# DETERMINATION OF SELECTED ACTIVE SUBSTANCE IN THE PREPARATION IX

Determination of related substances of Bromhexine Hydrochloride active substance using Ultra High-Performance Liquid chromatography

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A Thesis

Presented to the Faculty of Pharmacy of Charles University in Prague

Department of Pharmaceutical Chemistry and Drug Control

In Partial Fulfillment of the Requirements for the Degree of

Master of Pharmacy


May 2012, Hradec Kralove

I declare I processed this thesis on my own. All bibliographic sources and other materials that I used for this work are listed in the references and cited properly.

15 May 2012, Maltina Shkodra

ACKNOLEDGEMENTS

I would like to show my gratitude to my supervisor PharmDr. Petr Kastner, Ph.D., for his willingness and patience to guide me throughout the work of this thesis. His explanations from the initial to the final step have enabled me to develop a deep understanding of the subject.

PharmDr. Petra Kovařikova, Ph.D., and PharmDr. Radim Kučera, Ph.D., on teaching me the fundamentals of method validation for analytical procedures on the basis of the subject “Therapeutic Drug Monitoring”;

My sister Blerina for her encouragement and help during the laboratory work;

Lastly I offer my regards and blessings to my family and friends who has supported me in any respect during completion of this dissertation.

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# 1. INTRODUCTION

Liquid chromatography (LC) is an analytical chromatographic technique that has long been used in the separation of sample mixtures. As the first type of chromatography discovered, over time its advancement has lead to the improvement of chromatographic performance during analytical procedures. High-Performance Liquid Chromatography is one of the most widely used chromatographic techniques for determination of drugs in pharmaceutical preparations, drug bio-analysis and food products analysis. The latest upgrade on chromatographic systems entails that of Ultra High-Performance Liquid Chromatography (UPLC). It is one of the most sophisticated instruments utilized in analytical procedures nowadays. This avant-garde technology has successfully demonstrated an increase in laboratory productivity and chromatographic performance efficiency, which is the reason of its high applicability in world-leading scientific laboratories.

In this dissertation the development and validation of a new method for determination of related substances in an active pharmaceutical ingredient (Bromhexine Hydrochloride) using UPLC has been discussed. The method was transferred from the conventional procedure using HPLC guided by European Pharmacopeia. Several optimizations of chromatographic conditions have been carried out in order to combine the most optimal ones to be used for analysis. Testing the linearity, precision, accuracy and selectivity of analytical procedure ensured the validity of the newly developed method.

Furthermore, the apparatuses, principal of work and the differences on chromatographic performance of both instruments (HPLC and UPLC) have been described in detail.

# 2. AIM OF WORK

The objective of the work was to develop a new method for determination of Bromhexine Hydrochloride active pharmaceutical ingredient (API) using Ultra High-Performance Liquid Chromatography in favor of increasing the analytical performance efficiency in comparison to conventional method using HPLC. The newly developed method should spare the usage of large volumes of organic solvents and shorten the analysis time, while preserving a reliable chromatographic performance during method validation, with resulting satisfactory parameters to be used in Drug Control.

# 3. THEORETICAL PART

## 3.1 Instrumentation and Analytical Methods

### 3.1.1 High performance liquid chromatography

HPLC is a form of liquid chromatography and the most commonly used chromatographic technique for determination of drugs in pharmaceutical preparations, drug bio-analysis and food products analysis. It is a separation technique utilized to analyze, identify, quantify and purify the individual components of sample mixture.

#### *3.1.1.1 General Principle*

HPLC is a highly sophisticated system, consisting of four main parts: the solvent delivery system, the separation column, the detector and the data system. The column comprise of the stationary phase of densely packed particles. The mobile phase is pumped at a constant flow rate against the high pressure through the column and the sample mixture is injected into the flowing mobile phase just prior to the column inlet. Analytes are separated on the basis of their affinity to the stationary phase and depending on their individual migration speeds, they elute from the column at different times and are thus separated. The equilibrium distribution of each compound between the stationary phase and mobile phase determines the differential migration of each of them through column. The distribution constant ($K_C$) is dependent on the composition of both phases and also the temperature in the column oven.

$$Kc = \frac{Cs}{Cm}$$

**Equation 1. Distribution Constant[1]**

Where, Cs is the concentration of analyte on the stationary phase
$C_m$ is the concentration of analyte on the mobile phase

The pumps provide high operational pressure (instead of gravity) to deliver the mobile phase and sample contents through the porous material of stationary phase in the column, hence the name High Pressure Liquid Chromatography. The detector is a device used to visualize the analytes on a chromatogram. The computer system prints the resulting chromatograms and can also provide an automated process of the whole analysis, which is one of the main reasons why automated HPLC has become the most widely used technique in pharmaceutical analysis[1].

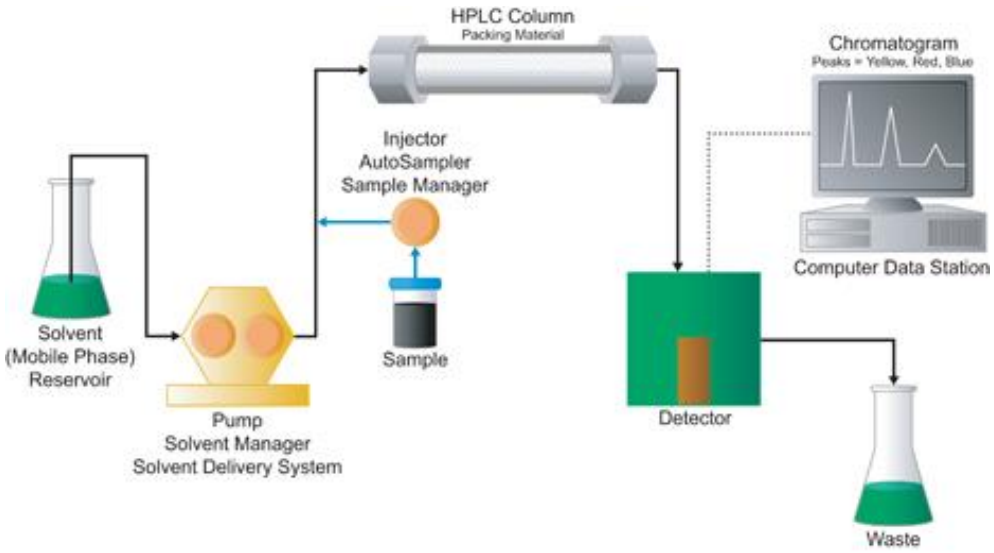



**Figure 3.1 HPLC apparatus diagram[2]**

***Retention time*** **($t_R$)** is the amount of time elapsed during which analytes travel from the injection site in the column to the detector. Retention times are depicted in chromatogram against the detector signal and they are different for each compound. ($t_M$) is the time elapsed for the mobile phase to pass through the column cavity.

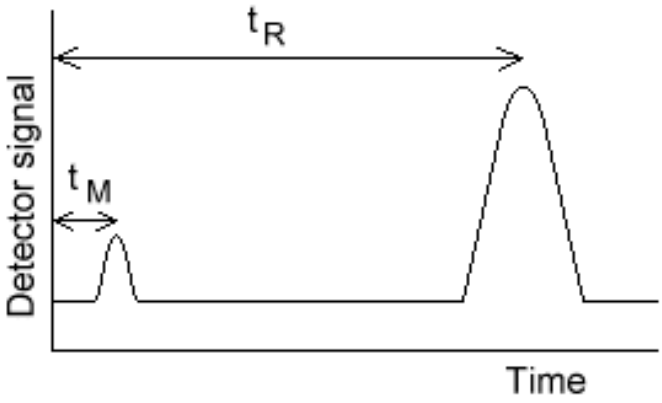



**Figure 3.2 Chromatogram with analyte and mobile phase retention times[3]**

#### 3.1.1.2 *Parameters affecting the chromatographic performance*

***Retention factor (k)*** also known as capacity factor, is the degree of retention of sample component in the column and is defined as the time the solute resides in the stationary phase **($t_R$),** relative to the time it resides in the mobile phase **($t_M$)[4]:**

$$k = \frac{t_{R-}t_M}{t_M}$$

**Equation 2. Retention factor (k)**

Retention factors are distinct for every chemical identity and they usually lie in the range between 2-5, except for samples of high molecular complexity, where the retention factor range needs to be higher to have satisfactory separation results.

***Selectivity factor*** also known as separation factor (α), describes the separation of two compounds. It measures the separation selectivity of components for the specific chromatographic method used:

$$\alpha = \frac{k_2}{k_1} \qquad \alpha > 1$$

**Equation 3. Selectivity factor**

The value of selectivity factor has to be more than 1, in order to have a good separation of the peaks in chromatogram.

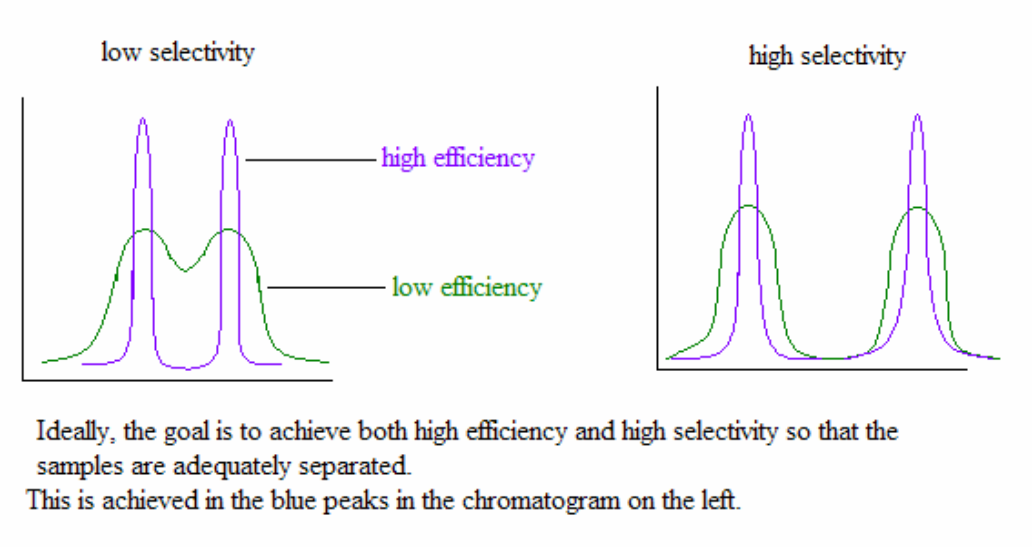



**Figure 3.3 Chromatograms showing the influence of selectivity factor on peak separation[5]**

Manipulating with the composition of mobile phase, column temperature and composition of stationary phase the selectivity factor can be increased to improve the separation process.

***Flow rate*** of mobile phase is adjusted before starting the analysis. Dependent on the diameter of the column, the flow rate should be set in order to have a linear velocity of mobile phase in the column. Linear velocity (V) is the speed at which the solvent front travels the length of the column (L), and is calculated by dividing the column length by the retention time ($t_0$) of an unretained component[6]:

$$V = L/t_0$$

**Equation 4 Linear velocity**

When the samples are injected in the column the analytes collect at the top and they descend by gradually blocking the column. This results in a an increase in backpressure, which can compromise the analysis process by decreasing the flow rate, thus it is important to keep the flow rate constant in order to have a constant gradual blockage of the column. The range of flow rate is 0.01-10ml/min, but the typical flow rate used is 0.5-2.0ml/min[1].

***Temperature*.** Column oven temperature should be kept constant to have satisfactory results of separation performance. Higher temperatures can improve the process of segregation, although temperatures above 60°C are rarely employed due to possible degradation of stationary phase and mobile phase evaporation. Unless, otherwise specified in individual monographs, columns are used at ambient temperatures[7].

***Particle size*.** Columns are composed of an inside porous material of packed micro-particles, with usual average particle diameter of 3μm, 5μm or 10μm. The particle size is an important parameter on controlling the backpressure of the column and the separation efficiency. Column backpressure and column efficiency are inversely proportional to the square of the particle diameter, meaning that as the particle size decreases, the column backpressure and efficiency increase[8]. The smaller particle size columns are more suitable for more complex mixtures with similar components due to higher separation efficiency, while the larger ones are used more frequently for analysis of compounds with greater structural differences.

***Physical dimensions***. Typical column lengths used in HPLC are 5, 10, 15, 25cm. Long columns provide increased efficiency and resolution, but their drawback stands in long retention times of analytes and increased backpressure. The internal diameter (**ID**) of the column is also very important factor influencing the performance. Columns of smaller

internal diameter size support higher sensitivity due to higher concentration of the analyte in mobile phase and their advantage stands on the reduced flow rate and mobile phase volume needed to reach the same optimal linear velocity without increasing the analysis time. A study on the efficiency of columns with different ID but same column length (238mm) at constant flow-rate shows the performance of columns with ID 4.6mm, 4.0mm, 3.2mm and 2.1. It was observed that in columns with ID 4.0mm and 3.2mm the reduction in plate height (HEPT) was the lowest, the 3.2mm ID column had higher reduction and the 2.1mm ID column had the highest reduction in HEPT. On the other hand columns with ID 4.0mm and 3.2mm showed increased efficiency for low concentrated sample, while the 4.6mm ID column showed highest load capacity and the 2.1mm ID column showed lowest load capacity[9]. Column length (**L**) and internal diameter (**ID**) determine the volume bed (**V**) i.e. the minimum volume of mobile phase required to elute an unretained analyte from the column[8].

***Column efficiency*** . The separation efficiency of analytes in the column is determined by the extent to which the compounds are spread in the column. If the analytes spread to a large extend in relation to their retention times, the peaks will have wider bands meaning that the column wasn't as efficient on separation. The narrow peaks indicate high column efficiency. In order to have optimal separation, peak broadening should be limited. Band broadening is expressed by the number of the theoretical plates (**N**) in the column. As the name suggests, these plates are theoretical separations of layers of columns, where the distribution of the sample and mobile phase undergo through separate equilibrations[10]:

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$$

**Equation 5. Theoretical plates**

$\mathbf{t_R}$ - retention time of analyte
$\mathbf{W_{1/2}}$ - the peak width at half-height from the baseline of chromatogram

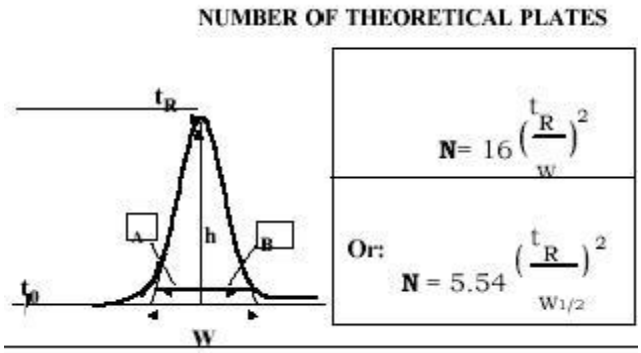



**Figure 3.4 Number of theoretical plates[11]**

The separation efficiency increases with the increasing number of theoretical plates, hence the formula for individual height of theoretical plates in relation to column length and plate number:

$$HETP = \frac{L}{N}$$

**Equation 6. Height equivalent to theoretical plates[10]**

**L**- length of column
**N**- number of plates

The smaller the **HETP**, the larger the value of **N** hence, the column efficiency increases.

***Resolution***- the degree of separation of two peaks is defined as resolution, $R_S$ [1]:

$$R_S = \frac{2\,(t_{R1} - t_{R2})}{W_1 + W_2}$$

**Equation 7. Peak resolution**

$\mathbf{t_{R1}}$ , $\mathbf{t_{R2}}$ - retention times of two components in chromatogram
$\mathbf{W_1}$, $\mathbf{W_2}$ - corresponding peak width at half peak height

There are three parameters to be considered to achieve high resolution:

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_2}{k'_2}\right)$$

**N** – reducing the particle size of stationary phase, also reduces the HETP will be reduced and in turn increases N;
**k'**– manipulating with composition of mobile phase to improve retention factor
**α** – optimize selectivity factor (mobile phase, column t (°C), stationary phase)

***Asymmetry factor*** **($A_S$)** is used to determine the peak symmetry:

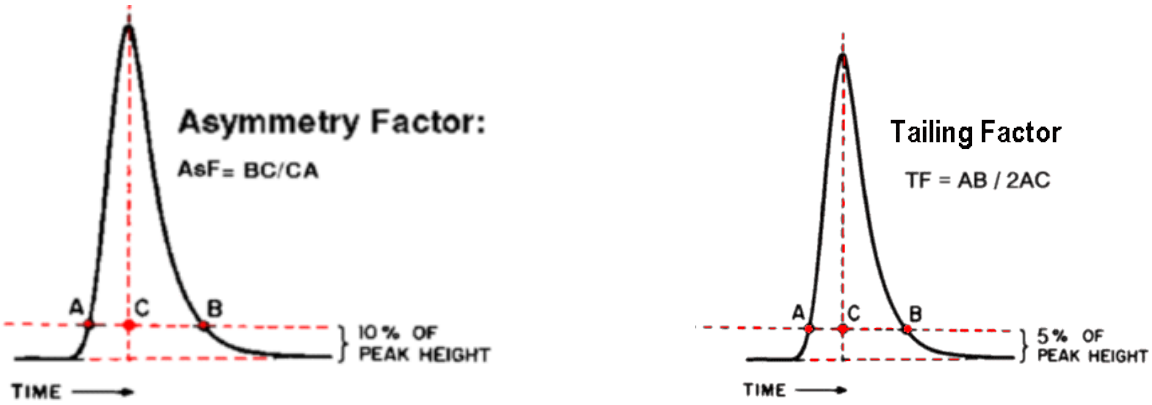



**Figure 3.5. Asymmetry factor; Tailing Factor[12]**

**$W_{0,05}$** - peak width in 1/20 of its height
**AC** - the distance between the perpendicular and the rising part of the peak
**BC** - the distance between the perpendicular and the decreasing part of the peak

**$A_S = 1$** -> peak is symmetric; **$A_S > 1$** -> peak is tailing; **$A_S < 1$** -> peak is fronting.

#### 3.1.1.3 *Operational modes and stationary phases*

The separations are achieved based on the different interactions of analytes to stationary and mobile phases. Depending on the type of the stationary and mobile phases used, there are several modes of separations:

- ***Ion exchange*** is based on charge-charge interactions between ionic groups of analytes and oppositely charged functional groups bound to the stationary phase. The mobile phase usually consists of aqueous buffers or ionic solutions and it's suitable for analysis of charged biological molecules such as: alkaloids [e.g. nicotine determination on hair of active smokers[13]]; proteins [e.g. detection of thalassemia/hemoglobin variants evaluated in a prospective study in a tertiary care center in north India [14]]; inorganic ions etc.

- ***Size exclusion*** chromatography (SEC) is based on the elution of solutes by mobile phase through the porous material of the column, without interacting with the stationary phase. In this manner the small molecules will be able to penetrate both the porous particles and the inter-particle volume, resulting in longer retention time; while the larger molecules can only penetrate the inter-particle volume due to their size, thus will be eluted faster from the column and be placed first in the chromatogram. Mobile phases used can be aqueous buffers or organic solvents. SEC is mostly used to determine large biological molecules and in polymer characterization [e.g. gyration distribution of polystyrene, polyphosphazenes and poly (ethylene oxide) using SEC[15]].
- ***Affinity*** chromatography is characterized with a specific ligand bound to the stationary phase. The protein(s) to be analyzed will therefore interact by means of hydrogen bonding, Van Der Waal's and/or electrostatic forces with their specific ligand and be successfully separated with the highest selectivity and resolution provided by this method. Now days there are many current studies aiming the design and manufacture of novel ligands tailored to specific biotechnological needs, by utilizing combinatory chemistry and molecular modeling[16].
- ***Chiral*** chromatography is solely employed to separate chiral molecules from their racemic mixture. The principle includes the usage of chiral stationary phases, i.e. a single enantiomer bound to the achiral matrix of stationary phase such as silica gel or various oligosaccharides (cellulose, cyclodextrines). When eluted through column, the enantiomers of the analyte will exert different affinity to the single enantiomer bound to the stationary phase, therefore their retention times will differ in the resulting chromatogram. Screening for new chiral stationary phases to improve the analysis and separation of chiral pharmaceutical drugs by different HPLC modes is increasing vastly. A strategy employing RP-HPLC using cellulose/amylose stationary phase, was successfully applied to set 37

diverse chiral pharmaceuticals. Satisfactory enantioselectivity was achieved for 89% of them[17].

- ***Adsorption***. The analytes along with a non-polar mobile phase (e.g. hexane) compete for adsorption sites of polar stationary phase (silica) in the column. Analytes with no affinity to polar sites will be retained from column together with mobile phase. Since adsorption is a reversible reaction, if polarity of mobile phase is increased, it would interfere the analyte-stationary phase interaction resulting in a faster elution of the compound.
- ***Partition*** is based in hydrophobic interaction of the non-polar stationary phase with analytes and the polar mobile phase. This is principle used very often in **Reversed Phase HPLC**

#### 3.1.1.4 Reversed Phase HPLC

***Stationary phase*** in most cases consists of derivatized silica with non-polar functional groups to the silanol moieties using chlorosilanes or other silane reagents[1]. There are many hydrophobic functional groups used to manipulate the silica matrix, with Octadecyl (C18) being the most favorable one considering it's high hydrophobicity. The free silanol groups left unreacted due to the steric hindrance of three alkyl groups in the silicum atom are treated further with trimethylsilylating reagents, in the process called *endcapping*. These reagents react with remaining silanol groups and they also provide protective shield on the stationary phase. From frequently used endcapping reagents, trimethylsilylphosphine has shown to be a more effective one, with improved efficiency i.e. reduced peak tailing for all solutes tested, compared to phases treated with trimethylchlorosilane, hexamethyldisilazane, or a combination of these two[18].

| |
|---|
| $CH_3 (CH_2)_{17}$ –; Octadecyl |
| $CH_3 (CH_2)_7$ - ; Octyl |
| $C_6H_5 (CH_2)_3$ - ; Phenyl |
| $CH_3 (CH_2)_3$ - ; Butyl |
| CN $(CH_2)_3$ - ; Cyanopropyl |

**Table 1. Non-polar functional groups ranked on declining hydrophobicity[1]**

The main disadvantage of silica-based stationary phase is that only mobile phases with pH range 2-8 can be used, due to the instability of siloxane bonds. However, polymeric-based and zirconium-based stationary phases with wide pH stability (1-11) are now available in market[12].

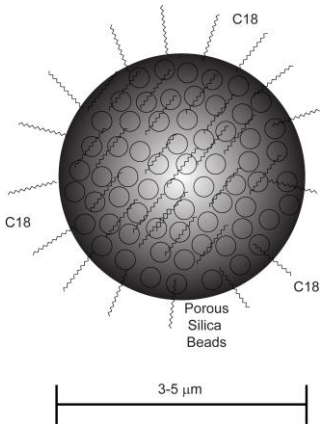



**Figure 3.6 Derivatized silica with C18[19]**

***Mobile phase***. The polar nature of mobile phase in RP-HPLC consists of mixtures of water and an organic solvent miscible with water. The strength of the mobile phase is determined according to the choice of organic solvent and its' percentage in the aqueous solution. Since the stationary phase is of non-polar nature, the increase of organic content, increases the strength of mobile phase, therefore desorbing the analytes from the hydrophobic stationary-

phase surface will result in shorter retention of compounds in column. The organic part of mobile phase is usually methanol, acetonitrile or tetrahydrofuran (their concentration ranging from ca. 15-90%) of etc. When performing a separation using a multi-solvent mobile phase system of these organic modifiers, the average retention times will be somewhat similar for all three of them, however the elution order between substances will be somewhat different because the selectivity in the three systems can be different[1].

#### 3.1.1.5 Pumps

Pumps are devices used to deliver the mobile phase at a constant flow rate into the column. They consist of piston(s), which are moved back and forth by the motor cam through the cylinder where the mobile phase enters through the inlet valve. When the piston is moved back the outlet valve closes and the mobile phase enters the cylinder; when the piston is moved forward the outlet valve opens forcing the entrance of mobile phase into the column. During pumping, the fluid flow pulsates, adding extra noise in the detector signal; therefore pulse dampeners are used to keep the fluid flow smoothly[1].

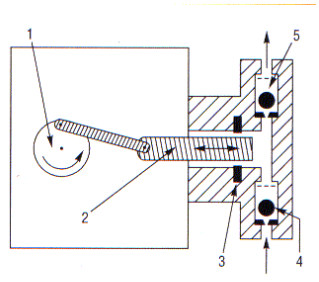



**FIGURE 1:** Schematic diagram of a generic LC pump. 1 = motor cam, 2 = piston, 3 = pump seal, 4 = inlet check valve, 5 = outlet check valve. (Reprinted courtesy of LC Resources Inc. [Walnut Creek, California])

**Figure 3.7. Cross-sectional view of pump work principle[20]**

When a constant composition of mobile phase is pumped through the column this elution is called i*socratic.* Conversely, when the composition of mobile phase is changed during the

analysis i.e. the analysis starts with a mobile phase of weaker eluting strength and it increases during the process, this is called *gradient elution*. The advantage of the gradient elution stands on the shortage of analysis time with better peak shapes, when components with big difference in retention times are being analyzed. The faster eluting analytes will have enough time for retention, thus proper separation and the later eluting substances will elute faster and have better peak shape in comparison to isocratic elution.

#### 3.1.1.6 Detectors

LC detectors give a visual representation of the sample being analyzed, by converting their response into electrical signals. These signals are depicted in chromatogram in the shape of peak areas and peak heights, which correspond to the concentration or masses of analytes. Depending on the chemical structure of substance being analyzed there are several detectors employed for detection in HPLC analysis: Spectrophotometric, Fluorescence, Mass spectrometric, Light Scattering.

Among these, the spectrophotometric and mass spectrometric detectors are the most commonly used.

***Spectrophotometric- UV/VIS detectors***

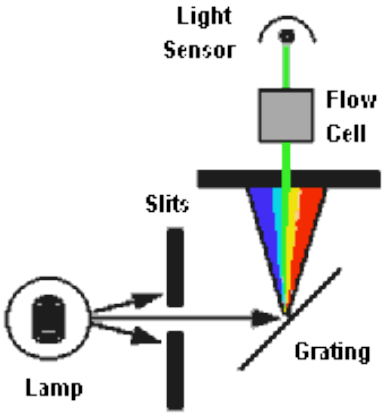
UV detectors are the most widely used detectors for both qualitative and quantitative determination on pharmaceutical analysis. Their frequent utilization as HPLC detector is based on their high sensitivity, ease of use and good stability. The principle of UV spectrophotometric detection relies on the absorption of UV light by the analyte. The Beer's law explains the absorption of radiation by a solution:

$$A = \varepsilon \, l \, c$$

**Equation 8. Beer's law**

According to this law the absorbance (A) is proportional to the path length through which the radiation passes (l) and the concentration of the substance in the solution (c). (ε) is the molar absorptivity of the substance which is constant and specific for every molecule.

The detector consists of a lamp that generates the radiation through the analyte in the flow cell. Depending on the substance to be detected there are two types of lamps used: the deuterium lamp- able to emit continuum light on wavelength range 190-400 nm, suitable for molecules containing a chromophore; and the tungsten lamp – which emits radiation at higher wavelengths of visible range up to 700 nm. On a *Variable UV detector* a monochromator ensures the correct direction of one selected wavelength of light to pass through the flow cell. Whereas on a *Photodiode Array detector* a polychromatic radiation is passed through the flow cell and the transmitted light is split in an optical lattice into the individual wavelengths, which can be measured by a number of photodiodes, giving a full UV spectrum of a peak in order to identify a substance[1]. The light sensor collects the maximum generated signal passed through the flow cell, which is electronically inverted by the data system resulting in the appearance of a positive peak in the chromatogram[21].



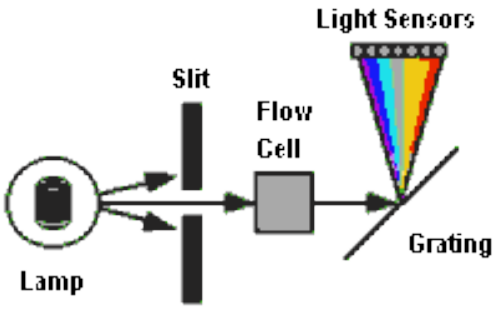




**Figure 3.8. A Variable UV detector;** **Photodiode Array UV detector**

The principle of quantitative analysis using UV detector is based on using the specific absorbance of the substance to calculate the unknown concentration as percentage (%), which is defined as:

$$A_{1cm}^{1percen} = \frac{10\varepsilon}{M_r}$$

$\mathbf{M_r}$ - relative molecular mass (molar mass)

**Equation 9. Specific absorbance[1]**

This refers to the absorbance of a solution containing 1g of the substance dissolved in 100ml of solvent measured at defined wavelength at a path of 1cm[1].

The principle for qualitative analysis using UV detector relies on identification of unknown molecules using a reference substance with identical absorption spectrum. The absorption of a substance is recorded as a function of wavelength[1]. Although a very useful method for identification, it is important to be careful on concluding the identification only by UV spectrophotometry due to similarities of different substances on their absorption spectrum.

***Mass spectroscopy detector***

Despite the high cost of instrument the mass spectroscopy detector has become very popular in liquid chromatography as a detection device in drug bio-analysis, environmental and pharmaceutical analysis. Its' high selectivity and sensitivity for identification and quantification of samples of low concentration and complex matrixes makes mass spectroscopic detector the detector of choice now days. The principle stands on ionization of sample with subsequent separation of ions based on their mass to charge ratio (m/Z).

Each MS detector consists of three main parts[12]:

- The sample ionization *source,* achieved by one of a variety of methods: atmospheric pressure ionization (API), electrospray ionization (ESI),

atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI)/

- The *analyzer* separates the ions according to their m/z under an electromagnetic field. There are several types of mass analyzers: quadruple mass filter, ion trap mass analyzers, time-of-flight mass analyzer etc. Each analyzer has it's own special characteristics and applications, as well as its own benefits and limitations[22].
- The *detector* records the charge induced when the ion passes by or the current produced when an ion hits a surface. The current generated by the passage of the ions is in turn amplified by an amplifier, which transfers the ratio output in a data acquisition system.

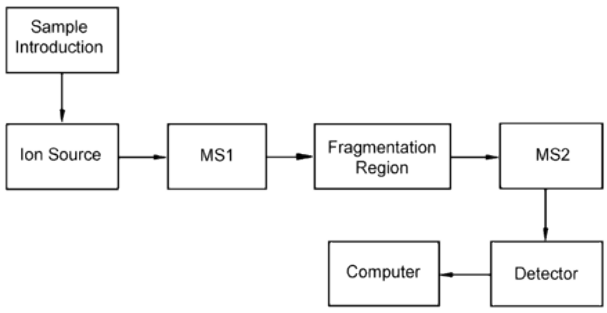



**Figure 3.9. Mass spectroscopic detector schematic diagram**

## 3.2 Ultra-fast performance liquid chromatography vs. HPLC

### 3.2.1 UPLC

The latest evolution in development of ultra-fast performance chromatography from conventional HPLC exploits the benefit of this state-of-the art technique in the field of analytical science in many folds. While HPLC is a deep-rooted technique for analysis of pharmaceutical compounds, it still has some shortcomings when it comes to analysis of high complexity samples, for example: samples containing around 0,1% relative impurities require high sample capacity in addition to high efficiency in order to be separated sufficiently and their concentration to be reliably measured[23]. By this saying, the needs for improvement of separation efficiency lead to the interest of developing new columns that would be able to generate higher resolution between the individual analytes in a sample and possibly reduce the analysis time. The advancement of columns used in UPLC has proven to not only increase the efficiency of separation of compounds, but also have satisfactory results of analysis in increased flow rates and linear velocities.

#### *3.2.1.1 The sub-2 µm particles*

The reduction in the densely packed particles' size inside the columns is the breakthrough strategy that implemented the basics of UPLC analysis. In comparison to HPLC particles' size (3 µm, 5 µm, 10 µm), the columns used in UPLC are packed with particles of sub-2 µm diameter. Since the particle size diameter (dp) is inversely proportional to the number of theoretical plates (N):

$$N \propto \frac{1}{dp}$$

**Equation 10. The inverse proportion of particle size (dp) and (N)**

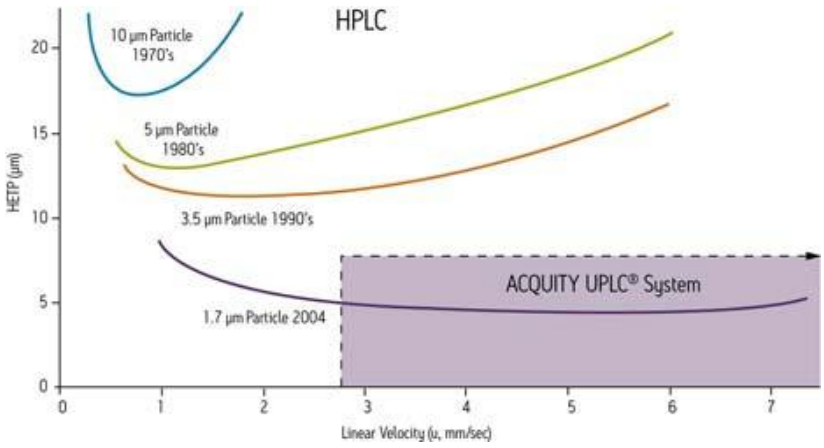
And resolution (R) is proportional to the square root of *N* (eq.7), then as the particle size is lowered by thrice i.e. from 5 µm to 1.7 µm, *N* is increased by three and the resolution by 1.7[24]. *N* is also inversely proportional to the square root of peak width:

$$\boldsymbol{N \propto \frac{1}{W^2}}$$

**Equation 11. The inverse proportion of *N* and *W***

Which indicates that the narrower the peaks the better the separation. And as the peak height is inversely proportion to peak width, this reduction in particle size also increases the sensitivity because narrower peaks are taller.

Van Deemter curve shows the amplification of chromatographic performance with usage of sub- 2 µm particles, enabling the increase of optimum flow (linear velocity) of mobile phase to reach the maximum *N[25]:*




**Equation 12. Van Deemter curve on different particle size[26].**

This graph shows that the curves are much steeper for particles of bigger size at high linear velocities, which means that in order to reduce the analysis time to acceptable values, columns packed with common particle size are often operated at linear velocities which do not give maximum efficiency[23]; in comparison to the use of columns packed with smaller particles size that are able to give high efficiency at high linear velocities.

#### 3.2.1.2 The need for high pressures

The main difficulty using smaller diameter packings is that the pressure required to pump the mobile phase through the column increases with the square of the particle diameter[27]. This necessity lead to the development of special equipment to generate high pressures. In comparison to HPLC, which have an operating pressure around 400bar, the UPLC systems are characterized with an ultra-high generating pressure of around1000bar (100MPa). The pumps are designed to withstand the high pressure while delivering the solvent smoothly and reproducibly, during both an isocratic or gradient elution[23]. The injection of sample is designated with fast injection cycles (around 25 s a cycle), low sample volumes with negligible carryover[28]. The signal detection of peaks in detectors is yet another critical adjustment on UPLC system. Considering the ability of columns packed with sub-2 μm packed particles to obtain very small peaks in less than one second, the detector sampling rate must be high enough to capture enough data points across the peak and have minimal dispersion (volume) to ensure a good separation efficiency[25]. Therefore the advancement of detectors employed in UPLC, are improved in order to have much higher sensitivities in sample detection. The spectrophotometric UV detectors used in UPLC have a smaller flow cell volume to maintain the concentration and signal strength.

#### 3.2.1.3 UPLC application

Ever since the UPLC instrument became available in the market, its application was increasing vastly. The advantages of short time analysis compared to conventional HPLC, without compromising the resolution were a huge incentive for pharmaceutical industry. Simultaneous determination of many active ingredients with high chromatographic efficiency is now much more convenient using UPLC. A study in procedure transfer from HPLC-MS to

UPLC-MS/MS for determination of antidementia drugs in human plasma concluded that both techniques are reliable however, the UPLC–MS/MS method is preferable with respect to specificity, sensitivity and speed selectivity[29]. Similar comparison study between HPLC and UPLC, using identical columns, tubing, flow rates, and pressures, and connected to the same tandem mass spectrometer (MS/MS), showed how UPLC can reduce the analysis time in half in immunosuppressant monitoring[30]. The use of UPLC is being largely employed in food analysis as well, such as: determination of sulfonamide residues present in meat[31]; analysis of mutagenic heterocyclic amines (HAs) in complex food samples[32]; determination of priority pesticides and transformation products in baby food[33].

The benefits of UPLC have also proved to provide a reliable technique on environmental analysis. The determination of estrogens in different water matrices using UPLC–Q-TOF-MS demonstrated the applicability of this newly developed method for quantitative and confirmatory analysis of estrogens investigation[34].

Forensic analysis studies on determination of amphetamine-type substances and ketamine show significant enhancement of separation efficiency in less than 3 min with acceptable resolution when using UPLC in toxicological analysis[35].

One of the most important utilization of UPLC/MS is its application in drug discovery for high-throughput solubility screening of potential drug candidates in the early stages of discovery. This method was concluded to be more suitable for high-speed solubility assays than traditional HPLC/UV technology[36].

### 3.3 Validation

According to *the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use* (ICH), the analytical procedure chosen for

the intended purpose should manifest its suitability of use. There are several performance characteristics used in method validation:

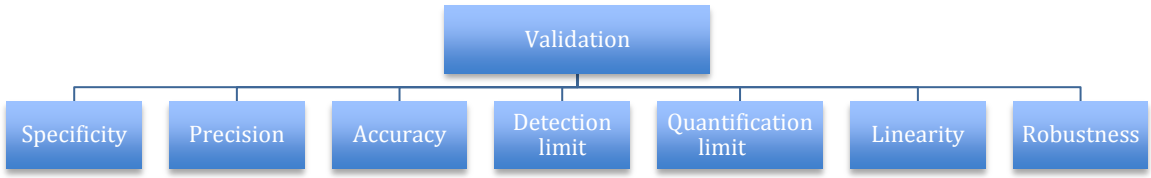



**Figure 3.10. Performance characteristics used in method validation**

***Specificity*** of an analytical method is defined as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be expected to be present in the sample matrix[37]. According to the ICH, in chromatographic procedures the chromatograms should demonstrate the degree of selectivity and peak purity test based on spectra (e.g. UV/VIS) are used to verify that the analyte peak doesn't correspond to more than one component[1].

***Precision*** of a method is the extent to which the individual test results of multiple injections of a series of standards agree[37]. It is expressed as the relative standard deviation (% RSD) of the mean (m) of a series of measurements: %RSD = RSD/m x 100% [1].

Precision can be measured in three levels:

*Repeatability*- precision measuring by the same analyst, with the same equipment used, same operating conditions over a short period of time, within the same laboratory. According to ICH, minimum nine determinations covering the specified range for the procedure should be used.

*Intermediate precision* (ruggedness) expresses the inter-laboratory variations, performed by different analysts and different equipment.

*Reproducibility* expresses the precision of method between different laboratories in a collaborative study[1].

***Accuracy*** of a method is the extent to which the test results generated by the method and the true value agree.[37]. When assessing an assay of drug substance, accuracy of the method can be determined by employing a certified reference substance of known purity, whereas in assay of a drug substance in a formulated preparation, a known amount of the drug substance (certified reference) is added to the blank formulated product (drug-free)[1]. The accuracy of a method is then presented as a percent recovery (ideally 100%), or as the difference between the mean and accepted value. According to the ICH, a minimum of nine determinations over a minimum of three concentration levels covering the specified range should be used to determine accuracy.

***Detection limit*** shows the minimal analyte concentration that can be detected during analysis and is expressed as the concentration of the analyte. In chromatography the detection limit is based on the signal to noise ratios (S/N ratio). S/N ratio is determined by measuring signals of samples with known concentrations and the baseline noise of blank samples. Ratios of 3:1 or 2:1 are generally accepted values for detection limit estimation[1].

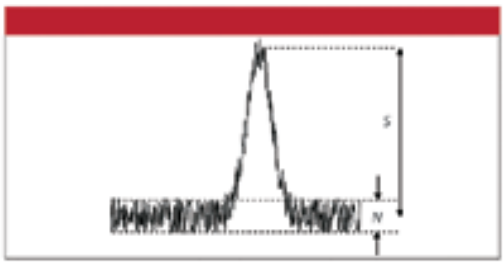



**Figure 3.11. S/N ratio[38]**

***Quantification limit*** is the lowest amount of an analyte in sample, which can be quantified accurately and precisely. In chromatography, the resulting peak of the analyte (H) should be ten times higher than the baseline noise (h) i.e. S/N is 10:1. The quantification limit can also be determined by employing the injected samples with decreasing amount of analyte from

precision data and the % RSD is plotted against the analyte concentration. The usual criteria to define limit of quantitation is set to 20%[1].

***Linearity*** The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analytes in samples within a given range[37]. According to ICH, determination of linearity should be based on a minimum of five concentrations, with specified ranges of concentration from 80-120% for assay of active substance or formulated product and 50-120% range for determination of impurity. Linearity can be evaluated graphically by plotting the deviations from the regression line versus the concentration or versus the logarithm of the concentration[37].

***Robustness*** is the evaluation the effect of variations of different operational parameters on the analytical method. Parameters like pH, column temperature, flow rate, detection wavelength etc., are changed and their variation is quantified. This difference should be within the specified range of robustness for the method to be considered correctly validated.

#### 3.3.1.1 System suitability test

It is a test of continuous evaluation of robustness during the method development. The mostly used parameters in chromatographic procedure are: Number of theoretical plates (N), Retention factor (k'), Resolution ($R_S$), Relative Retention and Asymmetry factor (A). In Ph.Eur and USP the requirements for system suitability test of HPLC methods are typically:

- %RSD of peak areas or peak heights are <1% (for drug substance assay)
- The resolution ($R_S$) is >2
- Asymmetry factor (A) is in the range 0.8-1.5
- The number of theoretical plates (N) are larger than 2000 (for HPLC)
- The retention factor (k') is >2.

### 3.3.2 Bromhexine Hydrochloride

#### 3.3.2.1 Physical and Chemical Properties

*Molecular formula:* C14H20Br2N2, HCl
*Molecular weight*: 412.6
*CAS Registry:* 611-75-6
*Chemical name:* N-[2-Amino-3,5-dibromobenzyl]-N-methylcyclohexanamine hydrochloride.
*Appearance and solubility:* A white or almost white crystalline powder. It exhibits polymorphism. Very slightly soluble in water; slightly soluble in alcohol and in dichloromethane. Protect from light.[39]

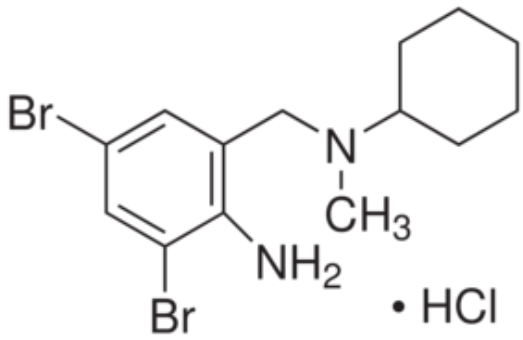



**Figure 3.12. Chemical structure of Bromhexine Hydrochloride**

#### 3.3.2.2 Pharmacology

Bromhexine Hydrochloride is a benzylamine derivative with expectorant/mucolytic activity. Its expectorant mechanism is explained by its ability to increase the water content in the airways secretion, thus hydrating the content of sputum, making it less sticky and easier for the bronchial cilia to expectorate the phlegm. The “mucoactive” effect reduces the viscosity of mucous by breaking down the mucin polymers (fibrin, DNA, F-actin) in the secretion of respiratory airways.

#### 3.3.2.3 Indications

It's pharmacological effect makes Bromhexine a useful prescribed medication for respiratory disorders associated with exorbitant mucus production. Several data explain the effect of Bromhexine in chronic bronchitis, which show that in patients with less severely impaired ventilatory capacity mucolytic therapy with Bromhexine is capable of producing objective as well as subjective improvement[40]. Unfortunately, the treatment didn't show satisfactory results in patients with more severely impaired respiratory capacity – patients with severely impaired ventilatory capacity when treated with Bromhexine did not improve objectively, but subjectively they felt better[40]. Some clinical observations showed that the usage of Bromhexine resulted in remission of lacrimal and salivary secretions in patients with Sjogren's syndrome. The usual dose of oral Bromhexine in the treatment of Sjogren's syndrome has been 16 milligrams orally three times daily.[41]

#### 3.3.2.4 Pharmacokinetics

Bromhexine Hydrochloride is rapidly absorbed from the gastrointestinal tract and it undergoes extensive first-pass metabolism in liver. Peak serum concentrations of Bromhexine occur approximately 1 hour following oral administration[42]. When administered orally, the bioavailability is around only 20%, because it bounds highly to plasma proteins and has a wide distribution on body tissues. It is excreted in urine mainly as metabolites, with Ambroxol being pharmacologically active among them.

#### 3.3.2.5 Adverse effects

During Bromhexine treatment infrequent gastrointestinal disturbances (nausea, epigastric pain, vomiting and diarrhea) have been noticed. A transient rise in serum aminotransferase values has been reported and other reported adverse effects include headache, vertigo (dizziness), sweating and allergic reactions.[43]

# 4. Experimental

## 4.1 Method development

A new method for determination of impurity content of Bromhexine Hydrochloride active pharmaceutical ingredient was developed using an Ultra High-Performance Liquid Chromatography. Bromhexine HCl (API) powder used for analysis was retrieved from the producer of preparation Bromhexine GALMED 8 – Galmed. The analytical procedure was transferred from the conventional method using HPLC described in European Pharmacopeia for determination of related substances on Bromhexine HCl (API). From the sample of test solution, two reference solutions were prepared:

- **Reference solution (a)** used for testing the optimization of chromatographic conditions on UPLC by evaluating the resolution ($R_S$), tailing factor (TF) and backpressure (P).
- **Reference solution (b)** used for the calculations during method validation.

### 4.1.1 Sample preparation

***Test solution***
The test solution was prepared according to the procedure in pharmacopeia:
50 mg of Bromhexine Hydrochloride was dissolved in methanol R. This solution was further diluted to 10.0 mL using the same solvent[39].

***Reference solution (a)***
According to pharmacopeia, 5 mg of Bromhexine impurity C CRS was dissolved in methanol. 1 mL of test solution was added and it was further diluted to 10.0 mL with the same solvent[39]. Due to restricted availability of this impurity in our laboratory, only 0.3 mg impurity C was used to prepare this reference solution with the same resulting concentration as the reference solution instructed by pharmacopeia. So 0.3 mg impurity C was dissolved in

methanol and the total volume of solution in order to have the same concentration was calculated to be 0.6 mL (as opposed to 10 mL). The calculated amount of test solution to be added was 0.06 mL; hence 0.54 mL of methanol was added to round up to 0.6 mL of reference (a) solution.

***Reference solution (b)***
1 mL of test solution was diluted to 100.0 mL methanol R. 1 mL of this solution was further diluted to 10.0 mL with the same solvent[39].

### 4.1.2 Solvents and reagents

***Mobile phase***
The composition of mobile phase used was also employed according to the procedure in pharmacopeia:

0.50 mL of *phosphoric acid R* was mixed in 950 mL of *water R*. The pH was adjusted to 7.0 with *triethylamine R* (about 1.5 mL) and was diluted to 1000 mL with water R. 20 volumes of this solution were mixed with 80 volumes of *acetonitrile R[39]*.

### 4.1.3 Optimization of chromatographic conditions

**"Prominence UFLC"** instrument by Shimadzu Corporation (Japan) was utilized to conduct analysis. The differences between HPLC columns used in analytical procedure from pharmacopeia and the UFLC columns used in our analysis are shown on the table below:

| Column specifications | HPLC (in Ph.Eur) | UFLC |
|---|---|---|
| ***Stationary phase description*** | End-capped C18 silica gel (3 µm) | **Kinetex 1.7 µm C18, 100A** |
| ***Column length*** | 120 mm | **150 mm** |
| ***Column Internal diameter*** | 4.6 mm | **2.1 mm** |

**Figure 4.1. Physical dimensions differences between HPLC and UFLC columns**

Optimization was tested on reference (a) solution. Two approaches were used to determine the chromatographic conditions for the new method, in order to utilize optimal conditions for analysis:

1. Adaption of different temperatures (from 25-40°C) at a flow rate 0.35 mL/min,
2. Adaption of different flow rates (from 0.35-0.6 mL/min) at 40°C temperature

Injection volume was **1.6µl**. The optimization was based on resulting resolution between the peaks of Impurity C and Bromhexine Hydrochloride, the tailing factors of peaks and the backpressure limit.

1. **Adaption of different temperature adjustments (from 25-40°C) at a flow rate 0.35 mL/min**

Reference (a) solution was injected once on four temperature adjustments of: 25°C, 30°C, 35°C and 40°C. The parameters depicting the method efficiency are shown on the table below:

| *t (°C) at flow rate 0.35 mL/min* | Resolution | Tailing factor Impurity C | Tailing factor Bromhexine HCl | Backpressure |
|---|---|---|---|---|
| **25** | 24.235 | 1.063 | 0.713 | 58.1 |
| **30** | 22.791 | 1.075 | 0.725 | 54.3 |
| **35** | 20.628 | 1.101 | 0.742 | 51.3 |
| **40** | 19.151 | 1.108 | 0.754 | 48.6 |

**Figure 4.2. Column efficiency at flow rate 0.35 mL/min at different temperature adjustments.**

Graphical depiction of resolution, tailing factors and backpressure:

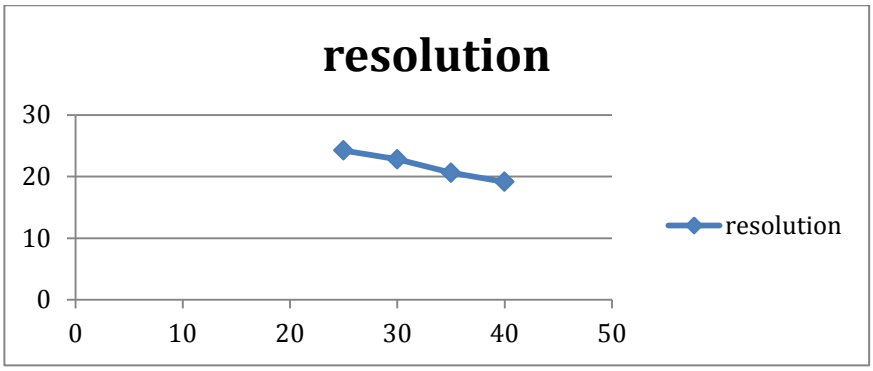



**Figure 4.3. Resolution plotted against temperature ranges**

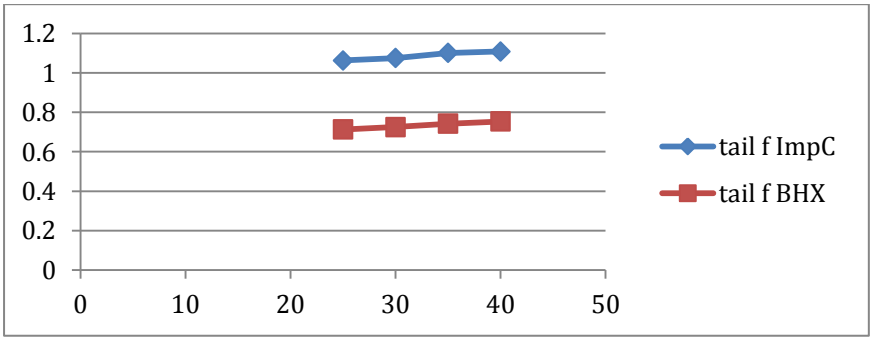



**Figure 4.4. Tailing factors of Bromhexine HCl and Impurity C plotted against different temperature adjustments**

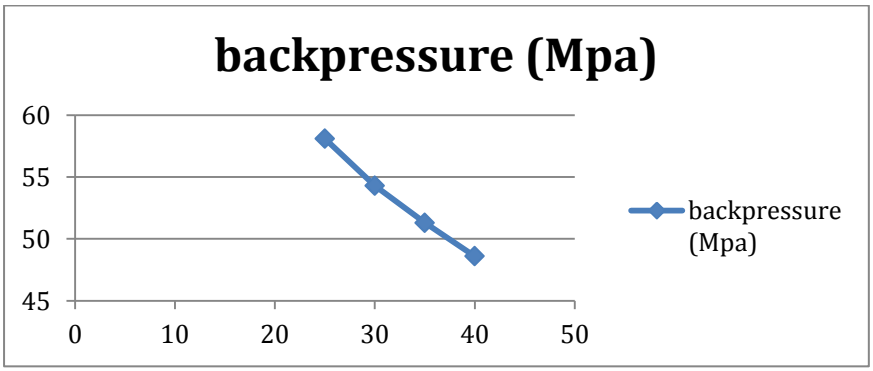



**Figure 4.5. Backpressure plotted against different temperature adjustments**

## 2. Adaption of different flow rates (from 0.35-0.6 mL/min) at 40°C temperature

Similarly, a single injection of reference (a) solution on six different flow rates at 40°C column temperature shows the resulting values of resolution, tailing factors and backpressure:

| *Flow rate (mL/min) at 40*°C Temperature | Resolution | Tailing factor Impurity C | Tailing factor Bromhexine HCl | Backpressure |
|---|---|---|---|---|
| **0.035** | 19.151 | 1.108 | 0.754 | 48.6 |
| **0.4** | 18.883 | 1.104 | 0.751 | 56.1 |
| **0.45** | 18.046 | 1.099 | 0.752 | 63.7 |
| **0.5** | **17.545** | **1.088** | **0.747** | **69.7** |
| **0.55** | 16.093 | 1.04 | 0.733 | 77.5 |
| **0.6** | 15.397 | 1.07 | 0.752 | 83.2 |

**Figure 4.6. Column efficiency at column temperature 40°C on different flow rate adjustments**

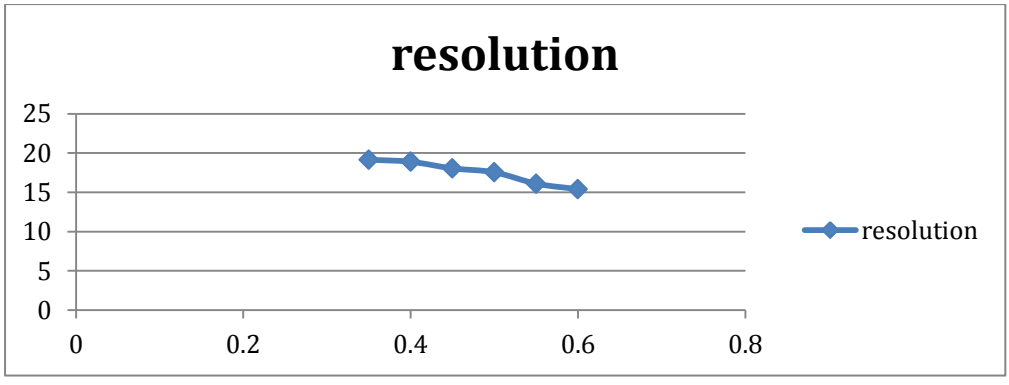



**Figure 4.7. Resolution on different flow rate adjustments**

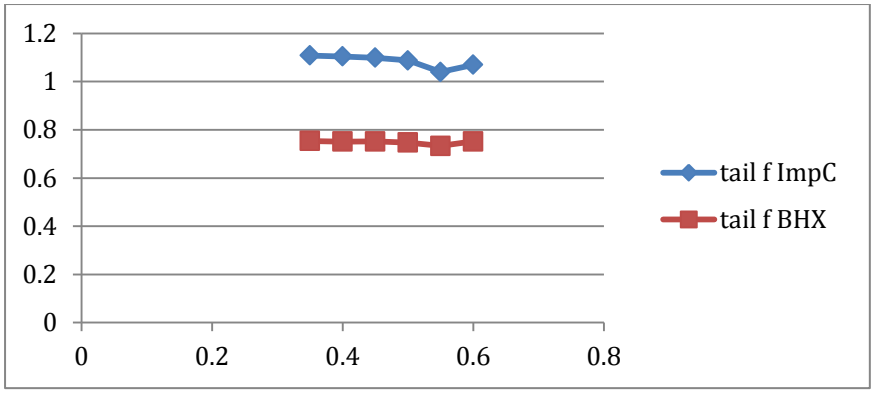



Figure 4.8. Tailing factors of Impurity C and Bromhexine on different flow rate adjustments

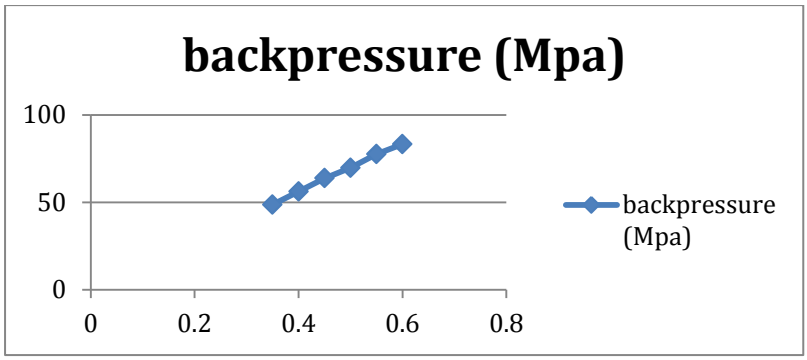



Figure 4.9. Backpressure on different flow rate adjustments

Based on the results obtained from these two approaches adopted to determine optimal chromatographic conditions for the newly developed method, it was concluded that the analysis conducted with **flow rate 0.5 mL/min at column temperature of 40°C** and **injection volume 1.6µl** showed the most optimal combination of resolution between the Impurity C and Bromhexine HCl peaks, their tailing factors and column backpressure limit. Compared to the method in Ph.Eur, the time of analysis was 3 times shorter; peak symmetry was in the limit (0.8-1.5), resolution of Impurity C and Bromhexine HCl was higher than 12 (which is the limit for system suitability test in Ph.Eur) and the pressure was below the limit (100MPa) given from directions of the column producer.

## 4.2 Method Validation

### 4.2.1 Linearity

Determination of linearity was based on seven different concentrations within a range of **0.02-0.3 %** of diluted test solution. Each solution was injected twice and their average areas were used to evaluate linearity graphically by plotting the deviations from the regression line versus the concentrations.

***Sample preparation***

1 ml of test solution was completed to 100 mL of methanol R.

0.2; 0.5; 1; 1.5; 2; 2.5; 3 ml from this solution was diluted to 10 ml with the same solvent.

The resulting concentration of Bromhexine HCl (BHX) in solutions of a range from 0.1 µm (0.02%)-1.5 µm (0.3%) were expressed in percentage:

| Linearity | L1a | L1 | L2 | L3 | L4 | L5 | L6 |
|---|---|---|---|---|---|---|---|
| **BHX %** | 0.02 | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 |

**Figure 4.10. Seven concentrations of test solution used to determine Linearity**

Areas of injection of seven concentrations of Bromhexine HCl test solution and the average for two injections of each concentration are shown below:

| Bromhexine | Area | Average Area |
|---|---|---|
| L_01a_001 | 5077 | |
| L_01a_002 | 5148 | 5112.5 |
| L_01_001 | 10648 | |
| L_01_002 | 10535 | 10591.5 |
| L_02_001 | 21075 | |
| L_02_002 | 21052 | 21063.5 |
| L_03_001 | 31138 | |
| L_03_002 | 31727 | 31423.5 |
| L_04_001 | 42886 | |
| L_04_002 | 43097 | 42991.5 |
| L_05_001 | 54304 | |
| L_05_002 | 54329 | 54316.5 |
| L_06_001 | 62698 | |
| L_06_002 | 62602 | 62650 |

**Figure 4.11. Linearity concentrations; areas; AVG area of two injections.**

The regression line is calculated by the method of least square where ***y*** is the peak area and ***x*** is the concentration of Bromhexine (BHX) in test solution. The square of the correlation coefficient $\boldsymbol{R^2}$= 0.99877, shows the high linearity of the peak areas with the corresponding concentrations.

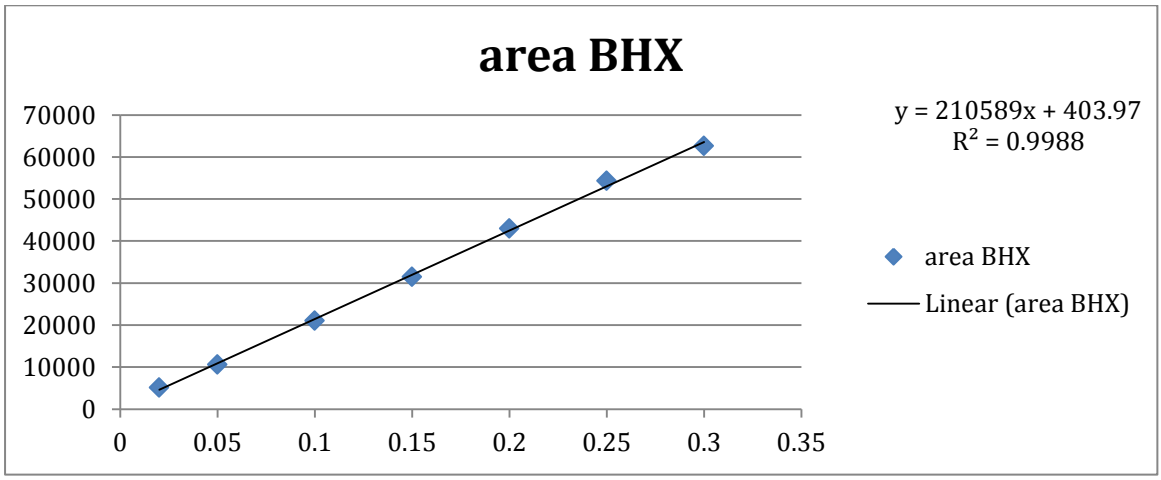



**Figure 4.12. Peak areas plotted against concentration**

### 4.2.2 Detection limit

The detection of lowest concentration of test solution was found using the signal-to-noise ratio *(S/N),* which shows the ratio between the peak height *(H)* and baseline noise height *(h)*. Since for detection of lowest amount of analyte *S/N*=2, then ***S/N=H/h=2***. Using the chromatogram from lowest concentration (0. 1µg/mL) solution in linearity, the value of *H* was measured to be 5.5cm, and *h* was 0.8 cm.

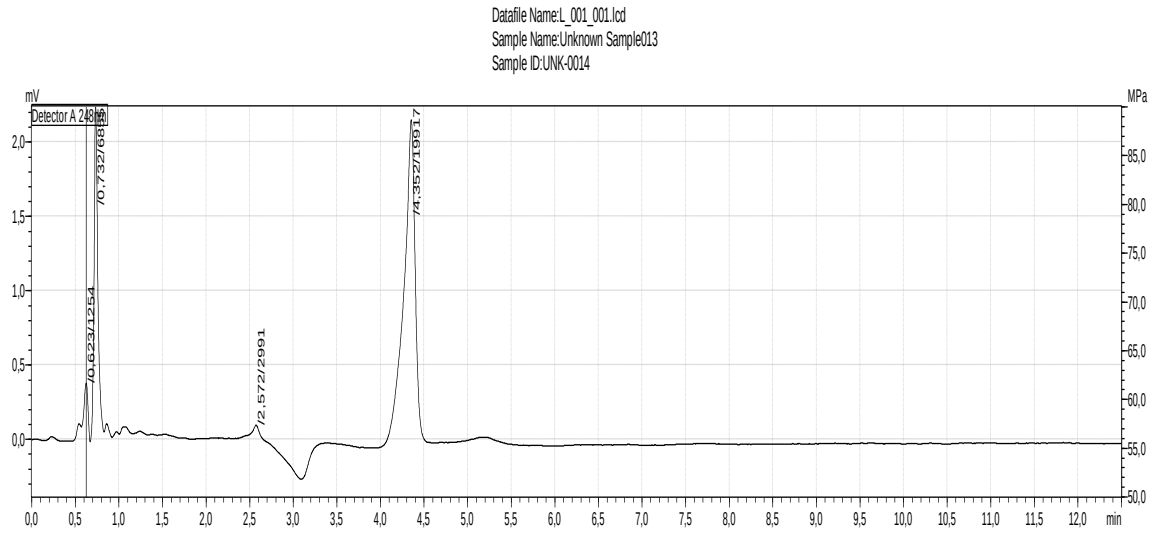



**Figure 4.13. Chromatogram of BHX HCl test solution with lowest c (µg/mL) obtained from linearity testing.**

According to the formula above: *H*= 2*h* the detection limit corresponds to the solution with peak height *H*= 2 x 0.8= 1.6cm and the concentration of test solution with peak height of 1.6 cm is:

0.1µg/mL x (1.6cm / 5.5cm) = 0.03µg/mL

This concentration accounts for 0.006 %

### 4.2.3 Quantification limit

The limit of quantification was determined similarly applying the signal to noise ratio (*S/N*) obtained from chromatogram of lowest concentration (0.1µg/mL) solution from linearity. Since the quantification limit is at *S/N* ratio of 10, the peak height *(H)* is:

H=10 x 0.8cm = 8cm,

Then: 0.1µg/mL x (8cm / 5.5cm) = 0.1µg/mL

From the above calculations, the quantification limit was found to be 0.1µg/mL, which accounts for 0.02%.

### 4.2.4 Precision

Repeatability was used to express the precision of method. The content of unknown impurities present on 6 samples of a ≈ 0.5 mg/mL of Bromhexine HCl (API) test solution (100%) was determined using two methods:

- The external standard method (as instructed in pharmacopeia) by employing the reference (b) solution with percentage area of Bromhexine HCl as 0.1% of the (100%) test solution to calculate the average percentage areas of 2 injections of unknown impurities from 6 six test solutions of similar concentration.
- The normalization method by directly calculating the average area percentage of impurities obtained from 2 injections of 6 test solutions.

Relative standard deviation (RSD 100%) was calculated to determine the precision of method.

***Sample preparation***

50 mg of Bromhexine HCl was measured six times and diluted to 10 mL with methanol R.

| Precision samples | P_01 | P_02 | P_03 | P_04 | P_05 | P_06 |
|---|---|---|---|---|---|---|
| Weights (mg) | 49.70 | 49.99 | 49.76 | 50.27 | 49.84 | 49.81 |

**Figure 4.14. Six measurements of BHX HCl for preparation of precision samples**

The tables below show the values of peak area percentage of Bromhexine HCl in ref. (b) solution and content of two unknown impurities present in the test solution calculated by both methods: external standard vs. normalization method. The resulting RSD % values are also shown for both methods used:

| Reference (b) solution | AVG area for 2 injections | Area % |
|---|---|---|
| Bromhexine (BHX) | 21223.5 | 100 |

Figure 4.15. Bromhexine peak area in ref. (b) solution for precision testing.

***Unknown impurity 1***- the average areas and area % of 2 injections from 6 test solutions:

| Unknown Imp.1 | AVG area | AVG area % (external standard method) | AVG area % (normalization method) |
|---|---|---|---|
| P_01 | 9629.5 | 0.045 | 0.048 |
| P_02 | 10047 | 0.047 | 0.049 |
| P_03 | 9159.5 | 0.043 | 0.046 |
| P_04 | 9836 | 0.046 | 0.047 |
| P_05 | 10111 | 0.048 | 0.050 |
| P_06 | 10395 | 0.049 | 0.052 |

Figure 4.16. Content of unknown impurity 1.

| Unknown Imp. 1 | AVG area % for 6 samples | RSD % |
|---|---|---|
| External standard method | 0.046 | **4.37** |
| Normalization method | 0.048 | **4.50** |

Figure 4.17. Relative standard deviation (RSD%) for content of unknown impurity 1

***Unknown impurity 2***- the averages area and area % of 2 injections from 6 test solutions:

| Unknown Imp.2 | AVG area | AVG area % (external standard method) | AVG area % (normalization method) |
|---|---|---|---|
| P_01 | 4939.5 | 0.023 | 0.024 |
| P_02 | 5103 | 0.024 | 0.025 |
| P_03 | 4585.5 | 0.022 | 0.023 |
| P_04 | 5150.5 | 0.024 | 0.025 |
| P_05 | 4775 | 0.022 | 0.023 |
| P_06 | 5254 | 0.025 | 0.026 |

**Figure 4.18. Content of unknown impurity 2.**

| Unknown Imp. 2 | AVG area % for 6 samples | RSD % |
|---|---|---|
| External standard method | 0.023 | **5.07** |
| Normalization method | 0.024 | **4.81** |

**Figure 4.19. Relative standard deviation (RSD%) for content of unknown impurity 2.**

### 4.2.5 Accuracy

Six samples of test solutions containing a known amount of Impurity C added were used to determine whether the results generated by the method and the true value agree. The true value was determined by employing the concentration of prepared solution of Impurity C in 10 mL methanol R (c=0.172 mg/mL) to calculate the concentration of Impurity C added (0.02 mL) in 1.02 mL of total volume of test solution. Lastly, accuracy was expressed as the percentage of recovery by the assay of known amount of impurity C added in the test solution.

***Sample preparation***

6 test solutions were prepared in the same manner as samples for precision calculation. 20µl (0.02 mL) of Impurity C solution with concentration 1.72mg / 10 mL was added to 1 mL of test solution.

The resulting volume of test solution for accuracy was 1.02mL.

The tables below show the results for accuracy determination using the *external standard method* to calculate the Impurity C content:

Bromhexine average peak area in accuracy testing:

| Reference (b) solution | AVG area for 2 injections | Area % |
|---|---|---|
| Bromhexine (BHX) | 21037 | 100 |

**Figure 4.20. Bromhexine peak area in ref. (b) solution for accuracy testing.**

The true values for 6 samples from accuracy and their corresponding recoveries of Impurity C:

| Impurity C | AVG area | AVG area % | Found value (mg/mL) | True value (mg/mL) | Recovery % |
|---|---|---|---|---|---|
| A_01 | 15126.5 | 0.072 | 0.00359 | 0.00337 | 106.60 |
| A_02 | 14067 | 0.067 | 0.00334 | 0.00337 | 99.14 |
| A_03 | 13854 | 0.066 | 0.00329 | 0.00337 | 97.63 |
| A_04 | 13928 | 0.066 | 0.00331 | 0.00337 | 98.16 |
| A_05 | 14284.5 | 0.068 | 0.00339 | 0.00337 | 100.67 |
| A_06 | 14291.5 | 0.068 | 0.00339 | 0.00337 | 100.71 |
| AVG recovery % | | | | | 100.48 |

**Figure 4.21. Impurity C accuracy testing using external standard method**

Accuracy determination using the *normalization method* to calculate the Impurity C content:

| Impurity C | AVG area | AVG area % | Found value (mg/mL) | True value (mg/mL) | Recovery % |
|---|---|---|---|---|---|
| A_01 | 15126.5 | 0.0715 | 0.00357 | 0.00337 | **106.00** |
| A_02 | 14067 | 0.069 | 0.00345 | 0.00337 | **102.30** |
| A_03 | 13854 | 0.0675 | 0.00337 | 0.00337 | **100.07** |
| A_04 | 13928 | 0.068 | 0.00345 | 0.00337 | **100.81** |
| A_05 | 14284.5 | 0.069 | 0.00345 | 0.00337 | **102.30** |
| A_06 | 14291.5 | 0.0685 | 0.00342 | 0.00337 | **101.55** |
| AVG recovery % | | | | | **102.17** |

**Figure 4.22. Impurity C accuracy testing using normalization method.**

### 4.2.6 Selectivity

The selectivity of method was tested in order to validate the method in case when Bromhexine HCl preparations (as opposed to API) are to be analyzed.

A batch with test solution (from precision and accuracy testing), reference (a) solution, reference (b) solution and a placebo, was run in order to evaluate the selectivity of the method. The peaks from each solution on the resulting chromatograms were investigated for any interference between retention times of substances present on solutions.

***Sample preparation***

The placebo solution was prepared according to the producer of Bromhexine HCl preparation (Bromhexine GALMED 8). 6.5 mL of placebo solution were completed to 10 mL of methanol R, in order to correlate to the 5mg/mL concentration of Bromhexine HCl in the test solution from our analysis.

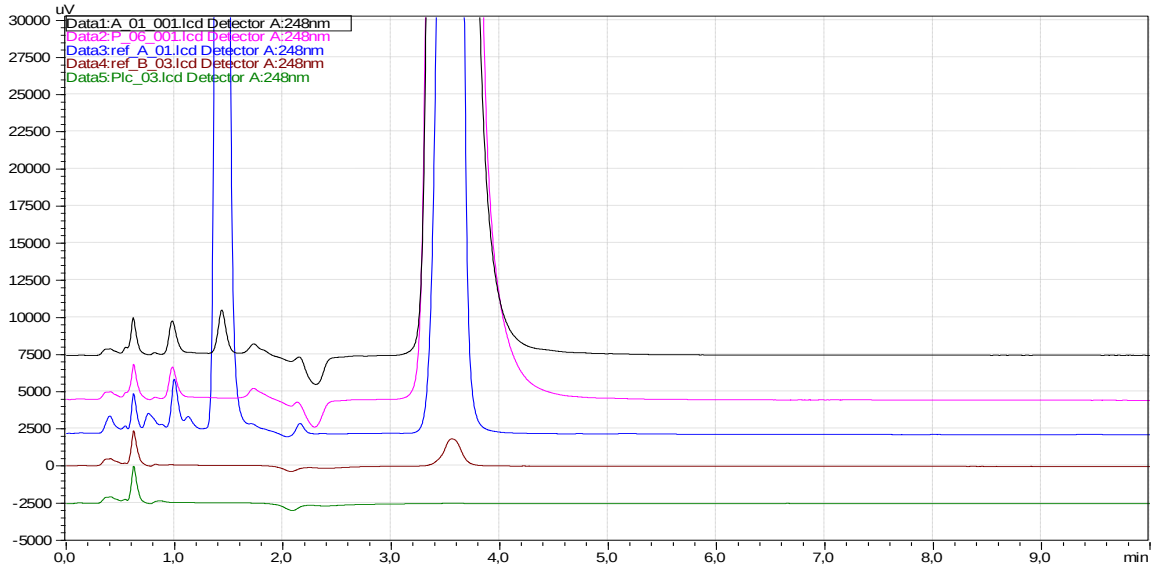



**Figure 4.23. Comparison of chromatograms for method selectivity**

As seen on the picture above, no peaks from placebo interfere with any peak of impurities and the active substance.

### 4.2.7 Results and discussions

The conventional HPLC method guided by European Pharmacopeia for determination of related substances of Bromhexine Hydrochloride (API) was successfully transferred to UFLC method for analysis. The newly developed method using UFLC with columns of sub-2 µm particle size of stationary phase and subsequently increased flow rate 0.5 mL/min at column temperature of 40°C was properly validated in order to ensure the suitability of the analytical procedure. The results for each validation method conducted are discussed below:

***-System suitability test:*** During the validation procedure, reference (a) solution was injected with every batch to ensure the suitability of test by measuring the resolution between the peaks of Bromhexine HCl and Impurity C. According to the procedure in pharmacopeia the minimum value of resolution between those peaks should be 12. The resolution value in our

analysis was **23**, which accounts for higher efficiency of the UPLC columns versus HPLC columns. Except resolution, the tailing factor was also used to ensure the suitability of test. The peak symmetry of both Impurity C and Bromhexine were within the limit range **0.8-1.5** during the whole analysis.

-***Linearity*** of the analytical procedure was affirmed based on the resulting correlation factor of $R^2$ =0.99877 (Figure 4.24.), which shows the high proportionality of seven concentrations (within a range of 0.02-0.3 mg/mL) of Bromhexine HCl in test solution to their corresponding areas in chromatogram.

According the European Pharmacopeia for Bromhexine HCl impurity content testing, any impurity peak area should not be greater than twice the area of the principal peak in chromatogram obtained from reference (b) solution **(0.2%)** [39]. Based on the results obtained from precision and accuracy, the impurity content in Bromhexine HCl was within the limited percentage.

***-Precision***. Two methods were employed to calculate the impurity content in order to evaluate precision (and accuracy): the external standard method and the normalization method. Based on the results obtained from both methods, we can say that both of them are suitable to give satisfactory results regarding the limits of impurity content. However, the external standard method can be considered less favorable for an analyst for a practical reason i.e. the necessity of preparation of the reference (b) solution needed for calculation of impurity content, as opposed to the normalization method where the impurity % was calculated directly from the peak areas in chromatograms.

According to the *ICH* the acceptable value for relative standard deviation (RSD%) in evaluation of precision in the case of impurity content in the active substance in concentration range from **LOQ** (**0.02** % in our case) to **0.1%** of impurity is **7%.**

The tables below show the comparison of two methods used to evaluate the results of RSD % obtained from our analysis of precision testing versus the acceptable values of RSD % for precision determined by *ICH:*

*-External method standard:*

| Impurities | AVG imp. content (%) | RSD % | RSD % (limit by *ICH*) |
|---|---|---|---|
| **Unknown Imp. 1** | 0.046 | **4.37** | **7%** |
| **Unknown Imp. 2** | 0.023 | **5.07** | **7%** |

**Figure 4.25. RSD limits for precision (external standard method).**

*-Normalization method:*

| Impurities | AVG imp. content (%) | RSD % | RSD % (limit by *ICH*) |
|---|---|---|---|
| **Unknown Imp. 1** | 0.048 | **4.50** | **7%** |
| **Unknown Imp. 2** | 0.024 | **4.81** | **7%** |

**Figure 4.26. RSD limits for precision (normalization method)**

***-Accuracy.*** Similarly as in precision testing, the two above mentioned methods for calculation of impurity % showed satisfactory recovery results within the required limits. Since the limits for determination of impurity content ($C_i$) for active substance is **LOQ%≤ $C_i$ ≤ 0.1%,** the recovery of impurities should be **85-115%.**

The tables below show the comparison of two methods used to evaluate the results of recovery (%) of Impurity C added to measure accuracy of method. Obtained values of

recovery from our analysis versus the acceptable values of recovery % for accuracy testing by *ICH* are as follow:

| Impurity C (%) | Recovery (%) | Recovery (%) -limit by *ICH* |
|---|---|---|
| External standard method | 100.486 | 85-115 |
| Normalization method | 102.173 | 85-115 |

**Figure 4.27. Differences on recovery values between two methods.**

***-Selectivity.*** In figure 4.28, the comparison of chromatograms of placebo, test solutions (from accuracy and precision), reference (a) solution and reference (b) solution show no peak interference of substances present in placebo solution with Bromhexine HCl or any of impurity peaks, proving the selectivity of method, hence the method is suitable for analysis of Bromhexine HCl preparations as well.

### 4.2.8 Conclusion

The development of a new method for determination of related substances in Bromhexine HCl active substance using Ultra High-performance Liquid Chromatography, with the aim of decreasing the analysis time and sparing the usage of large volumes of mobile phase while ensuring satisfactory peak resolution and overall chromatographic performance was successfully accomplished. The analytical tests conducted to assure the suitability of method proved that the newly developed method was properly validated and can therefore be used as an alternative analysis to HPLC. In comparison to the conventional method guided by European Pharmacopeia using HPLC, the utilization of UPLC in this analysis showed a much higher chromatographic efficiency. In conclusion, despite the high cost of this chromatographic instrument, its numerous advantages account for its high applicability in Pharmaceutical Analysis and Drug Control nowadays.

# 5. Bibliography


1. Hansen S.H. , P.B.S., Rasmussen K. E., *Introduction to Pharmaceutical Chemical Analysis*. 1st ed2012, UK: John Wiley & Sons Ltd. 494.
2. Waters. *High Performance Liquid Chromatography*. [cited 2012 3 May]; Available from: http://www.waters.com/waters/nav.htm?cid=10049055&locale=en_US.
3. *Chromatography, Introduction Theory*. [cited 2012 3 May]; Available from: http://teaching.shu.ac.uk/hwb/chemistry/tutorials/chrom/chrom1.htm.
4. Dong, M.D., *Modern HPLC for Practicing Scientists*, 2006, Jonh Wiley & Sons, Inc., Hoboken, New Jersey: UK. p. 285.
5. Laura Grell, A.B., *Proteomics/Protein Separations - Chromatography*, in *Proteomics*.
6. Science, T.B.L.T.C.o. *Basic Chromatography Calculations*. [cited 2012 1 May]; Available from: http://www.separations.us.tosohbioscience.com/ServiceSupport/TechSupport/ResourceCenter/ChromatographyCalculations/.
7. Pharmacopeia, U.S., *High Pressure Liquid Chromatography*. USP29-NF24. Vol. 31(3). 2005. 2639.
8. Grace. *Properties of HPLC columns*. [cited 2012 1 May]; Available from: http://www.discoverysciences.com/product.aspx?id=2604.
9. Atamna, I.Z., G.M. Muschick, and H.J. Issaq, *The Effect of Column Diameter on HPLC Separations Using Constant Length Columns.* Journal of Liquid Chromatography, 1989. **12**(3): p. 285-298.
10. Kovarikova, P., *Basic Chromatographic Parameters & System Suitability Test*, 2012, Charles University, Faculty of Pharmacy: Hradec Kralove CZ.
11. Rashmin *An introduction to analytical method development for pharmaceutical formulation*. 2008.
12. Kovařikova, P., *Basic Principles of HPLC.*, in *Therapeutic Drug Monitoring. Department of Pharmaceutical Chemistry and Drug Control. FaF, Charles University, CZ*2010.
13. Miller, E.I., et al., *Validation of a liquid chromatography-tandem mass spectrometry method for the detection of nicotine biomarkers in hair and an evaluation of wash procedures for removal of environmental nicotine.* J Anal Toxicol, 2011. **35**(6): p. 321-32.
14. Rao, S., et al., *Spectrum of haemoglobinopathies diagnosed by cation exchange-HPLC & modulating effects of nutritional deficiency anaemias from north India.* Indian J Med Res, 2010. **132**: p. 513-9.
15. Teresa, M., et al., *Polymer characterization by size-exclusion chromatography with multiple detection.* J Chromatogr A, 2001. **919**(1): p. 13-9.
16. Fassina, G., et al., *Novel ligands for the affinity-chromatographic purification of antibodies.* J Biochem Biophys Methods, 2001. **49**(1-3): p. 481-90.
17. Perrin, C., et al., *Screening approach for chiral separation of pharmaceuticals part II. Reversed-phase liquid chromatography.* J Chromatogr A, 2002. **966**(1-2): p. 119-34.
18. Marshall, D.B., C.L. Cole, and A.D. Norman, *The Use of Trimethylsilylphosphine as an End-Capping Reagent in RP-HPLC.* J Chromatogr Sci, 1987. **25**(6): p. 262-266.

19. *The cell biologist guide to protemics*. [cited 2012 3 May]; Available from: http://www.lamondlab.com/MSResource/LCMS/NanoflowHPLC/hplc.php.
20. Dolan, J.W. *Pump Preventive Maintenance*. LC Troubleshooting, 1997.
21. Resources, L. *Getting started in HPLC*. [cited 2012 5 May]; Available from: http://www.lcresources.com/resources/getstart/2e01.htm.
22. *Summary of the characteristics of different mass analyzers*. [cited 2012 5 May]; Available from: http://www.research.uky.edu/ukmsf/jeolanalyzers.pdf.
23. Wren, S.A.C. and P. Tchelitcheff, *Use of ultra-performance liquid chromatography in pharmaceutical development.* Journal of Chromatography A, 2006. **1119**(1–2): p. 140-146.
24. Skoog DA, H.F., Nieman TA, *An introduction to chromatographic separations. Principals of instrumental analysis*, 1998, Saunders college publishing: Florida.
25. Swartz, M.E. *Ultra Performance Liquid Chromatography (UPLC): An Introduction*. 2005.
26. Waters. *Solvents and Caveats for LC/MS*. [cited 2012 3 May].
27. L.R. Snyder, J.J.K., *Introduction to Modern Liquid Chromatography*, J.J.K. L.R. Snyder, Editor 1979, John Wiley & Sons, Inc.: New York.
28. A.B. Roge*, S.N.F., R. M. Dhane, V.J. Gunjkar, S M Vadvalkar, *Novel achievement of HPLC: UPLC* International Journal of PharmTech Research, 2011. **3**.
29. Noetzli, M., et al., *Simultaneous determination of antidementia drugs in human plasma: Procedure transfer from HPLC–MS to UPLC–MS/MS.* J Pharm Biomed Anal, 2012. **64–65**(0): p. 16-25.
30. Lindeman, N.I., S.M. Freeto, and P. Jarolim, *Immunosuppressant monitoring can be performed by UPLC–Tandem Mass Spectrometry in half the time needed for conventional HPLC–Tandem Mass Spectrometry, with comparable analytical performance.* Clinica Chimica Acta, 2008. **388**(1–2): p. 207-210.
31. Cai, Z., et al., *Simultaneous determination of 24 sulfonamide residues in meat by ultra-performance liquid chromatography tandem mass spectrometry.* Journal of Chromatography A, 2008. **1200**(2): p. 144-155.
32. Barceló-Barrachina, E., et al., *Ultra-performance liquid chromatography–tandem mass spectrometry for the analysis of heterocyclic amines in food.* Journal of Chromatography A, 2006. **1125**(2): p. 195-203.
33. Leandro, C.C., et al., *Comparison of ultra-performance liquid chromatography and high-performance liquid chromatography for the determination of priority pesticides in baby foods by tandem quadrupole mass spectrometry.* Journal of Chromatography A, 2006. **1103**(1): p. 94-101.
34. Farré, M., et al., *Comparative study of an estradiol enzyme-linked immunosorbent assay kit, liquid chromatography–tandem mass spectrometry, and ultra performance liquid chromatography–quadrupole time of flight mass spectrometry for part-per-trillion analysis of estrogens in water samples.* Journal of Chromatography A, 2007. **1160**(1–2): p. 166-175.
35. Apollonio, L.G., et al., *A demonstration of the use of ultra-performance liquid chromatography–mass spectrometry [UPLC/MS] in the determination of amphetamine-type substances and ketamine for forensic and toxicological analysis.* Journal of Chromatography B, 2006. **836**(1–2): p. 111-115.
36. Yamashita, T., et al., *High-speed solubility screening assay using ultra-performance liquid chromatography/mass spectrometry in drug discovery.* Journal of Chromatography A, 2008. **1182**(1): p. 72-76.

37. Huber, L. *Validation of Analytical Methods and Procedures*. 2007 [cited 2012 6 May]; Available from: http://www.labcompliance.com/tutorial/methods/default.aspx - 06_transfer.
38. Dolan, J.W. *Enhancing Signal-to-Noise*. 2012.
39. EDQM, *European Pharmacopeia*. 7th ed. Vol. 7. 2011, Strasbourg.
40. Christensen, F., et al., *Bromhexine in chronic bronchitis.* Br Med J, 1970. **4**(5727): p. 117.
41. Manthorpe, R., et al., *Bromhexine Treatment of Sjögren's Syndrome: Effect on Lacrimal and Salivary Secretion, and on Proteins in Tear Fluid and Saliva.* Scandinavian Journal of Rheumatology, 1981. **10**(3): p. 177-180.
42. Bechgaard, E. and A. Nielsen, *Bioavailability of bromhexine tablets and preliminary pharmacokinetics in humans.* Biopharmaceutics & Drug Disposition, 1982. **3**(4): p. 337-344.
43. Therapeutic Goods Administration, A.G., Department of Health and Ageing. *Core Bromhexine Product Information*. [cited 2012 30 April]; OTC template-pi-bromhexine]. Available from: http://www.tga.gov.au/rtf/forms/otc-template-pi-bromhexine.rtf.