1.0 Introduction and literature review

1.1 Introduction to HPLC

Chromatography has developed over the past century and has major input into many areas of modern science. Chromatography was defined in 1993 by IUPAC. According to the IUPAC definition, "Chromatography is a physical method of separation in which the components to be separated are distributed between two phases; one of which is stationary while the other moves in a definite direction". (IUPAC 1993)

Chromatography is a physical separation method in which the components to be separated are selectively distributed between two phases; a mobile phase is flowing through a stationary phase. Chromatography is named mainly by the nature of the mobile phase: gas chromatography (GC), liquid chromatography (LC) or supercritical fluid chromatography (SFC). The chromatographic process occurs as a result of repeated sorption/desorption steps during the movement of analytes along the stationary phase. (Meyer 1994)

There are many differences in chromatographic techniques (liquid, gas, paper, high class, ion exchange, supercritical fluids and electric chips), and there is a common property in chromatography techniques. The most widely used LC technology is HPLC, which appeared in 1974 and expanded very quickly. The abbreviation means high performance liquid chromatography. Since HPLC is fully controlled, LC is often used instead of HPLC. (Ahuja *et al*. 2005)

1.1.1 Separation mechanisms and LC modes

The classification of different LC techniques depends on the type of distribution mechanism applied in the separation. Individual HPLC columns may use any one of a number of different stages or processes to induce this resolution .A variety of chromatographic modes have been developed, on the basis of the mechanisms of retention and operation, which are considered as the more common classification. This classification scheme stems from the manner in which the analyte interacts with the stationary phase. The four major chromatographic modes that can be applied to the analysis of solutes in solution are: normal phase, reversed phase, ion-exchange

and size exclusion. In addition, a variety of sub-modes exist, such as hydrophobic or hydrophilic interactions, chiral separations, affinity, ion suppression and ion pairing (Boom *et al*. 2001; Skoog 2006). The following is a brief introduction to the basic principles and typical use areas for each of the above situations. Figure 1.1 illustrates the most important separation modes in HPLC.

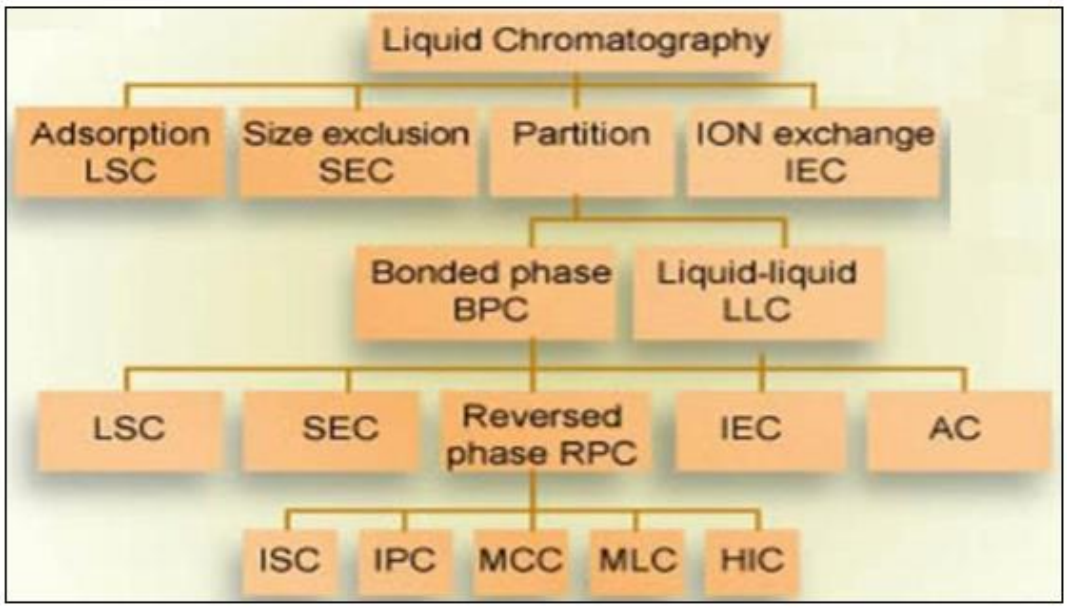


Figure 1.1: Modes of HPLC separation (Vonk 2008). Where, LS: Liquid solid, SE: Size-exclusion, IE: Ion-exchange, AC: Affinity chromatography, BP: Bonded phase, LL: Liquid liquid, RP: Reversed phase, IS: Ion suppression, IP: Ion pair, MC: Metal complexation, ML: Micellar liquid and HI: Hydrophobic interaction.

1.1.1.1 Normal phase HPLC (NP-HPLC)

NP-HPLC was discovered by Tswett in 1903 and represents the first type of HPLC techniques used. , And retention of the sample in this type depends on the polarity. Although NP chromatography can be performed using partition or adsorption mechanisms, the dominant retention mechanism is adsorption. As a consequence, NP chromatography is also known in the literature as adsorption chromatography or liquid solid chromatography. Normally bonded phase HPLC columns are composed of a stationary bed, which is strongly polar in nature, and a mobile phase that is nonpolar. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials. NP-HPLC is used when the analyte of interest is neutral species has a polar nature, and the separation on the basis of polarity (Snyder *et al*. 1997).

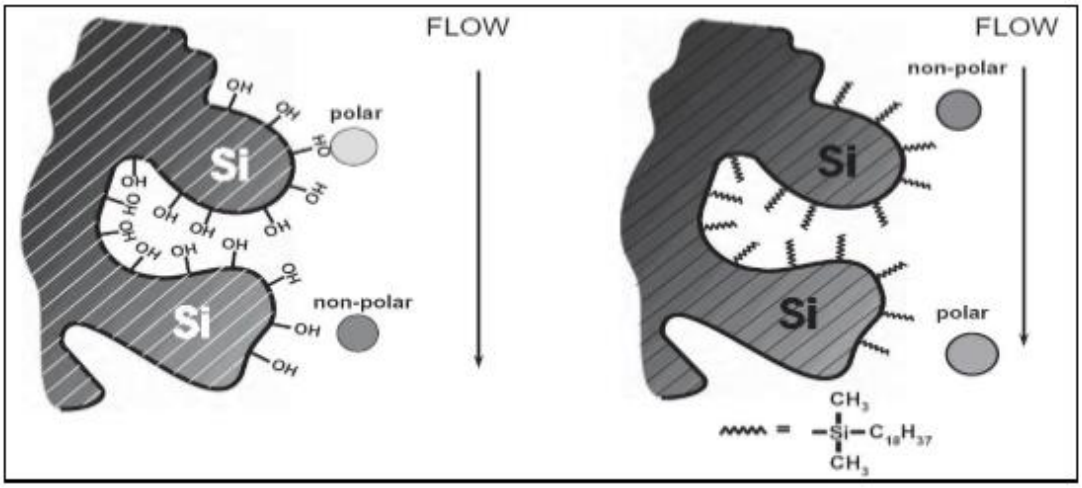


Figure 1.2: Schematic diagrams depicting separation modes of (left) NPC and (right) RPC

Figure 1.2 (left) shows a schematic diagram of part of porous silica particle with silanol groups (Si-OH) residing at the surface and inside its pores. Polar analytes migrate slowly through the column due to strong interactions with the silanol groups. Mobile phases in NP-HPLC are based on nonpolar solvents (such as hexane, heptane, octane, etc.) with the small addition of polar modifier (i.e., methanol, ethanol or isopropanol).

Packing materials traditionally used in NP-HPLC are usually porous oxides such as silica (SiO_2) or alumina (Al_2O_3). Silica is the preferred stationary phase owing to ready availability, low cost and known performance. Surface of these stationary phases is covered with the dense population of hydroxyl (OH) groups, which makes these surfaces highly polar. Analyte retention on these surfaces is very sensitive to the variations of the mobile phase composition. Chemically modified stationary phases can also be used in NP-HPLC. Silica modified with trimethoxy glycidoxypropyl silanes (common name: diol phase) is typical packing material with decreased surface polarity. The use of diol type stationary phase and low polarity eluent modifiers [esters (ethyl acetate) instead of alcohols] allow for increase in separation ruggedness and reproducibility, compared to bare silica. (Braithwaite 1999)

1.1.1.2Reversed phase HPLC (RP-HPLC)

The stationary bed in RP-HPLC is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid. RP chromatography is used for the separation of neutral species on the basis of their hydrophobicity.

RP chromatography typically refers to the use of chemically bonded stationary phases, where a functional group is bonded to silica, as illustrated in Figure 1.3. For this reason, RP chromatography is often referred to in the literature as bonded phase chromatography. Occasionally, however, polymeric stationary phases such as polymethacrylate or polystyrene, or solid stationary phases such as porous graphitic carbons are used (Bereznitski 1998).

Figure 1.3: Reaction of silica gel with a functional group to produce a RP stationary phase.

A simplified view of RP is shown in Figure 1.2 (right), where polar analytes elute first while nonpolar analytes interact more strongly with the hydrophobic C18 groups that form a liquid like layer around the solid silica support.

The mobile phases used in RP chromatography are based on a polar solvent, typically water, to which a less polar solvent such as acetonitrile or methanol is added. Solvent selectivity is controlled by the nature of the added solvent in the same way as was described for NP chromatography; the eluting strength of the solvent is inversely related to its polarity. Mobile phase is by far the major tool for the control of analyte retention in RP-HPLC. Variations of the eluent composition, type of organic modifier, pH and buffer concentration can have important effects on the analyte retention and selectivity in RP chromatography, which provide the chromatographer with a valuable set of variables for successful development of a separation method (Kazakevich 2001) The majority of packing materials used in RP chromatography are those in which a functional group is chemically attached to a silica support (chemically modified

porous silica bonded phases). The most popular bonded phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group, such as $-CH_3$, $-C_4H_9$, $-C_8H_{17}$ and $-C_{18}H_{37}$, phenyl ($-C_6H_5$) groups, cyano [$(-CH_2)_3CN$] groups and amino [$(-CH2)_3NH_2$] groups, with retention increasing exponentially with chain length. The properties of the silica surface and its modification methods have been studied for many years mainly as a direct result of growing popularity of RP-HPLC, and the technology of manufacturing porous spherical particles of controlled size and porosity is well developed. (Snyder 1997)

RP columns are quite difficult to damage compared with normal silica columns; however, they should never be used with strong aqueous bases as these will destroy the silica. They can be used with aqueous acid but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment. RP chromatography is the most popular mode for the separation of low molecular weight (<3000), neutral species that are soluble in water or other polar solvents. It is widely used in the pharmaceutical industry for separation of species such as steroids and vitamins. It is also used in other areas; for example, in clinical laboratories for analysis of catecholamines, in the chemical industry for analysis of polymer additives, in the environmental area for analysis of pesticides and herbicides, and in the food and beverage industry for analysis of carbohydrates, sweeteners and food additives. (Rustamov *et al*. 2001)

1.1.2 Columns and stationary phases

The column represents the heart of the HPLC where the sample mixture separation process occurs. Column packing materials are the media producing the separation, and properties of this media are of primary importance for successful separations. The selectivity, capacity and efficiency of the column are all affected by the nature of the packing material or the materials of construction.

Several thousands of different columns are commercially available, and when selecting a column for a particular separation, the chromatographer should be able to decide whether column type is needed and what the desired characteristics of the base material, bonded phase and bonding density of selected column is needed. Commercial columns of the same general type (e.g., C18) could differ widely in their

separation power among different suppliers. Basic information regarding the specific column provided by the manufacturer, such as surface area, percentage of carbon and type of bonded phase, usually does not allow prediction of the separation or for the proper selection of columns with similar separation patterns.

Great varieties of different columns are currently available on the market. Four distinct characteristics could be used for column classification:

(1) Type (porous; nonporous and monolithic).

(2) Geometry (surface area; pore volume; pore diameter; particle size; shape; etc).

(3) Surface chemistry (type of bonded ligands; bonding density; etc).

(4) Type of base material (silica; polymeric; alumina; zirconia; etc.

Where all four characteristics above are interrelated. Variations of porosity which include pore diameter can affect both the adsorbent surface area and the bonding density. The type of base material affects adsorbent surface chemistry. Most geometry related properties of packing materials such as particle size, particle shape, particle size distribution, packing density and packing uniformity, are related to the column efficiency and flow resistance. Surface chemistry related properties are mainly responsible for the analyte retention and separation selectivity. Adsorbent surface area, pore volume and pore diameter are the properties of significant importance. HPLC retention is generally proportional to the surface area accessible for a given analyte. Surface area accessibility is dependent on the analyte molecular size, adsorbent pore diameter and pore size distribution. The chemical nature of the ligands bonded on the surface of support material defines the main type of chemical interactions of the surface with eluent and analyte molecules, the interaction type and strength can significantly affect the analyte retention. (Wilson *et al*. 2002)

1.1.2.1 Type of packing materials (porous, nonporous, and monolithic)

In the case of porous particles with an average diameter between 3 and 10μm is the most packaging material used in HPLC. For most applications, it is recommended to use particle sizes of 3μm.Porosity provides the surface area necessary for the analyte retention (usually between 100 and 400m^2/g). Interparticle space is large enough to allow up to 1–3mL/min flow within acceptable pressure range. However, the pressure drop(back pressure or column pressure) across the column depends on the particle

size, length of column, temperature of separation and type of mobile phase composition.

Introduction of small nonporous spherical particles in the mid 1990's (Issaeva et.al 1999) was an attempt to increase efficiency by eliminating dual (particles) column porosity. In the column packed with porous particles, interparticle space is about 100 fold larger than pores inside the particles, and liquid flow around the particles is also faster; this leads to the significant band broadening. Unfortunately, elimination of particle porosity dramatically decreases adsorbent surface area, thereby decreasing the column loading capacity. Columns packed with small (1.5µm) nonporous particles also require ultra micro injection volumes and a corresponding increase of detector sensitivity.

Monolithic column technologies are one of the fastest separation types when using HPLC. Monolithic columns are formed from a block of continuous single piece materials made of highly porous rods with two types of pore structures (macropores and mesopores of different sizes), giving them greater porosity and permeability than conventional particle columns (Lubda *et al.* 2001). Comparison of the spherical packing material and monolithic silica is shown in Figure 1.4.

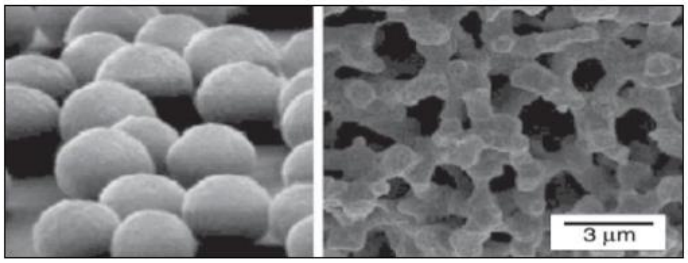


Figure 1.4: SEM pictures of HPLC silica particles (5µm) and silica monolith

1.1.2.2 Column dimensions

A modern HPLC column is stainless steel, plastic tubes or fused silica capillaries filled with the stationary phase and arranged with end fittings designed to provide sealed connection with the eluent inlet and outlet lines and to retain packing material inside while allowing liquid to pass through. The length, diameter and construction

material of the column affect the lifetime, efficiency and speed of separation. The size and nature of the packing material affect resolution.

The internal dimeter (i.d) of a column is a critical guise that determines quantity of analyte that can be loaded onto the column, the peak dilution and the flow rate. The larger the i.d., the greater is the loading capacity and the higher is the flow rate. However, peak dilution increases with i.d., and therefore mass sensitivity decreases. In contrast, smaller columns i.d. influences sensitivity and reduce the solvents consumption at the expense of loading capacity. Most analytical columns range from 2 to 5mm in diameter. Narrow bore (or smaller) columns with diameters of 2mm or less are used for applications where high sensitivity is required, the amount of sample is limited or solvent purchase and disposal costs are significant. Larger columns (preparative) are usually seen in industrial applications such as the isolation and purification of a drug product for later use. Bead and pore size of the stationary phase also play an important role in the separation process, small beads and pores generally provide more surface area and better separations (Snyder *et.al* 1997; Van Deemter *et al.* 1956).

Another factor that affects the efficiency and the speed of the separation is column length. Longer columns have higher plate numbers and yield better resolution but with longer analysis times. Note that column pressure drop is also proportional to the column length. However, the column efficiency tends to increase with length. In general, short columns are used for simple separations. Analytical columns can range from 30 to 300mm in length.

1.2 Capillary Liquid Chromatography

Capillary LC is a miniaturized HPLC technique performed using columns with an i.d. between 10– 500µm, in which, 150–500µm i.d. refers to micro, while as 10–150µm i.d. refers to nano LC. The name micro or nano capillary LC refers also to the mobile phase flow rate and sample injection volume which is in the micro or nano ranges. The main advantage of using smaller i.d. columns in HPLC is the increased detection sensitivity that can be obtained as a result of reduced sample dilution. For that reason,

capillary LC is ideally suited for the analysis of small sample amounts. In addition, the low flow rate used in capillary LC facilitates coupling to MS detectors.

1.2.1 Miniaturization LC

In recent years, the ID of LC columns has decreased from the standard 4.6 mm to 2.1mm ID conventional columns, and further down to 10 µm to 100 µm ID nanoLC columns (Lundanes et.al 2013; Saito 2004). Large columns are still frequently used for routine analyses due to well suited instrumentation for these columns, robustness and easy handling. Narrower columns would need more careful treatment and optimized connections (tubing, unions etc.) to avoid extra column band broadening (Stefanka 2006). Nevertheless, smaller ID columns provide other significant benefits . Most importantly, downscaling of column ID increases sensitivity for concentration sensitive detectors (Baker *et.al* 2010; Pruss *et.al* 2003; Wilson *et al*. 2015) as shown in (Figure 1.5).

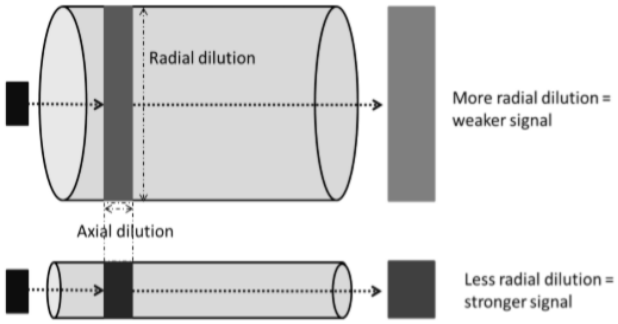


Figure 1.5 Dilution in chromatographic systems with a conventional (upper column) and narrow column (lower column).

Both columns have the same axial dilution (band broadening). However, the increased ID of the conventional column gives an increase in radial dilution, which will give a decrease in signal intensity (lower sensitivity for concentration sensitive detectors). Figure adapted with permission from (Wilson *et al*. 2015).

Downscales has recently become a very trendy word in science and technology, these have strikingly different properties due to their small size and thus are found to open many useful application fields (Dispenza *et al*. 2006).

Most of us think of low scales technology as something very modern and new, so it is hard to imagine that capillary HPLC method emerged more than 20 years ago. In 1988 Karl Karlsson and Milos Novotny published a paper in titled of "Separation efficiency of slurry packed liquid chromatography microcolumns with very small inner diameters" where they reported extremely high efficiencies with micro LC columns that had the i.d. of 44µm (Borges *et al*. 2007)

Since that time great efforts have been made to miniaturize LC instrumentation by carrying out theoretical, technological and methodological studies. Presently capillary LC is widely used as a complementary separation technique to conventional LC, providing a great number of important applications especially in proteomics.

Routine HPLC analyses are traditionally performed on columns with an i.d. of 4.6mm. These columns are robust, have a large sample capacity and are available in a broad range of stationary phases. Reduction of the column i.d. results in less sample dilution during the chromatographic process, thereby yielding higher detection sensitivity for concentration sensitive detectors such as UV and electrospray MS. For this reason, miniaturization of HPLC was already started in the early 1980's. As a result, a family of new HPLC techniques classified by the i.d. of the column exists nowadays which are listed in Table 1.1.

Table 1.1Names and definitions for HPLC techniques, relation between columns i.d., flow rate and sensitivity gain (Niessen *et.al* 2006; Sagliano *et al*. 1985).

HPLC technique	Column i.d.	Flow rate	Injection volume	Relative gain in sensitivity
Preparative	> 10mm	> 20mL/min	---	---
Semi-preparative	5.0–10mm	5.0–20mL/min	---	---
Conventional	3.2–4.6mm	0.5–2.0mL/min	100µL	1
Narrowbore	1.5–3.2mm	100–500µL/min	19µL	5
Microbore	0.5–1.5mm	10–100µL/min	4.7µL	21
Micro capillary	150–500µm	1–10µL/min	490nL	235
Nano capillary	10–150µm	10–1000nL/min	12nL	3800

These columns can be used for different applications according to its properties;

(1) Larger i.d. columns are used to purify the materials because of their large loading capacity.

(2) Analytical or conventional scale columns have been the most common type of columns, though smaller columns are rapidly gaining in popularity.They are used in traditional quantitative analysis of samples and often use a UV/VIS absorbance detector.

(3) Narrow or microbore columns are used for applications when more sensitivity is desired either with special UV/VIS detectors, fluorescence or LC/MS detection methods.

(4) Capillary columns which are used almost exclusively with alternative detection means such as MS. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

Capillary LC is one of the most extensively miniaturized HPLC techniques and is typically performed on columns of approximately 75µm i.d. using a mobile phase flow rate of around 250nL/min. In practice, the choice for a certain column i.d. depends on various considerations, such as amount of sample available, sample complexity and type of detector.

1.2.2 Advantages of using capillary LC

Because of high resolution, selectivity, sensitivity and speed, HPLC has become method of choice for a vast array of analytical separations. However, with growing environmental concerns over waste disposal, and the high cost of many biological samples, interest in miniaturization is increasing. Reduction of the column i.d. in HPLC can be attractive because it has increased detection sensitivity; easier coupling to MS interfaces and significantly reduces solvent and sample consumption. (Guillarmea *et al*. 2008 ; Kromidas 2006)

The increased detection sensitivity is the result of higher solute peak concentrations in the detector due to reduced sample dilution occurs in smaller i.d. columns. As the sample dilution is directly proportional to the volume of the column a straightforward approach to yield improved sensitivity for concentration sensitive detectors such as UV or ESI-MS, is to apply smaller i.d. columns.

Figure 1.6 below shows a sensitivity comparison that made by injecting the same sample mass of 2pmol of digested myoglobine on columns that have different i.d. The 4.6mm i.d. column has shown hardly any peaks, whereas the 75µm i.d. shows excellent peaks.

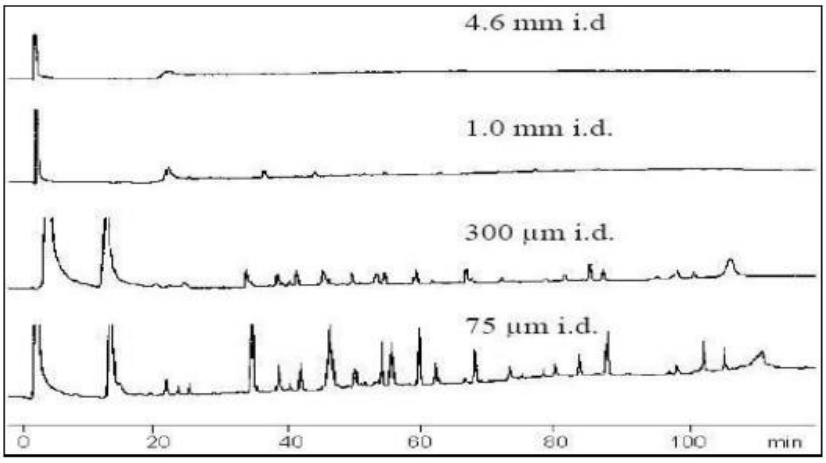


Figure 1.6: A sensitivity comparison of different column i.d. for digested myoglobine.

Other advantages for reducing columns i.d. include:

- Avoiding external peak broadening in order to maintain good resolution in the column (high separation efficiency and peak capacity).
- Perfect for handling minute and/or dilute samples.
- Decrease in the analysis time (Benkali *et.al 2008*, Meyring *et al*. 1999).
- Reducing the analytical cost. (Guillarmea *et al*. 2008 ; Kromidas 2006)
- Excellent repeatability and reproducibility with accurate performance.

1.2.3 Capillary LC columns

The performance requirements for capillary LC columns are similar as for standard HPLC columns with respect to efficiency, selectivity and stability. Capillary columns of (10–500µm) i.d. are commonly used in the capillary LC setups. They are typically made either from fused silica or PEEK (poly ether ether ketone) materials which are currently found in either everyday GC or HPLC.

A column is essentially a device that holds a stationary phase in place, allowing the mobile phase to carry an injected sample through and allowing analytes to interact with available surface. There are three basic types of capillary columns used in capillary LC; packed, open tubular and monolithic columns.

1.2.3.1 Packed capillary columns

Packed columns are made by stuffing the capillary with silica modified particles, commonly range from 3 to 5μm. Though recently, particles of even smaller sizes 1.5–1.8μm were successfully employed in UPLC systems. Such a small particle size provides capillary LC systems with higher efficiency, resolution, selectivity and shorter analysis time; however, it does increase the backpressure. Figure 1.7a shows schematic representation of a packed column cross section.

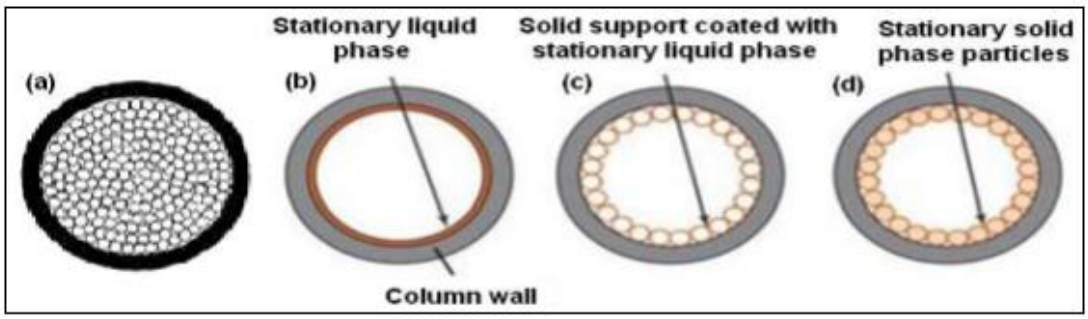


Figure 1.7: Schematic representation of (a) packed, (b) WCOT, (c) SCOT and (d) PLOT cross section columns.

Geometry of the packing material and uniformity of the column packing are the main factors defining the efficiency of particular column. The separation efficiency is related to the particle size of the stationary phase material; an increase of the efficiency can be expected with the decrease of the particle diameter, since the difference between the average size of the pores in the particles of the packing material and the effective size of interparticle pores decreases, which leads to the more uniform flow inside and around the particles. From Figure 1.8, it is obvious that the smaller the particles, the lower the theoretical plate height and the higher the efficiency (Dong *et al.* 1984). On the other hand, small particle columns suffer less efficiency loss at high flow rates since the van Deemter curved at high flow is

dominated by contribution from the C term, which is in turn proportional to the particle diameter square.

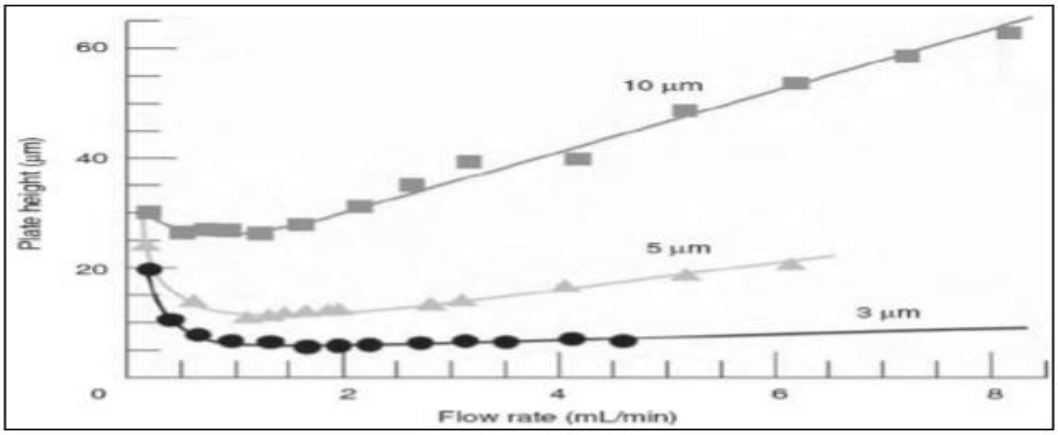


Figure 1.8: Experimental van Deemter curves, illustrate the dependence of the theoretical plate height on the flow velocity for columns packed with same type of particles of different average diameter

For these reasons, smaller particle columns are becoming popular in modern HPLC because of their inherent higher efficiency (Dong et al. 1984).They are particularly useful in Fast LC and in high speed applications such as high throughput screening.
Due to the high cost and limited types of stationary phases available, many research labs pack the columns "in-house". But it is a difficult and skill demanding process. The trick is to make the particles of the same diameter and to avoid undesirable void volumes. So far، the application of packed capillary columns is the most explored option in micro and nano LC.

1.2.3.2 Open tubular capillary columns

In open tubular LC column, the capillary wall is coated with highly permeable porous material that serves as the stationary phase. The open tubular capillary has lower sample loading capacity of the column, because only a small surface area is available for analyte interaction that can result in column overloading causing peak asymmetry and poor efficiency. Open tubular columns are divided into three classes:
(1) Wall coated open tubular WCOT columns, which are simply tubes coated with a thin layer of the stationary phase, this type is the most popular one (Figure 1.7b).

(2) Support coated open tubular SCOT columns, in which a stationary phase is a solid support film coated with stationary liquid phase (Figure 1.7c).

(3) Porous layer open tubular PLOT columns that designed to increase the loading capacity of the column, the inner surface of the capillary is lined with thin films of a support material, so this type of column holds more stationary phase and thus has a greater sample capacity (Figure 1.7d).

1.2.3.3 Monolithic capillary columns

Monolithic technology HPLC columns represent relatively new and innovative type of column for rapid chromatographic analysis. The concept of monolithic columns has been identified as an alternative to particle packed columns (Hjerten et al. 1989; Svec et al. 1992) In contrast to conventional HPLC columns, monolith columns are formed from a block of continuous single piece materials made of highly porous rods with two types of pore structures (macropores and mesopores of different sizes), giving them greater porosity and permeability than conventional particle columns. (Lubda *et al*. 2001)

In 1967, Kubin reported the first attempt to use a monolith material for separation (Kubin *et al*. 1967); several different monolithic supports were then described in the literature since the late 1980's or early 1990's. Two types of monolithic columns have been developed for chromatography (Minakuchi et al. 1998); macroporous organic polymer based monolithic columns produced by a simple molding process (Kubin *et al.* 1967), and silica based monolithic columns made by using the sol-gel approach (Wang *et al*. 1993).These stationary phases are basically synthesized from silica or organic monomers, such as acrylamide, styrene and methacrylate derivatives (Buszewski *et al*.2004 ;Hjerten *et al*. 1989). SEM images of styrene based and silica monoliths inside different capillary columns i.d. are shown in Figure 1.9.

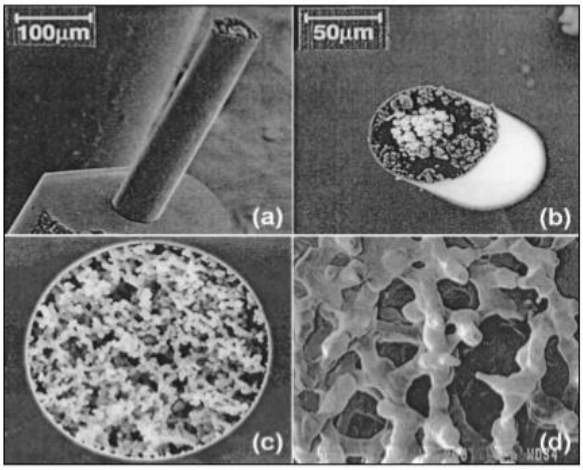


Figure 1.9: (a and b) SEM of a styrene based monolithic structure in a 75µm I.D. capillary column. (c and d) SEM of a silica monolith inside a 100µm i.d. capillary column.

Due to its unique structure as well as their ease of preparation, monolithic columns offer improving chromatographic performance and favorable properties for high efficiency and permeability, fast separations, high reproducibility, the absence of end frits, low backpressure drop across the column, fast mass transfer kinetics between the mobile and stationary phases and a high binding capacity .(Buszewski *et al*. 2004; Jiang *et al*. 2007; Yang *et al*. 2006)

1.2.4 The capillary LC system

The capillary LC system is mainly composed of the same components as the standard HPLC system. However, reducing the internal diameter of the separation column leads to the fact that all components of the HPLC device, including the injector, the pump, the pipe and the detector must be reduced.

1.2.4.1 Capillary LC pump

The flow rate of the mobile phase in the micro and nano LC is within 10-10,000 nL / min, and can be generated by standard HPLC pumps using flow splitting or by dedicated splitless pumps. Both types of pumps are able to deliver solvent gradients in the micro and nanoliter per minute range reliably. Splitless pumps have the advantage that less solvent is consumed. Standard pumps with a flow splitter can deliver a wide flow rate range and generally have a lower gradient delay time.

The performance of a capillary LC pump is determined by the accuracy and precision of gradient formation, flow delivery and the mixing efficiency. A significant improvement in flow rate accuracy and precision has been achieved by the incorporation of liquid flow sensors capable of measuring flow rates down to several micro or nanoliters per minute. (Wang *et al.* 1993)

1.2.4.2 Sample injection of capillary LC

The maximum injection volume that can be made on a column without causing dispersion is directly proportional to the column volume. The effect of the injector on the separation can be seen clearly in Figure 1.10.

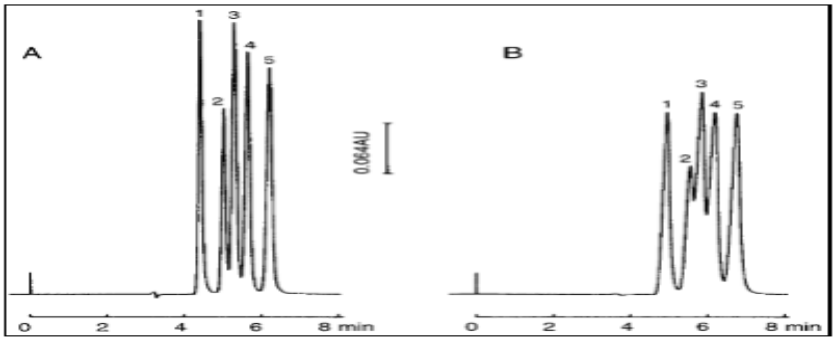


Figure 1.10: Chromatograms obtained with different injectors. (A) Chromatogram obtained using a micro valve injector. (B) Chromatogram obtained using a conventional injector. Conditions: column, C18 (250mm x 1.5mm i.d.), mobile phase, acetonitrile/water (90/10, v/v%), flow rate, 0.1mL/min, injection volume, 1mL, detection, UV absorbance at 250nm with 1mL cell, sample solvent: acetonitrile. Peaks: 1 benzene, 2 naphthalene, 3 biphenyl, 4 fluorene, 5 anthracene

Injectors with internal loop sizes as small as 4nL are currently available (Ishii et al. 1988), but the precise alignment of the small port boring and the extremely narrow loop groove is challenging. Another disadvantage is that the injection volume can only be changed by replacement of the rotor seal. Split loop injection can be applied with internal loop injectors with larger loop sizes but suffers from inaccurate and imprecise injection performance.

The heart of the column switching setup is a two position switching valve. This valve connects the trap column with the capillary LC separation column. The experimental setup for column switching in nano LC is shown in Figure 1.11.

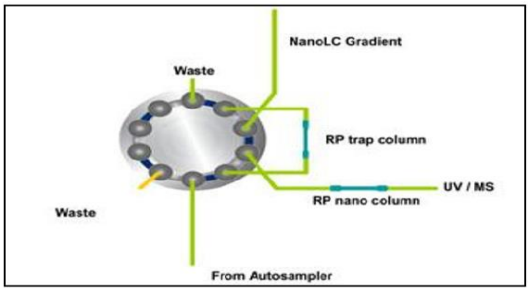


Figure 1.11: Schematic representation of the column switching system coupled with UV and/or MS.

The steps of column switching technique include:

(1) Sample injection by autosampler onto RP trap column with highly aqueous solvent.

(2) Sample preconcentration takes place due to extremely high retention factors.

(3) Valve switching to place trap column in series with RP capillary column.

(4) Solvent gradient started to elute compounds from RP column.

(5) Detection by UV and/or MS detectors.

(6) Valve switching to prepare for next injection.

In column switching the sample is injected onto a small trap column. The trap column has a low flow resistance, a small volume and a high sample capacity. The stationary phase is similar as that of the separation column. A second, isocratic pump is used to transport the sample to the trap column. The low flow resistance of the trap column allows the use of high flow rates to inject large sample volumes in a short time.

The column switching has the advantage that, by concentrating the sample onto the trap column, conventional size injection volumes can be made on capillary scale LC column. In addition, this setup can be used for various on-line samples clean up strategies, e.g. desalting of samples on C18 columns. (Orazio *et al.* 2008)

1.2.4.3 Capillary LC detectors

Almost all commonly applied detectors in conventional HPLC instruments have been miniaturized for use in capillary LC system. The volume of the detector cell must be adapted to the size of the peak volumes eluting from the column. To avoid dispersion during the detection of narrow band from a small volume column, the detector cell must be small enough to prevent diffusion. The detector variance increases as the flow rate increases.

Practically speaking, to minimize the extracolumn broadening by the detector, the maximum detection volume should be not more that 10% of the volume of the peak. If those dimensions are used, even if complete remixing of the solute band occurs in the detector cell, the increase in peak volume is less than 8% (Zhang *et al.* 2007). Thus, for 4.6mm i.d. column, with peak volume of 100µL, the maximum cell volume is 10µL. For 0.5mm i.d. microbore column, with peak volume of 1µL, the maximum cell volume is 0.1µL. Consequently, for analytes eluting from 75µm i.d. nanocolumn having peak volumes of around 50nL, the maximum detection volume is only 5nL (Levkin *et al.* 2008).

Obviously, as the column dimensions decrease, the maximum cell volume for optimum performance becomes prohibitively small. The effect of using an excessively large detector cell volume is illustrated in Figure 1.12.

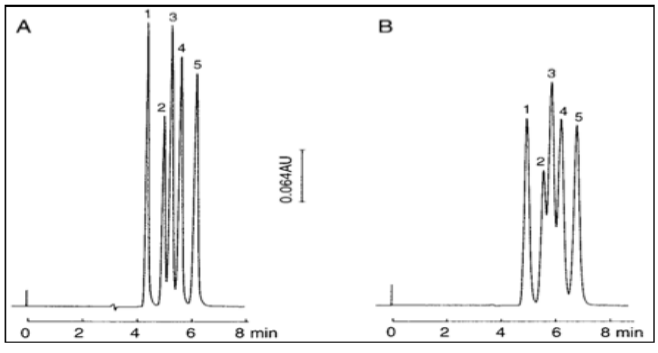


Figure 1.12: Effect of detector cell volume on separation efficiency: (A) 1µL cell; (B) 8µL cell. Experimental conditions: column, 250mm x 1.5mm i.d. C18 (5µm), mobile phase: acetonitrile/water (90/10, v/v%), flow rate, 100µL/min, injection volume, 1µL, detection, UV absorbance at 250nm. Peaks: 1 benzene, 2 naphthalene, 3 biphenyl, 4 fluorene, 5 anthracene.

To maximize the peak response, it is important to use the longest cell path length possible without introducing extracolumn dispersion. With spectrophotometric detection, the maximum signal, A, generated by the spectrophotometer is given by the Beer-Lambert law:

$A = \varepsilon c l$ …… Eq. (1.3.7)

Where: l is the cell path length, ε is the molar absorptivity of the solute and c is the solute concentration. From the Beer-Lambert equation, it can be seen that the signal and then sensitivity is directly proportional to the detector cell path length. Decreasing the volume of the detector cell by reducing the length of the detector cell, therefore, will result in a loss in sensitivity. Significant improvements in detection sensitivity can be achieved by extending the path length using specially Z- or U- shaped UV flow cells. Unlike ordinary on-column detection, where the path length of the light through the sample is equal to the i.d. of the capillary (0.02–0.15mm) (Noppe et al. 2006), these Z- or U- flow cells are made of fused silica, bends the capillary in order to provide an effective illuminated path length that can be varied from 10–30mm.

Another important factor is the detector time constant, which is the response time of the detector to the signal passing through it. A slower time constant will result in less apparent noise, but it will compromise signal and also resolution for closely eluting peaks. Therefore, for closely eluting peaks, it is important to use a faster time constant. If there is plenty of resolution, a slower time constant will provide a smoother baseline. Time constant effect is illustrated in Figure 1.13.

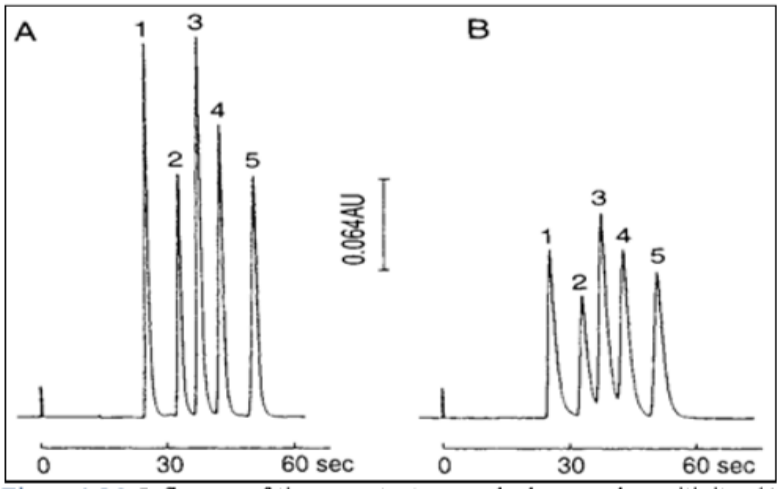


Figure 1.13: Influence of time constant on peak shape and sensitivity: (A) 0.2sec; (B) 2sec. Column, 50mm x 4.6mm i.d. C18 (3µm). Peaks: 1 α-tocopherol, 2 β-tocopherol, 3 γ-tocopherol, 4 δ-tocopherol

1.2.4.4Capillary LC tubing and connectors

The choice of connecting tubing and connectors is critical and important consideration in capillary LC system. Both the dimensions of the tubing and the manner in which it is connected are of very important when using small columns with small injection volumes, small detector cell volumes and low flow rates. The larger the amount of tubing between the injector and column and also between the column and the detector, in terms of diameter and length, the greater is the opportunity for dispersion. In addition, any diffusion effects will be magnified in the small volumes. Tubing that is not seated properly at the bottom of the connection holes, or tubing that has been cut at an angle, so that it cannot be seated flush with the bottom of the holes, will have a significant effect on the separation efficiency. Therefore, to minimize band broadening, it is important when using smaller i.d. columns, to use the narrowest tubing possible and to ensure that it is cut so that the end of the tubing is perfectly perpendicular to its length. The effect of improperly seating the tubing is illustrated in Figure 1.14.

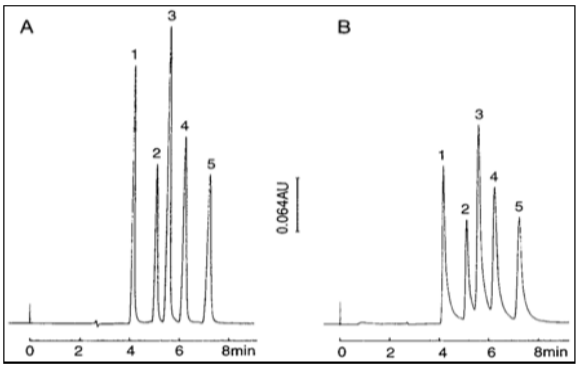


Figure 1.14: Effects of poor connecting tubing on separation efficiency: (A) properly connected tubing, (B) tubing seated improperly at either end of the column. Experimental conditions: column, 250mm x 1.5mm i.d. C18 (5µm), mobile phase, acetonitrile/water (90/10, v/v%), injection volume, 1µL, detection, UV absorbance at 250nm, detector cell volume, 1µL. Peaks: 1 benzene, 2 naphthalene, 3 biphenyl, 4 fluorene, 5 anthracene .

The best material for column tubing is glass, but careful handling is required due to its physical vulnerability. The most commonly used column material is stainless steel tubing, and the inner surface is polished like a mirror. These columns are physically stable and are compatible with a variety of compounds and eluent components. Some biological materials interact with metals; therefore, organic polymer and glass lined stainless steel tubes are preferable for biological samples, and also for eluents with high ionic strengths which can attack the steel. The design of the inlet and outlet connections of columns depends on the physical strength of the materials. However, different connectors and fittings from different systems should not be mixed. Different connectors sometimes damage the efficiency of a whole system because they can create significant extracolumn dead volumes. The best approach is to use one connection system, even with instruments from different manufacturers. Replacement of the connection system is now simple, and does not damage individual components. Finger tight polymer type PEEK connectors are desirable if the instrument is operated at usual pressures (up to 20MPa).

In particular tubing and connectors placed post-column, where no solute focusing is possible, need to be selected properly to avoid additional peak dispersion. Dispersion in cylindrical tubing is described by the Aris-Taylor equation (Reuter *et al*. 2007), and increases to the power of four with the i.d. of the tubing. Typically, as an example,

fused silica tubing with an i.d. of 20µm is used for connection of a 75µm i.d. capillary LC column to the detector (UV cell or MS orifice). Zero dead volume connections of fused silica tubing can be made with a piece of tefzel or teflon tubing, with a stainless steel or PEEK union. These union types of connections can withstand high backpressure and can also be used for pre-columns as shown in Figure 1.15.

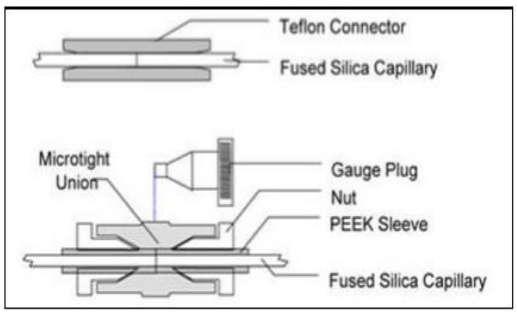


Figure 1.15: Types of connectors for fused silica tubing and columns (low and high pressure).

1.3 Monolithic Materials and Preparation

1.3.1 Definition

According to the IUPAC definition: "A monolith is a shaped, fabricated, intractable article with a homogeneous microstructure that does not exhibit any structural components distinguishable by optical microscopy". A number of alternative names to monolith have been coined in the literature concerned with this new generation of materials, such as continuous polymer bed (Hjerten et al. 1989) and rods (Kubin 1967) , macroporous polymer membranes (Minakuchi et al. 1996), porous silica rods (Steven 1996) or continuous column support (Guo et al. 2008). Obviously, these single words (monolith and monolithic) are handier than these multiword expressions. The popularity of these terms is now supported by trade names of commercial processes and products.

1.3.2 Brief history

The preparation of the separate species is from one piece to the late 1960s and early 1970s. Kubin, Spacek and Chromecek in the institute of macromolecular chemistry in Prague appear to be the first who prepared a swollen poly (2-hydroxyethyl

methacrylate) polymer for SEC to separate water soluble proteins under low pressure. However, the permeability of this continuous bed gel was far too low to make the material useful (Kubin et al. 1967). Some additional optimization slightly improved the separation properties of these materials; this first attempt was not very successful and has been forgotten for a number of years.

Ross and co-workers (Hileman *et al*. 1973;Ross *et al*. 1970; Schnecko *et al*. 1971) also prepared monolithic open pore polyurethane foam for both HPLC and GC in the early 1970's (Figure 1.16). Despite the excellent permeability, they were found to suffer from excessive swelling and softening in some solvents, which prevented their further use. Moreover, the polyurethane was stable at temperatures up to about 200^{o}C, a limit that seriously restricted its application in GC (Hileman *et al*. 1973).

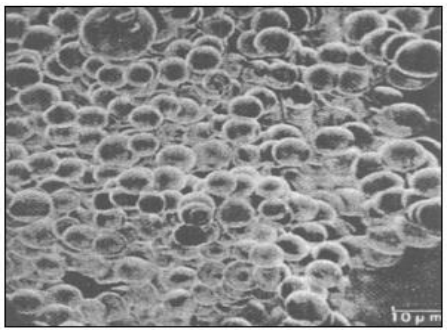


Figure 1.16: Photomicrograph of open pore polyurethane structure.

In addition to polyurethane, other foamed polymers were also used such as styrene copolymers, natural rubber and polyethylene. All these columns were used in gas solid GC (Schnecko *et al*. 1971).However; these columns were not widely accepted. Perhaps their preparation was too complicated compared to a simple packing of columns with particulate solids. Their thermal stability was also much lower than that of inorganic packings typically used in GC. Maybe, their fixed surface chemistry was less suitable for LC in a wide variety of separation modes. Most likely though, it was too difficult for these columns to compete at that time with the just establishing technologies relying on packed inorganic particles (Hileman *et al*. 1973).

After the initial efforts described in the previous sections, the interest in monolithic separation media faded for almost two decades (Hjerten *et al*. 1999). Successful

approaches towards continuous media emerged only in the late 1980's, including stacked membranes (Roper *et al*. 1995), rolled cellulose sheets (Kennedy et al. 1993), rolled woven matrices (Yang *et al*. 1992), compressed soft poly (acrylamide) gels (Hjerten *et.al* 1989), macroporous disks and organic foams (Hileman *et al*. 1973; Ross et al. 1970). The compressed soft gels called continuous beds developed by Hjerten et al. (Hjerten *et al*. 1989) in 1989 are the onset when continuous media were successfully used in chromatographic separation.

The major difficulty was to design such beds that simultaneously fulfilled two contradictory requirements in order to obtain the desired high resolution; a relatively low backpressure upon a strong compression of the beads. Continuing in these efforts, the workers observed again the counterintuitive increase in the resolution of peaks in columns packed with crosslinked nonporous agarose following the increase in the flow rate. Since a higher pressure has been used to achieve the desired flow rate and the beads were soft, the chromatographic bed was compressed under these conditions. The interstitial volumes between the beads became smaller and also changed the flow profile through the bed. Surprisingly, the coincidence of these effects was advantageous and significantly improved the separation (Hjertén *et al*. 1988).

In the early 1990's, Svec and Frechet (Svec *et al.* 1992) introduced an entirely new class of continuous media based on rigid macroporous polymer monoliths produced by a very simple molding process. Their unique properties allow these materials to be used in a broad variety of applications and gradually became the main stream of the research of continuous media family. Inorganic silica based monoliths were later reported by several groups starting in 1996 (Minakuchi *et al*. 1996;Steven *et al*. 1996), and interest in monolithic technologies has grown tremendously since that time. Actually, the monoliths media have become a rapidly burgeoning field in preparation of chromatographic stationary phases in recent years and even some of these have been commercialized (Majors *et al*. 2000). Some chromatography researchers consider them as the fourth generation chromatography sorbents (Iberer *et al.* 1999)

1.3.3 The causes and Impetus that led to the development of monoliths as stationary phases

Over the years, further improvements in column performance have arisen primarily from the development of spherical stationary phases of ever decreasing particle size and heterogeneity. However, since the pressure required driving a mobile phase through a packed bed is inversely proportional to the square of the particle diameter; further increases in column performance using this strategy are restricted by the pressure limits of current solvent delivery systems (Poppe *et al*. 1997). Other methods for improving column efficiency include open tube chromatography (Tock *et al.* 1989), CEC (Dittmann *et al*. 1996) and UPLC (MacNair *et al*. 1997). However, these methods currently require specialized equipment or may suffer from operational difficulties, making their wide scale acceptance and implementation problematic.

An alternative approach for addressing this operational conflict of increasing column pressures associated with decreasing particle size packings is the utilization of a monolithic continuous porous solid transversed by relatively large pores as the stationary phase (Peters *et al.* 1999; Tanaka *et al*. 2001), or alternatively, continuous beds (Hjerten *et al*. 1999). The interconnected network of large pores allows all the mobile phase to pass through such stationary phases using significantly lower pressures compared to their packed bed counterparts. The resulting convective flow greatly enhances the mass transfer rate of analytes in the fluid stream, thus increasing the column separation efficiency (Liapis *et al*. 1993, Rodrigues *et al.* 1993).

The first successful monolithic stationary phases were crosslinked synthetic organic polymers prepared by an in-situ polymerization process directly within the confines of an empty chromatography column. Since their introduction, numerous different materials have been prepared and tested, all of which fall into two broad categories: fully hydrated polymer beds (Ishizuka *et al.* 2000) and rigid macroporous polymers (Peters *et al.* 1999). The enhanced mass transfer properties of these species are clearly evident in their improved separations of both biological (Ishizuka *et al.* 2000 ;Wang *et al.* 1993) as well as synthetic macromolecules (Petro *et al*. 1996). However, these improved efficiencies did not fully extend to the separation of small molecules by

HPLC, probably due to the presence of micropores (pores less than 2nm in diameter) in the polymer matrix that restrict internal diffusion.

The push towards miniaturized capillary columns and separation methodologies such as capillary LC and CEC has provided a second strong impetus for the development of monolithic media. The benefits of miniaturized chromatography are well documented, and include higher sensitivities with both reduced sample and solvent consumption and the ease of coupling to MS. This miniaturization process gathered pace due to the advent of proteomics when reduced sample size was a significant problem. However, the homogeneous packing of narrow diameter capillaries with small particles and the production of end frits to retain these particles have proven difficult (Boughtflower *et.al* 1995; Schmeer *et al.* 1995). The use of monolithic stationary phases eliminates many of these technical problems.

Hjerten et al. (Hjerten *et al.* 1989) first introduced the use of monoliths with capillary LC in 1989, and since that time, monolithic columns have been extensively studied for use in capillary LC (Grafnetter *et al*. 2004; Holdšvendová *et al*. 2003; Minakuchi *et al*. 1996). Capillary LC is typically performed in columns that are 50–500μm i.d. However, the fabrication of such materials in small i.d. columns is not trivial .All packed columns need retaining frits and the ability to produce these with reproducible porosities, lengths and inertness is difficult. In contrast, because a monolith is a continuous porous material, it requires no retaining frits (the packing is covalently attached to the fused silica capillary wall) and because these can now be made by a one step process in-situ, their fabrication is considerably simpler and straightforward. Monolithic materials have some distinct advantages over their particulate counterparts.

Instead of packing preformed rigid particles, a solution of monomers and additives can easily be introduced into a fused silica capillary (Liao *et al*. 1996), or the channels of a microchip (Ericson *et.al* 2000; Fintschenko *et al*. 2001). The monolithic structure is then formed in-situ using a thermal (Svec *et al.* 1995), a chemical or light initiated (Viklund *et al.* 1997) polymerization process, and can be applied as the stationary phase itself, or alternatively serve as a retaining frit for a more traditional packed column (Chen *et al*. 2000). At present, monolithic continuous porous rods have

become frequented in separation science and proved to be sufficiently flexible and reliable as stationary phases for LC and CEC.

1.3.4 Preparation of monolithic columns

A monolithic stationary phase is the continuous unitary porous structure prepared by in-situ polymerization or consolidation inside the column tubing. A broad variety of tube sizes and materials, such as stainless steel, PEEK, fused silica and glass tubes, have been used as molds for the preparation of monoliths (Wang *et al*. 1993; Peters *et al.* 1999).If necessary, the surface of the monolith is functionalized to convert it into a sorbent with the desired chromatographic binding properties. Until now, a considerable variety of functionalized and non functionalized monolithic materials based on either organic or inorganic polymers are available. While inorganic monoliths are usually based on silica and may conveniently be prepared via sol-gel techniques, organic continuous beds are prepared almost exclusively by free radical polymerization. SEM images of different types of porous chromatographic materials are shown in Figure 1.17.

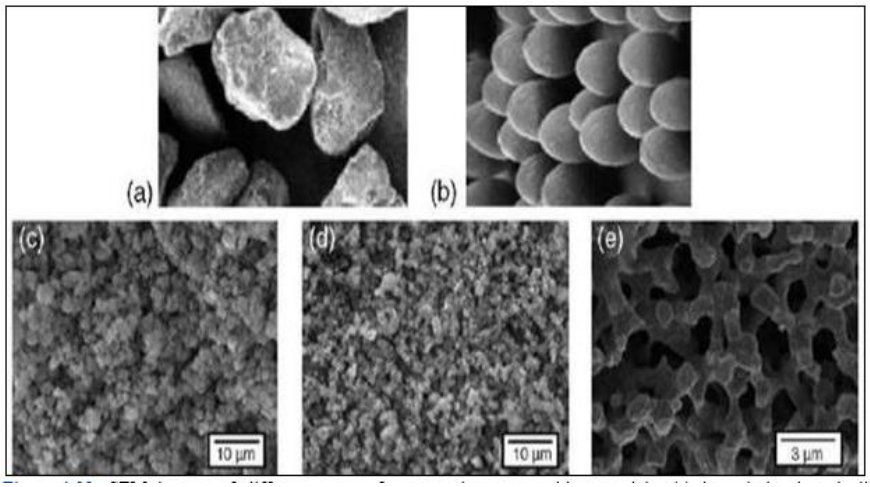


Figure 1.17: SEM images of different types of porous chromatographic materials: (a) irregularly shaped silica particles, (b) spherical silica particles, (c) organic polymer monolith A (UNO S), (d) organic polymer monolith B (CIM Disk) and (e) silica based monolith (Chromolith).

1.3.4.1 Preparation of inorganic monoliths

Although the continuous silica supports from silicate surfactant solutions had been reported at the end of the 1970's, useful silica monoliths for chromatographic applications had only emerged in 1996. So far, two methods have been developed for preparation of the silica monoliths.

One of the methods was introduced by Fields in 1996 (Pretorius *et al*. 1979), which was similar to that used to cast column end frits in fused silica tubing for packing capillary HPLC. A fused silica column was filled with a potassium silicate solution and heated at 100^{o}C for 1h. The column was then washed and dried with helium for 24h at 120^{o}C and then filled with a 10% solution of dimethyloctadecyl chlorosilane (ODS) in dry toluene and heated at 70^{o}C for 5h. After the reaction was finished, the column was washed and was used for RP-HPLC. Although the method can produce a continuous silica xerogel with about 2µm pore diameter, the morphology of the material was heterogeneous.

Another method, i.e. the sol-gel approach, was introduced by Nakanishi and coworkers (Nakanishi *et al*. 1991;1992) ; Then further developed by Tanaka and coworkers [98] and Cabrera and coworkers, this process based on the hydrolysis and polycondensation of alkoxysilanes in the presence of water soluble polymers, by which a more uniform structure of monolith could be constructed .In this method, a porous silica rod was prepared by hydrolytic polycondensation of alkoxysilane accompanied by phase separation in the presence of water soluble organic polymers. The process of gelation, ageing and drying was involved in the preparation procedure. In a typical procedure, tetramethoxysilane (TMOS) was added to a solution of poly (ethylene oxide) (PEO) in water and a suitable catalyst, such as acetic acid, was also added.

The mixture was stirred at 0^{o}C for 30min. The resultant homogeneous solution was poured into a cylindrical polycarbonate mould and allowed to react at 40^{o}C. The gelation occurred within 2h and the gelled sample was subsequently aged at the same temperature for 1 day. The formed wet silica rod was washed with distilled water and then immersed in an aqueous ammonium hydroxide solution in order to tailor the

mesopore structure. Evaporation drying and heat treatment were successively performed, which led to the decomposition of organic constituents and stabilization of the surface of the hydrophilic silica gel. Since the gel shrunk in the ageing and drying, the resultant silica gel had to be encased in heat shrinking poly (tetrafluoroethylene) (PTFE) tubing and compressed with external pressure to ensure that there was absolutely no void space between the silica rod and tube. The rod could be subsequently octadecylsilyated to C18 phase by an on column reaction. With this method, single piece of porous silica rods with a defined bimodal pore structure of 1–2μm throughpore size and 5–25nm mesopore size could be prepared, and the surface area of silica rods reached 300–400m^2/g (Cabrera *et al.* 1998). The silica based monolithic beds are now commercially available from Merck (Darmstadt, Germany) under the trade name of "Chromolith".

1.3.4.2. Preparation of organic polymer monoliths

The preparation of a polymer monolithic rod is relatively simple and straightforward compared to that of a silica rod. A mixture consisting of the monomers, crosslinker and initiator in the presence of at least one, usually two porogenic solvents was poured into a mold, typically a tube, which was sealed at one end, and then sealed at the other end. The polymerization was then triggereds frequently by heating (55–80^{o}C) or by UV light. The seals were replaced with the fittings and attached to a chromatographic pump, and a solvent was pumped through the column to remove the porogens and other soluble compounds that remained in the polymer rod after the polymerization was completed. Figure 1.18 shows the schematic for the preparation of monolithic columns. Monolithic columns could be classified for diverse chromatographic modes. Different monoliths modes have been prepared in the literature:

(1) Hydrophobic monoliths (polymethacrylate/acrylate (Podgornik *et al.* 2000; Peters *et al*. 1997; Wei *et al*. 2000; Svec *et al*. 1995) and polystyrene (Meyer *et al*. 1994; Peters *et al.* 1999; Tripp *et al*. 2000; Wang *et al*. 1995).

(2) Hydrophilic monoliths (polyacrylamides (Hoegger *et al*. 2003).

(3) Ion-exchange monoliths (Hutchinson *et al*. 2006; Liapis *et al*. 2007; Wei *et al*. 2006)

(4) Affinity monoliths (Noppe *et al*. 2006).

(5) Chiral organic polymeric monoliths (Han *et al*. 2006; Messina *et al*. 2006 ;Qin *et al*. 2006).
(6) Natural monoliths (agarose and cellulose) polymers (Gustavsson *et al*. 2001;).

In addition, monolithic molecularly imprinted polymers (Ou *et.al* 2007; Liu *et.al* 2004), and amphiphilic and/or zwitterionic monoliths (Al-Rimawi *et al*. 2006; Jiang *et al.* 2007), that contain hydrophilic and hydrophobic group which makes it possible to use them in both normal and reversed phase mode are also reported.

Polymer based monoliths can be prepared by numerous polymerization techniques such as free radical polymerization (Lee *et al*. 2004; Viklund *et al*. 2001), polyaddition (Tsujioka *et al*. 2005), polycondensation (Hosoya *et al.* 2006) and ring opening metathesis polymerization (ROMP) (Bandari *et al*. 2006; Lubbad *et al.* 2006;Sinner *et al*. 2008).

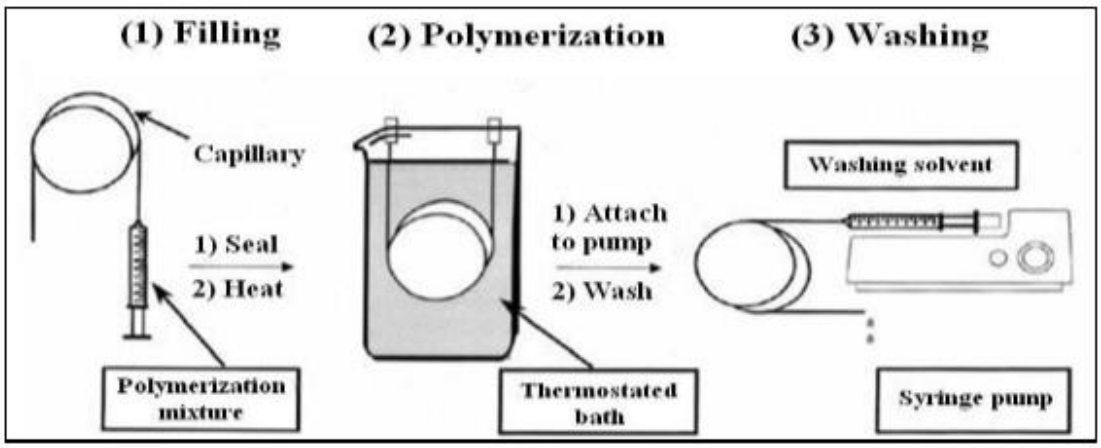


Figure 1.18: Schematics for the preparation of monolithic capillary columns

The prepared monolithic rods possess a bimodal pore size distribution consisting of both large µm sized through pores (macropores) and much smaller pores in the nm-size range (mesopores). The large pores allow liquid to flow through these materials under low pressures even at high flow rates, and the small pores are the most substantial contributions to the overall surface area.

The chemical and physical properties of the monolithic polymer depend on the type and concentrations of the monomer, crosslinker, porogenic solvent and initiator, in

addition to the preparation conditions such as the reaction time and temperature. Therefore, porous properties and morphology of the monoliths intended for use as separation media for chromatography and other applications have to be adjusted during their preparation. Although a number of parameters in the preparation of the monolithic rod affect the porous properties, some key variables such as the time and temperature of the polymerization reaction, composition of the porogenic agents and content of the crosslinker are frequently used to tune the pore size and other characteristics of the monolith (Viklund *et al.* 1996).

The effect of polymerization conditions on the porous properties of these monolithic rods is essentially similar. The polymerization temperature, through its effects on the kinetics of polymerization, is a particularly effective means of control, allowing the preparation of macroporous polymers with different pore size distributions from a single composition of the polymerization mixture. The effect of the temperature can be readily explained in terms of the nucleation rates (number of nuclei that result). As a rule, the higher the polymerization temperature, the smaller the pores, which can readily be explained by considering the number of polymer nuclei formed at different temperatures at constant initiator concentration as well as the rate of their formation(Viklund *et al.* 1996).

The first and most common initiation for the preparation of polymer based monolith is based on increased temperature. This process is very simple where a substance is added to the polymerization mixture which decomposes to form free radicals on a temperature increase (Tsujioka et al. 2005). From the point of view of the monolith homogeneity, UV radiation initiation is recommended (Hosoya et.al 2006), owing to a radial pore size distribution caused by a radial gradient of the degree of polymerization. However, this process has a limitation in that UV transparent liquids must be used and that brown polyimide coated capillaries, impenetrable for UV rays, are excluded Although ultraviolet light and thermal initiation are mainly used for this purpose, polymerization can also be induced by ionizing radiation (e.g., electron beam, γ and X-ray), infrared, microwave or even ultrasound (Zhang *et al*. 2007; 2008).

The choice and composition of porogenic solvents is another tool that may be used to control porous properties without changing the chemical composition of the final

polymer. The porogenic solvent controls the porous properties of the monolith through the solvation of the polymer chains in the reaction medium during the early stages of the polymerization (Lubbad *et al*. 2006). Porogens can be solvating or non solvating solvents for the polymer, or they can be soluble polymers, or mixtures of soluble polymers and solvents. In general, larger pores are obtained if poorer solvents are used because of an earlier onset of polymer phase separation. In addition to the high aliphatic alcohols usually used as the poor solvents for forming the large pores, linear polymers, supercritical carbon dioxide (Cooper *et al*. 1999) and solid granules (Sinner et.al 2008) can also be used to obtain the larger pores. Based on the existing knowledge of pore formation in monolithic materials (Viklund *et al*. 1996; Cooper et al. 1999), different mixtures of macro and microporogens were tested for their ability to afford the desired well defined microstructures. Macropore forming properties of methanol, 2-propanol, cyclohexanol, 1decanol and 1-dodecanol were investigated, while dichloroethane, dichloromethane and toluene were used as microporogens. Since tetrahydrofuran (THF) and dimethylsulfoxide (DMSO) are coordinating solvents that may reduce reactivity of the initiator, their use was avoided.

In contrast, increasing the proportion of the crosslinker in the monomer mixture affects not only the porous properties but also the chemical composition of the final monoliths, which also leads to a decrease in average pore size as a result of early formation of highly crosslinked globules with a reduced tendency to coalesce. This approach is useful for the preparation of monoliths with very large surface areas (Santora et al. 2001). In addition to the divinyl crosslinking monomers, the trivinyl monomer for example trimethylolpropane trimethacrylate (TRIM) is frequently used to provide a higher degree of crosslinking (Viklund *et al*. 1997)

On the other hand, the choice of the suitable initiator represents a crucial step in creating a well defined polymerization system in terms of initiation efficiency and control over propagation .Only if both quantitative and fast initiation occurs, the entire system may be designed on a stoichiometric base. This is important; since for control of microstructure, the composition of the entire polymerization mixture needs to be varied using extremely small increments.The catalyst needs to be carefully selected from both chemical and practical points of view. Concentration of initiator represents an important issue in the preparation of monoliths. For example, any uncontrolled

highly exothermic reactions must be strictly avoided. The total amount of initiator directly determines the number of growing nuclei that affect phase separation and micro globule size. However, for a desired in-situ derivatization, higher initiator concentrations are more favorable (Mayr *et.al* 2001; Sanford *et al*. 2001).

The effect of the concentration of initiator on the porous properties of the monoliths has also been reported by Xie et al. A higher concentration of initiator leads to a higher number of radicals being formed in the system and translates into a larger number of nuclei that result in the smaller pores. The polymerization time also affects the conversion of monomers and the use of shorter reaction times than required for complete monomer conversion leads to porous objects with larger flow through channels (Viklund *et al*. 2001)

The monolithic material may serve its purpose only if provided with a suitable surface chemistry, which depends on the desired application. The versatility of the preparation technique is demonstrated by its use with hydrophobic, hydrophilic, ionizable and zwitterionic monomers. The porous properties of the monolith can be controlled over a broad range. These, in turn determine the hydrodynamic properties of the devices that contain the molded media.

Several methods can be used to prepare monolithic columns with a wide variety of surface chemistry options. Examples of monomers, crosslinkers and initiators that have been used for the preparation of porous polymer monoliths are shown in Figures 1.19, 1.20 & 1.21, respectively.

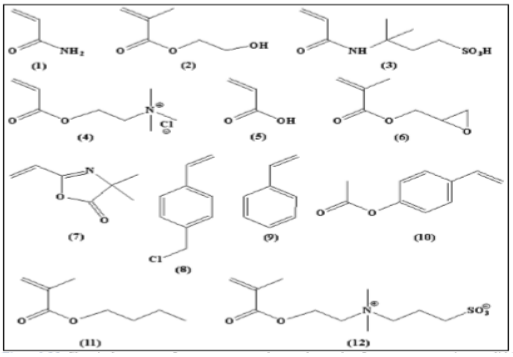


Figure 1.19: Chemical structure of some monomers that can be used to form porous organic monolithic structures.

A few examples of monomers that have been used for the preparation of porous monoliths are shown in Figure 1.19. The list of monomers includes a broad variety of chemistries varying from hydrophilic (acrylamide (1), 2-hydroxyethyl methacrylate (2)), ionizable (2-acrylamido-2-methyl 1-propanesulfonic acid (3), (methacryloyloxy)ethyltrimethylammonium chloride (4), acrylic acid (5)), reactive (glycidyl methacrylate (6), 2-vinyl-4,4-dimethylazlactone (7), chloromethylstyrene (8)), protected (4-acetoxystyrene (10)), hydrophobic (styrene (9), butyl methacrylate (11)) to zwitterionic (12) functionalities and also chiral monomers (Lämmerhofer *et al.* 2000;Peters *et al*. 1998;Viklund *et al*. 2000).

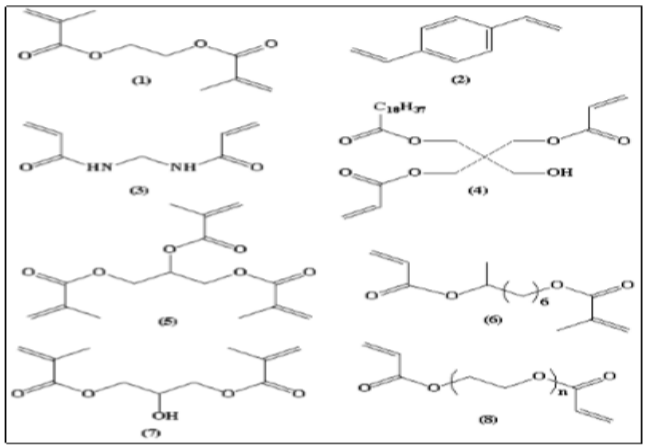


Figure 1.20: Examples of crosslinking monomers used for the preparation of porous polymer monoliths.

The number of most often used crosslinkers is limited (Figure 1.20). One of the reasons is commercial availability of these compounds. Ethylene dimethacrylate (1) is most often used in the acrylate/methacrylate family of monoliths, divinylbenzene (2) typically with styrenic monomers and methylenebisacrylamide (3) in aqueous systems. This does not mean that other crosslinkers were not used. For example: pentaerythritol diacrylate monostearate (4), Trimethylolpropane trimethacrylate (5), 2-methyl-1,8-octanediol dimethacrylate (6), 1,3-glycerol dimethacrylate (7), poly(ethylene glycol) diacrylate (8) 202](Chaisuwan *et al*. 2008; Chen *et al*. 2007; Gu *et al*. 2007; Oxelbark *et al*. 2007; Xu *et al*. 2009; Zhao *et al*. 2008).

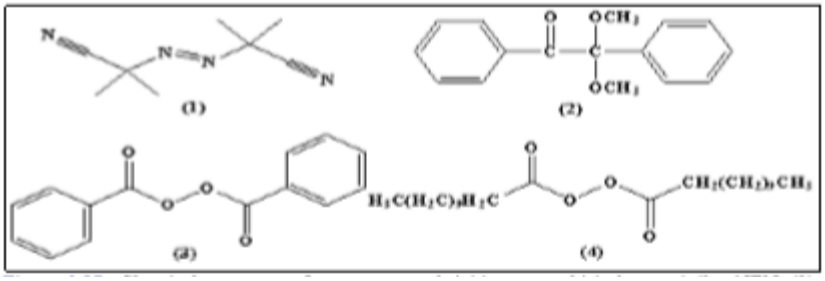


Figure 1.21: Chemical structure of some reported initiators: azobisisobutyronitrile AIBN (1) , 2,2dimethoxy-2-phenylacetophenone DMPA (2), dibenzoyl peroxide BPO (3) and lauryl peroxide LPO (4).

In addition, chemical modification is another route that increases the number of available chemistries, allowing the preparation of monoliths with functionalities for which monomer precursors are not readily available. These reactions are easily performed using monoliths prepared from monomers containing reactive group. Grafting (modification) is another method used in order to obtain monoliths with the desired properties. (Peters *et al.* 1997)

Several works have been done regarding the materials development and optimization for monolithic capillaries prepared from methacrylate ester monomers. Indeed, the number of publications describing the preparation, characterization and application of these monolithic materials has grown exponentially within the last years (Schlemmer *et al*. 2009; Svec *et al*. 2010; Ueki *et al*. 2006; Vlakh *et al*. 2009;Zhang *et al*. 2008). There are several advantages associated with using methacrylate based polymers as monolithic stationary phases, including high stability in a wide range of mobile phase pH (2–12), fast and simple preparation and easy functionalization. Methacrylate monolithic columns have also various selectivities towards monomers with wide ranging polarities. (Moravcová *et al.* 2003; Shu *et al*. 2004)

Some of the polymer monolith supports with different shapes were already put on the market from Bio-Rad (Hercules, USA) under the trade name "UNO", Knauer Saeulentechnik (Berlin, Germany) under the trade name of "QuickDisk" and by BIA Separations d.o.o. (Ljubljana, Slovenia) under the trade name "CIM" (Abou *et al.* 1991; Josić *et al*. 1992). Monoliths for large scale separations can be produced in three different geometric formats: disks, tubes and rods. (Iberer *et al*. 1999)

1.3.5 General properties and advantages of monolithic columns

A monolithic column can be defined as a column consisting of one continuous piece of solid with a defined pore structure that possesses interconnected skeletons and interconnected flow paths (throughpores) and mesopores. A monolithic column consists of micron sized skeletons (1–2µm), throughpores called macropores (in µm-size range, up to 8µm) and skeleton pores called mesopores (in nm-size range, 10–13nm) (Nakanishi et al. 1991;1992). The large macropores are responsible for a low flow resistance and therefore allow for the application of high eluent flow rates, while the small mesopores provide the needed surface area for retention. SEM photographs of monolithic silica columns have shown that both the silica skeletons and the throughpores are co-continuous. A monolithic column with small sized skeletons and large throughpores have a large (throughpore size)/(skeleton size) ratio, commonly 1–4, and can simultaneously provide high permeability and high column efficiency (Minakuchi *et al*. 1996; Motokawa *et al*. 2002; Steven *et al*. 1996) .SEM cross section image of silica based monolith show the bimodal pores (macro and mesopores) are illustrated in Figure 1.22.

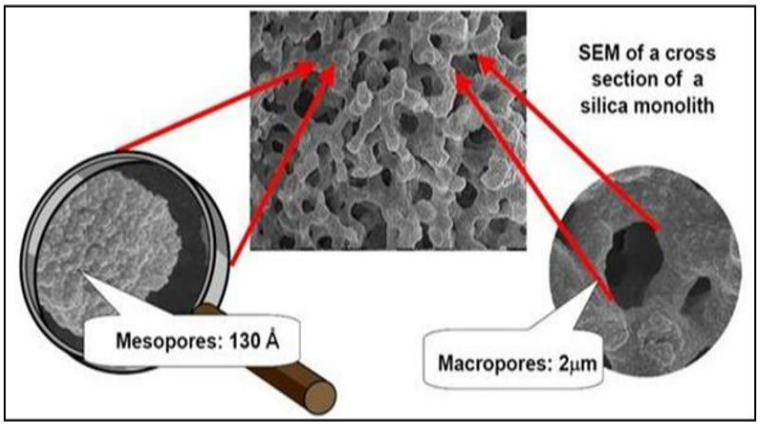


Figure 1.22: SEM image of the porous structure of a typical monolithic silica column (up), and enlarged view of both macro and mesopores.

When monoliths are prepared in a fused silica capillary, the silica network structure can be bonded to the tube wall. They can be used as a column directly after preparation and it is used after some other types of modification. Frits are no longer needed to retain the packing material within the column. The porosity of the

monolithic columns is much greater than that of a particle packed column (Asiaie et al. 1998). A major difference is seen in interstitial porosities: 65–70% for monolithic silica prepared in a mold and higher than 80% for those prepared in a capillary, compared to 40–60% for a particle packed column. Because they have a larger porosity, monolithic columns have a much larger permeability than packed columns, about 30–40% lower pressure drop (Hsieh et al. 2002). Figure 1.23 shows the dependence of the backpressure generated on the system as a function of the flow rate for packed column and a set of different monolithic columns. The slope on all monolithic columns is the same, and it is approximately five times lower than that on a packed column.

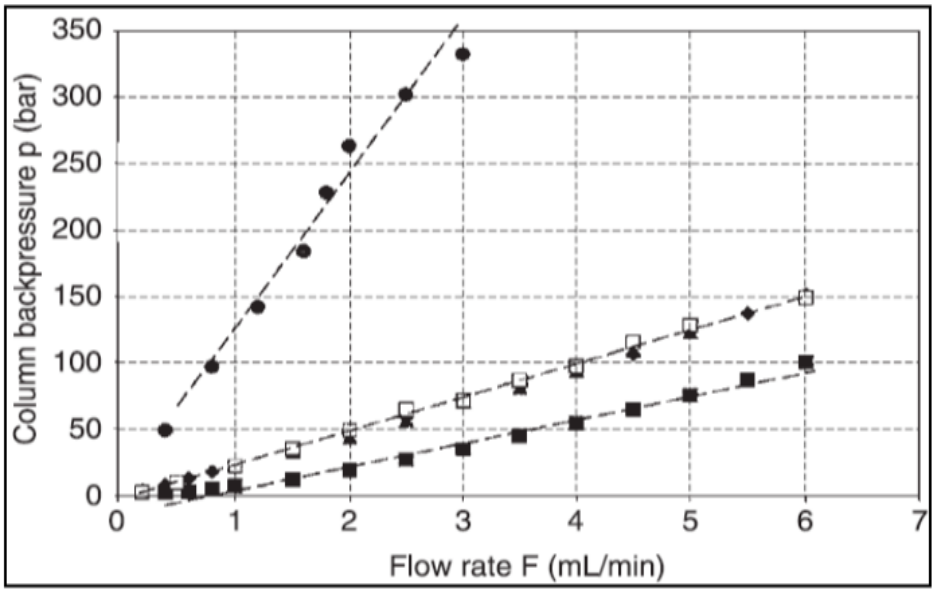


Figure 1.23: Column backpressure as a function of the flow rate. Backpressure comparison on conventional and monolithic column (silica) Circles denote Purospher C18 packed column; closed squares denote equipment pressure drop without the column; other symbols denote different monolithic columns, all in one line.

Monolithic columns consist of one large and continuous piece of porous material that is completely fills the interior of a separation column and sealed against the wall of a tube, as a result, almost all the stream of mobile phase cannot bypass any significant length of the bed but must percolate through it .(Figure 1.24 b). In a particulate column, mobile phase flow must be either throughpores within the particle or interstitial voids between particles (Figure 1.24a).

Continuous media differ from particle based supports in that their structure consists of a network of interconnecting channels through which the mobile phase flows. And because the monolith totally fills the column, this eliminates any interparticle void volume, which leads to differences in hydrodynamics. The problem of particulate separation media is their inability to completely fill the space within the chromatographic column, and the mass transfer between the surface and bulk liquid phase is restricted by hindered diffusion throughpores, for which the concentration gradient is the driving force. In contrast to diffusion, interphase mass transfer in monoliths is governed by convection, and the total pore volume is utilized (Afeyan *et al*. 1990; Wang *et al*. 1994). This allows a dramatic reduction in the time required for mass exchange between the mobile and stationary phases, i.e., reduced C term of the van Deemter equation and improve the separation efficiency.

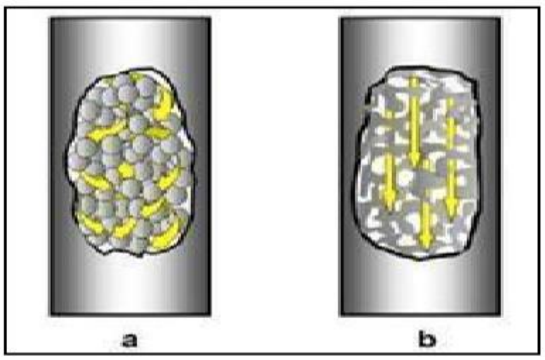


Figure 1.24: Representative passage of the mobile phase through particulate media (a) and monoliths continuous support (b).

It is known that, according to the van Deemter equation, the column efficiency depends on linear flow velocity, the efficiency of the HPLC separation deteriorates rapidly as the flow rate increases (when mass transfer becomes dominant HPLC. On the contrary, many studies for monolithic columns (Hahn *et al*. 2002 ;Ostryanina et al. 2002;Podgornik *et al*. 2000), reliably proved that the column efficiency and the dynamic binding capacity were not affected by flow. An explanation might be that some of the small pores, where the liquid is stagnant at low flow rates, become accessible at higher flow rates meaning the appearance of a convective flow. This allows higher flow rates than particulate columns at reasonable column backpressures. The length of column is not pressure limited, which provides better peaks resolution.

The separation times are consequently at least an order of magnitude faster on monoliths.

However, application of porous materials in chromatography relies on intimate contact with a surface that supports the interacting sites; so many applications require a large surface area in order to achieve a high loading capacity. This high surface area is generally a characteristic of porous material that contains smaller pores. The most substantial contribution to the overall surface area comes from the micropores, with sizes smaller than 2nm, followed by the mesopores ranging from 2 to 50nm. Larger macropores make only an insignificant contribution to the overall surface area. Therefore, a balance must be found between the requirements of low flow resistance and high surface area, and an ideal monolith should contain both large pores for convection and a connected network of shorter and smaller pores for high capacity.
Both chemical and thermal stability of monolithic materials is also very important. Monolithic columns are very stable, high resistance to blockage and have long columns lifetime, these are also advantages of high porosity. On practical basis, monolithic columns seem to have over packed columns the serious advantage of being more resistant to fouling by complex, real samples and of requiring a lesser degree of sample clean-up. This is particularly useful for samples of biological or clinical origin e.g., plasma samples. Moreover, it should be considered that in each run, one component of the mobile phase is a good swelling agent for the monolithic material of the column while the other is a precipitant.Although the high level of crosslinking does not allow extensive swelling of the monolith, even small volumetric changes of the matrix constitute a periodic stress for the column. However, this repeated stress had no effect on long term column performance. Great column stability was also demonstrated, different solvents such as THF, dichloromethane, methanol, acetonitrile, hexane and water with repeated changes in gradient composition without any adverse effects on the separations.

Data describing the reproducible preparation and operation of these columns are also extremely important in terms of further stimulation of both the development and the acceptance of this technology. As this technology has matured, increasingly more groups have reported data on column-to-column, run-to-run, day-to-day and batch-to-batch reproducibility of monolithic capillary columns (Buchmeiser *et al*. 2007; Barut

et al. 2005; Rieux et al. 2005; Ro et al. 2006; Wei et al.2006). Silica based monoliths are reproducible, while organic polymer monoliths are described much more empirically, there may be problems with reproducibility of their properties, but they offer great possibilities for tailoring stationary phases for given purposes.

Research on silica monoliths is restricted by the lack of availability of monoliths having different pore and domain size distributions or surface chemistry. Silica monoliths have proven to be difficult to prepare and often require a post functionalization step after their formation (Chen *et al.* 2001). Few scientists have reported on any successful attempts at reproducing the preparation methods (Kirsch *et al.* 2008 ;Nakanishi *et al*. 1991; Steven *et al*. 1996). These silica monolithic columns are protected by strong patents (Ho *et al*. 2008; Sinnaeve *et al*. 2004 ; Sihlbom et al.2008 ; Tanaka et al. 2001). Till now the stationary phase availability of monolithic columns is limited to normal silica, C8 and C18, with five diameters (0.1, 3, and 4.6, 10 and 25 mm) and short lengths (15cm for the narrow diameter model, 10cm for the other models). This limited choice has largely contributed to the development of polymer monolithic columns. It is probably not foreign that large biomolecules should be separated on polymer monolithic columns, not on silica monolithic columns. So that silica monolithic columns are essentially used for conventional RPLC separations and analyses of small or medium molecular weight range compounds.

In contrast to silica monoliths, the preparation of polymer monolithic columns is not exceedingly difficult , flexible, lack of criticality of many steps of the preparation and the ease for adapted to the preparation of stationary phases for nearly any of the different modes of chromatography, and to separate a wide variety of samples. A wide range of functionalities can directly be incorporated into a polymer monolith, enabling the one step preparation of stationary phases (Ericson *et al*. 1997; Lämmerhofer *et al*. 2000; Maruška *et al*.1999; Peters *et al*. 1998). For this combination of reasons, the area of biochemical separations is seeing an explosive growth of the applications of polymer monolithic columns, gel compressed columns and their like. On the other hand, although there are a few patents in this area (e.g., (Hjerten *et al*.1992; Ma, Qi-Feng No. 11/031,495.), their very number prevents them from locking up the entire field. The wide variety of possibilities offered by these columns and the immense

number of possible applications makes it challenging to define properly a first commercial product.

In spite of the previous notations, some authors have reported on the separations of peptides from proteins and of other bio-macromolecules that they were able to carry out on silica monolithic columns (Nakanishi *et al*.1997;Nogueira *et al*. 2006; Xiong *et al*.2004). On the other hand, small molecules have been separated on polymer monolithic columns by CEC and NP-LC (Lämmerhofer *et al.* 2001). Experimental results suggest that polymeric monolithic columns could be used for many of the same separations carried out with silica monolithic columns (Lämmerhofer *et al*. 2001; Svec *et al*.2005).

Although the morphology of silica and organic polymer based monoliths is completely different, both structures are characterized by large flow throughpores and high column permeability. The mass transfer of sample in the monolithic structure is primarily driven by convective flow instead of much slower diffusion resulting in highly efficient separations.

Finally, the last general advantage of the monolithic columns over the packed ones, narrow bore monolithic columns are easier to prepare and far more reproducible than narrow bore packed columns. Together with their other advantages, this explains why the use of narrow bore packed columns has been nearly abandoned. In contrast, large bore monolithic column for preparative applications are difficult, if not practically impossible to prepare, due to the exothermal character of the polymerization reactions. The drive to prepare them is limited further because they are less practical to use and store than particulate media.

In summary, the major goals of applying monolithic columns in HPLC were to achieve high speed separations, low column backpressure and fast mass transfer kinetics (Siouffi et al.2003). The HETP (height of effective theoretical plate)of the current commercial silica monolithic columns did match the performance of the columns packed with 3 to 5μm particles, with permeability similar to that of columns packed with 9μm particles (about $8x10{-}10cm^2$) [223, 258, 259](Kele *et al*.2002; Kobayashi *et al*. 2006; Motokawa *et al*.2002). But they do not match the performance

of the modern columns packed with the new generation of fine particles (below 2µm diameter), although these particles are difficult to pack. Monoliths can be cast directly in the chromatography column avoiding the time consuming steps of sieving and packing (Cavazzini *et al*.2007; Gritti *et al*. 2007;Hsieh *et al*.2002). The minimum HETP of polymeric monolithic columns seems to be on the average slightly larger than that of silica monolithic columns, although significant differences are found between the results of the many authors who have reported data (Eeltink *et al*. 2004). This is explained by the differences in the morphologies of the monoliths, in the average size of their throughpores and in the size distributions of these pores.

As a result of all their unique properties, the monolithic materials have attracted considerable attention as an alternative to the packed and open tubular columns from a number of different research groups. Especially, in the field of microscale separations, and as the rapid development of micro and nanoscale chromatographic separation systems such as capillary LC and CEC. Despite these promising characters, the number of monolithic columns that are currently commercialized is still small compared to the immense range of possibilities. More time is still needed to improve its applicability to official use and to evaluate result repeatability and reproducibility, and method transfer for different compounds, in addition to method developments.

1.3.6 Characterization of monolithic columns

The morphologies and pore structures of porous media are important in the design of chromatographic columns and separation capability due to their influence on hydrodynamic properties (e.g., flow properties), thermodynamic properties (e.g., loadability) and mass transfer kinetics (e.g., efficiency). The morphology of the monoliths is closely related to their porous properties, and is also a direct consequence of the quality of the porogenic solvent as well as the percentage of crosslinking monomer and the ratio between the monomer and porogen phases.

In contrast to particle columns preparation, where commonly particle size classification is required after polymerization is completed, synthesis of the stationary phase in monoliths is accomplished simultaneously with packing of the column. Monoliths are prepared with a bulk polymerization and their structure is defined

already by monomer composition and polymerization temperature without further processing (Svec *et al*. 1999), which means that morphology of the bed is created during the synthesis of the stationary phase. To obtain high efficiency, resolution, homogeneity and rigidity of the polymer bed, it is important to investigate and control the morphology and structure governing parameters during the synthesis of the monoliths.

The hydrodynamic properties expressed by the column pressure versus flow dependency provide an insight into the flow behavior. From these data, the column permeability can be calculated and compared with the expected value based on the average particle diameter and the column dimensions. Kinetic properties of the column expressing the mass transfer kinetics of analytes are a measure of the peak dispersion of a column. The peak dispersion is characterized by HETP and as the function of the linear velocity of the eluent. Thermodynamic properties are expressed by the retention coefficients and the selectivity coefficient of test solutes under constant conditions.

Several traditional methods are usually employed for determining pore size and morphology of the monoliths. Earlier, we have seen high resolution optical microscopy complementary to other microscopic techniques, such as SEM and also X-ray analysis, might be useful and convenient means for a simple and fast evaluation of the continuous bed morphology (Kornyšova *et al*.1999). It provides actual images of the surface which open a possibility for in-situ inspection of the bed homogeneity along the capillary, but no quantitative characterization of the surface area and pore volume. They are also quite involved, time consuming and expensive.

Bulk measurement techniques, such as nitrogen sorption porosimetry and mercury intrusion porosimetry (MIP) used together can rapidly and inexpensively determine the micro to macro porosities of materials in dry state. The mesopore size distribution (mode around 0.01µm) usually measured by nitrogen adsorption, while MIP technique is well suited for the determination of large pores (mode around 1.5µm), but its accuracy for the measurement of smaller pores is limited due to the compressibility of the polymeric matrix itself (Nakanishi *et al.* 1997). Furthermore, inverse size-exclusion chromatography (ISEC) often used for the determination of

pores smaller than about 50nm. The concept of ISEC is based on the measurement of pore volumes that are accessible to polymer standards (e.g., polystyrene in THF) of well defined molecular sizes (Knox *et al.* 1984; Maa *et al*.1988). Although somewhat controversial (Knox *et al*. 1984;1987), this method presents a suitable way to perform such measurements as it operates under conditions similar to those used in actual HPLC separations. Furthermore, the choice of polystyrene standards allows the determination of the specific surface area and porosity relevant for chromatographic separations (Mayr *et al*. 2001). In addition, the specific surface area of monoliths can be obtained from nitrogen adsorption/desorption isotherms. SEM, MIP and ISEC are four traditional methods for pore size characterization of packed and monolithic columns, which have been used for decades (Cantó *et al*. 2008; Wang *et al*. 1995).

Newer techniques, such as AFM, TEM (Courtois *et al*. 2007), total pore blocking (TPB) (Cabooter *et al*. 2007) and most recently, confocal laser scanning microscopy (CLSM) (Hlushkou *et al.* 2010) have been used to determine pore characteristics of packed and monolithic columns. CLSM is unique in that analysis based on quantitative physical reconstruction can be used to derive the complete three dimensional macropore morphology of monoliths (Fang *et al*. 2010).

For practical uses, further parameters should be characterized. The backpressure of a column should be as low as possible and the column should be mechanically stable. The first concern of a monolithic column is the permeability to mobile phase, which depends fully on the macropores of the medium. Prepared columns usually evaluated by measurement of the pressure drop across the column using different solvents and a wide range of flow rates. The linearity of backpressure drop versus flow rate plot confirms that the monolith is mechanically stable and not compressed at high flow velocities (Figure 1.23). Many applications of porous material are usually dependent on its surface area (Luo *et al*. 2001; Svec *et al*. 1995).

1.4 Analytical Performance and Method Validation

Validation is the process that helps establish, by laboratory studies, that the performance characteristics of a method meet the requirements for the intended application. It provides documented evidence that the method performs for the intended purpose (Skoog *et al*. 2003).

In order to develop a method effectively, most of the effort should be spent in method development and optimization as this will improve the final method performance. The method validation, however, should be treated as an exercise to summarize or document the overall method performance for its intended purpose.

Before beginning the method development, we need to review what is known about the sample; also the goal of the analysis should be defined at this point, and considerations must be given regarding how many samples will be analyzed and what HPLC equipment are available. The first consideration is to determine the solubility of the sample components, knowing the nature of the analytes will determine the most appropriate mode of HPLC to be used. The nature of the sample (e.g., whether it is hydrophilic or hydrophobic, its functional group and substituents) is also an important factor to consider. In our case, the targeted compounds are various types, e.g. aromatics, phenols, ketones and drug. For this reason, care should be taken into account when dealing with each type. Chemical purity of solvents is also an important factor, to avoid blocking of the column after repeated use and to prevent interferences. Analytes in a mixture should preferably be separated prior to detection. The chromatographic procedure for the separation of substance is based on differences in rates of migration through the column arising from different partition of the compounds between a stationary phase and a mobile phase transported through the system. Selecting an appropriate mobile and stationary phase can also help to improve the efficiency of method development.

There exist several different detectors suitable for detecting the analytes after the chromatographic separation. The choice of detector depends on the sample and the purpose of the analysis. The UV/Vis detectors are the most common HPLC detectors since they are robust, and since many solutes absorb light in the UV/Vis range. The

ordinary UV/Vis detector measures the absorbance at one single wavelength at a time. To change wavelength the monochromator setting must be changed, thereby introducing the problems of mechanical irreproducibility into the measurements. The purpose of sample preparation is to create a processed sample with simplified matrix that leads to better analytical results compared with the initial sample. The prepared sample should be compatible with the HPLC method and relatively free of interferences that may damage the separation column or limit analytical performance.
When the method has been developed it is important to validate it to confirm that it's suitable for its intended purpose. The validation shows how good the methods is, specifically whether it is good enough for the intended application. Today, method validation is an essential concern in the activity of analytical chemistry laboratories.
To help guarantee that a method is readily utilizable by any trained analyst, method validation has been defined by a number of scientific and regulatory bodies. Some documents were generated in close affiliation with governmental agencies [e.g., the United States Pharmacopoeia (USP) and United States Food and Drug Administration (FDA)], and some are the result of international cooperation between organizations [e.g., International Conference on Harmonization (ICH) and International Standards Organization (ISO). The intention of all the documents generated by these organizations is to give guidance to those analysts involved in the validation of a method. This guidance is meant to produce statistically verifiable and testable results, while at the same time allowing for as much scientific and flexible as possible.

Each parameter in method validation is generated from the statistical analysis (or the comparison of the parameter to an existing statistical limit) of the results generated during the validation run. ICH and USP have provided definitions of validation issues included in analytical procedures in several fields. The most common validation parameters will be briefly described below (ICH 1997;1999;USP 2003).

1.4.1 System suitability

System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being evaluated. In the case of

chromatographic procedures, system suitability test is performed from five or six replicate injections of standard working solution. (Stephan *et al*. 2002; Gustavo *et al.* 2007)

1.4.2 Limit of detection (LOD)

A statistical statement about the smallest amount (concentration or mass) of analyte in a sample that can be determined at a known confidence level (not necessarily quantitated as an exact value. More mathematically, it may be defined as that amount of analyte which produces a signal greater than the standard deviation of the background noise by a defined factor. This limit depends upon the ratio of the magnitude of the analytical signal to the size of the noise in the blank signal.

1.4.3 Limit of quantitation (LOQ)

The lowest concentration of analyte in a sample at which quantitative measurements with a suitable level of accuracy and precision can be made. The lower limit of quantitative measurements is generally taken to be equal to ten times the standard deviation of repetitive measurements on a blank:

1.4.4 Linearity

Linearity is the ability (within a given range) to obtain an output that is directly proportional to the input. Linearity should be evaluated over the range of the analytical procedure and equipment, ICH recommends a minimum of five points to demonstrate linearity by means of statistical evaluation (correlation coefficient) of the data. The range is the interval between the upper and lower limits for a parameter (including these upper and lower limits), for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.ICH recommends that a minimum of five concentrations is used to determine the linearity. Typically, acceptance values for the correlation coefficient are >0.999.

1.4.5 Specificity and selectivity:

Is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and excipients .In the case of the assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients (placebo) and demonstrating that the assay result is unaffected by the presence of these excipients. (Stephan *et al*. 2002; Gustavo *et al.* 2007)

1.4.6Accuracy

The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. In the documents of the (ISO), its termed trueness. Accuracy may be determined by application of the analytical procedure to an analyte of known purity (e.g., a Reference Standard) or by comparison of the results of the procedure with those of a second, well-characterized procedure, the accuracy of which has been stated or defined. In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. If it is not possible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product (i.e., “to spike”) or to compare results with those of a second, well characterized procedure, the accuracy of which has been stated or defined. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals. (Stephan *et al*. 2002; Gustavo *et al.* 2007;)

1.4.7 Precision (Repeatability and/or Reproducibility)

The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. (Stephan *et al*. 2002; Gustavo *et al.* 2007)

1.4.8 Robustness

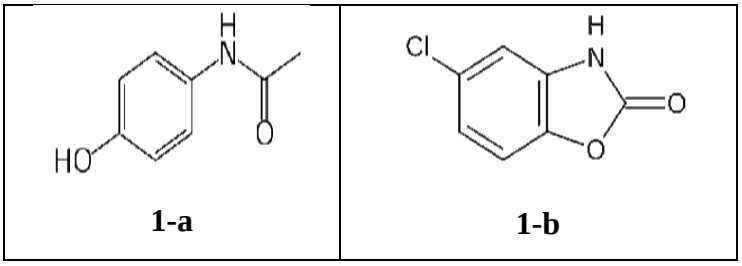
Robustness is a measure of the performance of a method when small, deliberate changes are made to the method conditions, these should be suitably controlled, or a precautionary statement should be included in the procedure to ensure that the validity of the analytical procedure is maintained. Typical variations are the pH of the mobile phase, the mobile phase composition, different lots or suppliers of columns, the temperature, and the flow rate. (Stephan *et al.* 2002; Gustavo *et al*. 2007)

1.5 Medical uses for PAR and CZN:

Paracetamol (p-hydroxy acetanilide) is a compound (Figure 1.26.a) with analgesic and antipyretic properties. It is much safer than aspirin in terms of gastric irritation, ulceration and bleeding (MADHUKAR et al. 2011). Paracetamol1,2 is a non-opiate, non-salicylate analgesic and antipyretic. Chemically, paracetamol is 4-hydroxyl acetanilide or N-(4-hydroxy phenyl) acetamide. Its empirical formula is $C_8H_9NO_2$ and molecular weight is 151.2. It acts by inhibiting prostaglandin synthetase centrally. Specifically, it is a potent inhibitor of cyclo-oxygenase in the CNS. (Krishnan et al.2008)

Chlorzoxazone (5-chloro-2(3H)-benzoxazolone) is a compound (Figure 1.26.b) with skeletal muscle relaxant property. It is used to decrease muscle tone and tension and thus to relieve spasm and pain associated with musculoskeletal disorders (MADHUKAR *et al*. 2011).Chlorzoxazone3 is 5-chloro-3H-benzooxazol-2-one. Its

empirical formula $C_7H_4NO_2Cl$ and molecular weight 191.5. It inhibits antigen-induced bronchospasms and hence, used to treat asthma and allergic rhinitis. Chlorzoxazone is also a centrally acting agent for painful musculoskeletal conditions. It also has sedative property. (Krishnan *et al.*2008)



1-a **1-b**

Figure 1.26 Chemical structures of (1a) paracetamol (1b) chlorzoxazone

1.6 Overview on the thesis

HPLC is one of the most widely used laboratory instrument. The literature on HPLC has exploded and almost every laboratory that deals with analytical problems has one or more LC instruments. Approximately one million LC analyses are performed daily around the world. The growth of HPLC has been phenomenal, and HPLC is using now routinely for all types of molecules from the smallest ions to macromolecules.

1.6 .1 Justification and aims of this work

Many of science and technology fields mentioned above, are becoming more sophisticated and facing new and continuous challenges; the need to analyze more complex substances, to reduce the analysis time and cost, and the necessity to enhance the sensitivity and resolution of the analysis have increased accordingly. On the other hand, such needs may increase as more and more materials and products are developed. Modern HPLC development concerns with creating new types of columns and improving the existing columns efficiency, reliability and reproducibility. Great efforts were made and a lot of useful columns and stationary phases were reported and became commercially available. In the few last years, capillary LC has been one of the most important developments in separation and analysis technology. Capillary HPLC offers several advantages over conventional normal scale HPLC. The advantages include increased chromatographic resolution, higher efficiency, lower

samples and solvents consumption, the ability to analyze and isolate rare compounds of interest, greater mass sensitivity and ease of on-line connection to mass spectrometer.

However, the successful development of these techniques is closely related to the technical challenges associated with the column manufacturing. Traditionally, capillary HPLC uses fused silica capillaries prepared with a variety of different stationary phases, these phases seem to be very promising in separating a wide variety of analytes in different application fields, including pharmaceutical, clinical, environmental, agrochemical and biological samples.

Monolithic stationary phases are relatively new structures, and have rapidly become highly popular and attractive as separation media in all areas of chromatographic methods as an alternative to particulate columns. Monolithic columns consist of one single continuous piece of highly porous material with bimodal pore size distribution, μm-sized throughpores (macropores) and nm-sized mesopores. Macropores dramatically increase the column porosity, thereby considerably reducing the analysis time, while mesopores form the fine porous structure and provide large active surface area for high efficiency separations.

The monolith structure does not contain interparticular voids. As a result, all the mobile phase must flow through the stationary phase. This unique structure of the monoliths as well as their ease of preparation, have offered improvement in chromatographic performance and favorable properties for high efficiency and surface area, fast separations, high reproducibility, low backpressure drop across the column, fast mass transfer kinetics between the mobile and stationary phases and a high binding capacity.

The main objectives of the present project can be summarized as follows:

(1) Preparation of two monolithic column one on capillary columns and another on stainless steel column. To prepare these types of columns, in the case of the capillary column, a mixture of monomer, initiator, crosslinker and porogenic solvents is poured into empty capillary tubing, after it has been conditioned by activation and

functionalization of its inner surface. The mixture is then heated to initiate polymerization reaction. The resulting monolith is then washed to remove any remaining monomers and porogen, the column is then connected to a suitable HPLC system to evaluate its ability to separate various standard solutes. The same steps are repeated in case of a stainless steel column. An important aspect of the use of capillary columns in micro and nano LC is the adaptation of the HPLC instrument which includes a drastic reduction of the flow rate, proper connections of the capillary to both the injector and detector as well as the miniaturization of the sample loop and detection cell.

(2) Characterization and investigation of the morphology of the prepared monolithic columns; this may be carried out using scanning and transmission electron and optical microscopy. Changing the porous and hydrodynamic properties; i.e. porosities including macropores, mesopores and permeability of the prepared monolithic columns could be thoroughly investigated. Different solvents may be used as mobile phases to demonstrate the permeability and mechanical stability of the resulting monoliths.

(3) Applying the prepared monolithic columns for validation studies of the proposed analytical procedures by determination of the limits of detection or quantitation, linearity, calibration, sensitivity, accuracy, precision and reproducibility, as well as the stability. Some applications of the validated methods will be carried out on various real samples, especially of pharmaceutical interest.

1.7 Literature review

1.7.1 Preparation of monolith column and its application

Zilin *et al.* (2002) developed new type of chiral monolith silica column for the chiral separation by micro high performance liquid chromatography (m-HPLC). The chiral monolith column with a continuous skeleton and a large through-pore structure was prepared inside a capillary of 100 mm I.D. by a sol–gel process, and chemically modified with chiral selectors, such as L-phenylalanin amide, L-alanin amide and L-prolin amide, on the surface of the monolithic silica column.

Jingwu *et al*. (2002) developed new method for the preparation of a silica monolithic capillary electrochromatography (CEC) column for the separation of enantiomers. The porous silica monolith was fabricated inside a fused-silica capillary column by using the sol-gel process.

Mohamed *et al*. (2002) prepared monolithic stationary phase by the in situ copolymerization of pentaerythritol diacrylate monostearate (PEDAS) and 2-acrylamido-2methyl-1-propanesulfonic acid (AMPS) in a ternary porogenic solvent consisting of cyclohexanol/ethylene-glycol/water.

Dana *et al*. (2003) prepared monolithic capillary columns in fused-silica capillaries by radical co-polymerization of ethylene dimethacrylate and butyl methacrylate in the presence of porogen solvent mixtures containing various concentration ratios of 1-propanol, 1,4-butanediol, and water with azobisisobutyronitrile as the initiator of the polymerization reaction.

Wenhui *et al*. (2004) prepared a biporous monolithic silica gel column possessing both micrometer sized through-pores and nanometer sized mesopores located in the silica skeletons by using high concentration porogen (e.g., 2 mol/L ammonium hydroxide solution) for increasing mesopore size in this work.

Chuanhui *et al*. (2005) proposed improved strategy for the preparation of monolithic silica ODS capillary column with high homogeneity. Column performance was evaluated by nanoscale liquid chromatography (μ-HPLC). The design for constructing an integrated nanoelectrospray emitter on the monolithic silica ODS capillary column was first introduced.

Xiaoli *et al*. (2008) prepared hydrophilic chiral capillary monolithic column for enantiomer separation in CEC by coating cellulose tris(3,5-dimethylphenyl-carbamate) (CDMPC) on porous hydrophilic poly(acrylamide-co-N,N0-methylene-bisacrylamide) (poly(AA-co-MBA)) monolithic matrix with confine of a fused-silica capillary.

Anastasiya *et al*. (2007) prepared Low capacity anion exchangers for IC by modification of nonporous uniformed silica MICRA microbeads and by modification of the organic polymeric monolithic matrixes prepared in situ in quarz capillary.
Violaine et al. (2008) prepared polyacrylate-based monolithic column bearing cationic functionalities and designed for capillary electrochromatography (CEC) via photopolymerization of a mixture of hexyl acrylate, butanediol diacrylate, 2-(acryloyloxy) ethyltrimethyl ammonium chloride (monomers), azobisisobutyronitrile (photoinitiator), acetonitrile, phosphate buffer, and ethanol (porogens).

Andreas *et al*. (2009) prepared monolithic poly(1,2-bis(p-vinylphenyl)ethane (BVPE)) capillary columns by thermally initiated free radical polymerization of 1,2-bis(p-vinylphenyl)ethane in the presence of inert diluents (porogens) and a,a9-azoisobutyronitrile (AIBN) as initiator.

Chiyang *et al*. (2009) prepared two types of monolithic silica capillary columns with an immobilized cellulose tris(3,5-dimethylphenylcarbamate) (CDMPC) selector for enantiomer separations in CEC. The monolithic columns were prepared by a sol-gel process in fused silica capillaries.

Hironobu *et al*. (2009) prepared newly monolithic wide-pore silica column it used for protein separation. The wide distribution of the pore sizes of monolithic columns was evaluated by mercury porosimetry. This column, as well as the conventional monolithic column, shows high permeability in the chromatographic separation of low-molecular-sized substances.

Minghuo *et al*. (2010) synthesized inorganic-organic hybrid monolithic capillary column was via thermal free radical copolymerization within the confines of a capillary using a polyhedral oligomeric silsesquioxane (POSS) reagent as the inorganic-organic hybrid cross-linker and a synthesized long carbon chain quaternary ammonium methacrylate of N -(2-(methacryloyloxy)ethyl)-dimethyl octadecyl ammonium bromide (MDOAB)as the organic monomer.

Zhenbin *et al*. (2011) prepared Perphenylcarbamoylatedβ-cyclodextrin-silica(Ph-β-CD-silica) hybrid monolithic columns for enantioseparation in capillary liquid

chromatography (CLC) by a “one-pot” approach via the polycondensation of alkoxysilanes and in situ copolymerization of mono (6A-N-allylamino-6A-deoxy)-Ph-β-CD and vinyl group on the precondensed siloxanes.

Zeid A. ALOthman et al. (2011) described the fabrication of long chain alkyl methacrylate monolithic materials for use as stationary phases in capillary liquid chromatography. After capillary inner wall surface activation with 3-(trimethoxysilyl)propyl methacrylate, monoliths were formed by copolymerization of either lauryl or stearyl methacrylate (LMA or SMA) with ethylene dimethacrylate (EDMA) as crosslinker, in the presence of azobisisobutyronitrile (AIBN) as initiator and a mixture of porogenic solvents including water, 1-propanol and 1,4-butanediol.
Huan et al. (2014) prepared tetrazolyl monolithic column through the combination of atom transfer radical polymerization (ATRP) and “click chemistry” technique. In the ATRP fabrication process, vinyl ester resin is used as both the monomer and the cross-linking agent, and cetyl alcohol is used as the porogen, carbon tetrachloride as the initiator and ferrous chloride as the catalyst.

Michał *et al*. (2014) synthesized monolithic polymer beds in fused silica capillaries using either trimethylolpropane trimethacrylate (TrIM) or a mixture of butyl methacrylate (BMa) with ethylene glycol dimethacrylate (EDMa) as monomers.

Hong *et al*. (2016) used a new device to prepare polymer monolith .And it effectively solves volume shrinkage during preparing a polymer monolith In the stainless steel-tube.

ÇELEBI *et al*. (2017) proposed new polyacrylate-based monosized-porous polymer beads were as a stationary phase for the separation of polar compounds by microbore reversed-phase chromatography.

SAID *et al*. (2016) compared monolithic and fused core stationary phases for fast separation of four fat-soluble vitamins.

Yu-Ru *et al*. (2017) determined 7-Aminoflunitrazepam (7-aminoFM2), a major metabolite of flunitrazepam (FM2) in urine samples by polymeric monolith-based capillary liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

1.7.2 Validation method of PAR and CZN

Altun *et al.* (2002) developed HPLC method for the analysis of Paracetamol, Caffeine and Dipyrone using a µ-Bondapack C8 column by isocratic elution with a flow rate of 1.0 ml/min. The mobile phase composition was 0.01 M KH2PO4−methanol-acetonitrile-isopropyl alcohol (420: 20: 30: 30) (v/v/v/v) and spectrophotometric detection was carried out at 215 nm.

Franeta *et al.* (2002) evolved HPLC method for simultaneous determination of acetylsalicylic acid, paracetamol, caffeine and phenobarbital in tablets, using chromatographic system consisting a Bio Rad 18 01 solvent pump, Rheodine 71 25 injector and Bio Rad 18 01 UV/Vis Detector. Separation was achieved using Bio SiL HL C18, 5 mm, 250/4.6 mm column. Mixture of acetonitrile/water (25:75 v/v) adjusted to pH 2.5 with phosphoric acid was used as a mobile phase at a flow rate of 2.0 ml min-1. UV detection was at 207 nm range 0.01 AUFS.

Krishnan *et al.* (2008) developed a simple, accurate, precise and reproducible high performance liquid chromatographic method for the simultaneous estimation of paracetamol, aceclofenac and chlorzoxazone in pharmaceutical dosage forms. A Phenomenex ODS C18 column (250 mm × 4.6 mm i.d., 3-5 mcm particle size) in gradient mode, with mobile phase acetonitrile and orthophosphoric acid (40:60) (v/v), the flow rate was 1 mL/min and effluent was monitored at 275 nm.

Venkatesh *et al*. (2009) evolved a rapid and sensitive high performance liquid chromatography for the quantification of paracetamol, chlorzoxazone and aceclofenac in dosage forms. The chromatographic separation was carried out on a Phenomenex Luna C18 column using a mixture of acetonitrile-0.05M disodium hydrogen orthophosphate (65:35) (pH adjusted to 3.0 using 10% orthophosphoric acid) as mobile phase with UV detection at 271 nm.

In 2010 Ramesh *et al*. developed two methods for simultaneous estimation of paracetamol, domperidone and tramadol HCl in pure and tablet dosage form by using 0.1N NaOH as a solvent. Paracetamol, domperidone and tramadol HCl show absorbance maximums at 256 nm, 289.6 nm and 218.4 nm respectively.

Suryan *et al*. (2011) evolved a simple, precise and rapid isocratic reverse phase high performance liquid chromatographic method for simultaneous determination of paracetamol, chlorzoxazone and diclofenac sodium from tablet dosage form. The chromatographic separation was performed on an inertsil C18 column (250 mm × 4.6 mm i.d 5 µm particle size).

Snehal *et al.* (2012) improved and developed a simple, precise, and accurate reversed-phase liquid chromatographic for the simultaneous determination of paracetamol (PCM), chlorzoxazone (CHZ), and nimesulide (NIM) in pharmaceutical dosage form. The chromatographic separation was achieved on a Thermo Hypersil GOLD C18 column (250 × 4.6mm i.d., 5µm particle size). The mobile phase consisted of water:acetonitrile(55:45v/v).The flow rate was set to1.2mLmin−1 and UVdetection was carried out at 275nm. The retention time (t_R) for PCM, CHZ, and NIM was found to be 2.69 ± 0.02, 4.61 ± 0.01, and 9.55 ± 0.02 min, respectively.

Rupali *et al.* (2012) evolved method for Simultaneous Estimation of Paracetamol, Chlorzoxazone and Ibuprofen by Validated two Spectrophotometric Methods. In this method Shimadzu UV 1700 was used for quantitation. Method I is three wavelength spectroscopy where absorbance of the sample solution was measured at 287.2 nm and 295.2 nm for the determination of paracetamol, chlorzoxazone, and at 221.8 nm for ibuprofen, respectively. Method II is multiwavelength spectroscopy where absorbance of standard solutions was measured at 221.8 nm, 256 nm, 287.2 nm and 295.2 nm and result of the sample solution obtained by statistical calculations.

In 2015 Khandker *et al*. developed a stability-indicating RP-HPLC method for routine analysis of Paracetamol (PARA), Caffeine (CAF) and Ibuprofen (IBU) in their combined solid dosage forms.

Eglal *et al*. (2015) developed two chromatgraphic methods for determination of Paracetamol (PCM) and 23 Pamabrom (PAM) in presence of P-aminophenol (PAP) and Theophylline (THEO) as potential impurities of both drugs respectively.

In 2017 Dnyanda *et al*. developed a validated procedure to quantify the assay of Paracetmol, Ibuprofen and chlorzoxazone tablet using a mobile phase containing mixture of acetonitrile: 0.02M potassium dihydrogen phosphate buffer (60:40) adjusted to PH 3with Orthophosphoric acid, at flow rate 1ml/min in isocratic mode and detector was set at 221nm.

2.0 Materials and methods

2.1 Chemicals

The chemicals used for monolithic materials preparation, in this work, were purchased from Aldrich (Steinheim, Germany) as follows:

- Ethylene dimethacrylate, used as crosslinkers
- 3-(trimethoxysilyl)propyl methacrylate (TMSM)98%
- Hexyl methacrylate (HMA) and glycidyl methacrylate(GMA) all were 98% assay and used as monomers.
- Azo-bis-isobutyronitrile (AIBN) as initiator.
- 1,4-butanediol used as porogenic solvent.

The inner surface of the fused-silica capillary (0.100 mm i.d. × 0.365 mm o.d., purchased from Polymicro Technologies, Phoenix, AZ, USA).

Sodium hydroxide, formic acid, hydrochloric acid, 1-propanol and acetic acid were provided from BDH (Lutterworth, UK).

HPLC grade solvents acetonitrile, acetone, ethanol and hexane were acquired from Fisher Scientific (Leicestershire, UK).

The purified water was obtained using Millipore system (Milli-Q Advantage Elix, Millipore S.A.S. 67120 Molsheim, France), then filtered with 0.2µm nylon membrane filter Whatman (Maidstone, UK). Always before use, the mixed mobile phases were filtered using Millipore vacuum glass filtration system Restek (Bellefonte, USA) through the same nylon membrane filters and degassed ultrasonically for 30min using Lab companion, Jeiotech UC-10 ultrasonic cleaner (Korea).

Working standards of PAR, CZN and excipients were supplied from Blue Nile Pharmaceuticals (Khartoum, Sudan). As a real sample, Relaxon tablets labeled 300 mg PAR and 250 mg CZN were collected from local a market in Riyadh, KSA.

2.2 Instruments

High Performance Liquid Chromatography (HPLC)

Company: Thermo Scientific

Origin: USA

Model: LC- Ultimate 3000 RSLC nanosystem

High Performance Liquid Chromatography

Company: Hitachi

Origin: Japan

Model: Model L-7000

Analytical balance

Company: Dietikon

Origin: Switzerland

Model:360 ES

Ultrasonic bath

Company: Jeiotech

Origin:Japan

Model:UC-10

Vortex Mixer

Company: JELO TECH

Origin: made in Corea

Model: VM-96B

Scanning electron microscope (SEM)

Company: JEOL

Origin: Japan

Model: JSM-6380

2.3 Glassware and apparatus

- 50-ml volumetric flask – Clas -A - Germany.
- 100-ml volumetric flask – Clas -A - Germany.
- 250-ml volumetric flask – Clas -A - Germany.
- 10-ml graduated pipette – Clas -A - Germany.
- Glass funnel – 6 cm diameter – Clas -A - Germany.
- Mortar - porcelain - 80 cm3 volume - Germany.
- Buchner system – quick fit – 1.25L volume - Germany.
- Syringe filter - nylon, 0.22micrometer porous- Germany.
- Nylon membrane filter 0.45micrometer porous – Germany.

2.4 Methods

2.4.1 Preparation of Hexyl Methacrylate Capillary Monolithic Column.

Since the columns was going to be used in HPLC mode, the monolith might to be firmly bounded to the capillary wall, to prevent displacement of the monolithic stationary phase by the mobile phase at high pressure. So that, before polymerization, the inner walls of the fused silica capillaries were modified to improve the adhesion of the monolith to the capillary walls.

In this work the inner wall of the fused-silica capillary (0.10 mm i.d. × 0.365 mm o.d., purchased from 105 Polymicro Technologies, Phoenix, AZ, USA) was activated by flushing with acetone, water, 0.20 mol/L sodium hydroxide, water, 0.20 mol/L hydrochloric acid and ethanol. This step was used to activate the capillaries inner surface and to convert their siloxane groups into silanol ones, as shown in Figure 3.1. The capillary was then flushed for 4 h with a solution of 3-(trimethoxysilyl)propyl methacrylate in ethanol 20% (v/v); the capillary was then rinsed with ethanol and dried with a highly pure nitrogen.

The polymerization mixture was prepared in the following weight percentages: 24% hexyl methacrylate as monomer, 16% ethylene dimethacrylate as cross-linker, 25% 1-propanol with 35% 1,4-butandiol as porogenic solvents and 1% azo-bis-isobutyronitrile as radical initiator. The reaction mixture was mixed into a homogenous solution, then filled inside the activated column and both ends were closed with small pieces of gas chromatography septa. The polymerization was performed in a water bath at 70^{o}C for 20 h, the Schematic representation of preparation procedure of monolithic columns as shown in Figure 3.2. Finally, both seals were removed, and the column was cut to 200 mm length, and washed overnight with acetonitrile at 0.1 µL/min flow rate.

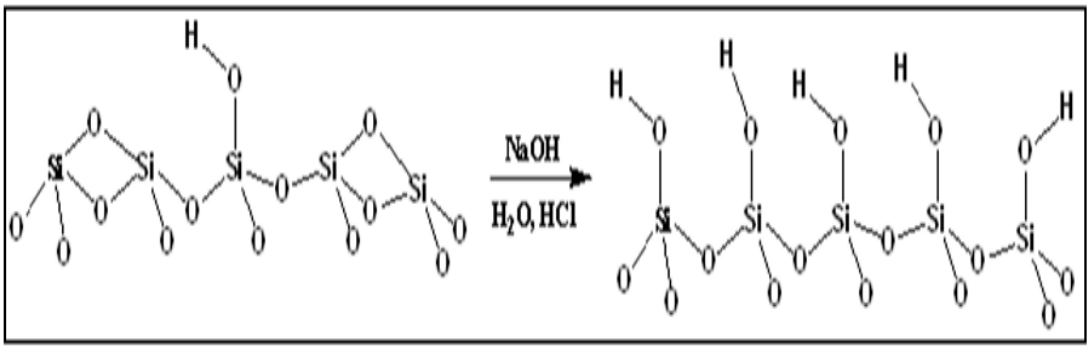


Figure 2.1: Activation of inner wall fused silica.

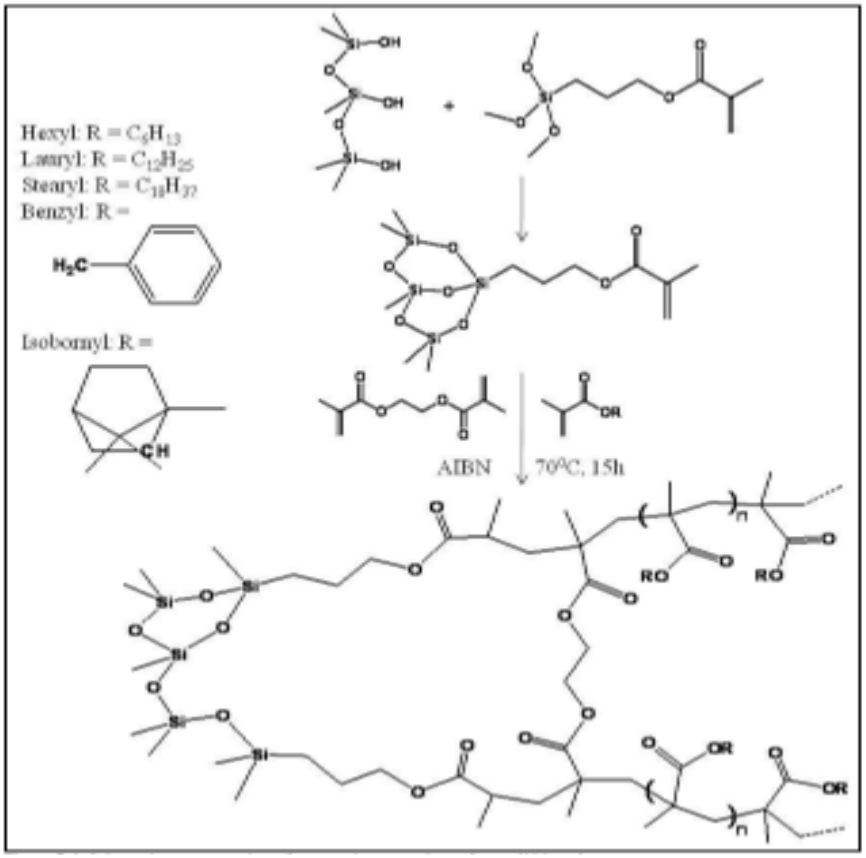


Figure 2.2: Schematic representation of preparation procedure of monolithic columns.

2.4.2 Preparation of Glycidyl Polymethacrylate Stainless Steel Monolithic Column.

Empty stainless steel column (3.2 i.d. x 100 mm length) was purchased from Restek, Bellefonte, PA, USA). The empty column was washed by acetonitrile. 0.02 g of AIBN was weighed in a vial, 600 µL of each 1,4-butanediol and propanol were added to the vial. The mixture was mixed by vortex mixer for 10 min. 480 µL of GMA and 320 µL EDMA were added to the vial. The mixture was mixed and purged by nitrogen gas for 5 min and sonicated in an ultrasonic bath set at 50°C for 10 min. The stainless steel column was filled with the mixture after the removal of both frits and placed in an oven maintained at 70ºC for 24 hours. The unreacted materials were removed by washing the prepared column by acetonitrile for 24 hours at 0.1 mL/min flow rate.

2.5 Characterization

2.5.1 Characterization of Hexyl Methacrylate Capillary Monolithic Column.

2.5.1.1 Hydrodynamic properties and porosity.

The total column porosity (ε_T) is an important parameter for column evaluation. In the literature, various methods are available to measure ε_T, such as the flow method, the conductivity method and the gravimetric method.

In this study, the flow method was used to evaluate ε_T. This method is based on the retention volume determination of an unretained marker and the geometrical volume of the empty column; since the column can be considered as a long cylindrical tube, after correction for extracolumn volume (void volume) contributions, depending on the end frits and connection tubes is used.

And since the column can be considered as a cylindrical tube, as stated before, the volume of the empty column V_c is the volume of the cylinder (Equation 3.1).

$V_c = \pi\,(d/2)^2\,L$ …… Eq. (3.1)

Where: d is the column i.d., L is the column length and $\pi = 3.14$.
The calculation of ε_T in this work was based on the Equation 3.2.

$$\varepsilon_T = \frac{V_0 - V_e}{V_c} = \frac{Ft_0 - V_e}{\pi(d/2)^2 L}$$ **…… Eq. (3.2)**

Where: V_0 is the volume of an unretained marker (pure acetonitrile or uracil were used in this work), V_e is the extravolume due to the fritting and tubes, F is the volumetric flow rate and t_o is the retention time of an unretained marker.

It is not easy to find a compound that is in the same time absolutely not retained on the stationary phase considered, not excluded from its internal pores and conveniently detected. The most common tracers used in RP-HPLC are uracil and thiourea. Both have a slight retention factor on most RP-HPLC phases.

The permeability (K_o) of a porous medium is a measure of its capacity to transmit a fluid driven by an imposed pressure drop across the column. Darcy's law links the solvent viscosity η and column porosity ε_T to K_o, which is calculated using Equation 3.3.

$$K^o = \frac{u\eta L}{\Delta P}$$ ……**Equation 3.3**

In this equation, u is the linear velocity of the mobile phase, η is the dynamic viscosity of the eluent, L is the column length and ΔP is the pressure drop. HPLC -

grade acetonitrile was used in this work. Viscosity value for this eluent was $3.41x10^{-4}$Pa.s. Pressure drops across the column have been evaluated at flow rates ranging from 0.10 to 3.0 µL/min. The permeability value of the prepared column was determined at 24ºC, while acetonitrile eluent was passed through the column at a 500 nL/min volumetric flow rate.

2.5.1.2 Monoliths morphology

Morphology of the monolith is one of the key factors affecting the separation capability of the polymeric monolithic column. To obtain high efficiency, homogeneity and rigidity of the polymer bed are needed. Therefore, it is important to investigate the properties and control the morphology governing parameters during the synthesis of the monoliths.

After all chromatographic experiments had been completed; the columns and monoliths were washed with acetonitrile, then dried with air and cut into pieces with a razor blade.The pore properties and microscopic morphology of the monolith polymers were examined by a Jeol (JSM-6380LA) analytical scanning electron microscope (Japan) at 5kV.

2.5.2 Characterization of Glycidyl Polymethacrylate Stainless Steel Monolithic Column.

2.5.2.1 Hydrodynamic properties and porosity

The permeability and the stability of the stationary phase inside the column were evaluated using both water and acetonitrile. Pressure drops across the column have been evaluated at different flow rates ranging from 0.10 to 2.0 mL/min. The permeability values of the prepared column were determined at 25ºC, while pure acetonitrile and water eluents passed through the column at a volumetric flow rate of 1.0 mL/min.The total porosity value was calculated using uracil as un-retained solute.

2.5.2.2 Monoliths morphology

After all chromatographic experiments had been completed; the columns and monoliths were washed with acetonitrile and dried with air. The pore properties and microscopic morphology of the monolith polymers were examined by a Jeol (JSM-6380LA) analytical scanning electron microscope (Japan) at 5kV.

2.6 Validation of assay method for Paracetamol and Chlorzoxazone using Capillary Monolithic Column

2.6.1 Optimized chromatographic conditions

Chromatographic analysis were carried out using a Thermo Scientific Ultimate 3000 RSLC nanosystem (Waltham, MA, USA), equipped with an electric actuator external

injector fixed with 4.0 nL inner sampling loop (Vici Valco, Houston, TX, USA) and a 3.0 nL Ultimate 3000 variable wavelength detection cell. Chromeleon 7.2 data package was used to control the nanosystem and to acquire the results. Microsoft Office XLSTAT software 2010 package was used for statistical parameters calculation. Simple isocratic elution consisting of aqueous formic acid solution (1% v/v): acetonitrile (40:60) mobile phase was used with flow rate of 1.0 µL/min. 4.0 nL of each standard and sample were injected by external injector and the active ingredients were detected at 270 nm. All analysis was performed at 50^{o}C column temperature.

2.6.2 Standard Stock Solution

PAR (0.300 g) and CZN (0.250 g) were weighed and transferred to the same 100 mL volumetric flask. The flask was partially filled with the same composition of the mobile phase and sonicated for 10 min, cooled to room temperature; then the volume was completed to the mark with the same solvent.

2.6.3 System Suitability

Series dilutions were made from the standard stock solution to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times for system suitability test.

2.6.4 Linearity, LOD and LOQ

The linearity of method for PAR and CZN was tested from 40–160% of the targeted level of the assay concentration for both compounds. All of the standard solutions containing 36–144 µg/mL of PAR and 24–96 µg/mL CZN in each linearity level were injected in triplicate. LOD and LOQ were calculated from the linear regression analysis.

2.6.5 Specificity

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times.

(b) Placebo

A placebo equivalent to average weight of one tablet was transferred to 100-ml volumetric flask. The flask was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature; the volume was completed to the mark with the

same solvent. Subsequent dilutions were made with mobile phase similar to those made for standard preparation.

(c) Sample

A placebo equivalent to that of one tablet's weight was transferred to 100 ml volumetric flask; PAR (0.300 g) and CZN (0.250 g) were weighed accurately and transferred quantitatively to the same flask which was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase to achieve same concentration of the standard.

2.6.6 Accuracy

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times.

(b) Samples

Seven 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 40%, 60%, 80%, 100%, 120%, 140% and 160% tablet's content of both PAR and CZN were each added to different flask. The flasks were half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions with mobile phase similar to those made for standard preparation were made. Each solution was injected three times. The results were collected and subjected to statistical treatments.

2.6.7 Precision

(a) Standard for Precision

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times.

(b) Samples for Precision

Three 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 80%, 100% and 120% tablet's content of both PAR and CZN was added each to a different flask. The flasks were half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same

solvent. Subsequent dilutions were made with mobile phase similar to those made for standard preparation.

In order to demonstrate the suitability of the optimized method using the monolithic prepared column, intra- and interday variability studies were carried out by analysis of three different concentration levels (80%, 100% and 120%) for PAR and CZN. The intraday study was performed using five replicates of the same concentration while as the interday precision was checked by repeating the injections on five consecutive days.

2.6.8 Robustness

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN.This solution was injected six times at each different condition.

(b) Samples

A placebo equivalent to one tablet's weight was transferred to 100-ml volumetric flask, the volume required to prepare 90 µg/mL for PAR and 60 µg/mL for CZN was transferred quantitatively from standard stock solution to the placebo flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature and the volume was completed to the mark with the same solvent. The standard was injected six times and the sample was injected three times at each of the following conditions relative to that of the optimum condition: three degrees more temperature, three degrees less temperature, 5% more organic solvent in mobile phase, 5% less organic solvent in mobile phase, 5% more flow rate of mobile phase, 5% less flow rate of mobile phase, 3nm above the detection wavelength and 3nm below the detection wavelength. The results were collected and subjected to statistical treatments.

2.6.9 Assay of real samples

(a) Standard preparation

Subsequent dilutions from the standard stock solution were made with mobile phase to give the concentration of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times.

(b) Assay preparation

Twenty tablets were weighed, transferred to a mortar and grinded. Average weight of tablet was transferred to 100-ml volumetric flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature and the volume was

completed to the mark with the same solvent, Subsequent dilutions were made with mobile phase similar to those made for standard preparation to achieve target concentration. The resulting solutions were filtered through a 0.45 µm nylon membrane filters. The recovered concentration was calculated by comparing the analyte response of the sample with that of the standard.

2.7 Validation of assay method for Paracetamol and Chlorzoxazone using stainless steel Monolithic Column

2.7.1 Optimized chromatographic conditions

Chromatographic analysis were carried out using a Hitachi HPLC (Japan), supported by equipped with UV-Vis detector and external injector with a fixed 5.0 µL loop. Microsoft Office Excel software 2016 package was used for statistical parameters calculation. Simple isocratic elution consisting of aqueous formic acid solution (1% v/v): acetonitrile (65:35) mobile phase was used with flow rate of 0.7 mL/min. 5.0 µL of each standard and sample were injected by external injector and both active ingredients were detected at 270 nm. All analysis was performed at 25oC column temperature.

2.7.2 Standard Stock Solution

PAR (0.300 g) and CZN (0.250 g) were weighed and transferred to the same 100 mL volumetric flask. The flask was partially filled with the same composition of the mobile phase and sonicated for 10 min, cooled to room temperature; then the volume was completed to the mark with the same solvent.

2.7.3 System Suitability

Series dilutions were made from the standard stock solution to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times for system suitability test.

2.7.4 Linearity, LOD and LOQ

The linearity of method for PAR and CZN was tested from 40–160% of the targeted level of the assay concentration for both compounds. All of the standard solutions containing 36–144 µg/mL of PAR and 30–120 µg/mL CZN in each linearity level were injected in triplicate. LOD and LOQ were calculated from the linear regression analysis.

2.7.5 Specificity

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times.

(b) Placebo

A placebo equivalent to average weight of one tablet was transferred to 100-ml volumetric flask. The flask was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature; the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for standard preparation.

(c) Sample

A placebo equivalent to that of one tablet's weight was transferred to 100 ml volumetric flask; PAR (0.300 g) and CZN (0.250 g) were weighed accurately and transferred quantitatively to the same flask which was half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature, and the volume was completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase to achieve same concentration of the standard.

2.7.6 Accuracy

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times.

(b) Samples

Seven 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 40%, 60%, 80%, 100%, 120%, 140% and 160% tablet's content of both PAR and CZN were each added to different flask. The flasks were half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions with mobile phase similar to those made for standard preparation were made. Each solution was injected three times. The results were collected and subjected to statistical treatments.

2.7.7 Precision

(a) Standard for precision

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times.

(b) Samples for precision

Three 100-ml volumetric flasks were labeled; a placebo equivalent to tablet's weight was transferred to each flask. A volume of standard stock solution required to produce 80%, 100% and 120% tablet's content of both PAR and CZN was added each to a different flask. The flasks were half filled with mobile phase, sonicated for 10 minutes, cooled to room temperature and completed to the mark with the same solvent. Subsequent dilutions were made with mobile phase similar to those made for standard preparation.

In order to demonstrate the suitability of the optimized method using the monolithic prepared column, intra- and interday variability studies were carried out by analysis of three different concentration levels (80%, 100% and 120%) for PAR and CZN. The intraday study was performed using five replicates of the same concentration while as the interday precision was checked by repeating the injections on five consecutive days.

2.7.8 Robustness

(a) Standard

Subsequent dilutions were made from the standard stock solution with mobile phase to give the concentrations of 90 µg/mL for PAR and 60 µg/mL for CZN.This solution was injected six times at each different condition.

(b) Samples

A placebo equivalent to one tablet's weight was transferred to 100-ml volumetric flask, the volume required to prepare 90 µg/mL for PAR and 60 µg/mL for CZN was transferred quantitatively from standard stock solution to the placebo flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature and the volume was completed to the mark with the same solvent. The standard was injected six times and the sample was injected three times at each of the following conditions relative to that of the optimum condition: three degrees more temperature, three degrees less temperature, 5% more organic solvent in mobile phase, 5% less organic solvent in mobile phase, 5% more flow rate of mobile phase, 5% less flow rate of mobile phase, 3nm above the detection wavelength and 3nm

below the detection wavelength. The results were collected and subjected to statistical treatments.

2.7.9 Assay of comerical samples

(a) Standard preparation

Subsequent dilutions from the standard stock solution were made with mobile phase to give the concentration of 90 µg/mL for PAR and 60 µg/mL for CZN. This solution was injected six times.

(b) Assay preparation

Twenty tablets were weighed, transferred to a mortar and grinded. Average weight of tablet was transferred to 100-ml volumetric flask which was half filled with mobile phase and sonicated for 10 minutes, cooled to room temperature and the volume was completed to the mark with the same solvent, Subsequent dilutions were made with mobile phase similar to those made for standard preparation to achieve target concentration. The resulting solutions were filtered through a 0.45 µm nylon membrane filters. The recovered concentration was calculated by comparing the analyte response of the sample with that of the standard.

3.0 Results, discussion and conclusion

3.1Characterization of the capillary monolithic column

HPLC-grade acetonitrile was used to check the mechanical stability and permeability of the prepared column. Pressure drops across the column have been evaluated at flow rates ranging from 0.10 to 3.0 µL/min as shown in Table 3.1. The column shows perfect mechanical stability and permeability over the investigated flow range with regression factor R^2 0.9994 as shown in Fig 31. The permeability value of the prepared column was determined at 24^{o}C, while acetonitrile eluent passed through the column at a 500 nL/min volumetric flow rate. The prepared column permeability value was 5.44×10^{-14} m^2 corresponding to the measured pressure drop of 189 psi (13 bar). The total porosity value is 0.79; it was calculated using uracil as un-retained solute.

Table: 3.1 Acetonitrile flow rate (µL/min) Vs Pressure drops across the capillary monolith column

Flow rate (µL/min)	ΔP ACN (psi)
0.1	105
0.2	127
0.3	142
0.4	165
0.5	**189**
0.6	208
0.7	226
0.8	247
0.9	266
1	281
1.5	378
2	474
3	656

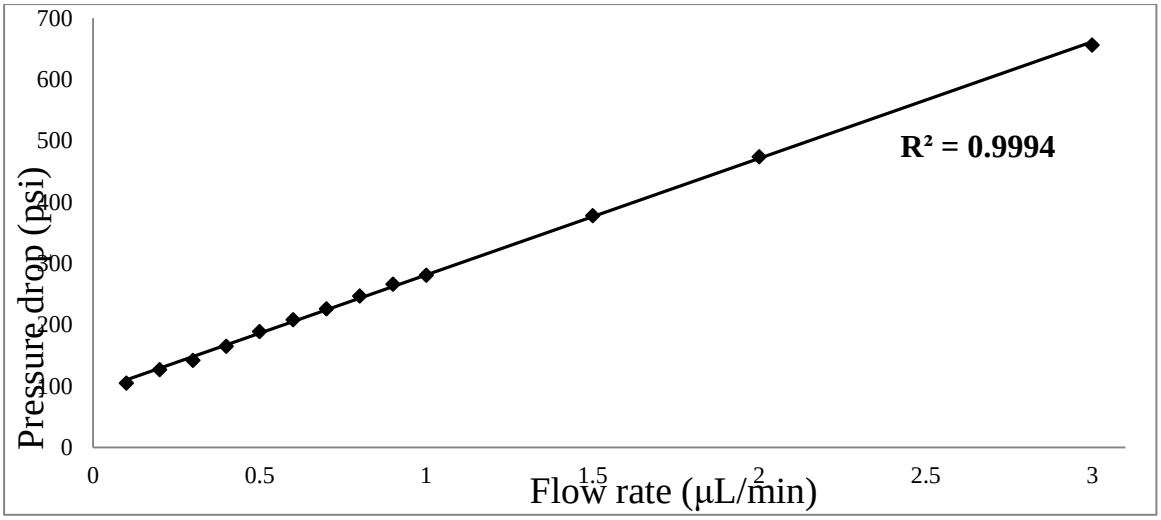


Figure 3.1 Mechanical stability plots of the prepared column, backpressure as a function of acetonitrile flow rates

Fig. 3.2 (A and B) illustrates the SEM micrographs of bulk region of the hexyl methacrylate-co-ethylene dimethacrylate. The SEM images show that the morphology of the synthesized monolith was permeable with a homogenous structure. The approximate diameter of the continuous monolith microglobules that appear in the figures ranged from 1–2 µm.

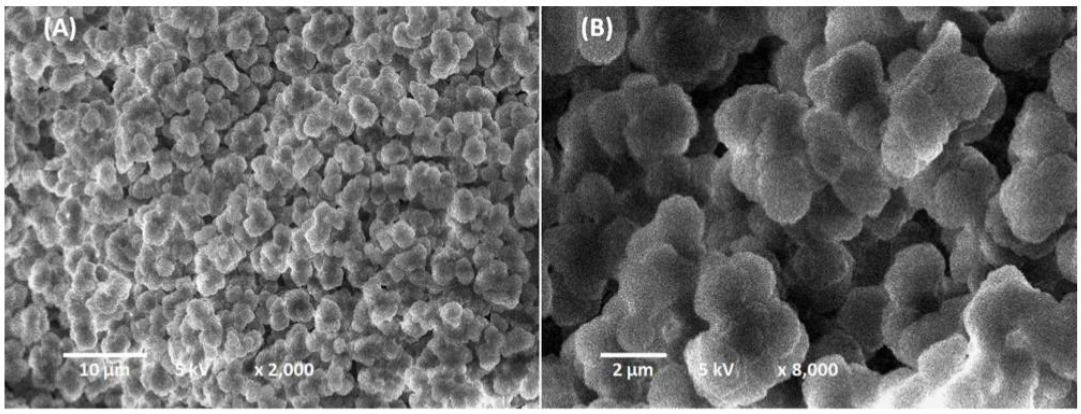


Figure3.2 SEM images of the synthesized monolith bulk region at (A) x 2,000 and (B) x 8,000 603 magnification powers.

3.2Characterization of the stainless steel monolithic column

The permeability and the stability of the stationary phase inside the column were evaluated using both water and acetonitrile. Pressure drops across the column have been evaluated at different flow rates ranging from 0.10 to 2.0 mL/min as shown in Table 3.2. The column shows stable permeability and perfect mechanical stability

over the investigated flow range with regression factor (R^2) of 0.9999 and 0.9997 for water and acetonitrile, respectively. The permeability values of the prepared column were determined at 25°C, while pure acetonitrile and water eluents passed through the column at a volumetric flow rate of 1.0 mL/min. The permeability values of the prepared column were 2.71×10^{-12} and 2.36×10^{-12} m^2 corresponding to the measured pressure drop of 435 psi (30 bar) and 993 psi (68.5 bar) for acetonitrile and water, respectively. Fig. 3.3 shows a directly proportional relationship between acetonitrile flow rate and columns backpressure at 25°C. The total porosity value was found to be 0.78; it was calculated using uracil as un-retained solute which is in accordance with the results obtained from SEM images Fig. 3.4.

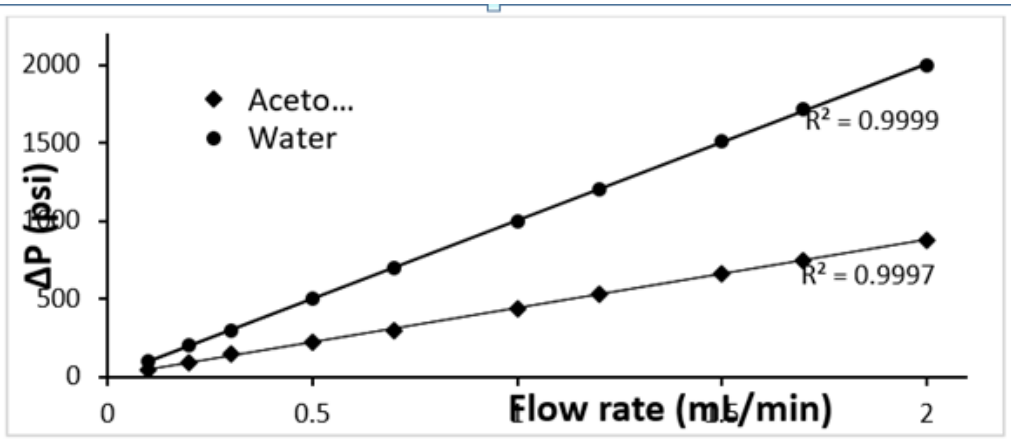


Figure 3.3 Mechanical stability plots of the prepared column, backpressure as a function of acetonitrile and water flow rates

Table: 3.2 acetonitrile and water flow rate (µL/min) Vs Pressure drops across the stainless steel monolith column

Flow rate (µL/min)	ΔP ACN (psi)	ΔP H_2O (psi)
0.1	48	99
0.2	93	201
0.3	143	299
0.5	219	507
0.7	301	700
1	435	993
1.2	531	1206
1.5	658	1515
1.7	747	1718
2.0	880	1998

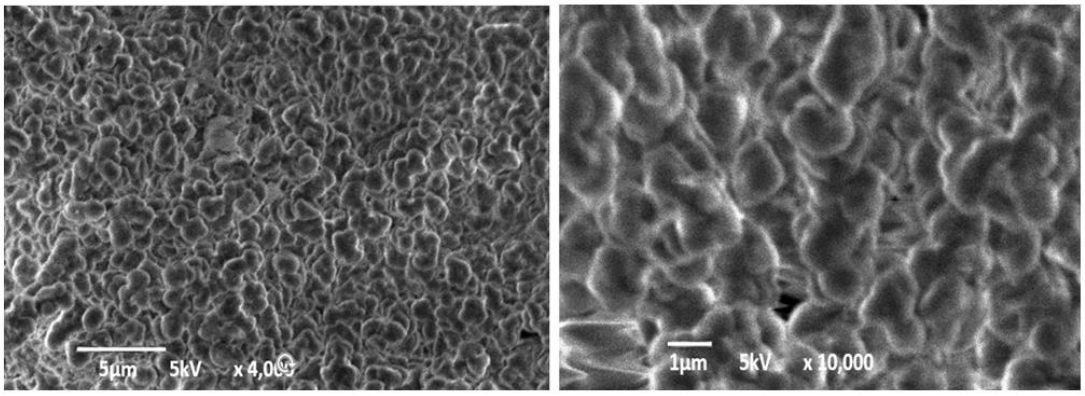


Figure3.4 SEM images of bulk region glycidyl polymethacrylate monolith column.

3.3 Validation results when using Capillary Monolithic Column

3.3.1 System Suitability

Example chromatogram for system suitability method showed in Appendix. A. System suitability results for paracetamol and Chlorzoxazone are shown in Table 3.3 and Table 3.4 respectively.

Table 3.3 System suitability results for paracetamol

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	1.0494	1.75	2.35	18958
2	1.0452	1.81	2.42	18654
3	1.0482	1.81	2.35	18594
4	1.0409	1.83	2.39	18772
5	1.0468	1.81	2.37	18954
6	1.0451	1.82	2.35	18746
Avg	1.0459	1.805	2.37167	18780
STDEV	0.003	0.028	0.02858	150.7656
RSD	0.2851			

Table 3.4 System suitability results for Chlorzoxazone

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5446	1.2	2.35	6437
2	0.5369	1.12	2.42	6529
3	0.5378	1.18	2.35	6382
4	0.5394	1.17	2.39	6473
5	0.5437	1.16	2.37	6360
6	0.5479	1.18	2.35	6433
Avg	0.5417	1.168	2.37167	6436
STDEV	0.0043			
RSD	0.8021			

3.3.2 Linearity, LOD and LOQ

i) Paracetamol

An example of chromatogram for linearity method is shown in Appendix. B. Table 3.5 shows linearity results for Paracetamol which then treated by XL 2010 program to predict linearity data that shown in Figure 3.5

Table 3.5 Linearity results for paracetamol

Conc µg/ml	**36 (40%)**	**54 (60%)**	**72 (80%)**	**90 (100%)**	**108 (120%)**	**126 (140%)**	**144 (160%)**
1	0.4247	0.6506	0.8492	1.0449	1.2377	1.4477	1.6671
2	0.422	0.6295	0.8386	1.0393	1.2342	1.4481	1.6734
3	0.4232	0.6193	0.8321	1.0327	1.2301	1.4492	1.6652
Avg	**0.4233**	**0.6331**	**0.8399**	**1.03896**	**1.234**	**1.4483**	**1.66856**

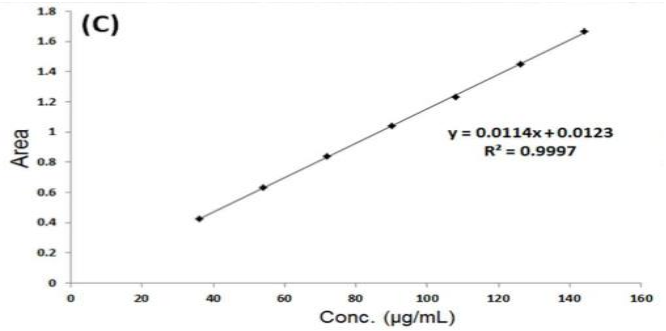


Figure 3.5 XL 2010 Graph of conc. in µg/ml Vs average area of paracetamol

Figure 3.5 shows the plot of average area versus concentrations for paracetamol in µg/ml, the linear regression equation:

Area = 0.0114*µg/ml + 0.0123

According to ICH guidelines, acceptance criteria are $R^2 \geq 0.997$.

Limit of detection and limit of quantitation

LOD= RMSE*3/slope

= 0.008*3/0.0114

LOD = 2.1053 µg/ml

% LOD =2.1053*100/90 = 2.34%

LOQ = RMSE *10 /slope

LOQ =0.008*10/0.0114

LOQ = 7.0175 µg/ml

% LOQ =7.0175*100/90 = 7.8 %

ii) Chlorzoxazone

Table 3.6 shows linearity results for Chlorzoxazone which then treated by XL 2010 program to predict linearity data that shown in Figure 3.6

Table 3.6 Linearity results for chlorzoxazone

Conc µg/ml	30(40%)	40(60%)	50(80%)	60(100%)	70(120%)	80(140%)	90(160%)
1	0.2134	0.3224	0.4362	0.547	0.6498	0.7739	0.8577
2	0.2122	0.3321	0.4366	0.5497	0.6646	0.7591	0.8978
3	0.2154	0.3255	0.432	0.5494	0.6574	0.7381	0.904
Avg	0.213667	0.326667	0.43493333	0.5487	0.65726667	0.757033333	0.8865

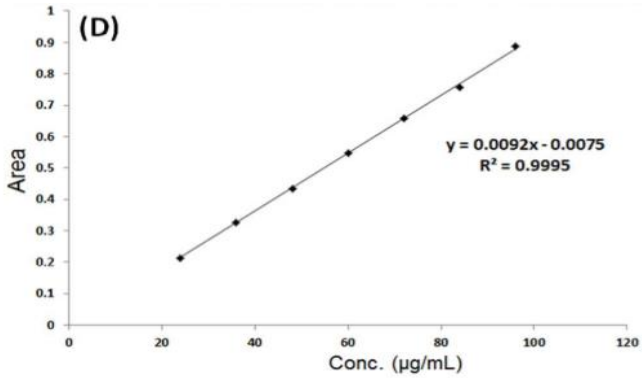


Figure 3.6 XL 2010 Graph of conc. in µg/ml Vs average area of Chlorzoxazone

Figure 3.6 shows the plot of average area versus concentrations for Chlorzoxazone in µg/ml, the linear regression equation:

Area = -0.0075+0.0092*µg/ml

According to ICH guidelines, acceptance criteria are $R^2 \geq 0.997$.

Limit of detection and limit of quantitation

LOD= RMSE*3/slope = 0.006*3/0.0092

LOD = 1.9565 µg/ml

% LOD = 1.9565 *100/60 = 3.2608%

LOQ = RMSE *10 /slope = 0.006*10/0.0092

LOQ = 6.5217 µg/ml

% LOQ =6.5217 *100/60 = 10.8695 %

3.3.3 Specificity

Figure 3.7 Figure 3.8 and Figure 3.9 show the specificity chromatograms for placebo, sample and standard, respectively, for paracetamol and chlorozxazone.

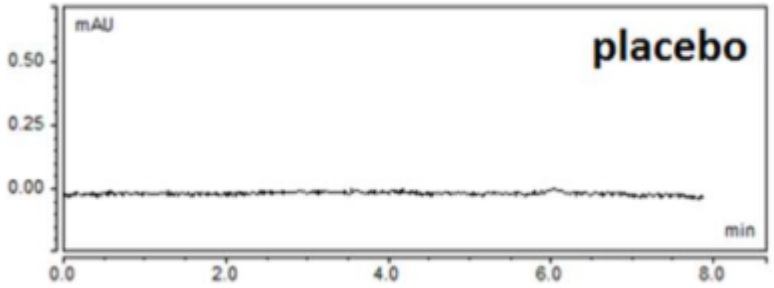


Figure 3.7 Specificity chromatogram for the Placebo of paracetamol and chlorozxazone

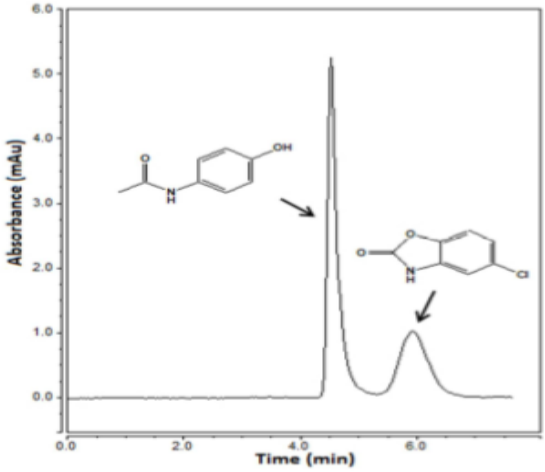


Figure3.8 Specificity chromatogram for the sample of paracetamol and chlorozxazone

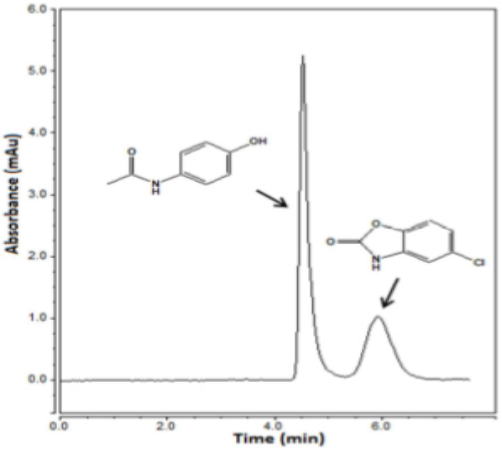


Figure 3.9 Specificity chromatogram for mixed standard of paracetamol and chlorozxazone

3. 3.4 Accuracy

An example of chromatogram for accuracy method is shown in Appendix. C.Table 3.7 shows the results of mixed standard of paracetamol and chlorozxazone , while the accuracy results for paracetamol and chlorozxazone samples were shown in Table 3.8 and Table 3.9, respectively; summary of accuracy results for both components is shown in Table 3.10.

Table 3.7 Accuracy results for paracetamol and chlorozxazone standards

No.	PAR	CZN
STD1	1.0494	0.5446
SDT2	1.0452	0.5369
STD3	1.0482	0.5378
STD4	1.0409	0.5394
STD5	1.0468	0.5437
STD6	1.0451	0.5479
Avg	1.04593333	0.54171667
STDEV	0.00298239	0.00434484
RSD	0.28514177	0.80205095

Table 3.8 Accuracy results for paractamol

Content	40	60	80	100	120	140	160
1	0.4247	0.6506	0.8492	1.0449	1.2377	1.4477	1.6671
2	0.422	0.6295	0.8386	1.0393	1.2342	1.4481	1.6734
3	0.4256	0.6193	0.8321	1.0327	1.2301	1.4492	1.6652
Avg	0.4241	0.6331	0.8399	1.0389	1.234	1.4483	1.6685
RECOVERY	40.5798	60.5328	80.3078	99.3339	117.9808	138.4728	159.5289
RECOVERY%	101.4496	100.8881	100.3848	99.3339	98.3172	98.9091	99.7056

Table 3.9 Accuracy results for chlorzxazone

Content	40	60	80	100	120	140	160
1	0.2134	0.3224	0.4362	0.547	0.6498	0.7739	0.8577
2	0.2122	0.3321	0.4366	0.5497	0.6646	0.7591	0.8978
3	0.2154	0.3255	0.432	0.5494	0.6574	0.7381	0.904
Avg	0.21366667	0.326666667	0.43493333	0.5487	0.65726667	0.75703333	0.8865
RECOVERY	39.442513	60.30212596	80.2879734	101.289112	121.330339	139.7471	163.646433
RECOVERY%	98.6062825	100.5035433	100.359967	101.289112	101.108616	99.8193573	102.27902

Table 3.10 Summary of accuracy results for paracetamol and chlorzxazone

Content%	RECOVERY %	
	PAR	CZN
40	101.449622	98.6062825
60	100.888096	100.5035433
80	100.384824	100.3599668
100	99.3339282	101.2891118
120	98.3172924	101.1086156
140	98.9091538	99.81935733
160	99.7056058	102.2790204
Avg	98.6132231	100.4639865
STDEV	0.418509245	0.911643273
RSD	0.424394652	0.907432907

3.3.5 Precision

i) Intraday Precision

An example of chromatogram for precision method is shown in Appendix D.Table 3.11 shows intraday precision results for paracetamol and chlorzoxazone mixed standard.

Table 3.11 intraday precision results for paracetamol and chlorzoxazone mixed standard.

Standards	PARA	CHLORO
No.	**Area**	Area
1	1.0723	0.563
2	1.0401	0.5412
3	1.0313	0.5598
4	1.0456	0.5436
5	1.0398	0.5454
6	1.0404	0.5648
Avg	1.044916667	0.552966667
STDEV	0.014181032	0.01068488
RSD	1.357144774	1.932282889

Tables numbered 3.12, 3.13 and 3.14 show intraday precision results for 80%, 100% and 120% of paracetamol, respectively, while tables numbered 3.15, 3.16 and 3.17 show intraday precision results for 80%, 100% and 120% of chlorozoxazone respectively. Table 3.18 show the summary of the previous six tables as well as the average and RSD of each five assays of the three concentrations of each active ingredient.

Table 3.12 Intraday precision results for 80% paracetamol

No.	**1st**	**2nd**	**3rd**	**4th**	**5th**
1st trial	0.848	0.8337	0.8335	0.8569	0.859
2nd trial	0.8232	0.8385	0.8237	0.851	0.853
3rd trial	0.8208	0.8347	0.8208	0.8504	0.8491
AVG	0.830666667	0.8356333	0.8208	0.8527667	0.8537
STDEV	0.015058995	0.0025325	0.0066551	0.0035921	0.00498698
RSD	1.81288063	0.3030583	0.8108033	0.4212314	0.58416107
RECOVERY	79.49597257	79.97129	78.551719	81.610974	81.7002951
RECOVERY %	99.36996571	99.964112	98.189648	102.01372	102.125369

Table 3.13 Intraday precision results for 100% paracetamol

No.	**1st**	**2nd**	**3rd**	**4th**	**5th**
1st trial	1.0466	1.0615	1.0524	1.0827	1.0833
2nd trial	1.0506	1.0708	1.0535	1.073	1.0861
3rd trial	1.0425	1.0528	1.0492	1.0708	1.0796
AVG	1.046566667	1.0617	1.0517	1.0755	1.083
STDEV	0.004050103	0.0090017	0.0022338	0.0063317	0.00326037
RSD	0.386989478	0.8478541	0.2124019	0.5887184	0.30104968
RECOVERY	100.1579073	101.60619	100.64917	102.92687	103.644629
RECOVERY %	100.1579073	101.60619	100.64917	102.92687	103.644629

Table 3.14 Intraday precision results for 120% paracetamol

No.	1st	2nd	3rd	4th	5th
1st trial	1.2367	1.2412	1.2926	1.2731	1.276
2nd trial	1.2316	1.2266	1.2698	1.2835	1.2709
3rd trial	1.2392	1.2344	1.2624	1.296	1.2782
AVG	1.235833333	1.2340667	1.2749333	1.2842	1.27503333
STDEV	0.003873414	0.0073057	0.0157408	0.011466	0.00374477
RSD	0.313425245	0.5920025	1.2346385	0.8928544	0.2937001
RECOVERY	118.2709945	118.10192	122.01292	122.89975	122.02249
RECOVERY %	98.55916208	98.418268	101.67743	102.41646	101.685408

Table 3.15 Intraday precision results for 80% chlorozoxazone

No.	1st	2nd	3rd	4th	5th
1st trial	0.4683	0.4458	0.4596	0.4519	0.4463
2nd trial	0.4547	0.4436	0.4497	0.4546	0.4442
3rd trial	0.4519	0.4478	0.4423	0.454	0.4363
AVG	0.4583	0.445733333	0.4505333	0.4535	0.4422667
STDEV	0.0087727	0.002100794	0.0086801	0.001417745	0.0052729
RSD	1.9141796	0.471311734	1.9266174	0.312622864	1.1922415
RECOVERY	82.880222	80.60763156	81.475677	82.01217674	79.98071
RECOVERY %	103.60028	100.7595395	101.8446	102.5152209	99.975888

Table 3.16 Intraday precision results for 100% chlorozoxazone

No.	1st	2nd	3rd	4th	5th
1st trial	0.5488	0.5578	0.5585	0.5545	0.5599
2nd trial	0.5592	0.5598	0.5536	0.5566	0.5502
3rd trial	0.558	0.5556	0.5572	0.5538	0.5449
AVG	0.555333	0.55773333	0.556433	0.55496667	0.551667
STDEV	0.0056898	0.002100794	0.0025384	0.001457166	0.0076068
RSD	1.0245661	0.376666298	0.4561862	0.262568238	1.3788753
RECOVERY	100.42799	100.862017	100.62692	100.3616855	99.764904
RECOVERY %	100.42799	100.862017	100.62692	100.3616855	99.764904

Table 3.17 Intraday precision results for 120% chlorozoxazone

No.	1st	2nd	3rd	4th	5th
1st trial	0.6689	0.6625	0.6787	0.6675	0.6641
2nd trial	0.6596	0.6576	0.6756	0.673	0.6573
3rd trial	0.6604	0.6666	0.6723	0.6725	0.675
AVG	0.6629667	0.662233333	0.6755333	0.671	0.6654667
STDEV	0.005154	0.004505922	0.0032005	0.003041381	0.0089288
RSD	0.7774091	0.680413051	0.4737769	0.453260993	1.341734
RECOVERY	119.8927	119.760082	122.16529	121.3454699	120.34481
RECOVERY %	99.910583	99.80006832	101.80441	101.1212249	100.28734

Table 3.18 Summery of intraday precession results for paracetamol and chlorozoxazone

	PAR			CZN		
	80%	100%	120%	80%	100%	120%
1st trial	99.36996571	100.1579073	98.55916208	103.6002773	100.4279945	99.91058332
2nd trial	99.96411197	101.6061887	98.41826834	100.7595395	100.862017	99.80006832
3rd trial	98.1896483	100.6491746	101.6774331	101.8445958	100.6269215	101.8044085
4th trial	102.0137172	102.9268682	102.4164606	102.5152209	100.3616855	101.1212249
5th trial	102.1253688	103.6446288	101.6854082	99.97588764	99.76490445	100.287339
AVG	100.3325624	101.7969535	100.5513465	101.7391042	100.4087046	100.5847248
STDEV	1.709887818	1.47740237	1.907330522	1.427053843	0.409320245	0.856453683
RSD	1.704220222	1.451322774	1.896872184	1.402660121	0.407654144	0.851474898

ii) Interday Precision

Table 3.19 shows results of interday precision test for mixture of paracetamol and chlorozoxazone standards.

Table 3.19 Interday precision test for mixture of paracetamol and chlorozoxazone standards.

	PAR			CZN		
	Day1	Day2	Day3	Day1	Day2	Day3
STD1	1.0723	1.1099	1.1262	0.563	0.5958	0.5935
STD2	1.0401	1.0961	1.1108	0.5412	0.5832	0.5819
STD3	1.0313	1.1023	1.1075	0.5598	0.5861	0.575
STD4	1.0456	1.0965	1.1114	0.5436	0.5801	0.5981
STD5	1.0398	1.1097	1.1004	0.5454	0.5829	0.584
STD6	1.0404	1.0929	1.1096	0.5648	0.5872	0.5989
AVG	1.044916667	1.101233333	1.110983333	0.552966667	0.585883333	0.588566667
STDEV	0.014181032	0.007295661	0.008459413	0.01068488	0.005471167	0.009709308
RSD	1.357144774	0.66249909	0.761434726	1.932282889	0.933832136	1.649653098

Tables numbered 3.20, 3.21and 3.22 show interday precision for 80%, 100% and 120% for each component, respectively. Table 3.23 shows the summary of interday precision results, the average and RSD of each three assays of the three concentrations for each active ingredient.

Table 3.20 Interday precision results for 80% of paracetamol and chlorozoxazone

	PAR			CZN		
	Day1	Day2	Day3	Day1	Day2	Day3
1st trial	0.8337	0.8693	0.8751	0.4458	0.4689	0.4764
2nd trial	0.8385	0.8639	0.8986	0.4436	0.4663	0.4709
3rd trial	0.8347	0.8758	0.8981	0.4478	0.4564	0.4759
AVG	0.835633333	0.869666667	0.8906	0.445733333	0.463866667	0.4744
STDEV	0.002532456	0.005958467	0.013425722	0.002100794	0.006595706	0.003041381
RSD	0.303058278	0.685143813	1.507491756	0.471311734	1.421896883	0.641100604
Recovery	79.97128958	78.97206163	80.16321877	80.60763156	79.17389696	80.60259387
Recovery %	99.96411197	98.71507703	100.2040235	100.7595395	98.96737121	100.7532423

Table 3.21 Interday precision results for 100% of paracetamol and chlorozoxazone

	PAR			CZN		
	Day1	Day2	Day3	Day1	Day2	Day3
1st trial	1.0524	1.1007	1.0962	0.5585	0.582	0.583
2nd trial	1.0535	1.0965	1.0957	0.5536	0.5896	0.5935
3rd trial	1.0492	1.0971	1.1129	0.5572	0.5917	0.5981
AVG	1.0517	1.0981	1.1016	0.556433333	0.587766667	0.591533333
STDEV	0.002233831	0.002271563	0.00978928	0.002538372	0.005103267	0.007739724
RSD	0.212401901	0.206863067	0.888641962	0.456186218	0.868247081	1.308417283
Recovery	100.6491746	99.71547053	99.15540287	100.6269215	100.3214519	100.5040494
Recovery %	100.6491746	99.71547053	99.15540287	100.6269215	100.3214519	100.5040494

Table 3.22 Interday precision results for 120% of paracetamol and chlorozoxazone

	PAR			CZN		
	Day1	Day2	Day3	Day1	Day2	Day3
1st trial	1.2926	1.3045	1.3437	0.6787	0.696	0.711
2nd trial	1.2698	1.3025	1.34	0.6756	0.713	0.7059
3rd trial	1.2624	1.3126	1.3315	0.6723	0.7078	0.7064
AVG	1.274933333	1.306533333	1.3384	0.675533333	0.7056	0.707766667
STDEV	0.015740817	0.005348208	0.006255398	0.003200521	0.008710913	0.002811287
RSD	1.234638472	0.409343435	0.467378786	0.473776886	1.234539781	0.397205309
Recovery	122.0129197	118.6427339	120.469854	122.1652903	120.4335334	120.252591
Recovery%	101.6774331	98.86894492	100.391545	101.8044085	100.3612778	100.2104925

Table 3.23 Interday precision results summery for each paracetamol and chlorozoxazone

	PAR			CZN		
	80%	100%	120%	80%	100%	120%
1st trial	99.96411197	100.6491746	101.6774331	100.7595395	100.6269215	101.8044085
2nd trial	98.71507703	99.71547053	98.86894492	98.96737121	100.3214519	100.3612778
3rd trial	100.2040235	99.15540287	100.391545	100.7532423	100.5040494	100.2104925
AVG	99.62773749	99.84001599	100.312641	100.160051	100.4841409	100.7920596
STDEV	0.799438059	0.754633788	1.405905691	1.032895799	0.153704802	0.879955555
RSD	0.802425187	0.755843016	1.401523953	1.031245281	0.15296424	0.873040554

3.3.6 Robustness:

The method was examined for robustness test under nine different conditions and its output under each condition is compared with that of the optimized conditions and with permissible limits according to ICH. Lastly the variation in method output is evaluated through calculation of RSD of the nine results obtained under the following different nine conditions. An example of chromatogram for robustness method is shown in Appendix. E.

i) Robustness at Optimized conditions

Standard solution was injected six times while sample solution was injected three times under optimized conditions. Results of paracetamol and chlorozoxazone standards are shown in Table 3.24 and 3.25, respectively; robustness results for both components standards shown in Table 3.26.

Table 3.24 Robustness results for paracetamol standards at optimum conditions

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	1.0257	1.89	2.36	3565
2	1.0303	1.93	2.35	20976
3	1.0286	1.91	2.3	58442
4	1.0249	1.97	2.3	95317
5	1.0267	1.93	2.3	154809
6	1.0313	1.94	2.31	212388
Avg	1.0279167	1.92833	2.32	90916.16667
STDEV	0.0025725	0.02714	0.02757	80540.04285
RSD	0.2502618			

Table 3.25 Robustness results for chlorozoxazone standards at optimum conditions

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5446	1.2	2.36	16043
2	0.5369	1.12	2.35	28316
3	0.5378	1.18	2.3	40948
4	0.5394	1.17	2.3	54403
5	0.5437	1.16	2.3	71132
6	0.5479	1.18	2.31	92962
Avg	0.541716667	1.168333333	2.32	50634
STDEV	0.004344844	0.027141604	0.02757	28335.42641
RSD	0.802050953			

Table 3.26 Robustness results for combined paracetamol and chlorozoxazone sample at optimum conditions

	PAR	CZN
1st trial	1.0449	0.547
2nd trial	1.0393	0.5497
3rd trial	1.0327	0.5494
Avg	1.038966667	0.5487
STDEV	0.006106827	0.001479865
Recovery	0.587778894	0.26970382
Recovery%	99.33392823	101.2891118

ii) Robustness at increased 3ºC temperature

Standard solution was injected six times while sample solution was injected three times after the column temperature had been raised up three degrees celsius, Results of paracetamol and chlorozoxazone standards are shown in Table 3.27 and 3.28, respectively; results of samples for both components are shown in Table 4.29.

Table 3.27 Robustness results for paracetamol standard at increased temperature

Injection No.	**Area**	**Asymmetry**	**Resolution**	**Theoretical plates**
1	1.0974	2.2	2.52	2994
2	1.0902	1.84	2.44	23032
3	1.0916	2.19	2.45	54938
4	1.0952	1.84	2.46	102873
5	1.0862	2.04	2.47	169486
6	1.0947	1.79	2.49	909958
Avg	1.09255	1.98333	2.47167	210546.8333
STDEV	0.0040476	0.18511	0.02927	347824.5899
RSD	0.370472			

Table 3.28 Robustness results for clorozoxzazone standard at increased temperature

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5754	1.18	2.35	7110
2	0.5751	1.25	2.42	28316
3	0.5772	1.24	2.45	40948
4	0.5729	1.18	2.46	54403
5	0.5798	1.21	2.47	71132
6	0.5724	1.18	2.49	92962
Avg	0.5754667	1.20667	2.385	49145.1667
STDEV	0.0027537	0.03204	0.0495	30656.10379
RSD	0.4785098			

Table 3.29 Robustness results for combined paracetamol and chlorozoxazone sample at increased temperature

	PAR	CZN
1st trial	1.0815	0.5742
2nd trial	1.0868	0.5789
3rd trial	1.0879	0.5752
Avg	1.0854	0.57705
STDEV	0.003421988	0.002616295
Recovery	0.315274344	0.453391403
Recovery%	99.34556771	100.275139

iii) Robustness at decreased 3ºC temperature

Standard solution was injected six times while sample solution was injected three times after the column temperature had been decreased three celsius degrees . Results of paracetamol and chlorozoxazone standards are shown in Table 3.30 and 3.31, respectively; results of samples for both components are shown in Table 3.32.

Table 3.30 Robustness results for paracetamol standard at decreased temperature

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	1.0915	2.06	2.4	3435
2	1.0906	1.85	2.41	23775
3	1.0865	1.92	2.41	63080
4	1.0952	1.89	2.43	120268
5	1.0886	2.22	2.39	195240
6	1.0895	1.99	2.39	288454
Avg	1.0903167	1.98833	2.405	115708.6667
STDEV	0.0029499	0.13586	0.01517	109557.2202
RSD	0.2705506			

Table 3.31 Robustness results for chlorozoxazone standard at decreased temperature

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5773	1.28	2.35	5300
2	0.5789	1.19	2.42	2681
3	0.5788	1.19	2.35	6347
4	0.5783	1.18	2.39	11681
5	0.5787	1.21	2.37	18269
6	0.5728	1.22	2.35	26048
Avg	0.5774667	1.21167	2.3717	11721
STDEV	0.0023602	0.03656	0.02858	8943.953824
RSD	0.4087207			

Table 3.32 Robustness results for combined paracetamol and chlorozoxazone sample at decreased temperature

	PAR	CZN
1st trial	1.0845	0.5786
2nd trial	1.0864	0.5698
3rd trial	1.0845	0.5755
Avg	1.085133333	0.574633333
STDEV	0.001096966	0.004463556
Recovery	0.101090389	0.77676596
Recovery%	99.52460294	99.50935119

iv) Robustness at increased 5% flow rate

Standard solution was injected six times while sample solution was injected three times after increasing the flow rate 5% of its optimized value. Results of paracetamol and chlorozoxazone standards are shown in table 3.33 and 3.34, respectively; results of samples for both components are shown in Table 3.35.

Table 3.33 Robustness results for paracetamol standard at increased flow rate

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	1.065	1.87	2.41	5381
2	1.0686	1.91	2.39	23353
3	1.0642	1.88	2.36	56576
4	1.063	1.87	2.37	125220
5	1.0621	1.92	2.33	255664
6	1.065	1.92	2.31	482051
Avg	1.06465	1.895	2.36167	158040.8333
STDEV	0.0022483	0.02429	0.0371	182937.7093
RSD	0.2111805			

Table 3.34 Robustness results for chlorozoxazone standard at increased flow rate

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5737	1.19	9.21	6780
2	0.5756	1.2	8.14	3227
3	0.5708	1.22	11.91	7222
4	0.5751	1.22	16.99	15158
5	0.5748	1.23	20.77	29813
6	0.5773	1.29	2.31	54540
Avg	0.57455	1.225	11.555	19456.6667
RSD	0.3798503			

Table 3.35 Robustness results for combined paracetamol and chlorozoxazone sample at increased flow rate

	PAR	CZN
1st trial	1.0641	0.5785
2nd trial	1.0652	0.5713
3rd trial	1.0656	0.5704
Avg	1.064966667	0.5734
STDEV	0.000776745	0.004439595
Recovery	0.072936118	0.774257861
Recovery%	100.0297437	99.79984336

v) Robustness at decreased 5% flow rate

Standard solution was injected six times, while sample solution was injected three times after decreasing the flow rate 5% of its optimized value. Results of paracetamol and chlorozoxazone standards are shown in Table 3.36 and 3.37, respectively; results of samples for both components are shown in Table 3.38.

Table 3.36 Robustness results for paracetamol standard at decreased flow rate

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	1.0257	1.89	2.36	3565
2	1.0303	1.93	2.35	20976
3	1.0286	1.91	2.3	58442
4	1.0249	1.97	2.3	95317
5	1.0267	1.93	2.3	154809
6	1.0313	1.94	2.31	212388
Avg	1.0279167	1.92833	2.32	90916.16667
STDEV	0.0025725	0.02714	0.02757	80540.04285
RSD	0.2502618			

Table 3.37 Robustness results for chlorozoxazone standard at decreased flow rate

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5391	1.17	2.36	6720
2	0.5361	1.13	2.35	2923
3	0.5365	1.23	2.3	7217
4	0.5371	0.121	2.3	11528
5	0.5315	1.18	2.3	18155
6	0.5388	1.22	2.31	24493
Avg	0.5365167	1.0085	**2.32**	**11839.3333**
RSD	0.510908			

Table 3.38 Robustness results for combined paracetamol and chlorozoxazone sample at decreased flow rate

	PAR	CZN
1st trial	1.0238	0.5354
2nd trial	1.0265	0.5368
3rd trial	1.0326	0.5344
Avg	1.027633333	0.535533333
STDEV	0.004508141	0.001205543
Recovery	0.438691568	0.225110685
Recovery%	99.97243616	99.81671896

vi) Robustness at increased 5% organic solvent

Standard solution was injected six times, while sample solution was injected three times after increasing of organic solvent in mobile phase 5% more than optimized value. Results of paraceamol and chlorozoxazone standards are shown in Table 3.39 and 3.40, respectively; results of samples for both components are shown in Table 4.41 .

Table 3.39 Robustness results for paraceamol standard at increased organic solvent

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	1.065	1.87	2.35	3581
2	1.0686	1.91	2.36	23353
3	1.0642	1.88	2.35	56576
4	1.063	1.87	2.36	125220
5	1.0612	1.92	2.32	255664
6	1.065	1.92	2.31	482051
Avg	1.0645	1.895	2.34167	157740.8333
1100RSD	0.2321688			

Table 3.40 Robustness results for chlorozoxazone standard at increased organic solvent

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5737	1.19	2.35	6780
2	0.5756	1.2	2.36	3227
3	0.5708	1.22	2.35	7222
4	0.5751	1.22	2.36	15158
5	0.5748	1.23	2.32	29813
6	0.5773	1.29	2.31	54540
Avg	0.57455	1.225	**2.3417**	**19456.6667**
STDEV	0.0021824			
RSD	0.3798503			

Table 3.41 Robustness results for combined paraceamol and chlorozoxazone sample at increased organic solvent

	PAR	CZN
1st trial	1.0641	0.5785
2nd trial	1.0652	0.5713
3rd trial	1.0656	0.5704
Avg	1.064966667	0.5734
STDEV	0.000776745	0.004439595
Recovery	0.072936118	0.774257861
Recovery%	100.043839	99.79984336

vii) Robustness at decreased 5% organic solvent

Standard solution was injected six times, while sample solution was injected three times after decreasing of organic solvent in mobile phase 5% more than optimized value. Results of paraceamol and chlorozoxazone standards are shown in Table 3.42 and 3.43, respectively; results of samples for both components are shown in Table 3.44 .

Table 3.42 Robustness results for paraceamol standard at decreased organic solvent

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	1.0494	1.75	2.35	144087
2	1.0452	1.81	2.42	258825
3	1.0482	1.81	2.35	376404
4	1.0409	1.83	2.39	522912
5	1.0468	1.81	2.37	673904
6	1.0451	1.82	2.35	909958
Avg	1.0459333	1.805	2.37167	481015
STDEV	0.0029824	0.02811	0.02858	281704.0847
RSD	0.2851418			

Table 3.43 Robustness results for chlorozoxazone standard at decreased organic solvent

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5446	1.2	2.35	16043
2	0.5369	1.12	2.42	28316
3	0.5378	1.18	2.35	40948
4	0.5394	1.17	2.39	54403
5	0.5437	1.16	2.37	71132
6	0.5479	1.18	2.35	92962
Avg	0.5417167	1.16833	2.371666667	**50634**
STDEV	0.0043448			
RSD	0.802051			

Table 3.44 Robustness results for combined paraceamol and chlorozoxazone sample at decreased organic solvent

	PAR	CZN
1st trial	1.0641	0.5389
2nd trial	1.0652	0.5387
3rd trial	1.0656	0.5415
Avg	1.064966667	0.5397
STDEV	0.000776745	0.00156205
Recovery	0.072936118	0.289429301
Recovery%	101.8197463	99.62772667

viii) Robustness at increased 3nm detection wavelength

Standard solution was injected six times, while sample solution was injected three times after increasing the 3nm more than the optimized detection wavelength. Results of paraceamol and chlorozoxazone standards are shown in Table 3.45 and 3.46, respectively; results of samples for both components are shown in Table 3.47.

Table 3.45 Robustness results for paraceamol standard at increased wavelength detection

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	1.0494	1.75	2.35	144087
2	1.0452	1.81	2.42	258825
3	1.0482	1.81	2.35	376404
4	1.0409	1.83	2.39	522912
5	1.0468	1.81	2.37	673904
6	1.0451	1.82	2.35	909958
Avg	1.0459333	1.805	2.37167	481015
STDEV	0.0029824	0.02811	0.02858	281704.0847
RSD	0.2851418			

Table 3.46 Robustness results for chlorozoxazone standard at increased wavelength detection

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5446	1.2	2.35	16043
2	0.5369	1.12	2.42	28316
3	0.5378	1.18	2.35	40948
4	0.5394	1.17	2.39	54403
5	0.5437	1.16	2.37	71132
6	0.5479	1.18	2.35	92962
Avg	0.5417167	1.16833	**2.3717**	**50634**
STDEV	0.0043448			
RSD	0.802051			

Table 3.47 Robustness results for combined paraceamol and chlorozoxazone sample at increased wavelength detection

	PAR	CZN
1st trial	1.0641	0.5487
2nd trial	1.0652	0.5428
3rd trial	1.0656	0.5462
Avg	1.064966667	0.5459
STDEV	0.000776745	0.002961419
Recovery	0.072936118	0.542483711
Recovery%	101.8197463	100.7722364

ix) Robustness at decreased 3nm detection wavelength

Standard solution was injected six times, while sample solution was injected three times after decreasing the 3nm less than the optimized detection wavelength. Results of paraceamol and chlorozoxazone standards are shown in Table 3.48 and 3.49, respectively; results of samples for both components are shown in Table 3.50.

Table 3.48 Robustness results for paraceamol standard at decreased wavelength detection

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	1.0494	1.75	2.35	144087
2	1.0452	1.81	2.42	258825
3	1.0482	1.81	2.35	376404
4	1.0409	1.83	2.39	522912
5	1.0468	1.81	2.37	673904
6	1.0451	1.82	2.35	909958
Avg	1.0459333	1.805	2.37167	481015
STDEV	0.0029824	0.02811	0.02858	281704.0847
RSD	0.2851418			

Table 3.49 Robustness results for chlorozoxazone standard at decreased wavelength detection

Injection No.	Area	Asymmetry	Resolution	Theoretical plates
1	0.5446	1.2	2.35	16043
2	0.5369	1.12	2.42	28316
3	0.5378	1.18	2.35	40948
4	0.5394	1.17	2.39	54403
5	0.5437	1.16	2.37	71132
6	0.5479	1.18	2.35	92962
Avg	0.5417167	1.16833	2.3717	50634
STDEV	0.0043448			
RSD	0.802051			

Table 3.50 Robustness results for combined paraceamol and chlorozoxazone sample at decreased wavelength detection

	PAR	CZN
1st trial	1.0641	0.5412
2nd trial	1.0652	0.54321
3rd trial	1.0656	0.5461
Avg	1.064966667	0.543503333
STDEV	0.000776745	0.002463135
Recovery	0.072936118	0.453195907
Recovery%	101.8197463	100.3298157

Summary of recovery for both components at the nine different conditions, average and RSD are shown in Table 3.51.

Table 3.51 Recovery results for paraceamol and chlorozoxazone at all robustness conditions

No.	Condition	PAR	CZN
1	Optimized Conditions	99.33392823	101.2891118
2	More 3 degree Celsius	99.34556771	100.275139
3	less 3 degree Celsius	99.52460294	99.50935119
4	5% More flow rate	100.0297437	99.79984336
5	5% less flow rate	99.97243616	99.81671896
6	5% more Organic solvent	100.043839	99.79984336
7	5% less Organic solvent	101.8197463	99.62772667
8	3nm Increased wave length	101.8197463	100.7722364
9	3nm Decreased wave length	101.8197463	100.3298157

3.3.7 Assay :

Standard solution and sample solution were prepared as described in section (2.6.9); standard solution was injected six times, while sample solution was injected three times, the average of each was used for assay calculations as shown in Table 3.52 and 3.53

Table 3.52 Assay results for paraceamol and chlorozoxazone standards

No.	**PAR**	**CZN**
STD1	1.0494	0.5446
SDT2	1.0452	0.5369
STD3	1.0482	0.5378
STD4	1.0409	0.5394
STD5	1.0468	0.5437
STD6	1.0451	0.5479
Avg	1.045933333	0.541716667
STDEV	0.002982393	0.004344844
RSD	0.285141766	0.802050953

Table 3.53 Assay results for combined paracetamol and chlorozoxazone samble

No.	**PAR**	**CZN**
trial-1	1.0449	0.547
trial-2	1.0393	0.5497
trial-3	1.0327	0.5494
Avg	1.038966667	0.5487
STDEV	0.006106827	0.001479865
RSD	0.587778894	0.26970382
Assay	99.33392823	101.2891118

3.4 Validation results when using stainless steel Monolithic Column

3.4.1 System Suitability

An example of chromatogram for system suitability method is shown in Appendix. F.System suitability results for paracetamol and Chlorzoxazone are shown in Table 3.54

Table 3.54 System suitability results for paracetamol and Chlorzoxazone

STD	Paracetamol			Chlorzoxazone			Resolution
	Area	Theoretical plates*	Asymmetry factor	Area	Theoretical plates*	Asymmetry factor	
STD 1	1316959	2300	1.67	837050	5000	1.45	1.97
STD 2	1322737	1700	2.40	845721	7100	1.17	2.01
STD 3	1321383	2100	1.50	834917	6400	1.20	2.04
STD 4	1319639	3500	1.75	839720	5300	1.27	1.86
STD 5	1322167	2100	2.75	843540	6400	1.20	2.03
STD 6	1330696	3500	1.40	839173	5300	1.19	1.86
AVG	1322264	2500	1.91	840020	5900	1.25	1.96
STDV	4629.07			4013.99			
RSD%	0.350			0.478			

3.4.2 Linearity, LOD and LOQ

i) Paracetamol

An example of chromatogram for system linearity method is shown in Appendix. G.Table 3.55 shows linearity results for Paracetamol which then treated by XL 2010 program to predict linearity data that shown in Figure 3.10.

Table 3.55 Linearity result for paracetamol

Content	40%	60%	80%	100%	120%	140%	160%
1	547347	794292	1104752	1301408	1619713	1822139	2201028
2	530324	795193	1077631	1344394	1583333	1775553	2071272
3	551613	788018	1046887	1342807	1535492	1855621	2137616
AVG	543094.7	792501	1076423	1322901	1579513	1817771	2136639
Recovery	41.09124	59.96165	81.44358	100.0924	119.508	137.5349	161.6608
Recovery%	102.7281	99.93608	101.8045	100.0924	99.58997	98.23921	101.038

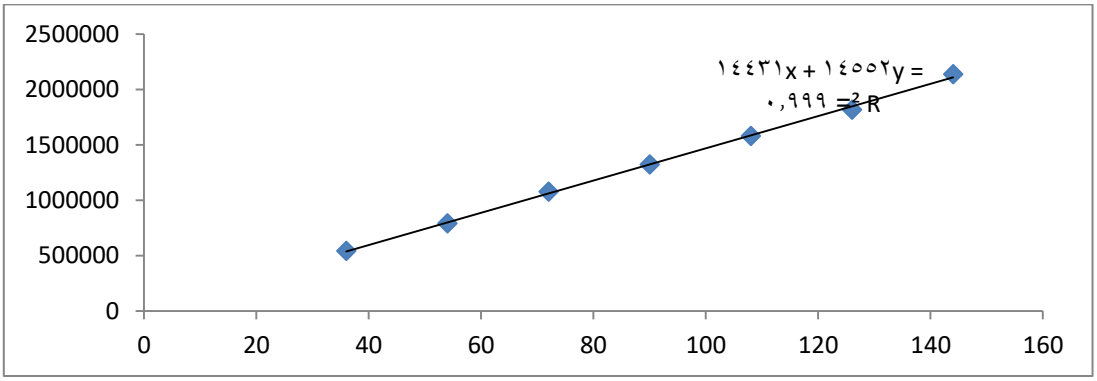


Figure 3.10 XL 2010 Graph of conc. Vs average area of paracetamol

Figure 3.10 shows the plot of average area versus concentrations for paracetamol in µg/ml, the linear regression equation:

Area 13097*µg/ml + 0.0123

According to ICH guidelines, acceptance criteria are $R^2 \geq 0.997$.

Limit of detection and limit of quantitation

LOD= RMSE*3/slope

= 19782*3/14552

LOD = 4.0782 µg/ml

% LOD =4.0782*100/90 = 4.5313 %

LOQ = RMSE *10 /slope

LOQ =19782*10/14552 = 13.594

% LOQ = 13.594*100/75 = 18.1253%

ii) Chlorzoxazone

Table 3.56 shows linearity results for chlorzoxazone which then treated by XL 2010 program to predict linearity data that shown Figure 3.11.

Table 3.56 Linearity results for chlorzoxazone

Content	40%	60%	80%	100%	120%	140%	160%
1	340118	513182	669004	799644	1020740	1149013	1368016
2	313116	482586	684480	846833	1028263	1140486	1286350
3	344123	509106	639929	842221	974024	1153214	1371205
AVG	332452.3	501624.7	664471	823238.5	1007676	1147571	1341857
Recovery	39.58895	59.73425	79.12625	98.03253	119.9956	136.6546	159.7904
Recovery%	98.97237	99.55709	98.90781	98.03253	99.99633	97.6104	99.86903

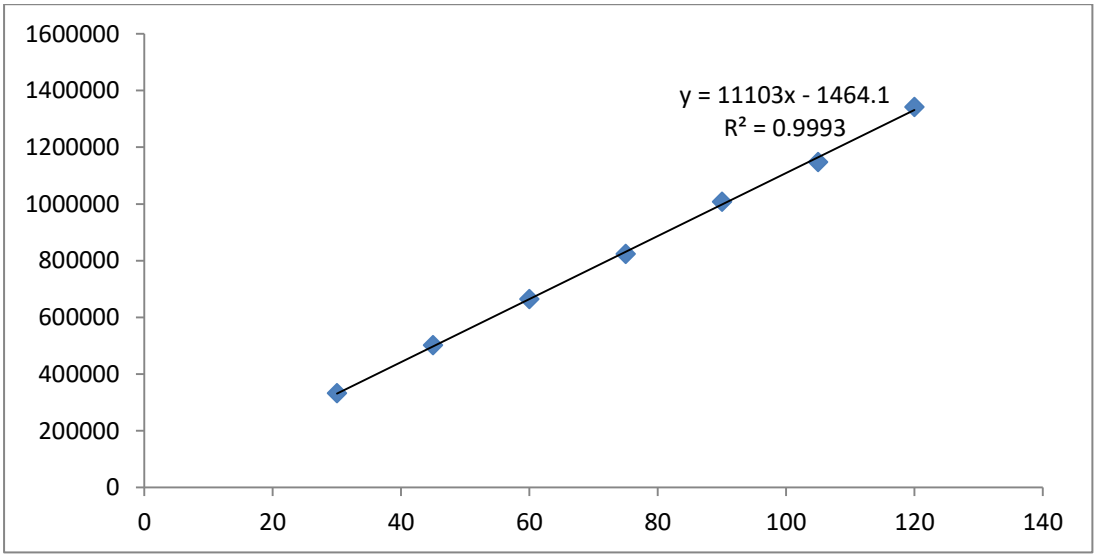


Figure 3.11 XL 2010 Graph of conc. In µg/ml Vs average area of Chlorzoxazone

Figure 3.11 shows the plot of average area versus concentrations for Chlorzoxazone in µg/ml, the linear regression equation:

Area = 11103 x+0.0092*µg/ml

According to ICH guidelines, acceptance criteria are $R^2 \geq 0.997$.

Limit of detection and limit of quantitation

LOD= RMSE*3/slope = 10734*3/11103

LOD = 2.9002 µg/ml

% LOD =2.9002 *100/75 = 3.87%

LOQ = RMSE *10 /slope = 10734*10 /11103

LOQ = 9.6676 µg/ml

% LOQ = 9.6676 *100/75 = 12.89 %

3.4.3 Specificity

Figure 3.12 Figure 3.13 and Figure 3.14 show the specificity chromatograms for placebo, sample and standard, respectively, for paracetamol and chlorozxazone.

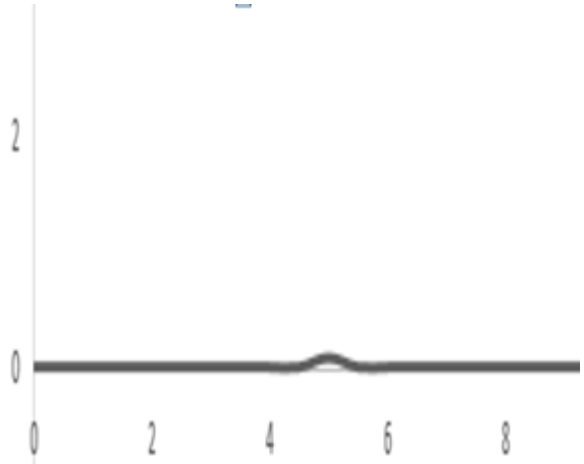


Figure 3.12 Specificity chromatogram for the Placebo of paracetamol and chlorozxazone

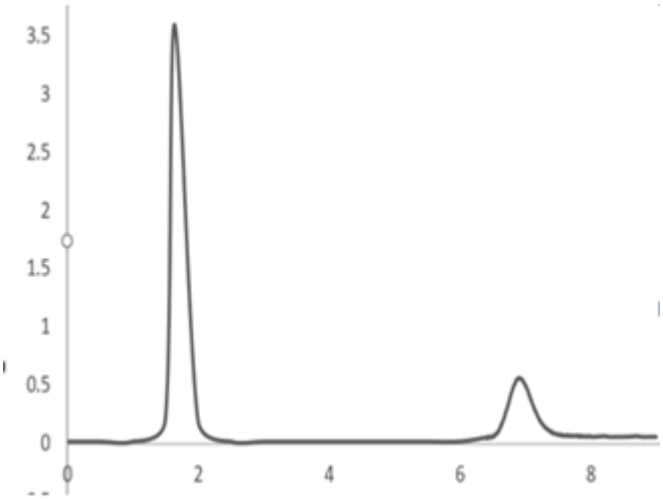


Figure 3.13 Specificity chromatogram for the sample of paracetamol and chlorozxazone

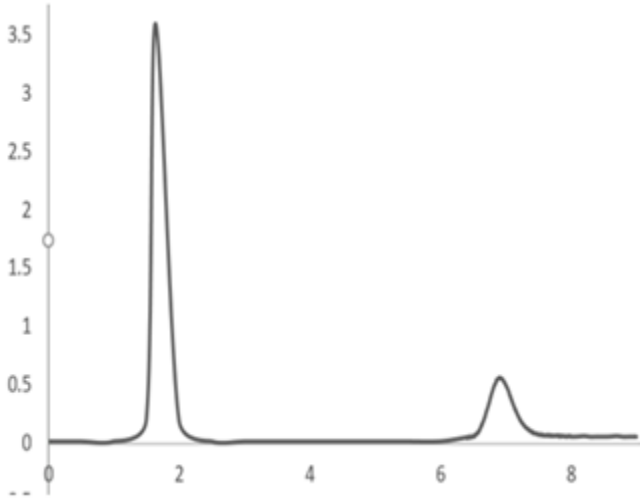


Figure 3.14 Specificity chromatogram for mixed standard of paracetamol and chlorozxazone

3.4.4 Accuracy

An example of chromatogram for accuracy method is shown in Appendix. H.Table 3.57 shows the results of mixed standard of paracetamol and chlorozxazone , while the accuracy results for paracetamol and chlorozxazone samples were shown in Table 3.58 and Table 3.59, respectively; summary of accuracy results for both components is shown in Table 3.60.

Table 3.57 Accuracy results for paracetamol and chlorozxazone standards

No.	PAR	CZN
STD1	1316959	837050
SDT2	1322737	845721
STD3	1321383	834917
STD4	1319639	839720
STD5	1322167	843540
STD6	1327194	837615
AVG	1321680	839760.5
STDEV	3414.684	4128.518
RSD	0.258359	0.49163

Table 3.58 Accuracy results for paractamol

Content	40%	60%	80%	100%	120%	140%	160%
1	547347	794292	1104752	1301408	1619713	1822139	2201028
2	530324	795193	1077631	1344394	1583333	1775553	2071272
3	551613	788018	1046887	1342807	1535492	1855621	2137616
AVG	543094.7	792501	1076423	1322901	1579513	1817771	2136639
Recovery	41.09124	59.96165	81.44358	100.0924	119.508	137.5349	161.6608
Recovery%	102.7281	99.93608	101.8045	100.0924	99.58997	98.23921	101.038

Table 3.59 Accuracy results for chlorzxazone

Content	40%	60%	80%	100%	120%	140%	160%
1	340118	513182	669004	799644	1020740	1149013	1368016
2	313116	482586	684480	846833	1028263	1140486	1286350
3	344123	509106	639929	842221	974024	1153214	1371205
AVG	332452.3	501624.7	664471	823238.5	1007676	1147571	1341857
Recovery	39.58895	59.73425	79.12625	98.03253	119.9956	136.6546	159.7904
Recovery%	98.97237	99.55709	98.90781	98.03253	99.99633	97.6104	99.86903

Table 3.60 Summary of accuracy results for paracetamol and chlorzxazone

Content%	RECOVERY %	
	PAR	CZN
40	102.7281065	98.97236573
60	102.7281065	98.97236573
80	102.7281065	98.97236573
100	102.7281065	98.97236573
120	102.7281065	98.97236573
140	102.7281065	98.97236573
160	102.7281065	98.97236573
Avg	100.489751	98.99222309
STDEV	1.493223613	0.906831215
RSD	1.485946177	0.916063

3.4.5 Precision

i) Intraday Precision

An example of chromatogram for precision method is shown in Appendix. I.Table 3.61 shows intraday precision results for paracetamol and chlorzoxazone mixed standard .

Table 3.61 Intraday precision results for paracetamol and chlorzoxazone mixed standard

Standards No.	PAR Area	CZN Area
1	1316959	837050
2	1322737	845721
3	1321383	834917
4	1319639	839720
5	1322167	843540
6	1327194	837615
Avg	1321679.833	839760.5
STDEV	3414.684314	4128.517736
RSD	0.258359417	0.49163038

Tables numbered 3.62, 3.63 and 3.64 show intraday precision results for 80%, 100% and 120% of paracetamol, respectively, while tables numbered 3.65, 3.66 and 3.67 show intraday precision results for 80%, 100% and 120% of chlorozoxazone respectively. Table 3.68 shows the summary of the results of the previous six tables as well as the average and RSD of each five assays of the three concentrations of each active ingredient.

Table 3.62 Intraday precision results for 80% paracetamol

No.	1st	2nd	3rd	4th	5th
1st trial	1104752	1043790	1106947	1106947	1074541
2nd trial	1077631	1062244	1079274	1079274	1070913
3rd trial	1046887	1020188	1046139	1064180	1088756
AVG	1076423	1042074	1083467	1078070	1078070
RECOVERY	81.44358	78.84466	81.97651	81.56817	81.56817
RECOVERY %	101.8045	98.55583	102.4706	101.9602	101.9602

Table 3.63 Intraday precision results for 100% paracetamol

No.	1st	2nd	3rd	4th	5th
1st trial	1324154	1322915	1324154	1315255	1309313
2nd trial	1344394	1398008	1328407	1287783	1387973
3rd trial	1301408	1216611	1358037	1317551	1364094
AVG	1323319	1312511	1336866	1306863	1353793
RECOVERY	100.124	99.3063	101.149	98.87894	102.4297
RECOVERY %	100.124	99.3063	101.149	98.87894	102.4297

Table 3.64 Intraday precision results for 120% paracetamol

No.	1st	2nd	3rd	4th	5th
1st trial	1619713	1698511	1522504	1546718	1669547
2nd trial	1583333	1535492	1566847	1569294	1620028
3rd trial	1592845	1541919	1597595	1592244	1459987
AVG	1598630	1591974	1562315	1569419	1583187
RECOVERY	120.9544	120.4508	118.2068	118.7442	119.786
RECOVERY %	100.7954	100.3757	98.50566	98.95353	99.82166

Table 3.65Intraday precision results for 80% chlorozoxazone

No.	1st	2nd	3rd	4th	5th
1st trial	669004	671957	681702	646890	650449
2nd trial	684480	667380	645234	672807	672392
3rd trial	639929	633966	663789	661280	687079
AVG	664471	657767.7	660325.7	669973.3	669973.3
RECOVERY	79.12625	78.32801	78.63262	79.78148	79.78148
RECOVERY %	98.90781	97.91001	98.29077	99.72685	99.72685

Table 3.66 Intraday precision results for 100% chlorozoxazone

No.	1st	2nd	3rd	4th	5th
1st trial	846265	825940	846265	810333	799406
2nd trial	846833	889046	769527	812917	858750
3rd trial	799644	768144	930451	854655	883567
AVG	830914	827710	848747.7	825968.3	847241
RECOVERY	98.94654	98.56501	101.0702	98.35761	100.8908
RECOVERY %	98.94654	98.56501	101.0702	98.35761	100.8908

Table 3.67 intraday precision results for 120% chlorozoxazone

No.	1st	2nd	3rd	4th	5th
1st trial	1020740	1064519	961270	992106	1021619
2nd trial	1028263	974024	965449	988820	994513
3rd trial	1001458	999213	1010637	971426	926715
AVG	1016820	1012585	979118.7	984117.3	980949
RECOVERY	121.0846	120.5803	116.595	117.1902	116.8129
RECOVERY %	100.9038	100.4835	97.16249	97.65853	97.34412

Table 3.68 Summery of intraday precession results for paracetamol and chlorozoxazone

	PAR			CZN		
	80%	100%	120%	80%	100%	120%
1st trial	101.8044713	100.1239962	100.7953599	98.9078136	100.9038027	100.9038027
2nd trial	98.55582775	99.30629947	100.3756709	97.91000926	100.483544	100.483544
3rd trial	102.4706374	101.1490049	98.50566012	98.29077259	97.16249124	97.16249124
4th trial	101.9602075	98.87893929	98.95353291	99.72684672	97.65853214	97.65853214
5th trial	101.9602075	102.4297488	99.82166214	99.72684672	97.34412371	97.34412371
AVG	101.3502703	100.3775978	99.69037719	98.91245778	98.71049874	98.71049874
STDEV	1.582282914	1.437390338	0.955816927	0.824299062	1.825117333	1.825117333
RSD	1.561202461	1.431983202	0.958785546	0.833362228	1.848959691	1.848959691

ii) Interday Precision

Table 3.69 shows results of interday precision test for mixture of paracetamol and chlorozoxazone standards.

Table 3.69 Interday precision results for mixture of paracetamol and chlorozoxazone standards

	PAR			CZN		
	1st Day	2nd Day	3rd Day	1st Day	2nd Day	3rd Day
STD1	1316959	1322167	1327194	837050	843540	837615
STD2	1322737	1316959	1322737	845721	837050	845721
STD3	1321383	1321383	1319639	834917	834917	839720
STD4	1319639	1327194	1321383	839720	837615	834917
STD5	1322167	1322737	1322167	843540	845721	843540
STD6	1327194	1319639	1316959	837615	839720	837050
AVG	1321679.833	1321582.4	1322624	839760.5	839004.6	840302.6
STDEV	3414.684314	3808.396881	2808.773042	4128.517736	4125.583268	4370.558351
RSD	0.258359417	0.288169461	0.212363683	0.49163038	0.491723558	0.5201172

Tables numbered 3.70, 3.71 and 3.72 shows interday precision for 80%, 100% and 120% for each component, respectively. Table 3.73 shows the summary of interday precision results, the average and RSD of each three assays of the three concentrations for each active ingredient.

Table 3.70 Interday precision results for 80% of paracetamol and chlorozoxazone

	PAR			CZN		
	1st Day	2nd Day	3rd Day	1st Day	2nd Day	3rd Day
1st trial	1104752	1074541	1092586	669004	650449	651587
2nd trial	1077631	1070913	1037895	684480	672392	645311
3rd trial	1046887	1088756	1066654	639929	687079	672961
AVG	1076423.333	1078070	1065712	664471	669973.3	656619.7
Recovery	81.44357704	81.57418	80.63906	79.12625	79.85336	78.14086
Recovery %	101.8044713	101.9677	100.7988	98.90781	99.8167	97.67607

Table 3.71 Interday precision results for 100% of paracetamol and chlorozoxazone

	PAR			CZN		
	1st Day	2nd Day	3rd Day	1st Day	2nd Day	3rd Day
1st trial	1324154	1324154	1365483	846265	846265	839975
2nd trial	1344394	1328407	1315480	846833	769527	812088
3rd trial	1301408	1358037	1361124	799644	930451	872006
AVG	1323319	1336866	1347362	830914	848747.7	841356.3
Recovery	100.124	101.1565	101.8704	98.94654	101.1613	100.1254
Recovery %	100.124	101.1565	101.8704	98.94654	101.1613	100.1254

Table 3.72 Interday precision results for 120% of paracetamol and chlorozoxazone

	PAR			CZN		
	1st Day	2nd Day	3rd Day	1st Day	2nd Day	3rd Day
1st trial	1619713	1698511	1635019	1020740	1064519	1012554
2nd trial	1583333	1535492	1583899	1028263	974024	973831
3rd trial	1592845	1541919	1551110	1001458	999213	953064
AVG	1598630	1591974	1590009	1016820	1012585	979816.3
Recovery	120.9544	120.4597	120.2163	121.0846	120.6889	116.6028
Recovery%	100.7954	100.3831	100.1802	100.9038	100.5741	97.169

Table 3.73 Interday precision results summery for each paracetamol and chlorozoxazone

	PAR			CZN		
	80%	100%	120%	80%	100%	120%
1st trial	101.8	100.12	100.8	98.91	98.95	100.9
2nd trial	101.97	101.16	100.38	99.81	101.16	100.57
3rd trial	100.8	101.87	100.18	97.68	100.13	97.83
AVG	101.52	101.05	100.45	98.8	100.08	99.77
STDEV	0.63216559	0.880170438	0.316438514	1.069252075	1.105848091	1.685299182
RSD	0.62	0.87	0.31	1.09	1.11	1.69

3.4.6 Robustness:

The method was examined for robustness test under nine different conditions, and its output under each condition is compared with that of the optimized conditions and

with permissible limits according to ICH. Lastly the variation in method output is evaluated through calculation of RSD of the nine results obtained under the following different nine conditions. An example chromatogram for robustness method is shown in Appendix. J.

i) Robustness at optimized conditions

Standard solution was injected six times while sample solution was injected three times under optimized conditions. Results of paracetamol and chlorozoxazone standards are shown in Table 3.74. Robustness results for both components standards shown in Table 3.75.

Table 3.74 Robustness results for for combined paracetamol and chlorozoxazone standards at optimum conditions

	PAR			CZN		
	Area	Theoretical plates*	Asymmetry factor	Area	Theoretical plates*	Asymmetry factor
1	1316959	2300	1.67	837050	5000	1.45
2	1322737	1700	2.40	845721	7100	1.17
3	1321383	2100	1.50	834917	6400	1.20
4	1319639	3500	1.75	839720	5300	1.27
5	1322167	2100	2.75	843540	6400	1.20
6	1327194	3500	1.40	837615	5300	1.19
AVG	1321680	2500	1.91	839760.5	5900	1.25
STDEV	3414.684					
RSD	0.258359					

Table 3.75 Robustness results for combined paracetamol and chlorozoxazone sample at optimum conditions

	PAR	CZN
1st trial	1324154	846265
2nd trial	1344394	846833
3rd trial	1301408	799644
Avg	1323319	830914
Recovery	100.124	98.94654
Recovery%	100.124	98.94654

ii) Robustness at increased 3ºC temperature

Standard solution was injected six times while sample solution was injected three times after the column temperature had been raised up three degrees celsius, Results of paracetamol and chlorozoxazone standards are shown in Table 3.76; results of samples for both components are shown in Table 3.77.

Table 3.76 Roubstness results for paracetamol and chlorozoxazone standards at increased temperature

	Area of PAR	Area of CZN
1	1358119	736694
2	1342262	705909
3	1343293	731503
4	1351501	723523
5	1346133	719279
6	1329028	702541
AVG	1345056	719908.2
STDEV	9809.702	13618.09
RSD	0.729316	1.891643

Table 3.77 Roubstness results for combined paracetamol and chlorozoxazone sample at increased temperature

	PAR	CZN
1st trial	1324154	846265
2nd trial	1344394	846833
3rd trial	1301408	799644
Avg	1323319	830914
Recovery	100.124	98.94654
Recovery%	100.124	98.94654

iii) Roubstness at decreased 3^{o}C temperature

Standard solution was injected six times while sample solution was injected three times after the column temperature had been decreased three celsius degrees . Results of paracetamol and chlorozoxazone standards are shown in Table 3.78, results of samples for both components are shown in Table 3.79.

Table 3.78 Roubstness results for paracetamol and chlorozoxazone standards at decreased temperature

	Area of PAR	Area of CZN
1	1320630	652843
2	1311637	626782
3	1339709	636862
4	1314532	631333
5	1336293	639365
6	1337674	646063
AVG	1326746	638874.7
STDEV	12597.42	9530.866
RSD	0.949498	1.491821

Table 3.79 Roubstness results for combined paracetamol and chlorozoxazone sample at decreased temperature

	PAR	CZN
1st trial	1326431	647051
2nd trial	1333260	649835
3rd trial	1333777	648898
Avg	1331156	648594.7
Recovery	100.3324	101.5214
Recovery%	100.3324	101.5214

iv) Roubstness at increased 5% flow rate

Standard solution was injected six times, while sample solution was injected three times after increasing the flow rate 5% of its optimized value. Results of paracetamol and chlorozoxazone standards are shown in Table 3.80; results of samples for both components are shown in Table 3.81.

Table 3.80 Roubstness results for combined paracetamol and chlorozoxazone standards at increased flow rate

	Area of PAR	Area of CZN
1	1271367	748799
2	1301203	765016
3	1300014	752550
4	1285709	759923
5	1279521	748076
6	1283309	743431
AVG	1286854	752965.8
STDEV	11718.17	8074.251
RSD	0.910606	1.072326

Table 3.81 Roubstness results for combined paracetamol and chlorozoxazone sample at increased flow rate

	PAR	CZN
1st trial	1232549	717396
2nd trial	1302718	760550
3rd trial	1263257	745189
Avg	1266175	741045
Recovery	98.39304	98.41682
Recovery%	98.39304	98.41682

v) Roubstness at decreased 5% flow rate

Standard solution was injected six times, while sample solution was injected three times after decreasing the flow rate 5% of its optimized value. Results of paracetamol and chlorozoxazone standards are shown in Table 3.82; results of samples for both components are shown in Table 3.83.

Table 3.82 Robustness results for combined paracetamol and chlorozoxazone standards at decreased flow rate

	Area of PAR	Area of CZN
1	1404761	883164
2	1392520	867256
3	1402393	860591
4	1398029	843807
5	1382209	900410
6	1416135	918045
AVG	1407967.5	867499
STDEV	13930.63	14113.06
RSD	0.9894142	1.626867

Table 3.83 Robustness results for combined paracetamol and chlorozoxazone samples at decreased flow rate

	PAR	CZN
1st trial	1423068	871467
2nd trial	1397260	843319
3rd trial	1427034	878709
Avg	1415787.3	864498.3
Recovery	100.007	101.8835
Recovery%	100.00703	100.007

vi) Roubstness at increased 5% organic solvent

Standard solution was injected six times, while sample solution was injected three times after increasing of organic solvent in mobile phase 5% more than optimized value. Results of paraceamol and chlorozoxazone standards are shown in Table 3.84; results of samples for both components are shown in Table 3.85 .

Table 3.84 Robustness results for combined paraceamol and chlorozoxazone standards at increased organic solvent

	Area of PAR	Area of CZN
1	1335843	721783
2	1331712	709349
3	1303611	694616
4	1324577	698153
5	1340374	719703
6	1355637	725817
AVG	1331959	711570.2
STDEV	17347.68	13006.27
RSD	1.302418	1.827826

Table 3.85 Robustness results for combined paraceamol and chlorozoxazone sample at increased organic solvent

	PAR	CZN
1st trial	1341339	722851
2nd trial	1353385	725328
3rd trial	1323856	707636
Avg	1339527	718605
Recovery	100.5682	100.9886
Recovery%	100.5682	100.9886

vii) Roubstness at decreased 5% organic solvent

Standard solution was injected six times, while sample solution was injected three times after decreasing of organic solvent in mobile phase 5% more than optimized value. Results of paraceamol and chlorozoxazone standards are shown in Table 3.86; results of samples for both components are shown in Table 3.87 .

Table 3.86 Robustness results for combined paraceamol and chlorozoxazone standards at decreased organic solvent

	Area of PAR	Area of CZN
1	1326460	704612
2	1334927	721846
3	1349662	718956
4	1323618	710064
5	1351055	716511
6	1325304	702440
AVG	1335171	712404.8
STDEV	12402.105	7929.939
RSD	0.9288777	1.113123

Table 3.87 Robustness results for combined paraceamol and chlorozoxazone sample at decreased organic solvent

	PAR	CZN
1st trial	1335698	714579
2nd trial	1358592	717788
3rd trial	1342657	717969
Avg	1345649	716778.7
Recovery	100.78477	100.614
Recovery%	100.78477	100.614

viii) Roubstness at increased 3nm detection wavelength

Standard solution was injected six times, while sample solution was injected three times after increasing the 3nm more than the optimized detection wavelength. Results of paraceamol and chlorozoxazone standards are shown in Table 3.88; results of samples for both components are shown in Table 3.89.

Table 3.88 Robustness results for combined paraceamol and chlorozoxazone standards at increased wavelength detection

	Area of PAR	Area of CZN
1	1158728	989044
2	1148433	1000090
3	1146246	1016956
4	1137473	969124
5	1155607	1006091
6	1155420	1019718
AVG	1150318	1000171
STDEV	7872.235	18902.91
RSD	0.684353	1.889969

Table 3.89 Robustness results for combined paraceamol and chlorozoxazone ample at increased wavelength detection

	PAR	CZN
1st trial	1176893	1036058
2nd trial	1134747	1012253
3rd trial	1139556	1008715
Avg	1150399	1019009
Recovery	100.007	101.8835
Recovery%	100.007	101.8835

ix) Roubstness at decreased 3nm detection wavelength

Standard solution was injected six times, while sample solution was injected three times after decreasing the 3nm less than the optimized detection wavelength. Results of paraceamol and chlorozoxazone standards are shown in Table 3.90; results of samples for both components are shown in Table 3.91.

Table 3.90 Robustness results for combined paraceamol and chlorozoxazone standards at decreased wavelength detection

	Area of PAR	Area of CZN
1	1594300	704194
2	1596629	714335
3	1595213	691692
4	1596006	707630
5	1585847	710138
6	1615639	697482
AVG	1597272	704245.2
STDEV	9828.182	8382.271
RSD	0.61531	1.190249

Table 3.91 Robustness results for combined paraceamol and chlorozoxazone sample at decreased wavelength detection

	PAR	CZN
1st trial	1584879	662961
2nd trial	1574806	674478
3rd trial	1585847	710138
Avg	1581844	682525.7
Recovery	99.03408	96.91592
Recovery%	99.03408	96.91592

Summary of recovery for both components at the nine different conditions, average and RSD are shown in Table 3.92.

Table 3.92 Robustness results for combined paraceamol and chlorozoxazone recovery at all robustness conditions

No.	Condition	PAR	CZN
1	Optimized Conditions	100.124	98.94654
2	More 3 degree Celsius	100.124	98.94654
3	less 3 degree Celsius	100.3324	101.5214
4	5% More flow rate	98.39304	98.41682
5	5% less flow rate	100.00703	100.007
6	5% more Organic solvent	100.5682	100.9886
7	5% less Organic solvent	100.78477	100.614
8	3nm Increased wave length	100.007	101.8835
9	3nm Decreased wave length	99.03408	96.91592

3.4.7 Assay :

Standard solution and sample solution were prepared as described in section (2.7.9); standard solution was injected six times, while sample solution was injected three times, the average of each was used for assay calculations as shown in table 3.93 and 3.94

Table 3.93 Assay results for combined paraceamol and chlorozoxazone standard

No.	PAR	CZN
STD1	1316959	837050
SDT2	1322737	845721
STD3	1321383	834917
STD4	1319639	839720
STD5	1322167	843540
STD6	1327194	837615
Avg	1321680	839760.5
STDEV	3414.684	4128.518
RSD	0.258359	0.49163

Table 3.94 Assay results for combined paracetamol and chlorozoxazone sample

No.	PAR	CZN
trial1	1324154	846265
trial2	1344394	846833
trial3	1301408	799644
Avg	1323319	830914
Recovery	100.124	98.94654
Assay	100.124	98.94654

3.5 Discussion

3.5.1 Hexyl methacrylate monolithic capillary column

Many previous works showed that in addition to the polymerization conditions, type and percentages of the monomeric mixture play an important role in the final characteristics and chromatographic performance of the fabricated column. In this work, a monolithic stationary phase was prepared by polymerizing of hexyl methacrylate with ethylene dimethacrylate and it chemically attaching to the inner surface of the capillary tube.

The prepared capillary column was evaluated by investigating the porosity, permeability and mechanical stability. Both porosity and permeability values of the prepared capillary column are in a good convergence. The column was used for separation of PAR and CZN standards. A theoretical plate, peaks asymmetry and chromatographic resolution have been measured for each standard at different mobile phase flow rates. According to the acceptance criteria set by ICH guidelines [37], all parameters proved the suitability of the prepared column and the optimized method for analysis of PAR and CZN.

Under the optimum conditions, PAR and CZN have been separated in time of 6.5 min with chromatographic resolution of 2.37. The mobile phase was composed of acetonitrile and 1% aqueous formic acid solutions in the ratio of 60:40 flowing at 1.0 µL/min (0.06 mL/h solvents consumption) which minimize the environmental impact. Furthermore, because of the requirements of much smaller amount of samples (4 nL sample injection volume) and stationary phase materials, preparation of monolith inside 0.1 mm i.d. scale column is also more cost efficient.

The chromatographic separation was optimized and the analytical method was completely validated in terms of system suitability, linearity, limit of detection and quantification, specificity, accuracy, inter- and intraday precisions and robustness tests. The developed assay exhibited good linearity in the proposed concentration range for each compound (36–144 µg/mL for PAR and 24–96 µg/mL for CZN). The regression coefficient values indicated excellent degree of method linearity when peak area was used for signal evaluation. A series of dilutions for the standard stock

solution were made to determine the detection and quantification limits based on the signal-to-noise ratios. Samples and placebo solutions were injected at the optimized conditions. The excipients did not affect the estimation of the PAR and CZN since no peaks were detected at their retention times. On the other hand, the perfect correlation between the retentions of PAR and CZN standards and active ingredients extracted from Relaxon tablets indicated the selectivity of the method. Satisfactory recovery values were obtained at different concentration levels indicating that the proposed method is accurate and reproducible for simultaneous chromatographic determination of PAR and CZN.

In comparison with the performance of conventional columns using particulate stationary phases applied earlier for the determination of PAR and CZN , that of the prepared capillary monolithic column showed greater advantages .The developed column lowers the consumption of solvent and samples leading to a remarkable decrease in cost of analysis and harmful impact on the enviroment. In conclusion, all parameters of method validation inferred that the prepared column and proposed method are applicable for quality control and routine analysis of PAR and CZN in their combined pharmaceutical formulations.

3.5.2 Glycidyl polymethacrylate monolithic stainless steel column

The permeability and the stability of the stationary phase inside the column were evaluated using both water and acetonitrile. Pressure drops across the column have been evaluated at different flow rates ranging from 0.10 to 2.0 mL/min. The column shows stable permeability and perfect mechanical stability over the investigated flow range with regression factor (R^2) of 0.9998 and 0.9997 for water and acetonitrile, respectively. The permeability values of the prepared column were determined at 25^{o}C, while pure acetonitrile and water eluents passed through the column at a volumetric flow rate of 1.0 mL/min. The permeability values of the prepared column were 2.71×10^{-12} and 2.36×10^{-12} m^2 corresponding to the measured pressure drop of 435 psi (30 bar) and 993 psi (68.5 bar) for acetonitrile and water, respectively. Fig.3.3 shows a directly proportional relationship between acetonitrile and water flow rate and columns backpressure at 25^{o}C. The total porosity value was found to be 0.78; it was calculated using uracil as un-retained solute which is in accordance with the results obtained from SEM images Fig. 3.4. Both permeability and total porosity

values are very close .After this preliminary investigation, the prepared monolith inside the conventional stainless steel column was used for separation of PAR and CZN standards. Fig.3.14 shows the separation chromatogram for targeted concentrations of the mixed standard solution at optimum chromatographic conditions. The two active ingredients were completely separated in about 9.0 min at 0.7 mL/min flow rate. In order to evaluate the column efficiency various chromatographic factors such as plate numbers, peaks asymmetry and resolution have been measured for each standard at different mobile phase flow rates. At 0.7 mL/min flow rate, the column exhibited an average efficiency of 2500 plates/m for PAR and 5900 plates/m for CZN, while the average chromatographic resolution for the six replicates was about 2.0.

To develop an effective method for the analysis of the drugs, preliminary tests were performed in order to select adequate and optimum conditions. Parameters such as detection wavelength, ideal mobile phase and its combination, optimum pH and concentration of the standard solutions were studied. HPLC method was found to be simple, accurate, economic and rapid for routine simultaneous estimation of PAR and CZN in tablet dosage forms. The conditions were optimized to obtain, an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried to separate drugs components. Mobile phase and flow rate selection were based on peak parameters particularly run time and resolution. Since the monolithic material is not affected by pH, the system with formic acid (1 v/v %): acetonitrile in the ratio of (65:35) with 0.7 mL/min flow rate is quite robust. The optimum wavelength for detection was 270 nm at which better detector response for drugs was obtained. The average retention times for PAR and CZN were found to be 1.675 and 5.995 min, respectively. According to USP, system suitability tests are an integral part of chromatographic method. They are used to verify the reproducibility of the chromatographic system. From this test, the %RSD values for the two active ingredients were found to be less than 2.0%; the low values of %RSD indicate the method is precise and accurate.

The developed assay was found to be linear in the proposed concentration range when peak area was used for signal evaluation. The calibration graphs were obtained using excel-2010 for PAR and CZN as shown in Fig.3.10. The regression coefficient values

R^2 were found to be 0.9990 and 0.9993, respectively; indicating an excellent degree of method linearity.

The detection limit and limit of quantification (LOD and LOQ) were calculated from linearity data according to ICH guidelines. LOD and LOQ represent the concentrations of the solutes that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ. A series of dilutions for the standard stock solution were made to determine the LOD and LOQ values.. In selectivity test, the placebo solution showed no peaks at the retention time of the PAR and CZN peaks. This indicates that the excipients used in the formulation did not interfere with the estimation of the active ingredients in the tablets. Also, based on Figs. 3.12, 3.13 and 3.14, the system suitability parameters in the respective chromatogram were almost identical to those of the standard chromatogram indicating that, excipients in the sample did not affect separation. On the other hand, there is perfect correlation between the retentions of the standards PAR and CZN and active ingredients extracted from Relaxon tablets**,** which indicates that the validated method is specific. The accuracy of the method was proven by recovery test. The method has shown good, consistent recoveries for PAR and CZN (98.24–102.73% and 97.61–99.99% respectively) which are close to 100%. The results of precision study indicate that the proposed method is reliable and reproducible.

The robustness of the method was checked by deliberately varying the mobile phase composition, flow rate, detection wavelength and column temperature which shows that the small changes of the method parameter does not affect the performance of our method. All the results presented in Table 3.92 were in accordance with the results for original conditions. The %RSD value obtained for the assay in the changed condition was less than 2% which indicates the robustness of the proposed method. In conclusion, all validation parameters permit to infer that the prepared column and proposed method are applicable for quality control and routine analysis of PAR and CZN in their combined pharmaceutical formulations.

3.6 Conclusion

In this work, hexyl methacrylate-co-ethylene dimethacrylate monolithic capillary column has been prepared and used for simple, green, efficient and reliable isocratic elution nano-LC-UV procedure to determine PAR and CZN in their pharmaceutical combination. When compared to the standard LC procedures, the most important advantages of the proposed method are its high recovery rates, low cost and green analytical approach with a mobile phase consumption of only 0.06 mL/h. The method was validated and showed good accuracy and precision. Therefore, this methodology based on application of capillary columns in nano-LC is highly recommended and might be suitable to be used for routine analysis of drugs as well as for research purposes.

Else in this work, Glycidyl methacrylate-co-ethylene dimethacrylate monolithic has been synthesized in stainless steel HPLC column and used for simple, efficient and reliable isocratic elution reversed phase HPLC-UV technique to assess PAR and CZN in their pharmaceutical combination. The method was validated and showed good accuracy and precision. Therefore, this methodology based on application of conventional HPLC which is available in almost all laboratories around the world, is highly recommended and might be suitable to be used for routine analysis of drugs as well as for research purposes.

Recommendations

Remarkable performance was revealed when capillary and stainless steel columns were prepared , even locally , using monolithic stationary phases .The fact that the simultaneous determination using these micro columns of PAR and CZN in their pharmaceutical formulations, shows also satisfactory validation parameters , invites further research work in the preparation of stationary phases to extend the application of monolithic HPLC to the vast areas of routinc pharmaceutical and biochemical analysis.

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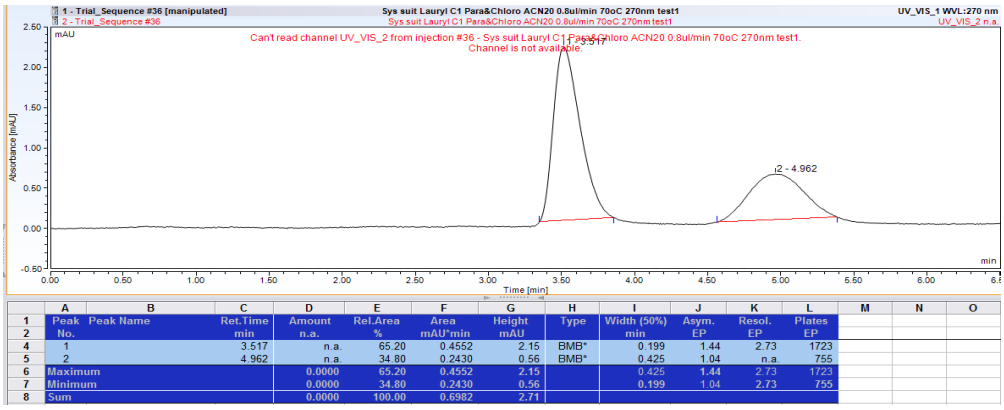
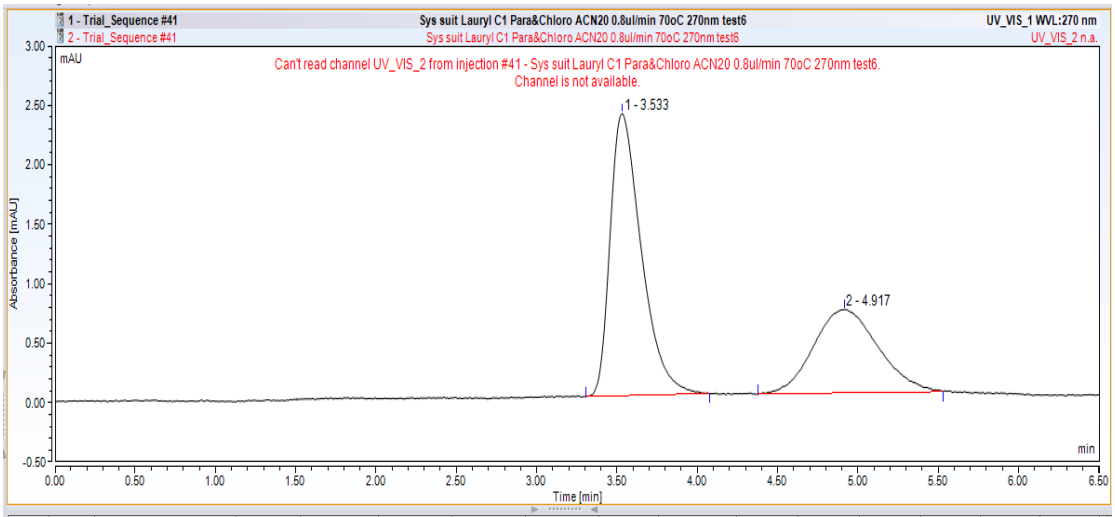
Appendixes



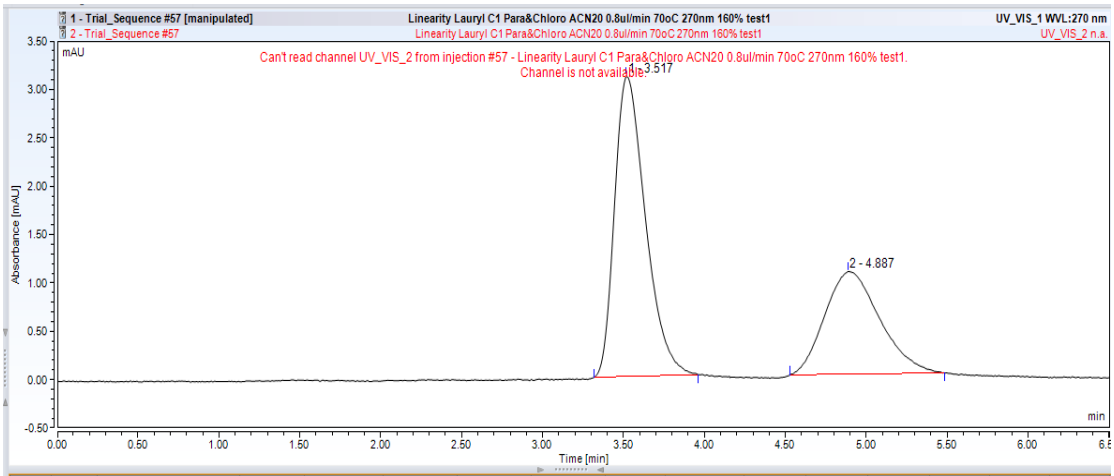
Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		3.517	n.a.	65.20	0.4552	2.15	BMB*	0.199	1.44	2.73	1723
2		4.962	n.a.	34.80	0.2430	0.56	BMB*	0.425	1.04	n.a.	755
Maximum			0.0000	65.20	0.4552	2.15		0.425	1.44	2.73	1723
Minimum			0.0000	34.80	0.2430	0.56		0.199	1.04	2.73	755
Sum			0.0000	100.00	0.6982	2.71					

Appendix. A chromatogram illustrating system suitability for paracetamol and chlorozoxazone (capillary monolithic column)



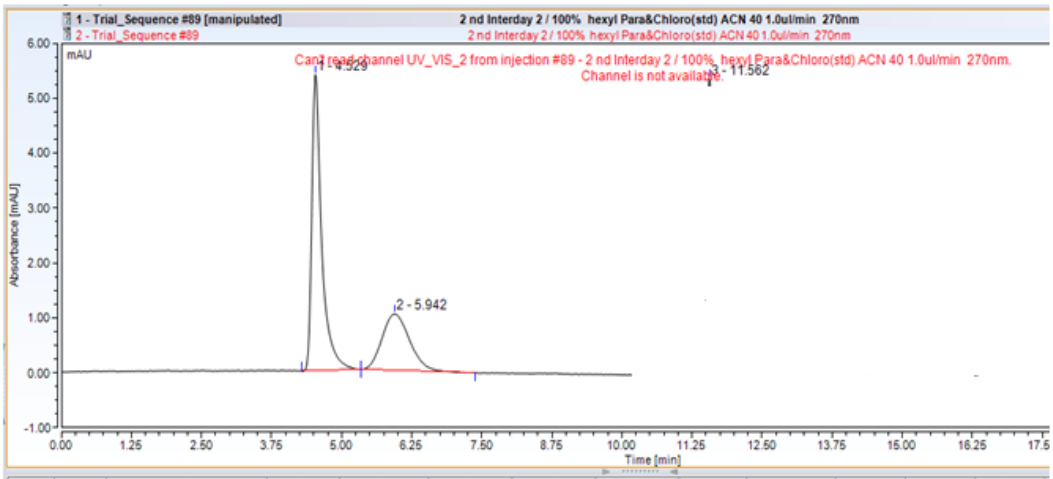
Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
1		3.533	n.a.	62.03	0.5349	2.37	BMB	0.205	1.54	2.55	1644
2		4.917	n.a.	37.97	0.3274	0.70	BMB	0.436	1.12	n.a.	705
Maximum			0.0000	62.03	0.5349	2.37		0.436	1.54	2.55	1644
Minimum			0.0000	37.97	0.3274	0.70		0.205	1.12	2.55	705
Sum			0.0000	100.00	0.8623	3.08					

Appendix.B chromatogram illustrating linearity for paracetamol and chlorozoxazone (capillary monolithic column)



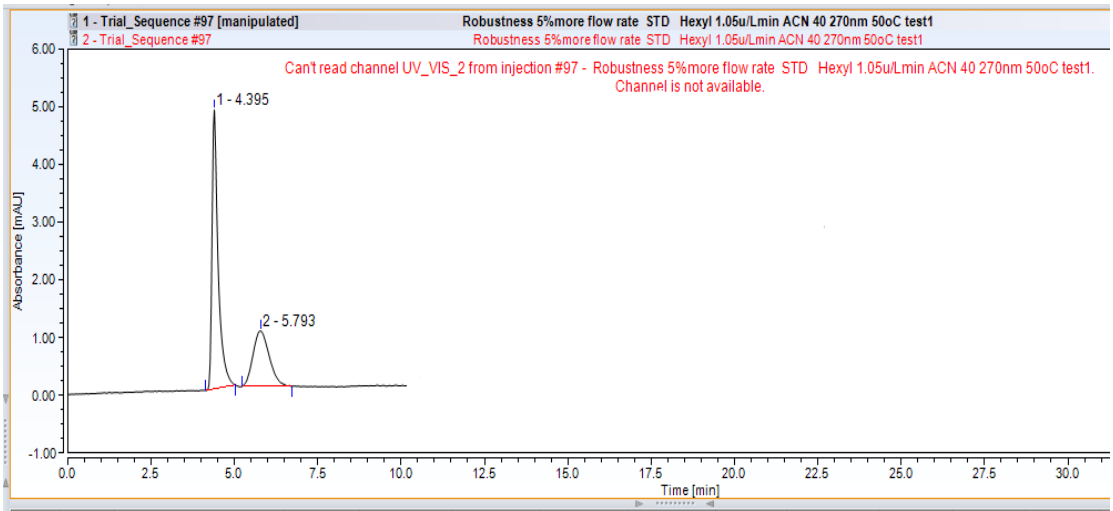
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Peak	Peak Name	Ret.Time	Amount	Rel.Area	Area	Height	Type	Width (50%)	Asym.	Resol.	Plates			
2	No.		min	n.a.	%	mAU*min	mAU		min	EP	EP	EP			
4	1		3.517	n.a.	62.19	0.6872	3.11	BMB*	0.204	1.48	2.82	1646			
5	2		4.887	n.a.	37.81	0.4178	1.06	BMB*	0.370	1.28	n.a.	966			
6	Maximum			0.0000	62.19	0.6872	3.11		0.370	1.48	2.82	1646			
7	Minimum			0.0000	37.81	0.4178	1.06		0.204	1.28	2.82	966			
8	Sum			0.0000	100.00	1.1050	4.17								

Appendix.C chromatogram illustrating accuracy for paracetamol and chlorozoxazone (capillary monolithic column)



	A	B	C	D	E	F	G	H	I	J	K	L
1	Peak	Peak Name	Ret.Time	Amount	Rel.Area	Area	Height	Type	Width (50%)	Asym.	Resol.	Plates
2	No.		min	n.a.	%	mAU*min	mAU		min	EP	EP	EP
4	1		4.529	n.a.	21.76	1.1007	5.39	BMB*	0.165	1.88	2.39	4154
5	2		5.942	n.a.	11.51	0.5820	1.03	BMB*	0.531	1.19	9.52	693
6	3		11.562	n.a.	21.68	1.0965	5.39	BMB*	0.166	1.80	2.41	26947
7	4		12.987	n.a.	11.66	0.5896	1.03	BMB*	0.532	1.17	9.43	3298
8	5		18.562	n.a.	21.69	1.0971	5.39	BMB*	0.165	1.84	2.39	69743
9	6		19.975	n.a.	11.70	0.5917	1.04	BMB*	0.531	1.19	n.a.	7832
10	Maximum			0.0000	21.76	1.1007	5.39		0.532	1.88	9.52	69743
11	Minimum			0.0000	11.51	0.5820	1.03		0.165	1.17	2.39	693
12	Sum			0.0000	100.00	5.0575	19.28					

Appendix.D chromatogram illustrating precission for paracetamol and chlorozoxazone (capillary monolithic column)



	A	B	C	D	E	F	G	H	I	J	K	L
1	Peak No.	Peak Name	Ret.Time min	Amount n.a.	Rel.Area %	Area mAU*min	Height mAU	Type	Width (50%) min	Asym. EP	Resol. EP	Plates EP
4	1		4.395	n.a.	65.55	1.0257	4.83	BMB*	0.173	1.89	2.36	3565
5	2		5.793	n.a.	34.45	0.5391	0.96	BMB*	0.526	1.17	n.a.	672
6	Maximum			0.0000	65.55	1.0257	4.83		0.526	1.89	2.36	3565
7	Minimum			0.0000	34.45	0.5391	0.96		0.173	1.17	2.36	672
8	Sum			0.0000	100.00	1.5648	5.79					

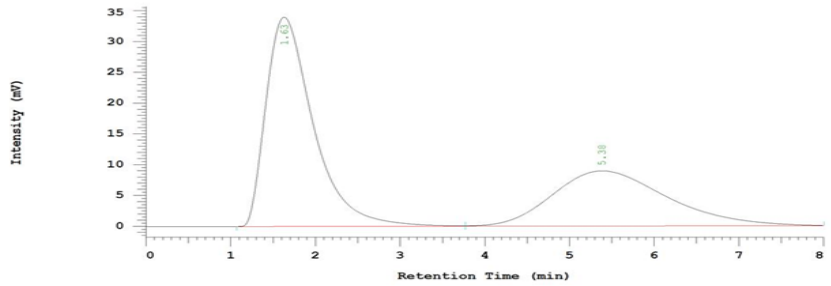
Appendix.E chromatogram illustrating robustness for paracetamol and chlorozoxazone (capillary monolithic column)

D-2000: Samples Series: 0305 Report Name: original System: Sys 1

D-2000 Elite HPLC System Manager Report

Analyzed Date and Time: 07/19/2017 05:36 PM
Reported Date and Time: 07/19/2017 05:44 PM
Processed Date and Time: 07/19/2017 05:44 PM
Data Path: C:\Win32app\D2000HSM\samples\DATA\0305\
Processing Method: para czn 10
System (acquisition): Sys 1
Series: 0305
Application(data): Samples
Vial Number: 1
Sample Name: UNKNOWN001
Vial Type: UNK
Injection from this vial: 1 of 1
Volume: 10.0 ul
Sample Description:

Chrom Type: HPLC Channel : 1



Processing Method: para czn 10
Column Type: Column
Method Developer:
Pump A: L-2130
Pump A Solvent A:
Method Description:

Chrom Type: HPLC Channel : 1
Peak Quantitation: AREA
Calculation Method: AREA%

No.	RT	Area	Area %	R-Factor	BC	Group	No.
1	1.63	1316959	61.140		BV		1
2	5.38	837050	38.860		VB		2
		2154009	100.000				

Peak rejection level: 0

Page Indicator: 1

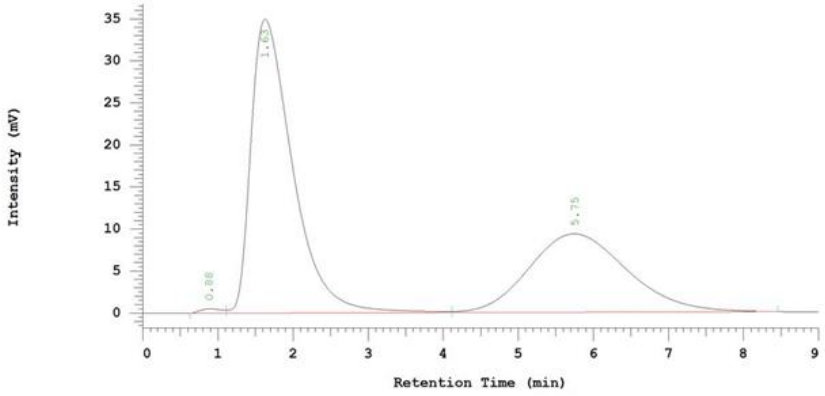
Appendix. F chromatogram illustrating system suitability for paracetamol and chlorozoxazone (stainless steel monolithic column)

D-2000: Samples Series: 0236 Report Name: original System: Sys 1

D-2000 Elite HPLC System Manager Report

Analyzed Date and Time: 07/18/2017 07:07 PM
Reported Date and Time: 07/18/2017 07:16 PM
Processed Date and Time: 07/18/2017 07:16 PM
Data Path: C:\Win32app\D2000HSM\samples\DATA\0236\
Processing Method: para czn 10
System (acquisition): Sys 1
Application(data): Samples
Sample Name: UNKNOWN001
Injection from this vial: 1 of 1
Sample Description:
Series: 0236
Vial Number: 1
Vial Type: UNK
Volume: 10.0 ul

Chrom Type: HPLC Channel : 1



Processing Method: para czn 10
Column Type: Column
Method Developer:
Pump A: L-2130
Pump A Solvent A:
Method Description:

Chrom Type: HPLC Channel : 1
Peak Quantitation: AREA
Calculation Method: AREA%

No.	RT	Area	Area %	R-Factor	BC	Group	No.
1	0.88	10392	0.472		BV		1
2	1.63	1344394	61.064		VV		2
3	5.75	846833	38.464		VB		3
		2201619	100.000				

Peak rejection level: 0

Page Indicator: 1

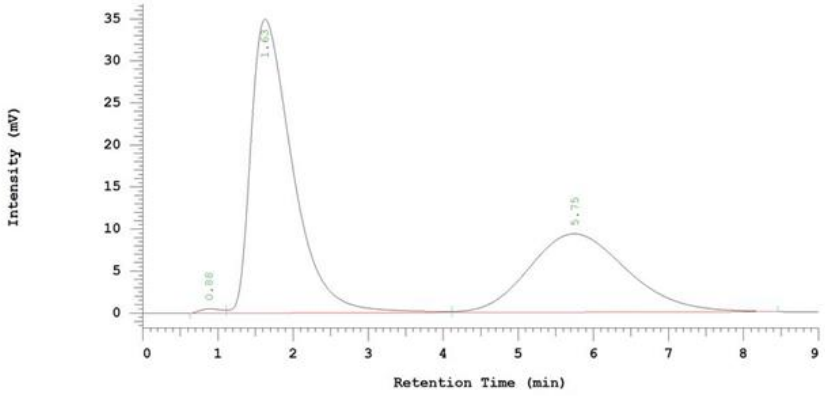
Appendix.G chromatogram illustrating linearity for paracetamol and chlorozoxazone (stainless steel monolithic column)

D-2000: Samples Series: 0236 Report Name: original System: Sys 1

D-2000 Elite HPLC System Manager Report

Analyzed Date and Time: 07/18/2017 07:07 PM
Reported Date and Time: 07/18/2017 07:16 PM
Processed Date and Time: 07/18/2017 07:16 PM
Data Path: C:\Win32app\D2000HSM\samples\DATA\0236\
Processing Method: para czn 10
System (acquisition): Sys 1
Application(data): Samples
Sample Name: UNKNOWN001
Injection from this vial: 1 of 1
Sample Description:
Series: 0236
Vial Number: 1
Vial Type: UNK
Volume: 10.0 ul

Chrom Type: HPLC Channel : 1



Processing Method: para czn 10
Column Type: Column
Method Developer:
Pump A: L-2130
Pump A Solvent A:
Method Description:

Chrom Type: HPLC Channel : 1
Peak Quantitation: AREA
Calculation Method: AREA%

No.	RT	Area	Area %	R-Factor	BC	Group	No.
1	0.88	10392	0.472		BV		1
2	1.63	1344394	61.064		VV		2
3	5.75	846833	38.464		VB		3
		2201619	100.000				

Peak rejection level: 0

Page Indicator: 1

Appendix.H chromatogram illustrating accuracy for paracetamol and chlorozoxazone (stainless steel monolithic column)

D-2000: Samples Series: 0254 Report Name: original System: Sys 1

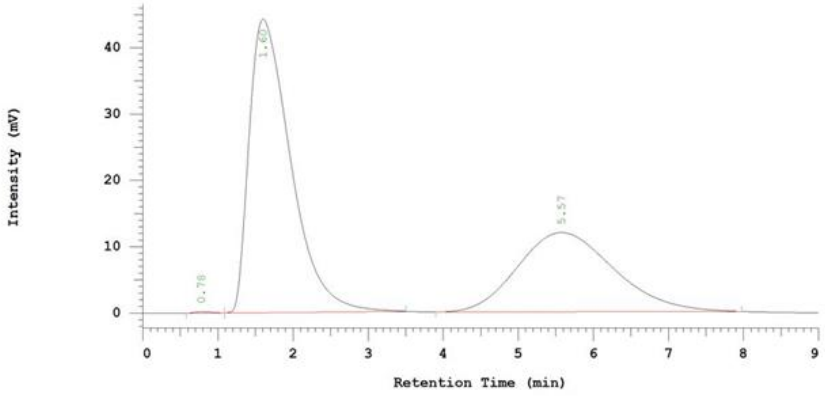
D-2000 Elite HPLC System Manager Report

Analyzed Date and Time: 07/18/2017 11:03 PM
Reported Date and Time: 07/18/2017 11:12 PM
Processed Date and Time: 07/18/2017 11:12 PM
Data Path: C:\Win32app\D2000HSM\samples\DATA\0254\
Processing Method: para czn 10

System (acquisition): Sys 1
Application(data): Samples
Sample Name: UNKNOWN001
Injection from this vial: 1 of 1
Sample Description:

Series: 0254
Vial Number: 1
Vial Type: UNK
Volume: 10.0 ul

Chrom Type: HPLC Channel : 1



Processing Method: para czn 10
Column Type: Column
Method Developer:
Pump A: L-2130
Pump A Solvent A:
Method Description:

Chrom Type: HPLC Channel : 1
Peak Quantitation: AREA
Calculation Method: AREA%

No.	RT	Area	Area %	R-Factor	BC	Group	No.
1	0.78	3915	0.142		BB		1
2	1.60	1698511	61.386		BB		2
3	5.57	1064519	38.473		BB		3
		2766945	100.000				

Peak rejection level: 0

Page Indicator: 1

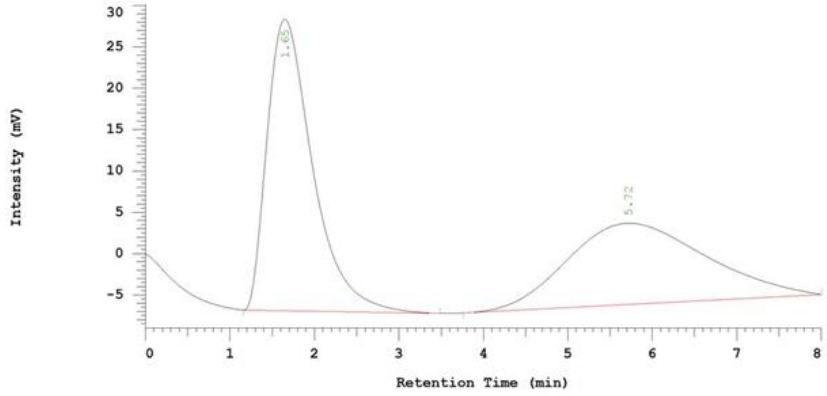
Appendix.I chromatogram illustrating precission for paracetamol and chlorozoxazone (stainless steel monolithic column)

D-2000: Samples Series: 0336 Report Name: original System: Sys 1

D-2000 Elite HPLC System Manager Report

Analyzed Date and Time: 07/20/2017 12:09 AM
Reported Date and Time: 07/20/2017 12:18 AM
Processed Date and Time: 07/20/2017 12:18 AM
Data Path: C:\Win32app\D2000HSM\samples\DATA\0336\
Processing Method: para czn 10
System (acquisition): Sys 1
Application(data): Samples
Sample Name: UNKNOWN001
Injection from this vial: 1 of 1
Sample Description:
Series: 0336
Vial Number: 1
Vial Type: UNK
Volume: 10.0 ul

Chrom Type: HPLC Channel : 1



Processing Method: para czn 10
Column Type: Column
Method Developer:
Pump A: L-2130
Pump A Solvent A:
Method Description:

Chrom Type: HPLC Channel : 1
Peak Quantitation: AREA
Calculation Method: AREA%

No.	RT	Area	Area %	R-Factor	BC	Group	No.
1	1.65	1320630	54.254		BB		1
2	5.72	1113522	45.746		BB		2
		2434152	100.000				

Peak rejection level: 0

Page Indicator: 1

Appendix.J chromatogram illustrating robustness for paracetamol and chlorozoxazone (stainless steel monolithic column)