Analysis of Drug Substances by Using New Concepts of HPLC

and Development of Some HPTLC Methods

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Amal Ammar and Peter Surmann, "*Improvement of etodolac purity test by reversed phase high-performance liquid cgromatography*". ***Die Pharmazie*** 12, **2008**, 913- 914.

- **Amal Ammar and Peter Surmann**, "*Development of the etodolac purity test by reversed phase high- performance liquid chromatography*". Deutsche Pharmazeutische Gesellschaft (DPhG), Frei Universität Berlin, **2006**. "Poster presentation"
- **Amal Ammar**, International Symposium for High performance Thin- Layer Chromatography, Berlin, **2006**. "Worckshope No.1"
- **Amal Ammar** , Chrom Forum Berlin, 2007. "Course about HPLC"
- **Amal Ammar and Peter Surmann**, "*Purity Assessment of Spiramycin by HPLC method*". 31st International symposium on high performance liquid phase separation and related techniques, Gent, Belgium, (**2007**). "Poster presentation"
- **Amal Ammar and Peter Surmann**, "*Development of the etodolac purity test by reversed phase high- performance liquid chromatography*". 31st International symposium on high performance liquid phase separation and related techniques, Gent, Belgium, (**2007)**. "Poster presentation"
- **Amal Ammar and Peter Surmann**, "*Fast analysis of Etodolac in the presence of its contaminant by using monolithic C18 HPLC column*". The 11th Scientific Congress of t he Association of the Colleges of Pharmacy in the Arab World, Dat El- Emad towers, Tripoli- Libya , **2007**. "Poster presentation"
- **Amal Ammar** , Chrom Forum Berlin, **2008**. "Kurse about HPLC"
- **Amal Ammar and Peter Surmann**, "*Developed an HPLC Method for Fast Analysis with Low Back Pressure by using Monolithic C18 Column*". Deutsche Pharmazeutische Gesellschaft (DPhG), Frei Universität Berlin **2008** "Poster presentation"
- **Amal Ammar and Peter Surmann**, "*Fused- Core Particle HPLC Column for High Speed and High Efficiency Separation*". Deutsche Pharmazeutische Gesellschaft (DPhG), Frei Universität Berlin **2008** "Poster presentation"

AF	Asymmetry Factor, AF= A/B At 10% Of Peak Height
A/B	A and B are tow half widths at each side of peak center
AUC	Area Under Curve
Bar	Atmospher
cm	Centimeter
DAD	Diode-Array Detector
DP	Particle Diameter
EC	Capillary Electrophoresis
F	Flow Rate
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
I.D.	Internal Diameter Of The Column
LC	Liquid Chromatography
LOD	Limit Of Detection
LOQ	Limit Of Quantitation
mg	Milligram
ml	Milliliter
mm	Milimeter
µ	Micro
min	Minute
M	Molar
ng	Nanogram
RP	Reversed-Phase
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
RSD	Relative Standard Deviation
RSE	Relative Standard Error
SD	Standard Deviation
SE	Standard Error
S/N ratio	Signal-To-Noise Ratio
TLC	Thin Layer Chromatography
UPLC	Ultra Performance Liquid Chromatography

UV/VIS	Ultraviolet/Visible
V/V	Volume-By-Volume
ETD	Etodolac
SPR	Spiramycin
SI	Spiramycin I
SII	Spiramycin II
SIII	Spiramycin III
TRX	Troxerutin
DOX	Doxycycline
OTC	Oxytetracycline
MTC	Metacycline
CLD	Clindamycin
ROX	Roxithromycin
CON	2-(7-Ethylindol-3yl)-Ethanol
EDTA	Ethylene Diamine Tetraacetic Acid
L	Column Length
N	Theoretical Plate Number, $N=16\ (T_r/W)^2$
$\mathbf{R_f}$	Retention Factor
$\mathbf{R_s}$	Resolution Value, $R_s = 2(T_{r2} - T_{r1}/W_2 + W_1)$
$\mathbf{R^2}$	Coefficient Of Determination
T	Absolute Temperature
$\mathbf{T_r}$	Retention Time
W	Peak Width At Base
$\mathbf{W_{1/2}}$	Peak Width At Half- Hieght

1. Introduction

1.1. Definition of High performance liquid chromatography (HPLC):

HPLC is a type of Liquid chromatography (LC) which is a separation technique where analyses are separated by virtue of differing solubilities between a liquid mobile phase and a liquid or solid stationary phase (Snyder and Kirkland, 1979). In HPLC the mobile phase is forced through a reusable column by means of a pumping system and the stationary phase is porous particles that are densely packed inside the column.

The reversed phase HPLC (RP-HPLC) is the most widely used in analytical techniques in the European pharmacopoeia (Pharm. Eur.). It is applied for identification; test the purity of bulk drugs and for quantitative analysis of the main compounds in the samples and their related impurities. In RP-HPLC the stationary phase is a hydrophobic legend chemically bonded onto a particulate support. RP-HPLC is generally used to separate small polar to semi-polar molecules (Snyder et al., 1997).

1.2. Definition of Thin layer chromatography (TLC):

TLC is a chromatography technique used to separate mixtures (Vogel, 1989). It involves a stationary phase consisting of a thin layer of adsorbent material, usually silica gel, aluminum oxide, or cellulose immobilized onto a flat, inert carrier sheet. A liquid phase consisting of the solution is drawn up the plate via capillary action. The separation is based on the polarity of the components of the compound in question. TLC is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture.

1.3. Definition of High-Performance Thin Layer Chromatography (HPTLC)

HPTLC is an analytical technique based on TLC, but with enhancements intended to increase the resolution of the compounds to be separated and to allow quantitative analysis of the compounds. Some of the enhancements such as the use of higher

quality TLC plates with finer particle sizes in the stationary phase which allow better resolution (Reich and Schibli, 2007).

1.4. Disadvantages of some official HPLC and TLC methods:

Some of the official HPLC methods which used for the analysis of some drugs in the Pharm. Eur. suffered from some drawbacks. Many of these drawbacks are related primarly to the use of the conventional columns which have low resolution power and consume long run time (e.g. the run time was 100 min. in HPLC official method for Roxithromycin (ROX). The column is the only device in HPLC system which actually separates an injected mixture. Column packing materials are the “media” producing the separation, and properties of this media are of primary importance for successful separations.

The use of the gradient elution is the other reason behind the disadvantages of some official HPLC analytical methods because of its own disadvantages as the long time needed for column (re-) equilibration, limited choice of detectors, base-line drift on varying the eluent, lower signal-to-noise and signal-to-background ratios, spur peaks (impurities in weak eluent) and increased instrument complexity (Katz, 2002).

Furthermore, some of official HPLC methods in Pharm. Eur. are using sample solvent different than that which used as mobile phase which can affects the analysis results of some drugs. In addition to unsuitable column temperature which is used in some official HPLC method (e.g. 15 Cº and 60 Cº for analysis of ROX and Doxycyclin (DOX) respectively).

Also, there are some drawbacks of the official TLC analysis of some drugs, as using a large developing chamber which requires large TLC pates and consume large quantity of the mobile phase as well as developing time. Complex mobile phase for some TLC purity test and type of sample solvent (e.g. it contains high quantity of water) are also important disadvantages.

1.5. Aim of the thesis:

1. Development and optimization of some official HPLC analytical methods of some pharmaceutical drugs which have important uses and difficult in the HPLC analysis.

2. Development and optimization of some official TLC methods of some pharmaceutical drugs and their related degradation, impurities and substances, which complain some disadvantages in TLC purity test. The choosen drugs are analyzed by both HPLC and TLC in the Pharm. Eur.

3. Validation of the developed methods.

4. Comparison between the TLC and HPLC methods as analytical tools used in terms of qualitative and quantitative analysis.

5. Comparison between different columns with different packing materials to prove what is the best for this study.

1.6. Proposed solutions for the problems of the official HPLC and TLC methods:

The disadvantages which are mentioned earlier in this chapter can be avoided by developing of the HPLC method e.g. by the effective use of a small diameter packing material and columns with short length which allow the analyst to perform the chromatographic separations faster and with better resolution.

One of the columns which can be used is the monolithic column which replaces the bed of packed particles. These columns are able to produce equivalent efficiencies of packed particle columns but with much lower flow resistances, enabling much higher velocities (Kazakevich and LoBrutto, 2007). Also, the fused-core particles have recently been introduced in chromatographic separations for the same reason.

Development of a suitable isocratic elution instead of gradient elution to avoid many disadvantages which are resulted from the use of gradient elution, and also the choice of the mobile phase system with lower viscosity to avoid the need for high column temperature.

The use of HPTLC plates with small horizontal chamber which have advantages as high efficiency of separation and also reduced analysis time with low consumbtion of mobile phase quantity. Moreover, the development of the mobile solvent and/or sample solvent is recommended to enhance the efficiency of separation of the drug.

2. Theoretical Part

2.1. Chromatography background:

At the beginning of the twentieth century, the Russian botanist Mikhail Tswett invented and named chromatography. He separated plant pigments by passing solution mixtures through a glass column packed with fine particles of calcium carbonate. The separation of those pigments appeared as colored bands on the column. Tswett named his separation method for the two Greek words “chroma” and “graphein,” which mean “color” and “to write,” respectively (Skoog et al., 1998). In the past six decades, chromatography has been extensively applied to all branches of science. The 1952 Nobel Prize in chemistry was awarded to A. J. P. Martin and R. L. M. Synge for their contributions to chromatographic separations, which tremendously impacted chemistry-related sciences. More impressively between 1937 and 1972, a total of 12 Nobel Prizes were based on work in which chromatography was a key tool.

In all chromatographic separations, the sample is carried by the mobile phase, which may be a gas, a liquid, or a supercritical fluid. The mobile phase is then percolated through an immiscible stationary phase that is fixed on a solid substrate. When the sample passes through the stationary phase, species are retained to varying degrees as a result of the physicochemical interaction between the sample species and the stationary phase. The separation of species appears in the form of bands or zones resulting from various retentions. Chemical information can thus be analyzed qualitatively and/or quantitatively on the basis of these separated zones.

Based on the physical means by which the stationary phase and mobile phase are brought into contact, chromatography can be classified as planar or column (Giddings, 1991). In planar chromatography the stationary phase is supported on a flat plate or a piece of paper, while the mobile phase is usually driven by capillary force, gravity, or an electric field. In a few cases, the mobile phase is forced under pressure, for example, in overpressure planar chromatography. When a tube holds the stationary phase, the chromatographic method is referred to as column chromatography. In column chromatography, the mobile phase is driven by pressure, gravity, or an electric field.

Because of its astonishing separation power, column chromatography has become the most frequently practiced means of analytical separation. Three types of mobile phases are used in column chromatography: liquids, gases, and supercritical fluids. Among these three types, liquids are the most frequently used. Therefore, LC is the predominant technique used in modern analytical separations. Early LC was operated in glass columns, and the mobile phase was driven by gravity. To ensure a reasonable flow rate (F), the column was packed with large particles in the 150 to 200 μm range. Such packing yielded poor results with long separation times, often several hours. Beginning in the late 1960s, small particles were packed in a steel tube, which was subjected to high pressure. Such a system dramatically improved the separation power of column chromatography; in the early years, “HPLC” stood for “high pressure liquid chromatography”. Three to ten micrometers particle diameter (dp) are commonly used as stationary phases in HPLC. Separation can thus be done in a high-performance mode, which means high resolution and short analysis time. Therefore, these newer procedures are termed “high-performance liquid chromatography” to distinguish them from the earliest methods.

2.2. Liquid chromatographic separation modes

2.2.1. Normal-phase chromatography (Adsorption Chromatography):

The principle of adsorption chromatography is known from classical column and TLC. A relatively polar material (water-soluble, hydrophilic) with a high specific surface area is used as the stationary phase, silica being the most popular, but alumina and magnesium oxide are also often used. The mobile phase is relatively non-polar (fat-soluble, lipophilic) as heptane or tetrahydrofuran. The different extents to which the various types of molecules in the mixture are adsorbed on the stationary phase provide the separation effect. Polar compounds are eluted later than non-polar compounds.

2.2.2. RP- Chromatography

Rp- chromatography is the term used to describe the state in which the stationary phase is less polar than the mobile phase. Chemically bonded octadecylsilane (ODS), an n-alkane with 18 carbon atoms, is the most frequently used stationary

phase. C8 and shorter alkyl chains and also cyclohexyl and phenyl groups provide other alternatives. Phenyl groups are more polar than alkyl groups.

The reverse of the above applies:

(a) The stationary phase is very non-polar.

(b) The mobile phase is relatively polar.

(c) A polar solvent such as water elutes more slowly than a less polar solvent such as acetonitrile. So, non-polar compounds are eluted later than polar compounds.

2.2.3. Chromatography with Chemically Bonded Phases

The stationary phase is covalently bonded to its support by chemical reaction. A large number of stationary phases can be produced by careful choice of suitable reaction partners. The RP method described above is the most important special case of chemically bonded-phase chromatography.

2.2.4. Ion-Exchange Chromatography

The stationary phase contains ionic groups (e.g. NR_3^+ or SO_3^-) which interact with the ionic groups of the sample molecules. The method is suitable for the separation of amino acids, ionic metabolic products and organic ions.

2.2.5. Ion Chromatography

Ion chromatography was developed as a means of separating the ions of strong acids and bases (e.g. Cl^-, NO_3^-, Na^+, K^+). It is a special case of ion-exchange chromatography but the equipment used is different.

2.2.6. Ion-Pair Chromatography

Ion-pair chromatography may also be used for the separation of ionic compounds and overcomes certain problems inherent in the ion-exchange method. Ionic sample molecules are ‘masked’ by a suitable counter ion. The main advantages are, firstly, that the widely available RP system can be used, so no ion exchanger is needed, and, secondly, acids, bases and neutral products can be analyzed simultaneously.

2.2.7. Size-Exclusion Chromatography

This mode can be subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solutions). Size-exclusion chromatography separates molecules by size.

2.2.8. Affinity Chromatography

In this case, highly specific biochemical interactions provide the means of separation. The stationary phase contains specific groups of molecules which can only adsorb the sample if certain steric and charge-related conditions are satisfied (cf. interaction between antigens and antibodies). Affinity chromatography can be used to isolate proteins (enzymes as well as structural proteins), lipids, etc., from complex mixtures without involving any great expenditure.

2.3. HPLC system:

The HPLC consists of pumps, an injector, column, mobile phase reservoir, oven, and detector. The injector introduces the sample into the HPLC system. This is either done by hand with a syringe, or automated with an auto-sampler. Figure 2.1 shows a simplified schematic of an HPLC system.

There are several different types of pumps available for use with HPLC. They include reciprocating pumps, which are the most common, syringe type pumps, and constant pressure pumps. The reciprocating pumps use a motor-driven piston to pump mobile phase into the column. On the backstroke, mobile phase is pulled in, and on the forward stroke, it is driven out to the column. These have the advantage of being able to achieve a wide range of flow rates. Dual and triple head pumps consist of identical units, which are 120 or 180 degrees out of phase. This type of pump system is significantly smoother.

Syringe type pumps, or displacement pumps, have a very small capacity, and are therefore most suited to small bore columns. They consist of a large syringe type reservoir, with a plunger that is activated by a motorized lead screw. The flow rate can be controlled by changing the voltage on the motor.

Constant pressure pumps use pressure from a gas cylinder to drive the mobile phase through the column. In order to generate high liquid pressures, a low-pressure gas source is needed. The solvent chamber has a low capacity, but a valve arrangement allows for rapid refill, and provides continuous mobile phase flow rate.

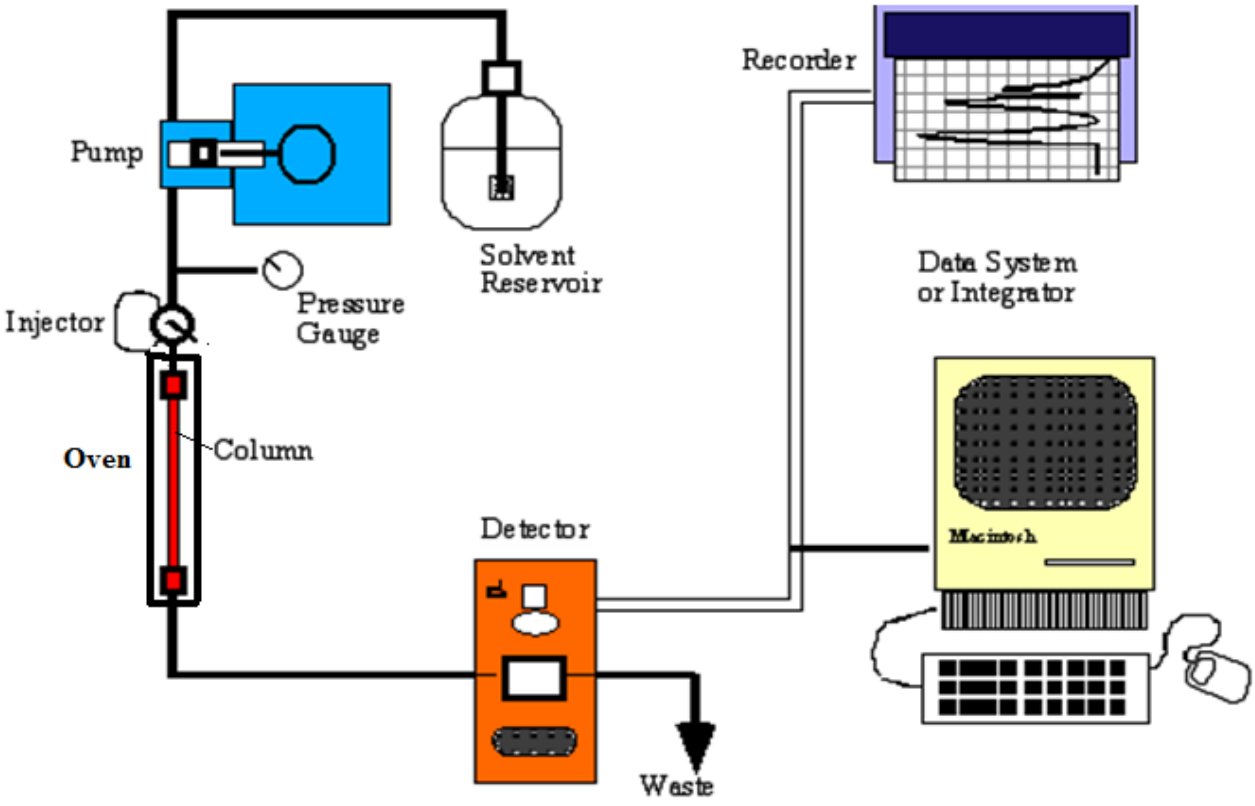


Figure 2.1: Simplified schematic of an HPLC system.(McNair, 1997)

HPLC columns are usually made of stainless steel tubing. There are two types of columns that are distinguished by the relative polarities of the mobile and stationary phases.

Guard columns are often used in front of the column. This short column helps protect the analytical column and increase its lifespan by removing larger particles and impurities before they can enter the column. The composition is similar to that of the analytical column.

There are many different detectors that can be attached to an HPLC. They include Ultra-Violet, Refractive Index, Fluorescent, Electrochemical, Mass Spectroscopy, and Light Scattering.

HPLC can be performed on three scales, preparative, analytical, or micro scale. Preparative HPLC is concerned with the isolation and/or purification of a target analyte,

While analytical and micro-HPLC involve the qualitative and/or quantitative analysis of a mixture of analyses.

Analytical HPLC generally uses column inner diameters (i.d.) of 2.1 to 4.6 mm and flow rate from 0.5 to 3 ml/min (Snyder et al., 1997). Micro-HPLC columns, on the other hand, are typically fashioned from fused silica capillaries (10 to 320 μm i.d) and use much lower F (0.1 to 100 μl/min) (Tsuda and Novotny, 1978) .

The performance of the chromatographic column is key in the separation process. Van Deemter plots (Van Deemter et al., 1956) are commonly used to describe column performance by plotting the height equivalent to a theoretical plate (HETP or H) against the average linear velocity (μ). The general form of the van Deemter equation is given by,

$$H = A + \frac{B}{\mu} + C\mu \qquad \text{eq. 1}$$

Where A, B and C are coefficients. The A term is a measure of packing efficiency and is a function of packing efficiency and particle size. The B term is a function of longitudinal diffusion, or diffusion in the mobile phase, while the C term is a function of the mass transfer between the stationary and mobile phase as well as within the mobile phase.

Figure 2.2 shows a diagram of the additivity of the three terms in the van Deemter equation. Note that the B term is dominant at low flow velocities while the C term is dominant at high flow velocities. The minimum of the van Deemter curve represents the ideal flow velocity where maximum column efficiency is obtained. It is a compromise between the B and C terms.

For most analyses and especially for fast analyses, it is desirable to operate at velocities well beyond the optimum. If the C term is minimized, band broadening (HETP) at higher velocities is minimized.

To generate a van Deemter plot, height equivalent to a theoretical plate (HETP or H) is plotted against average linear velocity (μ). H is calculated from column efficiency, N,

$$H = \frac{L}{N} \qquad \text{eq. } 2$$

Where L is the column length in micrometers and N is given by,

$$N = 5.54 * \left[\frac{tr}{w0.5}\right] 2 \qquad \text{eq. } 3$$

Where tr is the retention time in (minutes) and w0.5 is the width at one-half the height of the peak, also in (minutes). Linear velocity, μ, is reported in mm/sec and is classically calculated from the 'dead time', t_0, the time for the elution of an unretained compound. The column length, L, is reported in millimeters.

$$\mu = \frac{L}{t_0} \qquad \text{eq. } 4$$

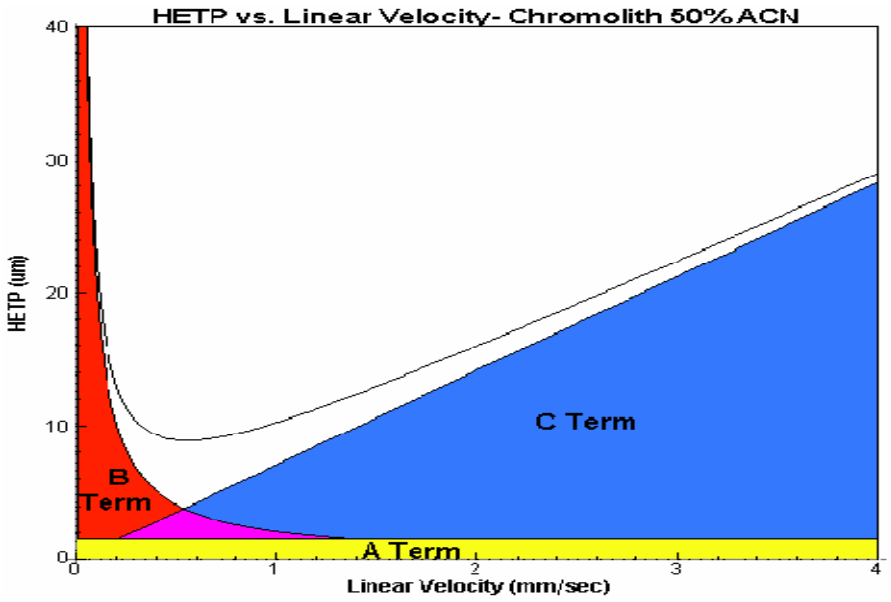


Figure 2.2: Diagram showing the additivity of the three terms of the van Deemter equation for a highly efficient column

2.4. The history of particulate columns

First, in the early years of HPLC, separations were believed to be a very slow and insensitive process. A routine HPLC separation could take several hours. In order to achieve high productivity, short, fast columns have been developed. Second, reproducible columns have been developed to improve reproducibility of separations. Efforts have been made to improve the recovery of the sample for the analysis of biological compounds. Third, durable columns have been embraced by the industry for the purpose of cost reduction. Narrow-bore and capillary columns have been developed to reduce the use of solvents. Fourth, the huge demand for separations has been boosted by the rapid development of biological analysis, which requires the analysis of complex samples, large molecules, and unstable compounds. Wide-pore, rugged, and biocompatible packing materials have been developed to meet the requirements of bioseparation.

The trend in particle-packed columns is toward the use of more uniform sized and finer particles to pack shorter columns. Shown in Figure 2.3 for the history of particle-packed columns (Majors, 2003).

In the 1950s and earlier, the column was packed with irregular-shaped nonporous particles. In 1967, spherical glass beads were first used as regular-shaped packing material.

The late 1960s and early 1970s saw the emergence of HPLC, which uses high-pressure pumps to drive the liquid phases through the column. These pumps made it possible to use fine particle-packed columns. Subsequently, the major development of packed columns has focused on decreasing the particle sizes. Small particles and short columns have dramatically improved the efficiency of HPLC separation. High throughput, high resolution, and fast columns have been developed to maximize the performance of the packed columns. Though particle-packed columns dominate the major applications, the hydraulic resistance resulting from particulate morphology is deemed to be the limit for the use of particles smaller than 1 microns. The monolithic column, which overcomes the limits of traditional columns imposed by the

backpressure, is considered to be the new generation of HPLC columns (Svec, 2003).

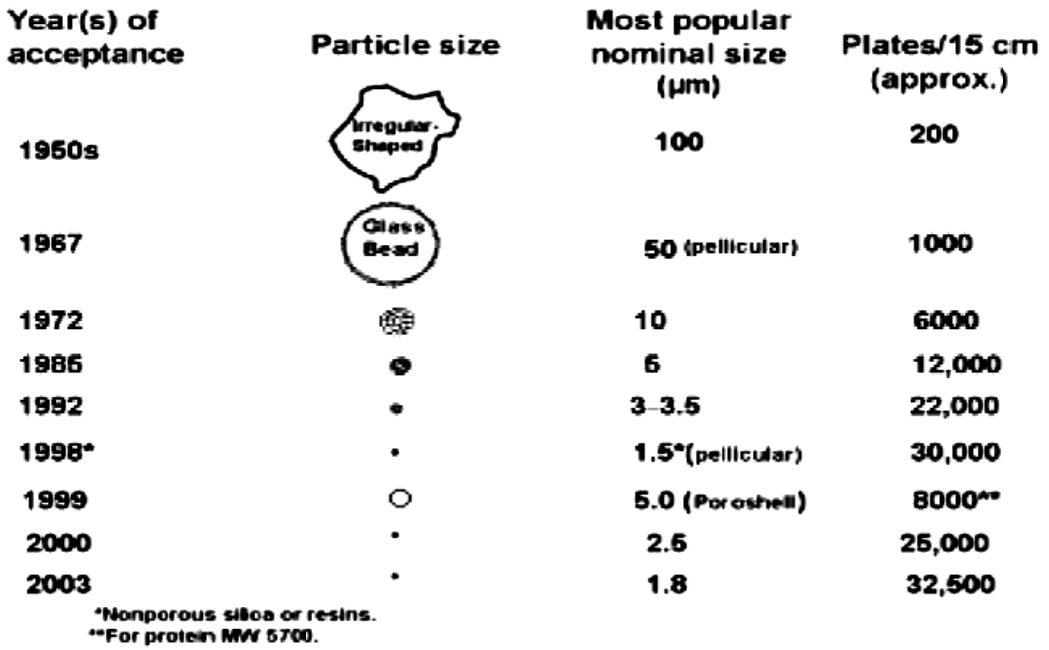


Figure 2.3. The history of packed columns (Majors, 2003).

2.5. Monolithic column

A monolithic column is a silica-based monolithic HPLC column. This technology creates highly porous rods of silica with a revolutionary bimodal pore structure. The single piece of high-purity polymeric silica gel is then clad in PEEK tubing to make the finished product.

Macroporous structure allows rapid flow (up to 9ml/min) at low pressures Each macropore is on average 2 µm in diameter and together form a dense network of pores through which the mobile phase can rapidly flow at low pressure dramatically reducing separation time.

The mesopores form the fine porous structure (130Å) of the column interior and create a very large surface area on which adsorption of the target compounds can occur. The unique combination of macropores and mesopores enables monolithic HPLC columns to provide excellent separations in a fraction of the time compared to a standard particulate column (Figure 2.4).

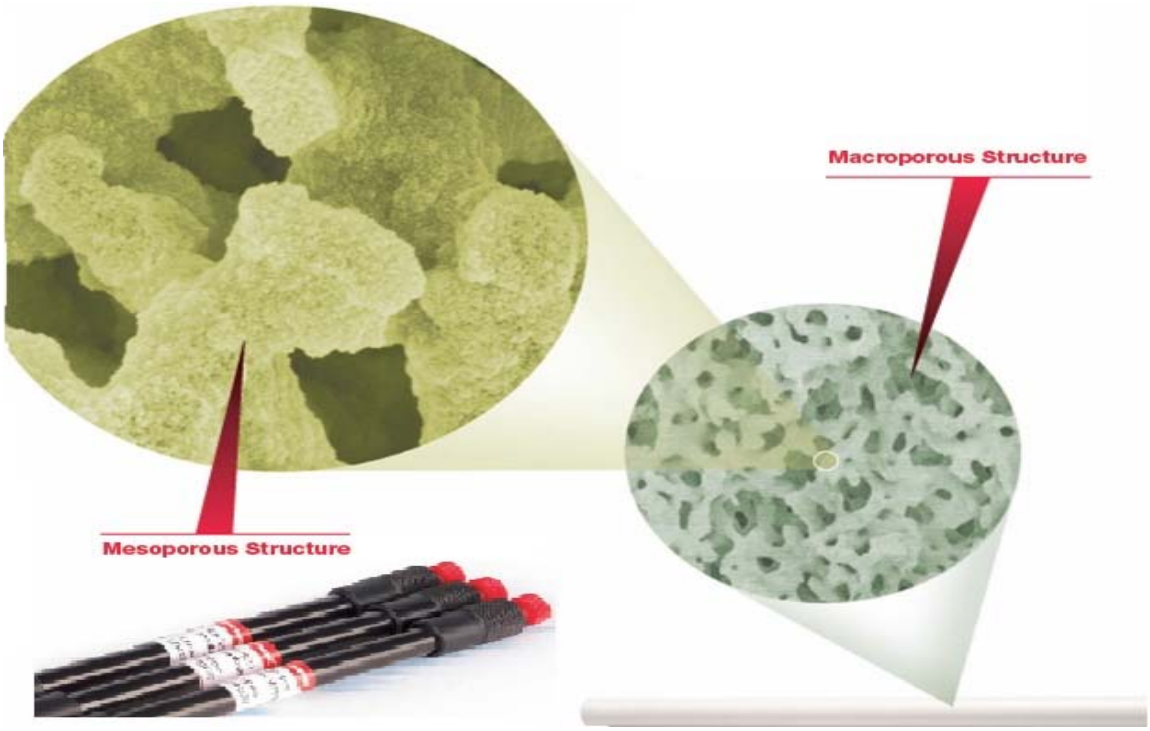


Figure 2.4. Monolithic columns.

The HPLC column has two kinds of porosity: external and internal. The external porosity of particulate columns is controlled by particle size, which is an important factor for column efficiency. Column efficiency is related to particle size: the smaller the particle, the higher the column efficiency and backpressure. Thus, improvement in column efficiency is achieved at the price of high pressure. The monolithic column succeeds in controlling external porosity. The efficiency of the monolithic column is determined by domain size, which is the sum of the skeleton and the channel. External porosity can be tuned without affecting the column efficiency by changing the channel size. Therefore, the external porosity can be optimized to produce better hydrodynamics. To maintain the same column efficiency, the monolithic column can be optimized to a much lower backpressure than the particulate column. With the hydrodynamic advantage, the monolithic column can be used for fast, high-resolution, and high-throughput separations. Figure 2.5 for comparison of the spherical packing material and monolithic silica with SEM pictures of HPLC silica particles (5μm) and silica monolithic.

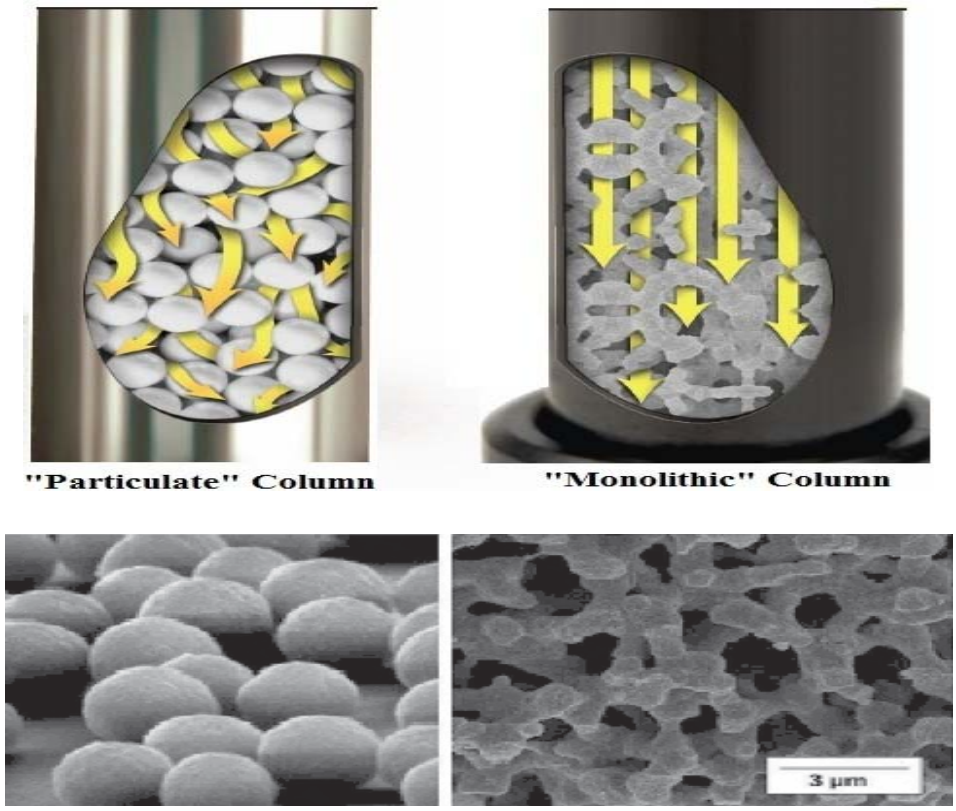


Figure 2.5. Comparison of particulate and monolithic columns. (adapted from (Leinweber and Tallarek, 2003).)

The main advantage of monolithic columns is to reduce analysis time by more than half. This advantage can be attributed to the ability to run high flow rates up to 9 ml/min with the same high quality resolution. Even at 9 ml/min, high backpressure is not a concern.

2.6. Fused-core column

Fused-Core particles (Cunliffe and Maloney, 2007) have recently been introduced as an alternative to using sub-2- µm particles in chromatographic separations. Fused-Core particles are composed of a 1.7 µm solid core surrounded by a 0.5 µm porous silica layer (dp = 2.7 µm) to reduce mass transfer and increase peak efficiency.

As early as the 1950s, van Deemter realized that peak efficiency could be improved by reducing particle size (Van Deemter et al., 1956, Van Deemter et al., 1995).

Since then, particle technology has advanced to offer a variety of stationary phases with sub-2-μm diameters.

Along with the improvement in peak efficiency, sub-2-μm particles offer higher resolution, shorter analysis times, and lower limits of detection compared to traditional HPLC particles with 3–5 μm diameters (Anspach et al., 2007a, MacNair et al., 1997, Nguyen et al., 2006). The improvement in peak efficiency, however, does come at a cost as the pressure (DP) required to flow mobile phase through a column increases as (dp) decreases, as shown in Eq. (5) (Nguyen et al., 2006, Giddings, 1991, Jerkovich et al., 2003).

$$\Delta\rho = \frac{\varphi\eta L\mu}{d_{p^2}} \qquad \text{eq. (5)}$$

Where φ is the flow resistance factor, η is mobile phase viscosity, L is the column length, and μ is the linear velocity.

Recently several column manufacturing vendors have taken a different approach and have aimed to balance efficiency and backpressure. These approaches have utilized a variety of particle sizes (1.8–2.7 μm), column lengths, and column diameters to reduce backpressure and minimize frictional heating effects while maintaining elevated performance (Anspach et al., 2007b, Way and Campbell, 2007, Nguyen et al., 2006). The most recent approach involves particles with a solid core and a superficially porous shell (Way and Campbell, 2007). These particles, termed Fused-Core.

A major benefit of the Fused-Core particle is the small diffusion path (0.5 μm) compared to conventional fully porous particles (Figure 2.6). The shorter diffusion path reduces axial dispersion of solutes and minimizes peak broadening. In fact, Ascentis Express columns are able to achieve efficiencies of 240,000 N/m, which is similar to that obtained with sub-2 μm particle columns, even though the backpressures are only 50% of that achieved under similar conditions with sub-2 μm particles.

Fused core and monolithic columns are used for the advantage of fast HPLC which will be explained in the following topic.

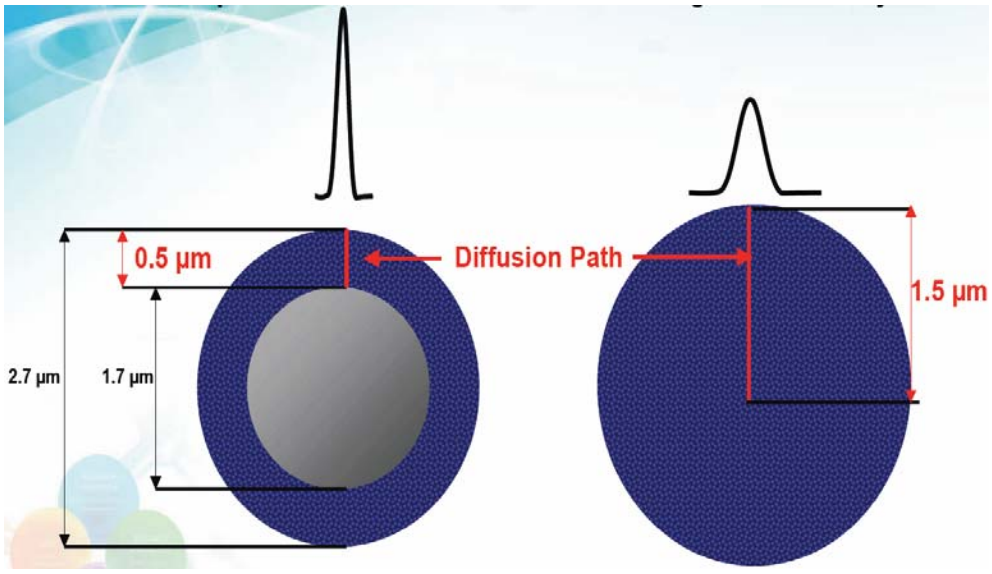


Figure 2.6. Comparison of particulate and fused core columns.

2.7. Fast HPLC

2.7.1. Definition of Fast HPLC

The term ‘fast HPLC’ is a relative one. Analysis time in of itself is a poor measurement of chromatographic performance; rather the important parameter is the number of peaks separated per unit time. For example, a 10 component run in ten minutes is more time efficient than a 2 component run in 10 minutes. Nevertheless, it should be noted that the

Terms ‘fast LC’, ‘fast HPLC’ ‘high-speed HPLC’ and ‘ultra-fast HPLC’ are commonplace in the literature without formal definition.

2.7.2. Factors affecting fast HPLC

Traditional approaches for decreasing HPLC analysis time include higher mobile phase F, shorter columns, reduced diameter and/or specialized particles and higher column temperatures (T). Each of these parameters is interrelated with the dependent parameters of analysis time, column backpressure and column efficiency. Table 2.1 lists the relationships among the six parameters, followed by a brief description on each parameter and its role in fast HPLC.

Table 2.1: Relationship between the independent parameters, L, F, dp and T with the dependent parameters of analysis time, column backpressure and column efficiency:

	(L)	**(F)**	**(dp)**	**(T)**
Analysis time	α L	α 1/F	Not Related	α $1/T^x$
Backpressure	α L	α F	α $1/(dp)^2$	α 1/T
Efficiency (N)	α L	By van Deemter	α 1/dp	α T

Perhaps the most obvious way to achieve faster HPLC analyses is to increase the mobile phase flow rate. Flow rate is inversely proportional to analysis time, so doubling the flow rate will result in halving the analysis time. Unfortunately, flow rate is also proportional to the pressure drop across the column, measured as system pressure or column backpressure.

Column lenght is directly proportional to both analyte retention time (t_R) and column efficiency. Reduction of L is acceptable as long as column efficiency remains sufficient for the separation. Column lenght is also proportional to column backpressure, so for fast HPLC shorter columns can be combined with smaller particles. The usual method for generating fast HPLC analyses is to use short columns with small particles at the highest possible flow rate.

An increase in column temperature is beneficial in at least two respects. First, increased tempreature reduces the viscosity of the mobile phase and therefore the column backpressure, permitting faster flow rate. Second, an increase in tempreature enhances analyte mass transfer, increasing efficiency at faster flow rate. The use of increased tempreature is limited, however, by the thermal stability of the analyte, the thermal stability of the stationary phase and the boiling point of the mobile phase.

2.8. TLC

2.8.1 Introduction to TLC

TLC is a LC method for separating mixtures of compounds. Although there have been many advances in sample application, chromatographic development, and detection since the first examples of thin layer separations in the early 1900s, the chromatographic principle remains the same. A small aliquot of a sample solution is applied in either a spot or band to a thin sorbent layer supported by a substrate (glass, plastic, aluminum foil) near one end of the TLC plate. After the sample has dried, the TLC plate is placed into a chamber where solvent is introduced to the end of plate where the sample was applied and capillary action wicks the solvent to the other side of the plate. Components of the sample mixture are separated-based on their different migration rates in the particular stationary and mobile phase combination. Differential migration is based on the relative affinity of each analyte for the stationary and mobile phases in the chromatographic system. Detection is often performed by visually observing the separated compounds, using either white or ultraviolet light, using necessary visualization agents to impart color or fluorescence to the compounds by using fluorogenic drivatizing agents.

TLC is employed in many areas where rapid, high-throughput, and inexpensive analysis is necessary. In the pharmaceutical field, TLC is used for identification, purity analysis, and concentration determination of active and inactive ingredients in drug preparations. TLC is used to measure active substances and their metabolites in biological matrices in clinical and forensic chemistry. TLC has many uses in the field of food chemistry. It is used to determine the concentration of pesticides and fungicides in water, fruits and vegetables, and meats. TLC is also used to determine the concentration of regulated substances in food, such as aflatoxins in milk products and grains and antibiotics in meats. In environmental analysis, TLC is used to measure groundwater and soil pollution.

In the early days of TLC, before the advent of HPLC, researchers experimented in their laboratories with developing chambers. Various chambers were used asending development, descending development and horizontal development (Hahn-Deinstrop

and Leach, 2007) . In this thesis horizontal chamber was used for all TLC experimental work. Therefore, in the following few paragraphs, an explanation about the horizontal development and horizontal chamber and its advantages will be discussed.

2.8.2 TLC developing chamber for horizontal development:

The horizontal chamber for TLC consists of a flat PTFE plate with five rectangular depressions: two containers of eluent and a central tray with three troughs and the chromatographic plate. The chamber is covered with a large cover plate.

Principle of action Development is started by shifting the plates to the chromatographic plate which brings a narrow zone of the absorbent layer on the plate into contact with the eluent from two sides. (Figure 2.7, A) shows the situation before chromatogram development and (Figure 2.7:B) during development. The eluent in containers is covered with the glass plates so that a vertical meniscus of the eluent is formed. Because the bottom of the containers is slightly slanted, the meniscus moves in the direction of the chromatographic plate during the development process, to the complete absorbtion of the eluent by the adsorbent layer.

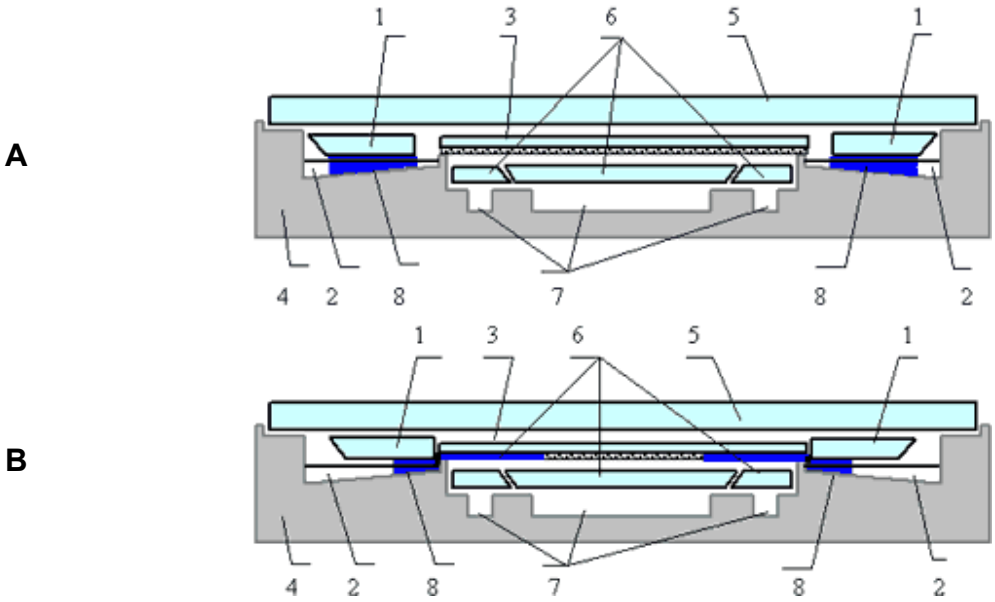


Figure 2.7: TLC Horizontal Chamber; where, 1 - cover plate of eluent reservoirs, 2 - eluent reservoirs, 3 - chromatographic plate, 4 - PTFE plate, 5 - large cover plate, 6 - cover plates of troughs, 7 - troughs for vapor saturation, 8 - eluent (blue area)

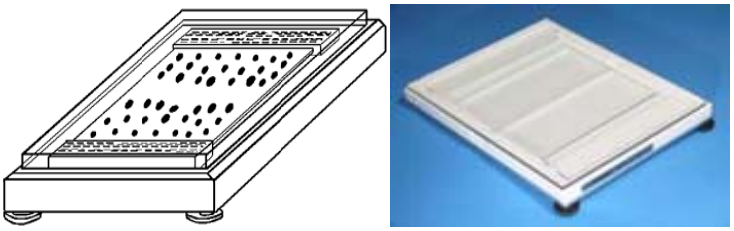


Figure 2.8 Axonometric view of the Horizontal DS-Chamber (model: DS-II-10x10)

The chamber permits the conditioning of the adsorbent layer with the solvent vapors by introducing several drops of the eluent or another solvent on the bottom of the troughs) before placing the chromatographic plate face down. The cover plates should then be removed from the chamber.

The Advantages of the chamber is extremely economical because:
1- The eluent from containers can be absorbed by the adsorbent layer to the last drop.

2- Development uses only 1/20 of the normal solvent consumption.

3- Conventional sandwich and conditioning modes can be applied, as well as stepwise and continuous gradient elution can be easily performed.

4-Two dimensional development of four samples on one plate can be performed.

5- Zonal application of the sample from the container can be carried out without any auxiliary equipment.

6- The chamber is designed for the optimization of eluent system because it enables a simultaneous development of the chromatographic plate with six different eluents.

7- It can be applied to the development of chromatograms on precoated glass carrier plates and foils, the adsorbent layer can be placed face up or down.

2.8.3. HPTLC

Although TLC soon enjoyed a wide application, it was essentially considered as a qualitative technique for the analysis or relatively simple mixtures. Further advances were directed toward instrumentation of TLC and on improvements in the technique

itself. Instrumentation was developed to permit more precise spotting of the sample onto the plates and the quantitative evaluation of the separated spots. Improvements in the technique itself resulted in higher separation power and faster analysis. Just as the name change of LC to (HPLC) character-ties, this improved TLC was also named HPTLC (Zlatkis and Kaiser, 1977).

The main difference between conventional and HPTLC was in the particle size and range of the adsorbent. The original "silica gel for TLC according to Stahl" had a fairly broad particle size range (10-60 µm), with an average of about 20 µm, but the material for HPTLC had a narrower range and an average particle size of only about 5 µm. The plates were also smaller, 10 x 10 cm against the conventional 20 x 20 cm, and the sample volume was reduced by an order of magnitude. The method of sample application was also improved with the design of mechanical applicators (dosimeters) permitting a reduction in the diameter of the starting spots. These improvements significantly reduced the time needed for an analysis, with a simultaneous increase of the separation efficiency.

The use of very fine particles, however, results in some additional problems. For example, the movement of the mobile phase on the plate will significantly slow down after a relatively short distance. On the other hand, as emphasized by Guiochon and co-workers (Guiochon, 1978b, Guiochon, 1978a, Guiochon et al., 1979, Guiochon et al., 1980), a fast and constant flow velocity of the mobile phase is needed to obtain an optimum efficiency. To overcome this problem, Kaiser started to apply pressure to the TLC plate. This then led to the development of the so-called forced-flow TLC technique.

2.9. Comparison between HPLC and TLC:

Whenever TLC is compared to HPLC, HPLC is usually considered the superior separation technique due to its higher separation capacity, automation, and its wide range of stationary phases and separation mechanisms.

However, TLC has many attributes that HPLC lacks, making the two techniques complementary rather than competitive (Poole, 1999, Poole and Poole, 1994).

The open sorbent bed and rectangular format of TLC plates allow the user to perform multiple separations in a single analysis. Up to thirty samples may be analyzed on a single plate, making TLC an excellent application in fields where many samples are routinely analyzed (Sherma and Fried, 2003). The high-throughput screening offered by TLC leads to a low cost analysis method. In addition, calibration standards and multiple unknown samples may be spotted on the same TLC plate and developed in parallel. This eliminates any variation that may occur when the samples and standards are run sequentially.

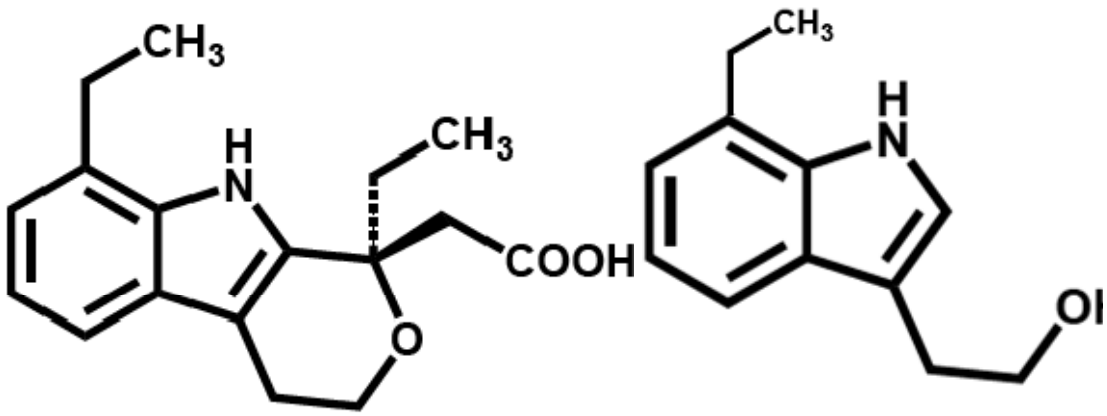
The relatively low cost of TLC plates means that they are single use items and not reused like HPLC columns. As a result, only minimal sample preparation is needed because fouling of the stationary phase will not impact future separations. Samples that contain suspended particulates or contain analyses that irreversibly bind to the stationary phase are not a concern because these will remain at the origin and not interfere with the separation. Samples of unknown matrix and composition may be run without worry of residual sample interfering with future analyses. In fact, TLC is often used for the simultaneous clean up and analysis of samples.

TLC operates in development mode whereas HPLC operates in elution mode. In development mode, all analyses have the same migration time but different migration distances. In elution mode, all analyses have the same migration distance but different migration times. At the end of a development in TLC, all of the analyses remain on the stationary phase. The solvent is evaporated off of the TLC plate allowing the samples to be analyzed without interference from the solvent. Post-chromatographic drivatization conditions may be optimized without time constraints, and multiple derivatizations may be performed to locate analyses with different functional groups. Multiple, nondestructive detection techniques may be used to gain the maximum information about each analyte. Because the samples remain on the stationary phase, the TLC plates may be saved to archive the separation, or separations may be performed at different times and locations than the detection (Poole, 1999).

3. Results and Discussion

3.1 Etodolac (ETD)

ETD is (1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]-indole-1-acetic acid), (Figure 3.1), belongs to the Non-steroidal anti-inflammatory class of drugs (NSAIDs) which are used in humans and domestic animals due to their anti-inflammatory, analgesic and anti-pyretic effects (Radi and Khan, 2006). ETD is indicated for the treatment of analgesia and for the signs and symptoms of rheumatoid arthritis and osteoarthritis (Boni et al., 1999). ETD has also found application in treatment of ankylosing spondylitis, postoperative pain as dental, obstetric, or orthopedic surgery, and non surgical pain as lower back pain, tendonitis, sports injury, or gout (Shah et al., 2002).



A **B**

(Figure 3.1) Chemical structure of A: ETD and B: CON

Several techniques have been reported for the analysis of ETD. HPLC is the most common technique (Becker-Scharfenkamp and Blaschke, 1993, Jamali et al., 1988, Wright and Jamali, 1993, Lapicque et al., 1989, Pirkle and Murray, 1990).

RP column was the main column which used to achieve separation of ETD from its sample or from its degradation products. Recently, ETD analysed by LC combined with tandem mass spectrometry (LC/MS/MS) (Lee et al., 2008), capillary electrochromatography– electrospray ionization mass spectrometry (CEC–ES-MS) and LC–ESI-MS methods (Strickmann and Blaschke, 2000). Chiral stationary phase was used for ETD enantiomers (Caccamese, 1992).

However, these methods suffer from a number of disadvantages as long run times and lower sensitivity analysis (Singh et al., 1986, Cosyns et al., 1983, Giachetti et al., 1994).

ETD was also analysed by gas chromatography with nitrogen- phosphours detection (GC- NPD) (Singh et al., 1986), gas chromatography–mass spectrometry (GC–MS) (Giachetti et al., 1994) and electrophoresis (Schmutz and Thormann, 1994, Mayer and Schurig, 1993). Spectrometry and spectrofluorimetry were used for ETD analysis (El Kousy, 1999).

TLC has been used to determine ETD (Ferdinandi et al., 1986). The method used silica gel plates and hexane-ethyl acetate-acetic acid (60:40:2, v/v). Another TLC method has been reported, which uses 0.25 or 2 mm silica gel plates as the stationary phase, and ethyl acetate:methanol (95:5, v/v) or toluene/ethyl acetate (7:3, v/v) as the developing agent (Cayen et al., 1981).

An established and validated HPTLC method has been developed for determination of ETD. The method uses aluminum- backed silica gel 60 HPTLC plates with n-hexan: ethyl acetate: glacial acetic acid, (6 : 2: 0.4, v/v/v) as mobile phase (Sane et al., 1998, Lalla et al., 1999).

The official chromatographic separation of ETD from its impurities in its bulk powder sample was achieved by Pharm. Eur. Method (Pharm.Eur., 2005). However, one of its impurities, 2-(7- Ethylindol-3-yl) ethalnol (CON) (Figure 3.1), was structurally similar to ETD that is why its peak separation from the main drug peak was difficult with bad resolution. Moreover, in the literature, there is no solution for this problem even in the last copy of the European Pharmacopoeia (Pharm.Eur., 2008) the conventional column and gradient elution system still used for ETD analysis. That cause long analysis time with the gradient elution drawbacks. Therefore, in this thesis, developments of new chromatographic analytical methods were estaplished to solve this problem. In the following two parts; HPLC and TLC, an analysis and separation of ETD and CON in ETD bulk drug sample will be disscused.

3.1.1 HPLC analysis

3.1.1.1. Optimization of the official HPLC method:

On applying the Pharm. Eur. method (Pharm.Eur., 2005) for ETD, the obtained chromatograms are showed in the figure 3.2.A. Broadening of the main drug peak and also for its impurity CON. Bad separation and resolution was also observed which can cause difficulty in their assay especially in the presence of each other.

The reason behind the broadening of the peak can be attributed to methanol which was used as solvent for the sample (ETD). This suggested reason can be explained by the fact that the injected solvent containing the sample is not immediately diluted with the mobile phase which is a mixture of solvent A (methanol and the buffer) and solvent B (acetonitrile) in the proportion 90:10 and therefore some of the sample might migrate down the column with stronger solvent, and other analyte molecules are diluted and migrate with the mobile phase, resulting in the distortion peak.

Therefore a change of the sample solvent from methanol to the mobile phase was examined. A much better peak shape (a well-shaped symmetric peak) was obtained for the main drug peak and its impurity as shown in the (figure 3.2. B). Therefore the proposed sample solvent will be miscible with the mobile phase immediately and consequently a distortion of the peak is avoided.

On the other hand, the aqueous solubility of ETD is pH dependence (Figure 3.3) According to this curve, the drug is predicted to exhibit a very low solubility at low pH; and its solubility dramatically increases at high pH. Therefore, it was very useful to use the mobile phase as a solvent (pH 7) for the ETD samples.

However the resolution of the observed peaks was not enough to assay a impurity which is usually the minor component from the main drug peak. Therefore a change in the gradient system was examined to optimize the resolution of the eluted peaks.

An optimum condition was observed with the use of solvent A and B starting with the ratio 96:4 till 92:8 through 20 min chromatographic run. The impurity

was used in a high concentration in the previous experiments that is why the method was applied on the actual concentration of the same impurity to test the sensitivity of the method to that compound. A good observed peak was eluted in the same retention time of the impurity and can be analysed in the presence of the main drug peak (Figure 3.2 C). The column resolution (Rs) is increased (Rs=2.5) compared to that obtained with the official method (Rs < 1). The developed method was efficient to detect and separate CON in CON/ETD mixture in which the CON and ETD concentrations was 0.01 and 1 mg/ml respectively.

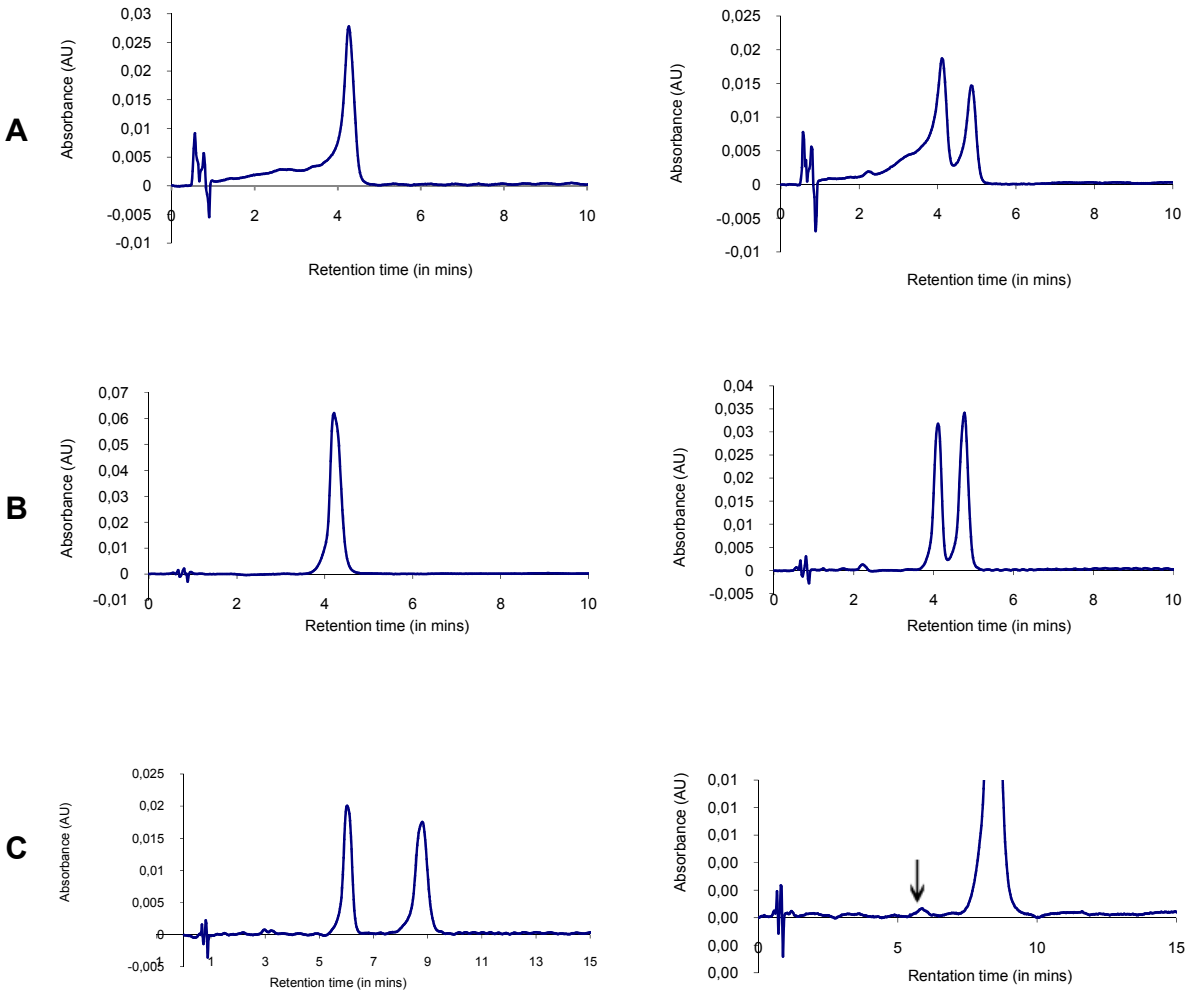


Figure 3.2. Optimization of the chromatographic analysis of ETD and CON mixture. A: Pharm. Eur. method, B: effect of sample solvent and C: effect of optimized gradient elution system on the resolution efficiency.

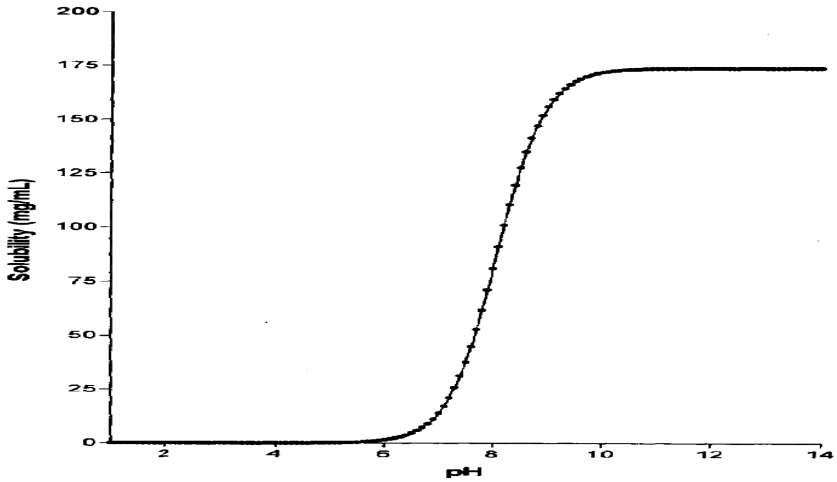


Figure 3.3. pH dependence of the aqueous solubility of ETD, as calculated using the ACD Solubility Suite 6.0 program (Advanced Chemistry Development, Toronto Canada).

3.1.1.2. Validation of the optimized HPLC method:

The optimized gradient elution system was validated with the following parameters:-

3.1.1.2.1. Precision

Precision which was an important topic of this study was carefully tested. To ensure assay precision within day (10 injections were performed) and between days (20 injections were taken randomly), precision was assessed at sample concentration 1mg/ml and the results are summarized in (Table 3.1).

Table 3.1: Within day and between days repeatabilities for ETD

Parameters	**Results**
Within day repeatability RSD% of AUC (n=10)	0.80
Within day repeatability RSD% of t_R (n=10)	0.65
Between days repeatabilities RSD% of AUC (n=20)	0.90
Between days repeatabilites RSD% of t_R (n=20)	0.72

3.1.1.2.2. Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

The calibration curve (peak area vs. concentration) for the ETD was investigated. Residual plot did not show any trend. Result was found to be linear

with high correlation coefficient. (LOD, S/N =3) and an estimate for the (LOQ, S/N = 10) for ETD. Results are in (Table 3.2).

Table 3.2: Linearity, LOD and LOQ (µg/ml)

Parameters	Results
R^2	0.9987
LOD	0.150
LOQ	0.500

3.1.1.3. Performance parameters of the optimized HPLC method:

Peak performance parameters for ETD were also calculated according to fundamental equations, results are in (Table 3.3).

Table 3.3: Performance parameters for ETD.

Parameters	Results
Theoretical plate N (Plate per column for ETD)	860
Asymmetry factor (AF) for ETD Peak	1.4
(Rs) ETD/CON	2.5
Run time	20 min

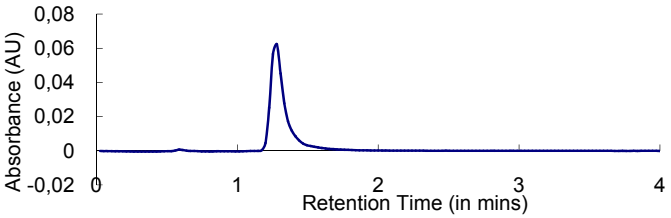
3.1.1.4. Development of UPLC- like method:

To avoid the disadvantages of gradient elution as mentioned in the introduction of the thesis it was suggested to change the elution mode of the HPLC method to isocratic elution using solvent A (buffer + methanol) : B (Acetonitrile) in a ratio 70:30 with F=1.2 ml/min. As a result of using that system a shorter analysis time of ETD was achieved. However a peak tailing was observed in the resulted chromatogram. The suggested reason behind that was the used stationary phase which was conventional RP silica C18. Therefore another stationary phase was tested to study the effect of the used stationary phase type on the peak shape particularly the peak tailing (Figure 3.4). Luna column was used instead of the conventional column and a high backpressure was observed with

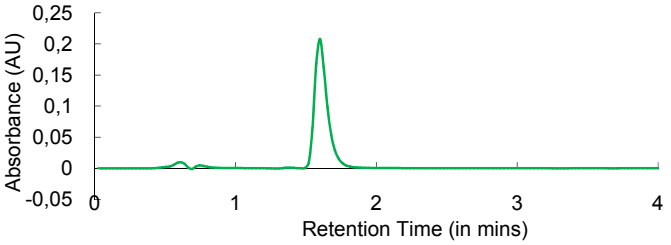
it which can be attributed to the fine size of its particles. Therefore decreasing in the applied flow rate was suggested to avoid such backpressure. A good peak shape with reduced peak tailing was obtained with luna column. However a bad resolution and separation of the drug and its impurity was observed. That is why it was necessary to replace the stationary phase with the modern stationary phases as monolithic and fused core columns. A much better peak shapes and good resolutions were observed with the use of monolithic column. On the other hand an optimum peak shapes and in the same time a good resolution was obtained with fused core column as a stationary phase in this method. The developed method was efficient to detect and separate CON in CON/ETD mixture in which the CON and ETD concentrations was 0.03 and 3 µg/ml respectively (Figure 3.5).

Another trial to examine the separation efficiency of all used columns was tested by the injection of a high concentration drug samples in order to magnify the content of the probable impurities and/or degradation products which can be found in the normal drug samples. Also the fused core column was the optimum column which can give the maximum number of well separated peaks in a reasonable run time (Figure 3.6).

A



B



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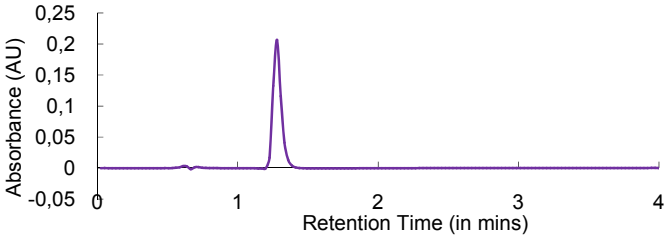


D

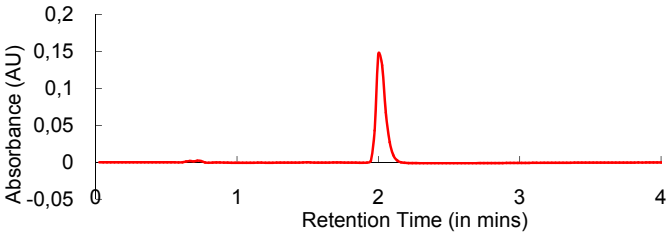


Figure 3.4: Representative chromatograms for determination of ETD according to developed method at sample concentration 0.003mg/ml by using: A; conventional column, B; luna column, C; monolithic column, D; fused core column.

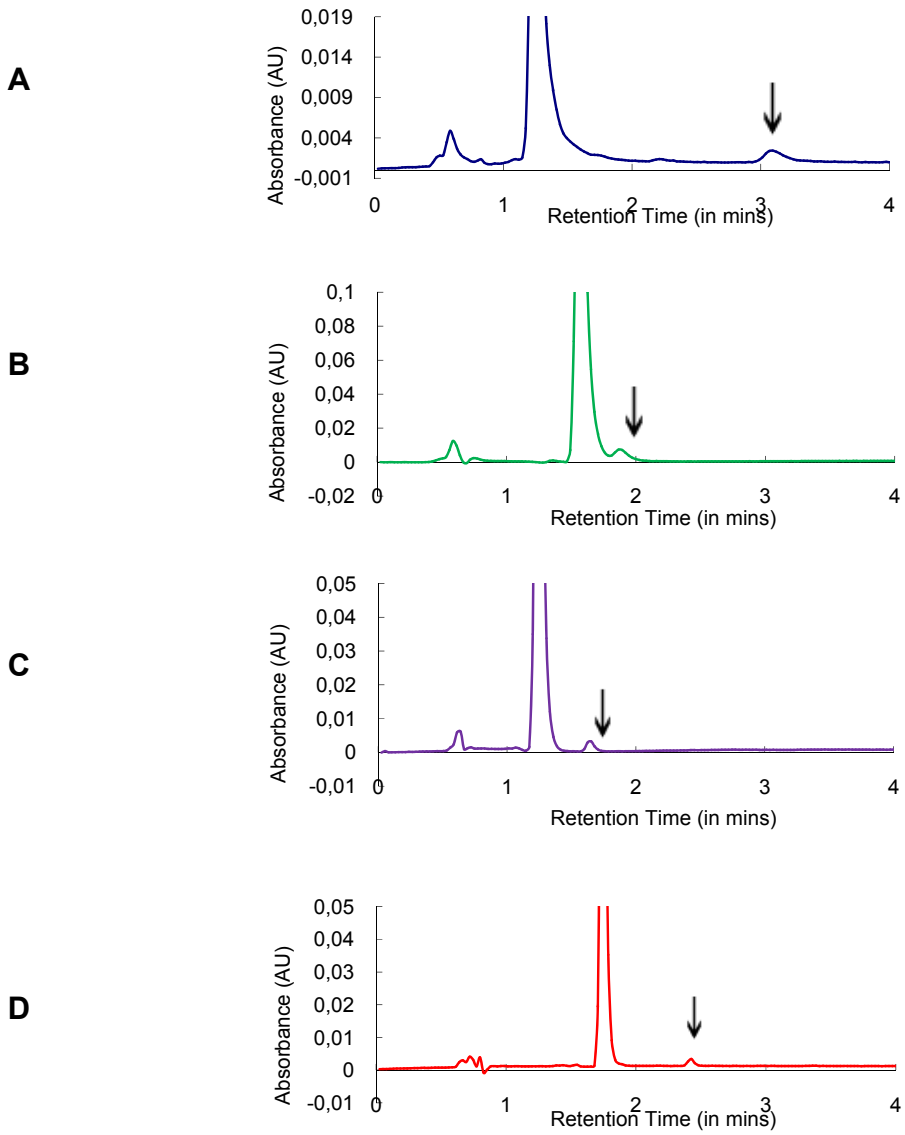


Figure 3.5: Representative chromatogram for the efficiency of developed method on the separation of CON in 0.3 µg/ 10 ml of ETD sample (0.003mg/ml) by using: A conventional column, B luna column, C monolithic column, D fused core column.

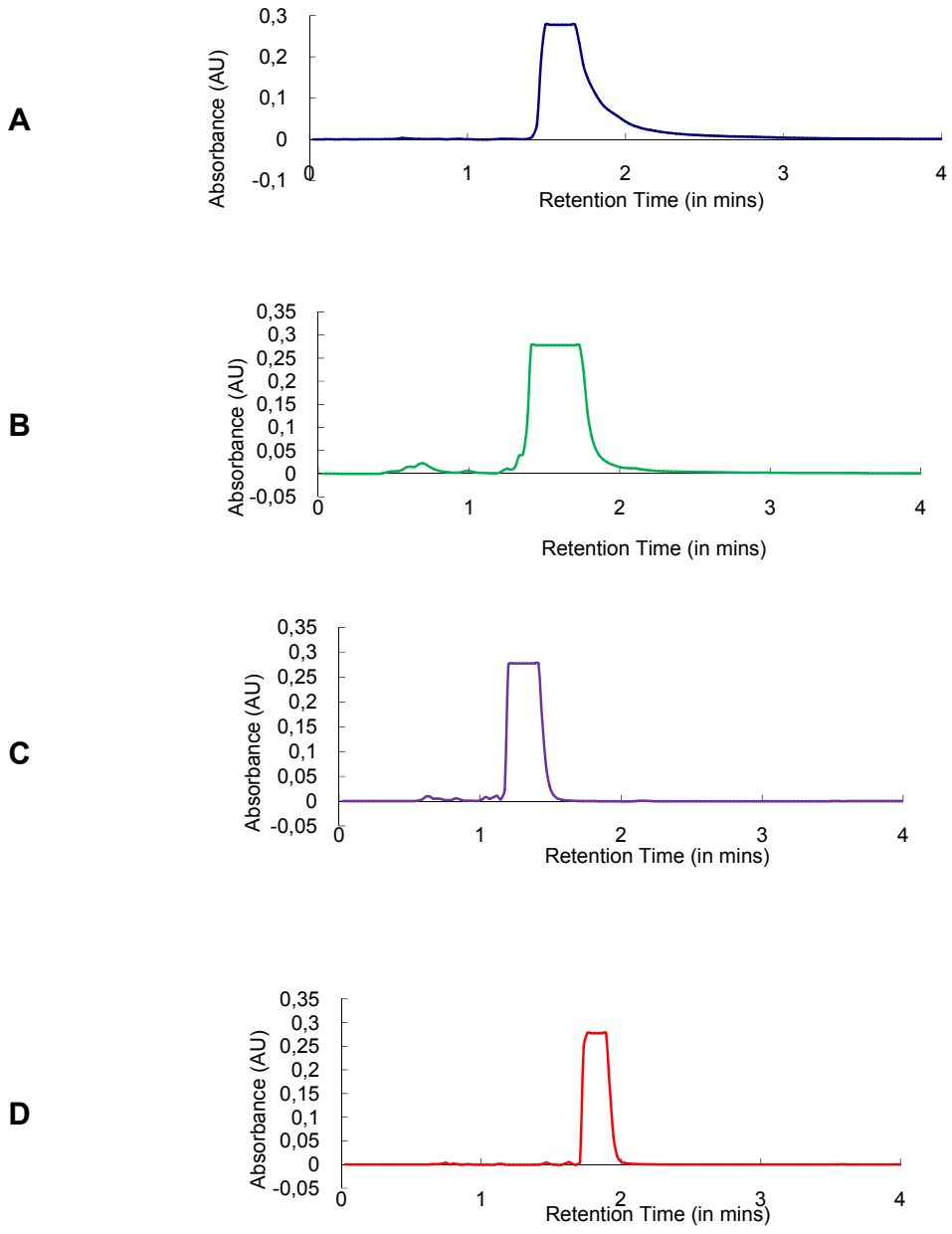


Figure 3.6: Representative chromatogram for analysis of ETD sample (0.1 mg/ml) according to developed method by using: A conventional column, B luna column, C monolithic column, D fused core column.

3.1.1.5. Validation of the UPLC-like method

The new HPLC developed method by using isocratic elution system was validated by:

3.1.1.5.1. Precision

To ensure assay precision within day repeatability (n=6) and between days repeatability (n=6) were assessed at 3 concentrations of ETD. The results in (Tables 3.4 and 3.5).

Table 3.4: Within day repeatabilities of ETD on conventional, monolithic , luna and fused core columns over a concentration range 0.003-0.1 mg/ml using n=6.

Column type	Within day repeatability RSD (%) of AUC			Within day repeatability RSD (%) of t_R
	0.003	0.01	0.1	
	(mg/ml)			(n=18)
Conventional	0.63	0.78	0.82	0.78
Monolithic	0.54	0.69	0.71	0.67
Luna	0.61	0.72	0.62	0.76
Fused core	0.60	0.55	0.52	0.48

Table 3.5: Between days repeatabilities of ETD on conventional, monolithic , luna and fused core columns over a concentration range 0.003-0.1 mg/ml using n=6

Column type	Between days repeatability RSD (%) of AUC			Between days repeatability RSD (%) of t_R
	0.003	0.01	0.1	
	(mg/ml)			(n= 18)
Conventional	0.90	0.99	1.25	0.86
Monolithic	0.56	0.73	0.88	0.83
Luna	0.62	0.87	0.85	0.80
Fused core	0.58	0.62	0.51	0.55

3.1.1.5.2: Linearity, LOD and LOQ

Calibration curve (peak area vs. concentration) for the analyzed ETD with new method was investigated over a concentration range of 0.003 - 0.1mg/ml. The (LOD, S/N =3) and an estimate for the (LOQ, S/N = 10) for the ETD were also in (Table 3.6).

Table 3.6: Linearity, LOD and LOQ of the ETD

Column type	LOD (µg/ml)	LOQ (µg/ml)	R^2
Conventional	0.006	0.02 0	0.9985
Monolithic	0.0033	0.0112	0.9995
Luna	0.0038	0.014	0.9984
Fused core	0.0030	0.01 0	0.9999

3.1.1.6. Performance parameters of the UPLC-like method:

Peak performance parameters were calculated according to fundamental equations (Table 3.7).

Table 3.7 : Performance parameters for ETD on conventional **(125 mm)**, monolithic **(50 mm)**, luna **(50mm)** and fused core **(100 mm)** columns.F ****0.5 ml/min** ***1.2ml/min**

Column type	Theoretical plate N (Plate per column for ETD)	AF for ETD Peak	Rs, ETD/CON	Backpressure (Bar)	Run time (min.)
Conventional*	151	1.66	6.0	110	3.5
Monolithic*	529	1.1	2.5	30	2
Luna **	455	1.5	1.35	102	2.5
Fused core*	1354	1.0	3.6	228	2.5

3.1.2 TLC analysis:

3.1.2.1. Development of a TLC purity test:

In the official TLC purity test of ETD there are two different mobile phases with two drying steps were used which time and cost are consuming. Therefore an easier TLC procedure was one of the main aims of this thesis.

The second solvent system of the official TLC method is a mixture of acetic acid 99% : absolute ethanol : toluene, (0.5 : 30 : 70, v/v/v) was used after the activation of the TLC plate by heating at 120 °C for 1hr. and applying the sample spots. A five minutes drying time of the plate and the isolated spots were detected by using U.V. lamp at 254 nm. Also the detection of the spots was examined with some drivatizing reagents such as anisaldahyde reagent, phosphomolybdic acid reagent and xanthydrole reagent.

A second trial to optimize the solvent system by omitting acetic acid from the mobile phase and using different ratios of absolute ethanol and toluene. R_f values were calculated and the best results were obtained with toluene: absolute ethanol (8:2, v/v). The later system was successively used in the separation of the drug from its impurity CON (Figure 3.7).No reaction was detected between the ETD spot with xanthydrole. The anisaldehyde reagent gave a dark brown background on the TLC plate. By using phosphomolybdic acid reagent a different coloured spots were detected for ETD and CON with circular blue spot and oval green spots respectively. These results facilitate the detection of that impurity in presence of the main drug by using the developed method.

The effect of alkaline or acidic substances as additives to the mobile phase was examined. Substance as diethyl amine and acetic acid was added in the 0.05 % separately to Toluene: acetone (5: 5, v/v) mobile phase. The separation of the spots was not affected in the case of addition of diethyl amine. However no improvement in the separation of the spots this is why there is no need for the addition of this basic additive to the mobile phase. On the other hand the CON spot couldn't be separated in the case of acetic acid (Figure 3.8). An exchange of absolute ethanol with acetone in addition to toluene at different ratios was examined. The optimum results were observed with 1:1, v/v toluene: acetone. The developed method was efficient to detect and separate CON in CON/ETD mixture in which the CON and ETD concentrations was 0.05 and 1 mg/ml respectively. On the other hand, the R_f values 0.54 and 0.67 for ETD and CON respectively by using HPTLC paltes (Figure 3.9) and the R_f values were 0.54 and 0.60 for ETD and CON respectively by using TLC plates (Figure 3.10). The developing time was 3 and 1.5 min by using TLC and HPTLC plates respectively.

Although for most qualitative analysis TLC plates can be used without any pretreatment. However, the impurities on the plate accumulate not only from the laboratory atmosphere but also from packing material such as shrink- wrapping foil. Therefore, it is important to consider a standardized cleaning procedure if the analytical method has to be validated and reproducible results are required as the developed method aim was for stability test.

Therefore, the effect of prewashing of TLC plates was studied. Methanol was used as a washing solvent (Figure 3.11) in a comparison between the washed and unwashed plates, the background was clear and the drug spots were with definite shape with a good contrast with the background.

The effect of activation of the TLC plate on the R_f values was tested. The activation process was done by heating the silica gel plates to 120 ºC in an oven to maximize activity for one hour. At that temperature, adsorbed water is completely removed from the surface. On the other hand, non-activated plate was used with the developed method (n=6) the means of R_f value was 0.59 for ETD which is more than in activated plate where R_f value was 0.54, i.e. the activity affects the R_f value of the analyte. These results agree with the reported fact that, the higher the activity, the lower is the R_f (Reich and Schibli, 2007).

During transport and sample application, the stationary phase is again in contact with the relative humidity of the environment. It is useful to equilibrate the active plate with the humidity of the surroundings by cooling it down to room temperature in a dust- and fume- free environment such as empty desiccators.

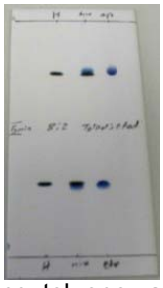


Figure 3.7: using toluene: absolute ethanol (8:2, v/v) as mobile phase for analysis of ETD.

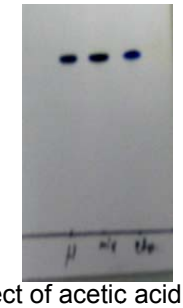


Figure 3.8: Effect of acetic acid in mobile phase on separation efficiency of ETD from CON (toluene: absolute ethanol: acetic acid, 5:5:0.05 v/v/v).

Figure 3.9: Optimum separation of ETD from CON by using mobile phase (toluene: acetone, 1:1 v/v) and HPTLC plates.

Figure 3.10: Separation of ETD from CON by using mobile phase (toluene: acetone, 1:1 v/v) and TLC plates.

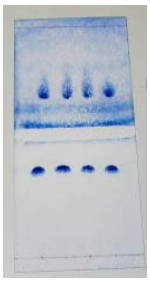


Figure 3.11: The effect of prewashing of TLC plates (A: unwashed part and B: washed part of plate.

3.1.2.2. Validation of the developed TLC method:

The developed TLC method for the analysis of ETD in presence of its impurity by using mixture of toluene: acetone, (1:1, v/v) and phosphomolibdic acid reagent was validated By:

3.1.2.2.1. Repeatability and Intermediate precision

To ensure assay precision within day repeatability (n=15) and between days intermediate precision (n=20) were assessed for ETD. The results are in Table 3.8.

Table 3.8: Repeatability and Intermediate precision for ETD

Parameters	ETD	CON
I.Repeatability at n=15 at the same day		
Mean R_f value	0.54	0.67
SD	0.02	0.016
RSD%	3.7%	2.39%
SE	0.005	0.004
RSE%	0.53%	0.59%
II.Intermediate precision at n=20 at different days		
Mean R_f value	0.54	0.67
SD	0.025	0.02
RSD%	4.6%	2.6%
SE	0.006	0.004
RDE%	1.1%	0.59%

3.1.2.2.2. Reproducibility and robustness:

Robustness is the ability of a method to tolerate variations of parameters without significant change in the result. With respect to TLC, the most important assessment to be made is that of the effects due to possible changes in relative

humidity. If plates from different manufactures are in use in the laboratory, it can be anticipated that substitution occurs. Particularly if the method will be used in more than one laboratory, this aspect may be evaluated in the robustness test.

By applying the same method for the determination of ETD in different labs and different times all over the year, the robustness was tested (n=30). The mean R_f value was 0.58 and standard deviation (SD = 0.03). The standard error (SE = 0.0056), the relative standard deviation (RSD = 5.2 %) and relative standard error (RSE =0.97 %).

For the CON, the mean R_f value was 0.70 and (SD = 0.023), (SE = 0.004), (RSD = 3.3 %) and (RSE = 0.54 %).

The method was applied using different TLC plates from different companies without change in the obtained results. Moreover, the developed method was applied with activated TLC plate without exposure to humid air and with another TLC plate which was left in the atmosphere to get moisture adsorbed to the silica surface. Therefore, the TLC system (layer and developing system) was not sensitive to small change in relative humidity. Therefore the method was robust.

3.2. Spiramycin (SPR) :

SPR, an antibiotic derived from the culture filtrate of streptomyces ambofaciens, is a member of the macrolide group of antibiotics (Reynolds, 1993). Antibiotics obtained from macrolide producing organisms consist usually of several homologous components and this is also the case with SPR, which consists predominantly of three closely related substances together with a number of other minor compounds (Kuehne and Benson, 1965). SI is the major component and constitutes about 91.4% of the mixture while S II and III constitute about 0.5 and 4.0% respectively (Figure 3.12). The members of this series are composed of 16-membered lactone ring with two or three sugar substituents, mycaminose, forosamine and mycarose, together with various other substituent groups.

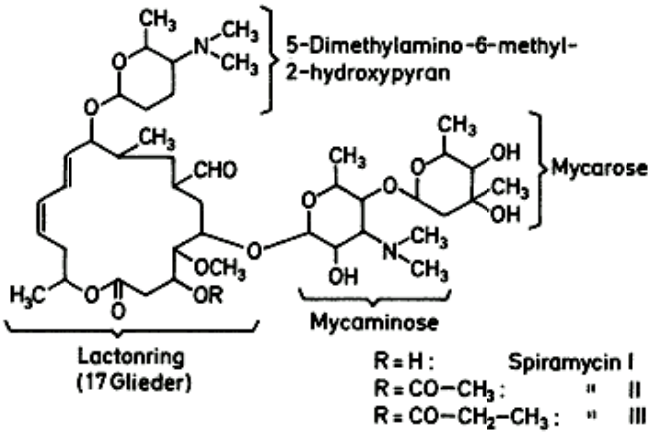


Figure 3.12. Chemical structure of SI, SII and SIII.

The chromatographic analysis of SPR has largely been concerned with separation of the major spiramycins to enable the analysis of these compounds with a sensitivity and selectivity which can not be afforded by traditional microbiological assays.

All the HPLC methods in the literature utilized both normal and RP chromatography with UV detection at about 230 nm, which is facilitated by a conjugated double bond within the lactone ring. A HPLC separation procedure of SI and III only is reported without quantitative data (Omura et al., 1973). developed a selective HPLC assay capable of resolving all three major componenets of SPR by using LiChrosorb RP-8, 10 µm column (Mourot et al., 1978). Separation of these three compounds was also accomplished on both RP C18 material at 50 °C and on silica gel at 25°C with the aid of an ion- pairing agent (diethyl- amine) in both instances (Bens et al., 1979). Particle beam mass spectrometry was used for the analysis of SPR with C18 stationary phase (Sanders and Delepine, 1994). The SPR was separated from other macrolides antibiotics by using end- capped high- purity silica- based C18 column (Leal et al., 2001). A RP HPLC method was used for determination of SPR with fluorecence detection (Gomis et al., 2004)

C8 stationary phase was combined with an ion- pairing agent, and UV detection, for the determination of SPR (Nagata and Saeki, 1986).

PS- DVB stationary phase has been used to identify and separate SPR and its related compounds present in raw materials (Liu et al., 1996, Liu et al., 1997b). RP C8, 5- µm material was used for HPLC analysis of SI and II (Dow et al., 1985).

LC/ MS was used to study the degradation of SPR (Shi et al., 2004). Gradient elution system was used for identification and quantification of SPR with HPLC - UV / DAD (Civitareale et al., 2004, Maher et al., 2008).

SPR was also determined by TLC coupled to microbiological detection (Vincent and Gizzi, 2007).

Till now, there are several publications can be seen for the separation of SPR by HPLC methods with different detectors (Wang and Leung, 2009, Furusawa, 1999, Pendela et al., 2007). However, in the literature there is no use of new stationary phases as monolithic and fused core columns, therefore an UPLC-like method and TLC method were developed to separate the three major components (SI, SII, and SIII) in short analysis time.

3.2.1. HPLC analysis:

3.2.1.1. Optimization of the official HPLC method:

The official method was applied mentioned in Pharm. Eur. 2005 and it was expected to get three peaks for SI, II and III. However just two peaks (for SI and II) were observed in the chromatogram throughout the chromatographic run time (40 min) (Figure 3.13 a). That was the first drawback of that official method. Therefore some modifications were examined to enhance the chromatographic separations to get SIII peak. The effect of temperature was tested, at 35 °C, and the required third peak (SIII) was observed at about 35 min however this was long t_R (Figure 3.13 b). Therefore, another modification in the mobile system was examined. An increase in the organic modifier percentage from 30% to 32% and a decrease in the buffer percentage from 70% to 68% as a result the required three peaks were observed in less than 33 min but with broad peaks of SII and SIIII (Figure 3.13 c). A gradient elution was tested and a much better result was obtained as the three peaks were observed in a 25 min

chromatographic run time with more sensitive results with sharp symmetric peaks (Figure 3.13 d).

Applying gradient elution system (Table 5.1) instead of isocratic elution system has improved the t_R as well as the peak shape of SII and SIII. The well-shaped and symmetrical peaks of SII and SIII can be explained by the fact that using gradient elution system decreased the volume of the peak (t_2-t_1) and increased the concentration of the peak (height of the peak) significantly. This finding is supported by (Civitareale et al., 2004).

3.2.1.2. Validation of optimised HPLC methods:

Both optimised HPLC methods with isocratic and gradient elution systems were validated by:

3.2.1.2.1. Precision:

To ensure assay precision within day (10 injections were performed) and between days (20 injections were taken randomly), precision was assessed at sample concentration 0.25 mg/ml (Table 3.9).

Table 3.9: Within day (n=10) and between day's (n=20) repeatability for SPR with isocratic and gradient elution systems.

Validation parameters	Isocratic elution	Gradient elution
Within day repeatability RSD% of AUC	0.67	1.0
Within day repeatability RSD% of t_R	0.40	0.75
Between days repeatabilities RSD% of AUC	1.64	1.3
Between days repeatabilites RSD% of t_R	0.59	0.91

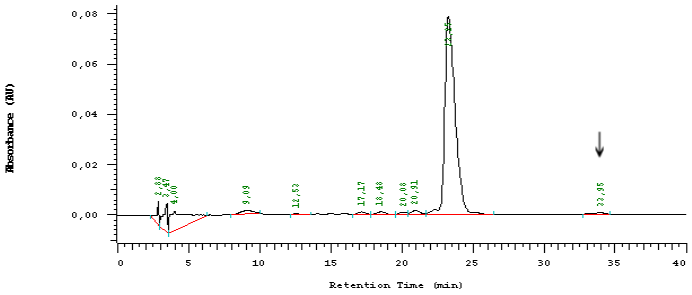
3.2.1.2.2. Linearity, LOD and LOQ

THe (LOD, S/N =3) and an estimate for the (LOQ, S/N = 10) for the SPR with isocratic and gradient elution systems were also in (Table 3.10).

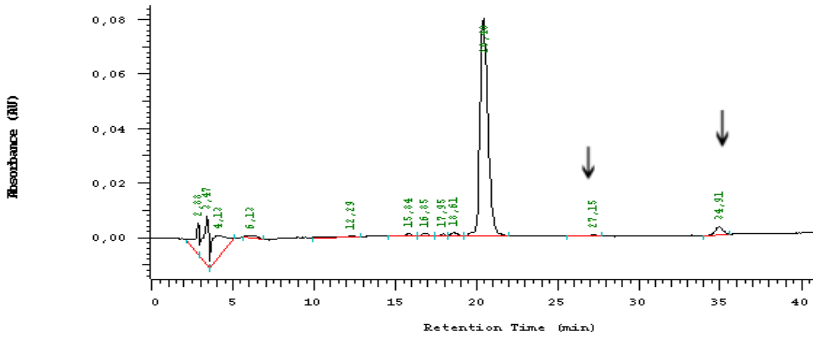
Table 3.10: Linearity, LOD and LOQ for spiramycin

Parameters	Isocratic elution	Gradient elution
R^2	0.9995	0.9996
LOD (µg/ml)	0.134	0.133
LOQ (µg/ml)	0.460	0.450

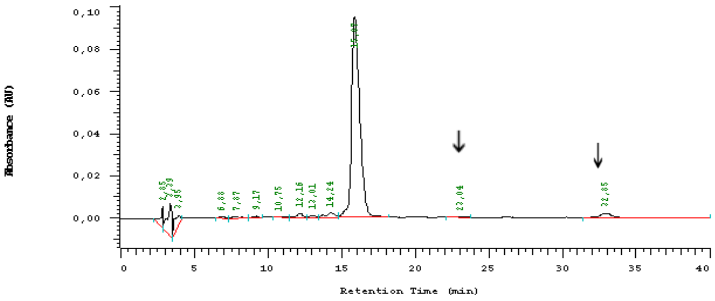
A



B



C



D

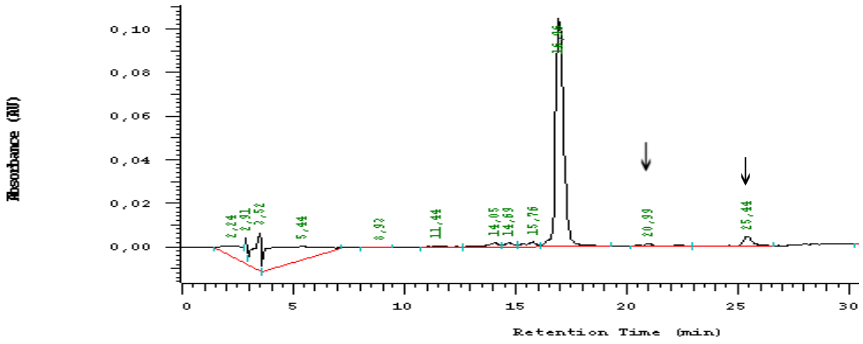


Figure 3.13. Representative chromatograms for analysis of SPR under different conditions
(A) Separation of SPR (0.25 mg/ml) according to isocratic elution system in Pharm. Eur. method (30:70 acetonitril: $NaClO_4$ x H_2O buffer pH 2.2).
(B) Representative chromatogram showing the influence of high temperature (35°C) on the separation.
(C) Representative chromatogram for the separation of SPR by modified isocratic elution system (32:68 acetonitril: $NaClO_4$ x H_2O buffer pH 2.2).
(D) Representative chromatogram for the separation of SPR by gradient elution system.

3.2.1.3. Performance parameters of optimised HPLC methods:

Peak performance parameters were calculated according to fundamental equations (Table 3.11).

Table 3.11: Performance parameters for SPR on conventional column with both isocratic and gradient elution systems.

Parameters	Isocratic	Gradient
Theoretical plate N (Plate per column for SI)	1791	4602
AF for SI Peak	1.4	1.2
(R_s) SII/ SIII	5.3	4.5
Run time	40 min	30 min

3.2.1.4. Development of UPLC-like method

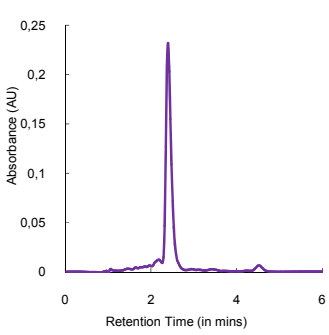
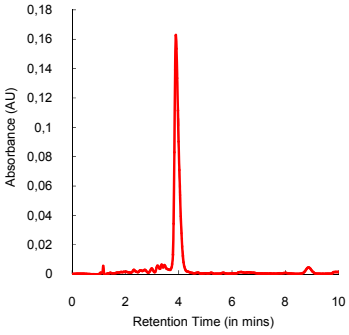
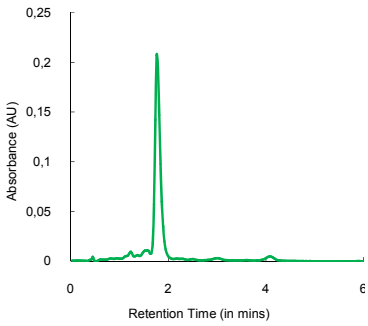
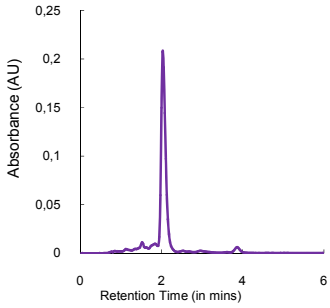
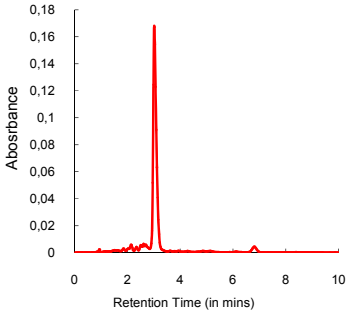
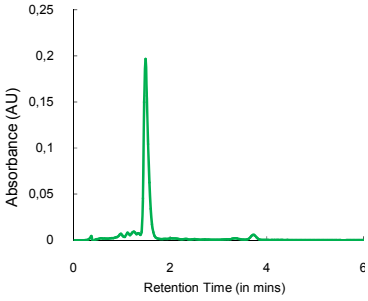
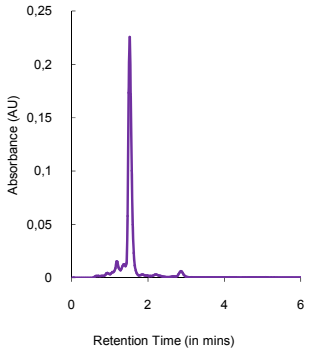
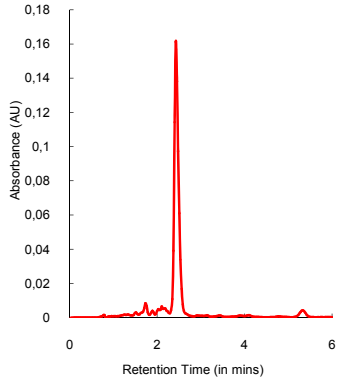
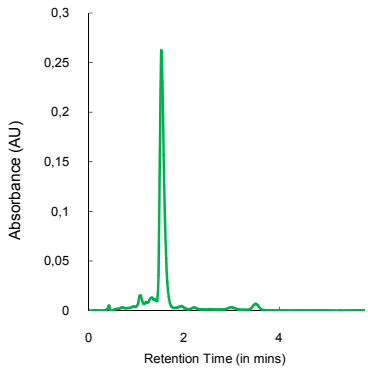
Different stationary phases were examined instead of RP C8. By using different columns (RP C18) as monolithic, fused core and luna at F=0.8 ml/min. a shorter chromatographic run time (5 min with monolithic and luna columns while 9 min with fused core column) was obtained with better results.

To examine the effect of increasing the flow rate of the mobile system on efficiency of separation, different flow rates (0.8, 1, 1.2, 1.4, 1.6, 1.8, 2.0 ml/min) was tested (Figure 3.14). A good separation of SI, II and III at all flow rates was observed. However, a high backpressure was observed with high flow rate using luna column. Moreover, with low volume column as fused core column the eluted peak height decreased with increasing flow rate more than 1.2 ml/min because the injected sample was 20 µl which is high sample volume.

A plot was constructed a comparison between the three used columns in terms of backpressure against the used flow rate (Figure 3.15). The monolithic column backpressure was very low due to the high permeability which offered by bimodal porous structure (mesopore and macropore).

The reason behind high backpressure with luna column was due to smallest particle size (2.5 µm) where the pressure and efficiency are inversely related to dp. On using fused core column the backpressure was reduced about 50% (of that resulted by luna column) which attributed to the new technology in particles (2.7 µm) with a high- capacity and high purity porous silica layer fused to a solid silica core.

Figure 3.14 Representataive chromatograms for the analysis of SPR using different RP C18 at different flow rate

F (ml/ min)	Monolithic Column	Fused Core Column	Luna Column
0.8			
1.0			
1.2			

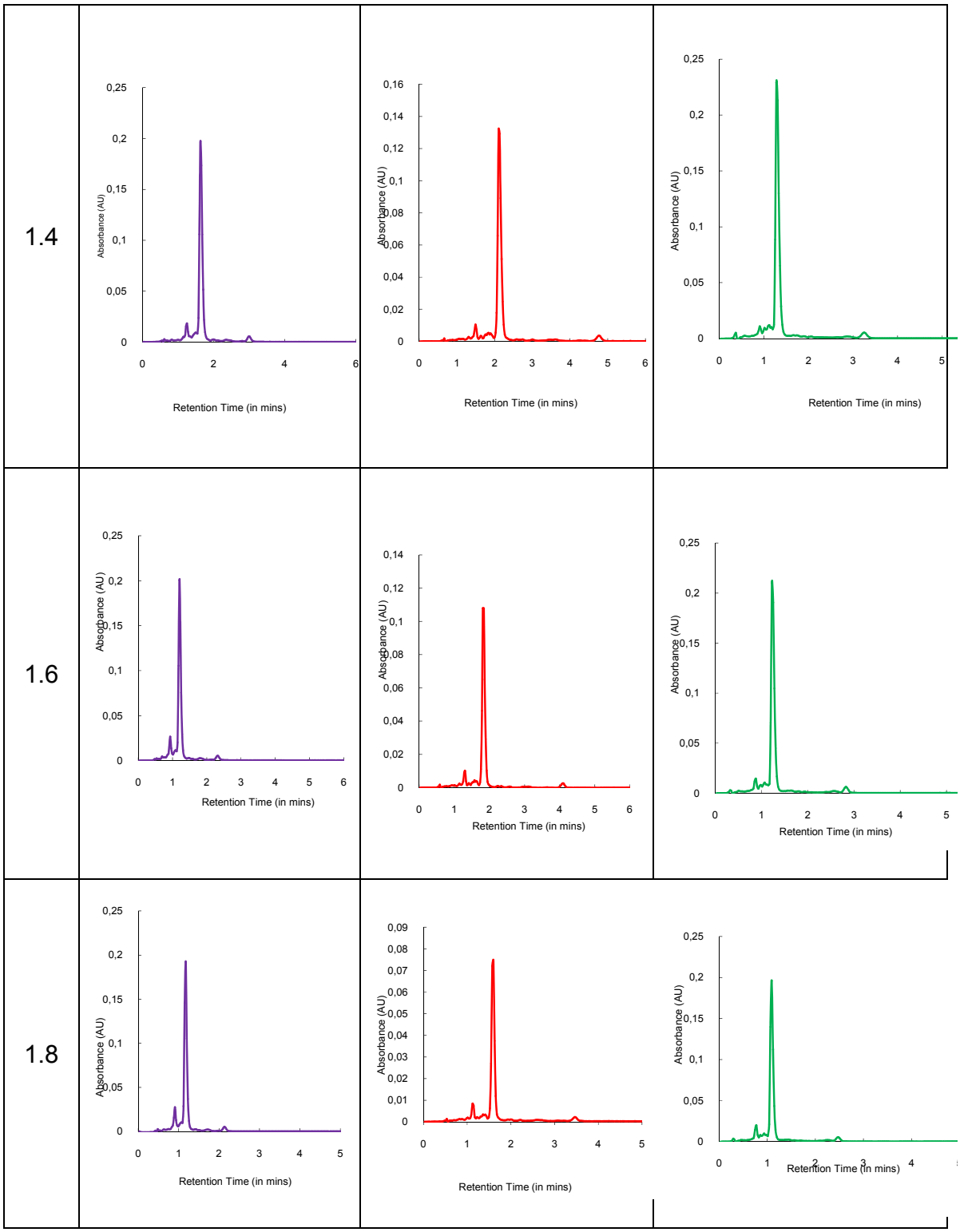


Figure 3.14 Representataive chromatograms for the analysis of SPR using different RP C18 at different flow rates.

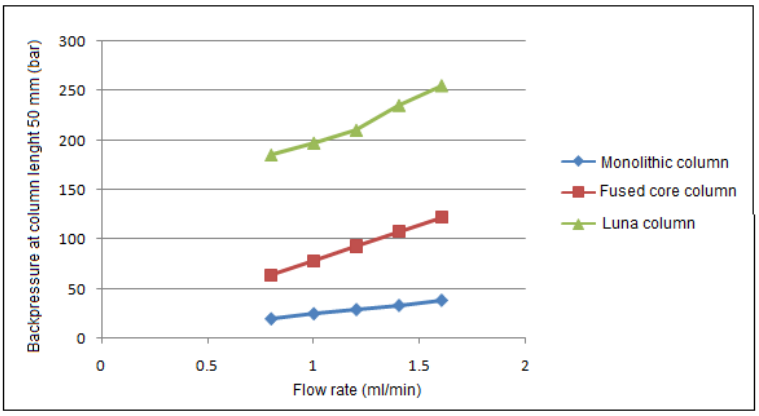


Figure 3.15: Diagram of Backpressure (bar) at L (50mm) against flow rate for different columns was used for analysis of SPR.

3.2.1.5. Validation of the optimised UPLC-like method:

The optimised UPLC method for analysis of SPR by using luna, monolithic, and fused core columns was validated by:

3.2.1.5.1. Precision

Precision was carefully tested. To ensure assay precision within day (10 injections were performed) and between days (20 injections were taken randomly), precision was assessed at sample concentration 0.25 mg/ml (Table 3.12).

Table 3.12: Within day (n=10) and between days (n=20) repeatabilities of SI on luna, monolithic, and fused core columns on concentration 0.25mg/ml and F =0.8 ml/min.

Column type	Within day repeatability RSD% of AUC	Within day repeatability RSD% of t_R	Between days repeatability RSD% of AUC	Between days repeatability RSD% of t_R
Luna	0.77	0.80	0.95	0.87
Monolithic	0.62	0.41	0.85	0.73
Fused core	0.58	0.45	0.86	0.60

3.2.1.5.2. Linearity, LOD and LOQ

The calibration curve (peak area vs. concentration) for the SPR was investigated.Residual plot did not show any trend. Result was found to be linear with high correlation coefficient. (LOD, S/N =3) and an estimate for the (LOQ, S/N = 10) for SPR. (Table 3.13).

Table 3.13: Linearity, LOD and LOQ

Column type	LOD (µg/ml)	LOQ (µg/ml)	R^2
Luna	0.131	0.440	0.9998
Monolithic	0.122	0.400	0.9999
Fused core	0.124	0.410	0.9999

3.2.1.6. Performance parameters for UPLC-like method:

Peak performance parameters were calculated according to fundamental equations and the run time with different columns (Table 3.14).

Table 3.14: Performance parameters for SI on monolithic (50 mm), luna (50mm) and fused core (100 mm) columns at F=0.8 ml/min

Column type	Theoretical plate N (Plate per column for SI)	AF for SI Peak	(R_s) S II/ SIII	Run time (min)
Luna	404	1.2	5.9	5
Monolithic	731	1.0	4.7	5
Fused core	568	1.1	7.2	9

3.2.2. TLC Analysis

3.2.2.1. Optimization of TLC Purity test:

The official TLC test for SPR was performed by using upper phase of the mixture which containing 2-propanol: ammonium acetate pH 9.6: ethyl acetate (4: 8: 9 v/v/v) (Figure: 3.16).

The sample was eluted for a long time (22 min) gave migration to 2 cm with 7.5 cm for the solvent front which means that the sample was retained alot on the surface of the silica as it is a highly polar substance. The used reagent for identifying the spots (anisaldehyde) gave a bad contrast between the spots and the background. The reaction between the reagent and the drug needs a heating for 5 min at 110°C after spraying with the reagent.

Optimization of the official method was needed. A multiple change of the ratio of the used mobile phase and different mobile systems components were examined. The best results were observed with the use of a modification of the official solvent to increase the polarity of the used solvent to isopropanol: ethyl acetate: ammonium acetate buffer pH 9.6 (11:9:8 v/v/v). The developing time reduced to 3 and 2 min by using TLC and HPTLC plates respectively . Another reagent was tested instead of anisaldehyde such as phosphomolybidic acid. A blue spots were observed without the need of heat. A better separation of the components of the spot into 7 spots which is more selective separation compared with the official method (Figure 3.17). There was no significant difference in the efficiency of separation between TLC and HPTLC plates.

However on dilution of the sample concentration from 4 mg/ml to 40 µg/ml, only one spot related to the main drug was noticed. That means the sensitivity of the SI to the proposed method is high as on its dilution to 1/100 concentration the drug spot gives positive color with the derivatizing reagent (Figure 3.18).

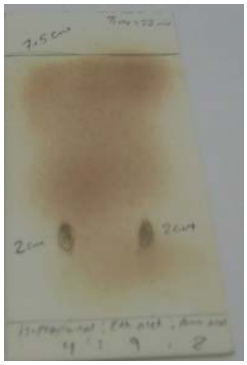


Figure 3.16: The official TLC test for SPR

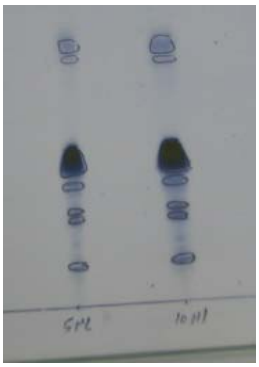


Figure 3.17: Separation of SPR with optimized TLC method

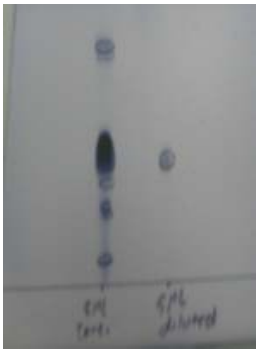


Figure 3.18: Detection of SI with optimized TLC method at sample concentration 40 µg/ml

3.2.2.2. HPLC analysis after separation by preparative TLC:

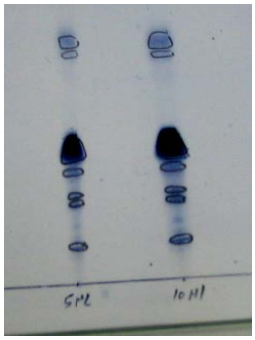
HPLC technique was used to examine the identity of the separated seven spots by TLC. A thick layer chromatographic analysis was used to separate a large amount (50 mg drug dissolved in 1 ml methanol) using the same solvent (mobile phase). Three representative bands were selected to test the presence of those seven different compounds by scratching each band and extract them in three successive steps firstly with dichloromethane (50 ml) then with 50 ml methanol. Finally, the analytes were extracted with 50ml dichloromethane. The extraction process followed filtration, washing. The collected filtrates were subjected to rota vapour instrument for evaporation of the solvent till dryness. The weight of the extracted compounds was calculated by subtracting the weight of the silica before and after the extraction process (Table 3.15). The obtained residue for each band was dissolved with sodium perchlorate monohydrate buffer adjusted with perchloric acid to pH 2.2 : acetonitrile in the ratio of 7:3 to get final concentration of the drug 250 ng / ml using the same method of gradient elution system which mentioned in the experimental part (section 5.2.1.4) to test how

many peaks will be observed with these bands in each chromatographic run. The first separated band which referred with number I (highly polar compounds = which more polar than SI). No definite peak can be showed in the obtained chromatogram with a sample concentration (250 ng / ml). Therefore more concentrated sample (5 µg/ml) was tested to see if there is an effect of the sample concentration or not. Four chromatographic peaks were obtained which can be suggested to be for the four spots below the main spot (SI) with TLC. Only one peak was obtained with the second band with the same t_R of SI. That is why a suggested name for this band was the main band as it carries the main compound. Therefore the most probably left compounds were the less polar compounds than SI. Two peaks were observed in the resulted chromatogram with the third band which suggested being for SII and SIII (Figure 3.19).

Table 3.15: The calculated weight of extracted components after analysis of SPR on preparative TLC**.**

The scratched band	**Weight of the silica**		**The calculated weight of the compounds**
	before the extraction process	after the extraction process	
High polar compounds	17.5169	17.4631	0.0538 g
Main band	16.8715	16.8616	0.0099 g
Less polar compounds	19.3308	19.3276	0.0032 g

A



B

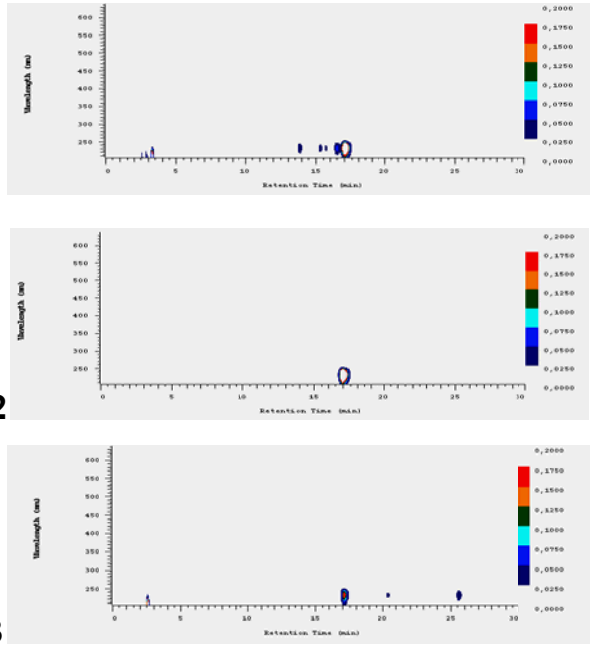


Figure 3.19: Representative TLC of the analysis of SPR, **A** and Representative chromatograms for HPLC analysis of SPR after separation by preparative TLC, **B,** 1. high polar compounds, 2.main compound and 3. less polar compounds bands .

3.2.2.3. Validation of the optimized TLC Purity test:

The optimized TLC purity test for SPR was validated by:

3.2.2.3.1. Repeatability

To ensure assay precision within day repeatability (n=15) was assessed for SPR (Tables 3.16).

Table 3.16: Repeatability for SPR at n=15 at the same day

Parameters	**SI**	**Less polar compound**	**More polar compound**
mean R_f value	0.43	0.75	0.24
SD	0.029	0.02	0.02
RSD%	6.75%	2.66%	8.3%
SE	0.007	0.005	0.005
RSE%	1.6%	0.66%	2.1%

3.2.2.3.2. Intermediate precision

Between days intermediate precision (n=20) were assessed for SPR (Tables 3.17).

Table 3.17: Intermediate precision for SPR (n=20) at different days

Parameters	SI	Less polar compound	More polar compound
mean R_f value	0.43	0.75	0.25
SD	0.038	0.026	0.026
RSD%	8.6%	3.46%	10.4%
SE	0.008	0.0058	0.0058
RSE%	1.66%	0.77%	2.3%

3.2.2.3.3. Reproducibility and Robustness

The developed method was applied in different laboratoriess, on different days. The reproducibility and robustness were tested and the results are summerised in table 3.18.

Table 3.18: Reproducibility and Robustness for SPR (n=30)

Parameters	SI	Less polar compound	More polar compound
mean R_f value	0.43	0.74	0.24
SD	0.037	0.025	0.027
RSD%	8.6%	3.5%	11.2%
SE	0.007	0.005	0.005
RSE%	1.6 %	0.65%	2.1%

3.3 Troxerutin (TRX)

Hydroxyethylrutosides is a standardized mixture of semisynthetic flavonoids obtained by substituting hydroxyethyl groups in the naturally occurring flavonol rutin. It acts primarily on the microvascular endothelium to reduce the hyperpermeability and friability of micrangium, inhibit platelet agglutination and erythrocyte aggregation, prevent the thrombosis and angiosclerosis, and is commonly used for the relief of oedema and related symptoms in patients with chronic venous insufficiency (Wadworth and Faulds, 1992). Because there are four dissociation hydroxyls in rutin, some fifteen kinds of hydroxyethylrutins can be theoretically synthesized (four mono-, six di-, four tri- and one tetra-hydroxyethylrutosides); the proportion of the individual composition in hydroxyethylrutosides is related to the reaction conditions. Other hydroxyethylated constituents, such as tetra-hydroxyethyl-quercetin, in which the sugar moiety is absent, are also present in small amounts. Therefore, 7,3',4'-hydroxyethylrutoside, namely 7,3',4'-tris[O-(2-hydroxyethyl)]rutin (TRX, Figure 3.20) has the highest potency.

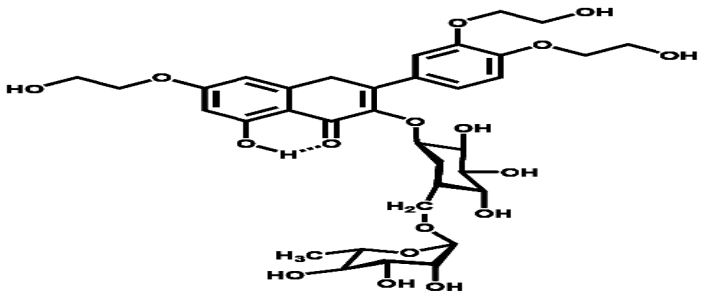


Figure 3.20: Chemical structure of TRX "Trihydroxyethylrutin; 3',4',7-Tris[O-(2-hydroxyethyl)]rutin"

The good quality of the raw material of a drug and the finished product must include the related impurities in an analytical investigation, and this seemed particularly important to the quality control of TRX. Unfortunately, it is difficult to find the standards of the derivative impurities on the market, which sometimes makes the investigation impossible. HPLC is the most commonly used method to control the quality of TRX and its preparations (Deng and Chao, 2001, Ding et al., 2002, Mei et al., 1998, Le Hoang et al., 1985, Kuhnz et al., 1983). The

separation and determination of TRX have been done on a VP-ODS column. The column was thermostatically controlled at 35°C (Yang et al., 2007).

Some other methods, such as TLC (Guo et al., 2004b) and CE (Guo et al., 2004a) were also reported for TRX analysis.

According to the literature, it is clear that, the chromatographic analysis of TRX still problematic as its sample can be considered as a complex sample as it contains plenty of different components. The new stationary phase, monolithic was reported in a combination with capillary electrochromatography for the determination of TRX (Guo et al., 2005). However, there is no new technology as fused core column was used. Therefore, in this study, fused core column with HPLC and TLC methods were developed to separate the major components of TRX bulk powder.

3.3.1 HPLC Analysis

3.3.1.1. Transfer of official HPLC method to UPLC-like method:

TRX is an official drug as there is a pharmacopoeal analytical method for it however the DAB was the only equivalent reference to the pharmacopoeia which publishes an analytical TLC method for TRX in 2005.

Octadecylsilane (ODS) was used as a HPLC column packing material for the official HPLC analysis of TRX in (Pharm.Eur., 2008) with isocratic elution using a sodium dihydrogen phosphate buffer (pH 4.4) : Acetonitrile (80:20). By the application of the practical official procedure a long chromatographic run was observed with poor resolution and the obtained peaks were with tailing. Therefore experimental trials for optimization of the analytical procedure were tested as changing the ratio of the mobile system components from 80:20 to 84:16 as a result the t_R of the analyses were decreased with a decrease in the total chromatographic run time from 40 min to 30 min (Figure 3.21). However the analysis time still long, therefore another trial was tested as a change of the used HPLC column to a fused core column a reduction in the chromatographic run time were observed to just 10 minutes and the obtained peaks were with greater peak heights which indicated a more sensitive detector response.

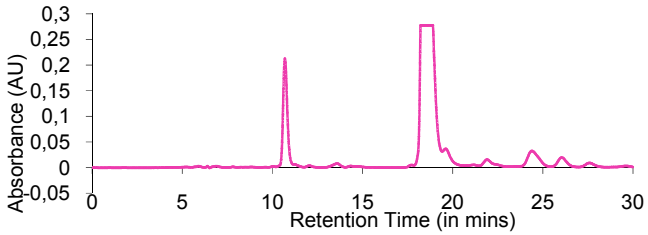


Figure 3.21: Representative chromatogram for optimized HPLC method to analysis of TRX (0.5mg/ml) with conventional column.

It was clearly observed that the chromatographic run time was significantly reduced to about 10 min with the use of fused core column (red chromatogram) compared with the conventional column (blue chromatogram). Moreover the peak height of the obtained components was increased as well by using fused core column which increase the sensitivity of the fused core column HPLC method to the analyses (Figure 3.22).

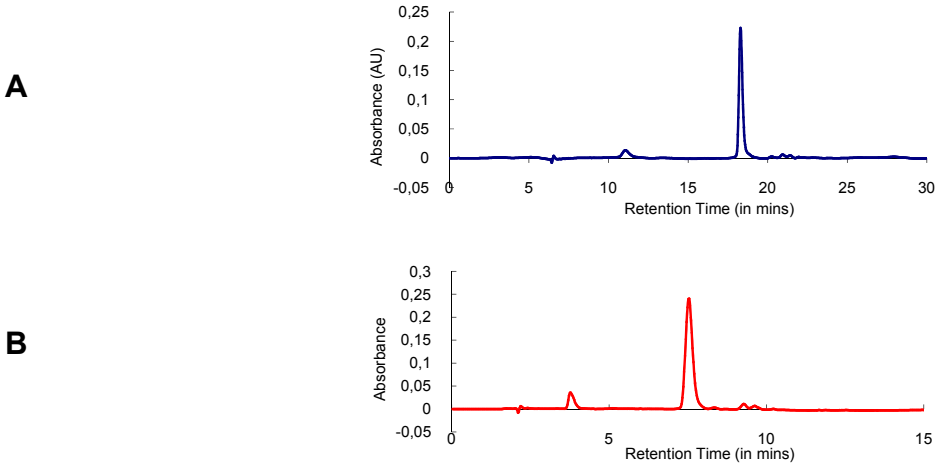


Figure 3.22: Representative chromatogram for analysis of TRX by A: conventional column, B: fused core column.

To examine the optimized procedure for the determination of all components in the presence of each other a large amount of Troxerutin (10 µg) was injected as a sample and as shown in the obtained chromatogram the separation efficiency of the fused core was much better than the conventional one, as extra peaks were observed with the use of fused core column compared with the conventional one.

It is important to notice the difference in the obtained chromatograms with the different sample sources (manufactures). Although both companies are prestigious companies however both gave different chromatograms even if the used columns, the mobile system and all the analytical procedure were the same as shown in the table. As a conclusion of that difference was the TRX is a difficult mixture of components to be analyzed as a different source of that analyte gave different components so that gave another challenge in its determination and/or separation of these components (Figure 3.23)

3.3.2 TLC Analysis

3.3.2.1. Development of TLC purity test:

According to procedure in DAB 2005, 1g of TRX was dissolved in 20 ml of water. 0.3 ml of the produced solution was completed to 10 ml with a mixture of methanol and water (1:1, v/v) to produce the sample solution which prepared for application on TLC and HPTLC plates. 1 and 3 µl of the last solution was applied for the development on HPTLC and TLC plates respectively as a spot by the use of glass microtube. A mixture of 0.1 M HCl, acetone, ethylacetat was used as a mobile phase in the ratio (3:10:10 v/v/v). A UV lamp (254 and 365 nm) was used for the detection of the developed spots at both wavelengths. The expected R_f value ranges for the tested mixture (Table 3.19).

Table 3.19: The expected R_f value ranges for the tested TRX sample

The compound	R_f value range
Tris(hydroxyethyl) rutosid (main spot) at 254 nm	(0.39 – 0.45)
Tetrakis (hydroxyethyl) rutosid (blue spot) weak at 254 nm and strong at 365 nm	(0.16 – 0.26)
Minor compound is isomer B (hydroxyethyl) rutosid-derivate weak at 254 nm	(0.44 – 0.64)

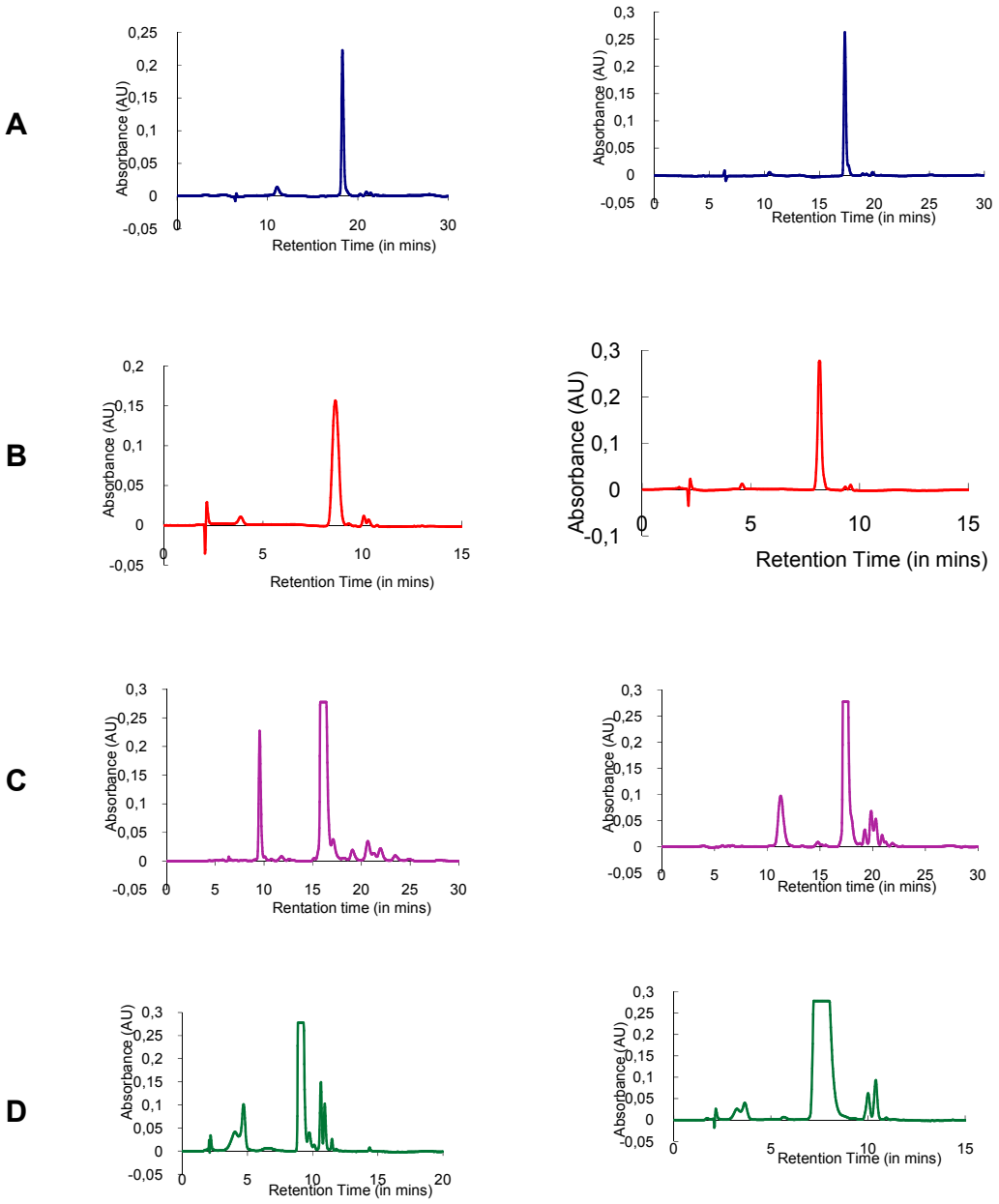


Figure 3.23: Representative chromatograms for analysis of TRX (0.05 mg/ml) A and B on conventional and fused core columns respectively, (0.5 mg/ml) C and D on conventional and fused core columns respectively. Left hand side; TRX 90%, Aldrich chemical (Milwaukee,USA) and right hand side;Trihydroxy ethylrutin ≥80% HPLC, Sigma-Aldrich (Belgium)

However the practically obtained R_f values for TRX sample (Aldrich) were 0.22 and 0.08 for the main spot and blue spot respectively. On the other hand the shape of the obtained spots was not rounded which may be due to a bad resolution and poor separation of the TRX components. This result was not promising for a good separation of the components of TRX as we obtained in the HPLC analysis. Therefore an improvement to the obtained R_f values as well as the shape of the separated spots was an important task by using TLC and HPTLC plates. First of all, the water was excluded from the sample solvent as a tested parameter for that improvement. Therefore a 0.3 ml of test sample was mixed with 10 ml of methanol then the procedure was completed as stated earlier. The R_f values were slightly increased to 0.236 and 0.09 for the main spot and blue spot respectively. That means the obtained R_f still lower than the expected range and the shapes of the spots still not suitable for the good detection (Figure 3.24).

Therefore a reduction of the sample solvent polarity was a second practical trial to improve both R_f values and the shape of the separated spots. 0.3 ml of test solution was diluted to10 ml with a mixture of methanol and chloroform (2: 8, v/v) which leads to a clear solution. 1 and 3 µl of the produced sample was applied on the HPTLC and TLC plates respectively then the procedure was completed as before. The obtained spots were small in size which is better in shape however the calculated R_f values was still less than expected values.

Therefore an increase of the polarity of mobile system was another practical test to increase the R_f values to be withen the expected ranges of DAB. An optimum mobile phase was with a mixture of 0.1 M HCl, acetone and ethylacetate (3: 9: 7, v/v/v) and the obtained R_f values were 0.43 and 0.24 for the main and blue spots respectively (Figure 3.25) and the developing time was 4 min by using TLC plates and only 2 min by using HPTLC plates.

However in all previous trials the minor compound couldn't be detected that may be attributed to the small drug concentration used as this component may be less than its LOD with the used drug concentration. That is why it was suggested to increase the sample content. A 0.6ml of test solution was

completed to 10 ml with a mixture of methanol and chloroform (4:6 v/v) to get final sample concentration 3 mg/ml. As observed in the used solvent the methanol volume was increased and the chloroform volume was relatively decreased and the reason behind that is to obtain the clear solution as used the test solution was more concentrated and the following procedure was completed as before. With the last conditions the third spot which related to minor compound was detected with R_f =0.57 (Figure 3.26).The TLC and HPTLC plates were activated 1 hr at 120 °C for all experiments.

Another tool of detection was used in both drug concentrations which is the use of the phosphmolibdic acid reagent. With the low sample concentration, an only one spot was observed as a result of the main compound with yellow colour. However at high sample concentration other 2 compounds appear.

The same procedure was applied on a sample from the other company, Trihydroxyethylrutin (Fluka) and the R_f values of 3 compounds were at normal range.Blue spot 0.24, Main spot 0.44, Minor compound 0.58.

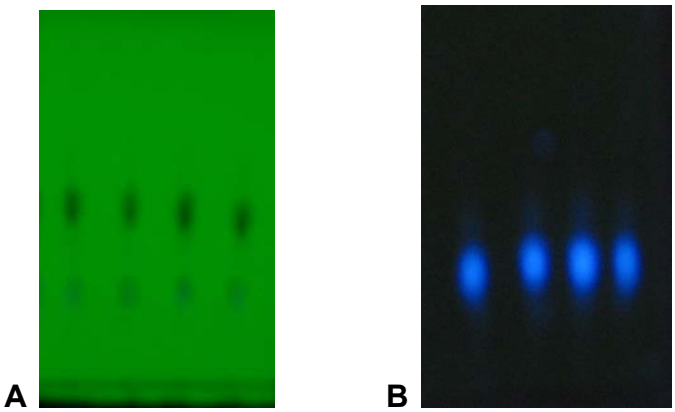


Figure 3.24: Representative figure for using methanol as sampl solven for TRX analysis, A at 254 nm and B at 365 nm

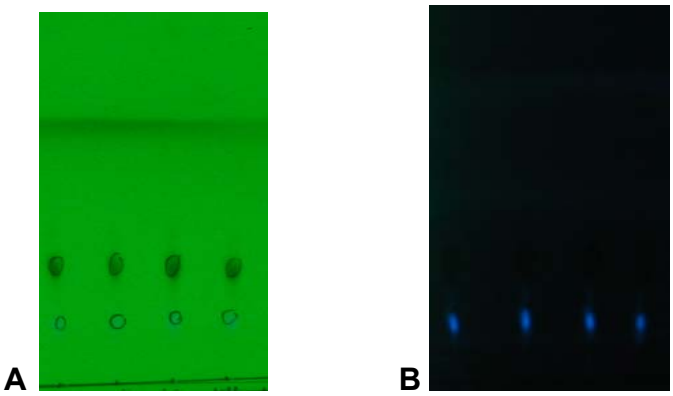


Figure 3.25: Representative figure for using the developed method for TRX analysis, A at 254 nm and B at 365 nm.

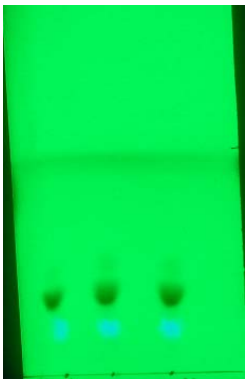


Figure 3.26: Representative figure for detection of minor compound in concentrated TRX sample (3mg/ml) at 254 nm, sample solvent is methanol/ chloroform (4:6 v/v).

3.3.2.2. Validation of developed TLC purity test:

The developed TLC method by using methanol / chloroform (2: 8 v/v) as sample solvent and a mixture of 0.1 M HCl, acetone and ethylacetate (3: 9: 7 v/v/v) as mobile phase was validated by:

3.3.2.2.1. Repeatability

The validation of the developed method was tested for the main spot by repeatability at the same day (n=15). The mean R_f value was 0.43 and SD = 0.007. SE = 0.002, RSD = 1.6 % and RSE = 0.46%.

Also, the validation of the developed method was tested for other compound (blue spot) by repeatability at the same day (n=15), the mean R_f value was 0.23 and SD = 0.008. SE = 0.002, RSD = 3.5 % and RSE = 0.87 %.

3.3.2.2.2. Intermediate precision:

The validation of the developed method was tested for main spot by intermediate precision at different day (n=20). The mean R_f value was 0.43 and SD =0.007. SE =0.0015, RSD = 1.6 % and RSE = 0.36 %.

Also, the validation of the developed method was tested for other compound (blue spot) by intermediate precision at different day (n=20), the mean R_f value was 0.23 and SD =0.008. SE = .0018, RSD = 3.5% and RSE = 0.78 %.

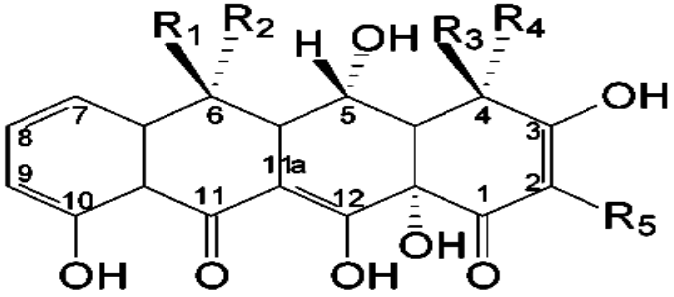
3.3.2.2.3. Reproducibility and Robustness

By applying the same method for the determination of TRX (main spot) in different labs, at different days, and by using TLC plates from different companies the reproducibility and robustness was tested (n=30). The mean R_f value was 0.43 and SD = 0.009. SE =0.0017, RSD = 2.1% and RSE = 0.51 %.

For the blue spot, the mean R_f value was 0.23 and SD =0.009. SE =0.0017, RSD =3.5 % and RSE = 0.71 %.

3.4 Doxycycline- Monohydrate (DOX)

DOX,1-dimethylamino-2,4a,5,7,12-pentahydroxy-11-methyl-4,6-dioxo-1,4a,11, 11a,12,12a-hexahydrotetracene-3-carboxamide (Figure 3.27), is a semi-synthetic broad spectrum antibiotic obtained from oxytetracyline (OTC) and it can exist in two forms DOX monohydrate and DOX hyclate. Metacyline (MTC) is an intermediate of the synthetic pathway. During the transformation of MTC into DOX, some 6 – epidoxycycline (6-EDOX) can also be formed. Upon storage of DOX in solution, it may epimerize to 4 – epidoxycycline (4-EDOX). For this compound, keto-enol tautomerism between C11 and C12 occurs (Naidong et al., 1993). 4,6-Epidoxycycline (4,6-EDOX), the epimer of 6-EDOX, is a derivative of minor importance. 2-Acetyl-2-decarboxamidodoxycycline (ADDOX) can be present as a result of the presence of its analogue in the starting material used for synthesis. Usually DOX is used in human and veterinary medicines or as feed additive, due to its activity against a wide range of Gram-positive and Gram-negative pathogens. (Joshi and Miller, 1997).



	R_1	R_2	R_3	R_4	R_5
DOX	H	CH_3	H	$N(CH_3)_2$	$CONH_2$
OTC	OH	CH_3	H	$N(CH_3)_2$	$CONH_2$
MTC		$=CH_2$	H	$N(CH_3)_2$	$CONH_2$
ADDOX	H	CH_3	H	$N(CH_3)_2$	$COCH_3$
6-EDOX	CH_3	H	H	$N(CH_3)_2$	$CONH_2$
4-EDOX	H	CH_3	$N(CH_3)_2$	H	$CONH_2$
4,6-EDOX	CH_3	H	$N(CH_3)_2$	H	$CONH_2$

Figure 3.27 Structures of DOX and its impurities.

The chemical structures of DOX and its related substances are shown in figure 3.27. The potential impurities are therapeutically inactive except OTC and MTC. Their content in commercial bulk DOX is restricted. The Pharm. Eur. sets the limits for 6-EDOX to be not more than 0.5% and any other impurity to be not more than 2% (Pharmacopoeia, 2005).

In the literature, TLC methods were established for determining of DOX (Choma et al., 1999, Oka et al., 1994).

Also, CE methods were used for the purity control of DOX and its impurities have been reported (Van Schepdael et al., 1995, Castellanos Gil et al., 2000). With the CE method described (Castellanos Gil et al., 2000), the above mentioned compounds are separated from each other including ADDOX, which is separated right after the main compound DOX.

LC methods have been described for the separation of DOX and its impurities (Dihuidi et al., 1985, Pharmacopoeia, 2005, Weng et al., 1990) using RP polymer based stationary phases, but these methods are unable to completely separate ADDOX from DOX. DOX was analysed on conventional columns by (Lu et al., 2004, Charoenraks et al., 2004, Castellanos Gil et al., 2000), also RP18- 25 mmx 4.6 mm monolithic column was applied for determination of DOX (Šatínskýa et al., 2005).

The DOX and its impurities were separated by an official method. However, some problems were faced by the analysts as high column temperature and broad chromatographic peaks were observed.

Recently, monolithic column was used as a stationary phase for the analysis of DOX. However, the elution was based on gradient system which has some drawbacks as mentioned in the theoretical part (Cristofani et al., 2009). Also, the monolithic stationary phase was used for the packing of the precolumn of solid phase extraction system of mixture of DOX and other tetracyclins (Sun et al., 2009).

Therefore, UPLC-like method with simple isocratic elution system was developed to separate and quantitate the OTC and MTC in the bulk DOX sample at very small quantity which is 1% of the DOX sample. The study was included a comparison between the monolithic and a new stationary phase (Fused core) in order to develop the optimum method of analysis.

3.4.1. Development of UPLC-like method:

We applied the official HPLC method (Pharm.Eur., 2005, Pharm.Eur., 2008) by using poly (styrene- divinylbenzene) (0.25 m, 4.6 mm, 8 µm at temp. 60°C) column for its determination however the obtained peak was very broad the drug's concentration was 0.8 mg/ml and the run time was 15 min (Figure 3.28). A good separation between DOX and its impurities "MTC and OTC" was obtained. The t_R of Dox, MTC and OTC were 11, 7 and 3 min respectively (Figure 3.29). Some problems were observed with the obtained chromatogram

as peak broadening and tailing. A time variation in the baseline of a chromatogram observed.

The effect of the decrease drug concentration on its peak broadening was tested. A 70 µg/ml of the drug were injected to the HPLC system and all obtained peaks were still broad (Figure 3.30).

However the official HPLC method was not suitable to elute the epimer's chromatographic peak may be because it was retained on the used column poly (styrene- divinylbenzene).

The effect of different packing material of the used analytical column on the peaks broadening was examined. Therefore, fused core, monolithic and luna columns were tested for that task.

In the case of fused core the used mobile phase was phosphate buffer pH 8.0 Acetonitrile (80 : 20) isocratic elution at F(0.5 ml/min). The sample concentration for DOX was 100 µg/ml and the peak broadening was much better than that which obtained from the official method. The t_R of the analyte was 6.0 min (Figure 3.31).

The effect of pH of the used mobile phase on the peak shape was tested. A trial with a same mobile phase with pH 7.0 the peak sharpness was better than that obtained with pH 8.0 however the baseline of the chromatogram was not constant. The t_R of the analyte was 8.0 min (Figure 3.32).

Another trial with pH 5.0 the obtained peaks were in the best conditions in terms of peak broadening, sharpness and baseline. However the peak tailing still observed in the chromatogram. The t_R of the analyte was 7.5 min (Figure 3.33).

A suggested mechanism for the observed peak tailing can be owned to the complexation between the drug and the packing material. Therefore a strong complexing agent was tried as ethylene diamine-tetra-acetic-acid (EDTA) in a concentration (0.2 mg/ml of solvent A) as a mobile phase additive and the peak tailing problem was completely solved. EDTA was suggested to replace the drug to liberate it without any effect on the t_R of the drug (Figure 3.34). A new peak

was observed at t_R 3.0. This peak suggested being the drug epimer. This suggestion is documented with the fact of instability of DOX at pH of 6.0 and below, DOX undergoes conversion into its epimer (McCormick et al., 1957, Stephens et al., 1963).

A 70 µg/ml of the DOX sample containing 0.7 µg/ml of each MTC and OTC (i.e. each impurity 1% of the sample) were injected to the HPLC system with the last optimum conditions with F=0.5 ml/min and a good separation was observed and all obtained peaks were symmetric, sharp without peak tailing.

For a robustness test the optimum mobile phase was tested with different packing materials as luna and monolithic columns (Figure 3.35).

With luna and monolithic column, the obtained peaks were sharp however the drug isomer's peak was not separated from the OTC's peak or almost poorly separated from each other (Figure 3.36). A trial to improve the resolution a decreasing in the F to 0.3 ml/min was tested. A better peaks separation was observed with monolithic column however luna column still bad peaks resolution (Figure 3.37).

Another trial to improve the resolution for the luna by increasing the retention with decreasing the organic modifier percent was examined. Therefore, the acetonitrile was used as 15% of the used buffer v/v with 0.5 ml/min and a much better separation was obtained (Figure 3.38).

The backpressure of different columns (luna, fused core, and monolithic) was tested at L (50 mm) and F= 0.5 ml/min for the analysis of DOX with new developed method. High backpressure was resulted with luna column due to the fine particle size followed with fused core column and the lowest backpressure was resulted with monolithic column which charachtrized with high permeability (Figure 3.39).

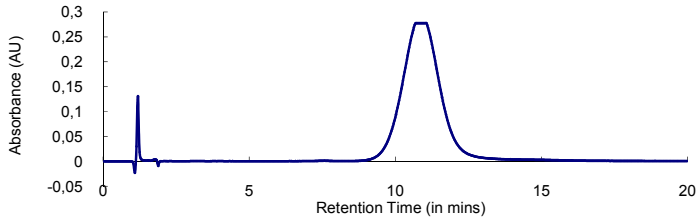


Figure 3.28: Representative chromatogram for determination of DOX (0.8mg/ml) according to the Pharm. Eur. method.

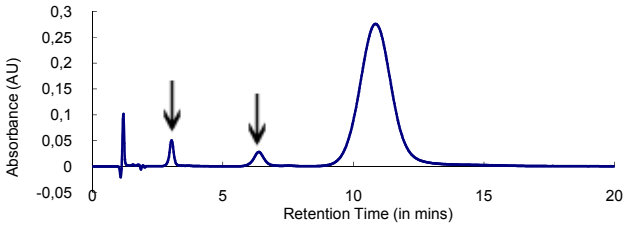


Figure 3.29: Representative chromatogram for separation of DOX (0.8mg/ml) from its impurities MTC (8µg/ml) and OTC (8µg/ml) according to the Pharm. Eur. method.

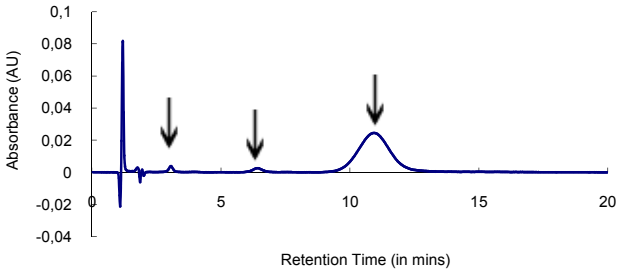


Figure 3.30: Representative chromatogram for separation of DOX (0.07mg/ml) from its impurities MTC (0.7µg/ml) and OTC (0.7µg/ml) according to the Pharm. Eur. method.

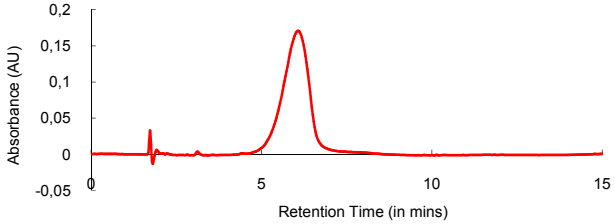


Figure 3.31: Representative chromatogram for determination of the DOX (0.1 mg/ml) by using fused core column with phosphate buffer pH=8 & acetonirtil 80:20 v/v.

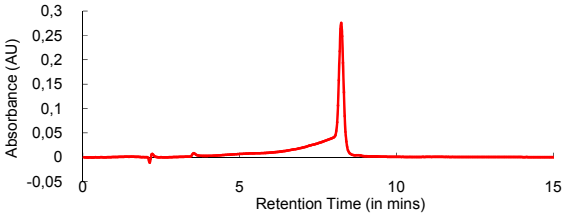


Figure 3.32: Representative chromatogram for determination of the DOX (0.1 mg/ml) by using fused core column with phosphate buffer pH=7 & acetonitril 80:20 v/v.

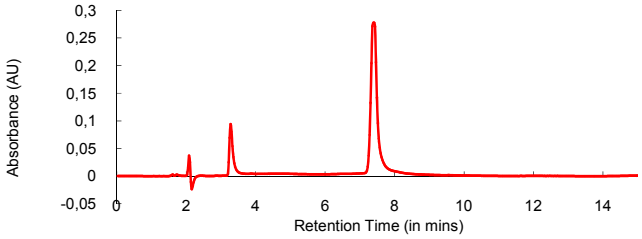


Figure 3.33: Representative chromatogram for determination of the DOX (0.1 mg/ml) by using fused core column with phosphate buffer pH=5 & acetonitril 80:20 v/v.

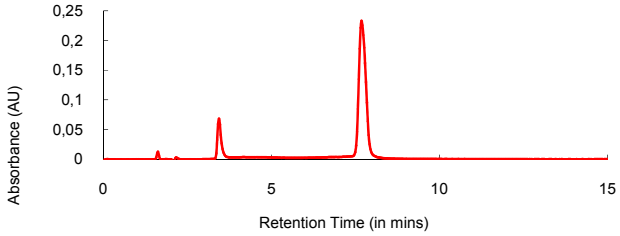


Figure 3.34: Representative chromatogram for determination of the DOX (0.07 mg/ml) by using fused core column with the developed method.

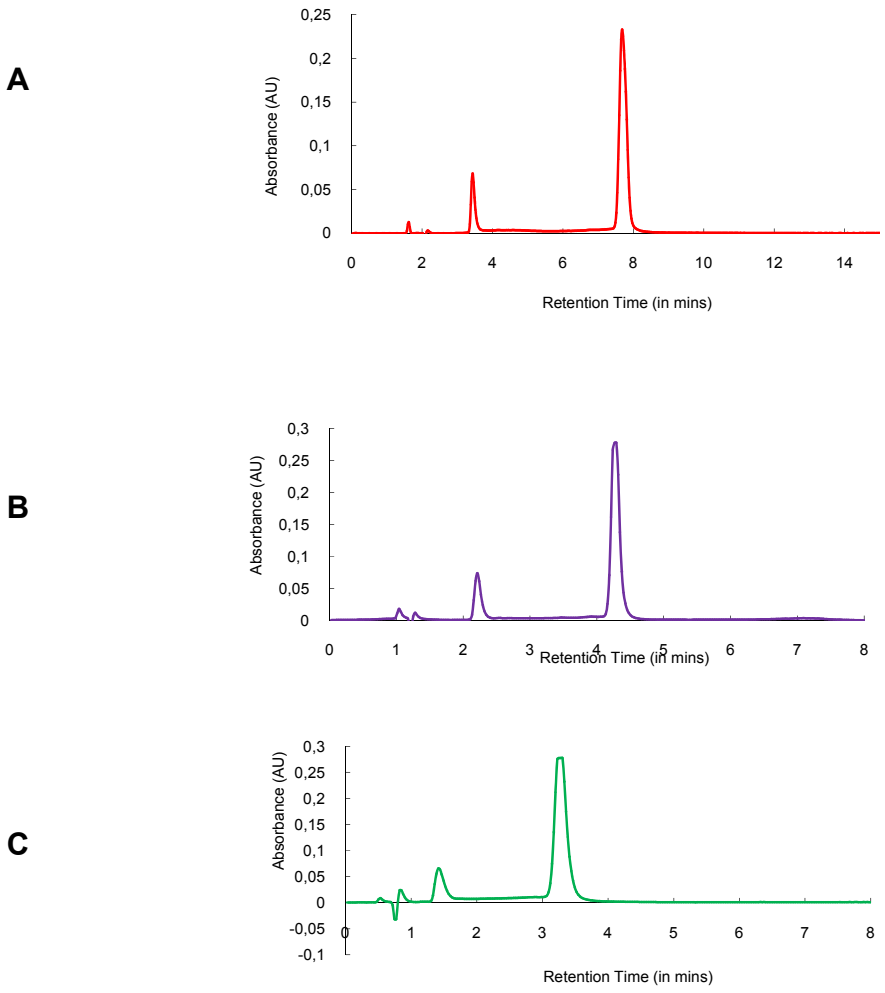


Figure 3.35: Representative chromatogram for determination of the DOX (0.07 mg/ml) by using developed method, and columns: A: fused core B: monolithic and C: luna columns.

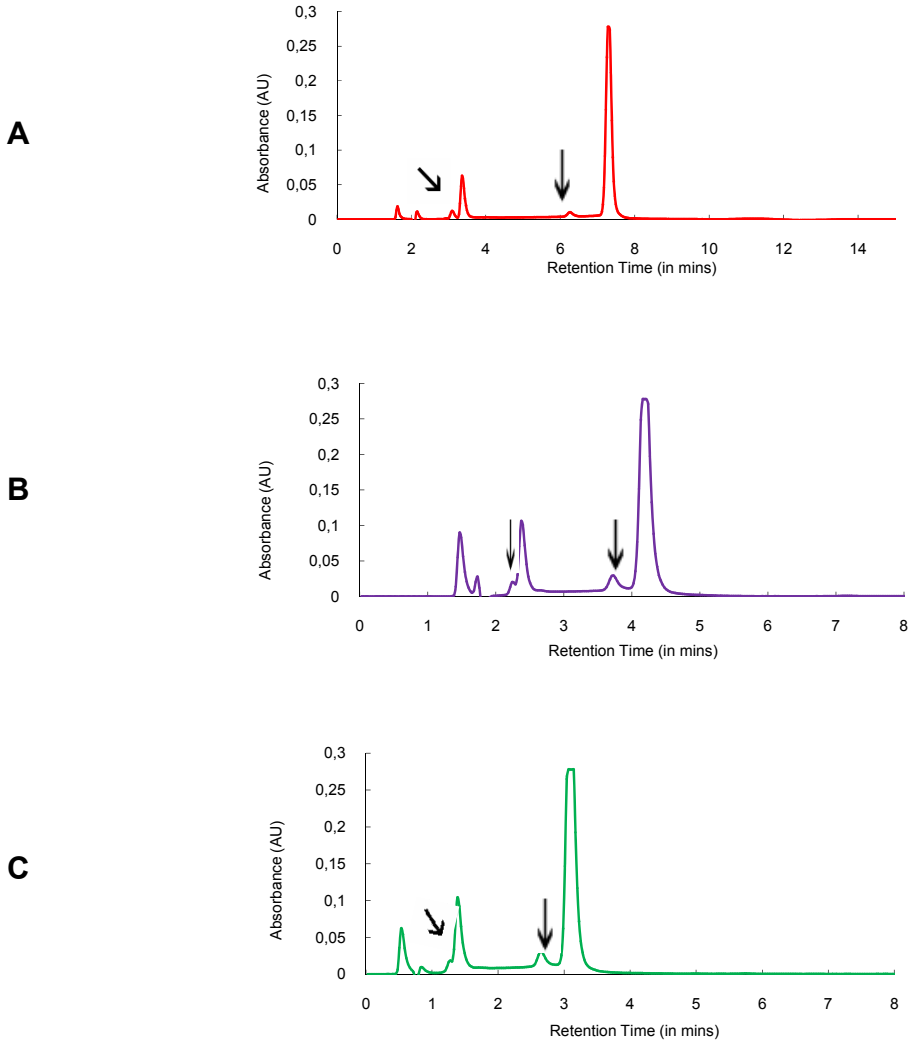


Figure 3.36: Representative chromatogram for the separation of DOX (0.07 mg/ml) from MTC (0.7µg/ml) and OTC (0.7µg/ml) by using the developed method, and A: fused core , B: monolithic, and C: luna columns.

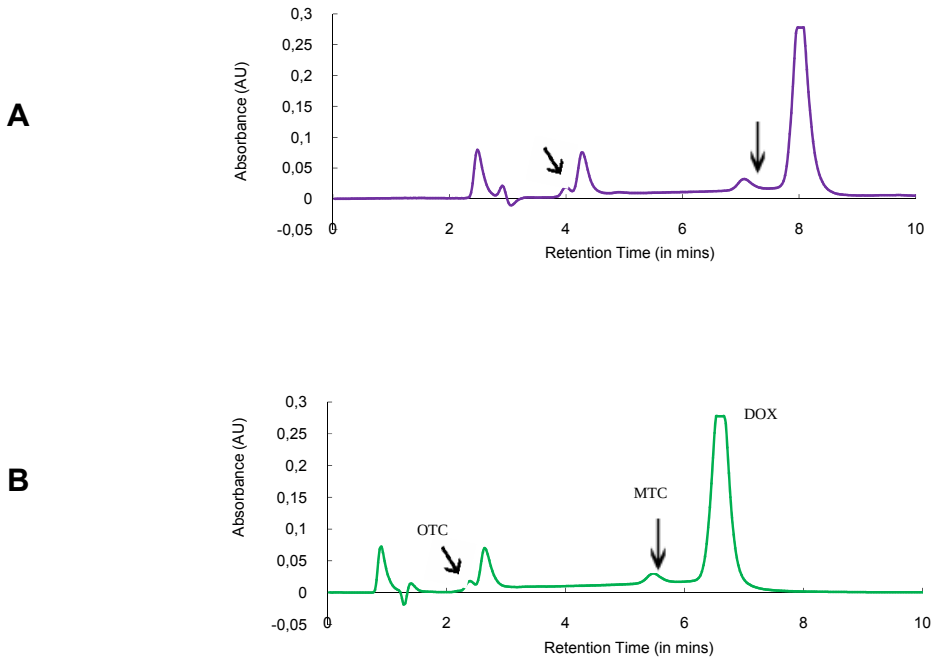


Figure 3.37: Representative chromatogram for the separation of DOX (0.07 mg/ml) from MTC (0.7µg/ml) and OTC (0.7µg/ml) by using the developed method, F=0.3ml/min and, A: monolithic, and B: luna columns.

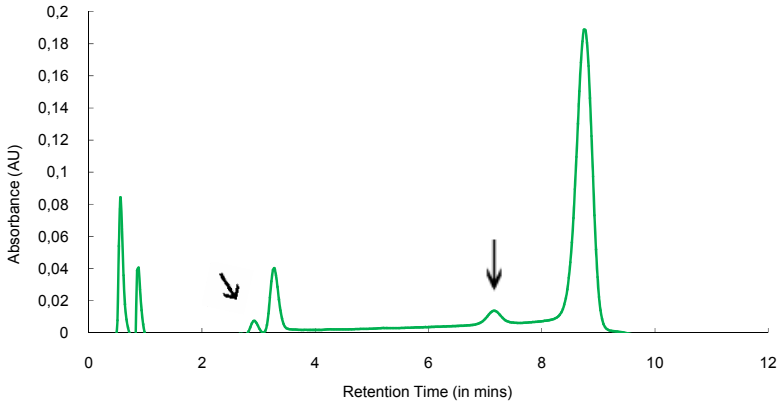


Figure 3.38: Representative chromatogram for the separation of DOX (0.07mg/ml). from MTC (0.7µg/ml) and OTC (0.7µg/ml) by using luna column with solvent A:B 85:15 v/v, F=0.5ml/min.

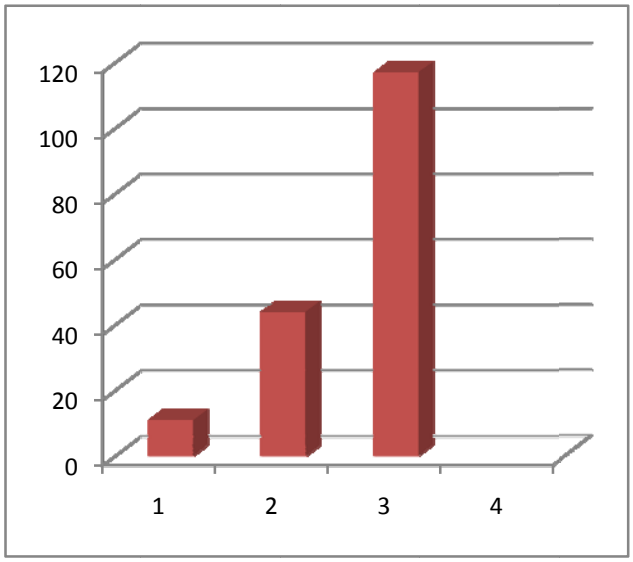


Figure 3.39: Plot of backpressure (bar) against different columns at L (50 mm) and F= 0.5 ml/min 1.Monolithic, 2. Fused core, and 3. Luna column.

3.4.2. UPLC separation of DOX and its epimer:

By appliying the official HPLC method using polymer stationary phase (polystyrene- divinylbenzene) the epimer peak couldn't be separated from the DOX peak. Therefore, the sample solvent was changed (Figure 3.40 and 3.41) as a trial to get the peak of epimer separated but it couldn't be observed in the obtained chromatograms. However, by using fused core, luna or monolithic columns an extra peak was observed which corresponds to the drug epimer.

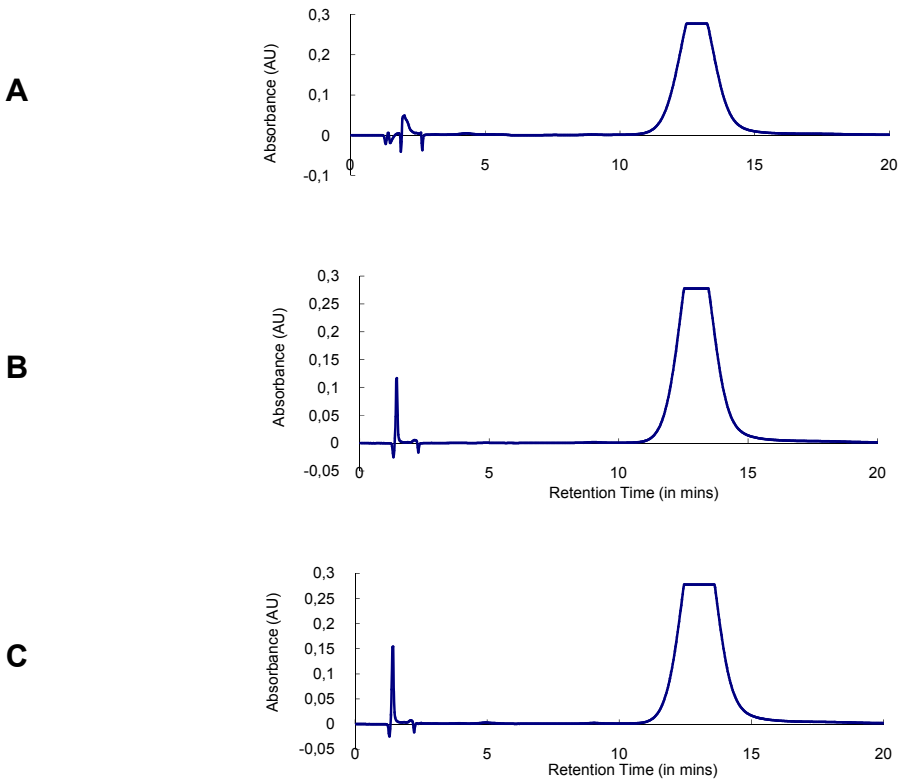


Figure 3.40: Representative chromatogram for analysis of DOX (0.8mg/ml) according to Pharm. Eur. method , sample solvent A: (as in developed method), B: 0.01 M HCl in refrigerator, C: 0.01 M HCl at room temperature 2 days.

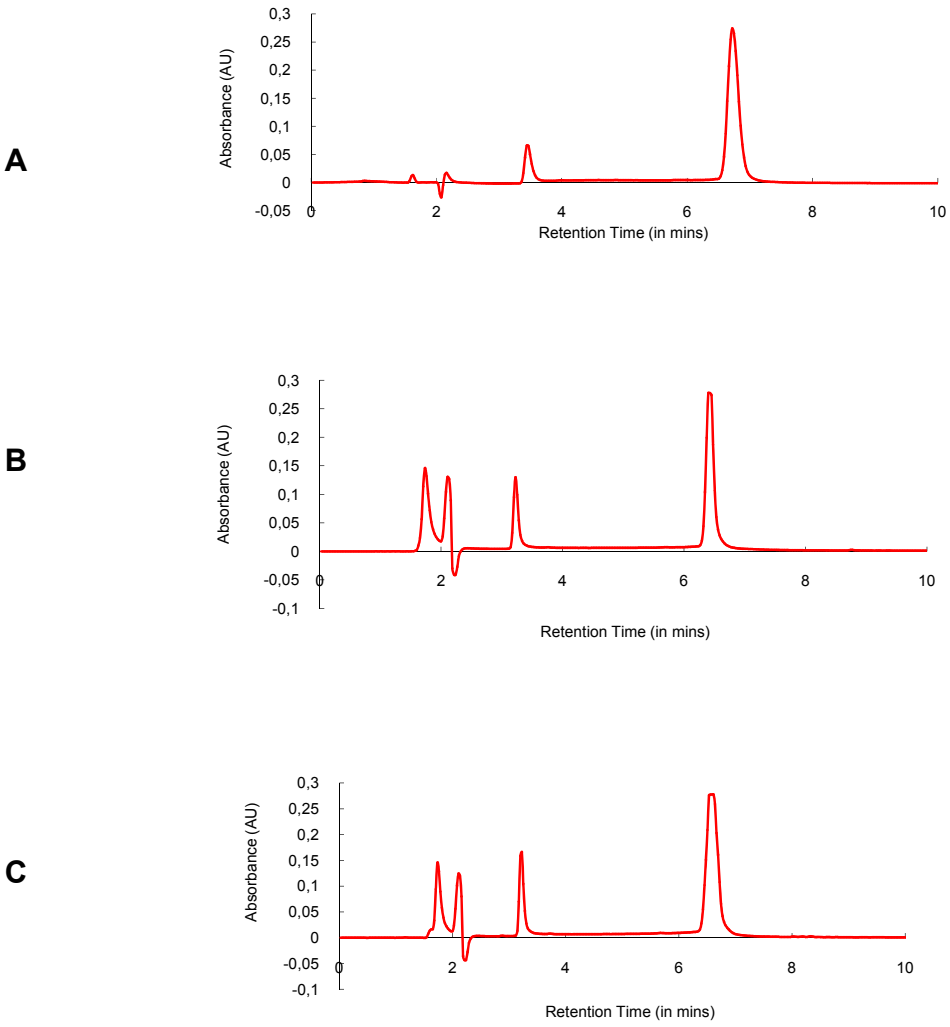


Figure 3.41: Representative chromatogram for analysis of DOX (0.07mg/ml) according to developed method by using fused core column, sample solvent :A (as in developed method), B: 0.01 M HCl (fresh sample), and C: 0.01 M HCl (one day at room temperature)

3.4.3. Validation of the developed method:-

The new developed HPLC method for analysis of DOX was validated by:

3.4.3.1. Precision

To ensure assay precision within day repeatability (n=6) and between days repeatability (n=6) were assessed at 3 concentrations of DOX (Tables 3.20 and 3.21).

Table 3.20 : Within day repeatabilities of DOX on luna , monolithic and fused core columns over a concentration range 0.07-0.3 mg/ml using n=6.

Column type	Within day repeatability RSD% of AUC			Within day repeatability RSD% of t_R (n= 18)
	0.07	0.1	0.3	
	(mg/ml)			
Luna	0.62	0.74	0.70	0.51
Monolithic	0.57	0.66	0.71	0.53
Fused core	0.73	0.53	0.38	0.42

Table 3.21: between days repeatabilities of DOX on luna, monolithic and fused core columns over a concentration range 0.07- 0.3 mg/ml using n=6.

Column type	Between days repeatability RSD% of AUC			Between days repeatability RSD% of t_R (n= 18)
	0.07	0.1	0.3	
	(mg/ml)			
Luna core	0.60	0.81	0.79	0.54
Monolithic	0.65	0.70	0.80	0.52
Fused core	0.59	0.62	0.56	0.50

3.4.3.2. Linearity, LOD and LOQ of the DOX

Calibration curve (peak area vs. concentration) for the analyzed DOX with new method was investigated over concentration range of 0.07- 0.3 mg/ml. The (LOD, S/N =3) and an estimate for the (LOQ, S/N = 10) for the DOX (Table 3.22).

Table 3.22: Linearity, LOD and LOQ of DOX

Column type	LOD (µg/ml)	LOQ (µg/ml)	R^2
Luna	0.041	0.120	0.9998
Monolithic	0.030	0.080	0.9999
Fused core	0.012	0.040	0.9999

3.4.4. Performance parameters of the developed UPLC-like method:

Peak performance parameters were calculated according to fundamental equations (Table 3.23).

Table 3.23: Performance parameters for DOX on monolithic (50mm), luna (50mm) and Fused core (100mm) columns at flow rate 0.5 ml/min.

Column type	Theoretical plate N (Plate per column for DOX	AF for DOX Peak	(Rs)DOX /MTC	Run time (min)
Monolithic	1841	1.1	1.5	5 min.
Luna	486	1.2	1.1	3.5 min.
Fused core	3775	1.1	2.5	8 min.

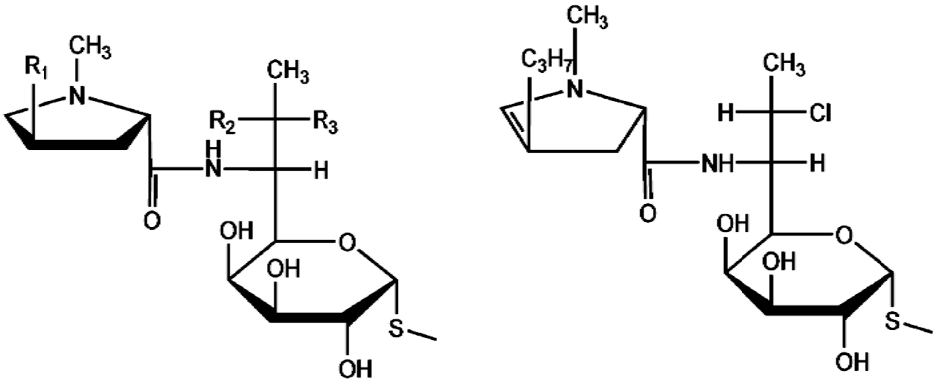
3.5 Clindamycin (CLD)

CLD (Figure 3.43) is an antibiotic that is highly effective against Gram-positive and Gram-negative anaerobic pathogens, as well as Gram-positive aerobes (Lafollette et al., 1988). CLD appears to inhibit protein synthesis in susceptible organisms by binding 50 S ribosomal subunits; the primary effect is inhibition of peptide bond formation (Gibson and Barker, 1988).

CLD is a semisynthetic antibiotic produced by a 7(S)-chloro-substitution of the 7(R)-hydroxyl group of the parent compound lincomycin by replacing its hydroxyl group at 7-position with a chlorine atom, resulting in an inversion of the configuration (Birkenme.Rd and Kagan, 1970).

Common impurities (Figure 3.42) in CLD bulk drug are CLD B, 7-epiclindamycin, and a small amount of the lincomycin starting material (Pharm.Eur., 2005). CLD

B is formed from lincomycin B which is a normal by-product of the fermentation; 7-epiclindamycin is produced during the synthesis of CLD (Landis et al., 1980).



		M.W.	R_1	R_2	R_3
1	Clindamycin	424	CH_3-CH_2-CH_2-	H	Cl
2	7-Epiclindamycin	424	CH_2-CH_2-CH_2-	Cl	H
4	Clindamycin B	410	CH_3-CH_2-	H	Cl
5	7-Epilincomycin	406	CH_3-CH_2-CH_2-	H	OH
6	Lincomycin	406	CH_3-CH_2-CH_2-	OH	H

3 Dehydroclindamycin (M.W. 422)

Figure 3.42: Stractures of CLD and related compounds

Further improvement in pharmaceutical properties of CLD is obtained by chemical modification to obtain the esters, CLD phosphate or CLD palmitate (Orwa et al., 1999)

It is used in the treatment of serious respiratory tract infections (e.g. empyema, pneumonia, lung abscess), serious skin and soft tissue infections, septicemia, intraabdominal infections and in infections of the female pelvis and genital tract caused by susceptible anaerobic bacteria (Phillips, 1971).

Several methods have been described in the literature for the determination of CLD in different sample matrices, including assays based on microbiology (Metzler et al., 1973), spectrophotometry (Elyazbi and Blaih, 1993), gas chromatography (Gatti et al., 1993), micellar electrokinetic chromatography (MEKC) (Dehouck et al., 2001), coupled (CE) with end-column electrogenerated chemiluminescence (ECL) (Wang et al., 2008), electrospray tandem mass spectrometry (Zhou et al., 2006), and chemiluminescence (Shao et al., 2006), have thus been reported for the determination of CLD in bulk drug, dosage forms and biological fluids.

A LC–tandem mass spectrometric (LC-MS/MS) method (Yu et al., 1999, Cherlet et al., 2002, Martens-Lobenhoffer and Banditt, 2001, Rechberger et al., 2003) was developed to determine CLD; however, these LC-MS/MS instruments are not yet readily available in all laboratories.

HPLC with UV detection (Batzias et al., 2004, Platzer and White, 2006), HPLC using either an RP18 column (Lafollette et al., 1988) or a coupled column (Fieger-Buschges et al., 1999) and other reported HPLC are mentioned in the table showing the column type used in each method (Table 3.24).

All HPLC reported methods were with conventional columns and long analysis time. In this thesis, the official HPLC method was transfered to UPLC- like method by using new stationary phase to get sensitive detection for CLD and its related substances with short time.

Table 3.24: Column types were used for CLD analysis

Column type	Year	References
RP C18 column (YMC-PACK ODS-A, 150 mm x 4.6 mm i.d., 5 µm, 120 A) at 25°C.	2006	(Zhou et al., 2006)
Spherisorb ODS-2 (250 mm x 4 mm i.d., 5 µm), C18 RP analytical column	2004	(Batzias et al., 2004)
Waters Xterra RP18 column (4.6mm i.d. x 100 mm, 3.5 µm).	2006	(Platzer and White, 2006)
RP C18 column Lichrospher, RP18, 5µm , 25cm x 4.6 mm i.d., phenomenex, USA)	2004	(Ye et al., 2004)
waters spherisorb (waters Assoc., Milford, MA, USA) CN (250 x 4.6 mm i.d., 5 µm) RP HPLC column at 35°C.	2005	(Cho et al., 2005)
5 µm, 25 cm x 4.6 mm i.d. ODS2 column	1997	(Liu et al., 1997a)
RP Nucleosil 100 C18 HD column	1999	(Fieger-Buschges et al., 1999)
5 µm- Hypersil column C18 (50 x 4.6 mm i.d.).	1999	(Yu et al., 1999)

3.5.1. Transfer of the official HPLC to UPLC-like method:

The method of analysis of CLD was adapted from the Pharm. Eur. (Pharm.Eur., 2005, Pharm.Eur., 2008) isocratic elution with a 45% acetonitrile concentration (v/v) in potassium hydrogen phosphate buffer, pH 7.5 and RP18 column specified in the Pharm. Eur. 2005 and the t_R of the drug was about 8.2 min (Figure 3.43 A) However, in our practical trials, some parameters were tested as the effect of the increase the organic modifier (about 10%). By increasing the amount of acetonitrile in the mobile phase from 45% to 55% v/v an decrease in the analysis time (about 2 min) was achieved and also an increase in the peak height was obtained (Figures 3.43 B, C).

Also a trials of changing the used column in the official method to shorter column as luna (sub-3µm), and also column with more advanced packing materials as monolithic, fused core columns were tested as well. As stated above that all reported HPLC methods previously described for the determination of CLD using conventional particle-packed C18 columns have been adapted to our conventional column, Spherisorb ODS2 RP-18 column (5µm particle size, 250mm x 4.6 mm, VDS optilab) (Figure 3.44).

Backpressure for different columns was tested at L (50 mm) and F (1 ml/min), however greater backpressure was resulted with luna column which packed with particles (2.5 µm), followed by fused core column (2.7 µm), and then conventional column (5 µm). The lowest backpressure was resulted by monolithic column due to high permeability (Figure 3.45).

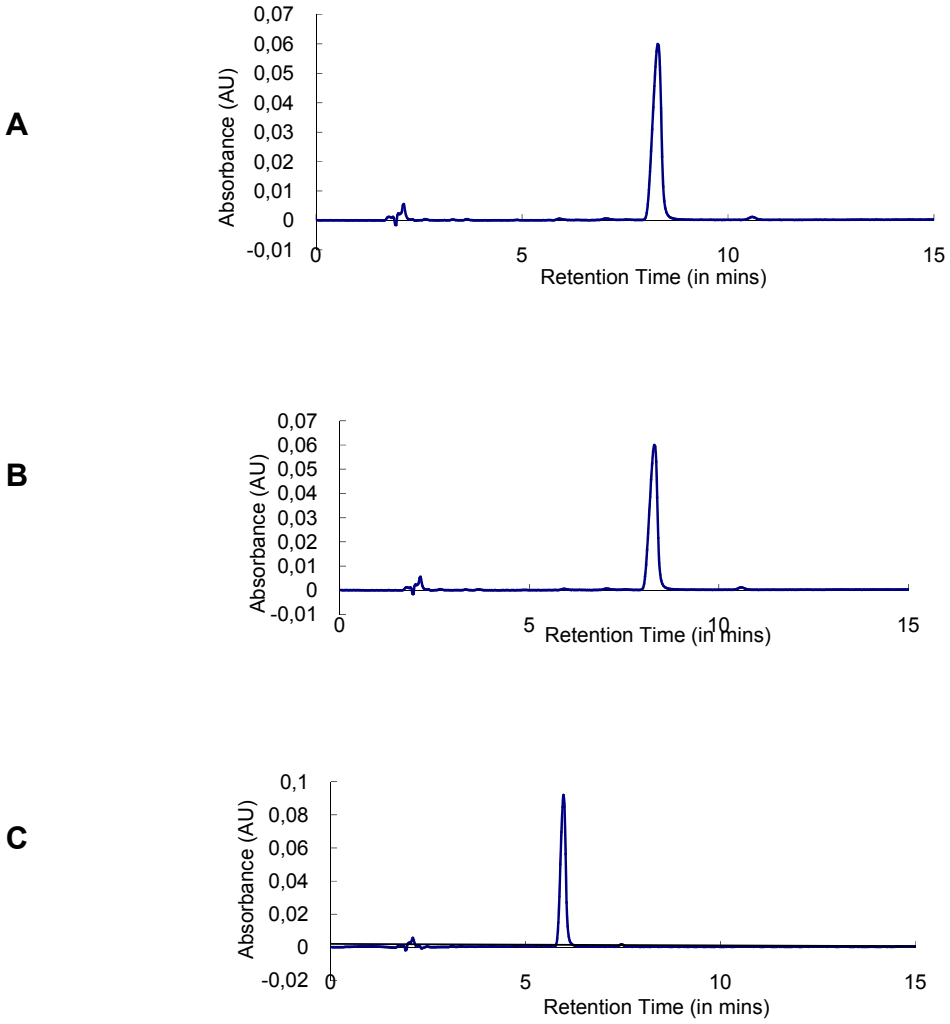


Figure 3.43: Representative chromatograms for analysis of CLD (1mg/ml), on conventional column at 1ml/min F. Mobile phase consists of a buffer pH 7.5: acetonitril **a)** (55:45, v/v), b**)** (50:50, v/v) and c**)** (45:55, v/v).

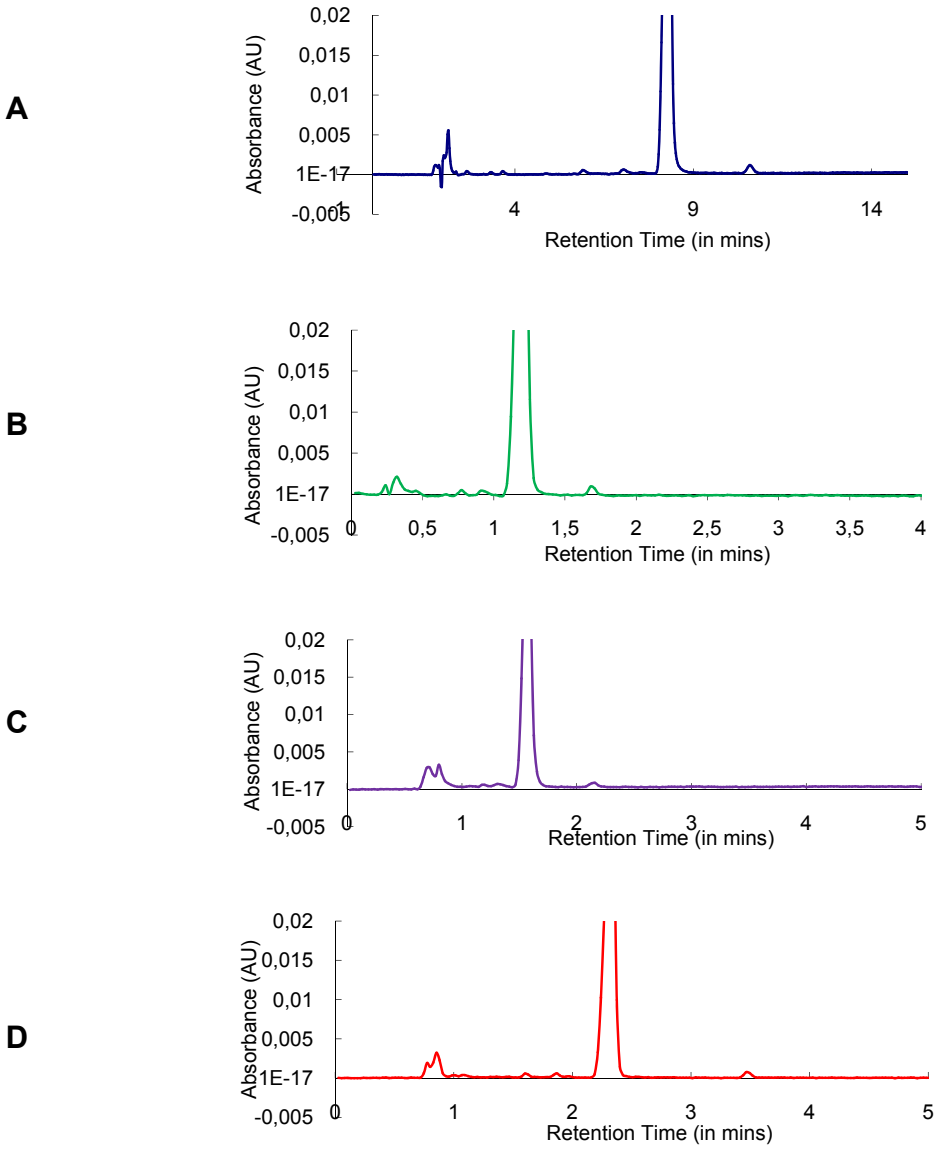


Figure 3.44: Representative chromatograms for CLD (1mg/ml), and its degradation products using Pharm. Eur. procedure with **a)** conventional column, **b)** luna column, **c)** monolithic column and **d)** fused core column.

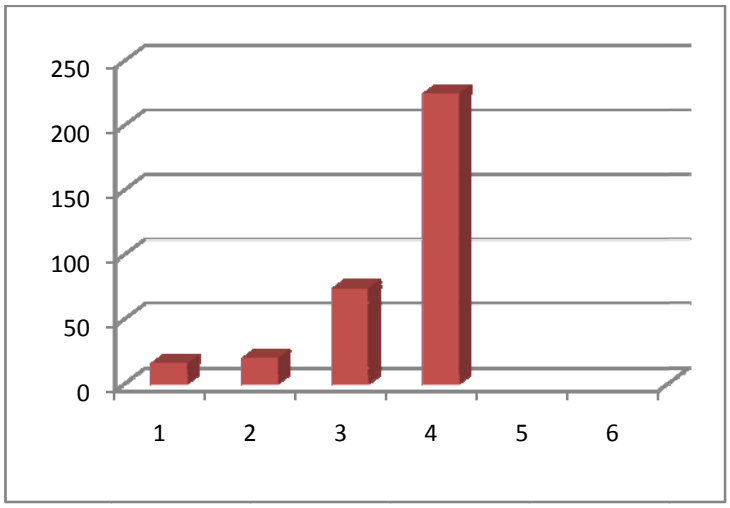


Figure 3.45. Plot of backpressure (bar) against different columns at L (50 mm) and F (1ml/min), 1) monolithic, 2) Conventional, 3) fused core, and 4) Luna columns.

3.5.2. Validation of the developed UPLC-like method:

The developed method by using different stationary phases was validated by:

3.5.2.1. Precision

To ensure assay precision within day and between days repeatability (n=10) for AUC and (n=15) for t_R were assessed on a concentration (1 mg/ml) of CLD (Table 3.25).

Table 3.25: Within day and between days repeatabilities of CLD on conventional, monolithic, luna and fused core columns on a concentration (1mg/ml)

Column type	Within-day repeatability		Between-days repeatability	
	RSD%ofAUC	RSD% of t_R	RSD%ofAUC	RSD% of t_R
	n=(10)	n=(15)	n=(10)	n= (15)
Conventional	0.88	0.52	0.90	0.66
Monolithic	0.54	0.45	0.53	0.53
Luna	0.51	0.50	0.80	0.67
Fused core	0.61	0.44	0.53	0.50

3.5.2.2. Linearity, LOD and LOQ of CLD:

Calibration curve (peak area vs. concentration) for the analyzed CLD with new method was investigated. The (LOD, S/N =3) and an estimate for the (LOQ, S/N = 10) for the CLD (Table 3.26).

Table 3.26: Linearity, LOD LOQ of the CLD

Column type	LOD (µg/ml)	LOQ (µg/ml)	R^2
Conventional	0.061	0.200	0.9992
Monolithic	0.012	0.040	0.9998
Luna	0.013	0.047	0.9996
Fused core	0.011	0.038	0.9999

3.5.2.3. Robustness

The effect of pH also was tested (Figure 3.46) and the effect of the type of the cations or anions of the used buffer was examined (Figure 3.47) The temperature effect on the chromatographic behavior of CLD was investigated in (Figure 3.48) in all conditions the flow rate was 1 ml/min and all chromatograms were monitored at 205 nm. The robustness of the method was tested at pH range (6-8) and the temperature to 35°C, also by changing positive ions or negative ions of buffer which used in the Pharm. Eur. It was no significant effect on the efficiency of separation.

3.5.2.3.1. Effect of buffer pH and its ions:

The change of pH was tested by decreasing the buffer pH from (7.5) to strong acidic (2.5) which lead to protonation of the major compound (CLD) and the high polarity of the resulted compound was decreased its t_R and sensitivity to the U.V. detector. Although Hornedo-Nuñez et al.(Hornedonunez et al., 1990) successfully applied a mobile phase with a pH 2.5. Adjustment was performed with the use of 1M *ortho*-phosphoric acid. Even in such pH values, silanols are expected to be substantially ionized. The addition of an ion-pairing agent in the mobile phase results in a relative “masking” of their negative charge and therefore, in a decrease in the electrostatic attraction between silanols and CLD. Furthermore, the theory of electrostatic repulsion of protonated analyses, like CLD, by positively charged tetra-*n*-butylammonium ions adsorbed onto the stationary phase has also been suggested (Fletouris et al., 1996). However. In

our study we develop the HPLC method by using as simple mobile phase as possible therefor higher pH of the buffer was tested. It was reported that an aqueous solutions of CLD show a maximum stability at pH (3.5–6.5) (Oesterling and Rowe, 1970, Nahata et al., 1993), therefor pH range (6-8) was tested and only small decrease in the sensitivity of CLD to U.V. at pH=6 it is suggested that the chemical structure of CLD was protonated or ionized in a functional basic group (Figure 3.46). However the efficiency of separation was roubst at pH range (6-8) and with changing the buffer ions except small peak broadening and tailing was resulted by using ammonium acetate instead of potassium hydrogen phosphate.

3.5.2.3.2. Temperature effect

The temperature of the column oven was set to 40°C because no decrease in t_R was observed at higher temperature. The effect of temperature was only minor but measurable at the low flow rate (1.0 ml/min). Representative chromatogram of the analyzed drug at flow rate of 1.0 ml/min using column oven set to 40 °C is shown in (Figure 3.48 B) the only notice is the high temperature led to the disappearance of the last small peak shown in (Figure 3.48 A) due to accelerated the elution of this compound to elute it with the major peak of CLD.

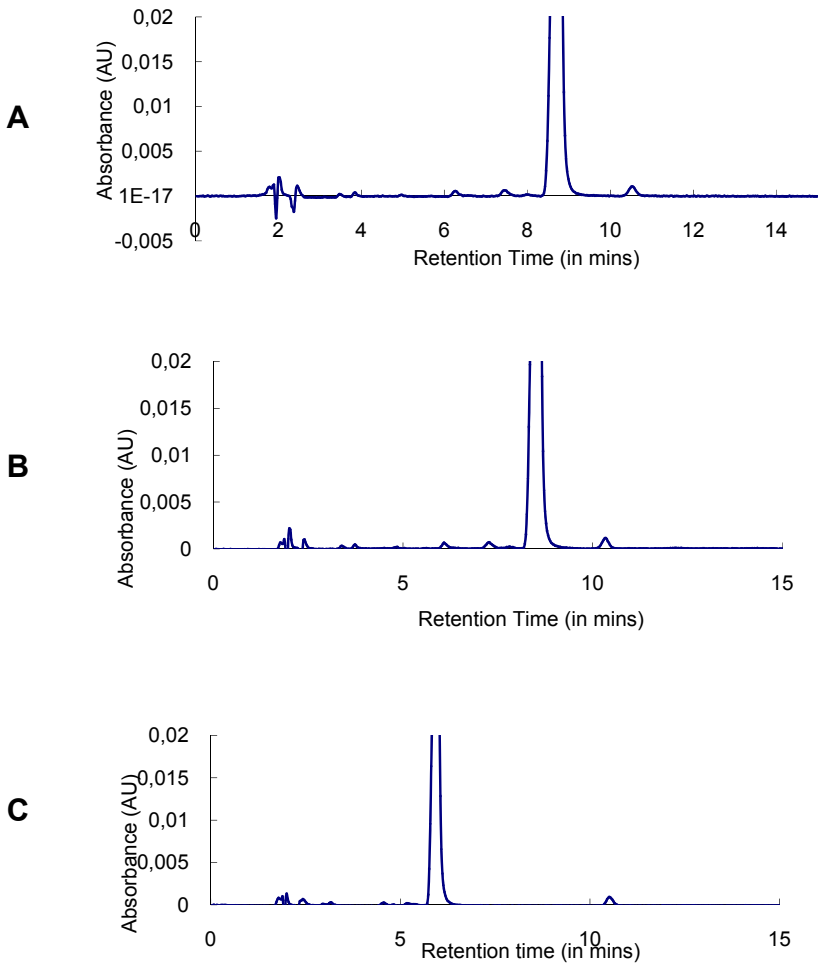


Figure 3.46: Representative chromatograms for CLD (1mg/ml) using conventional column, showing the effect of pH of the used mobile phase **a)** pH 7.5, **b)** pH 8.0 and **c)** pH 6.0

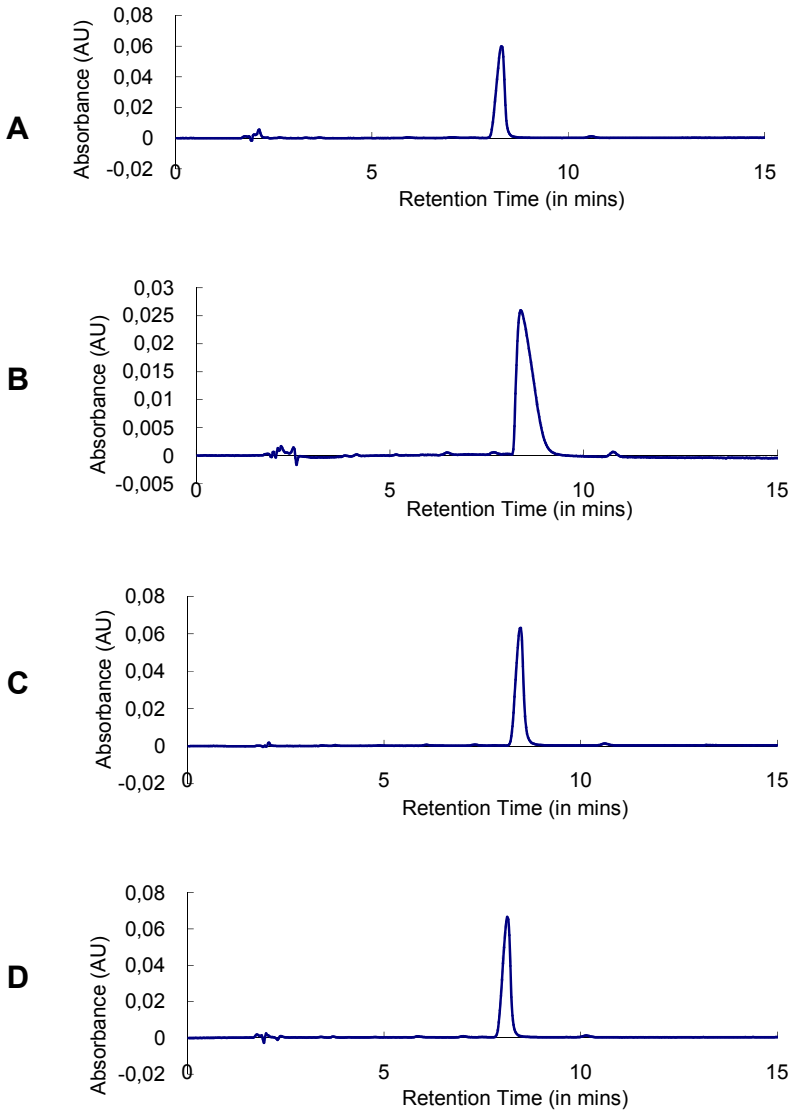
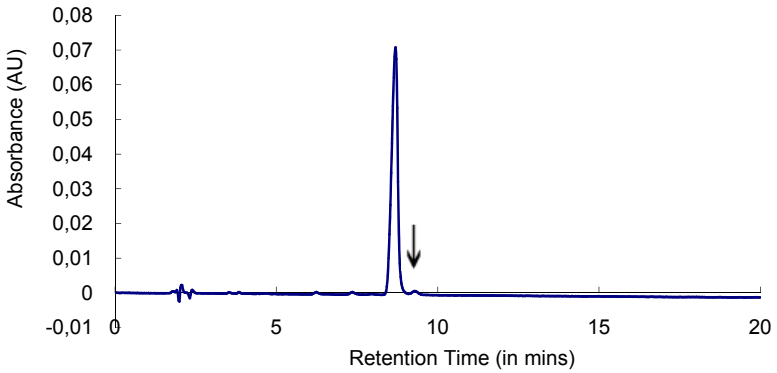


Figure 3.47: Representative chromatograms for CLD (1mg/ml) using conventional column, showing the effect of buffer ions of the used mobile phase with pH 7.5 in all cases: **A)** Potassium hydrogen Phosphate **B)** ammonium acetate **C)** Potassium acetate, and **D)** ammonium hydrogen Phosphate.

A



B

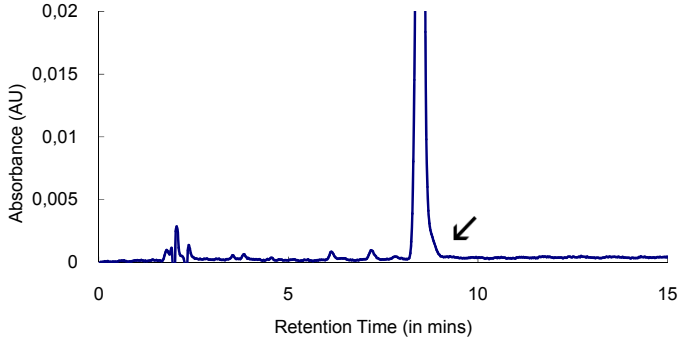


Figure 3.48: Representative chromatograms for CLD (1mg/ml), showing the effect of temperature on the chromatographic separation a) 35°C and b) 40°C

3.5.3. Performance parameters of the developed UPLC-like method:

Peak performance parameters were calculated according to fundamental equations (Table 3.27).

Table 3.27: Performance parameters for CLD on conventional (250 mm), monolithic (50 mm), luna (50 mm) and fused core (100 mm) columns (F=1 ml/min).

Column type	Theoretical plate N (Plate per column for CLD)	AF for CLD Peak	(Rs) CLD/its impurity	Run time (min)
Conventional	3051	1.5	4.6	11.0
Monolithic	631	1.1	3.37	2.5
Luna	576	1.1	3.2	2.0
Fused core	1378	1.0	6.57	4.0

3.6. Roxithromycin (ROX)

(ROX)-9-O- (2-methoxyethoxy- methyl)-oxime- erythromycin is a semi-synthetic, 14- membered ring macrolide antibiotic (Figure 3.49), in which the erythronolide A lactone ring has been altered to prevent inactivation in the gastric environment. It has proven clinical efficacy against some Staphylococcus spp. and many Streptococcus spp. A (Markham and Faulds, 1994).

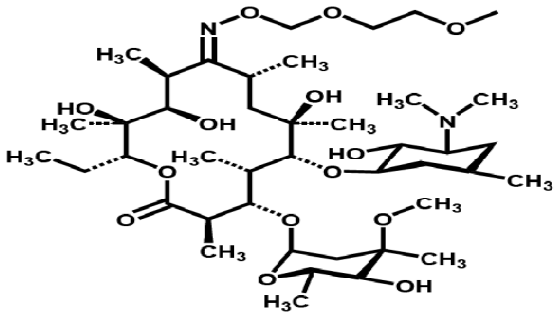


Figure 3.49: Chemical structure of ROX

ROX was analysed by HPLC using different detectors as spectrophotometric detection (Demotes-Mainaird et al., 1989), mass spectrometry detection (Dubois et al., 2001, Lim et al., 2000, Wang et al., 2005), and recently by fluorescence detection (Glźwka and Karazniewicz-Lada, 2007). RP- 18 columns were used in all previous analysis and others used RP-8 for its separation from other macrolides (Gonzalez de la Huebra et al., 2004).

The previously reported LC-MS methods for ROX determination in the literature were generally applied to antibiotic analysis (Lim et al., 2000, Yang and Carlson, 2004, Schlüsener et al., 2003, Li et al., 2001).

In this thesis, UPLC- like method was developed for fast analysis of ROX by using modern columns.

3.6.1. Disadvantages of the official HPLC method

Due to the observed disadvantages of the official Pharm. Eur. Method (Pharm.Eur., 2008, Pharm.Eur., 2005) of ROX as long run time (100 min), very high buffer concentration for HPLC (0.5M), too low working temperature (15 °C), the used sample solvent was different than the used mobile phase which should affect the baseline in the obtained chromatogram and also with the drawbacks of gradient elution. Therefore, a new method was necessary to be developed to get a better way of analysis.

All published and reported methods for the analysis of the drug didn't solve all these problems also didn't use a new packing material as a trial for the solution of these problems. Therefore it was necessary to try the modern columns.

3.6.2. Development of UPLC-like analysis method:

The developed method by applying isocratic elution system using a simple mobile phase consists of ammonium dihydrogen phosphate buffer / acetonitrile 70/30 v/v. It was noticed that the optimum concentration of the used buffer is 0.05M (pH 4.3 adjusted by phosphoric acid) as less than this concentration broad peaks were observed. Different columns were examined which are luna, monolithic and fused core columns (Figure 3.50). At high sample drug concentration (2 mg/ml) some extra peaks (corresponds to the drug impurities) were observed with the use of fused and monolithic columns however it couldn't be observed in the obtained chromatograms when luna column was used (Figure 3.51). The injected drug sample was dissolved in the mobile phase. The detection wavelength was 205 nm. All chromatographic runs were at ambient T.

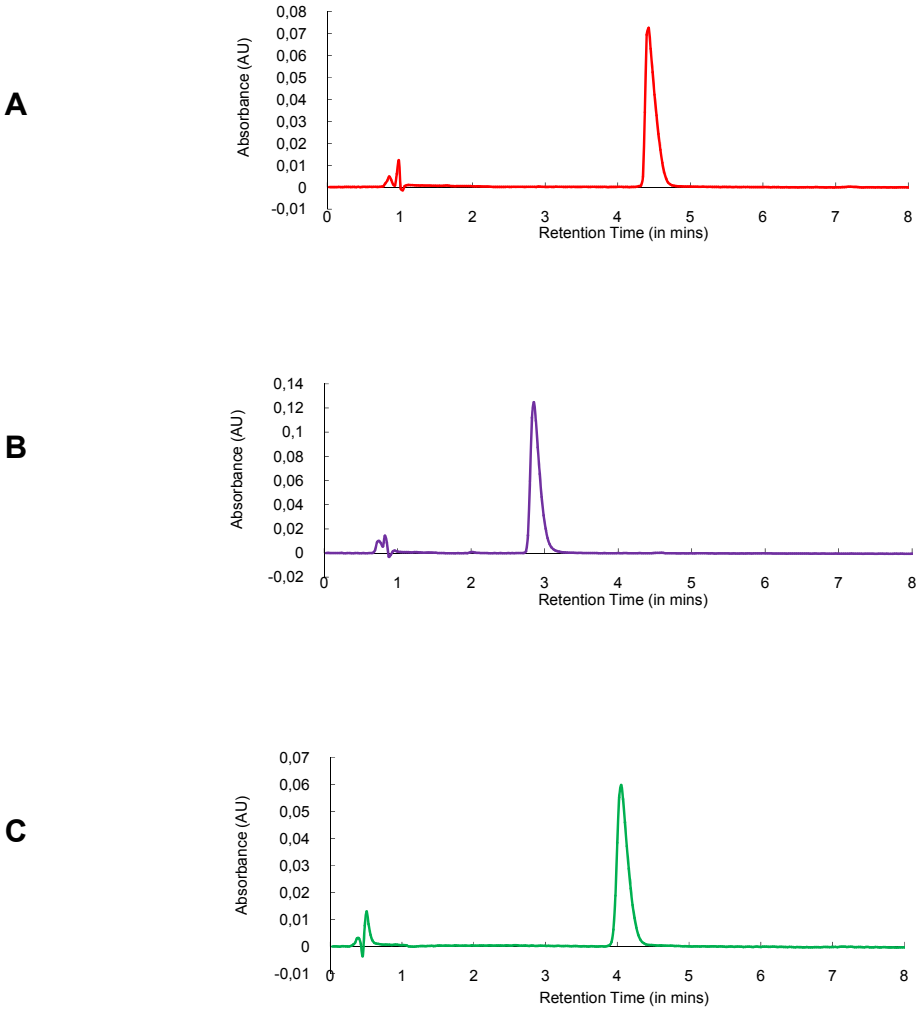


Figure 3.50: Representative chromatogram for analysis of ROX (0.1 mg/ml) by developed method by A: fused core column, B: monolithic column, C: luna column.

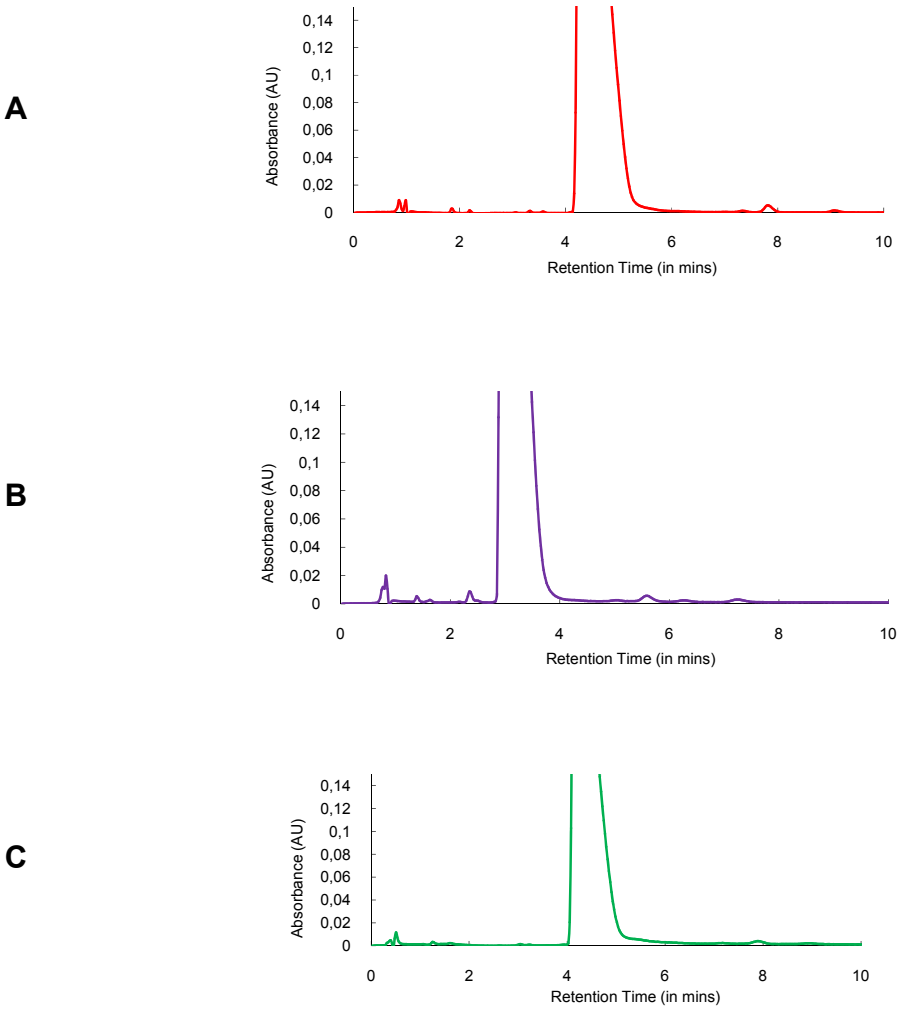


Figure 3.51: Representative chromatogram for separation of other compounds from ROX sample (2mg/ml) by: A: fused core, B: monolithic, and C: luna columns.

3.6.3. Validation of the developed method

The developed UPLC- like method for analysis of ROX was validated by:

3.6.3. 1. Precision

To ensure assay precision within day repeatability (n=6) and between days repeatability (n=6) were assessed at 3 concentrations of ROX (Tables 3.28 and 3.29).

Table 3.28 : Within day repeatabilities of ROX on monolithic , luna and fused core columns over a concentration range 0.1-2 mg/ml using n=6.

Column type	**Within day repeatability RSD% of AUC (n=6)**			**Within day repeatability RSD% of t_R** (n= 18**)**
	0.1	**0.5**	**2**	
	(mg/ml)			
Monolithic	0.78	0.60	0.56	0.55
Luna	0.87	0.84	0.60	0.65
Fused core	0.75	0.65	0.45	0.42

Table 3.29: Between days repeatabilities of ROX on monolithic , luna and fused core columns over a concentration range 0.1-2 mg/ml using n=6.

Column type	**Between days repeatability RSD% of AUC (n=6)**			**Between days repeatability RSD% of t_R (n=18)**
	0.1	**0.5**	**2**	
	(mg/ml)			
Fused core	0.89	0.95	0.75	0.65
Monolithic	0.94	0.84	0.72	0.70
Luna	0.96	0.94	0.80	0.79

3.6.3. 2. Linearity, LOD and LOQ of the ROX

Calibration curve (peak area vs. concentration) for the analyzed ROX with new UPLC- like method over a concentration range of 0.1 - 2 mg/ml was investigated. The (LOD, S/N =3) and an estimate for the (LOQ, S/N = 10) for the ROX (Table 3.30).

Table 3.30: Linearity, LOD and LOQ of the ROX

Column type	**LOD (µg/ml)**	**LOQ (µg/ml)**	**R^2**
Monolithic	0.1250	0.420	0.9998
Luna	0.180	0.600	0.9995
Fused core	0.1220	0.400	0.9999

3.6.4. Performance parameters of UPLC-like method:

Peak performance parameters were calculated according to fundamental equations (Table 3.31).

Table 3.31: Performance parameters for ROX on monolithic (50mm), luna (50mm) and fused core (100mm) columns. F **0.8 ml/min *1ml/min.

Column type	**Theoretical plate N (Plate per column for ROX)**	**AF for ROX Peak**	**Backpressure (bar)**	**Run time (min)**
Monolithic*	668	1.3	24	7.5
Luna **	641	1.5	190	17
Fused core*	609	1.0	171	10.5

4. Summary

Antibiotics and anti-inflammatory drugs are two main categories which are widely used in treatment of different diseases. That is why pharmaceutical analysts are trying to develop different analytical methods for their determination. The Pharmacopoeias are the main source to get the analysis methods of most of the official drugs. Therefore Pharm. Eur. was selected to get the official quantitative and qualitative method of analysis

Some representative drugs were chosen for the study in this thesis. Spiramycin, Doxcyclin, Clindamycin and Roxithromycin were examples for antibiotics. For anti-inflammatory drugs, Etodolac and Troxerutin were the selected examples. According to the Pharm. Eur., HPLC and TLC techniques were the official analytical methods for assay, identification and purity test for these drugs.

Through the literature of the official and nonofficial HPLC analysis of all these drugs, conventional HPLC column was the only stationary phase which used for their analysis. There are many disadvantages for the use of that type of columns such as long analysis time. To the best of our knowledge there is no reported UPLC-like method for any of these drugs. UPLC is a new type of fast HPLC technique which characterised by the short analysis time. Therefore it was important to transfer these official HPLC methods to UPLC-like method by the use of new analytical columns which packed with more advanced stationary phases such as monolithic and fused core columns. Moreover comparisons were established among all selected stationary phases in terms of chromatographic run time, different flow rates, the produced column back pressure, different types of elution (gradient and isocratic), different mobile system composition, and different sample solvents. Performance parameters as AF, Rs and the theoretical plate number were also studied. The validation parameters (linearity, precession, LOD, LOQ, robustness, reproducibility) were therefore examined for the optimum conditions in each case. The aim of the work was to get the most suitable chromatographic method which can be used instead of the official HPLC method without affecting the resolution of peaks, sensitivity of the method or all validation parameters.

Very short analysis time was obtained with all developed methods by using modern columns. For example, the official chromatographic run time for the analysis of CLD was (12 min) and on its transfer to UPLC-like method the analysis time was reduced to (2 min), for SPR the run time reduced from official method (40 min) to developed method (5 min), while for ROX run time was very long with official method (100 min) but by new developed method the run time was only (5 min).

Validation parameters were tested for all developed methods as Precision. The within-day repeatability was in the range (0.52- 0.88%) with conventional column and (0.41- 0.87%) with the modern columns. Furthermore, the between-days repeatability was in the ra nge (0.66 –1.25%) with conventional and (0.50 – 0.96%) with modern columns.

Modern columns gave more precise integration than that of the conventional columns which can be attributed to the better peak shape and reduced baseline noise.

In fact, peak tailing in RP HPLC is particularly prevalent when separating basic compounds. It causes a number of problems including lower Rs, reduced sensitivity and poor precision and quantitation. However symmetric peaks were obtained with the other used stationary phases. The highest AF value was obtained with the use of conventional stationary phase. For example, for Pharm. Eur. Analysis of ETD, CLD or SPR, the obtained AF was 1.4, 1.4 and 1.5 respectively. However, with the developed UPLC-like methods AF was reduced to (1.0 – 1.1) by using fused core and monolithic columns respectively.

In the official HPLC methods of some of the selected drugs, the used temperature was not suitable for our lab work. For example, in ROX or DOX official analysis, the temperature were 15 °C and 60°C respectively. However in the developed methods, an ambient temperature was the optimum for the analysis. Moreover, ROX was efficiently separated from its other components in the concentrated sample by the applying the developed method.

Acceptable resolution values were obtained with all developed methods. ETD analysis as a representative example, poor separation of ETD from its impurity CON was observed on applying the official method and the obtained Rs was (Rs < 1). On

the other hand, on applying the developed gradient elution method, the Rs was raised to (Rs=2.5). Also, a developed UPLC-like method with isocratic elution system for analysis of ETD and its impurity, the Rs values were 1.4, 2.5 and 3.6 by using luna, monolithic and fused core columns respectively.

The developed methods were sensitive with low LOD and LOQ. For example, the LOD and LOQ in the analysis of ETD by official method were 0.15 and 0.5 µg/ml respectively, while with the developed method LOD values were reduced to 6, 3.8, 3.3 and 3 ng/ml was by using conventional, luna, monolithic and fused core columns respectively. Also, the LOQ values were reduced to 20, 11.2, 14, and 10 ng/ml by using conventional, luna, monolithic and fused core column respectively. In the analysis of CLD by the official method, the LOD and LOQ values were 61 and 200 ng/ml respectively. However, on transferring to UPLC-like method the LOD values were reduced to 13, 12 and 11 ng/ml by using luna, monolithic and fused core columns respectively. LOQ values were reduced as well to 47, 40 and 38 ng/ml by using luna, monolithic and fused core columns respectively. The low LOD and LOQ values which obtained with the use of monolithic and fused core columns can be attributed to the obtained low background noise.

The obtained column backpressure readings were watched. For example, with CLD assay, the flow rate was 1ml/min with column lenght (50 mm), the column backpressure readings were 16, 20, 74, 225 bar by using monolithic, conventional, fused core and luna respectively. Generally higher backpressure resulted with the use of luna because of the small particle size (2.5 µm dp). On using fused core column the backpressure was reduced which attributed to the new technology in particles (2.7 µm) with a high-capacity and very pure porous silica layer which fused to a solid silica core. Monolithic column showed the lowest backpressure reading due to the high permeability of its bimodal structure.

For some of the selected drugs “Etodolac, Spiramycin and Troxerutin”, it was found that the official TLC methods were not able to get efficient separation of the drug and its impurities spots. Therefore developments of new TLC methods with the use of horizontal developing chamber were studied.

TLC mobile phase and the used sample solvent were optimized. In TRX, The decrease in polarity of the sample solvent showed better results. With the other drugs (SPR and ETD), phosphomolibdic acid reagent was used as new derivatizing reagent with clear background of plate. That gave better detection of the analyte spots rather than using UV lamp detection only which was used in the official TLC method for ETD and anisaldhyde reagent for SPR.

In the official TLC purity test of Etodolac there are two different mobile phases with two drying steps were used which time and cost are consuming. One hour analysis time was consumed for the official method procedure. On the other hand, in developed method, the analysis time was reduced to 3 and 1.5 min by using TLC and HPTLC plates respectively, with toluene and acetone in the ratio 1:1 v/v as a mobile system. The developed methods were effective to separate CON and ETD by using TLC and HPTLC plates. The measured TLC R_f values 0.54 and 0.60 for ETD and CON respectively. On the other hand, HPTLC R_f values were 0.54 and 0.67 for ETD and CON respectively.

No separation of components of SPR sample was observed by applying official TLC method with 22 min analysis time. However, seven different spots (which related to SI, II, III and other four components in the same sample mixture) were observed by using the developed method and the analysis time was reduced to 3 and 2 min by using TLC and HPTLC plates respectively.

According to DAB 1999 procedure, TRX was analysed by TLC methods. Poor shaped spots were observed with R_f values 0.22 and 0.08 for the main spot and blue spot respectively which are less than normal R_f range. That may be due to a poor resolution and separation of the TRX components. By reducing the sample solvent polarity and increasing of the polarity of mobile system, the R_f values and the shape of the separated spots were improved. The obtained R_f values were 0.43 and 0.24 for the main spot and blue spot respectively to be within the expected ranges of DAB, and the developing time was 4 and 2 min by using TLC and HPTLC plates respectively. However, the developed method with using concentrated sample was able to separate the minor compound.

The developed TLC method for analysis of SPR was applied to preparative TLC. The developed bands were scratched, extracted and analyzed with HPLC for comparison and confirmation. The obtained results were matched. By using new developed HPLC method with isocratic elution system for ETD analysis with different types of columns and the run time was 2 min.

The method was efficient to separate 0.03 µg of CON/ ml of ETD sample in a concentration 0.003 mg/ml. However, the developed TLC and HPTLC methods were also efficient to separate CON from ETD but in 0.05 mg of CON/ml of ETD sample (1mg/ml) within 3 and 1.5 min developing times respectively.

By using the UPLC- like method for the analysis of SPR (0.25 mg/ml) at F= 0.8 ml/min, many peaks were observed within 5 min run time. On the other hand, by applying the developed TLC and HPTLC methods, seven different spots were developed with SPR (4 mg/ml) within 3 and 2 min developing times respectively.

In all examined official HPLC and TLC methods a significant improvement could be obtained by using UPLC-like methods and optimized conditions of HPTLC methods respectively.

Zusammenfassung

Antibiotika und entzündungshemmende Arzneistoffe sind zwei Substanzklassen, die in der Behandlung der verschiedensten Krankheiten am meisten verschrieben werden. Das Bestreben der pharmazeutischen Analytiker ist es daher, verschiedene analytische Methoden für ihre Untersuchung zu entwickeln. Die Arzneibücher sind die Hauptquelle, um sich über die Analysenmethoden der meisten zugelassenen Arzneistoffe zu informieren. Folglich wurde Pharm. Eur. gewählt, um die vorgeschriebenen quantitativen und qualitativen Analyseverfahren anzuwenden.

In dieser Doktorarbeit wurden einige repräsentative Arzneistoffe gewählt. So waren Spiramycin, Doxcyclin, Clindamycin und Roxithromycin Beispiele für Antibiotika. Testsubstanzen für entzündungshemmende Arzneistoffe waren Etodolac und Troxerutin. Entsprechend der Pharm. Eur. waren HPLC- und DC-Techniken die gängigen analytischen Methoden zur Identitäts- und Reinheitsprüfung dieser Substanzen.

In der Literatur ist die HPLC-Analyse all dieser Arzneistoffe mit konventionellen HPLC-Säulen als einzige stationäre Phase durchgeführt worden. Es resultieren jedoch zahlreiche Nachteile beim Gebrauch dieser Art von Säulen, wie z.B. die lange Analysenzeit. Nach bestem Wissen wurden keine UPLC-ähnlichen Methoden einer dieser Drogen veröffentlicht. Die UPLC ist eine schnelle HPLC-Technik, die sich in ihrer kurzen Analysenzeit wiederspiegelt. Folglich war es wichtig, diese gängigen HPLC-Methoden auf UPLC-ähnliche Methoden unter Anwendung von neuen analytischen Säulen zu bringen, die mit neuartigen stationären Phasen gepackt sind, wie z.B. die monolithischen Säulen und die Fused Core-Säulen. Außerdem wurden Vergleiche unter allen ausgewählten stationären Phasen hinsichtlich der chromatographischen Laufzeit, der verschiedenen Fließgeschwindigkeiten, dem produzierten Rückstau der Säule, den verschiedenen Arten der Eluierung (Gradienteneluierung und isokratische Eluierung), dem unterschiedlichen Aufbau des beweglichen Systems und der verschiedenen Lösungsmittel gemacht. Verschiedene Leistungsparameter wie AF, Rs und die Zahl der theoretischen Böden wurden auch untersucht. Die Validierung (Linearität, Präzision, LOD, LOQ, Robustheit,

Reproduzierbarkeit) wurde für jeden Fall mit den optimalen Bedingungen überprüft. Das Ziel der Arbeit war es, die beste chromatographische Methode zu finden, die anstelle der vorgeschriebenen HPLC-Methoden angewendet werden kann, ohne die Auflösung der Peaks, die Empfindlichkeit der Methode oder die Validierungsparameter zu beeinflussen.

Sehr kurze Analysenzeiten wurde mit allen weiterentwickelten Methoden erhalten, indem man moderne Säulen verwendete. So wurde die chromatographische Laufzeit für die Analyse von CLD (12 Minuten) durch Anwendung UPLC-ähnlicher Methoden auf eine Analysenzeit von 2 Minuten gebracht, während die Laufzeit von SPR von 40 Minuten auf 5 Minuten verringert wurde. Bei ROX war die Laufzeit nach Pharm. Eur. sehr lang (100 Minuten), durch die neu entwickelte Methode betrug die Laufzeit lediglich 5 Minuten.

Die Validierungsparameter wurden für alle entwickelten Methoden überprüft. Die Wiederholpräzision lag mit konventionellen Säulen im Bereich von 0.52 - 0.88 % und von 0.41 - 0.87 % mit modernen Säulen. Außerdem lag die Tag-zu-Tag-Präzision im Bereich von 0.66 - 1.25 % mit konventionellen und von 0.50 - 0.96 % mit modernen Säulen.

Moderne Säulen gaben eine präzisere Integration als konventionellen Säulen. Durch ihnen erhielt man eine bessere Peakform und geringeres Grundlinienrauschen.

Das Peaktailing überwiegt in der RP HPLC in der Tat, wenn man einige Verbindungen trennt. Man hat hierbei Probleme hinsichtlich eines niedrigeren Rs, einer verringerten Empfindlichkeit, einer geringen Präzision und der quantitativen Bestimmung. Jedoch wurden symmetrische Peaks mit den hier verwendeten stationären Phasen erreicht. Der höchste AF-Wert wurde beim Gebrauch von konventionellen stationären Phasen erhalten. So wurden z.B. für die Analyse von ETD, CLD und SPR nach Pharm. Eur. folgende AF-Werte erreicht: 1.4, 1.4 und 1.5. Jedoch wurde bei der Entwicklung UPLC-ähnlicher Methoden, d.h. durch die

Anwendung der Fused-Core-Säulen beziehungsweise der monolithischen Säulen, der AF-Wert auf 1.0 - 1.1 verringert.

In den vorgeschriebenen HPLC-Methoden einiger Arzneistoffe war die verwendete Temperatur nicht für unsere Laborarbeit verwendbar. So wurden z.B. ROX und DOX nach Pharm.Eur. bei einer Temperatur von 15°C bzw. 60°C analysiert. In den entwickelten Methoden war die Raumtemperatur optimal für die Analyse. Außerdem konnte ROX in der hochkonzentrierten Probe effizient von seinen anderen Bestandteilen durch das Anwenden der entwickelten Methode getrennt werden.

Mit allen entwickelten Methoden konnten akzeptable Auflösungen erreicht werden. So wird bei der Analyse von ETD nach Pharm. Eur. eine schlechte Trennung von seiner Verunreinigung CON erreicht. Der Rs-Wert beträgt lediglich Rs < 1. Bei Anwendung der weiterentwickelten Methode der Gradienteneluierung konnte der Rs-Wert erhöht werden (Rs=2.5). Auch die entwickelte UPLC-ähnliche Methode mit isokratischer Eluierung ergab bei der Analyse von ETD und seiner Verunreinigung bessere Rs-Werte. Bei Verwendung der Luna-, monolithischen und Fused-Core-Säulen betrugen diese 1.4, 2.5 und 3.6.

Die entwickelten Methoden hatten niedrige LOD- und LOQ-Werte. So betragen die LOD- und die LOQ-Werte bei der Analyse von ETD nach Pharm.Eur. 0.15 und 0.5 µg/ml, während mit der entwickelten Methode die LOD-Werte verringert wurden und bei 6, 3.8, 3.3 und 3 ng/ml lagen (bei der Anwendung konventioneller, Luna-, monolithischer und Fused-Core-Säulen). Auch die LOQ-Werte wurden auf 20, 11.2, 14 und 10 ng/ml durch die Anwendung konventioneller, Luna-, monolithischer und Fused-Core-Säulen verringert. Bei der Analyse von CLD nach Pharm.Eur. betrugen die LOD- und LOQ-Werte 61 bzw. 200 ng/ml. Bei Anwendung UPLC-ähnlicher Methoden wurden die LOD-Werte auf 13, 12 und 11 ng/ml durch die Anwendung der Luna-, der monolithischen sowie der Fused-Core-Säulen verringert. Außerdem konnten die LOQ-Werte auf 47, 40 und 38 ng/ml gebracht werden. Die niedrigen LOD- und LOQ-Werte, die beim Gebrauch monolithischer und Fused-Core-Säulen

erreicht werden konnten, können dem niedrigen Grundrauschen zugeschrieben werden.

Die erhaltenen Rückstauwerte wurden beobachtet. So wurde bei der Analyse von CLD bei einer Strömungsgeschwindigkeit von 1ml/min und einer Säulenlänge von 50 Millimeter folgende Werte erhalten: 16, 20, 74, 225 bar (beim Gebrauch monolithischer, konventioneller, Fused-Core-und Luna-Säulen). Generell erhielt man wegen der Teilchengröße (2.5 µm DP) einen höheren Rückstau beim Gebrauch von Luna-Säulen. Bei Verwendung von Fused-Core-Säulen wurde der Rückstau verringert, welches der neuen Technologie der Partikeln (µm 2.7) aufgrund ihrer hohen Kapazität und der sehr reinen porösen Silikonschicht zu verdanken ist, die zu einem festen Silikonkern verschmilzt. Die monolithische Säule führte zum niedrigsten Rückstau aufgrund der hohen Permeabilität seiner bimodalen Struktur.

Bei den Arzneistoffen Etodolac, Spiramycin und Troxerutin wurde gefunden, dass sich die in Pharm.Eur. vorgeschriebenen DC-Methoden als nicht sinnvoll erwiesen, da es nicht möglich war eine leistungsfähige Trennung der Arzneistoff und seiner Verunreinigungen zu erhalten. Folglich wurden neue DC-Methoden durch den Gebrauch von horizontalen Kammern studiert.

Hier wurden die mobile Phase und das Lösungmittel der Probe optimiert. Bei TRX beobachtete man bessere Resultate bei abnehmender Polarität des Lösungsmittels. Mit den anderen Arzneistoffen (SPR und ETD) wurde Molybdatophosphorsäure als neues derivatisierendes Reagens für einen klaren Hintergrund der Platte benutzt. Dies führte zu besserer Detektion der Analytpunkte als unter Verwendung der UV-Lampendetektion, die in Pharm.Eur. für ETD und für das Anisaldhyd-Reagens für SPR verwendet wird.

Bei der Reinheitsprüfung von Etodolac gibt es nach Pharm.Eur. zwei verschiedene mobile Phasen, die zuvor getrocknet werden müssen, so dass dies zeit- und kostenraubend ist. Die Analysenzeit beträgt hier eine Stunde. Durch den Gebrauch von DC-und HPDC Platten (mit Toluol und Aceton im Verhältnis-1:1 v/v als mobile Phase) in unserer weiterentwickelten Methode konnte die Analysenzeit enorm verringert werden auf Werte von 3 und 1.5 Minuten. Die entwickelten Methoden waren wirkungsvoll, um CON und ETD voneinander zu trennen, indem man DC-und HPDC Platten verwendete. Die gemessenen DC R_f-Werte betrugen 0.54 und 0.60 für ETD bzw. CON. Die erhaltenen HPDC R_f-Werte betrugen 0.54 und 0.67 für ETD und CON.

Es wird keine Trennung in die Bestandteile der SPR-Probe beobachtet, wenn man die im Pharm. Eur. vorgeschriebene DC-Methode mit einer Analysenzeit von 22 Minuten anwendet. Jedoch wurden sieben verschiedene Punkte bei Anwendung der weiterentwickelten Methode beobachtet, die zu SI, II, III und anderen vier Bestandteilen in der gleichen Probenmischung gehören. Die Analysenzeit konnte auf 3 (DC) bzw. 2 (HPDC) Minuten verringert werden.

Entsprechend dem DAB 1999 wurde TRX mit DC analysiert. Man erhielt schwach ausgebildete Punkte mit R_f-Werten von 0.22 für den Hauptpunkt und 0.08 für den blauen Punkt. Die unüblich geringen R_f-Werte resultieren wahrscheinlich von der schwachen Auflösung und Trennung der TRX-Komponenten. Durch Verringerung der Polarität des Lösungsmittels und Erhöhung der Polarität der mobilen Phase wurden die R_f-Werte und die Form der getrennten Punkte verbessert. Die erhaltenen Rf-Werte waren 0.43 für den Hauptpunkt und 0.24 für den blauen Punkt und liegen somit innerhalb des erwarteten Bereichs des DAB. Die Analysenzeit betrug hier 4 Minuten für DC- und 2 Minuten für HPDC-Platten. Mit der weiterentwickelten Methode konnte bei Verwendung der konzentrierten Probe das Nebenprodukt vom Hauptprodukt getrennt werden.

Die entwickelte DC-Methode wurde für die Analyse von SPR für die präparative DC angewendet. Die entwickelten Spots wurden ausgekratzt, extrahiert und mit Hilfe der HPLC zum Vergleich und zum Nachweis analysiert.

Die erzielten Resultate wurden für die ETD-Analyse bei Anwendung der neu entwickelten HPLC-Methode mit isokratischer Eluierung und verschiedenen Arten von Säulen gezeigt (Laufzeit 2 Min.). Mit dieser Methode war es möglich 0.03 µg CON pro ml von der ETD Probe der Konzentration 0.003 mg/ml zu trennen. Mit den entwickelten DC-und HPDC-Methoden war es auch möglich CON von ETD zu trennen, jedoch in höherer Konzentration (0.05 mg CON/ml der ETD Probe (1mg/ml) innerhalb von 3 bzw. 1.5 Minuten).

Bei Verwendung UPLC-ähnlicher Methoden konnten für die Analyse von SPR (0.25 mg/ml) bei F= 0.8 ml/min viele Peaks innerhalb einer Laufzeit von 5 Minuten beobachtet werden. Währenddessen konnte bei Anwendung der weiterentwickelten DC-und HPDC Methoden sieben verschiedene Punkte beobachtet werden, wobei SPR (4 mg/ml) innerhalb von 3 bzw. 2 Minuten analysiert wurde.

Bei allen überprüften im Pharm.Eur. vorgeschriebenen HPLC- und DC-Methoden konnte eine deutliche Verbesserung erzielt werden, indem man UPLC-ähnliche Methoden und optimierte Bedingungen der HPDC-Methoden anwendete.

5. Experimental Part

5.1. Materials

Acetonitril HPLC grade	Baker (Deventer- Holland)
Methanol HPLC grade	Baker (Deventer- Holland)
Phosphoric acid 85%	Riedel-de Haen (Germany)
Acetic acid	Riedel-de Haen (Germany)
Tert-Butanol	Sigma-Aldrich (Germany)
Tetrabutyl ammonium hydrogen sulphate	Sigma-Aldrich (Switzerland)
Potassium dihydrogen phosphate	Merck (Darmstadt,Germany)
Potassium acetate	Merck (Darmstadt,Germany)
Ammonium acetate	Merck (Darmstadt,Germany)
Sodium hydroxid	Merck (Darmstadt,Germany)
Diammonium hydrogen phosphate	Merck (Darmstadt,Germany)
Potassium hydroxide	Ferak (Berlin,Germany)
Perchloric acid 70%	Ferak (Berlin,Germany)
Ammonia solution 25%	Ferak (Berlin,Germany)
Sodium Perchlorate	Janssen Chemical (Belgium)
Sodium dihydrogen phosphate-1-hydrat	Germed(Germany)
Disodium edetate. 2 H_2O	Sigma-Aldrich (USA)
2-(7-ethylindol-3-yl) ethanol	gift from prof. Dr. löewe
Etodolac	CILAG AG (Schlaffhausen)
Spiramycin	grünenthahl Gm bh (Aachen, Germany)
Troxerutin 90%	Aldrich chemical (Milwaukee,USA)
Trihydroxy ethylrutin ≥80% HPLC	Sigma-Aldrich (Belgium)
Clindamycin HCl 93.4%	Salutas Pharma GmbH
Roxithromycin 99.8%	Salutas Pharma GmbH
Doxycyclin- H_2O 95.9%	Salutas Pharma GmbH
Metacyclin	Dr.Ehrenstorfer GmbH
Oxytetracyclin	(Augsburg,Germany)
Methanol	Caesar and Loretz GmbH Hilden
Hydrochloric acid	Baker (Deventer- Holland)

Acetone	Baker (Deventer- Holland)
Diethyl amine	Baker (Deventer- Holland)
Toluene	Baker (Deventer- Holland)
Potassium hydroxide	Baker (Deventer- Holland)
Aluminium chloride	Ferak (Berlin, Germany)
Sulphuric acid	Ferak (Berlin, Germany)
Acetic acid	Riedel- De Haen (Hanover, Germany)
Phosphoric acid	Riedel- De Haen (Hanover, Germany)
Chloroform	Riedel- De Haen (Hanover, Germany)
Ethyl acetate	Merck (Darmstadt, Germany)
Molybdate phosphoric acid- hydrate	Merck (Darmstadt, Germany)
Hexane	Merck (Darmstadt, Germany)
Methyl sulphonic acid	Merck (Darmstadt, Germany)
Potassium hydrogen phosphate	Merck (Darmstadt, Germany)
2-Propranol	Merck (Darmstadt, Germany)
Ammonium acetate	Merck (Darmstadt, Germany)
Sodium hydroxide	Merck (Darmstadt, Germany)

LichroCART RP-18 column	(5µm partical size, 125mm x 4mm, Merck)
LiChrospher 100 RP-8 column	(5 µm partical siza, 250 mm x 4 mm, Merck)
Spherisorb ODS2 RP-18 column	(5µm particle size, 250mm x 4.6 mm, VDS optilab)
Onxy monolithic RP-18 column	(50 mm x 4.6 mm Phenomenex)
Ascentis Express C18 column	(10cm x 4.6 mm, 2.7 µm, Sigma Aldrich)
Luna 2.5µm C18 column	(50 x 3.00 mm, 2.5 µm, Phenomenex)
Polystyrene Divinylbenzene (PSDVB) 100A column	(150 x 4.6 mm, 5 µm, Phenomenex)
Silica Gel 60 TLC plates	(0.25 mm layer thickness) , Merck (Darmstadt, Germany)
Silica Gel 60 TLC plates	(0.25 mm layer thickness) , Macherey Nagel(Düren,Germany)
Silica Gel 60 HPTLC plates, Thick layer chromatography	Merck

(20 x 20 cm)	Merck
Embty desecator	(Glas werk, Werheim)
Micro pippete 0.5-10 µl	(Eppendorf-Netheler-Hinz)
Micro pippete 200-1000 µl	(Eppendorf-Netheler-Hinz)

5.2. Instrumentation

Merck Hitachi HPLC system

Interface: D-6000 Interface (Merck/Hitachi)

Pump: L-6200A Intelligent Pump (Merck/Hitachi)

Detector: L-4500 UV-VIS (diode array) detector

Column oven T-6300 (Merck/Hitachi)

Data analysed D7000 HSM software (Merck/Hitachi)

CAMAG horizontal developing chamber Merck

(10 x 10 cm) and (5 x 5 cm).

Sensitive Scale	Horst Schirmer, Germany
Chromatography Desaga	Glas werk, werheim
Sonorex R K 100	W, Germany
UV. Vis Lamp	Camag
Hot plate	Gerhardt, Germany
Rotary evaporator	Büchi, Swiss
Digital pH meter pH 522	WTW

5.3. Developed methods

5.3.1. Etodolac

5.3.1.1. HPLC analysis

5.3.1.1.1. Stock and working sample solutions

Stock solution was prepared by accurately weighing 10 mg pure ETD and quantitatively transferred into a 10-ml volumetric flask and complete the volume with mobile phase.

For the preparation of the working solutions serial dilution was done to get three different concentrations (0.1, 0.5, 1 mg/ml) for gradient elution method. Also, another serial dilution was done to get three different concentrations (0.003, 0.01, 0.1 mg/ml) for isocratic elution method.

Stock and working solutions of CON were prepared with the same procedure of ETD solutions preparation**.** For preparation of the drug-impurity mixture, the CON impurity solution was prepared as 1 % v/v in ETD sample solution.That done by transferring 100 µl impurity working solution by micropipette to 10-ml volumetric flask and complete the volume with ETD working solution. (working solution for ETD and CON should be at the same concentration)

5.3.1.1.2. Potassium dihydrogen phosphate buffer:

Accurately weighed 13.6 g potassium dihydrogen phosphates were quantitatively transferred to one-liter beaker by small amount of bi-distilled water. The obtained solution was completed to 900 ml bi-distilled water to get clear solution. The pH was adjusted to 7 by addition of drops of potassium hydroxide solution (300g/L) (5.6 M).

5.3.1.1.3. Mobile phase:

a. Solvent A: Accurately measured 688 and 312 ml of potassium dihydrogen phosphate buffer and methanol respectively were transferred to one-Liter volumetric flask. Bi-distilled water was added to complete to the volume.

b. Solvent B: was acetonitrile HPLC-grade.

The mobile phase was filtered through 0.45 µm millipore membrane filters and degassed by sonication in an ultrasonic bath before use.

5.3.1.1.4. Chromatographic conditions of gradient elution system:

Solvent A and B mixture was used as a mobile phase which pumped with flow rate (1.5 ml/min) in a time programming starting with 96:4 till 92:8 for A: B ratios along 20 min run time. The stationary phase was LichroCART RP-18 column. Twenty microliters was the sample volume and the detection wavelength was 225 nm. The column temperature was 40 $C^{0.}$

5.3.1.1.5. Chromatographic conditions of isocratic elution system:

The isocratic mobile phase consists of solvent A and B, 70:30 v/v, which pumped at F= 1.2 ml/min. The stationary phases were LichroCART RP-18, monolithic column, fused core, and luna columns. The Injection volume was 20 µl and 225nm was used as detection wavelength. All separations were performed at ambient temperature.

5.3.1.2. TLC analysis

5.3.1.2.1. Sample preparation:

Ten milligrams pure ETD powder were accurately weighted and transferred quantitatively to 10-ml volumetric flask. The drug powder was dissolved in acetone and complete to volume with acetone giving 1 mg/ml solution.

The sample of ETD- impurity CON was prepared with the same procedure and at the same concentration of ETD sample. The drug-impurity mixture, was prepared by transferring 500 µl impurity sample (1mg/ml) by micropipette to 10-ml volumetric flask and complete the volume with ETD sample (1mg/ml) to get 0.05mg of CON/ ml of ETD sample.

5.3.1.2.2. Phosphomolibdic acid reagent

Accurately weighed 4 g of phosphomolybdic acid were dissolved in distilled water and the volume is completed to 40 ml with distilled water. Sixty ml of sulfuric acid was

added drop by drop under ice bath to obtaine the final concentration 40 mg/ml.The reagent was freshly prepared at each time of usage.

5.3.1.2.3. Chromatographic conditions

TLC and HPTLC plates were pre-washed with methanol, and then activated at 120 °C for 1 hr. The plates were left in desicator for 15 min to lower its temprature to room temperature. Three and five microliter sample volumes were spotted on the HPTLC and TLC plates respectively by the use of glass micro tubes. The mobile phase consists of toluene: acetone, 1: 1 (v/v).The plates were dried by using hot air (hair dryer). After drying stage the plates were placed in a horizontal chamber which contained 2 ml of the same used mobile phase for 3 and 1.5 min by using TLC and HPTLC plates respectively. The plates then removed from the chamber and dried again in a current of hot air. Detection was performed with a U.V. lamp at wavelength of 254 nm. The plates then sprayed with freshly prepared phosphomolybdic acid solution.

5.3.2. Spiramycin

5.3.2.1. HPLC analysis:

5.3.2.1.1. Stock and working sample solution

Accurately weighed 25 mg SPR powder was quantitatively transferred to 100-ml volumetric flask with small amount of the mobile phase. The volume was completed with mobile phase to get 250 µg/ml drug sample concentration.

5.3.2.1.2. Mobile phase:

a. Solvent A: Accurately weighed 9.3 g of sodium perchlorate hydrate was quantitatively tranfered by dissolving in small amount of bi-distilled water to one-liter beaker and the volume was completed by bi-distilled water (0.07 M). The pH was adjusted to 2.2 by addition of drops of diluted perchloric acid (10%).

b. Solvent B: acetonitrile HPLC-grade.

The mobile phase was degassed by sonication before use.

5.3.2.1.3. Chromatographic conditions of isocratic elution system

Mobile phase was a mixture of solvent A and B; 68:32 v/v. The flow rate was 0.8 ml/min and the stationary phases were Lichrospher 100 RP-8 and luna columns, while different F= (0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 ml/min) were applied the other used columns (monolithic and fused core columns). The injection volume was 20 µl and the detection wavelength was 232 nm. All separations were performed at ambient temperature.

5.3.2.1.4. Chromatographic conditions of gradient elution system

Solvent A and B mixture was used as a mobile phase which pumped with 0.8ml/min flow rate in a time programming along 30 minutes run time as indicated in Table 5.1. The sample volume was 20 µl and the chromatographic separation was at room temperature.

Table 5.1:-The gradient elution system time programming:

Time (min.)	Solvent A%	Solvent B%
0	70	30
10	66	34
20	62	38
30	58	42

5.3.2.2. TLC analysis:

5.3.2.2.1. Sample preparation:

Stock solution: accurately weighed 20 mg of pure SPR powder were quantitatively transferred by dissolving the weighed amount in methanol to 5-ml volumetric flask and the volume was completed by addition of methanol to get drug sample solution with 4 mg/ml concentration.

Working solution: 1 ml of the stock solution was transferred to 100-ml volumetric flask by pippet and the volume was completed with the addition of methanol to obtain 40µg/ml drug sample concentration.

For the analysis of SPR on thick layer chromatography; accurately weighed 250 mg of pure SPR powder was quantitatively transferred to 5 ml volumetric flask by dissolving the weighed amount in methanol and complete the volume with methanol to get drug concentration of 50mg/ml.

5.3.2.2.2. Ammonium acetate buffer:

Hundred and fifty g were accurately weighed and quantitatively transferred to one-liter volumetric flask, 3 ml of acetic acid was added and complete volume with distelled water (1.5 M). The pH was adjusted at 9.6 by 10 M NaOH solution (42g /100 ml).

5.3.2.2.3. Chromatographic conditions

The plates were pre-washed with methanol, and then activated at 120 °C for 1 hr. The plates were left in desicator for 15 min to lower its temprature to room temperature. The mobile phase was the upper layer produsedfrom mixing of isopropanol: ethyl acetate: ammonium acetate buffer pH 9.6 (4:9:8 v/v/v).

Five and three microliters sample volumes were spotted on the TLC and HPTLC plates respectively. After drying of the spots under a current of hot air the plate was placed in a horizontal chamber which contained 2 ml of the mobile phase. The plates were developed in 3 and 2 minutes the TLC and HPTLC plates respectively. The plates were again removed and dried in a current of hot air. Detection was done by spraying the plates with freshly prepared phosphomolybdic acid solution.

5.3.3. Troxerutin

5.3.3.1. HPLC analysis

5.3.3.1.1. Mobile phase:

a. solvent A: accurately weighed 13.8 g of sodium dihydrogen phosphate hydrate and transferred to one- liter volumetric flask and complete volume with distelled water (0.1M). The pH was adjusted to 4.4 by addition of diluted NaOH solution 2M (8.5 gm/100ml water) or phosphoric acid (10%).

b. solvent B: acetonitrile HPLC-grade.

The mobile phase consists of a mixture between solvent A and B in (84 :16 v/v ratio). The mobile phase was degassed by sonication before use.

5.3.3.1.2. Sample preparation:

Stock solution: Ten milligrams of TRX were accurately weighed and transferred quantitatively to 10-ml volumetric flask by dissolving in small amount of the mobile phase and the volume was completed with the mobile phase.
For the preparation of the working solutions serial dilution was done to get three different drug concentrations (0.05, 0.1, 0.5 mg/ml).

5.3.3.1.3. Chromatographic conditions

The separation was performed with fused core and Spherisorb ODS2 columns and the injection volume was 20 µl. The flow rate was 0.5 ml/min and the detection wavelength was 350 nm. All separations were performed at ambient temperature.

5.3.3.2. TLC analysis

5.3.3.2.1. Preparation of sample

Stock solution: One gram pure TRX or Trihydroxy ethylrutin was accurately weighed and quantitatively transferred to 20-ml volumetric flask by dissolving in small amount of water and complete the volume with distelled water.

Working solution: 0.3 ml of stock solution was transferred to 10-ml volumetric flask and the volume was completed with a mixture of methanol: chloroform (2:8 v/v) to get 1.5 mg/ml drug concentration.

5.3.3.2.2. Chromatographic conditions

TLC and HPTLC plates were pre-washed with methanol, and then activated at 120 °C for 1 hr. The plates were left in desicator for 15 min to lower its temprature to room temperature. The mobile phase consists of 0.1 HCl: acetone: ethyl acetat 3: 9:7 (v/v/v). 1 and 3 µl of the final sample (1.5 mg/ml) was spotted on the HPTLC and TLC plates respectively by the use of glass micro tubes. The plates were dried by using hot air (hair dryer). After drying stage the plates were placed in a horizontal chamber which contained 2 ml of the same used mobile phase for 4 and 2 min for the TLC and HPTLC plates respectively. The plates then removed from the chamber and dried again in a current of hot air. Detection was performed with a U.V. lamp at 254 and 365 nm wavelengths.

5.3.4. Doxycyclin

5.3.4.1. Mobile phase:

a. solvent A (Accurately weighed 0.686 and 0.2 g of potassium dihydrogen phosphate and sodium edetat respectively were quantitatively transferred to 1- liter beaker by dissolving in small amount of distilled water and complete to 1- Liter with distilled water to get 0.005 M buffer with pH=5 .

b. solvent B: acetonitrile HPLC-grade.

The mobile phase consists of a mixture of solvent A and B in the ratio 80:20 v/v. The mobile phase was degassed by sonication before use.

5.3.4.2. Sample preparation:

Stock solutions: Hundred milligrams of DOX or its impurities (MTC or OTC) were accurately weighed separately and quantitatively transferred to three different 100-ml volumetric flasks by dissolving each compound separately in small amount of mobile phase and complete to the volume of each flask with the mobile phase.

Working solutions: Serial dilutions for each compound were done to get three different drug concentrations 0.07, 0.1, 0.3 mg/ml.

Hundred microliters of MTC and OTC working solutions were accurately transferred by micropipette to 10-ml volumetric flask and complete to the volume with DOX working solution to get a sample mixture of DOX, MTC, and OTC solution in the ratio 98:1:1 v/v/v respectively (0.7 µg/ml of MTC and 0.7µg/ml of OTC in DOX sample 0.07 mg/ml).

5.3.4.3. Chromatographic conditions

The separation was performed on fused core, monolithic and luna columns with flow rate of 0.5 ml/min. All separations were performed at ambient temperature and 254 nm was the detection wavelength. Injection volume was 20 µl.

5.3.5. Clindamycin

5.3.5.1. Mobile phase:

a. solvent A (Accurately weighed 6.8 g of potassium dihydrogen phosphate was quantitatively transferred to 1-liter beaker by dissolving in small amount of distilled water and complete to the volume with distilled water to get 0.05 M buffer and the pH was adjusted to 7.5 by addition of drops of potassium hydroxide solution 4.5 M(250 g/ L) .

b. solvent B: acetonitrile HPLC-grade.

The mobile phase was prepared by mixing solvent A and B in the ratio of 55:45 v/v. The mobile phase was degassed by sonication before use.

5.3.5.2. Preparation of sample

Stock solutions: Hundred milligrams of CLD powder were accurately weighed separately and quantitatively transferred to 100-ml volumetric flasks by dissolving in small amount of mobile phase and complete to the volume of the flask with the mobile phase to get 1 mg/ml concentration.

5.3.5.3. Chromatographic conditions

The separation was performed on a Spherisorb ODS2, monolithic column, luna column, and fused core column. The flow rate was 1ml/min for all columns. The injection volume was 20 µl and the detection wavelength was 210 nm. All separations were performed at ambient temperature.

5.3.6. Roxithromycin

5.3.6.1. Mobile phase:

a. solvent A (Accurately weighed 6.595 g of di-ammoniumhydrogen phosphate was quantitatively transferred to 1-liter beaker by dissolving in small amount of distilled water and complete to the volume with distilled water to get 0.05 M buffer and the pH was adjusted to 4.3 by addition of drops of diluted phosphoric acid (10%).

b. solvent B: acetonitrile HPLC-grade.

The mobile phase was prepared by mixing solvent A and B 70:30 v/v. The mobile phase was degassed by sonication before use.

5.3.6.2 Sample preparation:

Stock solutions: Hundred milligrams of ROX powder were accurately weighed separately and quantitatively transferred to 100-ml volumetric flask by dissolving each compound in small amount of mobile phase and complete to the volume of the flask with the mobile phase to get 1 mg/ml concentration.
Working solutions: Serial dilution was done to get three different drug concentrations 0.1, 0.5 , 2 mg/ml.

5.3.6.3. Chromatographic conditions

The separation was performed on monolithic and fused core columns at F=1 ml/min, while it was 0.8 ml/min on luna column. The injection volume was 20 µl and the detection wavelength was 205 nm. All separations were performed at mbient temperature.

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Bescheinigung

Gem. § 5 Abs. 4 und Abs. 2b der Promotionsordnung des

Fachbereichs Biologie, Chemie, Pharmazie

Hiermit versichere ich, dass ich meine Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt habe.

Berlin, den