DIPLOMA THESIS

DETERMINATION OF SELECTED ACTIVE SUBSTANCE IN THE PREPARATION

VIII

QUETIAPINE FUMARATE

DETERMINATION OF RELATED SUBSTANCES IN THE PREPARATION

USING ULTRA-FAST LIQUID CHROMATOGRAPHY

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“I declare that this thesis is my original copyrighted work. All literature and other resources I used while processing are listed in the bibliography and properly cited.”
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1 INTRODUCTION

Pharmaceutical analysis provides information on the identity, purity, content and stability of starting materials, excipients and active pharmaceutical ingredients (APIs). It is a discipline that in its course provides information and guidance on the exploitation of sophisticated technological instrumentation and advanced laboratory methodologies in the analysis of drug substances, with the purpose of achieving its’ ultimate objective to improve the quality of life with better and safer drugs. A medicinal preparation is a substance or a combination of substances which is used in humans and/or animals in order to restore correct or modify a physiological function by exerting pharmacological, immunological or a metabolic action. Pharmaceutical control of medicinal preparations is necessary and crucial to the public health to ensure that these medicines meet the acceptable standards of quality, safety and efficacy. The medicinal products are available in different dosage forms (ointments, tinctures, pills, lotions, suppositories, infusions, drops, etc.) and consist of the active pharmaceutical substance, pharmaceutical excipient(s) and, commonly, impurities that usually appear during the synthesis of the pharmaceutical ingredient; they are usually monitored according to the guidelines of the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) and the pharmacopoeias above.
Pharmacopoeias are official reference manuals published by the authority of the government according to the World Health Organization directives; it contains detailed monographs on the requirements and test methods for active ingredients and other products used for therapeutic purposes, and is essential for guidance in the production, testing or marketing of medicinal products.

In drug control, the chromatographic separation methods are among the most important laboratory techniques used to achieve its' course objective. High Performance Liquid Chromatography (HPLC) is the most used analytical technique in drug analysis, environmental analysis and food products analysis. It provides both quantitative and qualitative data in one run. Although it is one of the most sophisticated technologies in the field of analysis, the increasing efforts nowadays for the evaluation of trace impurities to improve the safety and quality of pharmaceuticals and food products, has further encourage technology expert to design a better LC-system that would increase the separation performance, decrease the analysis time and generate a higher procedure efficiency. Nowadays, chromatographic methods among other application are massively employed in pharmaceutical industry, especially in the quality control (QC) and quality assurance (QA) monitoring processes; they are integral tools to ensure the compliance of the manufacturers with the current Good Manufacturing Practice and Good Laboratory Practice.
Quetiapine fumarate is novel atypical antipsychotic used to treat symptoms of psychiatric disorders such as schizophrenia and bipolar disorders. It is among the newest antipsychotic drugs, which in the recent conducted clinical trials has shown improved tolerance of the drug for longer treatments and fewer neurological side effects compared to the older agents. In this thesis, the determination of the active ingredient of the novel antipsychotic drug and other related substances was carried out using Ultra-Fast Liquid Chromatography as a method with higher sensitivity of trace impurities, shorter analysis time and overall higher efficiency procedure compared to the conventional HPLC method.
2 AIM OF WORK

The aim of this work is to convert conventional HPLC analytical method to the conditions of Ultra-Fast Liquid Chromatography. The analytical method is adopted to determine Quetiapine related substances in the preparation. New method should need less organic solvents, should save time, it should be validated with satisfactory parameters to use it for drug control.
3 THEORETICAL PART

3.1 QUETIAPINE FUMARATE

Quetiapine fumarate is a second-generation antipsychotic medication launched in the market by the laboratories of AstraZeneca pharmaceutical company under the brand name Seroquel™. It is indicated for the treatment of schizophrenia and acute manic episodes of bipolar disorders⁷. Quetiapine was approved by the US Food and Drug Administration in 1997.

3.1.1 THE CHEMISTRY OF QUETIAPINE FUMARATE

Quetiapine fumarate, 2-[(2-(4-dibenzo [b,f][1,4]thiazepine-11-yl-1-piperazinyl)ethoxy)ethanol fumarate (Fig. 1) belongs to a new chemical class, the dibenzothiazepine derivatives⁸.

Figure 3.1 The chemical structure of quetiapine fumarate
The synthesis of quetiapine was derived from dibenzothiazepines.³

![Quetiapine synthesis](image)

*Figure 3.2 Quetiapine synthesis*

### 3.1.2 THE PHARMACOLOGY OF QUETIAPINE FUMARATE

#### 3.1.2.1 MECHANISM OF ACTION AND INDICATIONS

Quetiapine fumarate is believed to mediate its effect by acting at multiple neurotransmitter receptors in the brain. It antagonizes the dopamine and serotonin receptors, specifically Dopamine D(1) and D(2), serotonin 5-HT(1A) and 5-HT(2), histamine H(1), adrenergic alpha(1) and alpha(2) receptors. The efficacy in the treatment of the symptoms of schizophrenia; bipolar depression and bipolar mania is supposed to be due to a combination of D(2) and 5-HT(2) receptors.⁴

In contrast to the older antipsychotic agents, quetiapine has a better therapeutic profile in that it is less associated with the common adverse effects witnessed in most of the antipsychotic medications. The drug has minimal activity on dopamine receptors in the nigrostriatal dopamine system, the part of the brain responsible for the extrapyramidal side
effects; and also has minimal effects on the tuberoinfundibular dopamine system, thereby avoiding the problem of hyperprolactinemia, frequently associated with antipsychotic therapy. Patients on long-term treatment with quetiapine have reported high compliance, increased ability to function and improvement on the symptoms accompanying the disorder, therefore its use is particularly appropriate in patients sensitive to side effects (e.g. children and elderly patients)\textsuperscript{6}.

In the off-label use of atypical antipsychotics, quetiapine has shown to be beneficial in the treatment of generalized anxiety disorder, when compared to placebo in three large controlled trials\textsuperscript{12}.

3.1.2.2 ADVERSE EFFECTS

The histamine H(1) antagonism is believed to be responsible for the most common adverse effects of the medication which are dizziness and somnolence\textsuperscript{13}. Other common side effects that have been reported during treatment with quetiapine are:

- fatigue, dry mouth, sore throat, dizziness, abdominal pain, constipation, upset stomach, orthostatic hypotension, inflammation or swelling of the sinuses or pharynx, increased appetite, and weight gain\textsuperscript{10}.

Serious but rare side effects that have been reported in less than 1% of the patients are: prolonged QT interval, syncope, leukopenia, neutropenia, pancreatitis, seizures, and suicidal thoughts.

Rhabdomyolysis has also been considered as one of the rare but serious
adverse events related to quetiapine and therefore should be monitored during treatment\textsuperscript{14}.

3.1.2.3 CONTRAINDICATIONS

Apart from the hypersensitivity to this drug and/or this class of chemicals, no other specific contraindications have been determined so far.

3.1.2.4 PREGNANCY AND BREAST FEEDING

In an observational study of 54 childbearing women under antipsychotic treatment, among different antipsychotics (olanzapine, haloperidol, risperidone) quetiapine has shown the lowest placental passage with 24.1\%, a significant difference compare to olanzapine 72.1\% being the highest\textsuperscript{15}. In one case report the use of 300 to 400 mg quetiapine in a pregnant woman has shown no infant abnormalities after birth and at six month of age the infant was developing normally\textsuperscript{16}. However, quetiapine is considered a category C according to the FDA’s Pregnancy Category and should therefore be used only if the potential benefits outweigh the potential risks. Although the limited data on the safety of quetiapine during breastfeeding show no evidence of toxicity, infant risk cannot be ruled out and therefore the infants’ quetiapine plasma levels should be monitored carefully\textsuperscript{17}. 
3.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography is a laboratory technique used for separating complex mixtures into individual substances. It is a highly improved form of column chromatography that has found an extensive use in the pharmaceutical analysis for a variety of applications, for example: isolation of natural pharmaceutically active compounds; identifying undesirable impurities in pharmaceutical substances; assay of pure drugs; and determination of the related substances present in various drug dosage forms. Its’ detection methods are highly automated and sensitive and instead of using gravity for the extraction of the solvent, it runs under high pressure up to 400 atmospheres which makes it much faster.

**PRINCIPLE:** A mixture of components or a drug sample is dissolved in a proper organic solvent and injected into a moving liquid known as the "mobile phase". The mobile phase is pumped through a column, a solid tube composed of densely packed particles called "stationary phase". The components of the drug sample are transported by the mobile phase to the column and brought into contact with stationary phase. Based on the physical characteristics of their molecule entities, the stationary phase will retard their migration at different speeds. The separation of the drug components is a result of differences in their movement through a two-phase system and differences in the interaction of the components with the stationary phase.
3.2.1 CHROMATOGRAPHIC MODES

According to its’ physical principles the separation operates in different mechanism:

- **Size exclusion**: separation of constituents is based on the radius of the molecule;
- **Ion exchange**: used for the positively or negatively charge molecules (proteins, peptides, nucleic acids) and is based on their ability to exchange anions or cations;
- **Hydrophobic Interaction**: is based on the non-polar interaction between the molecule and the stationary phase, but in contrast to RPC (see below), with a much lower density of the hydrophobic interaction;
- **Affinity chromatography**: is used for the biologically active compounds that have highly specific interaction with a specific ligand, and is therefore used whenever a suitable ligand is available for the compound of interest\(^{18}\).
- **Reverse Phase Chromatography**: is based on the interaction of the non-polar regions of the molecule with the non-polar stationary phase. In contrast to the ‘normal phase’, the polar constituents are eluted from the column with high organic mobile phase, while the non-polar constituents are retained on the hydrophobic stationary phase;

Reversed phase chromatography is the most frequent separation mechanism used in drug analysis. It is an elution procedure in which the
mobile phase is significantly more polar than the stationary phase\textsuperscript{19}; the more hydrophobic the compound the longer the retention time.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{reverse_phase_chromatography.png}
\caption{Reverse-Phase Chromatography Mechanism}
\end{figure}

3.2.2 INSTRUMENTATION

A modern HPLC apparatus consist of the following essential compartments:

- Solvent reservoir and degasser system,
- Pumps and sample injection system,
- Columns,
- Detectors,
- Strip-chart recorder,
- Data handling device and microprocessor control\textsuperscript{2}.
3.2.3 THE STATIONARY PHASE

The most frequently used type of the stationary phase for reverse-phase liquid chromatography is composed of silica particles which are thinly coated with long-chain hydrophobic alkyl groups (e.g. C4, C8 or C18) to interact with the analyte. Particle size range from 3 to 10 µm, sometimes the size goes up to 50µm or more for preparative columns. The main drawback of this type of composition is its’ limited stability of pH (2-8). Polymeric based stationary phase is a matrix that can be modified with different chemical groups. The advantage of this stationary phase is its’
wide range of pH stability (1-11), but the limited availability in the market is a drawback.

**Zirconium based stationary phase** is a novel type of sorbent with higher pH stability and a unique selectivity for particular compounds. It is ZrO$_2$ matrix which can be modified by e.g. polystyrene, polybutadiene or C18. Other types of stationary phases: underivatized graphitized carbon; TiO$_2$ based stationary phase$^4$.

### 3.2.4 COLUMN

The columns in HPLC are utilized with densely packed sorbent particles which provide a great surface area for interaction of the particles and make possible of the partitioning of the components in the mixture. Most common packing material consists of silica particles. The columns are characterized by the particle size (3 to 10µm) and the particle pore size ranging from 100 to 1000 angstroms that make up for the dimension of the column with an internal diameter ranging from 2 to 5mm (typical 4.6mm), length ranging from 10-30cm (typical 25cm) and. Usually, smaller particles generate a higher pressure than the greater particles. In addition, they also account for a better efficiency in the separation. Silica materials are sensitive to extremes of pH especially when combined with the high temperature; hence it is recommended that the organic solvent should not exceed the pH of 7 in order to avoid damaging of the column particles$^{21}$. The columns are constructed of highly polished stainless
steel, an inert material capable of resisting the corrosive properties of organic solvents.

### 3.2.4.1 Chromatographic parameters

#### 3.2.4.1.1 Retention factor ($k'$)

The retention factor for a given solute (also known as the capacity factor ($k'$)) is a function of the mobile phase polarity\(^2\). The migration of individual solutes of the sample through the column depends on the equilibrium distribution of each solute between the mobile phase and the stationary phase. When the difference in the retention factors is sufficiently large, the solutes elute from the column at different times and are separated. The distribution of the solute between the two-phase systems is given by the distribution constant:

$$K_c = \frac{C_s}{C_m}$$

The retention factor shows how the amount of the substance is distributed between the stationary phase and the mobile phase and depends on the distribution constant ($K_c$) and the volume of the stationary phase and the mobile phase\(^2\):

$$k' = K_c \frac{V_m}{V_s}$$

When the retention factor is equal to 1, the compound will be distributed equally between the stationary phase and the mobile phase. If $k'$ value is
less than 1, the elution is so fast that determining an accurate retention
time is very difficult. When the retention factor is equal to 5, there will
be 5 times more of the compound in the stationary phase than in the
mobile phase. If $k'$ values are too high (greater than 20) the elution will
take very long. Ideally, $k'$ value for a given analyte is between 1 and 5.
Each analyte in a sample will have a different retention factor which can
be determined from the chromatogram using the following equation:

$$k' = \frac{t_R - t_M}{t_M}$$

Where $t_R$ is the time between sample injection and an analyte peak
reaching the detector at the end of the column; $t_M$ is the time taken for
the mobile phase to be eluted from the column. Both these parameters
can be easily obtained from the chromatogram$^{23}$:

![Chromatogram of a sample and parameters used to measure the retention factor](image)
3.2.4.1.2 Brand broadening \((N)\)

A column's efficiency is explained by the Theoretical Plate Model which supposes that the column contains a large number of separate layers. The peak broadening expresses how efficiently a compound is transported through the column. In order to obtain an optimal separation with sharp and symmetrical peaks, peak broadening should be limited. The wider the peak, the longer the retention time and the smaller will be the number of components that can be separated in a given time. Band broadening is expressed by the parameter \(N\), called the number of theoretical plates.

The plate number depends on the length of the column, that is to say that the longer the column the larger the number of the plates and thus the better the separation. The height of a single theoretical plate \(H\) (Height Equivalent to a Theoretical Plate), is a measure of the efficiency of a column per unit length of the column. The smaller the \(H\) value, the larger will be the number of plates.

**Efficiency Parameters**

![Diagram](image)

- **Theoretical plates**:
  \[
  N = 16 \left( \frac{t_R}{W_b} \right)^2 = 5.545 \left( \frac{t_R}{W_h} \right)^2
  \]

- **Plate height**:
  \[
  \text{HETP} = H = \frac{L \epsilon}{N}
  \]

*Fig. 3.2.4.2 The Gaussian peak and parameters used to measure peak width*
3.2.4.1.3 **Selectivity (α)**

The separation of two different analytes in the column is described by the separation factor, which is a measure of the selectivity of a chromatographic system. The separation factor has values greater or equal to 1. If \( \alpha \) equals to 1 separation is not possible; the greater the separation factor the easier the separation\(^{22}\).

\[ \alpha = \frac{t'_{R(B)}}{t'_{R(A)}} = \frac{k_{R(A)}}{k_{R(B)}} \]

- \( \alpha \) larger than 1.2 is desirable

*Fig. 3.2.4.3 Selectivity parameters\(^{24}\)*

3.2.4.1.4 **Peak symmetry (A\(_s\))**

Asymmetrical peaks can be a result of low quality or polluted columns. The symmetry factor (A\(_s\)) is used to indicate the peak symmetry:

\[ A_s = \frac{w_{0.05}}{2f} \]

When \( A_s \) equals 1.0 it signifies symmetry; when \( A_s > 1.0 \) the peak is tailing; when \( A_s < 1.0 \) the peak is fronting.
W_{0.05} – peak width in 1/20 of its height

f – the distance between the perpendicular and the rising part of the peak

3.2.4.1.5 Resolution (R_s)

Resolution is a measure of how well the peaks in the chromatogram are separated:

\[ R_s = 1.18 \frac{t_{R2} - t_{R1}}{w_{h1} + w_{h2}} \]

\( t_{R2} \) and \( t_{R1} \) are the retention times of the peak 2 and peak 1; \( w_{h1} \) and \( w_{h2} \) are the peak widths at the half height of peak 2 and 1.

\[ R_s = \frac{2 (t_{R2} - t_{R1})}{w_1 + w_2} \]

The equation above is used when peak widths are measured at the base of peak 2 and 1.
To improve the separation of the peaks the following equation uses the adjustable variables such as the retention factor, plate number and separation factor to control the resolution:

$$R_s = \frac{1}{4} \sqrt{N} \left( \frac{a-1}{a} \right) \frac{k}{(k+1)}$$

The plate number ($N$) can be manipulated by reducing the height equivalent to a theoretical plate by reducing the size of the stationary phase particles; the retention factor ($k$) can be optimized by changing the composition of the mobile phase; the separation factor ($\alpha$) can also be manipulated but if $\alpha$ is close to 1, optimizing $k'$ and increasing $N$ is not sufficient for a good separation\(^4\).

### 3.2.4.1.6 Relative retention ($r$)

In chromatography, the relative retention is the ratio of the adjusted retention factor of a substance relative to that of a standard, obtained under identical conditions\(^25\).

$$r = \frac{tR_2 - tM}{tR_1 - tM} \quad \text{or} \quad r = \frac{tR_2}{tR_1}$$
3.2.4.1.7 The Signal/Noise Ratio

To get a sharp, symmetrical peak the S/N ratio is used to separate the signal of interest from the unwanted signals (noises); it separates the desired signal from the background noise:

\[ S/N = \frac{2H}{h} \]

where \( H \) is the height of the peak and \( h \) is the height of the noise.

3.2.5 SOLVENTS AND SAMPLE PREPARATION

When using reversed phase chromatography the mobile phase is a mixture of aqueous buffer and an organic solvent. The organic solvent has to be pure and miscible with water. Furthermore, it should be inert towards the analyzed compounds. Most widely used organic solvents in RPC are acetonitrile, methanol and tetrahydrofuran (THF). Ethanol and isopropanol are also used in some cases.

The elution of the analytes can be performed as isocratic elution or as gradient elution. In the former mode the water-solvent composition does not change during the separation mechanism, while in the later the
composition of the mobile phase can be changed during the separation process.

In order to get a good separation with accurate retention times, there are several techniques that can be employed in sample preparation prior to the analysis. To obtain a desirable k' value for a solute, the polarity of the solute should be close to that of the organic solvent. One way to achieve this is by modifying the composition of the mobile phase, i.e. increasing the portion of the organic solvent (commonly 1:5 or 1:10)\(^4\).

In cases where the pH of the mobile phase interferes with the sample, such as with compounds of weak acidic or weak basic character (most of the pharmaceutical drugs), the k’ value can be increased using ion-pairing chromatography. The addition of ion-pairing agents in the mobile phase will suppress the analytes’ ionization. They form ionic interaction with the analyte and are extracted into the organic solvent as an electroneutral ion-pair. In this way they modify the analytes’ hydrophobicity and consequently their retention factors\(^26\). Commonly used ion-pairing agents are:

- **Inorganic:** Cl\(^-\), Br\(^-\), ClO\(_4\)^-  
- **Organic:** alkylammonium ions (for extraction of acids): tetrabutylammonium-iodide, cetyltrimethylammonium-bromide; alkylsulfonates (for extraction of bases): salts of pentasulfonic, hexasulfonic, heptasulfonic acids.

Desalting effect is another laboratory technique used to increase the k’ when preparing the sample for reversed phase chromatography. Adding
inorganic salt in the mobile phase will decrease the availability of the aqueous phase and thus resulting in the extraction of the solute in the organic phase. Salting-out agents are: buffers (5-0.01 mol/L), inert neutral salts e.g. NaCl, Na₂SO₄ etc⁴.

3.2.6 PUMPING SYSTEM

The pumps run under high pressure up to 40 MPa (400 atmospheres) and are responsible for delivering the mobile phase from the solvent reservoir to the column at constant flow rates with minimal fluctuation. The modern HPLC apparatus consists of one or two pumps that can be programmed to vary the mobile phase components, as is required for the gradient chromatography.

3.2.7 DETECTOR

LC-detectors are important accessories of the HPLC instrument. When the analytes are eluted from the column, they reach the detector which in turn converts electrical signals of the analytes into visual responses. The visual data are collected by a data acquisition system and are depicted in the chromatogram as peak areas or peak heights. Their operation is very important and largely applied in quantitative analysis.

The most frequently used detectors in liquid chromatography are the UV-VIS detector, the Fluorescence detector and the electrochemical detector. Other less frequently used but important detectors are: mass spectrometry (MS) detector and refractive index detector.
3.2.7.1 *The Ultraviolet-visible detector*

UV detectors are used in 75-80% of HPLC analysis. Its’ universal application is based on its simple working principle; it analyzes substances based on their absorption of the UV light. These substances are required to contain a chromophore, i.e. containing at least one double bond or unshared electrons, which is the case with most compounds. The UV wavelength range is from 180 to 340nm and 400 to 700nm for the colored compounds that absorb in the visible region. A UV-VIS detector consists of a lamp (deuterium lamp for UV spectra and/or the tungsten lamp for the visible spectra) which is the source of the radiation; the monochromator that narrows the emission of the radiation and ensures that the UV radiation of the correct wavelength is directed through the flow cell; the flow cell (detector cell) through which the eluent passes is designed in a Z-shape with dimensions of 10 mm length and 8µL volume capacity; a light-sensor (photodiode array) that converts the photons into current or voltage; an amplifier that modifies the output coming from the flow cell; and a data acquisition system that records the signal. The UV rays emitted by the lamp pass through the sample in the detector flow cell and fall onto the photodiode array producing electrons whose current is recorder. The absorbance of the UV radiation is proportional with the concentration of the sample passing through the cell. This relationship is given by the Lambert’s Beer Law:

\[ A = ε l c \]
\( \varepsilon \) - the molar absorptivity, an intrinsic property of the compound which measures how much light will be absorbed by 1 cm in a 1 M solution of the sample; \( l \) is the length of the flow cell and \( c \) accounts for the concentration of the sample.

![UV detector diagram](image)

**Fig. 3.2.7.1 UV detector diagram**

### 3.2.7.2 Mass spectrometry

Nowadays, mass spectrometers are common instruments in biochemical, pharmaceutical and analytical laboratories. The increasing demands for MS spectrographs are attributed to their advantage in providing both qualitative (identification and structure elucidation of chemical compounds) and quantitative analysis. Laboratories around the world are employing the use of MS instruments specifically because it complements well with the latest UPLC-system. Its sensitivity for detecting low concentrations takes advantage of the narrow peak width generated by the UPLC.

**Principle:** after the sample is eluted from the column, it reaches the ionizer where it is nebulized; ions are then separated and detected according to their mass-to-charge ratio.
(m/Z). A mass spectrometer consists of three main parts: (a) an ionizer; (b) a mass selector; and (c) a detector.

In LC-MS, the column effluent is directed into the ionizer where it is nebulized under atmospheric pressure (Atmospheric Pressure Ionization). The ionization mechanism is achieved by either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The ESI is considered a liquid-phase ionization technique; the ions in the solution are disperse by electrospray into aerosol, evaporated into gas-phase and subsequently analyzed; whereas the APCI is considered a gas-phase ionization technique based on the gas-phase ion-molecule reaction between the analyte molecules and the solvent-based reagent gas; these reactions are initiated by electrons from the corona discharge needle. After nebulization of the sample, the ions are directed into the mass selector which separates the ions by manipulating their trajectories under electric and/or magnetic field according to their m/Z ratio. The analyzer operates under vacuum so that the ionized particles run freely in the chamber without hitting air molecules. The separated ions are detected by the detector and the signals are processed into mass spectra.
3.2.8 QUANTIFICATION AND QUALITY OF DATA ANALYSIS

3.2.8.1 METHOD DEVELOPMENT

When working with testing equipment it is important to ensure that when used as measurement equipment they will measure correctly. Performing a successful chromatographic method and generating quality data requires selection of a suitable method for the analysis and also an experienced staff. Hence, before beginning with the analysis the performance of the instrument system should always be checked. Measuring instruments are calibrated with the help of various equipment or in the case of HPLC by comparing its responses with known standards. When evaluating chromatograms and instruments operation there are several important questions that need to be addressed: Do the peaks look normal (Gaussian)?; Is the response obtained comparable to the response from previous calibrations?; Are non-target peaks present in calibration analyses?; Are contaminants present in the blanks?; Is the injector leaking?; Does the HPLC guard column need replacement?31.

There are several methods used to calibrate LC-instruments prior to analysis.

3.2.8.1.1 Internal standard calibration

An internal standard in analytical chemistry is a chemical substance that is added in a constant amount to samples, the blank and calibration standards in a chemical analysis32. The internal standard calibration is used to compare the instrument response between the reference standard
and the targeted compound. The internal standard should show similar but not completely identical behavior to the target compound; it should be recovered to a similar extent as the target compound and be separated from all other substances in the sample. The calibration is done by plotting the analyte/internal standard peak area or peak height ratio\textsuperscript{22}. This ratio is called the relative response factor (RRF) and is used to obtain the analyte concentration from the calibration curve:

\[
\text{RFF} = \frac{A_c \times C_{is}}{A_{is} \times C_c}
\]

\(A_c\) – the peak area of the target analyte
\(A_{is}\) – the peak area of the internal standard
\(C_c\) – the concentration of the target analyte
\(C_{is}\) – the concentration of the internal standard

The internal standard is a certified reference substance of the compound to be determined which is added at known concentration to standard solutions and to sample solutions prior to the analysis. This method has shown to compensate for the analytical errors due to sample losses and variable injection volumes compared to the external standard method\textsuperscript{33}.

3.2.8.1.2 **External standard calibration**

The external standard is similar to the internal standard in that the sample solutions are compared with reference solution, but the key difference is that the external standard is not added to the sample solution but instead run separately under identical conditions.
3.2.8.1.3 Standard addition

This method is used to determine the concentration of an analyte which is part of a complex matrix (e.g. biological fluids) and contain substances that may interfere with the detectors' response. In this case, plotting a calibration curve based on the analytes concentration would be incorrect due to the interferences of substances from the matrix. Instead, a sample solution is “spiked” with a known analyte concentration and the change between the sample solution and the “spiked” sample is monitored in the instruments response. This change is assumed to be only due to change in analyte concentration.

3.2.8.1.4 Normalization

The normalization method is the most straightforward and easiest technique used in accessing quantitative analysis. It is applied in a limited number of analyses where the detectors’ response is the same for all the analytes present in the sample. The results are obtained by expressing the area of a given peak as a percentage of the sum of all peak areas:

\[ A(\%) = \frac{A}{\sum A_i} \times 100 \]

3.2.8.2 METHOD VALIDATION

Validation is a compulsory task for every analytical procedure in pharmaceutical analysis. The requirements for validating a method are recommended by the International Conference on Harmonization of Technical
Requirements of Pharmaceuticals for Human Use (ICH), based on the document Validation of Analytical Procedure: Text and Methodology. Validation is a method used to demonstrate that an analytical procedure is suitable for its intended use. It comprises of a series of characteristics:

3.2.8.2.1 Accuracy

Accuracy of an analytical method describes the closeness between the results obtain from the procedure to that of the true value (true concentration) of the analyte. Accuracy should be reported in either of two ways:

- The percentage of recovery by the assay of the known added amount of the analyte in the sample;
- The difference between the mean and the accepted true value.

For a method to be considered accurate, the limit deviation of the mean and the true value is 85-115%, except for the LLOQ (low limit of quantification) which is 80-120%.

3.2.8.2.2 Precision

Precision of an analytical method is usually expressed as the variance, or as the standard deviation between a series of measurements obtained from a multiple sampling of the same homogenous sample under the prescribed conditions. It is considered at three levels:

- **Repeatability** is expressed as the precision under the same operating conditions over a short interval of time;
- **Intermediate precision** expresses within laboratories variations: different days different analysts, different equipment, etc.;

- **Reproducibility** expresses the precision between laboratories\(^{34}\);

The limit for the coefficient variation or the relative standard deviation is 85-115\%, except for the LLOQ in which case it is 80-120\%.

### 3.2.8.2.3 Specificity

Specificity or selectivity is the ability of an analytical method to assess the analyte concentration regardless of the presence of possible interfering substances (impurities, endogenous matrix, degradants).

### 3.2.8.2.4 Detection limit

The limit of an analytical procedure is the lowest amount of the analyte that can be detected but not necessarily quantified as an exact value\(^ {34}\).

### 3.2.8.2.5 Quantification limit

Limit of quantification is the lowest limit for a given analyte that can be quantitatively determined by an analytical procedure. The response (peak area) of the lowest standard should be 5 times higher than the response of a blank sample and should be reproducible with a precision of 20\% and accuracy of 80-120\%\(^ {4}\).

### 3.2.8.2.6 Linearity

The linearity (calibration or standard curve) shows the relationship of the instruments’ response and the known concentration of the analyte\(^ {4}\).
According to the ICH, to establish the linearity a minimum of five concentrations should be used.

Figure 3.6 Example of a calibration curve with five sample concentrations

3.2.8.2.7 Range

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity \(^3\).

3.2.8.2.8 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Examples of typical variations in liquid chromatography are: variations of pH in the mobile phase; variations in composition of the mobile phase; variations in temperature and flow rate.
3.3 ULTRA-FAST LIQUID CHROMATOGRAPHY

3.3.1 The Evolution of smaller particles and the advantages of UFLC

Ultra-fast performance liquid chromatography is the newest separation technique in liquid chromatography. Although the HPLC was a proven technique that was routinely used in laboratories around the world, the need for improving separation efficiency has led to the development of a more sophisticated and advantageous instrument such as the UFLC. The principle on which the improvement of the separation efficiency lies is the decrease in size of the packing material in the column. The decrease of particle size is explained by the van Deemter equation which describes the relationship of the decrease of particle size with the linear velocity (flow rate) of the mobile phase moving through the column and the plate height (HETP or column efficiency).35.

![Figure 3.7 Van Deemter curve that explains the effect of decreasing particle size in linear velocity and column efficiency](image-url)
The increased flow rate does not affect the resolution and the sensitivity of the method, in fact it improves it. This is explained from the fundamental resolution equation:

\[ R_s = \frac{1}{4} \sqrt{N} \frac{(\alpha-1)}{\alpha} \frac{k}{(k+1)}, \]

which shows that the resolution is proportional to the square root of plate number (N). But since N is inversely proportional to the particle size (N=\( \frac{1}{dp} \)), if dp is decreased by a factor of three, for example, from 5 µm (HPLC scale) to 1.7 (UFLC scale), then N is increased by three and \( R_s \) is increased by the square root of three or 1.7. Another aspect that explains the increase in resolution is that N is also inversely proportional to the peak width (N = 1/\( w^2 \)), as the particle size is decreased, the plate number increases and the peak width (\( w^2 \)) decreases; meaning that the narrower the peaks are, the easier they are to be separated from one another. In addition, the peak height is inversely proportional to peak width; the narrower the peaks, the taller the peaks and thus the higher the sensitivity\(^{35}\).

The use of smaller particles required for utilization of higher pressure in order to push the mobile phase through the packed material in the column. UFLC allows the use of pressure up to 1000 bar which is one order of magnitude greater than pressure generated by the HPLC\(^{36}\). The drawback of the small particles was in the ability of the other compartments of the instruments to withstand the high back-pressures.
As a result pumping system and sample injector had to be customized. In a work done by MacNair\textsuperscript{36}, they invented a static-split injector which could inject samples in much higher speed, were able to work under the highest pressure ever generated in liquid chromatography. In addition, the high pressure accounted for increased heat in the column which would cause decomposition of the mobile phase and/or the sample molecules. The problem was solved in the work of Wu\textsuperscript{37} were heat dissipation was achieved by using capillary columns.

The evolution of smaller particles allowed for a better resolution, an increase in sensitivity and faster analysis\textsuperscript{38}.

The first ultra-fast liquid chromatography apparatus was ACQUITY UPLC® designed by Waters. The system adjustments involved a binary pump system that could withstands pressures up to 1000 bar, an autosampler and a sample injector that could perform fast injection cycles, low injection volumes with minimum carryovers and temperature control (4 - 40ºC)\textsuperscript{39}. 
4 EXPERIMENTAL PART

Apparatus

Nexera Ultra-Fast Liquid Chromatography system with gradient elution capability and spectrophotometric UV detector, Shimadzu, Japan.

Analytical column: KINETEX® Column Protection KrudKatcher™ Ultra, fittings Sur-Lok™ and Traditional; column length 150 mm; internal diameter 2.1 mm, particle size 1.7µm C18, 100Å

Conditions

Mobile phase: mixture: eluent A (buffer) : eluent B (50 : 50); the mobile phase was sonicated for a few minutes

Flow rate: 0.15 ml/min

Detection UV/Vis: 250 nm

Injection volume: 3µL

Acquisition time: 35 minutes

Column oven temperature: 40°C

Diluent: eluent B

Data comparison between HPLC and UFLC and method conversion

The method transfer from conventional HPLC to UFLC apparatus was achieved by using an online method transfer calculator⁴⁰.
**Preparation of buffer (eluent A)**

1.54 mg of Ammonium acetate was weighted into a beaker containing 500 ml of water. The pH of the solution was adjusted to 9.0 ± 0.5 using Triethyl amine solution. The eluent was filtered through a 0.45µm membrane and sonicated in an ultrasonic bath for 5 minutes. The eluent was prepared just before use in order to avoid instabilities of the solution and possible errors during the analysis.

**Preparation of eluent B**

To prepare a 500 ml of eluent B, Acetonitrile (150 ml) and Methanol (350 ml) were mixed together.
Preparation of standard solutions

Standard solution (a): 8.91 mg of quetiapine fumarate working standard were weighted accurately into a 25 ml volumetric flask, sonicated and made up to volume with diluent.

c=0.3564 mg/ml

Standard solution (b): 1 ml of the standard solution (a) were diluted to 250 ml.

c=0.0014 mg/ml

Preparation of the system suitability test solution (SST):

Approximately 1 mg of each of the working standard substances of dibenzo impurity, triethoxy impurity and quetiapine fumarate were weighted into a 100 ml volumetric flask, initially dissolved with 50 ml of methanol and subsequently diluted to make 100 ml solution.

4.1.1 System Suitability Test

Three injections of system suitability test solution and three injections of quetiapine standard solution were run to compare retention times, check the tailing factors and resolution between peak areas.

<table>
<thead>
<tr>
<th></th>
<th>Area</th>
<th>Retention time</th>
<th>Tailing factor</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibenzo</td>
<td>6777</td>
<td>9,441</td>
<td>0,701</td>
<td>4,826</td>
</tr>
<tr>
<td>Quetiapine</td>
<td>14188471</td>
<td>22,034</td>
<td>0,845</td>
<td>3,076</td>
</tr>
<tr>
<td>Triethoxy</td>
<td>811</td>
<td>24,088</td>
<td>1,751</td>
<td>3,806</td>
</tr>
</tbody>
</table>
**Evaluation:** according to the Ph.Eur. and USP requirements, the tailing factors of the peak areas obtained are within the limit range which is 0.8 – 1.5. The resolution of the peak areas also complies with the pharmacopoeia requirement, where \( R_s \) of the three peaks is greater than 2.

### 4.1.2 Linearity

To test the linearity (response) of the detector five concentrations of the sample were prepared. The conventional value for the minimum limit of impurities in a sample is 0.05%; in cases where the obtained value is lower than 0.05% the presence of the impurities is considered to be irrelevant. The conventional value for maximum limit of impurities in a sample is plus 50% of the minimum limit of impurities.

According to the manufacturer of Questax® the value for maximum limit of impurities is 0.2%. The maximum value for limit of impurities chosen in this work was 0.3%.

Range concentration of the sample was divided in five parts as follows: 0.05% (the minimum limit); 0.075%; 0.15%; 0.225% and 0.3% (the maximum limit).

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>c</td>
<td>0.05%</td>
<td>0.075%</td>
<td>0.15%</td>
<td>0.225%</td>
<td>0.3%</td>
</tr>
</tbody>
</table>
Sample preparation

Sample concentration of 100% quetiapine (150 mg of quetiapine in 250 ml) is $c=0.6$ mg/ml.

- For 0.3% of quetiapine the concentration was calculated to be $c=0.0018$ mg/ml.
- For 0.05% of quetiapine the concentration was calculated to be $c=0.0003$ mg/ml.

Procedure of preparing the range of five different concentrations:

0.5 ml were taken from the standard solution of quetiapine with concentration $c=0.3$ mg/ml and diluted with diluent to make 50 ml quetiapine solution ($c=0.003$ mg/ml). Furthermore, 6 ml were taken from the above solution and diluted with diluent to make 10 ml solution of 0.3% quetiapine ($c=0.0018$ mg/ml, the maximum limit of impurities);

4.5 ml were taken from the standard solution and diluted with diluent to make 10 ml solution of 0.225% quetiapine;

3 ml were taken from the standard solution and diluted with diluent to make 10 ml solution of 0.15% quetiapine;

1.5 ml were taken from the standard solution and diluted with diluent to make 10 ml solution of 0.075% quetiapine;

1 ml was taken from the standard solution and diluted with diluent to make 10 ml solution of 0.05% quetiapine ($c=0.0003$ mg/ml, the minimum limit of impurities).
A batch with the vials containing five different concentrations were each injected twice for the analysis.

![Calibration curve](chart.png)

**Figure 4.1** Calibration curve obtained from the range of five different concentrations of the sample; \(c_1=0.05\%, c=0.075\%, c=0.15\%, 0.225\%, 0.3\%\); each sample was injected twice under the conditions described in the method above.

<table>
<thead>
<tr>
<th>Linearity</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1-001</td>
<td>5700</td>
</tr>
<tr>
<td>L1-002</td>
<td>5240</td>
</tr>
<tr>
<td><strong>AVG</strong></td>
<td><strong>5470</strong></td>
</tr>
<tr>
<td>L2-001</td>
<td>9418</td>
</tr>
<tr>
<td>L2-002</td>
<td>9303</td>
</tr>
<tr>
<td><strong>AVG</strong></td>
<td><strong>9360.5</strong></td>
</tr>
<tr>
<td>L3-001</td>
<td>20967</td>
</tr>
<tr>
<td>L3-002</td>
<td>20612</td>
</tr>
<tr>
<td><strong>AVG</strong></td>
<td><strong>20789.5</strong></td>
</tr>
<tr>
<td>L4-001</td>
<td>32641</td>
</tr>
<tr>
<td>L4-002</td>
<td>33051</td>
</tr>
<tr>
<td><strong>AVG</strong></td>
<td><strong>32846</strong></td>
</tr>
<tr>
<td>L5-001</td>
<td>44076</td>
</tr>
<tr>
<td>L5-002</td>
<td>44298</td>
</tr>
<tr>
<td><strong>AVG</strong></td>
<td><strong>44187</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CONCENTRATION</th>
<th>AREA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>5470</td>
</tr>
<tr>
<td>0.075</td>
<td>9360.5</td>
</tr>
<tr>
<td>0.15</td>
<td>20789.5</td>
</tr>
<tr>
<td>0.225</td>
<td>32846</td>
</tr>
<tr>
<td>0.3</td>
<td>44187</td>
</tr>
</tbody>
</table>
**Evaluation:** the regression curve was calculated by the method of least squares: \( y = 155282x -2314.5 \), where \( y \) is the peak area and \( x \) is the concentration. The square of R is the correlation coefficient between these two variables and for the two variables to be in correlation \( R^2 \) should be higher or equal to 0.99. The square of the correlation coefficient obtained from this batch \( R^2 = 0.9999 \) shows that the two variables are highly correlated which indicated that the detector was linear and ready to continue for further analysis.

### 4.1.3 Quantification Limit (LOQ)

The lowest limit of the sample concentration that was able to be quantified by the instrument was calculated by the signal-to-noise ratio. The S/N ratio was measured from the chromatogram showing the peak area of the lowest concentration of the sample solution (c=0.05%).

![Chromatogram of the peak area of the lowest limit of quantification](image_url)

*Figure 4.2 Chromatogram of the peak area of the lowest limit of quantification*
Evaluation: The calculation was done using the formula: \( S/N = 2H/h \); the peak height (H) and the noise height (h) were measured and retrieved from the chromatogram.

\( S/N = 16.44 \) for \( c = 0.0003 \) mg/ml; so for \( S/N = 10 \) which is the conventional pharmacopeia value for the lowest concentration that the instrument can quantify, the limit of quantification (LOQ) was found to be 0.0002 mg/ml and this value refers to the concentration of 0.03%.

4.1.4 Detection Limit (LOD)

Similarly, the S/N ratio was measured from the chromatogram showing the peak area for the lowest limit of concentration; and for \( S/N = 3 \) which is the conventional pharmacopoeia value for the lowest concentration that the instrument can detect, the limit of detection (LOD) was found to be 0.00005 mg/ml. The value corresponds to approximately 0.01%.

4.1.5 Selectivity

A batch containing the sample solution, quetiapine standard solution, SST solution and two placebo solution was run for the analysis to test the selectivity of the method. Chromatograms of each injection were compared. Placebo solutions were prepared according to the recommendations of the manufacturer: 50 mg of placebo were weighted into a 50 ml volumetric flask, dissolved and diluted to the mark with diluent.
Figure 4.3 Data comparison of the peaks obtained from the sample solution, the standards alongside placebo.

- Sample solution of quetiapine fumarate
- Quetiapine base standard solution
- SST solution
- Placebo solution of 100 mg quetiapine tablet
- Placebo solution of 25 mg quetiapine tablet

**Evaluation:** according to the chromatograms, auxiliary substances present in Questax® 100mg and Questax® 25 mg tablets do not interfere with the active substance and its impurities and the analytical method was evaluated to be selective.

### 4.1.6 Precision

The relative standard deviation was calculated to measure the extent of the precision to which individual test results of multiple injections of a series of standard agree.

In this work, precision was considered by testing the repeatability of the method.
Sample Preparation

The following procedure was done to prepare six samples:

74 mg of crushed powder of 25 mg tablet were weighted into a 50 ml volumetric flask. Thereafter, 30 ml of diluent (mobile phase) were added to dissolve the powder. The mixture was sonicated for 15 minutes and then left to cool down at room temperature. After cooling, the mixture was filled up to the mark with diluent and filtrated through a 0.45 µm membrane.

The six samples where each injected twice. The average peak area of two injections was calculated. The response factor for dibenzo impurity is 1.38 and for triethoxy impurity is 0.85. Assay for known individual impurities was calculated using the formula:

\[
\% = \frac{\text{Area}}{Rf} \times \frac{\text{concentration of quetiapine base}}{\text{Area of Quetiapine standard}} \times \frac{\text{concentration of Quetiapine fumarate}}{100}
\]

<table>
<thead>
<tr>
<th>Dibenzo impurity</th>
<th>Peak Area</th>
<th>Area/Response factor</th>
<th>Impurity content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>6780.5</td>
<td>4913.405797</td>
<td>0.037306</td>
</tr>
<tr>
<td>P2</td>
<td>6434.5</td>
<td>4662.681159</td>
<td>0.035402</td>
</tr>
<tr>
<td>P3</td>
<td>6380</td>
<td>4623.188406</td>
<td>0.035102</td>
</tr>
<tr>
<td>P4</td>
<td>6242</td>
<td>4523.188406</td>
<td>0.034343</td>
</tr>
<tr>
<td>P5</td>
<td>6188</td>
<td>4484.057971</td>
<td>0.034046</td>
</tr>
<tr>
<td>P6</td>
<td>6693</td>
<td>4850</td>
<td>0.036824</td>
</tr>
<tr>
<td>total</td>
<td>6453</td>
<td>4676.086957</td>
<td>0.035504</td>
</tr>
</tbody>
</table>
### Triethoxy impurity

<table>
<thead>
<tr>
<th></th>
<th>Peak Area</th>
<th>Area/Response factor</th>
<th>Impurity content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>821</td>
<td>965.88</td>
<td>0.0073</td>
</tr>
<tr>
<td>P2</td>
<td>859</td>
<td>1010.59</td>
<td>0.0077</td>
</tr>
<tr>
<td>P3</td>
<td>831.5</td>
<td>978.23</td>
<td>0.0074</td>
</tr>
<tr>
<td>P4</td>
<td>899</td>
<td>1057.68</td>
<td>0.0080</td>
</tr>
<tr>
<td>P5</td>
<td>920.5</td>
<td>1082.94</td>
<td>0.0082</td>
</tr>
<tr>
<td>P6</td>
<td>889</td>
<td>1045.88</td>
<td>0.0078</td>
</tr>
<tr>
<td>total</td>
<td>870</td>
<td>1023.53</td>
<td>0.0077</td>
</tr>
</tbody>
</table>

Precision is usually expressed by the relative standard deviation:

\[
\% \text{ RSD} = \frac{\text{sample standard deviation}}{\text{mean standard}} \times 100
\]

The calculations were done in excel:

<table>
<thead>
<tr>
<th></th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibenzo impurity</td>
<td>3.26</td>
</tr>
<tr>
<td>Triethoxy impurity</td>
<td>4.42</td>
</tr>
</tbody>
</table>

Requirements for precision for related substances in preparation are:

<table>
<thead>
<tr>
<th>c</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,0 % ≤ Ci</td>
<td>5</td>
</tr>
<tr>
<td>0,1 % ≤ Ci &lt; 1,0 %</td>
<td>7</td>
</tr>
<tr>
<td>LOQ % ≤ Ci &lt; 0,1 %</td>
<td>10</td>
</tr>
</tbody>
</table>
4.1.7 Accuracy

Sample preparation

The procedure of preparing the samples for accuracy was the same as in samples preparation for precision, with the exception of adding known amount of impurities to the sample solution, which had to be calculated. Firstly, the concentration of each impurity in the SST solution was calculated according to the formula:

\[
 c (\text{mg/ml}) = \frac{\text{Area} \times \text{concentration of Quetiapine base standard}}{\text{Area of Quetiapine standard}}
\]

Dibenzo impurity \( c = 0.0146 \); Triethoxy impurity \( c = 0.0084 \).

Secondly, the amount of each of the impurities to be added to the SST solution was calculated as follows:

- the concentration of 100% quetiapine in SST solution was \( c = 0.6 \) mg/ml. The percentage of impurities present in the SST solution has to be within the range of the lowest LOQ and the highest limit on the calibration curve. The chosen point to be taken as the true value for triethoxy impurity was 0.05%, for which the concentration was found to be 0.01512 mg/50 ml. Because 0.01512 mg was a very small amount to weigh, instead, the ml needed to be added in the sample were calculated as follows: if 1ml of SST solution contains 0.0084 mg of triethoxy, then 0.01512 mg accounts for 1.8 ml of SST.
The amount of dibenzo impurity in 1.8 ml of SST solution, with a concentration of 0.0146 mg/ml, was found to be 0.02628 mg. If a 100% solution of quetiapine standard contains 30 mg of quetiapine (0.6 mg/ml x 50 ml), percentage of dibenzo impurity in 50 ml of sample solution was calculated as 0.08732%.

True values that were added to the sample: Triethoxy 0.050531%; Dibenzo 0.08732%.

The tables below show the comparison between found value and the true value and also, the recovery of the assay of the known added amount of impurities in the sample was calculated with the formula:

\[
\% = \frac{\text{found value}}{\text{true value}} \times 100
\]

<table>
<thead>
<tr>
<th>Dibenzo impurity</th>
<th>Peak Area</th>
<th>Area/Response factor</th>
<th>% with added impurity</th>
<th>% without added impurity</th>
<th>Found added value</th>
<th>True value</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>21,161</td>
<td>15333.70</td>
<td>0.1163</td>
<td>0.0373</td>
<td>0.0810</td>
<td>0.0873</td>
<td>92.80</td>
</tr>
<tr>
<td>A2</td>
<td>20819.5</td>
<td>15086.60</td>
<td>0.1144</td>
<td>0.0354</td>
<td>0.0792</td>
<td>0.0873</td>
<td>90.66</td>
</tr>
<tr>
<td>A3</td>
<td>21436</td>
<td>15533.33</td>
<td>0.1178</td>
<td>0.0351</td>
<td>0.0825</td>
<td>0.0873</td>
<td>94.54</td>
</tr>
<tr>
<td>A4</td>
<td>21470</td>
<td>15557.97</td>
<td>0.1180</td>
<td>0.0343</td>
<td>0.0827</td>
<td>0.0873</td>
<td>94.76</td>
</tr>
<tr>
<td>A5</td>
<td>21564</td>
<td>15626.10</td>
<td>0.1185</td>
<td>0.0340</td>
<td>0.0832</td>
<td>0.0873</td>
<td>95.35</td>
</tr>
<tr>
<td>A6</td>
<td>21228.5</td>
<td>15382.97</td>
<td>0.1167</td>
<td>0.0368</td>
<td>0.0814</td>
<td>0.0873</td>
<td>93.24</td>
</tr>
<tr>
<td>average</td>
<td>21,280</td>
<td>15,420</td>
<td>0.1170</td>
<td>0.0355</td>
<td>0.0822</td>
<td>0.0873</td>
<td>93.56</td>
</tr>
</tbody>
</table>

*Figure 4.4 Table showing the average of 2 injections of six samples prepared for accuracy and the resulting recovery % for the dibenzo impurity.*
### Evaluation:

The percentage of recovery complies with the requirements for accuracy for related substances in preparation. For concentrations:

<table>
<thead>
<tr>
<th>c</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1,0 % \leq C_i$</td>
<td>$90 – 110 %$</td>
</tr>
<tr>
<td>$0,1 % \leq C_i &lt; 1,0 %$</td>
<td>$85 – 115 %$</td>
</tr>
<tr>
<td>$\text{LOQ} % \leq C_i &lt; 0,1 %$</td>
<td>$80 – 120 %$</td>
</tr>
</tbody>
</table>

Figure 4.6 The EU-Phár and USP requirements of accuracy for related substances in the preparation.
5 RESULTS AND CONCLUSION

The method was developed for determination of related substances of Quetiapine Fumarate (Questax®) by Ultra-fast liquid chromatography system with gradient elution capability and spectrophotometric UV detector, particle size of 1.7 µm with flow rate of 0.15 ml/min. The suitability of the method was validated by the parameters of linearity, LOQ and LOD, precision and accuracy and the results of the experiment are shown below:

System suitability test: an SST solution containing both the active substance and the impurity contents was injected with each batch during the validation procedure. The method was shown to be suitable as the tailing factor of the peaks was kept within the limit range of 0.8 – 1.5; also, the resolution of each of the peak areas was in all cases greater than 2, which complies with the Ph.Eur. and USP requirements.

Linearity: the validity of the method was further ensured as the correlation coefficient (R²) obtained from the procedure was 0.999, which shows that the two variables, peak areas and the range of five different concentrations of the sample highly correlate with each other. For the method to be linear, the correlation coefficient should be higher or equal to 0.99.

Quantification Limit: the lowest quantification limit was measured using the S/N ratio of the peak area with the lowest concentration of the sample solution (0.05%). The concentration was found to be 0.0002 mg/ml which refers to 0.03% of the sample solution.
**Detection Limit:** the lowest detection limit was measured similarly as for LOQ and the lowest concentration to be detected was found to be 0.00005 mg/ml, which is approximately 0.01% of the sample solution.

**Selectivity:** the specificity of the analytical method was determined by injecting two placebo solutions simultaneously with the SST and standard solutions, and according to the chromatograms of the peak areas, the excipients of the preparation do not interfere with neither with the active substance nor with its impurities.

**Precision:** the extent of the precision of the method was measured by the relative standard deviation (%RSD) of multiple injections of a series of standards. The RSD of two impurities with contents lower than 0.1%, were 3.27% for dibenzo and 4.42% for triethoxy. According to the requirements for precision for related substances in preparation, for substances with concentration lower than 0.1% the RSD value should be more than 10%.

**Accuracy:** the accuracy of the method was conducted by measuring the percentage of recovery of the known added amount of the impurity to the sample solution. The limit of deviation between the mean value and the true value obtained from the procedure was RSD = 93.56% for dibenzo impurity (c (%) = 0.0355%) and RSD = 105.90% for triethoxy impurity (c (%) = 0.0077). According to the requirements for accuracy for related substances in preparation, for substances with c <0.1% the RSD value should be in the range limit of 80 – 120%.

**CONCLUSION:** The newly developed method of UFLC was successfully validated as shown in the results in the validation procedure. The results obtained in this work
show that the method transfer from conventional HPLC to UFLC method was successful in that it shortened the analysis time for up to 30 minutes and the resolution of the method was increased by 1.7 which accounts for the improvement of the separation efficiency. Overall, the applicability of UFLC system compared to that of the conventional HPLC system, despite the high cost of the instrument, is highly more advantageous and should be employed whenever possible as the LC-system of the choice.
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