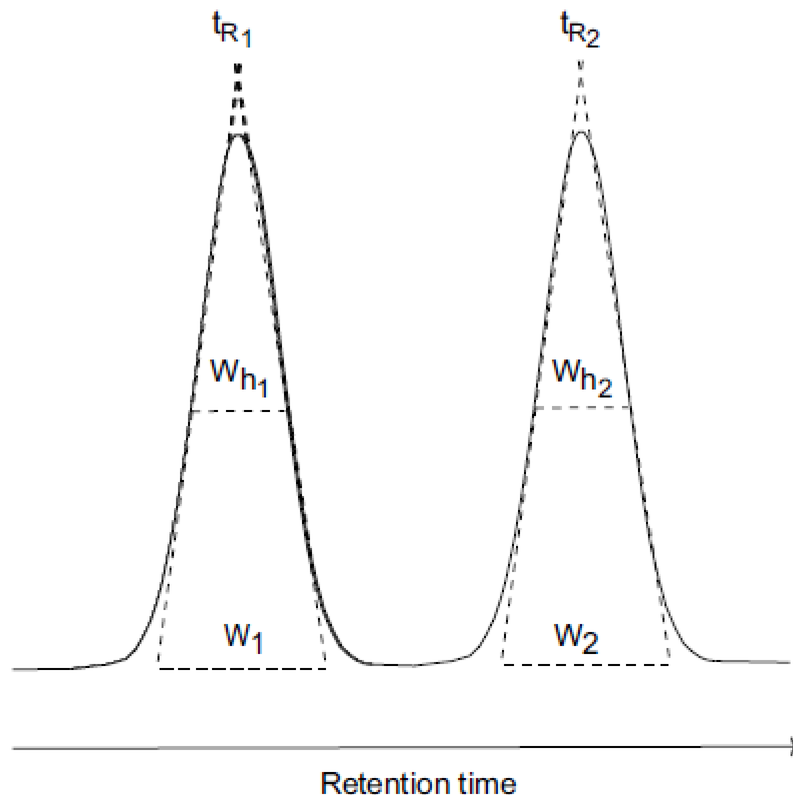


Figure 19: Parameters for Equation 16 (9)

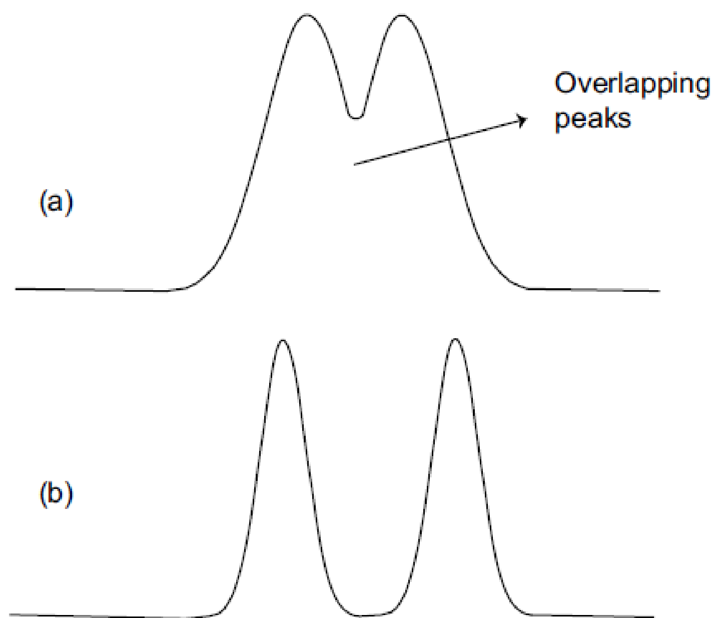


Figure 20: Non-baseline (a) and baseline (b) separation (9)

2.2.4 Separation Principles

2.2.4.1 Normal phase chromatography

It is a system with a polar stationary (most often silica) phase using nonpolar mobile phase (liquid like heptane). Higher affinity to the polar silica is due to analytes having polar functional groups which interact with the stationary phase and retain in the system. The analytes attach to the absorption sites of the surface of silica and the interaction is reversible. Increasing polarity in mobile phase (by adding some polar additives, amine or acid) will lead to weakening the interaction between analytes and silica and decreasing in retention. It is typical mode for TLC but sometimes also performed in HPLC. (9)

- **Stationary phases**
  - Silica

Silica gel or silica is H_2SiO_4, silicic acid but partly dehydrated, polymerized, highly purified after pulverization and drying process and fractionated into particles of particular sizes. It has a large surface area between 200-800 m^2/g thanks to porous structure like a sponge. The pore diameter reaches 60-150Å (one angstrom is 10^{-10} m) in small particles. (9) (11)

It is covered with silanol groups (–Si–OH) acting like weak acids and making the surface polar. Some kinds of silica surface are shown in Figure 21. The interactions between silica and functional groups could be dispersion, dipole-dipole, hydrogen bonding, or ionic interactions. The last two mentioned are the strongest. (9)

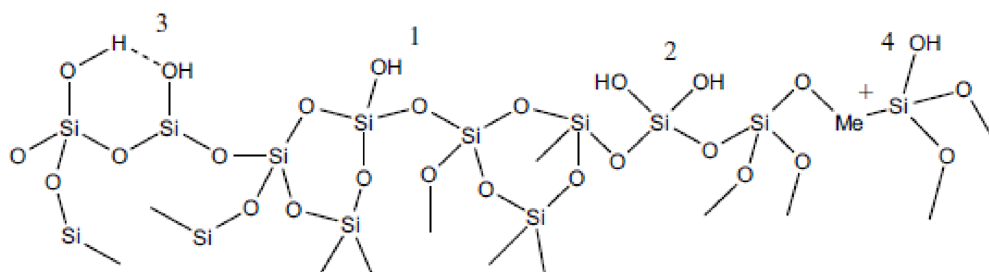


Figure 21: Types of silanol groups: 1 – free, 2 – germinal, 3 – associated, 4 – metal activated (9)

Retention depends on the number and interaction strength between the stationary phase and the polar functional groups of the substance (see Figure 22). In addition, the substance size plays a role in retention. Generally, a small analyte with many polar groups has a strong retention. (9)

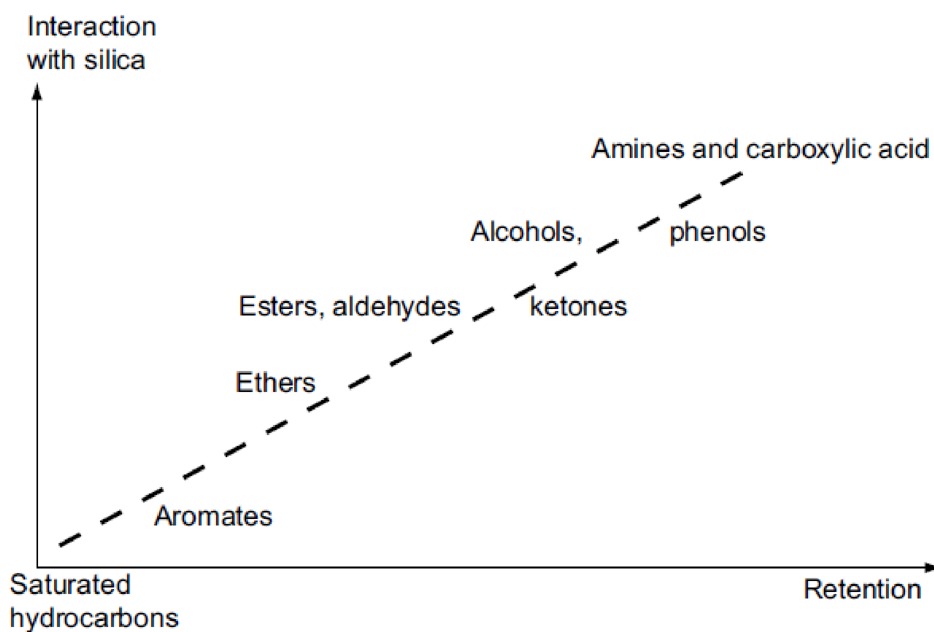


Figure 22: Some functional groups with their retentions (9)

- Other SPs used in normal phase chromatography

Polar materials such as aluminium or magnesium silicates are other SPs used in normal mode LC. Derivatization of silica with CN, NH_2, and diol groups brings also other kinds of polar SPs which can provide changes in the elution order in comparison with silica. (9)

➢ Mobile phases

Mobile phases are used to keep the mixtures in consistency of a solution, to transport the analytes throughout the system and to compete with the analytes for absorption sites on the surface of stationary phase.

They are mostly made of a mixture of organic solvents (pentane, heptane, toluene, chloroform, triethylamine, acetone, ethyl acetate, acetonitrile) and therefore it is less polar than stationary phase. (9)

2.2.4.2 Reverse phase chromatography

It is the most used in HPLC.

➢ **Stationary phases**

Stationary phases (SPs) are hydrophobic due to derivatization on the silica surface usually with chlorosilanes. Some reagents are shown below, Figure 23, where the most hydrophobic is C18 – octadecyl and the most hydrophilic is CN – cyanopropyl. (9)

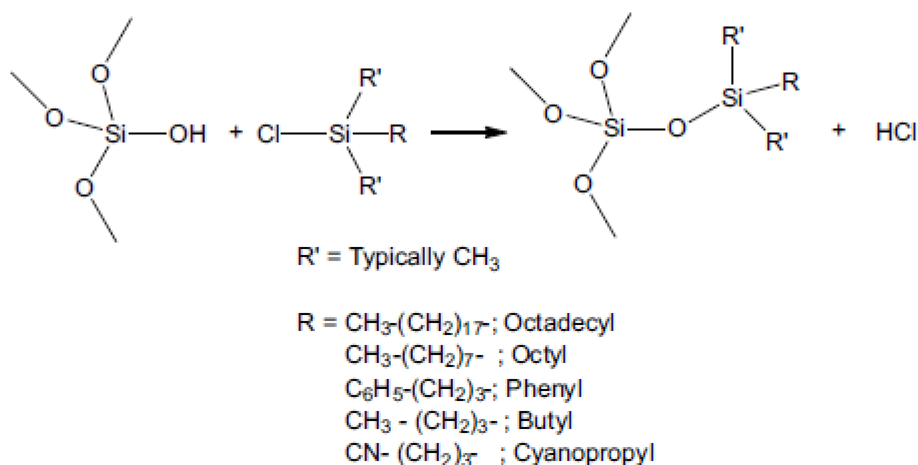


Figure 23: Chlorosilane reagent derivatizing silica (9)

After the derivatization there are still some free silanol groups. To minimize the amount of these free Si–OH groups the reagent trimethylchlorosilane is applied. The procedure is called endcapping. Afterwards there could still be a little percentage of free silica silanol groups capable of polar interactions. (9)

The most often used phase is the octadecyl modified silica which works within 2-8 pH range. Higher or lower pH can destroy the SP. Although SPs based on polymerized silica tolerate the whole pH scale, for example a polystyrene–divinylbenzene copolymer SP.

The main mechanism is done by hydrophobic interactions like Van der Waal's forces, but size of the compound takes place in it as well. Therefore, very polar substances are eluted quicker than nonpolar compounds which retain on the column. The free silanol groups can contribute to separation of hydrophilic analytes. Ionization state influence retention of the ionizable substances. The more ionized it is, the weaker interactions with the SP, thus lower retention. (9)

- **Mobile phases**

For reverse phase, mixture of water and some organic solvents like methanol, acetonitrile, tetrahydrofuran, is used. Greater part in organic character of MP increases its strength and so decreases the retention of analytes.

The pH does not influence neutral compound retention but for ionizable substances it makes big difference in separation and selectivity. Hence, ionization rate for acids or bases is controlled with buffers addition to the MP. (9) (11)

2.3 SEPARATION OF ENANTIOMERS

The process of separating a mixture of enantiomers is called the resolution of enantiomers.

Historically the first chemist, the French chemist Pasteur, performed the separation of enantiomers with tartaric acid salt racemate in 1848. He concluded his observation of chiral molecules forming different crystals with measuring their optical activities.

Tartaric acid is found in nature in grapes. And actually, the terms "racemic" and "racemate" comes from an old name of tartaric acid = racemic acid (racemus, Latin, cluster of grapes).

Anyway, more suitable resolution strategy of racemic mixture is built on the difference of physical properties of diastereomers or using special chiral stationary phase (CSP).

There are numerous methods for separation of a racemic mixture, mostly involving chromatography and electromigration techniques. Procedures such as high-performance liquid (HPLC), gas (GC) chromatography, super- and subcritical fluid chromatography (SFC), capillary electrophoresis (CE), and others are able to separate chiral drugs. (4) (5)

2.3.1 Chromatographic separation

Beginning in this field, all enantiomeric mixtures had to be converted to diastereomeric mixtures by derivatization with a chiral reagent. Afterwards, regular/achiral stationary phase could be used for this separation type by both mentioned chromatography techniques. This approached is called indirect method.

Newer procedures also called direct analyses can be done without derivatization of enantiomers but either chiral stationary phase (CSP) or chiral mobile phase is required.

All these methods form either stable, or transient diastereomers. The difference of stereoisomeric separation is made by adsorption, stability and solubility. (4) (6)

2.3.1.1 Separation using Achiral Stationary Phase

Indirect chromatographic separation

When a mixture of enantiomers is to be separated in achiral environment, it has to undergo the change of derivatization by a chiral agent resulting in a mixture of diastereomers. Diastereomers can be separated using an achiral stationary phase with an achiral solvent, due to the distinguishability of their physicochemical properties.

The principle is shown below (Figure 24). At the picture, the racemic mixture of enantiomers brings on a single peak (a), while the resolution of diastereomers converted by a chiral reagent is conducted with success and separated into two peaks on an achiral stationary phase (b). (4) (6)

The agent refers to an enantiopure molecule also called as unichiral reagent or chiral derivatizing reagent (CDR). The requirement of high purity of the CDR is important for the analysis. Contamination of the CDR with even a small amount of the other enantiomer of reagent will lead to not accurate results. If a CDR does not have a uniform configuration, the reaction with chiral analyte develops a mixture of four different diastereomers. These 4 diastereomers form two racemic mixtures, two pairs of

enantiomers, and would be separated only into two peaks on an achiral stationary phase. This makes the quantification inapplicable. Therefore, only one and very pure enantiomer can be used for transformation in indirect chromatographic resolution of enantiomers (Figure 25). (4) (6)

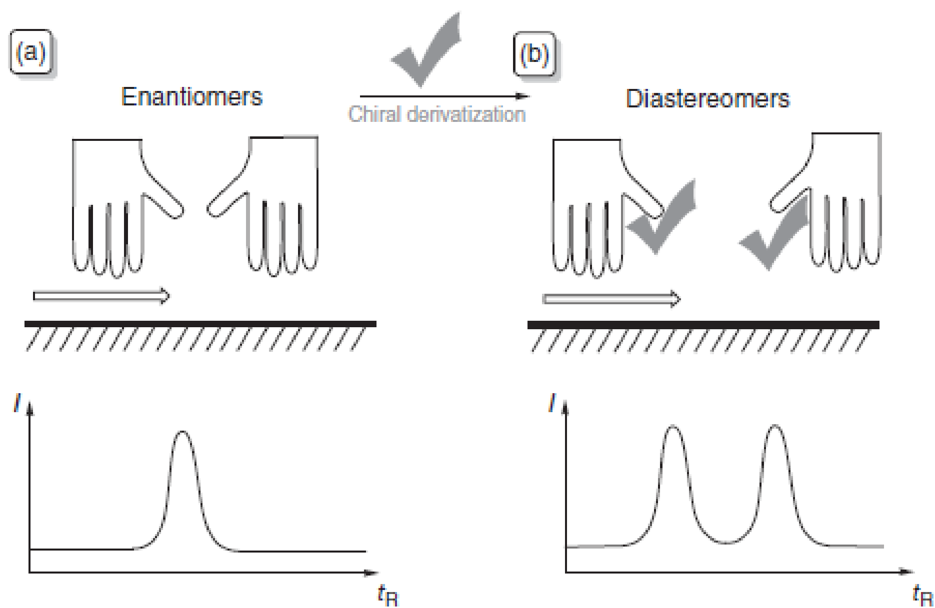


Figure 24: The influence of chiral derivatization on resolution of enantiomers (6)

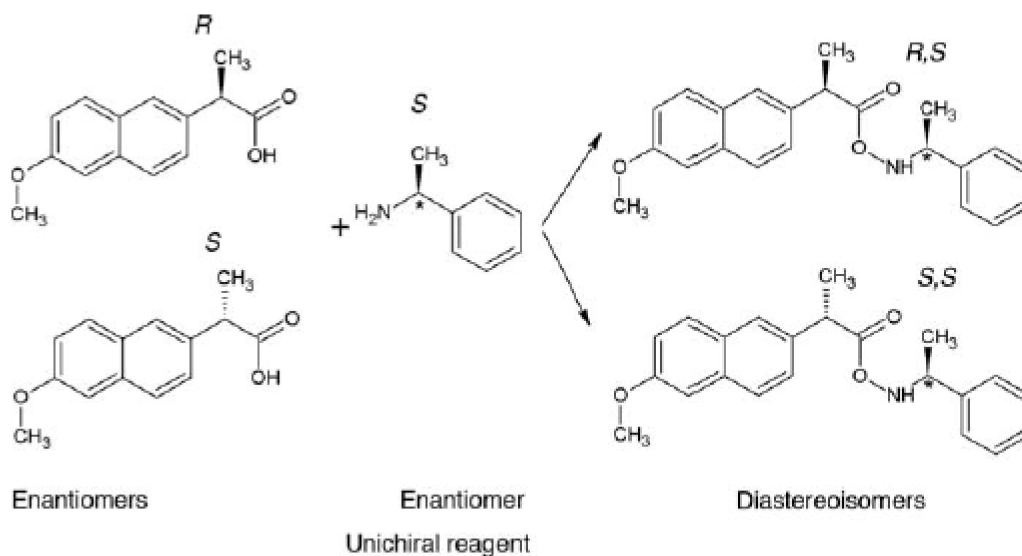


Figure 25: The important CDR purity resulting in applicable resolution of products of derivatization reaction in indirect measurements (4)

2.3.1.2 Separation using Chiral Stationary Phase

Direct chromatographic separation

Direct separation (without prior derivatization) of enantiomers can be provided by chiral environment, using such a mobile phase or a chiral stationary phase (CSP). CSPs are used more often than chiral mobile phases though.

Enantiomers are injected into the system directly and they bind with CSP and create temporary diastereomers. Physicochemical parameters of these transient diastereomers are the key of efficiency of separation. (4) (6)

Illustration of the principle of this method is in Figure 26.

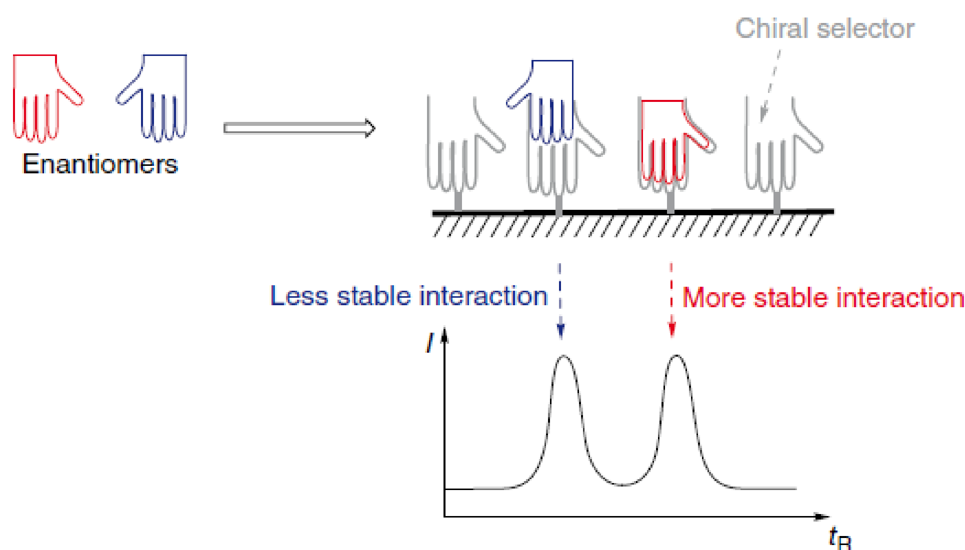


Figure 26: The principle of direct chromatographic separation (6)

CSPs consist of polymer bases like silica, methacrylate, cellulose. Chiral molecules of different kinds as proteins, antibiotics, helical methacrylates, polysaccharides, cyclodextrins, and others are attached to the polymer bases and so built different chiral stationary phases. This provides distinct selectivity. The development and the significant impact of CSPs has arrested the attention in drug stereochemistry, indeed in entire pharmaceutical industry. (4) (6)

The chiral stationary phase can be characterized mainly by capacity (efficiency) and selectivity of performed separation. As apparent from Figure 27, numbers of active

sites on the CSP determine the capacity of separation, (a) and (b). Nevertheless, enantiopurity of CSP is also of concern (c).

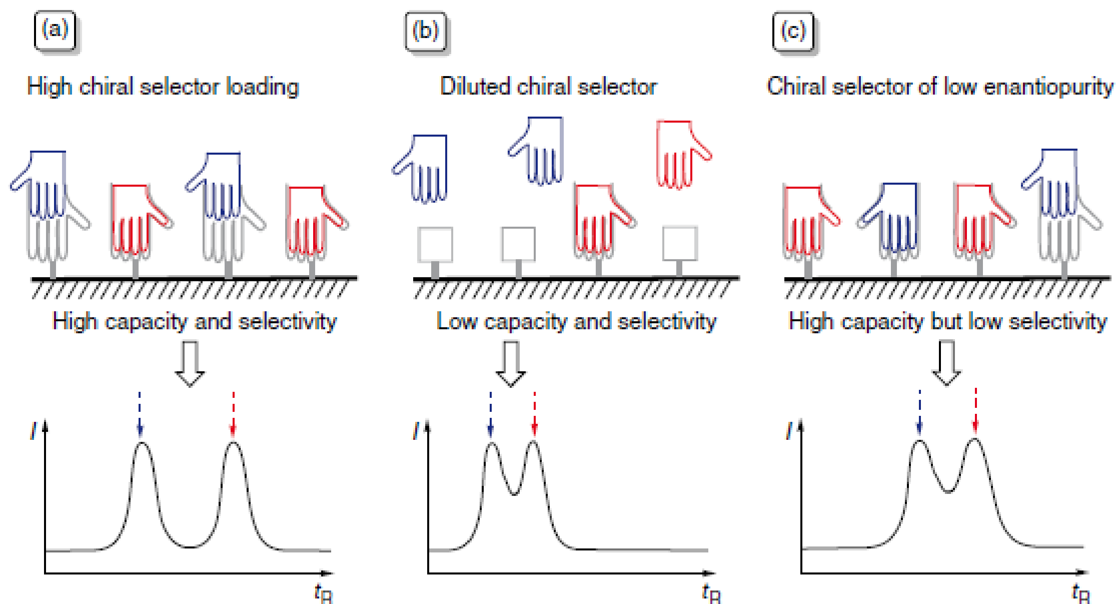


Figure 27: Comparison of capacity and selectivity of separation using CSP (6)

Chiral selector can be mostly prepared in both (R and S) enantiomeric configurations. If a racemic mixture is tested on both types of configuration of CSP, retention times are in reverse, as in Figure 28 – column (a) and column (b). (4) (6)

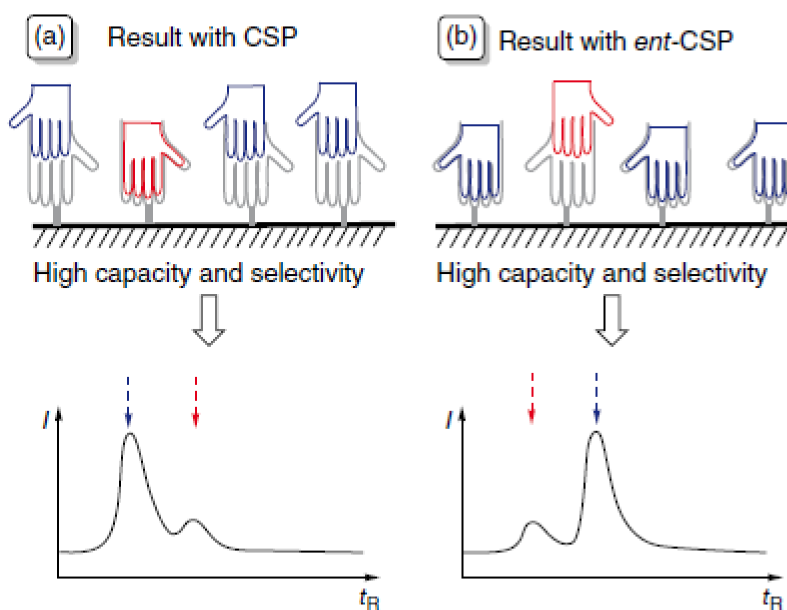


Figure 28: Comparison of CSPs related as mirror images (6)

2.3.1.3 Chiral Stationary Phases (CSPs)

Nowadays, many CSP types are commercially available.

One of the first CPS was **Pirkle-type** in the early 1980s which had an electron-deficient dinitrobenzoyl group (Figure 29) and therefore it was able to separate only compounds with electron-rich aromatic groups, like a naphthalene and mostly neutral substances. (4) (9) (14)

Later on, around 1985, **Hermansson′s Enantiopac CSP** was introduced (Figure 29). It is based on **plasma protein** α_1-acid glycoprotein (AGP) with a wide range of chiral separations, mostly for basic drugs but also a few neutral and acidic drugs can be separated. They work well in reverse phase chromatography. Other proteins of this CSP kind like a human serum albumin or a cellobiohydrolase – stable enzyme showed good separation activity as well. (4) (9)

Late 1980s were years of **cyclodextrin CSP,** originally used in normal phase chromatography, and **free or derivatized polysaccharides CSP,** such as cellulose and amylose. They have helical structures but are available only as a single enantiomer of SP.

It was followed by **higher-generation Pirkle CSP** and derivatized polysaccharides CSP with carbamate base rather than ester derivatization. (4) (9) Thanks to the availability of different CSP, it was no problem to separate chiral molecules and it became a part of guidelines for production of enantiomeric drugs.

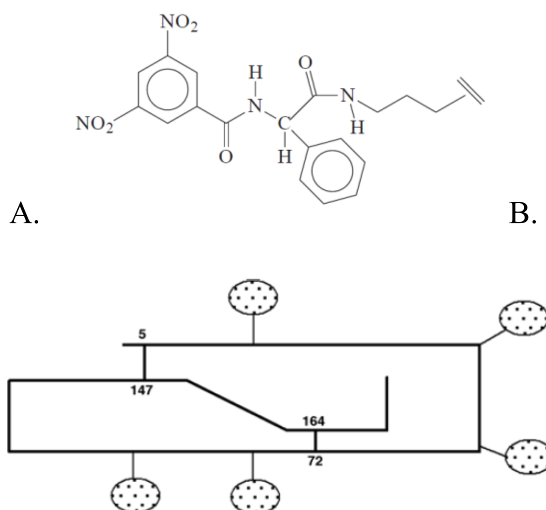


Figure 29: A. Pirkle-type CSP, B. Hermansson's Enantiopac CSP (4)

Characterization of selected CSPs

Immobilized derivatized polysaccharides as CSPs (Chiralcel OD, Chiralpak AD, Figure 30) were introduced later on. They can be used with a broader range of mobile phases and brought larger robustness. (4) (9) (15)

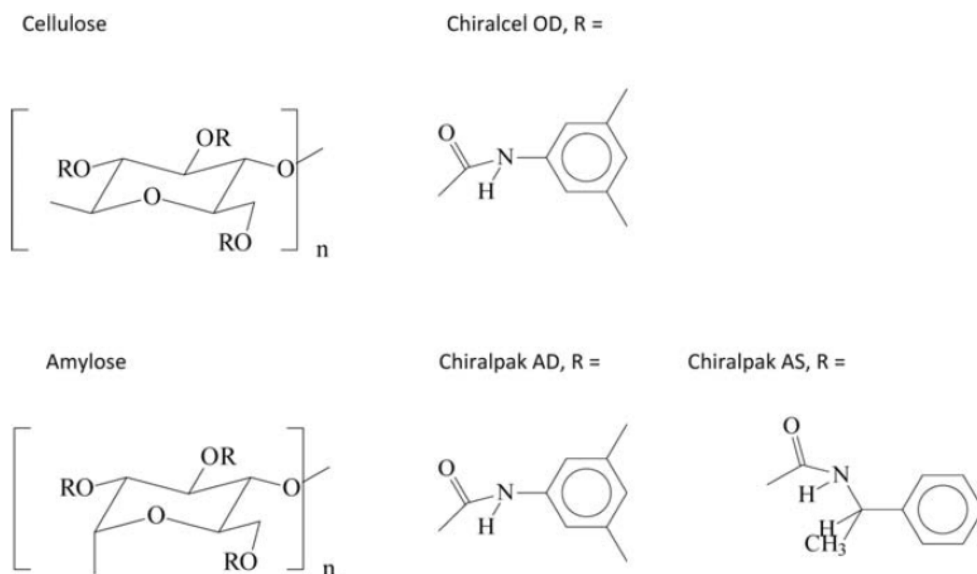


Figure 30: The polysaccharides CSP with the derivatizing groups (4)

They were prepared by coating the stationary phase with chiral selector that means there is not much chance for samples to get contaminated with chiral selectors. (4) (9)

Lux Cellulose columns from Phenomenex company (Figure 31) belong to these group of immobilized derivatized polysaccharides. Especially a column Lux Cellulose-4 is built up from cellulose tris(4-chloro-3-methylphenylcarbamate). (9) (16)

It is worth to mention also a **chiral ion-exchange CSP**. They are weak anion-exchange CSPs, based on O-9 (tert-butylcarbamoyl) quinine or quinidine (Figure 32). Very useful for separation of analytes containing acidic groups like carboxylic, phosphonic, phosphinic, phosphoric, sulfonic and especially for non-steroidal anti-inflammatory drugs. Quinidine and quinine are enantiomers to each other therefore applying them to chromatography, they will give reverse elution order. (4) (9)

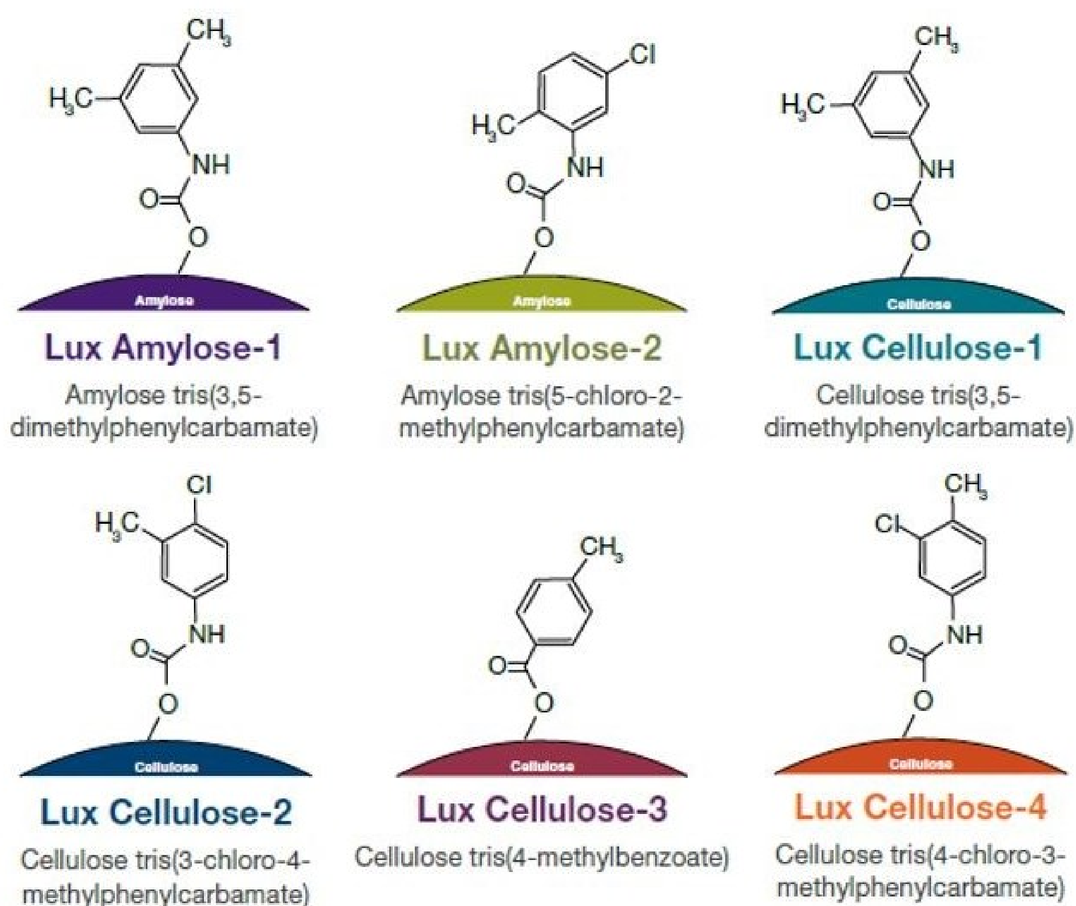


Figure 31: New immobilized Lux cellulose and amylose columns (16)

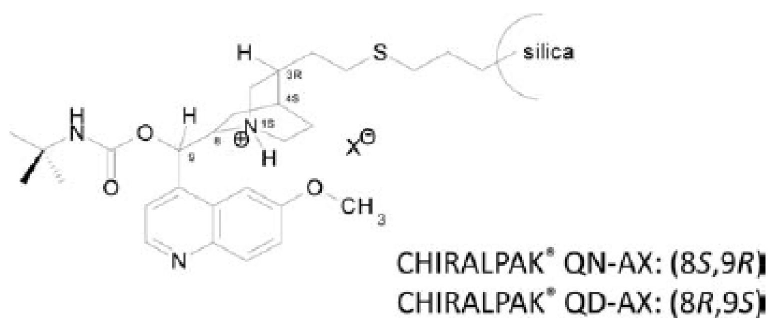


Figure 32: Chiral anion-exchange CSPs (4)

Macrocyclic antibiotics are other CSPs and have peptide/amide and sugar residues (Figure 33). They were used in reverse phase and later in normal phase chromatography too and are able to separate a wide scale of substances, even large molecules like proteins. (4) (9)

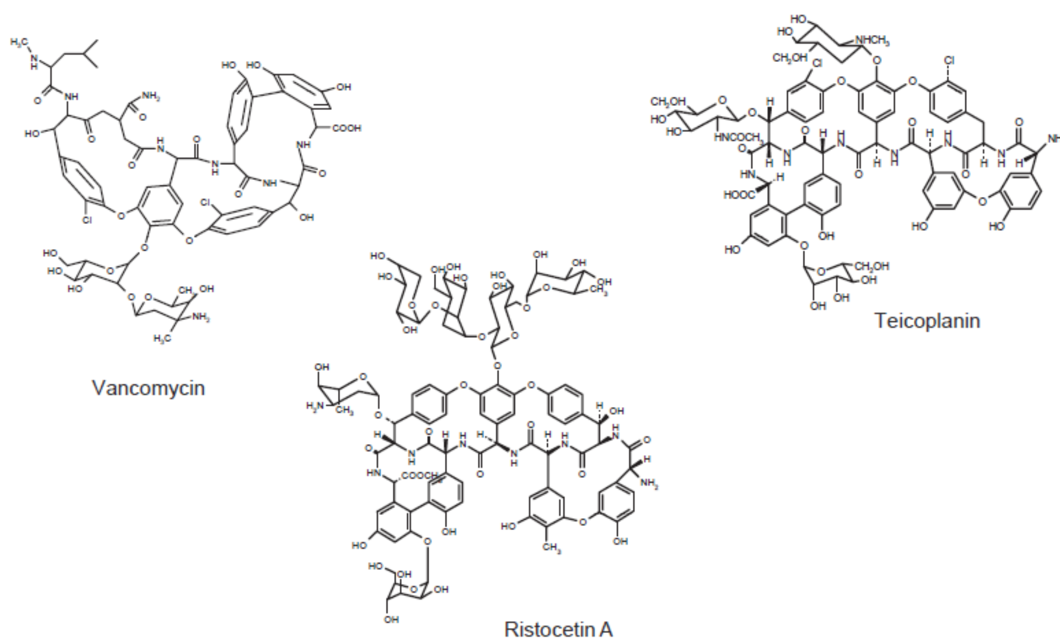


Figure 33: Macrolides as CSPs (4)

Oligosaccharides like **cyclodextrins** were one of the first CSPs as was stated earlier. They were used in native forms but later they subphase has been derivatized. Newer oligosaccharides are **cyclofructans** with a special use for primary amines. They vary in mobile phases, no needs for highly acidic and aqueous MP. **Aromatic functionalized cyclofructans** (Figure 34) separate many substances including secondary amines, tertiary amines, sulfonamides and compounds with acid groups too. They are intended mostly for normal phase chromatography. (4) (9)

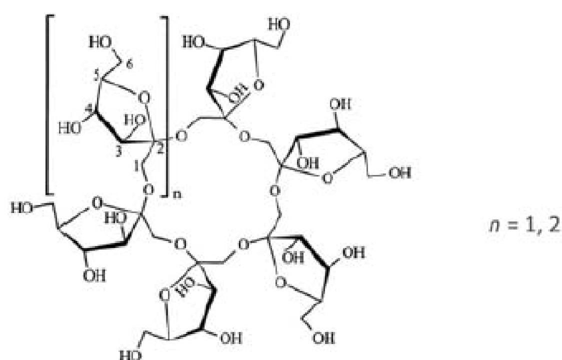


Figure 34: Cyclofructans (4)

2.4 TESTS FOR IMPURITIES IN PHARMACEUTICAL MATERIALS

Tests for impurities are performed to ensure that no material is contaminated by unwanted compounds. These purity tests belong usually to limit tests. They prevent the contamination of the pharmaceutical raw material higher than the limit and thus ensure the purity, quality and safety of pharmacotherapy. (9) (17)

Pharmacopeia monographs can contain 3 – 12 impurity tests for identification and limitation. (9)

2.4.1 Tests for Related Substances

These tests evaluated the quantity of impurities which are structurally related to the active pharmaceutical ingredient (API) which can be part of final pharmaceutical products. It is necessary because related substances, notably degradation products, can decrease the efficiency of API and subsequently pharmaceutical products as well.

They are performed to control new batches of API or pharmaceutical products but also during stability tests to establish the shelf life of a product over the time. These tests are mostly performed by HPLC. Usually, the concerned analyte is dissolved in particular solvent and filtrated before analysis. The related substances differ in retention time from API. Each impurity is quantified, and it must be below its acceptable limit.

They are followed by GMP = Good Manufacturing Practice to meet guideline requirement from ICH = International Conference On Harmonisation for new drug substances - Q3A(R2) and new drug products - Q3B(R2). (9) (17)

2.4.2 Quantification of impurities

Peak height or peak area are used for analyte quantification. To do so, four main methods are involved:

1. External standard method
2. Internal standard method
3. Standard addition
4. Normalization – used in this Diploma Thesis. (9) (17)

2.4.2.1 Normalization

It is a technique for quantitative analysis of a mixture. The sample′s concentration is usually of 1 mg/ml. Ratio of a given peak area (A) and sum of all peak areas in the mixture (A_i) is a percentage value (A (%)) of the substance in the mixture. (9)

$$A\,(\%) = \frac{A}{\sum A_i} \times 100 \qquad (17)$$

2.5 THE INTERNATIONAL PHARMACOPOEIA (PH. INT.)

The International Pharmacopoeia is a collection of analytical procedures to determine pharmaceutical substances or dosage forms for World Health Organization (WHO) Member State.

In comparison with the other pharmacopoeias, this one watches also over the needs of developing countries. Its priority are medicines under WHO health programs like treating malaria, tuberculosis or HIV/AIDS. These medicines are included in WHO Model List of Essential Medicines which had 433 drugs in 2017.

Its specific goal is to provide quality control specifications and so to enable quality medicines all over the world.

The first Ph. Int. was published in 1951 and now 8th edition has been available since 2018. (18) (19) (20)

2.6 DRUG DOLUTEGRAVIR SODIUM

Dolutegravir sodium (DTG) or (4R,12aS)-N-[(2,4-Difluorophenyl) methyl]--7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2Hpyrido[1',2':4,5] pyrazino-[2,1-b] [1,3]oxazine-9-carboxamide sodium salt (Figure 35) has a molecular formula $C_{20}H_{18}F_2N_3O_5Na$ and its molecular weight is 441.36. (2)

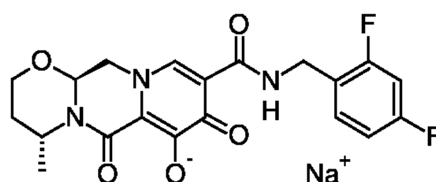


Figure 35: Chemical structure of DTG (21)

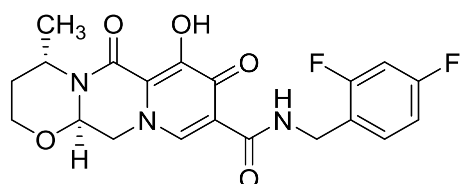
It is an integrase inhibitor which blocks the transfer step of the integration of viral genom into host cell. It is used for treatment of HIV infection. DTG has a unique resistance profile in patients who have been previously treated with raltegravir and their treatments failed. (22) (23) (24)

It also disposes of a great tolerability and minimum of toxicity for human host cells, suitable for pediatric patients too. (25) (26) Other advantages are the dosage - once per day and rapid absorption. (27)

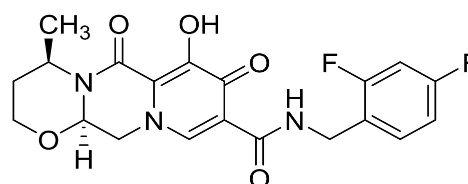
Dolutegravir sodium got its first approval in 2013 by FDA (Food and Drug Administration). (28) It is available as a single substance table - Tivicay® or in combination with other HIV drugs - Dovato®, Juluca®, Triumeq®. (29) Although it has no monograph in any of the pharmacopoeia so far. (1)

2.6.1 Related substances of DTG

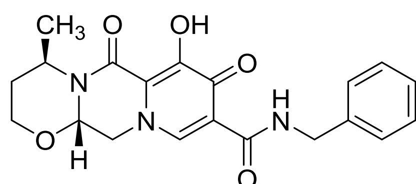
The following compounds (Figure 36) were identified as related substances of dolutegravir (2):



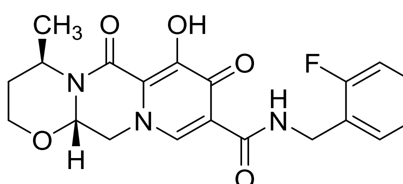
A. Enantiomer (4S, 12aR)



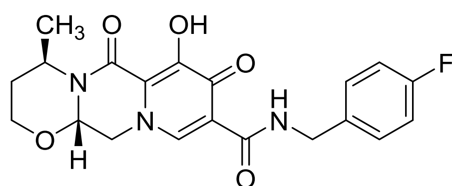
B. Diastereomer (4R, 12aR)



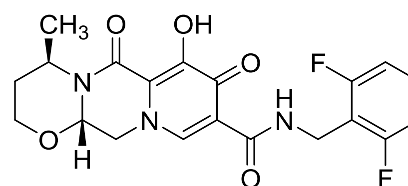
C. Desfluoro dolutegravir



D. 2-Fluoro dolutegravir



E. 4-Fluoro dolutegravir



F. 2,6-Difluoro dolutegravir

Figure 36: DTG's related substances

2.6.2 Chromatographic separation of DTG

There are various HPLC methods for DTG determination in plasma samples, even with very small volume of samples, for instance 20 µl of human plasma is processed with protein precipitation and separated on C18 column. Also, other methods by HPLC and TLC were published but there was no method for chiral separation. (30) (31)

Recently, a novel validated HPLC chiral method was published to separate an enantiomer and a diastereomer and other related substances of DTG using a column Lux cellulose – 4 (250 mm x 4.6 mm, 5µ). MP used for separation is a mixture of acetonitrile, water and orthophosphoric acid (980:40:2 v/v/v). The resolution of the peaks reaches very satisfying value of 3.0. (2) (32) (33)

3 AIM OF THE PROJECT

The aim of the project was to develop suitable HPLC method for separation of chiral impurities of DTG to be capable of introduction to the International Pharmacopoeia.

Afterwards, the method was applying for analysis of a few batches of dolutegravir API, racemic mixture and tablets.

4 EXPERIMENTAL PART

4.1 SAMPLES

4.1.1 Drug samples

The all drug samples were provided by companies Aurobindo and Cipla (Hyderabab, India). (34) (35) They were:

Three batches of API:

- Dolutegravir Sodium Aurobindo 34
- Dolutegravir Sodium Aurobindo 35
- Dolutegravir Sodium Cipla

One batch of racemic mixture:

- Dolutegravir Sodium racemic Cipla

One batch of tablets:

- Dolutegravir Tablets 50mg, Aurobindo, TBL DUSA 17034-A

The following tables present information of batch numbers, expiration/retest dates and sources/companies of drug samples (Table 1) and impurities standards (Table 2).

Compound	Batch No.	Exp./retest	Source
Dolutegravir sodium 34	1811104234	Retest 21/2/2020	Aurobindo
Dolutegravir sodium 35	1811104235	Retest 23/2/2020	Aurobindo
Dolutegravir sodium Cipla	LDC180088	Exp. 23/02/2023	Cipla
Dolutegravir sodium Racemic	TS/C/D210/02	Exp. 18/03/2021	Cipla
Dolutegravir (TBL) 50 mg	DUSA17034-A	Exp. 9/2019	Aurobindo

Table 1: Drug samples

4.1.2 Standards of impurities

One batch of each impurity from Aurobindo company was applied:

- Impurity A = Dolutegravir (4S, 12aR) – Enantiomer
- Impurity B = Dolutegravir (4R, 12aR) – Diastereomer
- Impurity C = Desfluoro dolutegravir
- Impurity D = 2-Fluoro dolutegravir
- Impurity E = 4-Fluoro dolutegravir
- Impurity F = 2,6-Difluoro dolutegravir

Compound	Batch No.	Exp./retest	Source
Enantiomer (Imp. A)	DOL (1470) 68	Retest 6/2019	Aurobindo
Diastereomer (Imp. B)	DOL (1470) 84	Retest 6/2019	Aurobindo

Table 2: Impurities standards

4.2 CHEMICALS

- Methanol (later only MeOH), super gradient grade for HPLC, HiPerSolv CHROMANORM®, either Sigma-Aldrich or VWR, Germany
- Ethanol (later only EtOH), super gradient grade for HPLC, HiPerSolv CHROMANORM®, either Sigma-Aldrich or VWR, Germany
- Acetonitrile (later only ACN), super gradient grade for HPLC, HiPerSolv CHROMANORM®, either Sigma-Aldrich or VWR, Germany
- Milli-Q water (later only H_2O), prepared with Milli-Q water machine in the insstitute laboratory
- o-phosphoric acid (later only OPA), 85%, ρ = 1,7 g/ml, ROTH, Germany
- Ethylenediaminetetraacetic acid disodium salt dihydrate (later only EDTA), Merck, Germany

4.3 LABORATORY INSTRUMENTS AND EQUIPMENT

4.3.1 High performance liquid chromatography system

- Two pumps: LC-10AS
- Auto injection system: SIL-10A
- UV-VIS detector: SPD-10A
- System controller: SCL-10A VP
- Column oven: CTO-20AC
- LabSolutions software, Shimadzu

All components were from company Shimadzu, Kyoto, Japan.

4.3.2 The column

- Lux® Cellulose-4, LC Column 150×4,6mm, Phenomenex®, USA
- Security Guard Cartridge Kit KJ0-4282 (Security Guard Cartridges, AJ0-8627, Lux® Cellulose-4, 4×3,0mm ID, Phenomenex®, USA)

4.3.3 Other equipment

- Vortex Genie 2TM, Bender & Hobein AG, Zurich, Switzerland
- Analytical balance, Mettler Toledo, USA
- Analytical balance, Sartorius AG Göttingen, MC 5, max. 5,1g, Germany
- Ultrasonic bath, Merck eurolab, Germany
- Syringe filters, PP 0,22μm, Simplepure™, USA
- Vacuum system, PC 2001 Vario, Vacuubrand, Germany
- Filters for vacuum system, Membrane Filters (Mixed cellulose ester), ME 25, 0.45μm, diameter 47mm, GE Healthcare, Whatman^TH, UK
- Vials, snap cap, 32 × 11,6 (644), VWR, Germany
- Snap ring cap 11mm transp., soft version, VWR, Germany
- Micro Insert, clear glass, 0,2ml, flat bottom, 31 × 6 mm, VWR, Germany
- Pipettes and pipette tips (Eppendorf, Germany), filtrate paper, laboratory glass, mortar and pestle

4.4 PREPARATION

4.4.1 Mobile phase preparation

The mobile phase (MP) consists of acetonitrile, Milli-Q water and o-phosphoric acid. During this project following MPs were tested:

MP0 = ACN:H_2O:OPA 980:40:2 (v/v/v) = MP original

MP1 = ACN:H_2O:OPA 940:80:2 (v/v/v)

MP2 = ACN:H_2O:OPA 900:120:2 (v/v/v)

MP3 = ACN:H_2O+EDTA(0,186g/l):OPA 980:40:2 (v/v/v)

<u>Flushing MP</u> (to switch from normal phase to reversed phase) = MeOH:EtOH 90:10 (v/v)

These mobile phases were prepared according to the following protocol:

<u>Flushing MP</u> was prepared just by measuring 90 ml of MeOH and 10 ml of EtOH in measuring cylinders and mixing them together.

For preparation of other MPs, Milli-Q water was filtrated with vacuum system on membrane filter ME 25, 0.45µm, diameter 47mm, (mixed cellulose ester), from WhatmanTH. Next, the accurate volumes of water (980 or 940 or 900 ml) and ACN (40 or 80 or 120 ml) were measured in measuring cylinders and mixed together.

At the end, OPA was added to the MP. In published article, there was OPA of 1,44g/ml density. (2) For this project, OPA of 1,7g/ml was used and therefore the exact volume needed to be calculated by the densities and indirect proportion. According to the

calculations, 1694 µl of OPA and 306 µl of H_2O were added to every 1020 ml mixture of H_2O and ACN.

For EDTA-MP, the solution of 1000 ml was prepared by weighing 0,186 g of EDTA powder on the analytical balance Sartorius and resolving it in 1000 ml of H_2O. Then 40 ml of this solution was mixed with 980 ml of ACN and 1694 µl of OPA and 306 µl of H_2O.

All MP were ultrasonicated before use in Ultrasonic bath, Merck eurolab to get rid of all bubbles for a period of cca 1 hour.

4.4.2 Stationary phase preparation

Before the first use, the column needed to be switched from shipping solvent to the solvent compatible with reverse phase analysis. It was performed by flushing the column with the flushing MP with at least 10 column volumes (e. i. for 1 hour at the flow rate of 0.5 ml/min.

Next, the column was equilibrated with either MP0 or the other type of MP (MP1, MP2, MP3) at least for an hour at flow rate of 0.5 ml/min and prepared for samples analysis.

4.4.3 Chromatographic conditions

The initial chromatographic conditions were taken from the published paper (2), but they were needed to be adjusted for a shorter column (Lux® Cellulose-4, 150×4,6mm, Phenomenex®).

The system was running at different flow rates from 0.3ml/min to 1.2ml/min and with different MPs (as mentioned earlier). Isocratic mode was applied for all separations. The optimized flow rate of 1 ml/min and mobile phase composed of a mixture of acetonitrile, water, orthophosphoric acid in ration of 980:40:2 (v/v/v). The injection volume was 10 µl. The temperature of column oven was set for 25ºC. UV detection was used at 258 nm. One analysis took from 20 to 120 min according to the MP, final method lasted for 40 min.

4.4.4 Sample preparation

For preparation of samples always the analytical balance for weighing was used and diluent of ACN:H_2O 50:50 (v/v). The diluent was prepared by taking exact volume of 50 ml of ACN and 50 ml of H_2O in measuring cylinders and mixing them together. The diluent was ultrasonicated the same way as MPs and used for sample dissolution and for dilution of solutions.

4.4.4.1 Preparation of API, racemic mixture and impurities

Samples as **APIs, racemic mixture and impurities** which were already in powder form were just weighed and diluted with diluent to exact concentration.

The stock solutions, mostly 1mg/ml were prepared by dissolving of appropriate amount of API in dilution solvent. This solution was further used to prepare the working solutions of 10µg/ml or 1µg/ml or other concentrations, that were used for either peak identification or finding of detection limit. Racemic mixture was prepared as solution of 100µg/ml concentration. All solution concentrations are always written close to each chromatogram.

4.4.4.2 Preparation of TBL

Tablets needed following preparation:

Five TBLs containing 250 mg of dolutegravir (5 x 50 mg) were crushed and grinded in mortar with pestle into a fine powder. Now the powder containing 250 mg of DTG was weighed. This powder of 1551,5 mg weight was used for solutions.

To prepare **Solution (1) = S(1)**, a quantity of powdered tablets (y), nominally equivalent to 35.0 mg dolutegravir was transferred to a 50 ml volumetric flask. The substance was dissolved with about 35 ml of the diluent using sonicator for 5 minutes. Then the volume was completed using the same solvent and finally it was carefully mixed and filtrated with accordion filtrate paper.

Then concentration of **S(1)** became 35mg/50ml which is 0,7mg/ml.

To prepare **Solution 2 = S(2)**, firstly, 1 ml of S (1) was diluted to 100 ml with the diluent and secondly, 10 ml of this solution was diluted to 50 ml.

The final concentration of **S(2)** was 1,4µg/ml.

Solution (3) = S(3) was prepared similarly as **S(1)** but with target to achieve final solution concentration of 1mg/ml by dissolving TBL powder equivalent to 10.0 mg dolutegravir to 10 ml volumetric flask.

S(3) became 10mg/ml concentrated which is 1mg/ml.

4.5 QUANTIFICATION OF IMPURITIES BY NORMALIZATION

Normalization was applied for quantification of impurities in these samples. The sample solutions (DTG-API or DTG-TBL) had 1mg/ml concentration. The impurity limit was established to 0.15%. Thus, any impurity being of 0.15% of areas sum had to be the maximum. After substitution of 0.15% value to the Equation 17 (in the chapter 2.4.2.1 Normalization), now Equation 18 calculating limit of impurity must be true.

$$0.15\,\% > \frac{A}{\sum A_i} \times 100 \qquad (18)$$

At least 3 measurements of each sample were taken, and the average values were submitted to the regulatory body.

4.6 RECOVERY TEST – CALCULATION

Recovery test is one of accuracy testing of the analytical technique. It is proceeded by spiking (adding) known amount of analyte to the sample and comparing it with the result of just plane sample. (37)

In this case, the impurity A and B, each of 1µg/ml concentration, were added to solution of dolutegravir API batch Aurobindo34 (1mg/ml). To calculate recovery of impurities, the area data of unspiked sample analysis must be subtracted from the spiked/added one. And the outcome is the amount which recovered from the measurement. The difference between areas of spiked and unspiked samples should be the area of spiked amount and so the area of spiked impurities. The more of spiked impurities amount recovers, the more accurate the analytical technique is.

5 RESULTS AND DISCUSSION

At the beginning the chromatographic conditions were set to the particular published as in the literature (2), with flow rate that should be optimal for this column length. However, the separation was not satisfying, and the analytes (DTG and impurities) were not base line separated at all (Figure 37). Thus, different flow rates and mobile phase compositions (as specified in paragraph 4.4.1) were tested. Because no of the conditions provided acceptable separation, the decision was to flush the column once more with flushing MP for an hour and half. This action had changed the separation completely and allowed of getting base line separation as well as acceptable resolutions of the peaks ($R_s > 1,5$). From that time continuing the column kept its separation with conditions of mobile phase composed of a mixture of acetonitrile, water, orthophosphoric acid in ration of 980:40:2 (v/v/v) at flow rate 1ml/min (Figure 38).

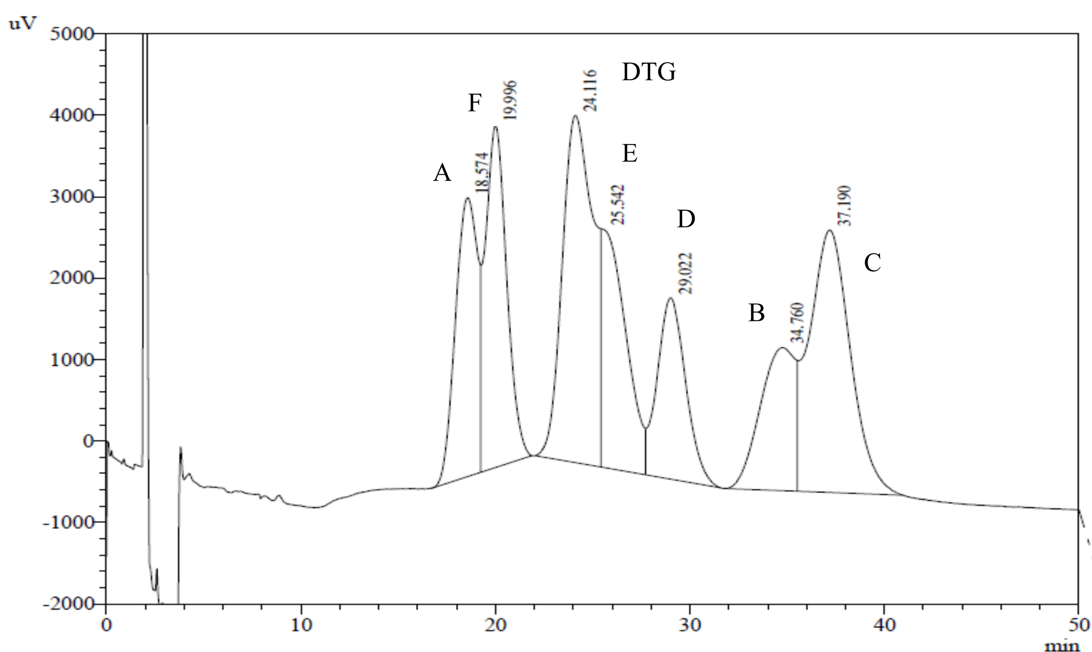


Figure 37: Chromatogram of analysis before second flushing of the column

5.1 ANALYSIS OF DTG WITH ALL IMPURITIES

This column is able to separate DTG and impurity A, impurity B, impurity C, impurity D, impurity F. At the beginning, the analysis of each substance (standard) was run individually for determinatio DTG ition time. Dolutegravir Sodium Aurobindo34 was employed as standard for DTG and all provided samples of impurities were employed as their standards. It was found that the peak corresponding to impurity E coeluted with DTG. Therefore, in Figure 38, peak of impurity E is most likely to be covered with DTG peak. However, impurity E is not a concernment of this project. Table 3 shows selected chromatographic parameters.

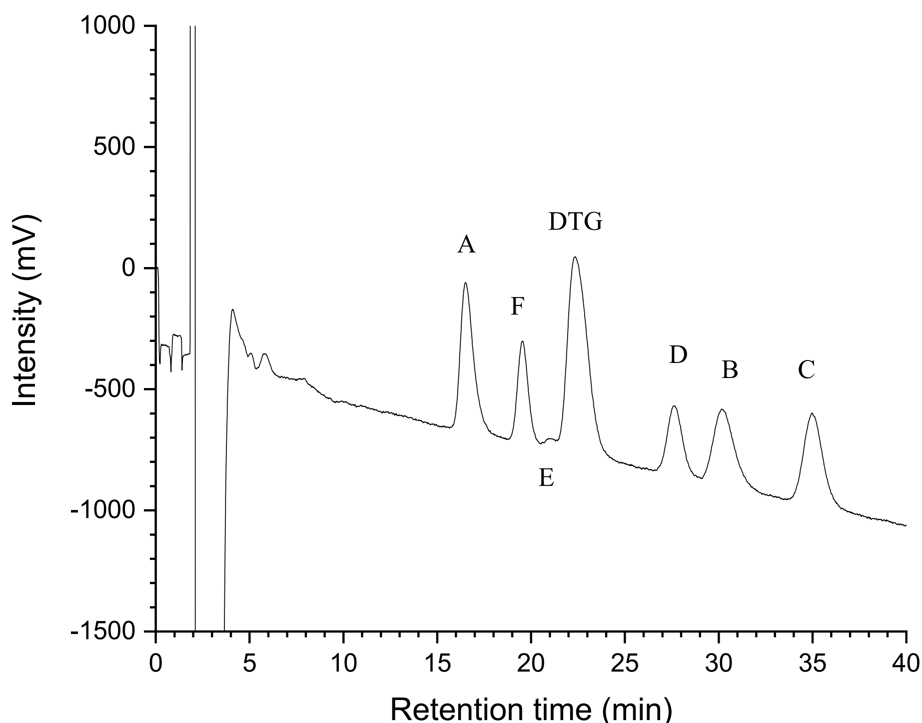


Figure 38: Chromatogram of analysis of DTG and all impurities (each at concentration of 1µg/ml and run at flow rate 1ml/min)

Peak #	Name	Ret. time	Resolution
1	Imp. A	16.61	0.00
2	Imp. F	19.57	2.39
3	DTG	22.36	1.89
4	Imp. D	27.60	3.08
5	Imp. B	30.15	1.54
6	Imp. C	34.97	2.43

Ret. time = retention time

Imp. = impurity

Table 3: Chromatographic parameters for analysis of DTG and all impurities presented at Figure 38

5.2 ANALYSIS OF DTG WITH IMPURITY A AND B

Even though the optimized chromatographic conditions are able to separate all impurities, it is not in concern of this Diploma Thesis. Separation of the mixture of DTG and its enantiomer (impurity A) and its diastereomer (impurity B), which is a spotlight of this thesis is shown again but without other impurities in Figure 39, Table 4. All substances have concentration of 15.15µg/ml and run at 1ml/min flow rate.

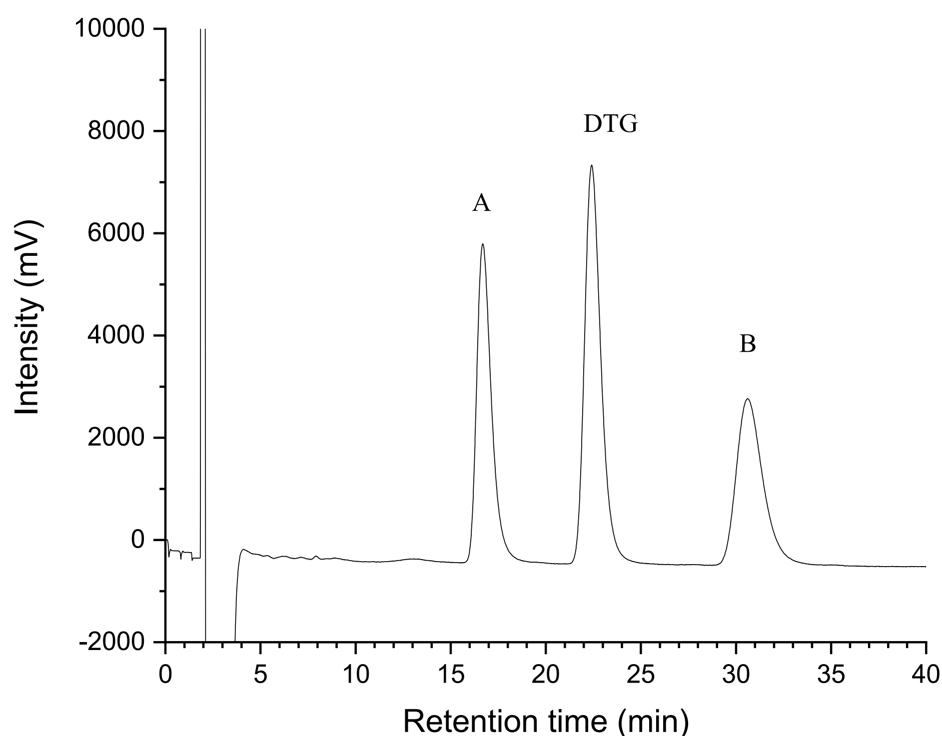


Figure 39: Chromatogram of analysis of DTG and imp. A and imp. B (each at concentration of 15,15 µg/ml and run at flow rate 1ml/min)

Peak #	Name	Ret. time	Resolution
1	Imp. A	16.68	0.00
2	DTG	22.41	1.89
3	Imp. B	30.61	1.54

Table 4: Chromatographic parameters of DTG and imp. A and imp. B (Figure 39)

The chromatogram (Figure 39) has proven that these chromatographic conditions are capable of successful chiral separation of dolutegravir.

System suitability test and recovery test were other required tasks from regulatory body WHO to be undertaken.

5.3 SYSTEM SUITABILITY TEST

System suitability test (SST) is also interpreted as a check of the system before the use. Some selected characteristic of the system is set as criteria for its proper separation conditions. (36)

The resolution between two closest peaks (in this case, impurity B and impurity D) were chosen like a significant marker in SST. Figure 40 shows a chromatogram of SST test and the concentration of impurity B is 50µg/ml as well as of impurity D. And the resolution there is above value of 1.5 which correspondents to base line separation and so satisfying result, see Table 5. This concentration of 50µg/ml has given a proof that even so high concentration can be base line separated.

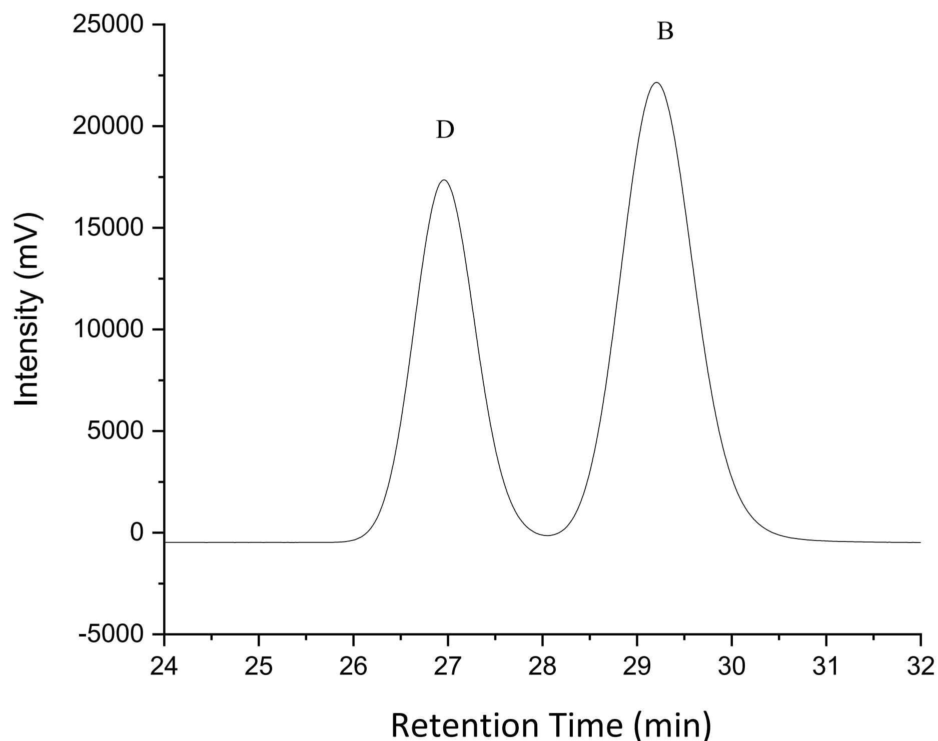


Figure 40: Chromatogram of analysis of SST – imp. B and imp. D (each at concentration of 50 µg/ml and run at flow rate 1ml/min)

Peak #	Name	Ret. time	Resolution
1	Imp. D	26.95	0.00
2	Imp. B	29.20	1.62

Table 5: Chromatographic parameters of SST – imp. B and imp. D (Figure 40)

5.4 ANALYSIS OF REAL DRUG SAMPLES

Before every measurement, blank was run and checked for clear base line to ensure further correct quantification (Figure 41). Normalization was applied for quantification of impurities in these real samples, with the impurity limit of 0,15% (calculated from 1mg/ml solution).

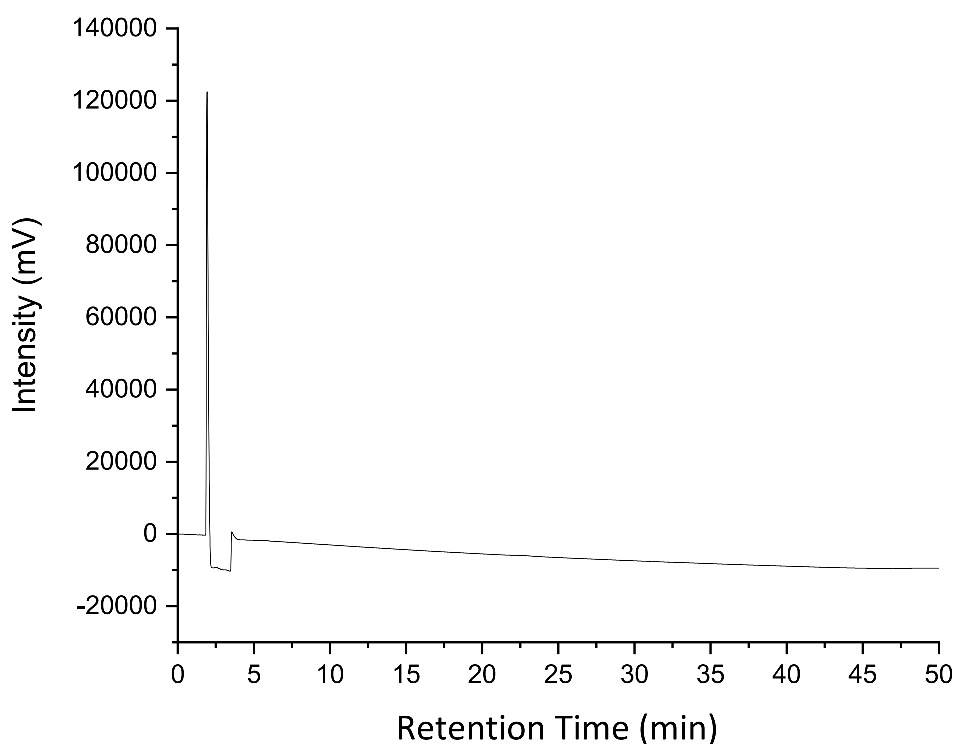


Figure 41: Chromatogram of analysis of the blank

5.4.1 Dolutegravir API

1st tested API batch was **Dolutegravir Sodium Aurobindo 34** of 1mg/ml concentration (Figure 42). **2nd** tested was **Dolutegravir Sodium Aurobindo 35** of 1mg/ml concentration (Figure 43) and **3rd** was **Dolutegravir Sodium Cipla** of 1mg/ml concentration (Figure 44).

Impurity A, B and D were found in each sample batch. All of them were below the limit (see the value of Area% in Table 6 for the batch of Aurobindo 34, Table 7 for Aurobindo 35, and Table 8 for Cipla batch) and so all batches of API passed the test for enantiomer and diastereomer purity.

In summary, all Dolutegravir API batches passed this purity test. Impurities A and B were below the 0,15% - the required limit. The average values of at least three measurements are presented in Table 9.

API batches *1mg/ml = 100% of API*	Area %				Result
	Imp. A	DTG	Imp. D	**Imp. B**	Limit: 0.15%
Aurobindo34	**0.005**	99.879	0.028	**0.087**	**passed**
Aurobindo35	**0.005**	99.890	0.025	**0.080**	**passed**
Cipla	**0.006**	99.893	0.009	**0.092**	**passed**

Table 9: Summary of impurity content for DTG - API batches

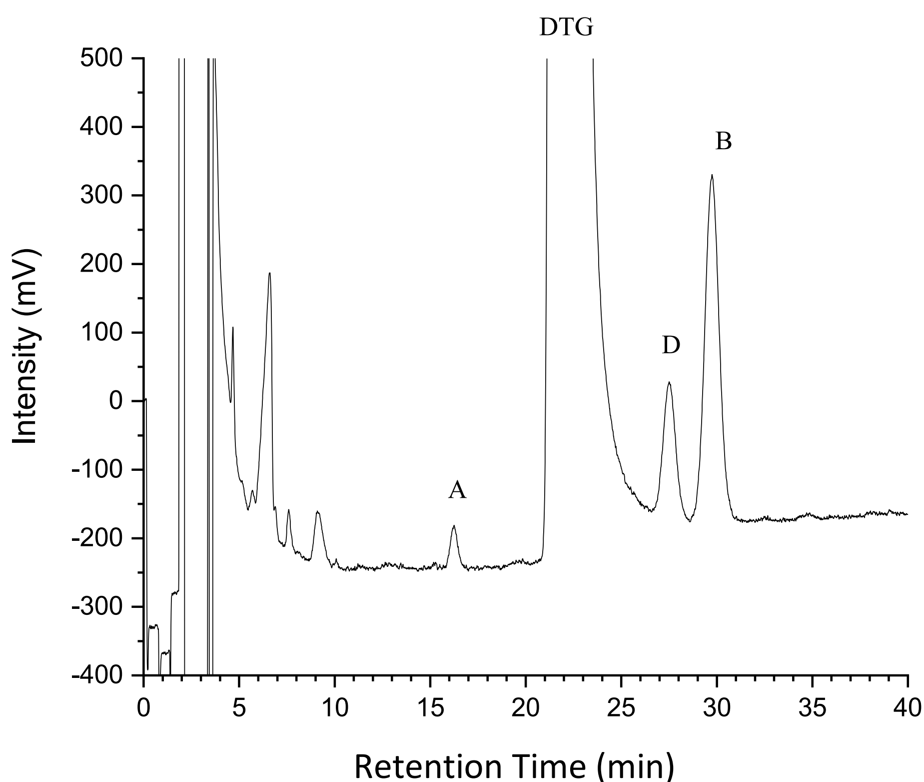


Figure 42: Chromatogram of analysis of API - DTG_Aurobindo34
(at concentration of 1mg/ml and run at flow rate 1ml/min)

Peak #	Name	Ret. time	Area	Resolution	Area %
1	Imp. A	16.24	1575	0.000	**0.005**
2	DTG	22.03	30068366	6.86	99.881
3	Imp. D	27.51	8042	5.05	0.027
4	Imp. B	29.75	26073	1.78	**0.087**
Total			30104056		100.000

Table 6: Results of analysis of API - DTG_Aurobindo34 presented at Figure 42

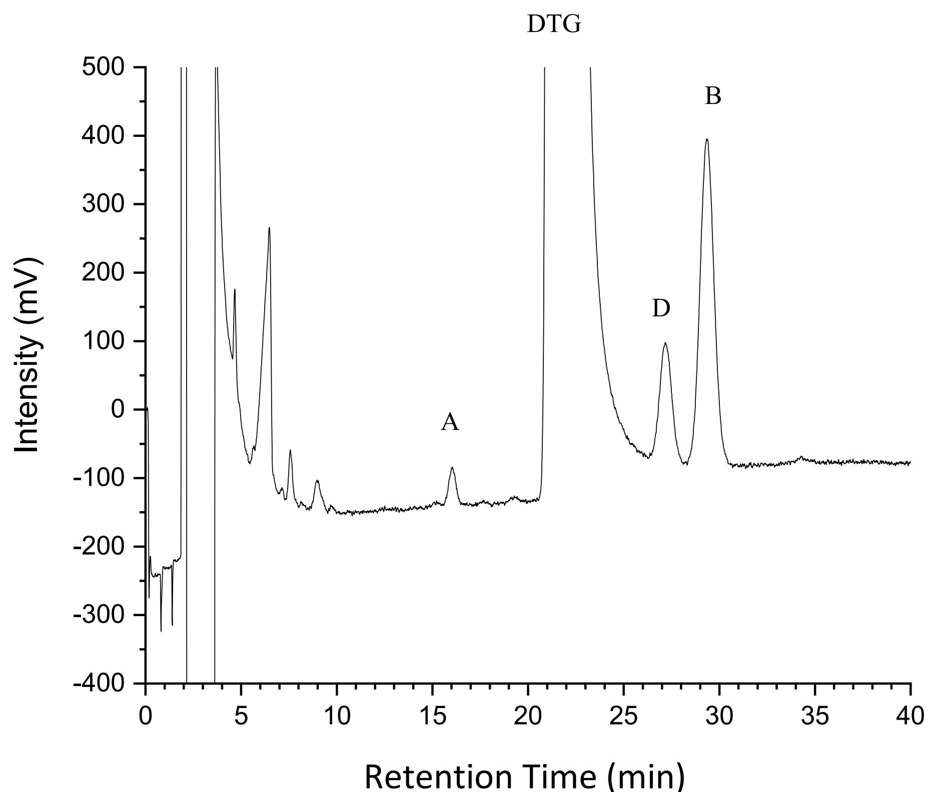


Figure 43: Chromatogram of analysis of API - DTG_Aurobindo35 (at concentration of 1mg/ml and run at flow rate 1ml/min)

Peak #	Name	Ret. time	Area	Resolution	Area %
1	Imp. A	16.02	1424	0.00	**0.005**
2	DTG	21.78	29794154	6.90	99.890
3	Imp. D	27.17	7579	5.08	0.025
4	Imp. B	29.33	23837	1.77	**0.080**
Total			29826994		100.000

Table 7: Results of analysis of API – DTG_Aurobindo35 presented at Figure 43

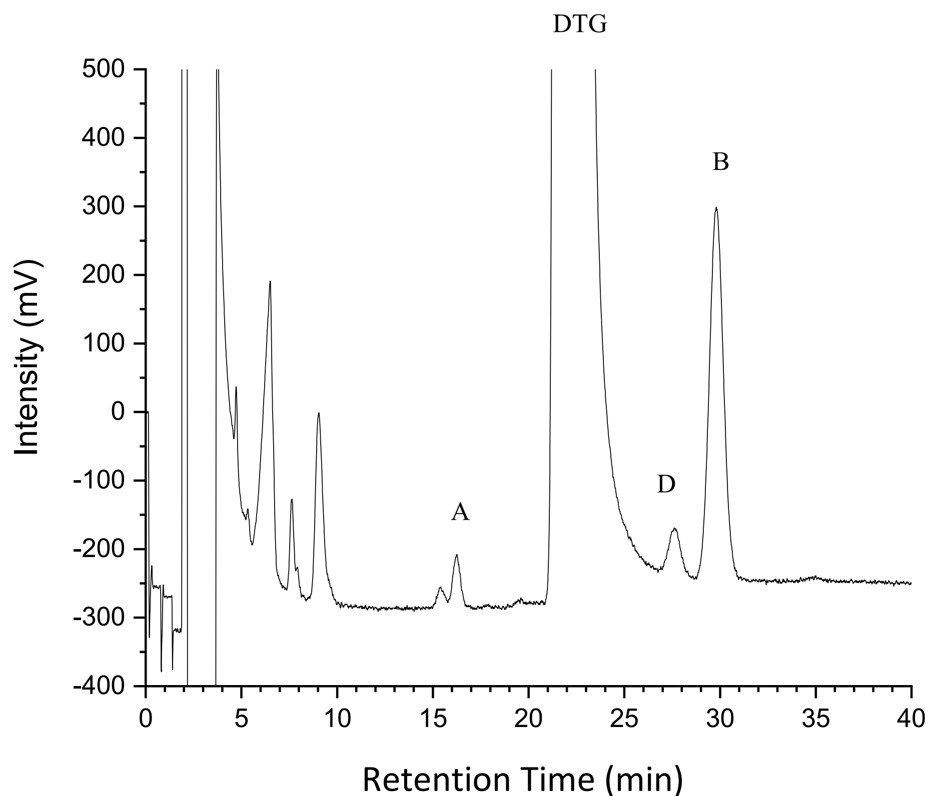


Figure 44: Chromatogram of analysis of API - DTG_Cipla (at concentration of 1mg/ml and run at flow rate 1ml/min)

Peak #	Name	Ret. time	Area	Resolution	Area %
1	Imp. A	16.23	1786	0.000	**0.006**
2	DTG	22.12	30583523	6.88	99.894
3	Imp. D	27.61	2505	5.36	0.008
4	Imp. B	29.79	28016	1.86	**0.092**
Total			30615830		100.000

Table 8: Results of analysis of API - DTG_Cipla presented at Figure 44

5.4.2 Dolutegravir racemic mixture

Cipla company declared purity of 49,8% of this racemic mixture. The result of this sample analysis was similar, around 50% of DTG and 50% of enantiomer impurity (see Table 10). Figure 45 is a chromatogram of this racemic mixture, 100µg/ml concentrated.

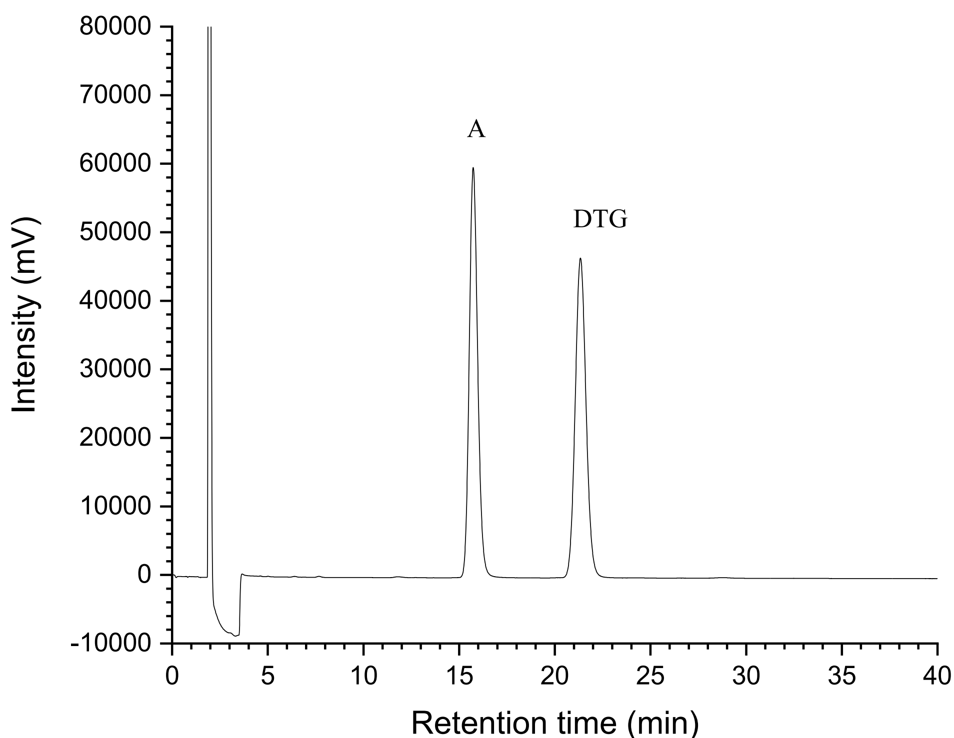


Figure 45: Chromatogram of analysis of DTG - racemic mixture (at concentration of 100µg/ml and run at a flow rate 1ml/min)

Peak #	Name	Ret. time	Area	Resolution	Area %
1	Unknown	11.79	3400	0.000	0.093
2	Imp. A	15.73	1831920	5.097	**49.902**
3	DTG	21.34	1835711	6.078	**50.005**
Total			3671031		100.000

Table 9: Results of quantification of DTG - racemic mixture presented at Figure 45

5.4.3 Dolutegravir TBL

In order to assay impurity in the TBLs, three extracts S(1), S(2), S(3) were prepared ahead and the followed measurements started with the samples of the lowest concentration to the highest concentration, in this order: S(2) = 1,4µg/ml, then S(1) = 700µg/ml and S(3) = 1mg/ml at the end.

The concentrations of solutions were chosen for following reasons. S(1) was an intermediate state for dilution, the 700µg/ml concentration was not directly used for analysis, but it can be potentially useful when one can check if any impurity already appeared at this concentration.

Meanwhile, S(2) represented the limit of impurity and so could be applied for this purpose. Because potential 1,4µg/ml concentration of impurity would take an area of 0,14% in a solution of 1000µg/ml concentration (S(3)), according to Equation 17 (see chapter 2.4.2.1 Normalization). Therefore, the area value of S(2) could be used as impurity limit.

Measurements of S(3) samples of 1mg/ml concentration were the actual analysis of the impurity detection in the TBLs, either proving or disproving the applicability of pharmaceutical products.

Figure 46 shows an example of S(2) chromatogram, where only DTG was detected and Table 11 provides main chromatographic parameters.

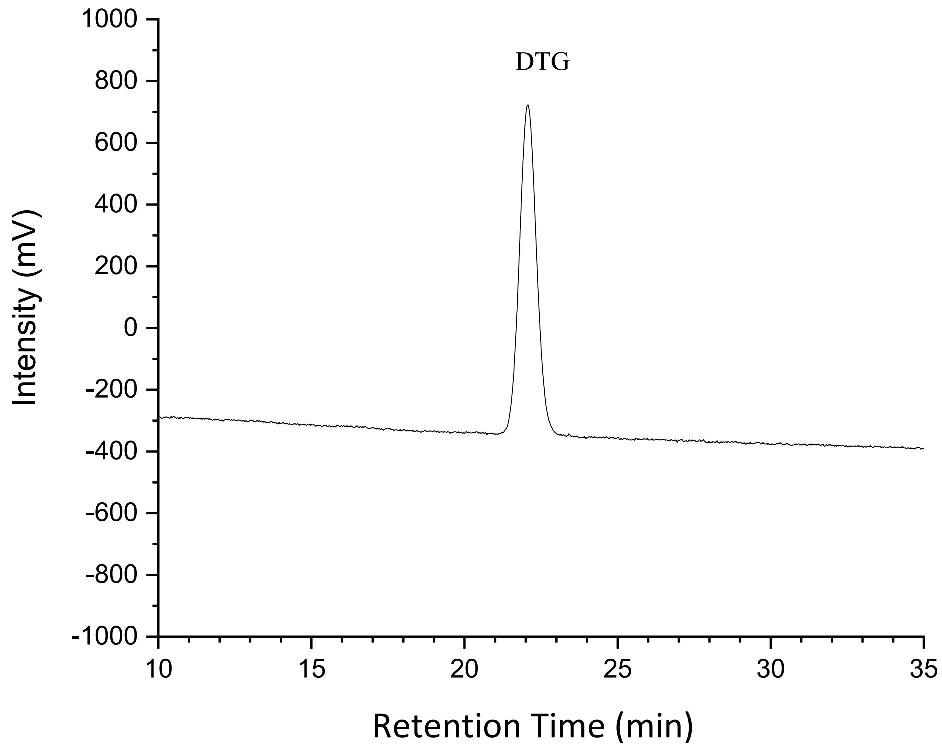


Figure 46: Chromatogram of analysis of DTG – TBL - S(2) = 1,4µg/ml

Peak #	Name	Ret. time	Area
1	DTG	22.07	41928

Table 10: Chromatographic parameters of DTG – TBL - S(2) = 1,4µg/ml presented at Figure 46

An example of S(1) chromatogram is presented as Figure 47 and the calculated content of impurity B and D is provided in Table 12.

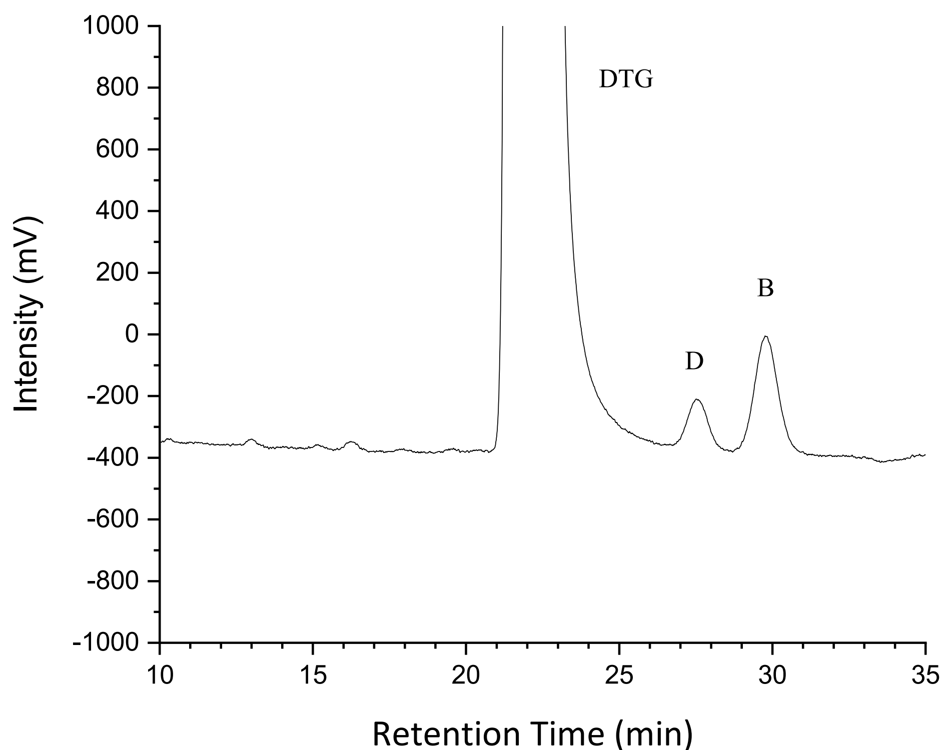


Figure 47: Chromatogram of analysis of DTG – TBL - S(1) = 700µg/ml

Peak #	Name	Ret. time	Area	Resolution	Area %
1	DTG	22.06	22205351	0.000	99.883
2	Imp. D	27.52	6733	4.991	0.030
3	Imp. B	29.76	19223	1.767	**0.086**
Total			22231307		100.000

Table 11: Chromatographic parameters of DTG – TBL - S(1) = 700µg/ml presented at Figure 47

Analysis of solutions S(3) = 1mg/ml corresponded to the essential analysis of TBL. Figure 48 shows a chromatogram of S(3) separation. Dolutegravir, impurity A and

B and D were detected in these TBL samples. Importantly, both impurities, impurity A and B were below the permitted maximal limit (Table 13). Thus, the result of the analysis proved proper purity characteristics in certain way of these dolutegravir tablets.

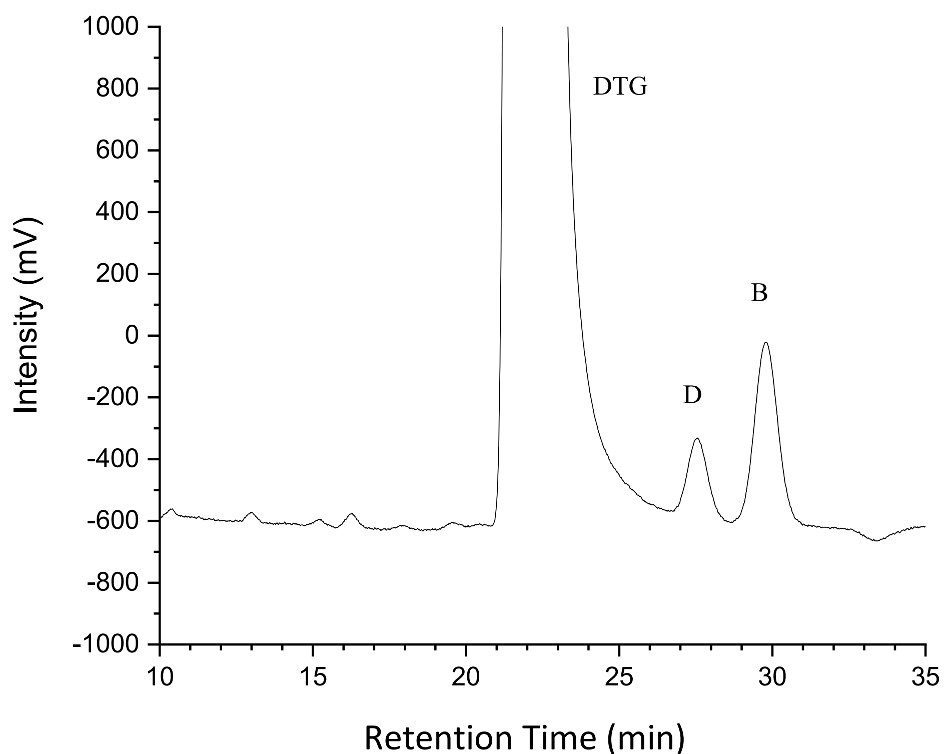


Figure 48: Chromatogram of analysis of DTG – TBL - S(3) = 1mg/ml

Peak #	Name	Ret. time	Area	Resolution	Area %
1	Imp. A	16.27	1201	0.000	**0.003**
2	DTG	22.07	34698137	6.877	99.876
3	Imp. D	27.54	11385	4.965	0.033
4	Imp. B	29.79	30406	1.771	**0.088**
Total			34741129		100.000

Table 12: Chromatographic parameters of DTG – TBL - S(3) = 1mg/ml presented at Figure 48

In Summary: The tested batch of dolutegravir TBL passed this purity test. Impurities A and B were below the 0,15% - the required limit. The average values of at least 3 measurements are presented in Table 14.

TBL	Area %				Result
	Imp. A	DTG	Imp. D	**Imp. B**	Limit: 0,15%
S(2) = 1,4µg/ml	NF	100.000	NF	NF	**passed**
S(1) = 700µg/ml	NF	99.884	0.030	**0.086**	**passed**
S(3) = 1mg/ml	**0.003**	99.877	0.033	**0.089**	**passed**

NF = not found

Table 13: Summary of impurities content in DTG – TBL

For summation of all impurities: according to the average area value of S(2), which was 42693, all areas of impurities in S(3) did not reach this value by far. It was another proof of these TBL purity.

5.5 Recovery Test

Recovery was tested for the impurity A and B, each of 1µg/ml concentration was added to solution of dolutegravir API batch Aurobindo 34 (1mg/ml).

The average areas of all peaks detected in concentrated dolutegravir API Aurobindo34 solution (1mg/ml) is in Table 15. As shown earlier, in Figure 41, this sample contained also impurity A, B and impurity D.

Peak #	Name	Ret. time	Area	Area %
1	Imp. A	16.29	1610	**0.005**
2	DTG	22.11	31085942	99.88
3	Imp. D	27.59	8807	0.028
4	Imp. B	29.77	27147	**0.087**
Total			31123506	100.000

Table 14: Average of API - DTG_Aurobindo34 (at concentration of 1mg/ml and run at a flow rate 1ml/min)

Theoretically, spiked samples should have the same area values as unspiked samples but plus some extra area of 1µg/ml spiked addition. Predicted situation of spiked excess is listed in Table 16. A chromatogram of recovery test is shown in Figure 49.

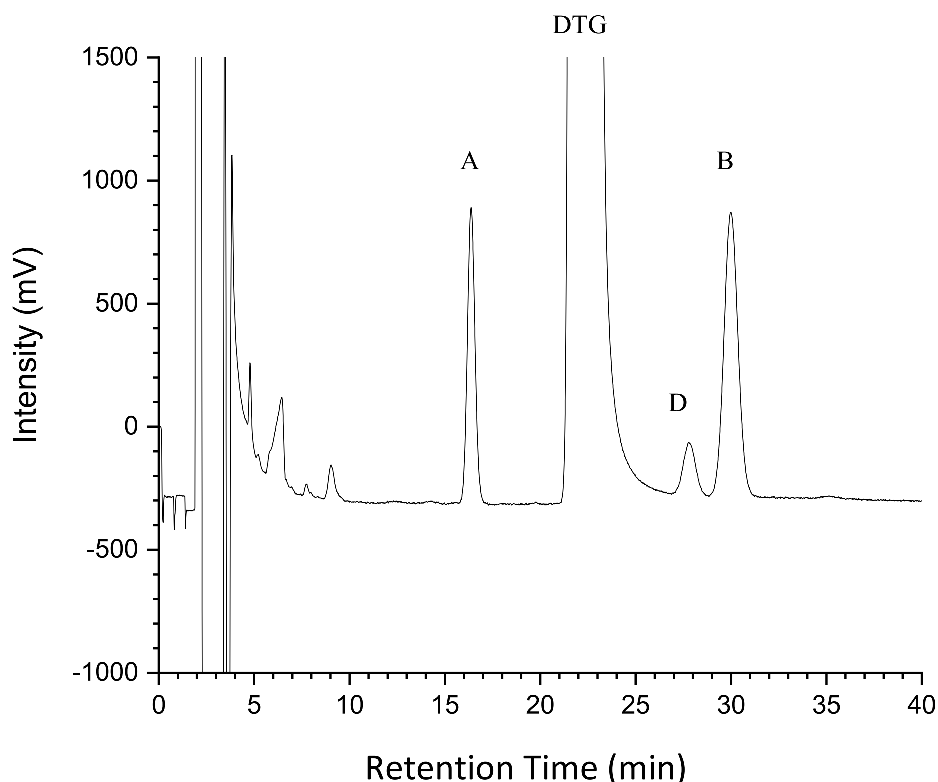


Figure 49: Chromatogram of analysis of Recovery test = DTG_Aurobindo34 and spiked impurities A and B (DTG at concentration of 1mg/ml, impurities each at concentration of 1µg/ml and run at flow rate 1ml/min)

Peak #	Name	Ret. time	Area	Resolution	Area %
1	Imp. A	16.37	33536	0.000	**0.105**
2	DTG	22.27	31919766	6.676	99.682
3	Imp. D	27.78	9318	5.025	0.029
4	Imp. B	29.98	58983	1.731	**0.184**
Total			32021603		100.000

Table 15: Chromatographic parameters of recovery test presented at Figure 49

Now, knowing all values, the recovered amount can be calculated, see Table 17. A simple calculation (Equation 17 in chapter 2.4.2.1 Normalization) could be done to realize that 1µg/ml of spiked impurity in 1mg/ml sample actually owns the area of 0,1%. And so, for instance, there was 0,087% of impurity B occurring in API sample, when the 1µg/ml of impurity B was spiked to the sample, then the samples had 0,184% of impurity B. Therefore, the value after subtraction (0,184 - 0,087 = 0,096%) was pointing out that the sample of 1mg/ml really had been enriched with the spiked amount of 1µg/ml (0,1%) and recovered the most of it (0,096%). Thus, the analysis resulted in high accuracy of the technique.

Recovery	Area %			
	Imp. A	DTG	Imp. D	**Imp. B**
Recovery test (RT) = API + spiked impurities	**0.106**	99.682	0.029	**0.184**
API	**0.005**	99.879	0.028	**0.087**
Difference (= RT - API) = recovered/spiked amount	**0.101**			**0.096**

Table 16: Calculation for recovered amount of Figure 49

5.6 CONCLUSION

The suitable HPLC method for separation of chiral impurities (A and B) of dolutegravir was optimized. Apart from these chiral impurities the method was capable of separation of other related products (impurity C, D, E and F). The method was successfully applied for analysis of selected drug samples provided from pharmaceutical companies (three batches of API, racemic mixture and tablets). Every analysis resulted in lower content of impurities A and B then it was required (limit 0,15%). These data as well as data of system suitability test and recovery test were demanded from regulatory body. The optimized HPLC method presented in this thesis will be incorporated to the novel monograph in The International Pharmacopeia. The monograph of Dolutegravir Sodium is supposed to be included in its ninth edition.

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8 ABREVIATIONS

α	Separation factor
A	Area
A_i	Sum of all areas in the mixture
A_S	Symmetry of a peak
ACN	Acetonitrile
AIDS	Acquired Immune Deficiency Syndrome
API	Active pharmaceutical ingredient
C18	Octadecyl silica column
CDR	Chiral derivatizing reagent
CE	Capillary electrophoresis
CSP	Chiral stationary phase
d	Diameter
D	Dextrorotary
DTG	Dolutegravir
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FDA	Food and Drug Administration
GMP	Good Manufacturing Practice
GS	Gas chromatography
H	Height of a single theoretical plate
H_2O	Water
HIV	Human Immunodeficiency Virus
HPLC	High performance liquid chromatography
ICH	International Conference On Harmonisation
Imp.	Impurity
k	Retention factor
K_C	Distribution constant
L	Length of a column

L	Levorotary
LC	Liquid chromatography
MeOH	Methanol
MP	Mobile phase
N	Number of theoretical plates
OPA	Orthophosphoric acid
Ph. Int.	The International Pharmacopoeia
R	Rectus, right
R_S	Resolution
RT	Recovery test
S	Sinister, left
S(1 or 2 or 3)	Solution (1 or 2 or 3)
SP	Stationary phase
SST	System suitability test
TBL	Tablet
TLC	Thin layer chromatography
t_M	Hold-up time
t_R	Retention time
UV	Ultraviolet light
VIS	Visible light
V_m	Volume of the mobile phase
V_M	Hold-up volume
V_R	Related volume
V_s	Volume of the stationary phase
v/v	Volume per volume
W	Width of the peak
W_h	Width at half-height
WHO	World Health Organization

9 REFERENCE

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