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Stability indicating RP-UPLC Method for estimating sofpronium by quality by design approach.

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Abstract : A simple, Accurate, precise method was developed for the estimation of the Sofpironium in bulk and pharmaceutical dosage form. Chromatogram was run through ACQUITY UPLC BEH C18 Column, 1.7 µm, 2.1 mm X 50 mm. Mobile phase containing 0.1% AmmoniumFormate: Methanol taken in the ratio 73.6 (%v/v) and 26.3 was pumped through column at a flow rate of 0.28 ml/min. Temperature was maintained at 29.21°C. Optimized wavelength selected was ACQUITY TUV ChA 219.0 nm. Retention time of Sofpironium was found to be 1.814 min. %RSD of the Sofpironium was found to be 0.4%. %Recovery was obtained as 99.94% for Sofpironium. LOD, LOQ values obtained from regression equations of Sofpironium were 0.05, 0.15. Regression equation of Sofpironium is y = 52995x + 2524.1.Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

IndexTerms - Sofpironium, QbD Approach, Method development, RP-UPL1

INTRODUCTION

Most medications in multicomponent dosage forms may be analysed by the UPLC system thanks to its many benefits, including speed, specificity, consistency, accuracy, precision, and simplicity of automation. The UPLC method saves repeating extraction and isolation procedures. There are several modes of differentiation in UPLC. These are Size Exclusion Chromatography, Chromatography of Reversed Phase Ion Phase, Chromatography of Affinity, Normal Phase Mode, and Inverted Phase Mode.

The effectiveness and safety of a medicine are significantly influenced by the quality of the drug. For customers to have access to safe and effective medicinal formulations, quality assurance and control of pharmaceutical and chemical formulations are crucial. Hence When determining whether a chemical is suitable for use in patients, analysis of both the pure drug material and its pharmaceutical dose forms is crucial. The calibre of the processes used to generate the data are what determines the calibre of the analytical data (1). To ensure that pharmaceuticals and their formulations are legally certified by regulatory bodies, it is crucial to establish tough and reliable analytical procedures.

Depending on the type and characteristics of the substance, a broad range of obstacles are encountered when creating the procedures. This, along with the significance of achieving selectivity, speed, cost, simplicity, sensitivity, reproducibility, and accuracy of results, provides researchers with an opportunity to find solutions to the problems associated with getting new analytical techniques adopted by the chemical and pharmaceutical industries. Different physico-chemical methods (1) are used to study the physical phenomenon that

occurs as a result of chemical reactions. Among the physico-chemical methods, the most important are optical (refractometry, polarimetry, emission and fluorescence methods of analysis), photometry (photocolorimetry and spectrophotometry covering UV-Visible, IR Spectroscopy and nepheloturbidimetry) and chromatographic (column, paper, thin layer, gas liquid and high performance liquid chromatography) methods. Methods such as nuclear magnetic resonance (NMR) and para magnetic resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy (MS) with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures which are based on complex formation; acid-base, precipitation and redox reactions. Titrations in non-aqueous media and complexometry have also been used in pharmaceutical analysis. The number of new drugs is constantly growing. This requires new methods for controlling their quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of Pharmacopoeia.
3. The analysis should be economical.
4. The selected method should be precise and selective.

1.1CHROMATOGRAPHY

Chromatography (Chroma means ‘color’ and graphein means to ‘write’) is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase,(2-4) which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Differences in compounds partition coefficient results in differential retention on the stationary phase and thus changing the separation.

Different types of chromatographic techniques were summarized in

Table No. 1.1Different types of chromatographic techniques

Sl. No	Basic principle involved	Type of Chromatography
1.	Techniques by chromatographic bed shape	Column chromatography
		Paper chromatography
		Thin layer chromatography
2	Techniques by physical state of mobile phase	Gas chromatography
		Liquid chromatography
3	Affinity chromatography	Supercritical fluid chromatography
4	Techniques by separation mechanism	Ion exchange chromatography
		Size exclusion chromatography
5	Special techniques	Reversed phase chromatography
		Simulatedmoving-bed chromatography
		Pyrolysis gas chromatography
		Fast protein liquid chromatography
		Counter current chromatography
		Chiral chromatography

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a

mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportion of analytes in a mixture.

INTRODUCTION TO UPLC

UPLC:

UPLC is an emerging area of analytical separation science which retains the practicality and principles of UPLC while increasing the overall interlaced attributes of speed, sensitivity and resolution. Speed and peak capacity can be extended to new limits, termed Ultra Performance Liquid Chromatography, or UPLC by using fine particles. UPLC takes full advantage of chromatographic principles to run separations using columns packed with smaller particles and/or higher flow rates for increased speed, sensitivity and superior resolution.

In this article we explored the potential of UPLC to improve the analysis of the samples that are encountered during pharmaceutical development and manufacturing. Particular emphasis has been placed on determining whether UPLC can reduce analysis times without compromising the quantity and quality of the analytical data generated compared to UPLC.

Here particular emphasis is given on principle involved, instrumentation encountering different UPLC columns, different particle chemistries, detectors and various applications. UPLC generated higher separating efficiencies through the use of a smaller diameter particle packing and higher operating pressures. A commercial system capable of generating much higher pressures (1000 bar) than used in standard UPLC has been evaluated to determine its potential in routine analysis. UPLC has been shown to generate high peak capacities in short times and this is found to be quite beneficial in analyzing the complex mixtures that constitute metabolism samples. The application of UPLC resulted in the detection of additional drug metabolites, improved the spectrum quality and separation efficiency[1,2].

PRINCIPLE

The UPLC is based on the principal of use of stationary phase consisting of particles less than 2µm, while UPLC columns are typically filled with particles of 3 to 5µm. The underlying principles of this evolution are governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency) [3]. The Van Deemter curve, governed by an equation with three components shows that the usable flow range for a good efficiency with a small diameter particles is much greater than for larger diameters[4,5].

$$H = A + \frac{B}{\mathrm{v}} + C\mathrm{v}$$

Where A, B and C are constants and v is the linear velocity, the carrier gas flow rate. The A term is independent of velocity and represents "eddy" mixing. It is smallest when the packed column particles are small and uniform. The B term represents axial diffusion or the natural diffusion tendency of molecules. This effect is diminished at high flow rates and so this term is divided by v. The C term is due to kinetic resistance to equilibrium in the separation process. The kinetic resistance is the time lag involved in moving from the gas phase to the packing stationary phase and back again. The greater the flow of gas, the more a molecule on the packing tends to lag behind molecules in the mobile phase. Thus this term is proportional to v.

Chemistry of Small Particles

Smaller particles provide not only increased efficiency, but also the ability to work at increased linear velocity without loss of efficiency, providing both resolution and speed. Efficiency is the primary separation parameter behind UPLC since it relies on the same selectivity and retentivity as UPLC. In the fundamental resolution (Rs) equation:

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

Resolution is proportional to the square root of N. But N is inversely proportional to particle size (dp):

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

N is also inversely proportional to the square of the peak width:

$$N \propto \frac{1}{w^2}$$

This illustrates that the narrower the peaks are, the easier they are to separate from each other. Also, peak height is inversely proportional to the peak width:

$$H \propto \frac{1}{w}$$

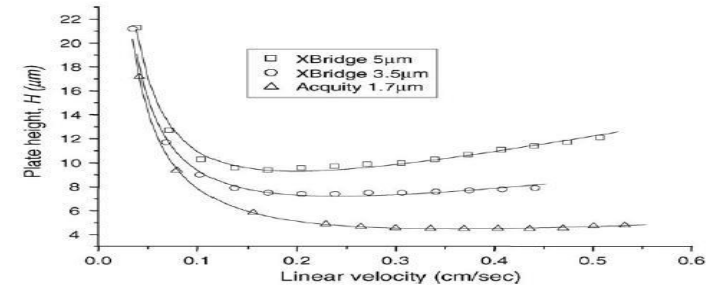
So as the particle size decreases to increase N and subsequently Rs, an increase in sensitivity is obtained, since narrower peaks are taller peaks. Narrower peaks also mean more peak capacity per unit time in gradient separations, desirable for many applications. Eg: peptide maps.

Efficiency is proportional to column length and inversely proportional to the particle size[3]:

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

Therefore, the column can be shortened by the same factor as the particle size without loss of resolution. Using a flow rate three times higher with smaller particles and shortening the column by one third (again due to the smaller particles), the separation is completed in 1/9 the time while maintaining resolution. An underlying principle of UPLC dictates that as column packing particle size decreases, efficiency and thus resolution also increases. As particle size decreases to less than 2.5µm, there is a significant gain in efficiency and it doesn't diminish at increased linear velocities or flow rates according to the common Van Deemter equation[6].

The Van Deemter equation shows that efficiency increases with the use of smaller size particles but this leads to a rapid increase in back pressure, while most of the UPLC system can operate only upto 400 bar. That is why short columns filled with particles of about 2µm are used with these systems, to accelerate the analysis without loss of efficiency, while maintaining an acceptable loss of load. The effect of particle size on HETP and linear velocity was illustrated using Van Deemter plot in Fig.1.



H–u plots obtained for acetophenone on Acquity and XBridge columns. Columns: Acquity BEH C18, 1.7 µm, 10 cm × 2.1 mm ID; XBridge C18, 3.5 µm, 15 cm × 4.6 mm ID; XBridge C18, 5 µm, 25 cm × 4.6 mm ID.

Fig.1: Van Deemter Plot illustrating the effect of particle size on plate height.

INSTRUMENTATION

The specifications used for UPLC and UPLC for different characteristics have been listed in Table 1.

Table 1: Comparison between HPLC and UPLC

Characteristics	HPLC	UPLC
Particle size	3 to 5µm	Less than 2µm
Maximum backpressure	35-40 MPa	103.5 MPa
Analytical column	Alltima C_{18}	UPLC BEH C_{18}
Column dimensions	150 X 3.2 mm	150 X 2.1 mm
Column temperature	30 °C	65 °C
Injection volume	5µL (Std. In 100% MeOH)	2µL (Std.In100% MeOH)

Sample Injection

In UPLC, sample introduction is critical. Conventional injection valves, either automated or manual, are not designed and hardened to work at extreme pressure. To protect the column from extreme pressure fluctuations, the injection process must be relatively pulse-free and the swept volume of the device also needs to be minimal to reduce potential band spreading. A fast injection cycle time is needed to fully capitalize on the speed afforded by UPLC, which in turn requires a high sample capacity. Low volume injections with minimal carryover are also required to increase sensitivity [3]. There are also direct injection approaches for biological samples [7,8].

UPLC Columns

Separation of the components of a sample requires a bonded phase that provides both retention and selectivity. Recently, columns used in UPLC are packed with particles produced through different technologies like Ethylene Bridged Hybrid [BEH] particle technology, Charged Surface Hybrid [CSH] particle technology, High Strength Silica [HSS] particle technology. An internal dimension (ID) of 2.1mm column is used. For maximum resolution, choose a 100mm length and for faster analysis, and higher sample throughput, choose 50mm column.

Ethylene Bridged Hybrid [BEH] Particle Technology

Four bonded phases are available for UPLC separations: UPLC BEH C_{18} and C_8 (straight chain alkyl columns), UPLC BEH Shield RP_{18} (embedded polar group column) and UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C_6 alkyl) [3]. Each column chemistry provides a different combination of hydrophobicity, silanol activity, hydrolytic stability and chemical interaction with analytes.

For more than a decade, hybrid particle technology [HPT] has delivered unsurpassed versatility and performance, enabling chromatographers to push the limits of LC separations. This first generation organic/inorganic methyl hybrid was illustrated in Fig.2 which provides significant improvement to the most problematic characteristics plaguing silica-based column: poor peak shape for basic compounds and column longevity due to chemical instability. The XTerra particle was the first commercially available option to improve these issues without the drawbacks of unpredictable selectivity produced by alternative materials such as organic polymers, zirconia and graphitic carbon. With the commercialization of 2.5µm XTerra particles, the concept of fast UPLC with small particles was born, improving the productivity of chromatographic laboratories globally. Different columns produced through this BEH particle technology is represented in Table 2.

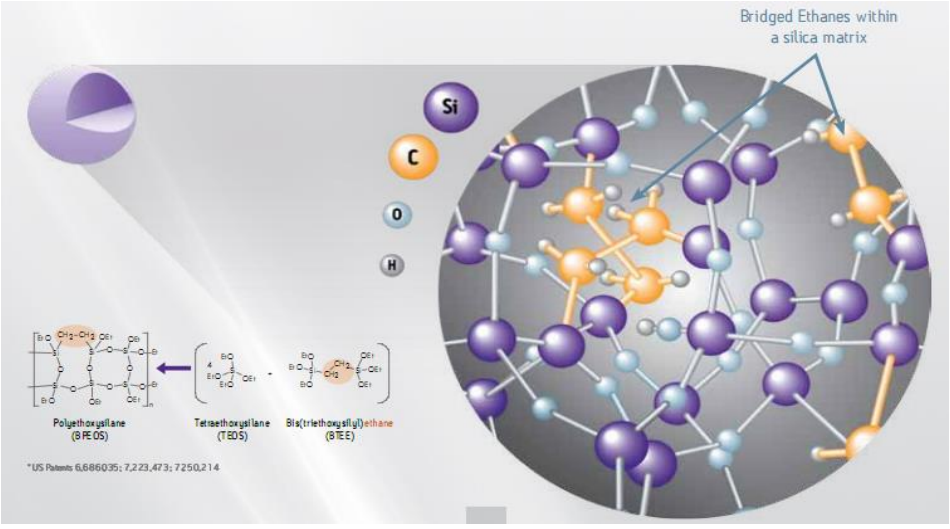
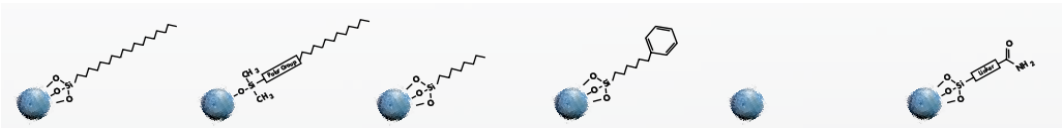


Fig. 2: UPLC column of BEH Particle Technology

Table 2: Different columns of BEH Particle Technology



	BEH C_{18} 1.7µm	BEH ShieldRP18 1.7µm	BEH C_8 1.7µm	BEH Phenyl 1.7µm	BEH HILIC 1.7µm	BEH Amide 1.7µm
Ligand Type	Trifunctonal C_{18}	Monofunctio-nal Embedded Polar	Trifunctional C_8	Trifunctional Phenyl-Hexyl	Unbonded BEH Particle	Trifunctional Carbamoyl
Ligand Density	3.1µmol/m^2	3.3µmol/m^2	3.2µmol/m^2	3.0µmol/m^2	n/a	7.5µmol/m^2
Carbon Load	18%	17%	13%	15%	Unbonded	12%
Endcap Style	Proprietary	TMS	Proprietary	Proprietary	n/a	none
USP Classification	L1	L1	L7	L11	L3	-
pH Range	1-12	2-11	1-12	1-12	1-9	2-11
Low pH Temp.Limit	80ºC	50ºC	60ºC	80ºC	60ºC	90ºC
High pH Temp.Limit	60ºC	45ºC	60ºC	60ºC	45ºC	90ºC
Pore Diameter	130Aº	130Aº	130Aº	130Aº	130Aº	130Aº
Surface Area	185m^2/g	185m^2/g	185m^2/g	185m^2/g	185m^2/g	185m^2/g
UPLC Column Equivalent	XBridge BEH C_{18}	XBridge BEH Shield RP18	XBridge BEH C_8	XBridge BEH Phenyl	XBridge BEH HILIC	XBridge BEH Amide
UPLC Particle Sizes	2.5,3.5,5,10µm	2.5,3.5,5,10µm	2.5,3.5,5,10µm	2.5,3.5,5µm	2.5,3.5,5µm	2.5,3.5µm

Forced degradation studies are performed to identify likely degradation products and establish degradation pathways as well as the intrinsic stability of a drug substance. Forced degradation analysis of Glimepiride on BEH C_{18} was shown in Fig.3. In the later stage of drug development, forced degradation studies are used to distinguish between degradation products related to the drug substance in formulation from excipients. The increased resolution capability of UPLC technology enables an improved characterization of complex samples.

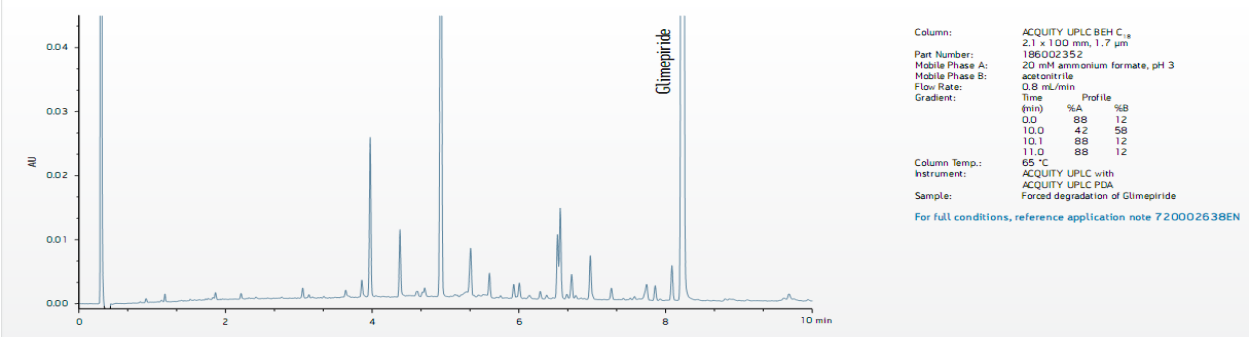


Fig.3: High Resolution Analysis of Glimepiride Forced Degradation on BEH C_{18}

Charged Surface Hybrid [CSH] Particle Technology

The progression and evolution of materials science has led to significant advances in chromatographic materials, the most recent of which being hybrid particle technology. Hybrid-based packing materials offer exceptional peak shape and efficiency as well a industry-leading chemical stability. Charged Surface Hybrid [CSH] Technology as shown in Fig.4 is the latest advancement in hybrid materials that utilizes a controlled, low-level surface charge to provide enhanced selectivity and exceptional peak shape, particularly in low-ionic-strength mobile phase.

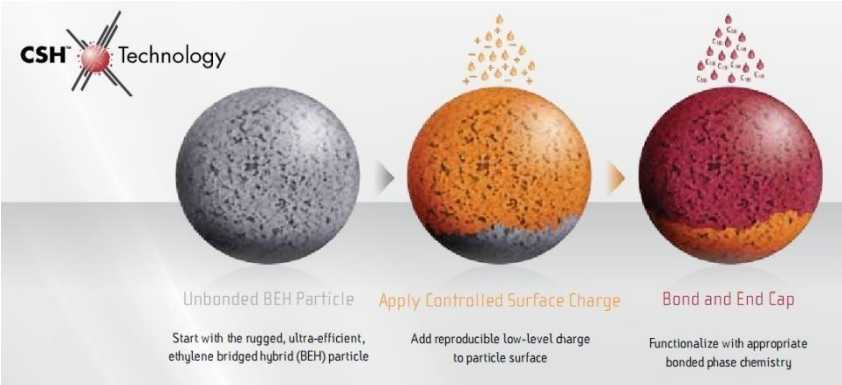


Fig.4: Acquity UPLC column of CSH Particle Technology

Advantages of CSH Technology include:

■ Unique column selectivity with industry-leading reproducibility.

■ Exceptional peak shape and loading capacity for basic compounds at low and high pH without the need for ion-pair reagents.

■ Exceptional stability and advanced column equilibration at low and high pH.

Ziprasidone is an anti-psychotic drug primarily used to treat the symptoms of schizophrenia, mania and bipolar disorder by altering the activity of specific natural chemicals present in the brain. The UPLC CSH C_{18} was used to successfully characterize the peroxide degradation products of ziprasidone in a simple formic acid mobile phase while demonstrating exceptional peak shape and peak-to-peak resolution in a rapid analysis time as shown in Fig.5.

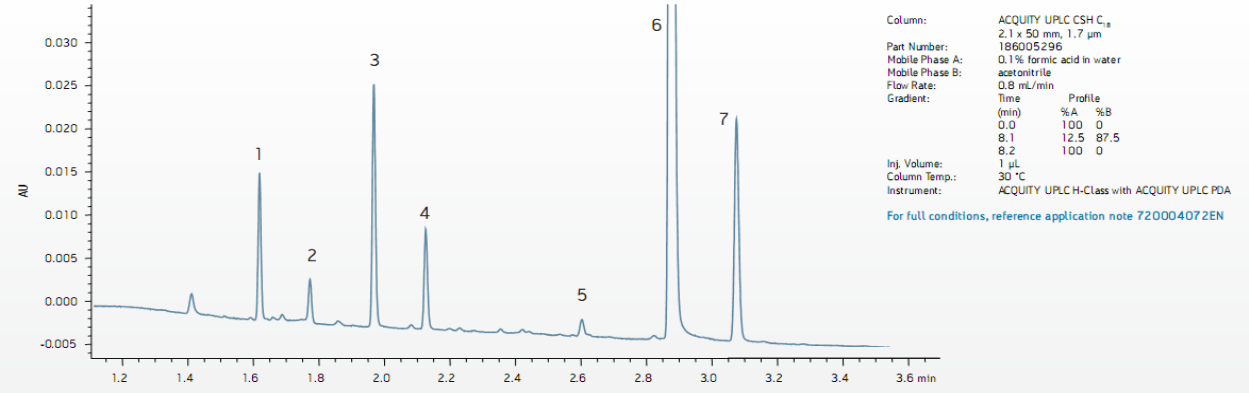


Fig. 5: Analysis of Ziprasidone Peroxide Degradation on CSH C_{18}

High Strength Silica [HSS] Particle Technology

To complement revolutionary Hybrid Particle Technology [HPT], a mechanically tolerant, silica-based material as shown in Fig.6 was designed to withstand UPLC pressures. High Strength Silica [HSS] particle technology was born from an innovative synthetic process that significantly increases the mechanical stability of silica while maintaining pore volumes similar to that of UPLC silica-based materials. The result is a novel particle technology that provides increased retentivity compared to hybrid particles while serving as the ideal substrate to create stationary phases that provide alternate selectivity.

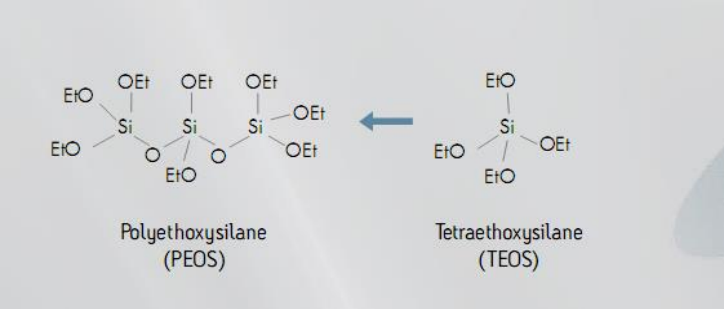


Fig.6: UPLC column of HSS Particle Technology

Xanthine alkaloids are commonly used as stimulants of the central nervous system to temporarily reduce fatigue and drowsiness. Additionally, this class of compounds has been used as bronchodilators for the treatment of asthma. The UPLC HSS C_{18} Column allows for the rapid analysis of several xanthine alkaloids as shown in Fig.7 that are part of the metabolic pathway of caffeine.

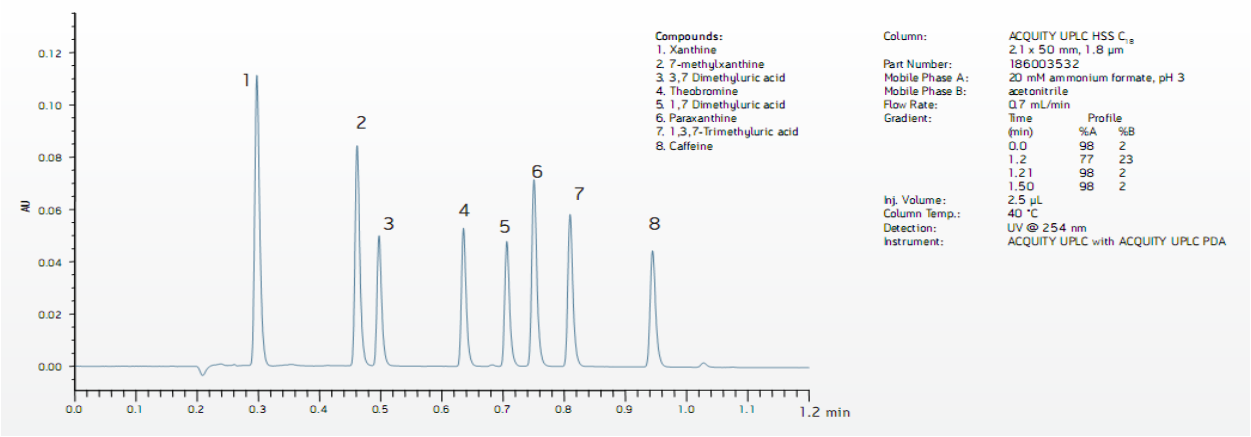


Fig.7: Separation of Xanthine Alkaloids on HSS C_{18}

Column Choices for Alternate Selectivity

When selecting a column for a specific separation, it is important to match the properties of your analytes with the separation capabilities of a specific column. When screening multiple columns, it is important to select columns that provide differences in selectivity and hydrophobicity to maximize the potential separation capability. Fig.8 represents the reversed-phase column selectivity chart that compares these differences under low-pH conditions. The further apart the columns are on the y-axis, the more different in selectivity they are. Additionally, the columns are plotted in increasing hydrophobicity (retention) from left to right on the x-axis.

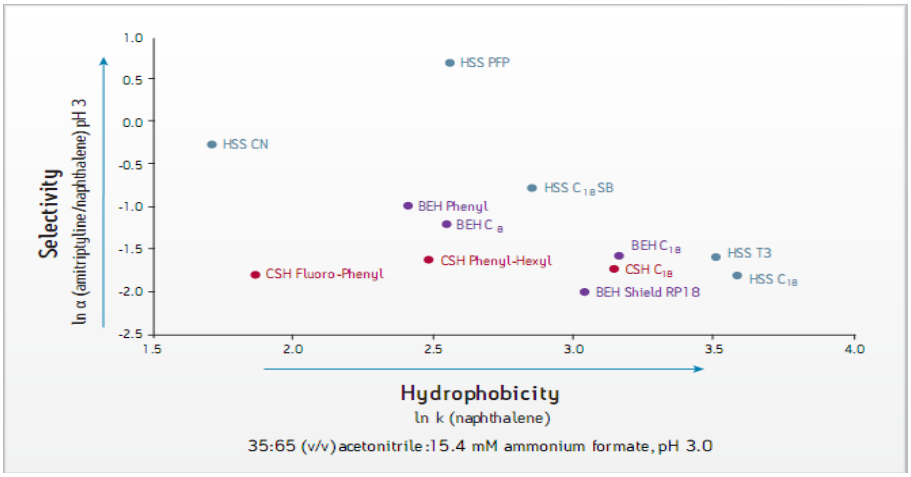


Fig. 8: Chart representing the UPLC column selectivity

UPLC Column Protection – VanGuard Pre-Columns

Contamination resulting from the analysis of samples present within complex matrices, or that are particulate-laden, may result in reduced column lifetime if not properly addressed. VanGuard Pre-Columns are ideally suited for the physical and chemical protection of UPLC Columns. The key features and benefits of this VanGuard Pre-columns are listed in Table 3.

Directly compatible with UPLC pressures upto 18,000psi [1241bar], this ultra-low dispersion direct connect guard column is specifically engineered to preserve the lifetime of an UPLC Column without negatively impacting its separation performance[9].

Table 3: Key Features and Benefits of VanGuard Pre-Column

Feature	**Benefit**
First pre-column for UPLC applications	Guaranteed compatibility with pressures upto 18,000psi
Patent pending, ultra-low volume design	Minimal chromatography effects
Manufactured using UPLC column hardware, particles and chemistries	Superior UPLC column protection and performance
Connects directly to UPLC column	Leaks and connection voids are eliminated

The UPLC System consists of a binary solvent manager, sample manager including the column heater, detector, and optional sample organiser. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient. There are built-in solvent select valves to choose upto four solvents. There is a 15,000psi pressure limit (about 1000bar) to take full advantage of the sub-2µm particles. The sample manager also incorporates several technology advancements. Using pressure assisted sample introduction, low dispersion is maintained through the injection process, and a series of pressure transducers facilitate self-monitoring and diagnostics. It uses needle-in-needle sampling for improved ruggedness, and needle calibration sensor increases accuracy. Injection cycle time is 25 seconds without a wash and 60sec with a dual wash used to further decrease carry over. A variety of microtiter plate formats likedeep well, mid height, or vial can also be accommodated in a thermostatically controlled environment. Using the optional sample organiser, the sample manager can inject upto 22 microtiter plates. The sample manager also controls the column heater. Column temperatures upto 65°C can be attained. To minimize sample dispersion, a “pivot out” design allows the column outlet to be placed in closer proximity to the source inlet of an MS detector.

Detectors

Half-height peak widths of less than one second are obtained with 1.7µm particles, which gives significant challenges for the detector. In order to integrate an analyte peak accurately and reproducibly, the detector sampling rate must be high enough to capture enough data points across the peak. The detector cell must have minimal dispersion (volume) to preserve separation efficiency. Conceptually, the sensitivity increase for UPLC detection should be 2-3 times higher than UPLC separations, depending on the detection technique. MS detection is significantly enhanced by UPLC; increased peak concentrations with reduced chromatographic dispersion at lower flow rates promotes increased source ionization efficiencies. UPLC detectors enhance ability to analyze a variety of compounds. It includes- The Photodiode Array (ACQUITY TUV), Tunable UV (TUV) and Evaporative Light Scattering (ELS).

Tunable UV (TUV):

The Tunable UV/Visible detector cell consists of a light guided flow cell equivalent to an optical fibre. Light is efficiently transferred down the flow cell in an internal reflectance mode that still maintains a 10mm flow cell path length with a volume of only 500mL. Tubing and connections in the system are efficiently routed to maintain low dispersion and to take advantage of leak detectors that interact with the software to alert the user to potential problems[10].

UPLC is an ideal inlet for the sensitivity and specificity offered by mass spectrometry. The low dispersion, high-speed detection

performance of MS Technologies, in combination with the performance characteristics of UPLC, can dramatically extend detection capabilities.

Electronic Tools to Facilitate Method Transfer

Based on the concept of maintaining column length [L] to particle size [dp] ratio [L/dp], the UPLC Columns Calculator enables methods to be transferred from UPLC to UPLC or from UPLC to UPLC while preserving the integrity of the separation as shown in Fig.9. In addition, this intuitive software program compensates for differences in gradient dwell volume, thus replicating the gradient profile independent of the LC system type being used.

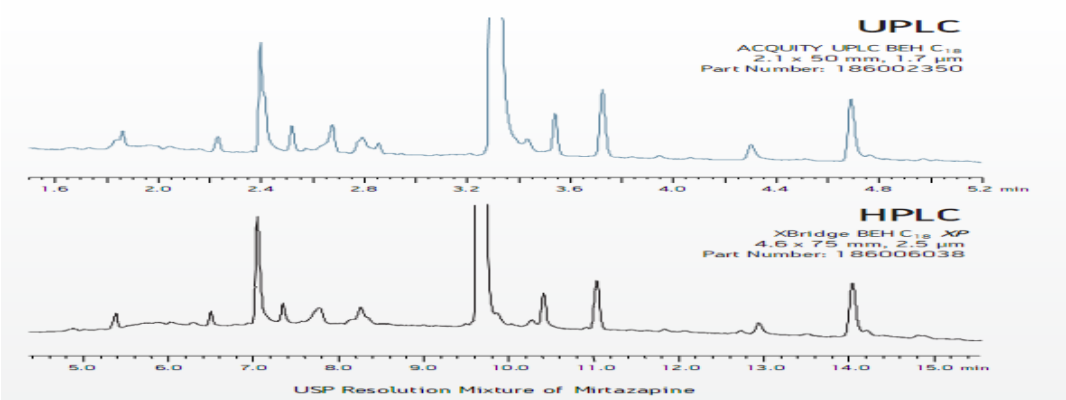


Fig.9: Chromatogram of HPLC and UPLC indicating method transfer.

ADVANTAGES

• Drastically decreases the run time compared to UPLC.

• Provides the selectivity, sensitivity, and dynamic range of LC analysis.

• The time spent on optimizing new methods can also be greatly reduced.

• Expands the scope of Multi residue methods.

• UPLC's fast resolving power quickly quantifies related and unrelated compounds.

• Use of very fine particle size of novel separation materials reduces the analysis time.

• Operation cost is reduced and less solvent consumption.

• Increases sample throughput and enables manufacturers to produce more material that consistently meet or exceeds the product specifications, potentially eliminating variability, failed batches, or the need to re-work material[2,4].

• The time needed for column equilibration while using gradient elution and during method validation is much shorter.

DISADVANTAGES

One of the major disadvantages in UPLC is the higher back pressures compared to conventional UPLC which in turn may reduce the life of the columns. This backpressure can be reduced by increasing the column temperature.

In addition, the phases of less than 2µm are generally nonregenerable and thus have limited use[11,12].

APPLICATIONS

1. Enhanced efficiency compared to UPLC

The analysis time and solvent consumption is greatly reduced in UPLC compared to UPLC as depicted in Fig.10.

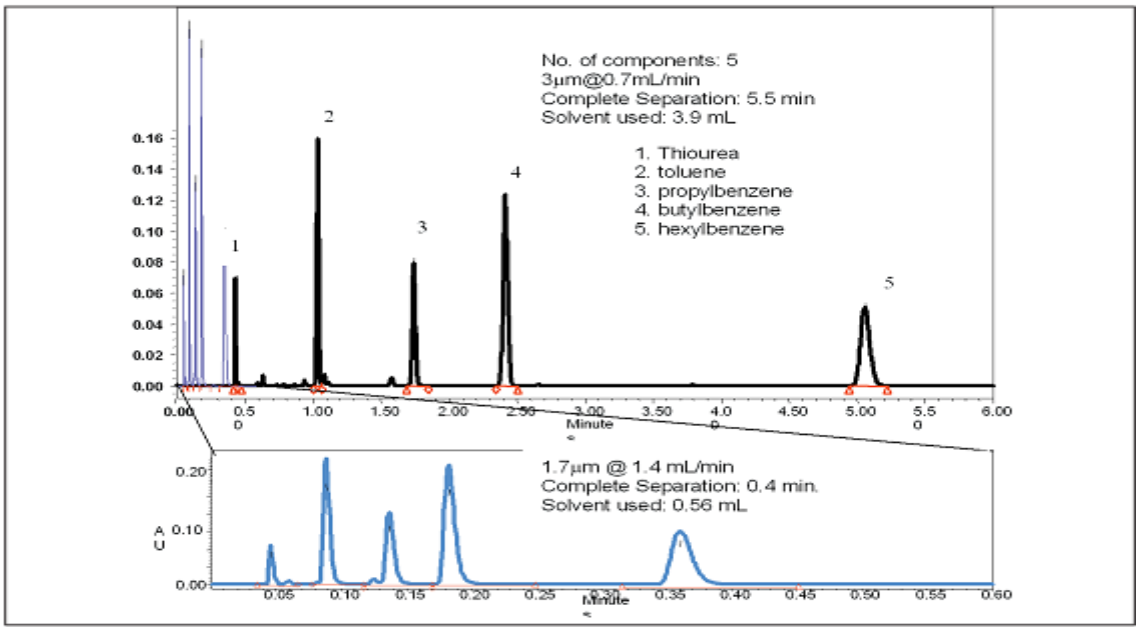


Fig.10: The top chromatogram is an overlay of both conventional (3 µm) UPLC and 1.7µm UPLC for a five component sample mixture[13]. The bottom is an expansion of the first 0.6 minutes of the overlay to show the increased speed of UPLC, while resolution is still maintained. Solvent use is also greatly reduced with UPLC.

Another example of an reduced run time in UPLC by using 2.1 by 100mm 1.7 mm ACQUITY BEH C_{18} at 288C and gradient solvent at a flow rate of 0.3mL/min as compared to UPLC using 2.1 by 100mm 5.0mm prototype BEH C_{18} at 288C and gradient solvent at a flow rate of 1.0mL/min is shown in Fig.11.

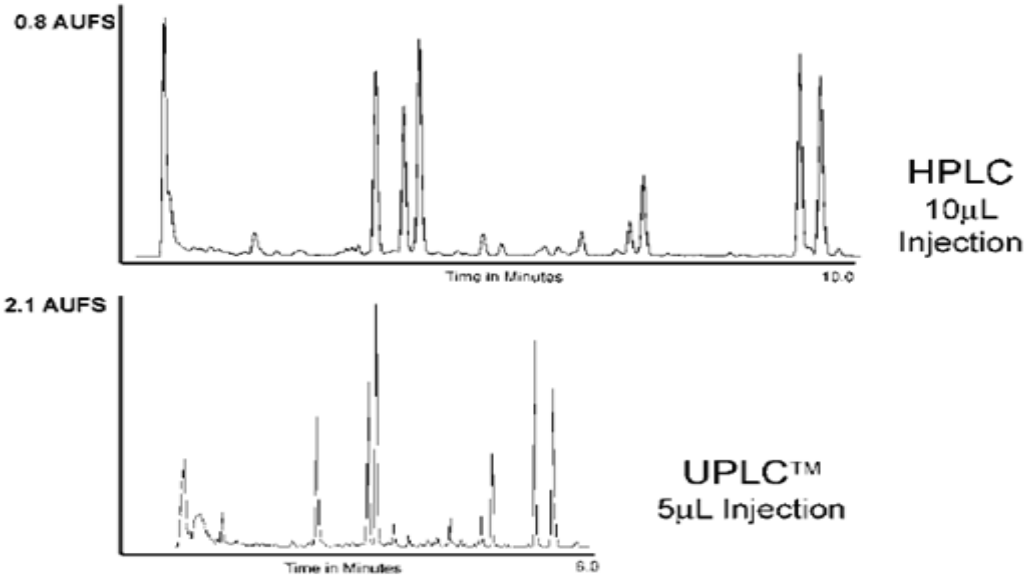


Figure .11: **Comparison of HPLC and UPLC for the separation of a ginger root extract**[14].

2. **Analysis of Traditional Chineese Medicines**

Ultra Performance Liquid Chromatography (UPLC), through its chromatographic principles, was used in the quality control of Panax ginseng[15].

Table 4: Gradient Mobile Phases of HPLC and UPLC

Mobile phase	Acetonitrile (%)	18	18	29	29	40	19	19
	Water (%)	82	82	71	71	60	81	81
Elution	UPLC (min)*	0	35	55	70	100	101	110
Time	UPLC (min)^	0	8.8	13.8	17.5	25	25.1	27
(min)	UPLC (min)#	0	3.7	5.8	7.4	10.5	10.6	11.5
*Flowrate=1.0mL/min ^Flowrate=0.21mL/min #Flowrate =0.50mL/min								

In comparison to UPLC, the UPLC has obvious advantages in the TCM analysis. The main advantage is that the significant reduction of analysis time as shown in Table 4 and Fig.12, which means reduction in solvent consumption but keeping the equivalent separation power.

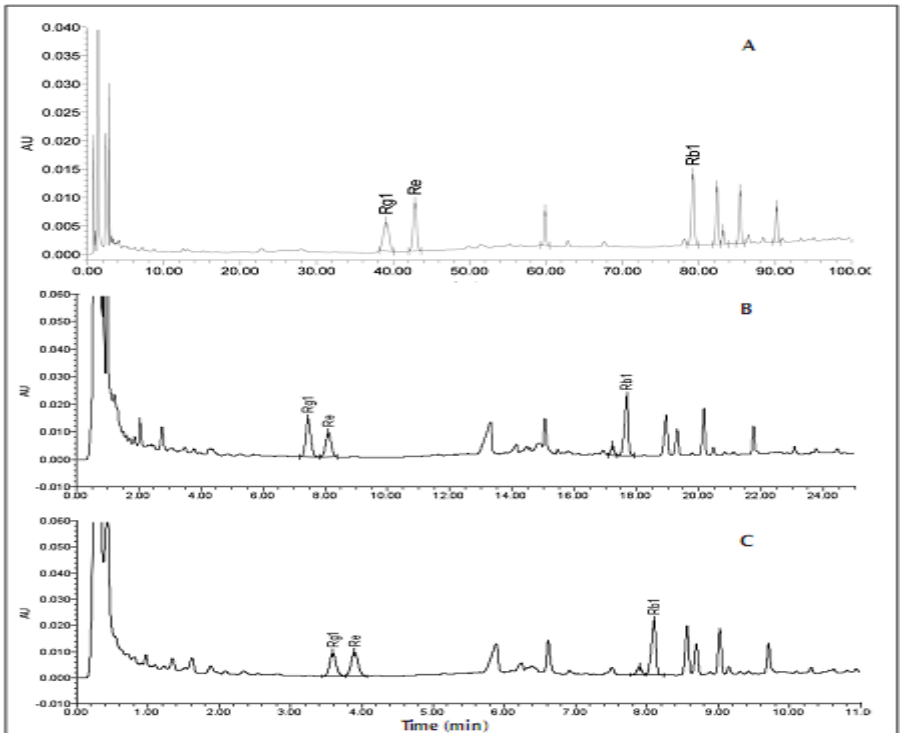


Figure 12: The (A) HPLC and (B and C) UPLC analysis of Panax ginseng.

Conditions A: Hypersil ODS-2 column(200 mm × 4.6 mm, 5 µm); the mobile phases shown in Table 4; flow rate 1.0mL/min; detection 203nm; temperature 25°C; injection 10µL; acquisition rate 1Hz. Conditions B: Acquity BEH C_{18} column (50mm×2.1 mm,1.7µm); the mobile phases shown in Table 4; flow rate 0.21mL/min; detection 203nm; temperature 25°C; injection 2µL; acquisition rate 20Hz. Conditions C: flow rate 0.5mL/min (other conditions same as conditions B).

3. Application of UPLC Amino Acid Analysis Solution to Foods and Feeds

Amino acid analysis has been used in the food and feed industries to determine and characterize materials and processes[16].

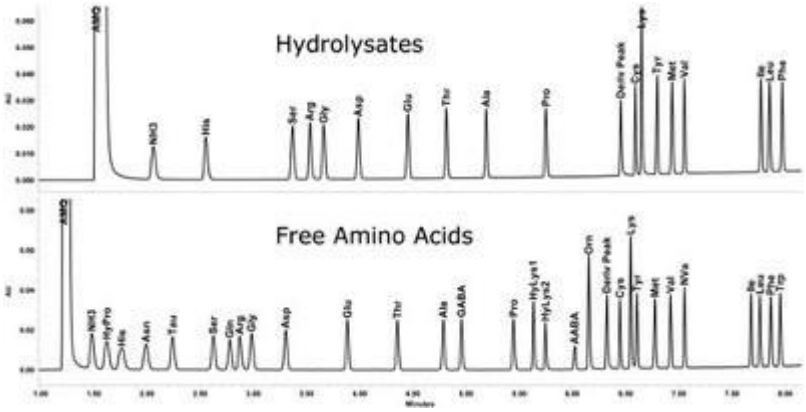


Fig.13: Representative chromatograms for hydrolysate amino acids and free amino acids using the UPLC Amino Acid Analysis Solution methods, both at 10pmol on column.

Amino acid content has been determined by a variety of techniques. With the continuing need for improved accuracy, precision, and ruggedness, preferably with higher throughput Ultra Performance LC has been combined with the well-established pre-column derivatization of AccQ·Tag Ultra. A robust turnkey solution, the UPLC Amino Acid Analysis Solution, has been developed to meet the needs of the food and feed industries. The improved resolution and sensitivity provide unequivocal identification and consistent quantitation of the amino acids. Standard methods are involved in the analysis of hydrolysate amino acids as well as for the separation of a larger set of free amino acids. These standard separations are shown in *Figure 13*.

Three applications indicate the utility of this method. The amino acid composition of a hydrolyzed poultry feed was measured which determine its nutritional content. Starting fermentation barley malts were identified and characterized through the analysis of free amino acids to characterize the raw materials used in a manufacturing process. Lastly, the progress of fermentation was monitored with amino acid analysis at different stages to illustrate the role of metabolic processes.

4. **Application of UPLC-SRM/MS method** to quantify urinary eicosanoidsEicosanoids are considered to be the key mediators and regulators of inflammation and oxidative stress often used as biomarkers for diseases and pathological conditions such as cardiovascular and pulmonary diseases and cancer. Analytical quantification of different eicosanoid species in a multi-method approach is problematic as most of these compounds are relatively unstable and may differ in their chemical properties. Here a novel ultra-performance liquid chromatography-selected reaction monitoring mass spectroscopy (UPLC-SRM/MS) method for simultaneous quantification of key urinary eicosanoids, including the prostaglandins (PG) tetranor PGE-M, 8-iso-, and 2,3-dinor-8-iso-$PGF_{2\alpha}$; the thromboxanes (TXs) 11-dehydro- and 2,3-dinor-TXB_2; leukotriene E_4; and 12-hydroxyeicosatetraenoic acid[17].Chromatographic separation was performed on a Waters (Eschborn, Germany) Acquity ultra performance liquid chromatography (UPLC) BEH C_{18} column (2.1 × 50 mm) with a 1.7µm particle size. The column was maintained at 30°C, and the injection volume was set to 5µl. Eluent A consisted of 0.1% formic acid in water; eluent B was 0.1% formic acid in acetonitrile.An effecient chromatographic separation is crucial for eicosanoid analysis because these compounds often occur in isobaric forms. In addition, there may occur ion suppression and misquantification due to interfering matrix components present within urine. Previous studies have shown that RP UPLC is an adequate technique for separation of most eicosanoid species. Based on these data, chromatographic separation using a C_{18} RP analytical column was established. In contrast to previously published methods, short UPLC column containing very small particles (50×2.1mm; 1.7µm) allowing shorter runtimes (14min including re-equilibration compared with >20min [UPLC column; 100 × 3.0 mm; 3.5 µm]) are proved to be beneficial.

5. Iodinated disinfection by product Iodinated disinfection by products (DBPs) are generally more toxic than their chlorinated and brominated analogues. Until now, only a few iodinated DBPs in drinking water have been identified by gas

chromatography/mass spectrometry. Faster detection of polar iodinated DBPs was developed using an electrospray ionization-triple quadrupole mass spectrometer (ESI-tqMS) by conducting precursor ion scan of iodide at *m/z* 126.9. Through this pictures of polar iodinated DBPs in chlorinated, chloraminated, and chlorine-ammonia treated water samples were achieved. By coupling ultra performance liquid chromatography (UPLC) to the ESI-tqMS, tentatively structures of 17 iodinated DBPs were proposed. The results demonstrate that, with respect to the DBP number/levels among the three disinfection processes, chloramination generated the highest iodinated DBPs, chlorination produced the lowest iodinated DBPs, and chlorine-ammonia sequential treatment formed iodinated DBPs lying in between. The ratio of total organic iodine levels in chlorine-ammonia sequential treatment and chloramination could be expressed as a function of the lag time of ammonia addition[18].

6. Identification of Metabolite Biotransformation of new chemical entities (NCE) is necessary for drug discovery. Compound after reaching the development stage, metabolite identification becomes a regulated process. It is of utmost importance to detect and identify all circulating metabolites of a candidate drug. Discovery studies are generally carried out in vitro to identify major metabolites so that metabolic weak spots on the drug candidate molecule can be recognized and protected by structure changes of the compound. In metabolite identification major aspect is maintaining high sample throughput and providing results as soon as they are available to medicinal chemists. UPLC/MS/MS addresses the complex analytical requirements of biomarker discovery by offering unmatched sensitivity, resolution, dynamic range, and mass accuracy.

7. Study of Metabonomics / Metabolomics

Development of new medicines are carried out in labs to accelerate the Metabonomics studies. The ability to compare and contrast large sample groups provides insight into the biochemical changes that occur when a biological system is exposed to a new chemical entity (NCE). A rapid and robust method for detecting these changes was provided by metabonomics, improves understanding of potential toxicity, and allows monitoring the efficacy. The perfect implementation of metabonomic and metabolomic information helps similar discovery, development, and manufacturing processes in the biotechnology and chemical industry companies. Taking these into consideration, scientists are better able to visualize and identify fundamental differences in sample sets. The UPLC/MS System combines the benefits of UPLC analyses, high resolution exact mass MS, and specialized application managers to rapidly generate and interpret information-rich data, allowing rapid decisions to be made.

8. Bioanalysis / Bioequivalence Studies For pharmacokinetic, toxicity, and bioequivalence studies, quantitation of a drug in biological samples is an important part of development programs. The drugs are usually of low molecular weight and are tested during both preclinical and clinical studies. Several biological matrices are used for quantitative bioanalysis, among which the most common being blood, plasma, and urine19.The primary technique for quantitative bioanalysis is LC/MS/MS. The sensitivity and selectivity of UPLC/MS/MS at low detection levels generates accurate and reliable data that can be used for a wide variety of purposes, including statistical pharmacokinetics (PK) analysis. 9. Forced Degradation Studies The analytical technique most commonly used for monitoring forced degradation experiments is UPLC with UV and/or MS detection for peak purity, mass balance, and identification of degradation products but the main drawback is that these UPLC-based methodologies are time-consuming and provide only medium resolution to ensure that all of the degradation products are accurately detected. ACQUITY TUV /MS (photodiode array and MS), allows for faster and higher peak capacity separations, for complex degradation product profiles also. Combining the speed, resolution, and sensitivity of UPLC chromatographic separations with the high-speed scan rates of UPLC-specific photodiode array and MS detection helps in the identification of degradation products and thus shortening the time required to develop stability-indicating methods.

CONCLUSION

UPLC extends and expands the utility of chromatography compared with conventional UPLC as it increases productivity in both chemistry and separation barriers. The main advantage is that it provides more information per unit of work as it gives increased resolution, speed, and sensitivity for liquid chromatography. During separation of Phthalates, UPLC and UPLC were compared and this

study showed analysis time was reduced by a factor of 2.5 and solvent consumption by a factor of 6.4 with UPLC. Analysis time, solvent consumption, and analysis cost are considered very important in many analytical laboratories. Sensitivity can be compared by studying the peak width at half height. It was found that UPLC sensitivity was much higher than that of conventional UPLC. Tailing factors and resolution were found to be similar for both techniques. Peak area repeatability (as RSD) and peak retention time repeatability (RSD) were also similar for both techniques. Overall, it can be stated that UPLC can offer significant improvements in speed, sensitivity and resolution compared with conventional UPLC.

Table 1.1: System Suitability Parameters and their recommended limits.

Parameter	**Recommendation**
Capacity Factor (K')	The peak should be well-resolved from other peaks and the void volume, generally K' > 2
Repeatability	RSD ≤ 1% N ≥ 5 is desirable
Relative Retention	Not required as the resolution is stated
Resolution(R_S)	R_S of > 2 between the peak of interest and the closest eluting potential interferent
Tailing Factor(T)	T ≤ 2
Theoretical Plates(N)	In general should be > 2000

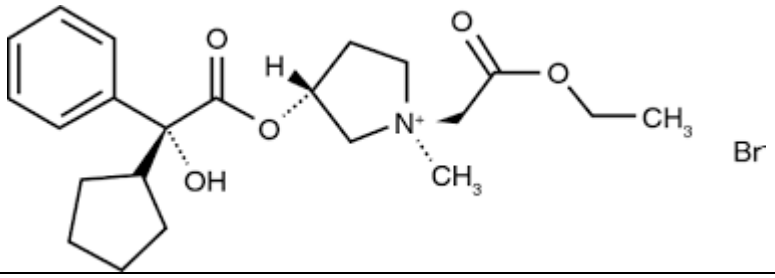
Table 1.2: Characteristics to be validated in UPLC.

Characteristics	**Acceptance Criteria**
Accuracy/trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate Precision	RSD < 2%
Specificity / Selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	Correlation coefficient R^2 > 0.999
Range	80 –120 %

1.4. Drug information

Drug name: Sofpironium

Description:
Sofpironium is a topical anticholinergic used to treat primary axillary hyperhidrosis.
Indication:
Sofpironium bromide is indicated for the treatment of primary axillary hyperhidrosis in patients ≥9 years of age
Structure:



CAS number	:	1628251-49-9
Molecular Weight	:	Average: 390.499
Molecular formula	:	$C_{22}H_{32}NO_5$
Physical state	:	Solid
Solubility	:	freely soluble

Mechanism of Action: Sofpironium is a competitive inhibitor of muscarinic acetylcholine receptors are located on certain peripheral tissues, including eccrine sweat glands. By inhibiting the activity of these receptors, sofpironium indirectly reduces the rate of sweating in patients with axillary hyperhidrosis.
Pharmacodynamics: Binding studies at human muscarinic receptors (M1–M4) and guinea-pig ileum assays have found sofpironium bromide to have potency close to glycopyrronium with a shorter duration of action. Despite its topical application, sofpironium bromide exhibits some degree of systemic absorption that can result in anticholinergic adverse effects like urinary retention and heat illness.
Adsorption: the mean plasma C_{max} was 2.71 ng/mL
Protein Binding: 34.8 - 37.8%
Metabolism:
Sofpironium is metabolized by non-enzymatic hydrolysis, oxidative metabolism via CYP2D6 and CYP3A4, and glycine conjugation
Route of elimination: Urinary excretion of sofpironium and BBI-4010 were less than 0.5% of the applied dose
Half-life: 3.86 hrs
Brand name: Sofdra

3. Hypothesis

A robust UPLC method will be developed by using QbD approach. The developed UPLC method will be capable to identify all the possible degradation products of Sofpironium. The degradation pathways of Sofpironium under forced degradation conditions will be established.

4. Objectives

To develop a stability indicating assay method (SIAM) for

Sofpironium by QbD approach.

Conduction of forced degradation studies under different stress conditions as per ICH guidelines

To validate UPLC method for Sofpironium as per ICH guidelines.

5. MATERIALS AND METHOD

5.1. Chemicals and reagents

Pure Sofpironium was procured from Spectrum pharma lab (Hyderabad). Hydrochloric acid AR grade (HCL) and sodium hydroxide AR grade (NAOH) were obtained from rankem, India. Hydrogen Peroxide (H2O2) was purchased from Qauligens. Acetic acid AR grade was purchased from Fisher scientific, India and S.D. Fine chem Ltd. Respectively. Potassium dihydrogen orthophosphate and orthophosphoric acid were obtained from S.D. Fine chem Ltd and Merck India Pvt Ltd. Respectively. UPLC grade Acetonitrile (ACN)

and methanol (MeOH) were purchased from Fischer scientific. UPLC grade water used throughout analysis was obtained from the Merck milli-Q water purification unit.

5.2. Apparatus and Equipment

UPLC studies were carried out on WATERS UPLC 2965 SYSTEM with a Photo diode array detector (PDA) set at 220 nm for uv detection. columns, viz; Agilent C18 (150×4.6 mm, 5 µm), Discovery C18(150*4.6mm,5 µm), Zodiac (150*4.6mm,5 µm) ,BDS (150*4.6mm,5 µm) and Phenomenex (150*4.6mm,5 µm)column were utilized in the study. Design Expert® (11.0.0) modeling software (Stat-Ease Inc., Minneapolis, MN, USA) was used for generation of contour plots and 3D space.

pH meter (Eutech instruments pH tutor, pH meter, India) was used to check the pH of all solutions.

Other equipment sonicator (ePEI ultrasonic generator), Analytical balance (Mettler Toledo), vortex meter (IKA Vortex), Hot air oven (Yorco scientific).

5.5 Simple and Robust RP-UPLC method development by DOE approach.

5.5.1. Preparation of drug solution

Preparation of Standard stock solutions: Accurately weighed 10mg of Sofpironium and transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (200µg/ml of Sofpironium)

Preparation of Standard working solution (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (20µg/ml of Sofpironium).

Preparation of Sample stock solutions: 10 mg of tablet were taken and calculate the average weight of each tablet and Weight equivalent to 1 tablet was transferred into a 100mL volumetric flask, 50mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. (1000µg/ml of Sofpironium)

Preparation of Sample working solution: From the filtered solution 0.2 ml was pippeted out into a 10 ml volumetric flask and made upto 10ml with diluent. (20µg/ml of Sofpironium).

Methodology for Validation Parameters:

System suitability parameters:

The system suitability parameters were determined by preparing standard solutions of Sofpironium (20ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined.

The % RSD for the area of six standard injections results should not be more than 2%.

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision:

Preparation of Standard stock solutions: Accurately weighed 10mg of Sofpironium and transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (200µg/ml of Sofpironium)

Preparation of Standard working solution (100% solution): 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (20µg/ml of Sofpironium).

Preparation of Sample stock solutions: 10 mg of tablet were taken and calculate the average weight of each tablet and Weight equivalent to 1 tablet was transferred into a 100mL volumetric flask, 50mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. (1000µg/ml of Sofpironium)

Preparation of Sample working solution: From the filtered solution 0.2 ml was pippeted out into a 10 ml volumetric flask and made upto 10ml with diluent. (20µg/ml of Sofpironium).

Linearity:

Preparation of Standard stock solutions: Accurately weighed 10mg of Sofpironium and transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (200µg/ml of Sofpironium)

25% Standard solution: 0.25ml from standard stock solution was pipette out and made up to 10ml. (5µg/ml of Sofpironium)

50% Standard solution: 0.5ml from standard stock solution stock solutions was pipetted out and made up to 10ml. (10µg/ml of Sofpironium)

75% Standard solution: 0.75ml from standard stock solution stock solutions was pipetted out and made up to 10ml. (15µg/ml of Sofpironium,)

100% Standard solution: 1.0ml from standard stock solution stock solutions was pipetted out and made up to 10ml. (20µg/ml of Sofpironium)

125% Standard solution: 1.25ml from standard stock solution stock solutions was pipetted out and made up to 10ml. (25µg/ml of Sofpironium)

150% Standard solution: 1.5ml from standard stock solution stock solutions was pipettede out and made up to 10ml. (30µg/ml of Sofpironium)

Accuracy:

Preparation of Standard stock solutions: Accurately weighed 10mg of Sofpironium and transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (200µg/ml of Sofpironium)

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Acceptance Criteria:

The % Recovery for each level should be between 98.0 to 102

Robustness: Small deliberatechanges in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

Robustness conditions like Flow minus (0.1ml/min), Flow plus (0.3ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much effected and all the parameters were passed. %RSD was within the limit.

LOD sample Preparation: 0.25ml Standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.25ml Sofpironium, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents

LOQ sample Preparation: 0.25ml standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.9ml Sofpironium of, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Degradation studies:

Oxidation:

To 1 ml of stock solution of Sofpironium, 1 ml of 20% hydrogen peroxide (H2O2)was added separately. The solutions were kept for 30 min at 600c. For HPLC study,the resultant solution was diluted to obtain 20µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:

To 1 ml of stocks solution Sofpironium, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 600c.The resultant solution was diluted to obtain 20µg/ml solution and 10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:

To 1 ml of stock solution Sofpironium, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 600c. The resultant solution was diluted to obtain 20µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:

The standard drug solution was placed in oven at105°C for 1h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 20µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the 200µg/ml solution to UV Light by keeping the beaker in UV Chamber for 1hrs or 200 Watt hours/m2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 20µg/ml solutions and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxingthedruginwaterfor1hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to 20µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

5.5.2. Preparation of buffer

5.5.2.1. 0.01N Potassium dihyrogen ortho phosphate

Accurately weighed 1.36gm of Potassium dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1ml of Triethylamine then PH adjusted to 3.0 with dil. Orthophosphoric acid solution

5.5.2.2. 0.1% Ortho phosphoric acid buffer

1ML of Ortho phosphoric acid solution in a 1000ml of volumetric flask add about 100ml of milli-Q water and final volume make up to 1000 ml with milli-Q water

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5.5.3. Initial UPLC runs of Sofpironium

Initial UPLC runs of Sofpironium of 20 µg/mL concentration were performed using

Different buffer viz, Potassium dihyrogen ortho phosphate and Ortho phosphoric acid.

Different organic modifier viz, acetonitrile and methanol

Different columns such as Symmetry C18 (150×4.6 mm, 5 µm), Agilent C18 (150×4.6 mm, 5 µm), Discovery C18(150*4.6mm,5 µm), Zodiac (150*4.6mm,5 µm), BDS (150*4.6mm,5 µm) and Phenomenex (150*4.6mm,5 µm)column

5.5.4. Optimization of method

The method was optimized using central composite design (CCD). The initial trials are needed to optimize the final method. Total Three factors viz; % Organic concentration, Flow rate and column temperature were needed to be optimized. So CCD was used to optimize these parameters which were varied over three level (high, mid and low).different ranges of four parameters ranging from 63.27-76.73%,

0.1%Ammonium Formate, ml/min flow rate 24.95 and 35.05 0C and 0.24-o.35 ml/min flow rate respectively were taken and counter and 3D surface plot showing the effect of each parameter on Retention Time, Area, Theoretical plates and Asymmetry (CQA) were generated. A desirability function applied to the optimized conditions to predict retention time, asymmetry, theoretical plates and peak area.

Table 6. Design summary of CCD

Design Summary					
File version: DX 11.0.0 Study Type: Response surface Design Type: central composite design Design Model: Quadratic			ATP: Robustness CQA: Retention time, Area, Theoretical plates and Asymmetry Runs: 24		
CMPs	Unit	Type	Subtype	Min.	Max.
column temperature	0C	Numeric	Continuous	26.64	33.36
Flow rate	ml/min	Numeric	Continuous	0.6636	1.34
%Org ratio	%	Numeric	Continuous	36.59	53.41

5.6 Stress study

5.6.1. Generation of stress samples of Sofpironium

5.6.2.1. Acid Hydrolysis

To 1 ml of stock solution of 1ml of 2N HCl solution was added. These degradation samples were kept in Radley apparatus (Veego) with continuous stirring at 70º C for 1 hrs in 2N HCl. These samples were neutralized to pH 7, diluted and analyzed by the UPLC system.

5.6.2.2. Base hydrolysis

To 1 ml of stock solution of 1ml of 2N NAOH solution was added. These degradation samples were kept in Radley apparatus (Veego) with continuous stirring at 70º C for 1 hrs in 2N NaOH solution. These samples were neutralized to pH 7, diluted and analyzed by the UPLC system.

5.6.2.3. Neutral hydrolysis

10 mg of Sofpironium was weighed and dissolved in 50 ml of water. These degradation samples were kept in Radley apparatus (Veego) with continuous stirring at 70º C for 24 hrs. These samples were diluted and analyzed by the UPLC system.

5.6.2.4. Oxidative study

To 1 ml of stock solution of 1ml of 20% H2O2 solution was added. These degradation samples were kept in dark area without disturbance at room temperature for 24 hrs. These samples were diluted and analyzed by the UPLC system.

5.6.2.5. Thermal degradation

10mg Sofpironium was kept in a Petri dish and kept in hot air oven at 70ºC for 1 day. Sampling was done at multiple time points. Samples were dissolved in methanol, diluted 10 times and analyzed by the UPLC system.

5.6.2.6. Photo degradation

10mg Sofpironium was uniformly spread in a Petri dish and was exposed to directly sunlight for 24 hrs. Sampling was done at multiple time points and analyzed by the UPLC system.

5.7 Method validation

The final optimized chromatographic analytical method was validated as per the International Conference on Harmonization (ICH) Q2(R1) guidelines for system suitability, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness. Standard

stock solution was prepared by dissolving 10mg of Sofpironium in 50 mL of diluents to a final concentration of 200µg/ml. then 1ml stock solution is transferred into 10 v/f and made upto the volume to get 20µg/ml.

5.7.1 Linearity

Standard calibration curves were generated with seven different concentrations including the LOQ by making serial volume to volume dilution of stock solution I over the range of 25-150 µg/ml. Linear calibration curves were generated between peak area and drug concentration. The linearity was examined using linear regression, which was calculated by the least square regression method.

5.7.2 Accuracy

The accuracy of developed analytical method was analyzed by developed method, accuracy experiments were carried out using standard addition method. Three different level concentrations (50%, 100%, and 150%) of standards were added to pre-analyzed samples in triplicate. The percentage accuracy of Sofpironium at each level and each triplicate were calculated and the mean of percentage accuracy (n=9) and the relative standard deviation was determined.

5.7.3 Precision

The precision of the developed analytical method was determined by repeatability (intraday) and intermediate precision (inter-day).Repeatability defines the use of the analytical procedure within a laboratory over a short period of time that was examined by assaying the samples during the same day. Intermediate precision was evaluated by comparing the assays on different days. SD and %RSD were determined.

5.7.4 Limits of detection and quantitation

Limits of detection (LOD) and limit of quantitation (LOQ) were determined from the signal-to-noise ratio. The detection limit was refer to as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantitation limit was refer to as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than ten.

5.7.5 System suitability

The system suitability was determined by taking six replicates of the drug at same concentration of 100µg/ml. The acceptance criteria was ± 2% for the percent coefficient of variation (% CV) for the peak area, retention time of drug, USP Plate Count, and asymmetry.

5.7.6 Robustness

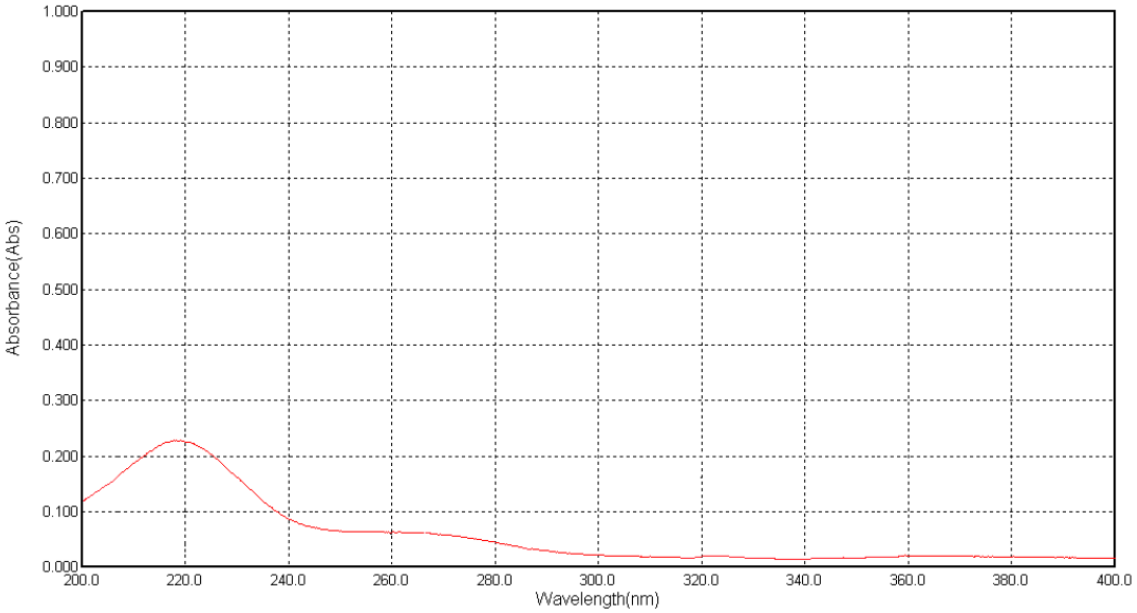
The Robustness is one of the validation parameter, it measure of method capacity to remain unaffected by small, deliberate changes in chromatographic conditions was studied by testing the influence of small changes in the organic content of mobile phase (±10%), flow rate (±10%) and pH (±10%) and.

Results and discussion

6.2 Authentication and identification of received API

6.2.1. Authentication by UV-VIS spectra

After scanning from 400 to 200nm in UV-VIS spectrophotometer, Sofpironium was showed absorption maxima at 219.0 nm in Methanol 100% UV spectra of drug given in figure 3**.**



6.3. Simple and Robust RP-UPLC method development by DOE approach

6.3.1 Parameter selection

Various preliminary UPLC trials were carried out for selection of Column and organic modifier. The choice of C18 column based on the preliminary investigation was done using Symmetry C18 (150×4.6 mm, 5µm), Azilent C18 (150×4.6 mm, 5µm), Discovery (150×4.6 mm, 5µm) and BDS(150×4.6 mm, 5 µm) columns. Symmetry C18 column having less tailing, higher theoretical plate and good shape of drug peak as compare to the Azilent, BDS and Discovery column. (Appendices 1) Selection of a suitable organic modifier is also important to get better selectivity with adequate separation of all analytes. Commonly used organic solvents for the reversed phase UPLC include Acetonitrile and Methanol,from that trials Acetonitrile showed to be an ideal and suitable organic modifier compared to acetonitrile, because Sofpironium was solubilized in acetonitrile compare to methanol. Therefore, acetonitrile was selected and finalized as the organic modifier for further optimization study.

6.3.2. Optimization of method

The method was optimized using central composite design (CCD). The initial trials are needed to optimize the final method. Organic concentration, Flow rate and column temperature were needed to be optimized. So CCD was used to optimize these parameters which were varied over three level (high, mid and low).different ranges of four parameters ranging from 40-50%, 0.01N potassium dihydrogen orthophosphate, column temperature 28^0C and 32 ^{0}C and 0.8-1.20ml/min flow rate respectively were taken and counter and 3D surface plot showing the effect of each parameter on Retention Time, Theoretical plates and Asymmetry (CQA) were generated. A desirability function applied to the optimized conditions to predict retention time, asymmetry, theoretical plates and peak area.

Table 9. Design summary of CCD

Design Summary	
File version: DX 11.0.0	ATP: Robustness
Study Type: Response surface	CQA: Retention time, Area, Theoretical plates and Asymmetry
Design Type: central composite design	
Design Model: Quadratic	Runs: 24

CMPs	Unit	Type	Subtype	Min.	Max.
Temp	-	Numeric	Continuous	28 ^{0}C	32 ^{0}C
Flow rate	ml/min	Numeric	Continuous	0.8	1.20
%Org ratio	-	Numeric	Continuous	40	50

Table 10. Box-Behnken experimental design matrix with response

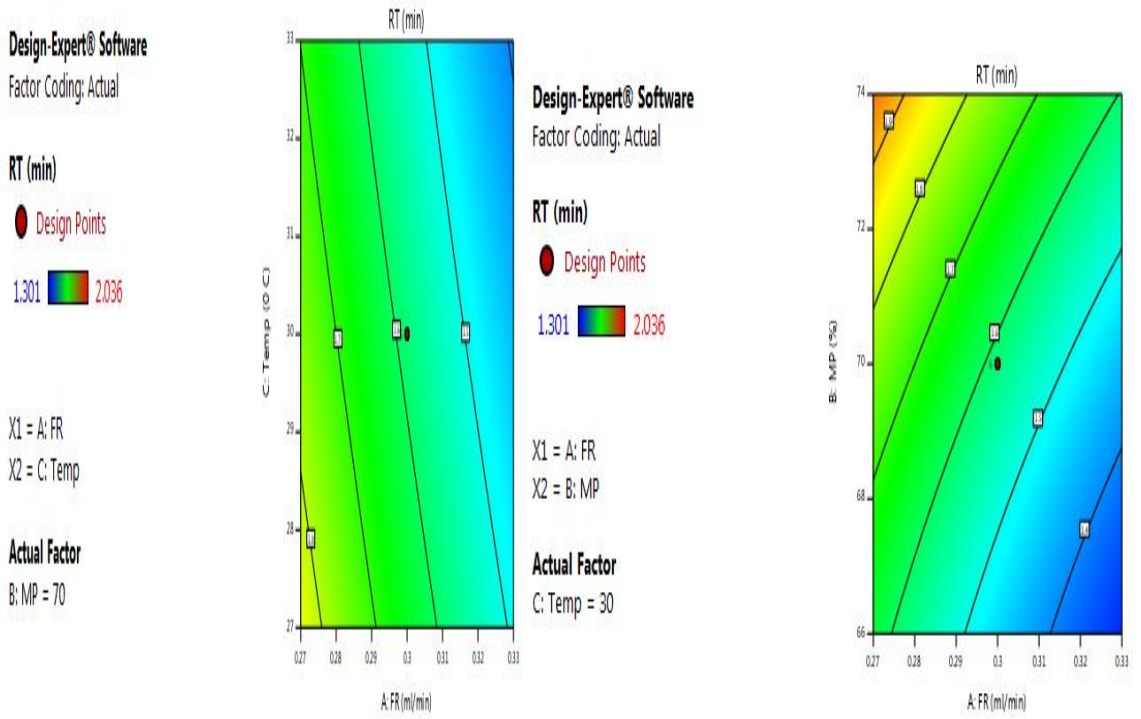
		Factor 2	Factor 1	Factor 3	Response 1	Response 2	Response 3
Std	Run	B:Flowrate	A:Mobilephase	C:Temperature	RT	NTP	TF
		%	ml/min	0 C	min	num	num
1	7	0.27	66	27	1.695	7990	1.03
2	11	0.33	66	27	1.38	7440	1.07
3	18	0.27	74	27	2.036	8445	1.04
4	9	0.33	74	27	1.653	7863	1.09
5	12	0.27	66	33	1.578	8005	1.08
6	15	0.33	66	33	1.301	7657	1.1
7	3	0.27	74	33	1.884	8466	1.07
8	5	0.33	74	33	1.554	8155	1.1
9	19	0.249546	70	30	1.907	8078	1.04
10	1	0.350454	70	30	1.357	7184	1.1
11	16	0.3	63.2728	30	1.395	7560	1.07
12	17	0.3	76.7272	30	1.898	8485	1.07
13	14	0.3	70	24.9546	1.702	8197	1.05
14	10	0.3	70	35.0454	1.478	8456	1.1
15	8	0.3	70	30	1.578	7658	1.07
16	20	0.3	70	30	1.58	7659	1.07
17	6	0.3	70	30	1.58	7652	1.07
18	13	0.3	70	30	1.581	7676	1.07
19	2	0.3	70	30	1.587	7641	1.07
20	4	0.3	70	30	1.592	7683	1.07

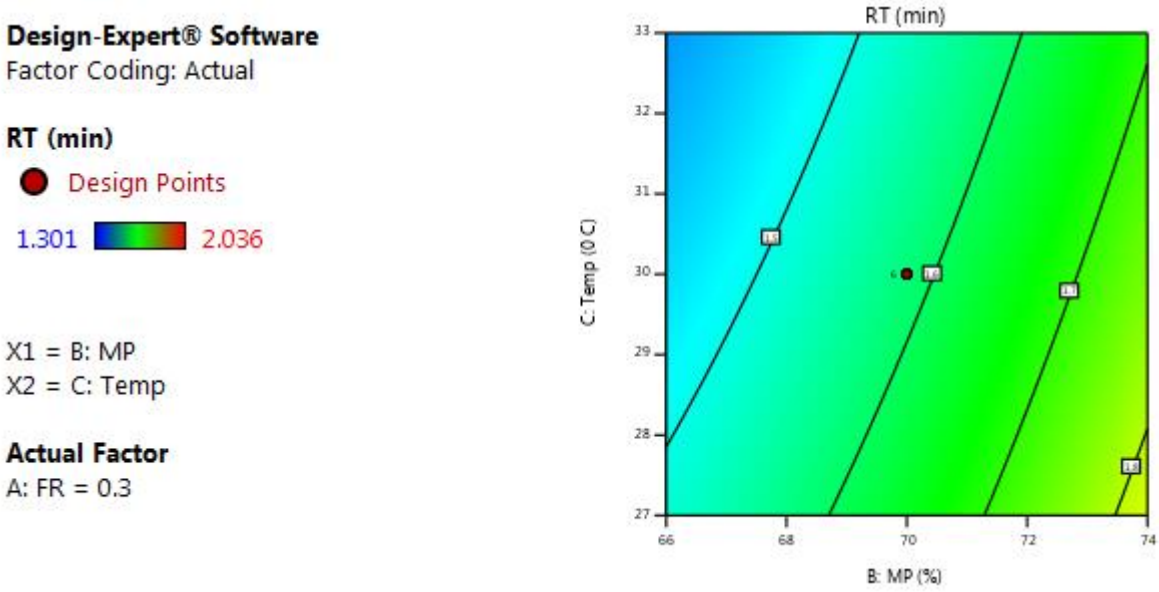
Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	FR	ml/min	Numeric	0.2495	0.3505	-1 ↔ 0.27	+1 ↔ 0.33	0.3000	0.0254
B	MP	%	Numeric	63.27	76.73	-1 ↔ 66.00	+1 ↔ 74.00	70.00	3.39
C	Temp	0 C	Numeric	24.95	35.05	-1 ↔ 27.00	+1 ↔ 33.00	30.00	2.54

Response	Name	Units	Observations	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Transform	Model
R1	RT	min	20	Polynomial	1.301	2.036	1.62	0.1959	1.56	None	Quadratic
R2	NTP	num	20	Polynomial	7184	8485	7897.50	378.04	1.18	None	Quadratic
R3	TF	num	20	Polynomial	1.03	1.1	1.07	0.0203	1.07	None	Quadratic

Table 11. ANOVA table for Retention time using CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.7284	9	0.0809	906.15	< 0.0001	significant
A-FR	0.3641	1	0.3641	4076.88	< 0.0001	
B-MP	0.2985	1	0.2985	3341.73	< 0.0001	
C-Temp	0.0497	1	0.0497	556.27	< 0.0001	
AB	0.0018	1	0.0018	20.49	0.0011	
AC	0.0010	1	0.0010	11.59	0.0067	
BC	0.0004	1	0.0004	4.23	0.0667	
A^2	0.0054	1	0.0054	60.78	< 0.0001	
B^2	0.0087	1	0.0087	97.13	< 0.0001	
C^2	0.0003	1	0.0003	3.35	0.0970	
Residual	0.0009	10	0.0001			
Lack of Fit	0.0007	5	0.0001	5.20	0.0472	significant
Pure Error	0.0001	5	0.0000			
Cor Total	0.7293	19				


Design-Expert® Software
Factor Coding: Actual
RT (min)
Design Points
1.301
2.036
X1 = A: FR
X2 = C: Temp
Actual Factor
B: MP = 70
RT (min)
C: Temp (0 C)
A: FR (ml/min)
Design-Expert® Software
Factor Coding: Actual
RT (min)
Design Points
1.301
2.036
X1 = A: FR
X2 = B: MP
Actual Factor
C: Temp = 30
RT (min)
B: MP (%)
A: FR (ml/min)


Design-Expert® Software
Factor Coding: Actual
RT (min)
Design Points
1.301
2.036
X1 = B: MP
X2 = C: Temp
Actual Factor
A: FR = 0.3
RT (min)
C: Temp (0 C)
B: MP (%)

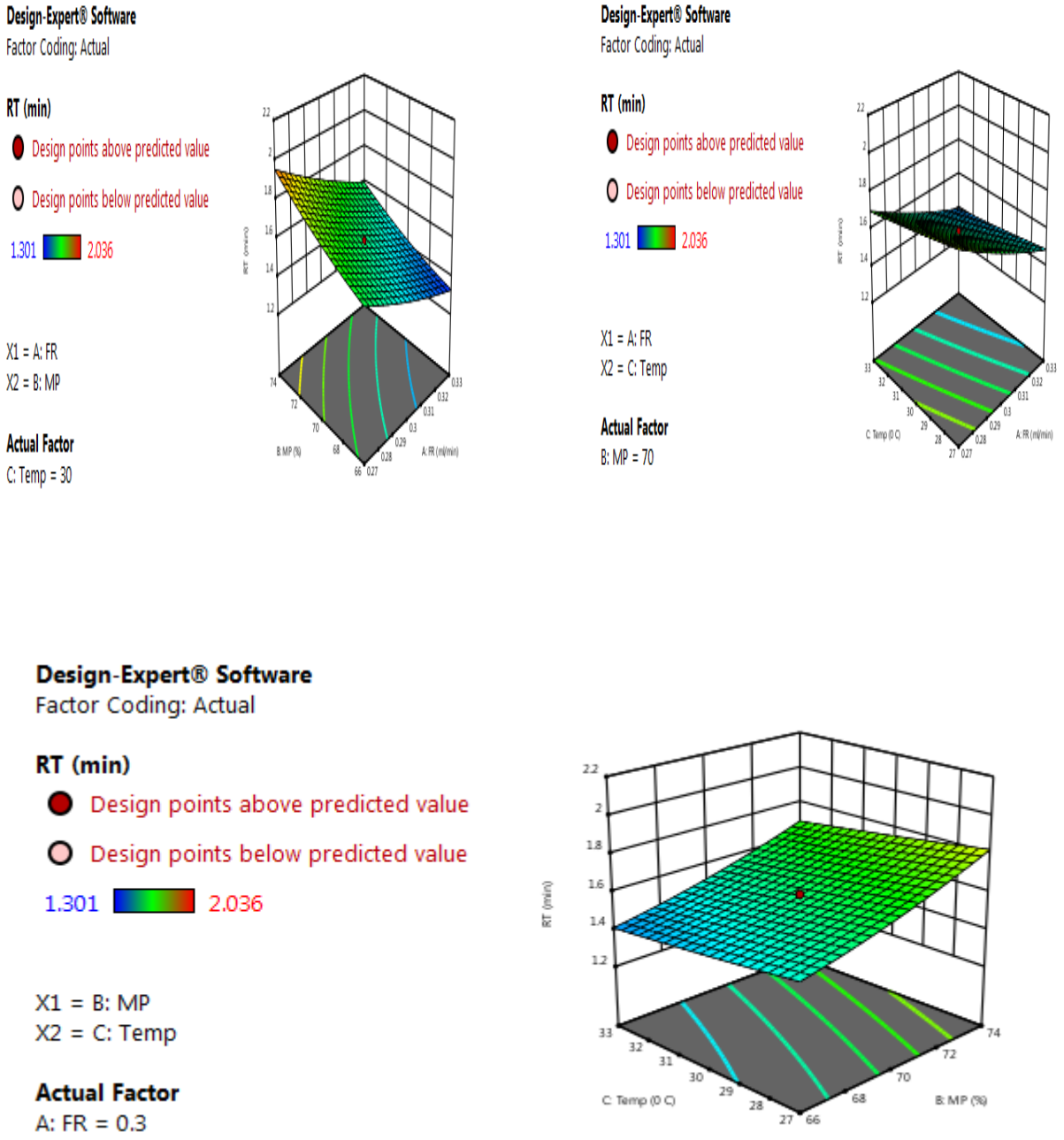


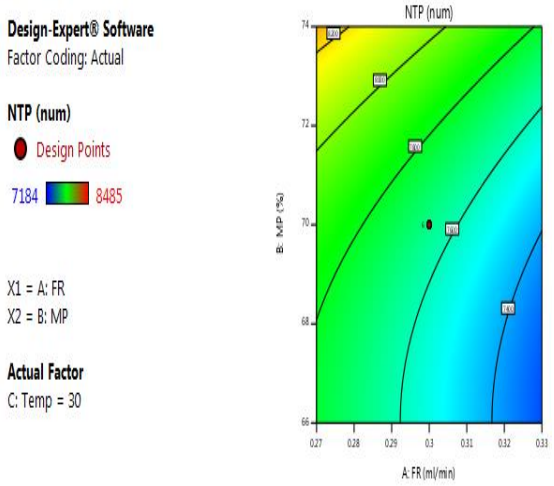
Fig 7. 2D contour plots of retention time as a function of Column temperature, Flow rate and organic ratio.

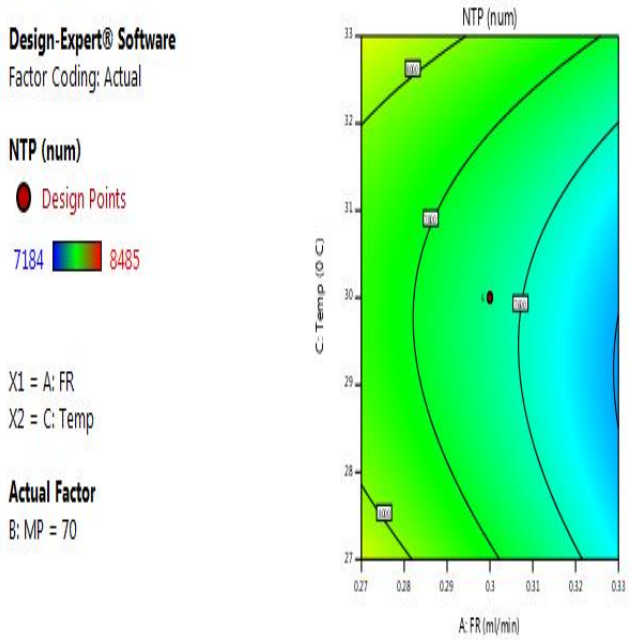
In order to understand the results, contour plots and 3D plot were generated after processing all data using the Design Expert® software (Fig.7 and 8). It shows the two dimensional contour plot as a function of Column temperature, Flow rate and organic ratio. Based on the color code, the working region can be easily identified. Retention time maps represent the value of the retention time, with warm "red" colors indicating larger retention time, cold "blue" colors lower and light green to yellow color represent intermediate retention time.

Table 12. ANOVA table for Theoretical plates using CCD

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.700E+06	9	3.000E+05	198.99	< 0.0001	significant
A-FR	7.948E+05	1	7.948E+05	527.11	< 0.0001	

B-MP	8.428E+05	1	8.428E+05	558.98	< 0.0001	
C-Temp	70407.55	1	70407.55	46.70	< 0.0001	
AB	3.13	1	3.13	0.0021	0.9646	
AC	27966.12	1	27966.12	18.55	0.0015	
BC	820.13	1	820.13	0.5439	0.4778	
A^2	2461.93	1	2461.93	1.63	0.2302	
B^2	2.264E+05	1	2.264E+05	150.17	< 0.0001	
C^2	7.812E+05	1	7.812E+05	518.12	< 0.0001	
Residual	15077.74	10	1507.77			
Lack of Fit	13876.24	5	2775.25	11.55	0.0089	significant
Pure Error	1201.50	5	240.30			
Cor Total	2.715E+06	19				





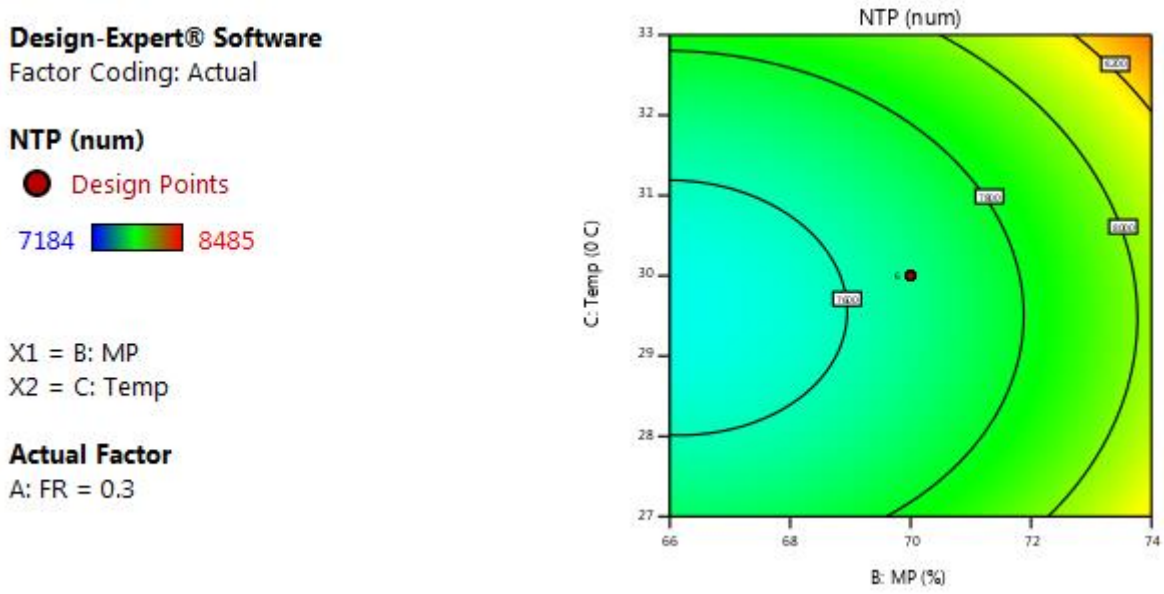
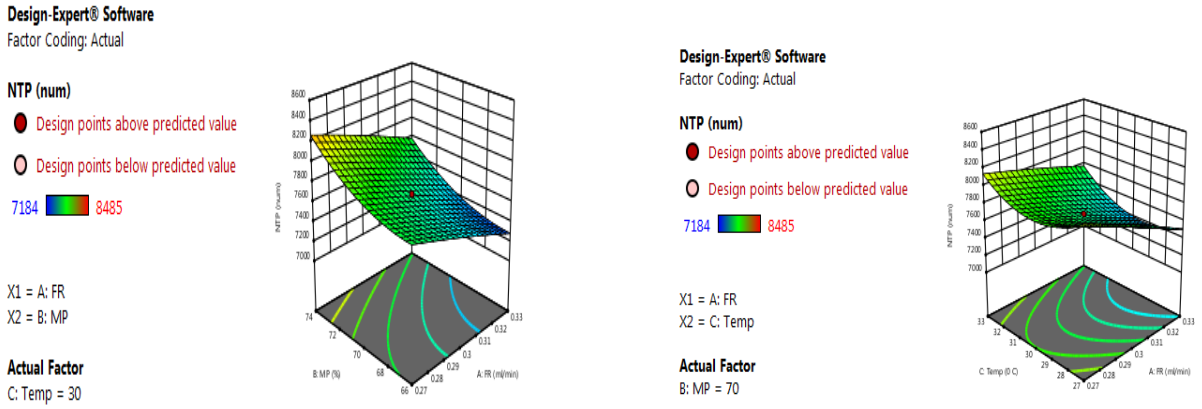


Fig 9. 2D contour plots of Theoretical plates as a function of Column temperature, Flow rate and organic ratio.



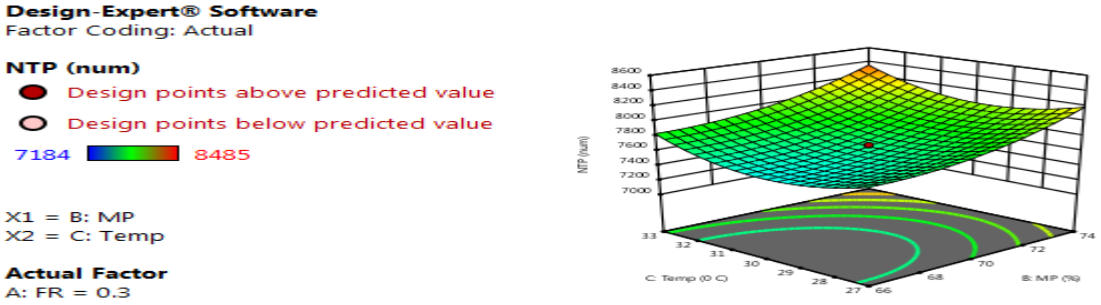


Fig 10. 3D contour plots of Theoretical plates as a function of Column temperature, Flow rate and organic ratio.

Table 13. ANOVA table for Asymmetry using CCD

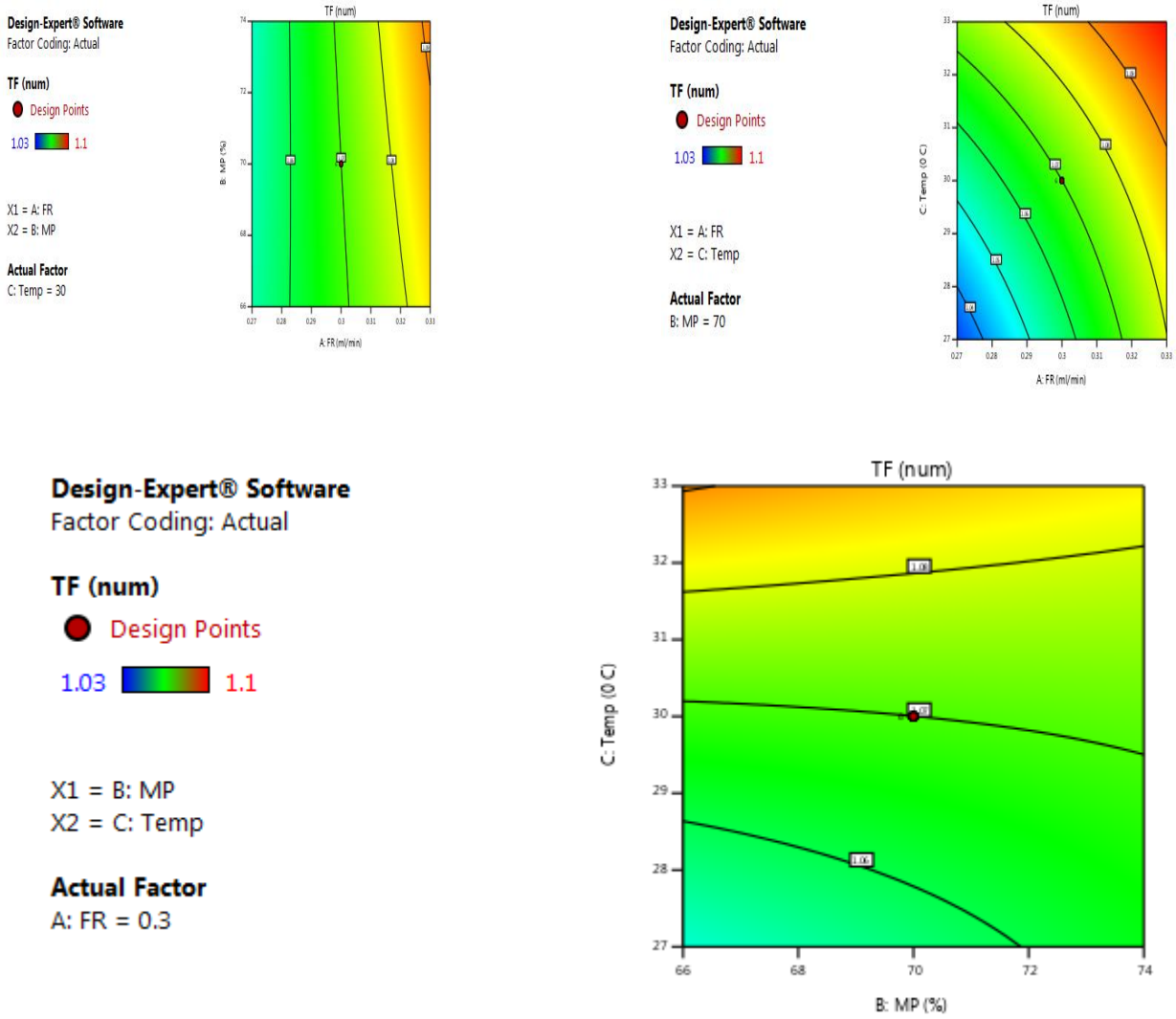
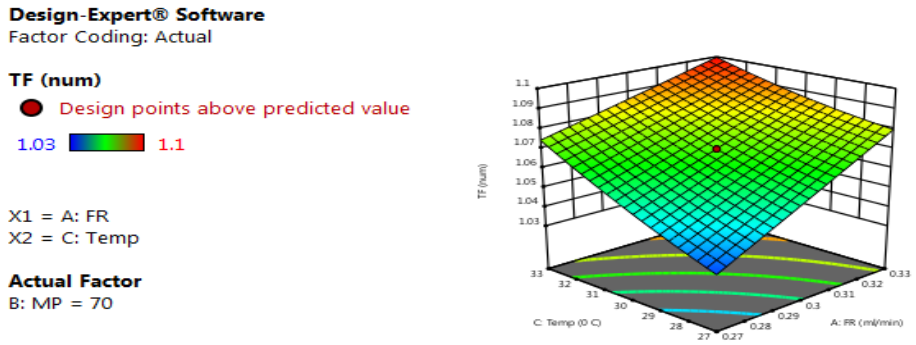


Fig 11. 2Dcontour plots ofasymmetryas a function of Column temperature, Flow rate and organic ratio.



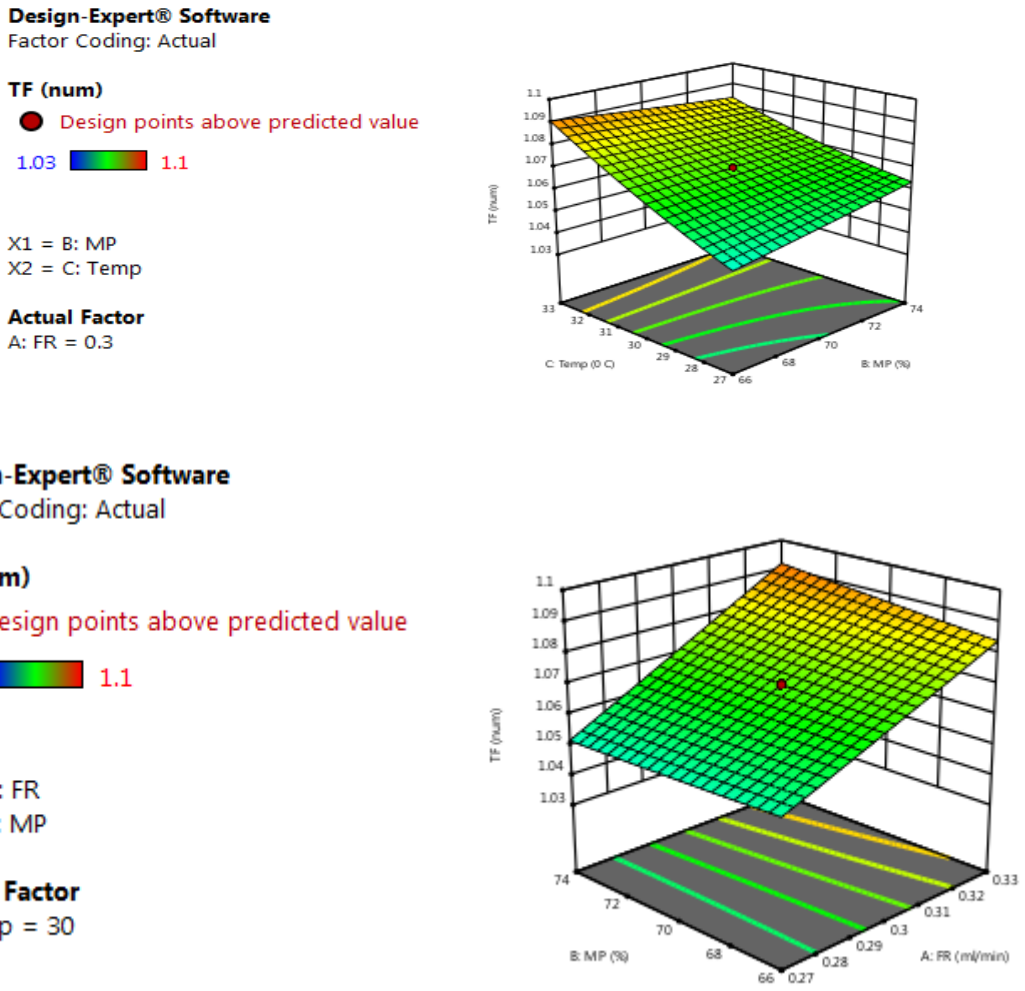


Fig 12. 3D contour plots of asymmetry as a function of Column temperature, Flow rate and organic ratio.

.3.3. Final developed UPLC method

In order to understand the results, 3D space was obtained after processing all data using the software. A composite desirability was applied to get an optimum set of conditions based on the specified goals and boundaries for the each response. This desirability function was depends on a scale of desirability function ranges between d = 0, for a completely undesirable response, to d = 1 for a fully desirable response Based on the specified goals and boundaries for the retention time, area, Asymmetry and a composite desirability (D) of 1 was obtained, which gave the optimal flow rate of 0.28 ml/min. To confirm these optimum set of conditions, three replicate injections of 20 µg/ml Sofpironium was analyzed to determine if their observed retention time, asymmetry and theoretical plates were within the predicted ranges. It was observed that the differences between the observed and predicted peak response were less than 5%.

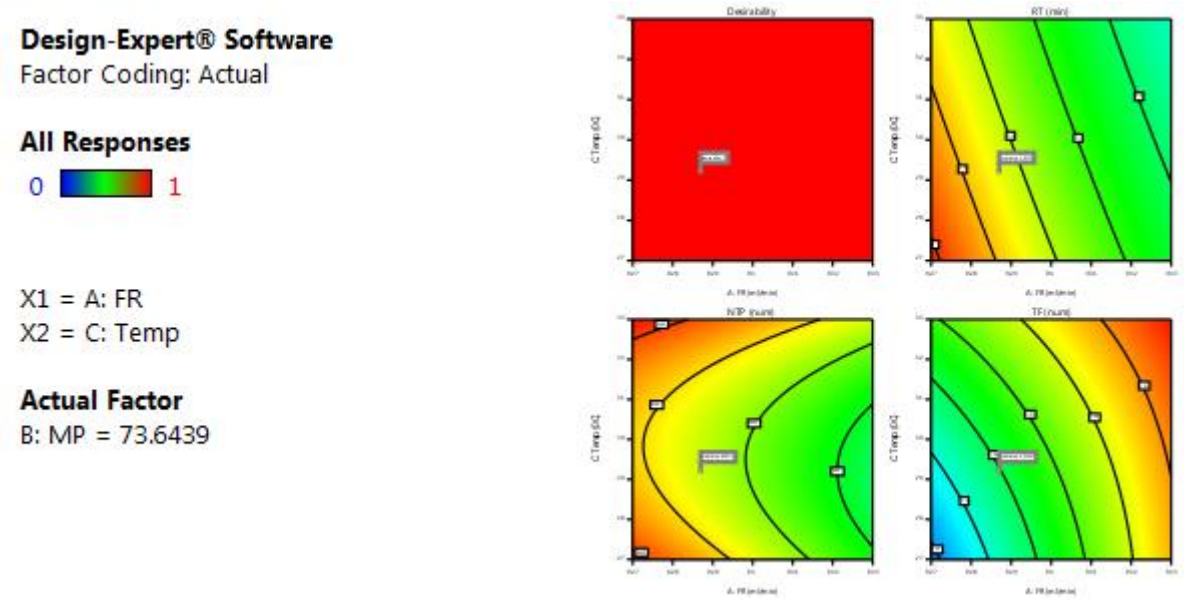


Fig 15. Overall desirability of final method

Table 15. Final developed and optimized UPLC method parameter

Factor	Name	Level	Low Level	High Level	Std. Dev.	Coding
A	FR	0.2870	0.2700	0.3300	0.0000	Actual
B	MP	73.64	66.00	74.00	0.0000	Actual
C	Temp	29.21	27.00	33.00	0.0000	Actual

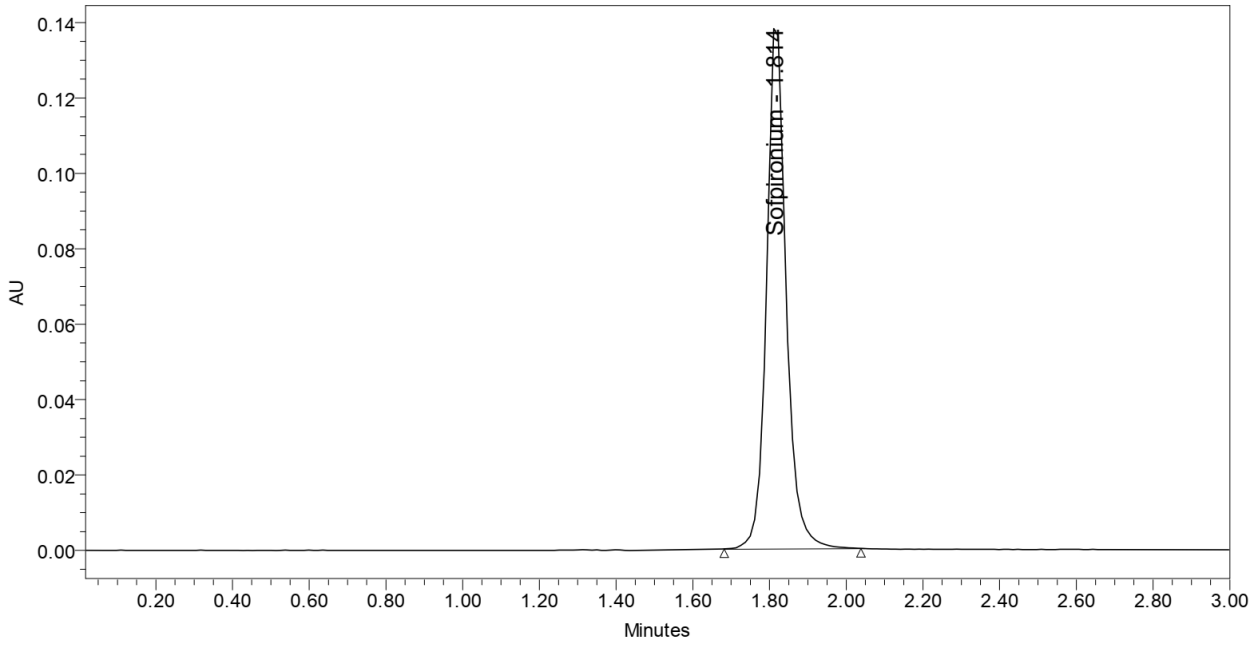


Fig 16. Chromatogram of final optimized method

6.4 Stress degradation study ***6.4.1 Acid hydrolysis***

The drug substance was exposed to 2N HCl, kept for reflux in Radley apparatus at 70 ^{0}C temperature for 8 hrs, it was showing there is 4.02% of Sofpironium degradation in acid hydrolysis. The blank solutions also subjected to stress study in the same fashion as the drug solution. The exposed stress sample and blank solutions were analyzed by UPLC system.

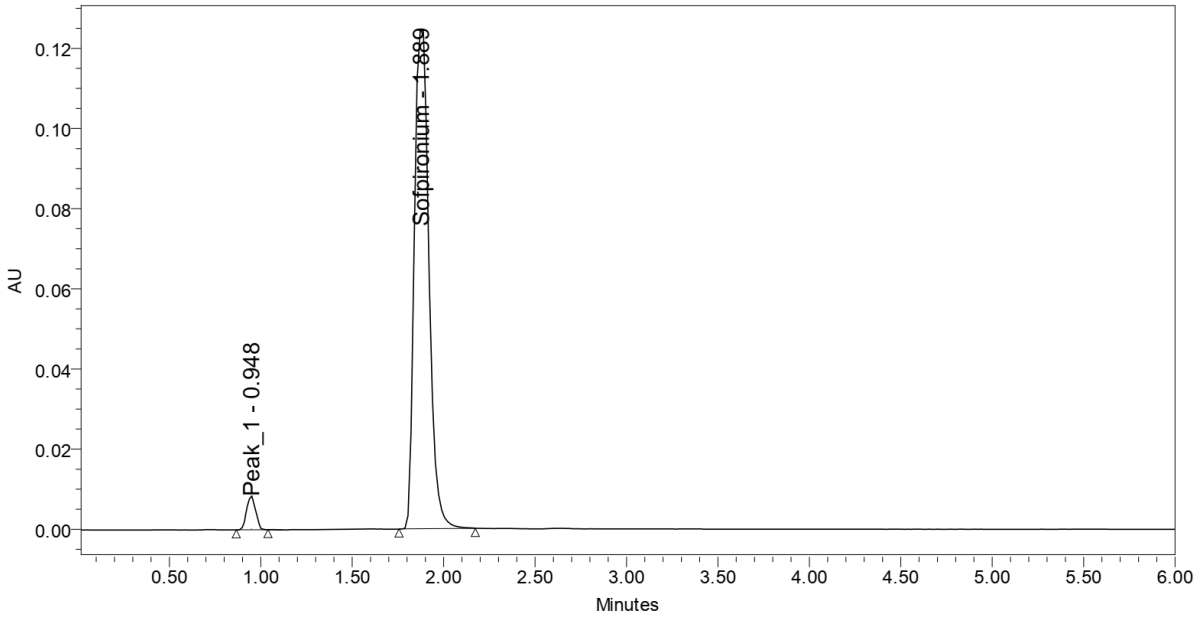


Fig 18. Chromatogram of drug in 2N HCl at 70 ^{0}C temperature for 8 hrs

6.4.2 BASE HYDROLYSIS

The drug substance was exposed to 2N NaOH, kept for reflux in Radley apparatus at 70 ^{0}C temperature for 8 hrs, it was showing 4.59% of Sofpironium degradation in base hydrolysis with two degradation products. The blank solutions also subjected to stress study in the same fashion as the drug solution. The exposed stress sample and blank solutions were analyzed by UPLC system.

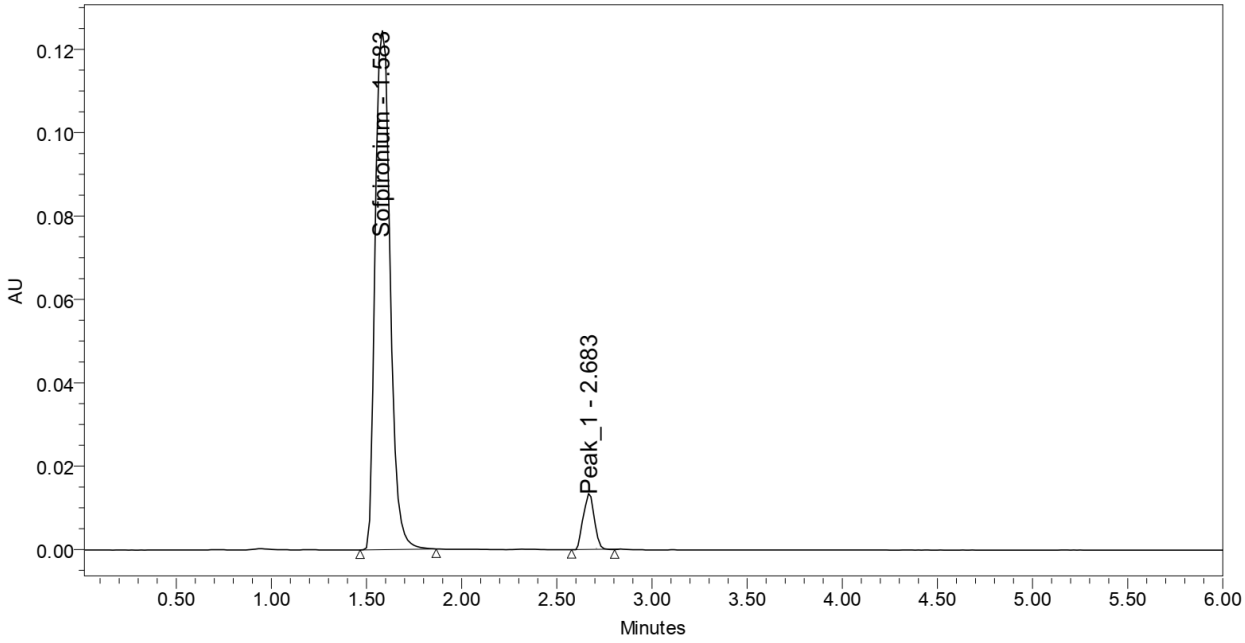


Fig 20. Chromatogram of drug in 2N NaOH at 70 ^{0}C temperature for 8 hrs

6.4.3. NEUTRAL HYDROLYSIS

The drug substance was exposed to water, kept for reflux in Radley apparatus at 70 ^{0}C temperature for 24 hrs; it was showing there was 0.27% degradation in neutral hydrolysis. The blank solutions also subjected to stress study in the same fashion as the drug solution. The exposed stress sample and blank solutions were analyzed by UPLC system.

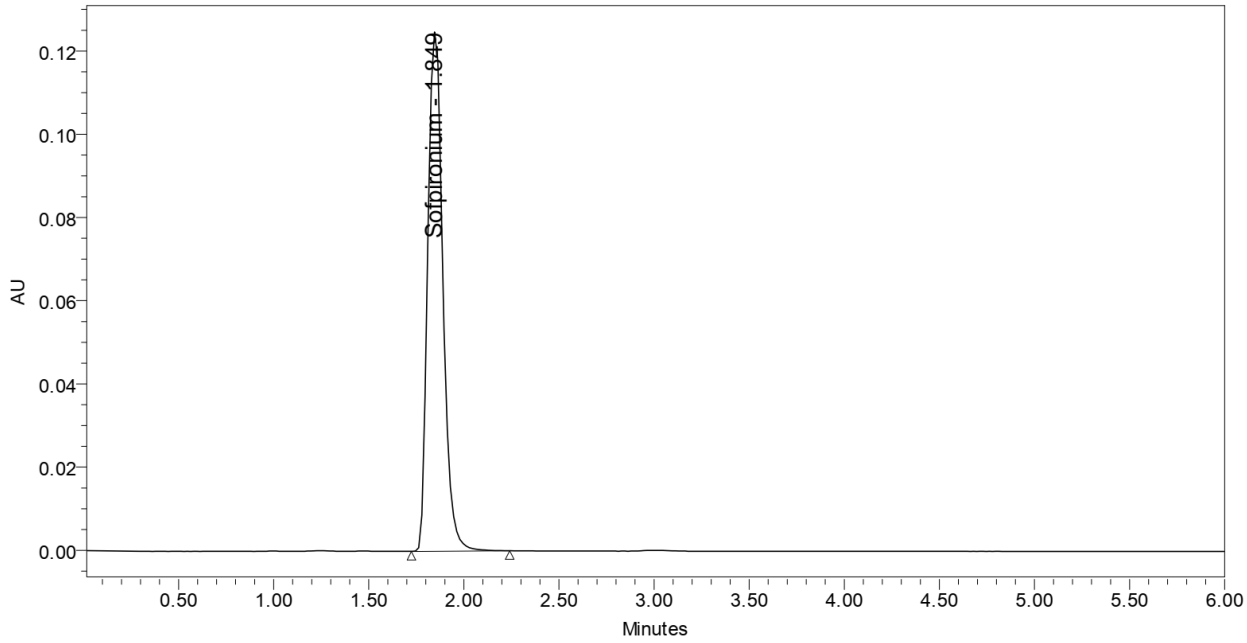


Fig 22. Chromatogram of drug in water at 70 ^{0}C temperature for 24 hrs

6.4.4 OXIDATIVE DEGRADATION

The drug was exposed to 20% H_2O_2, at room temperature for 24 hours. Samples were withdrawn at different time intervals and injected into the UPLC system and chromatogram was recorded 4.71% of Sofpironium degradation in 20% H2O2 solution at the end of 24 hrs .The blank solutions also subjected to stress study in the same fashion as the drug solution. The exposed stress sample and blank solutions were analyzed by UPLC system.

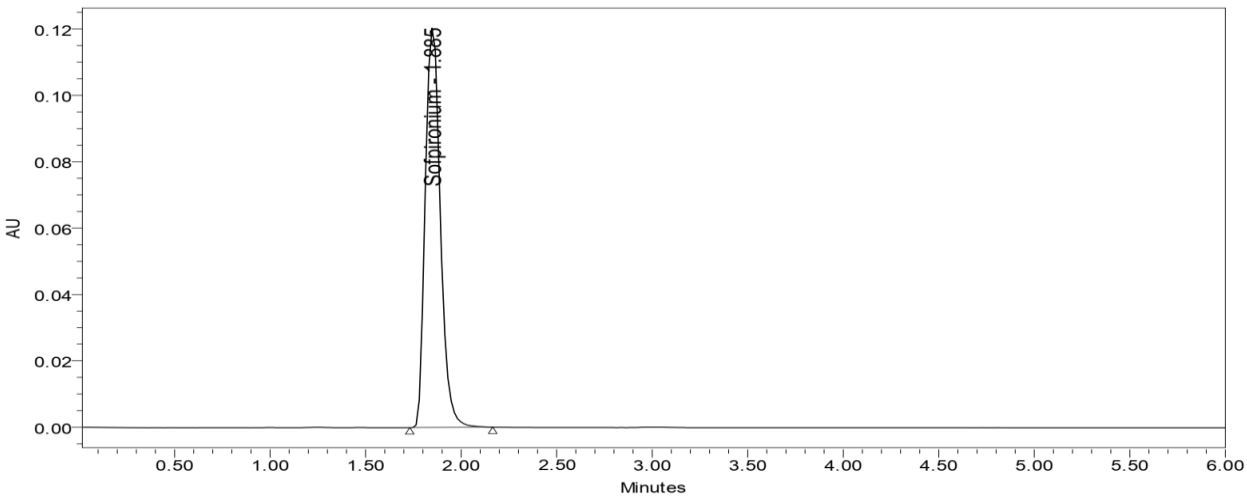


Fig 24. Chromatogram of drug in 20% H_2O_2 at room temperature for 24 hrs

6.4.5. Thermal degradation

The drug sample was exposed to 70˚C for 1 day in a hot air oven and samples were withdrawn at different time intervals from 1day. Samples were injected into the UPLC system and chromatogram was recorded. 1.06% of Sofpironium degradation was found at the end of 3 days of exposure. Hence the drug can be regarded as thermostable at 70˚C.

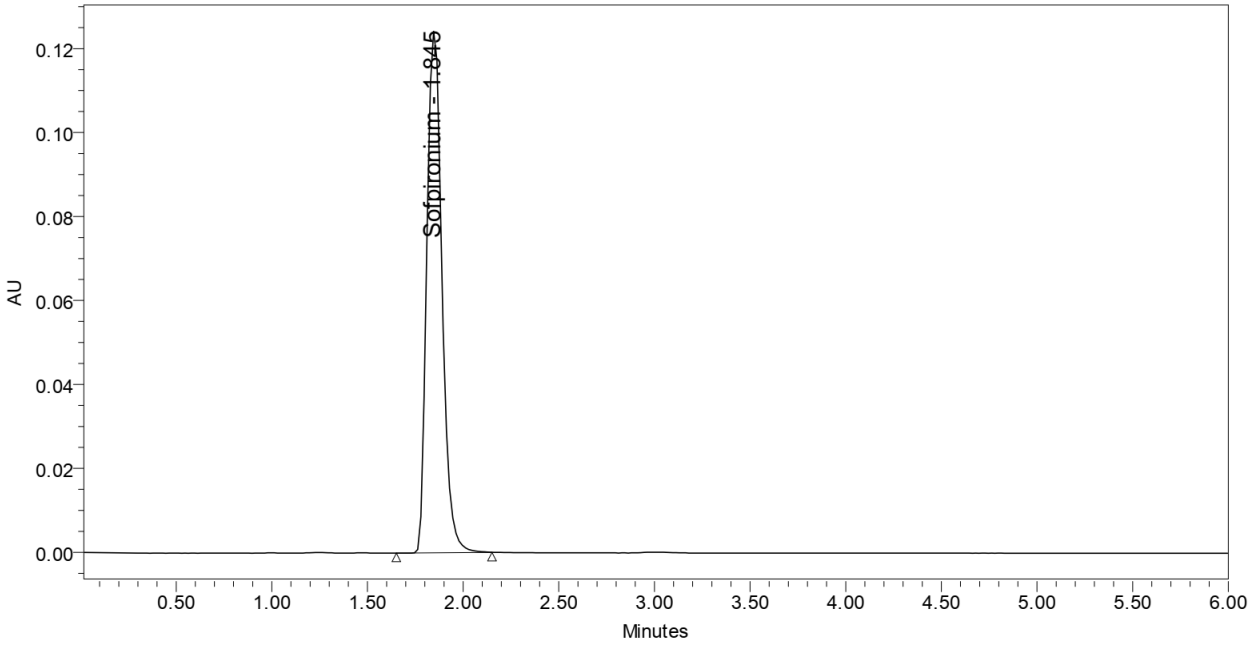


Fig 24. Chromatogram of drug at 70 ^{0}C temperature for 24 hrs

6.4.6. Photo degradation

The drug sample was exposed to direct sunlight for 24 hours. Samples were injected into the UPLC system and chromatogram was recorded. 1.06% of Sofpironium degradation was found at the end of 24hrs of exposure. Hence the drug can be considered as photostable.

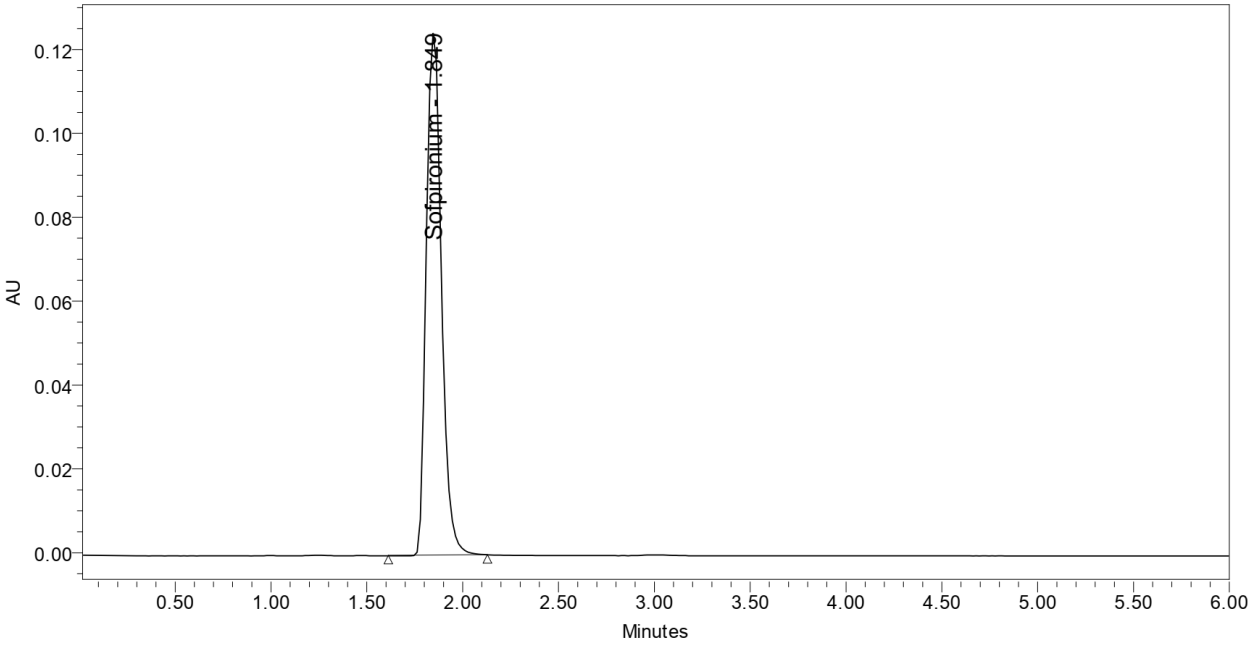


Fig 26. Chromatogram of drug after 24hrs exposure in direct sunlight

Table 16. Summary of degradation study

Sr.No.	Condition of degradation study	% of drug degraded	% of drug Undegraded
1.	2N HCl, 8 hrs	4.02	95.98
2.	2N NaOH, 8hrs	4.59	95.41
3.	Oxidative degradation, 24 hrs	4.71	95.29
4.	Thermal degradation, 1 days	1.06	98.94
5.	Photo degradation, 24 hrs	0.97	99.03
6.	Neutral hydrolysis, 24 hrs	0.27	99.73

6.5 Method validation

Validation:

Specificity:

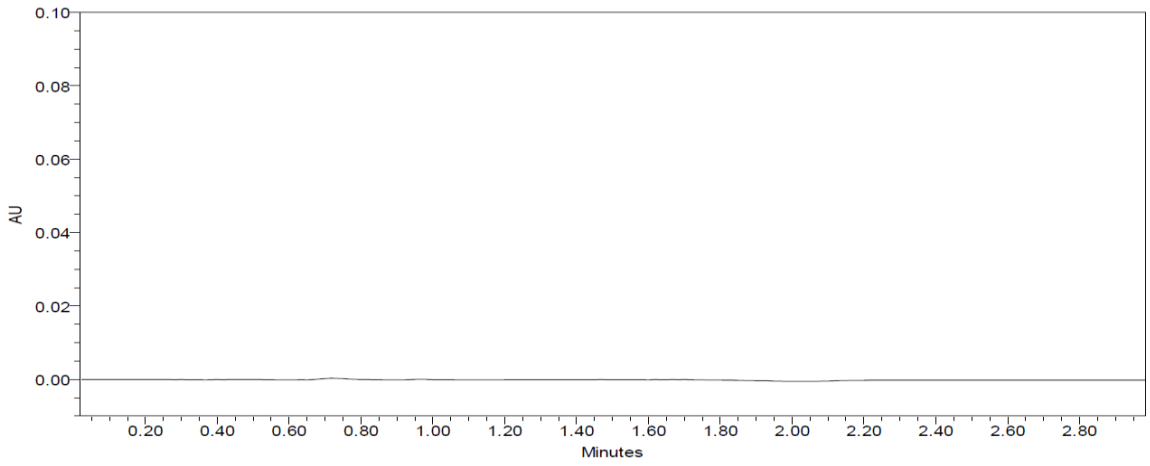


Fig 6.7 blank Chromatogram

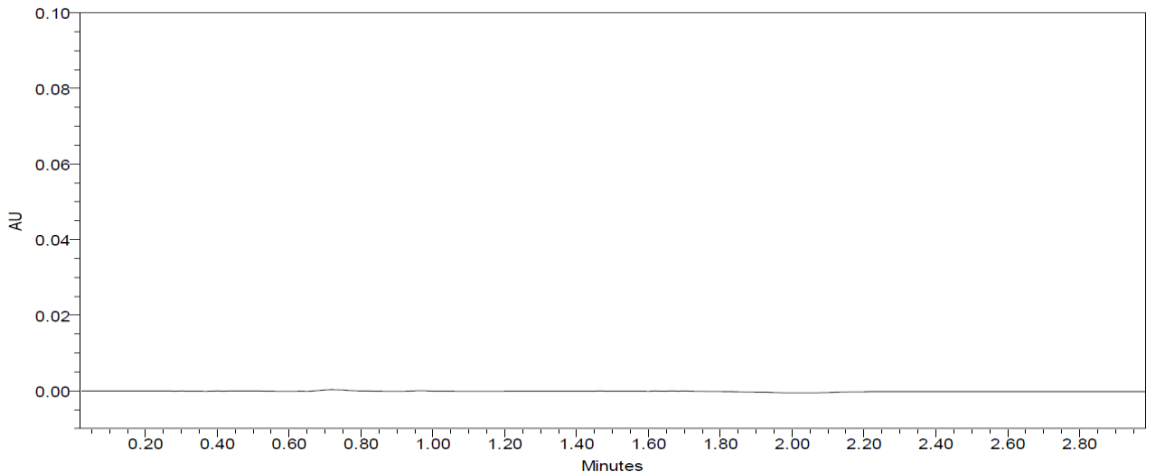


Fig 6.8 Placebo Chromatogram

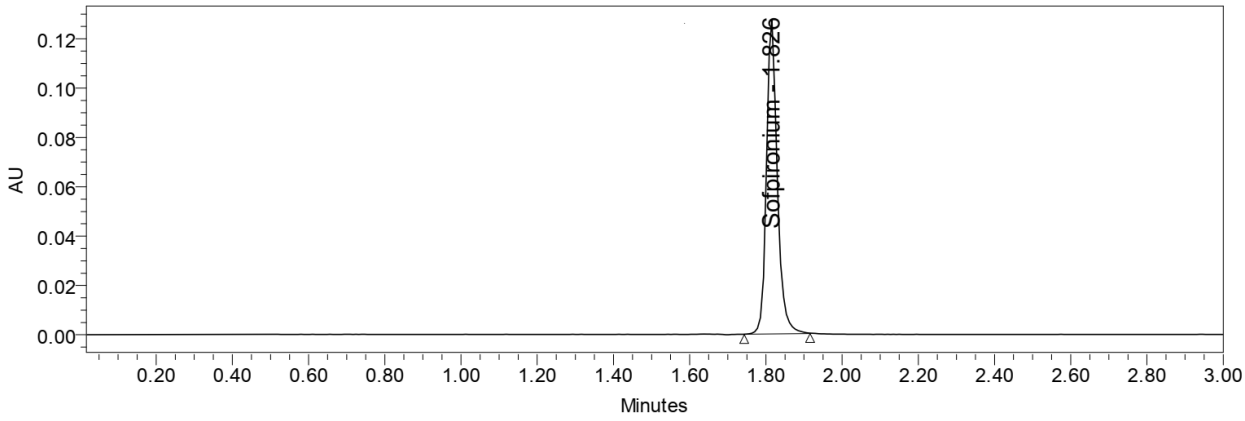


Fig 6.9 Standard Chromatogram

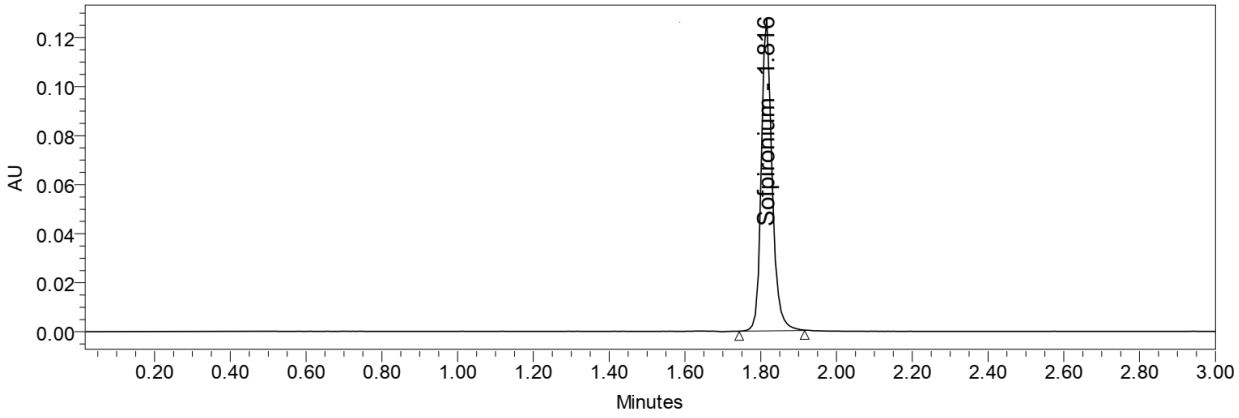


Fig 6.10 Sample Chromatogram

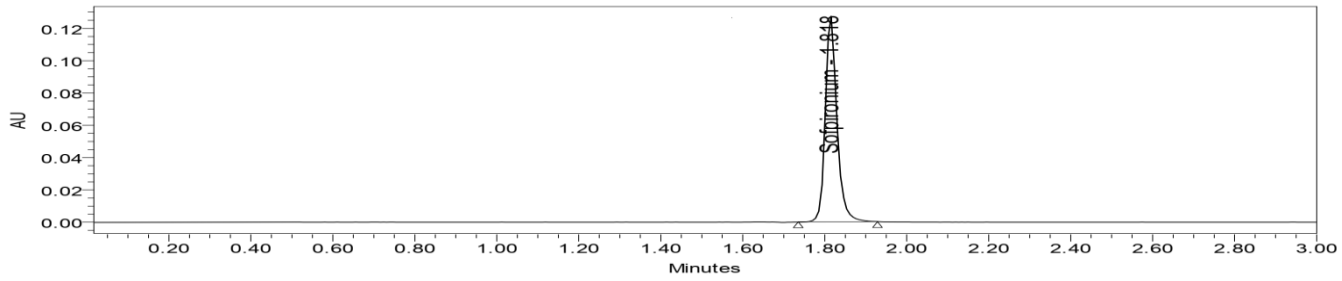
Discussion: Retention time of Sofpironium was 1.821 min. We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific

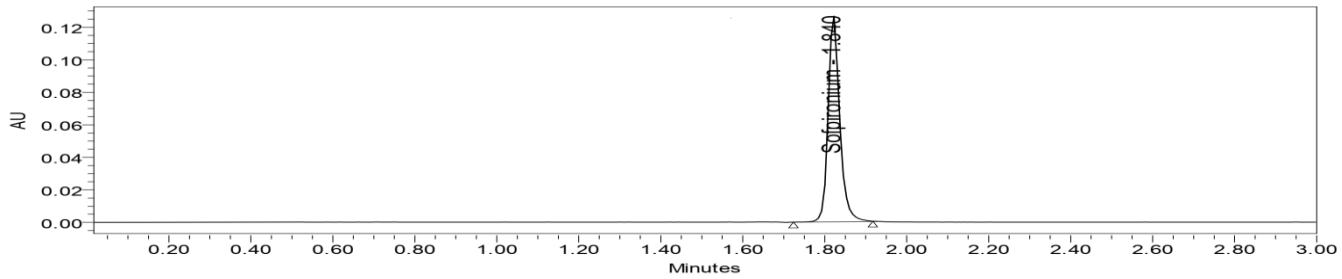
6.2 Precision:

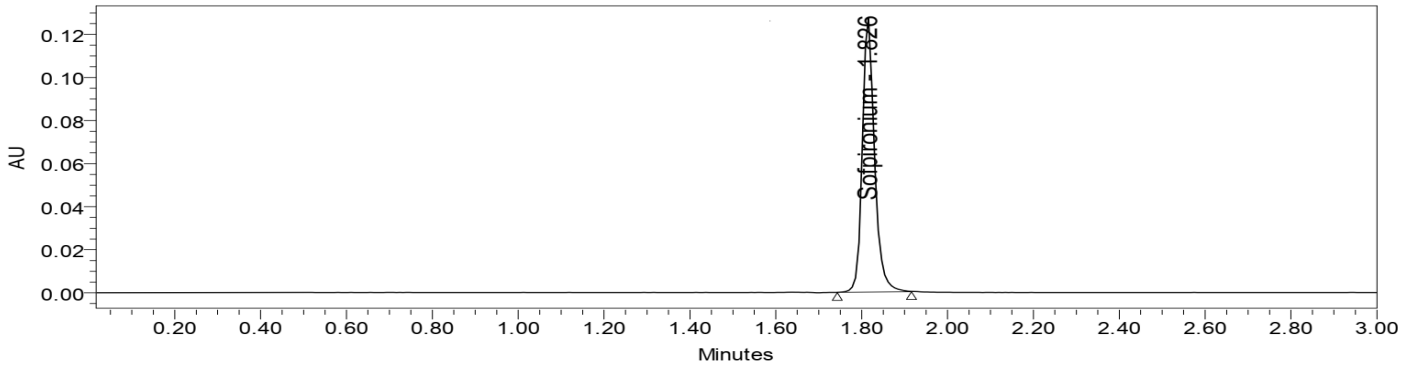
System Precision: Six working sample solutions of 20ppm are injected and the % Amount found was calculated and %RSD was found to be 0.4 and chromatogram was shown in fig

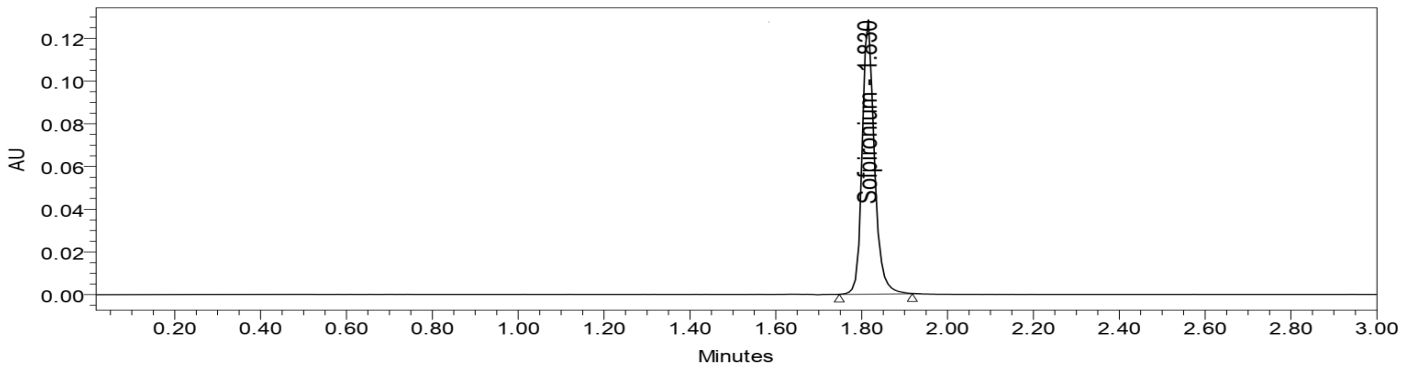
6.2. **Table 6.2 SystemPrecision data**

S.No	Peak Area
1	1071324
2	1066647
3	1069843
4	1075862
5	1068874
6	1062264
AVG	1069136
STDEV	4561.0
%RSD	0.4


Sofpironium - 1.818
AU
Minutes


Sofpironium - 1.840
AU
Minutes


Sofpironium - 1.826
AU
Minutes


Sofpironium - 1.830
AU
Minutes

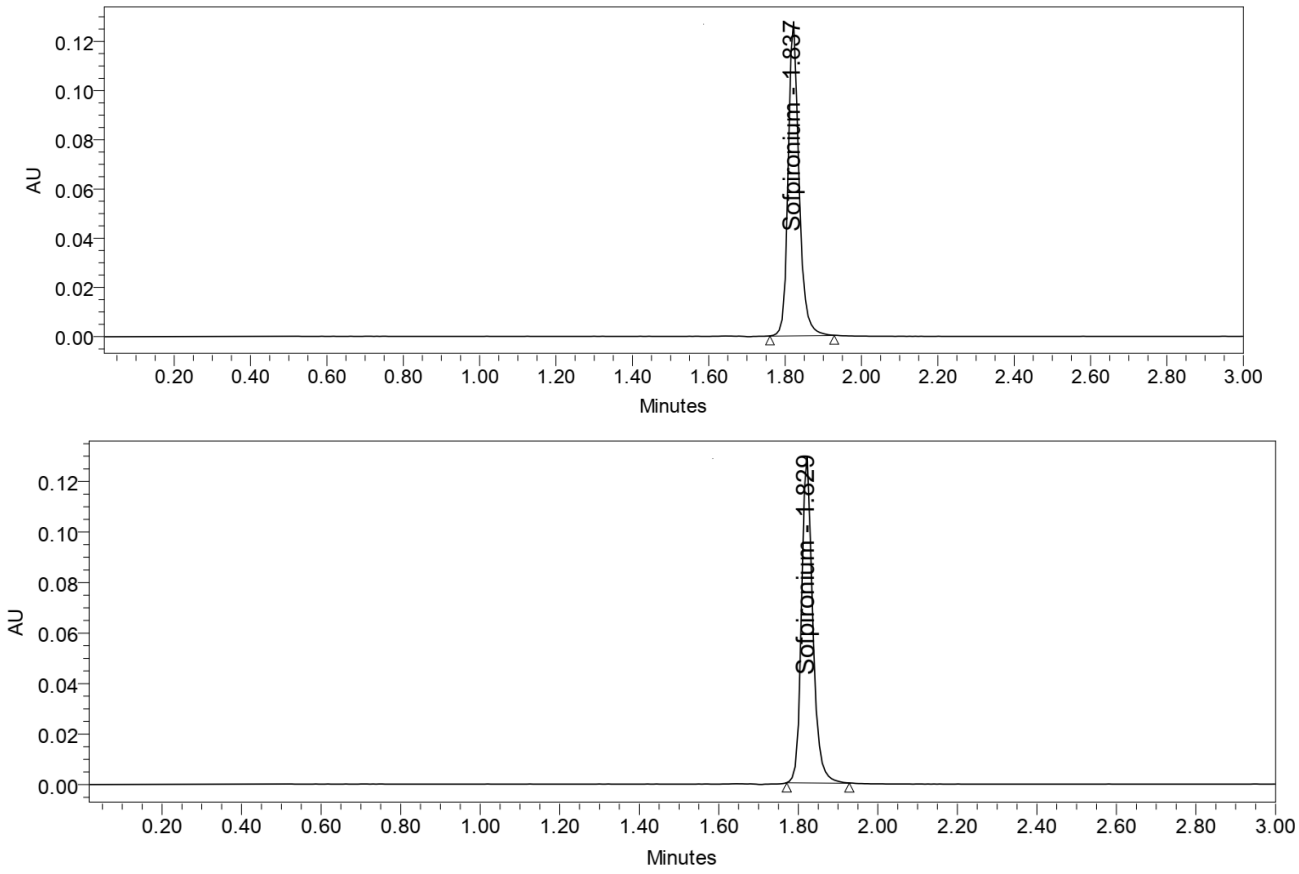
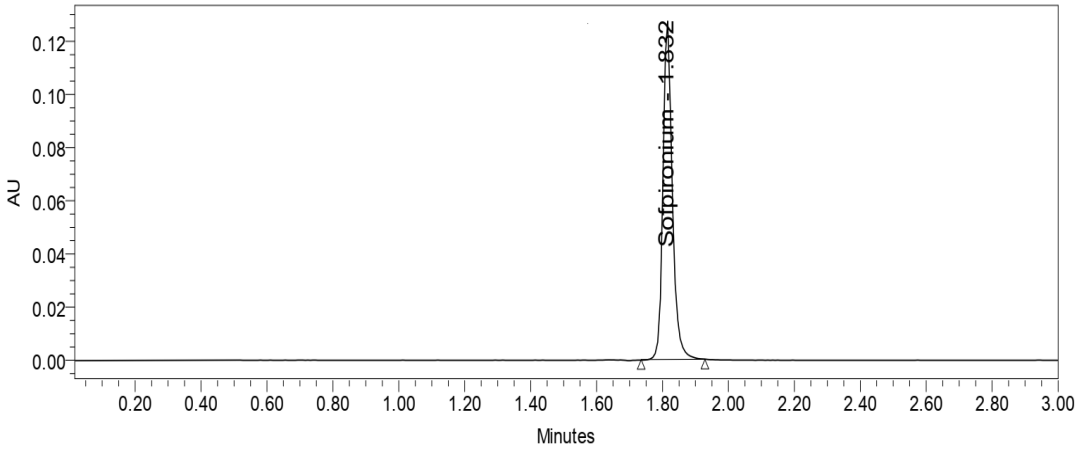


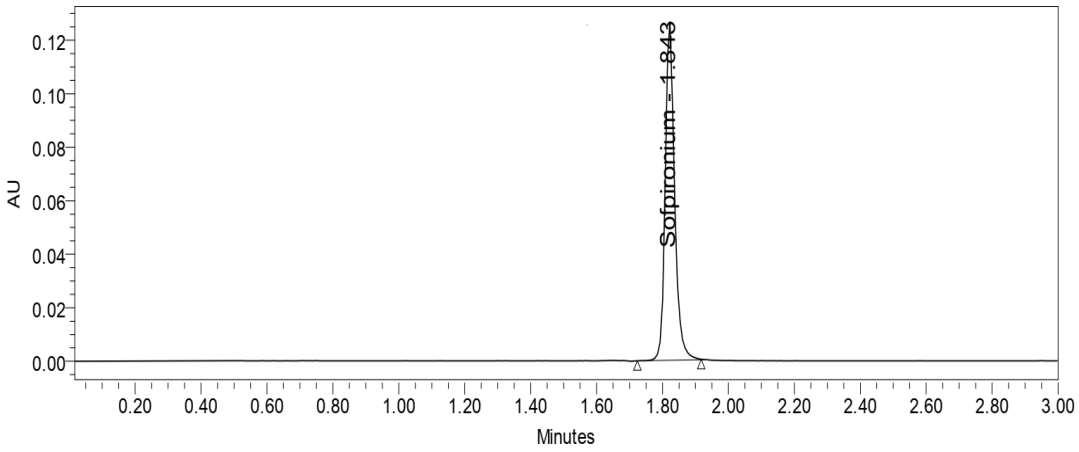
Fig 6.11 System precision Chromatogram

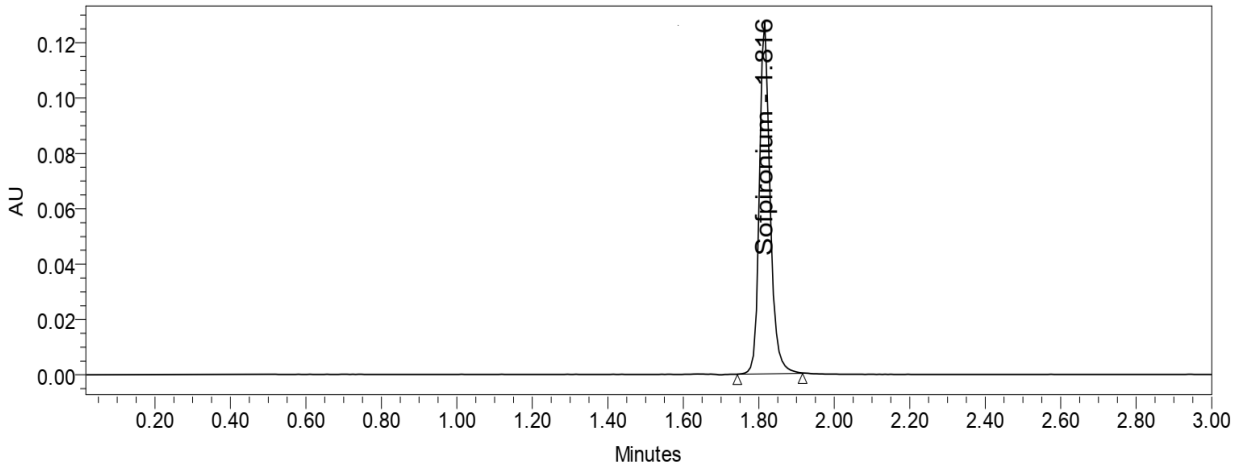
Method precision: Six working sample solutions of 20ppm are injected on the next day of the preparation of samples and the % Amount found was calculated and %RSD was found to be 1.0 and chromatogram was shown in fig 6.3.

Table 6.3 Method precision data

S.No	Peak Area
1	1074976
2	1059172
3	1065933
4	1058407
5	1068799
6	1044806
AVG	1062016
STDEV	10455.7
%RSD	1.0


Sofpironium - 1.832
AU
Minutes


Sofpironium - 1.843
AU
Minutes


Sofpironium - 1.816
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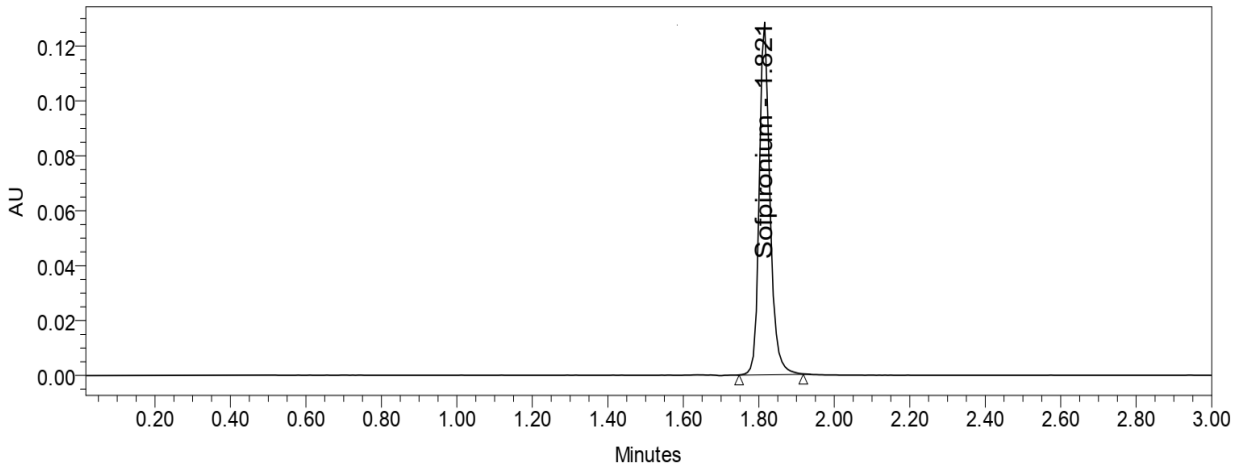
Minutes


Sofpironium - 1.821
AU
Minutes

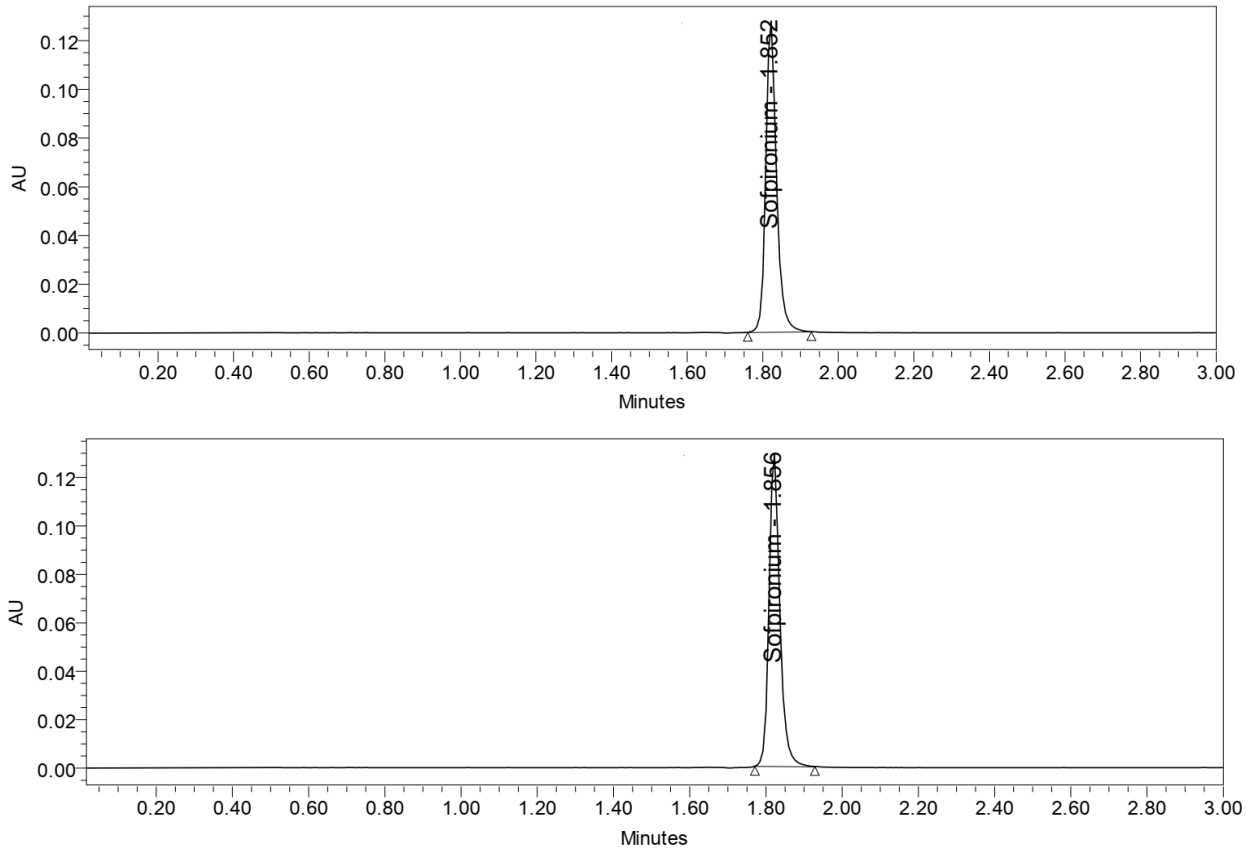
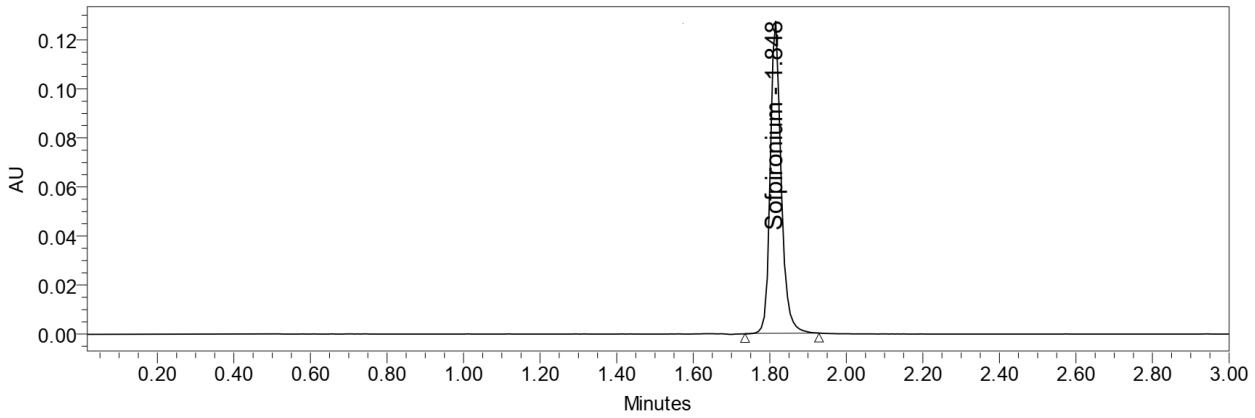


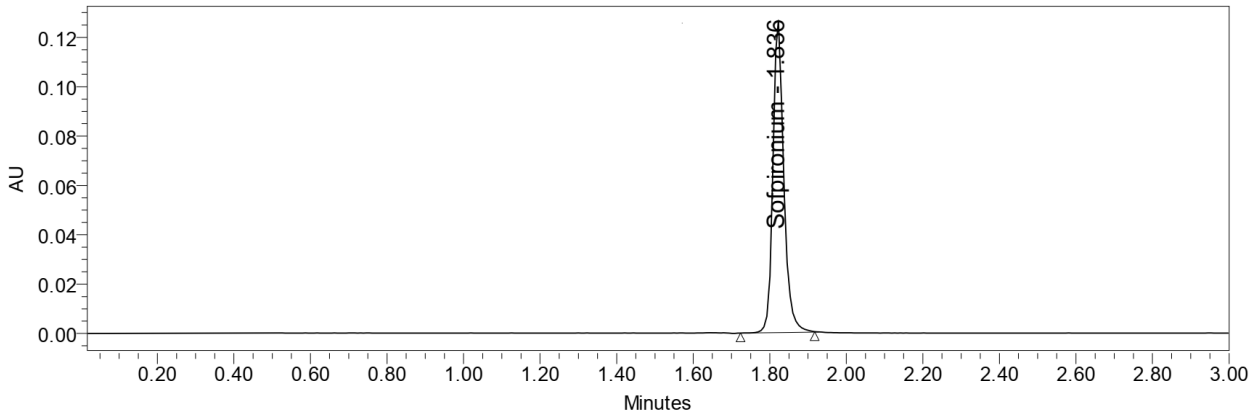
Fig 6.12 Method precision Chromatogram

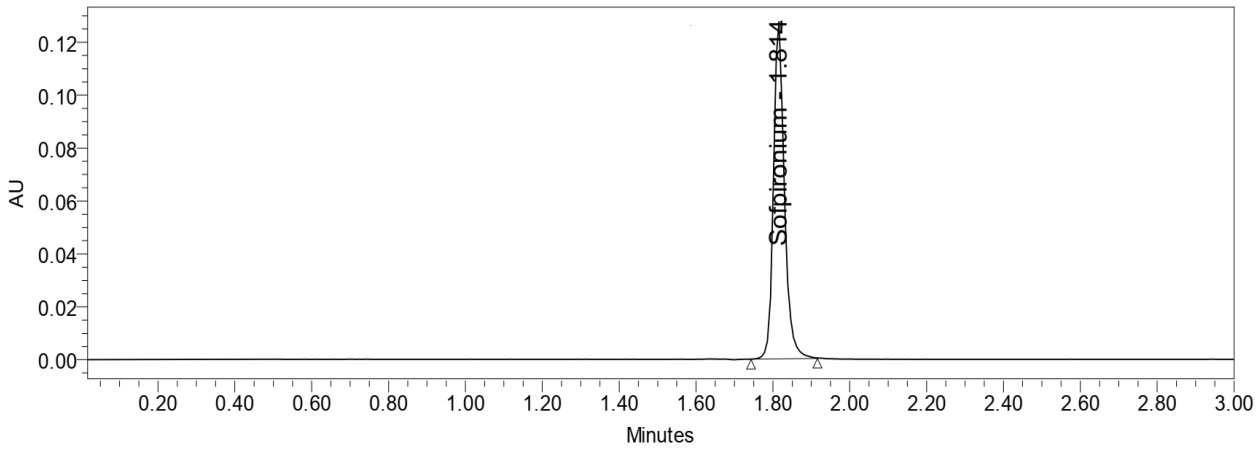
Intermediate precision: Six working sample solutions of 20ppm are injected on the next day of the preparation of samples and the % Amount found was calculated and %RSD was found to be 1.2 and chromatogram was shown in fig 6.3.

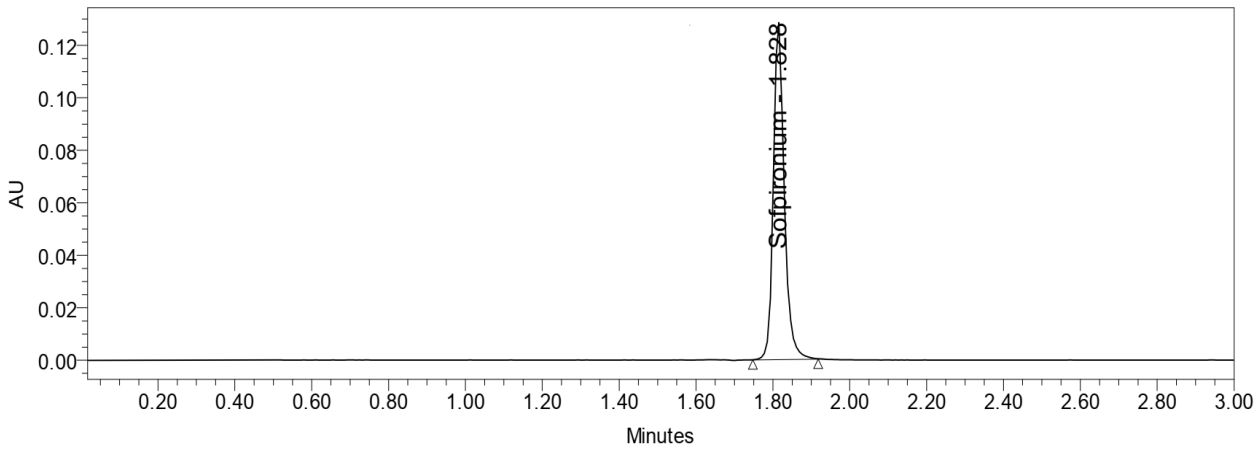
Table 6.3 Intermediate precision data

S.No	Peak Area
1	1014552
2	1021286
3	1016125
4	1032367
5	1005632
6	1038785
AVG	1021458
STDEV	12213.6
%RSD	1.2


Sofpironium - 1.848
AU
Minutes


Sofpironium - 1.836
AU
Minutes


Sofpironium - 1.844
AU
Minutes


Sofpironium - 1.828
AU
Minutes

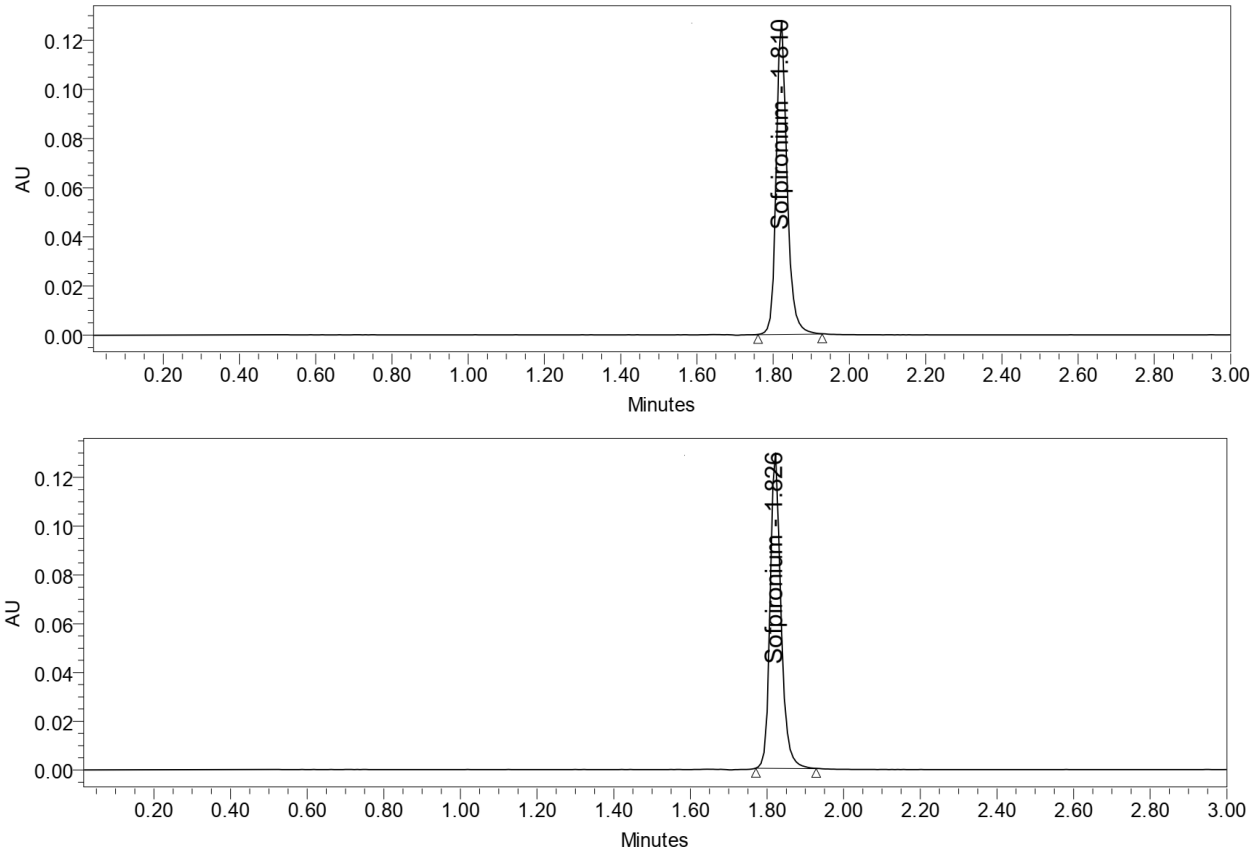


Fig 6.12 Intermediate precision Chromatogram

6.3 LINEARITY:

To demonstrate the linearity of assay method, inject 6 standard solutions with concentrations of about 5ppm to 30ppm of Sofpironium. Plot a graph to concentration versus peak area. Slope obtained was y = 52995x + 2524.1 and Correlation Co-efficient was found to be 0.999 and Linearity plot was shown in Fig 6.15.

Table 6.4 Linearity Concentration and Response

Linearity Level (%)	Concentration (ppm)	Area
0	0	0
25	5	266684
50	10	532281
75	15	805170
100	20	1057956
125	25	1333080
150	30	1586923

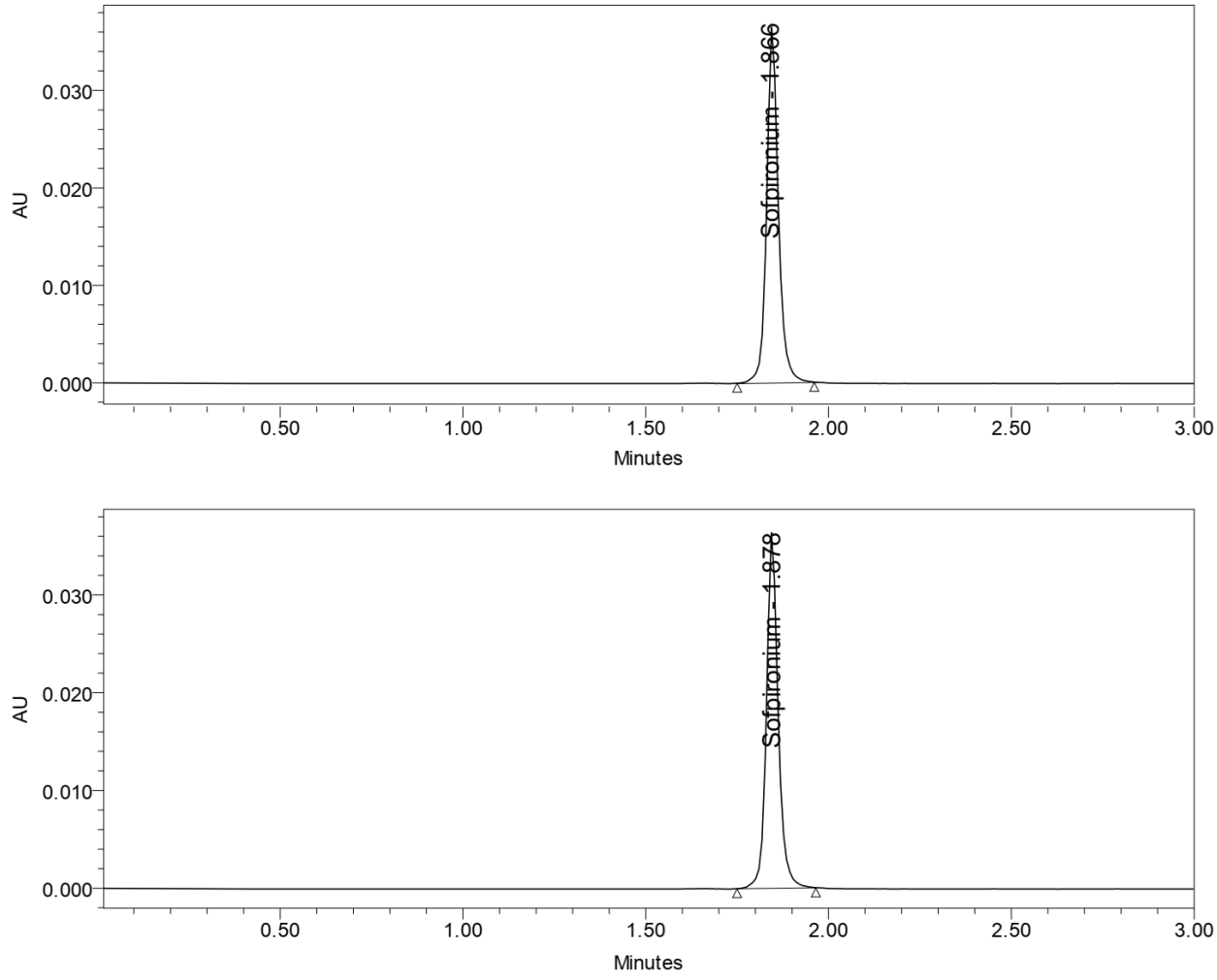
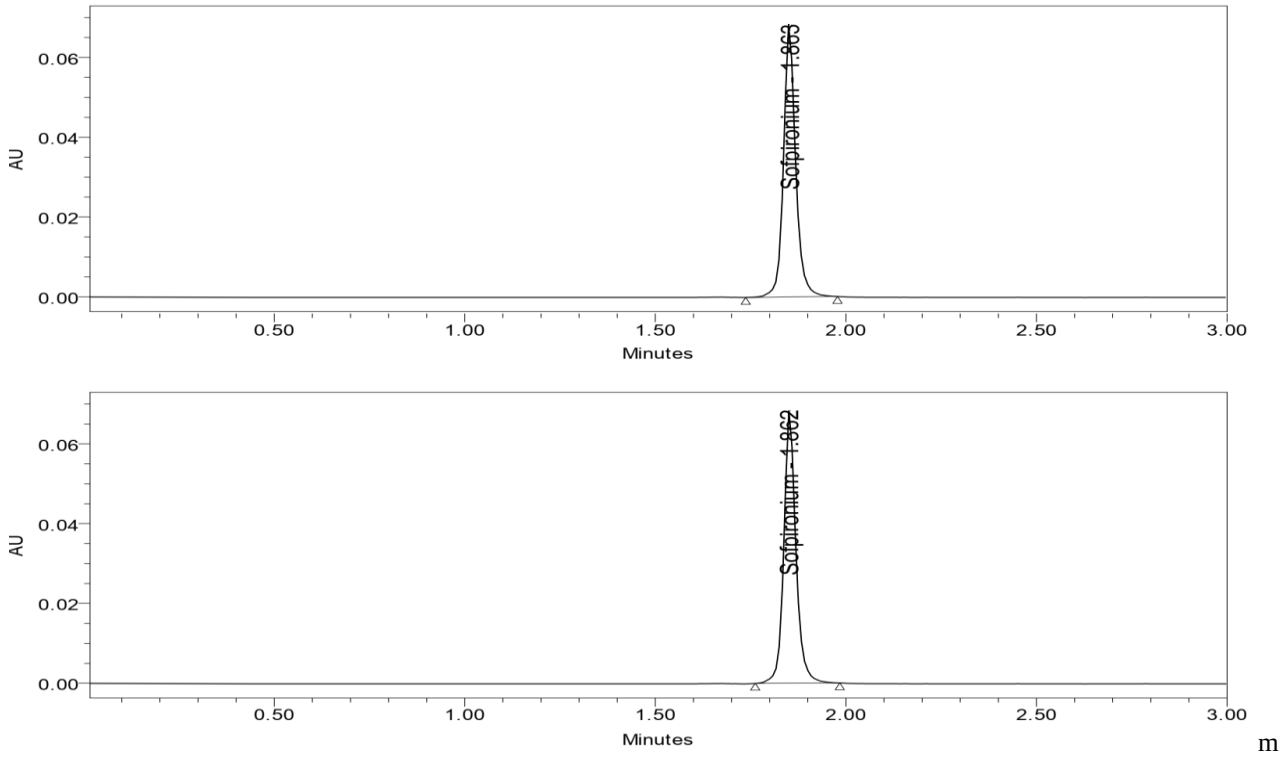


Fig 6.13 Linearity 25% Chromatogra



m

Fig 6.14 Linearity 50% Chromatogram

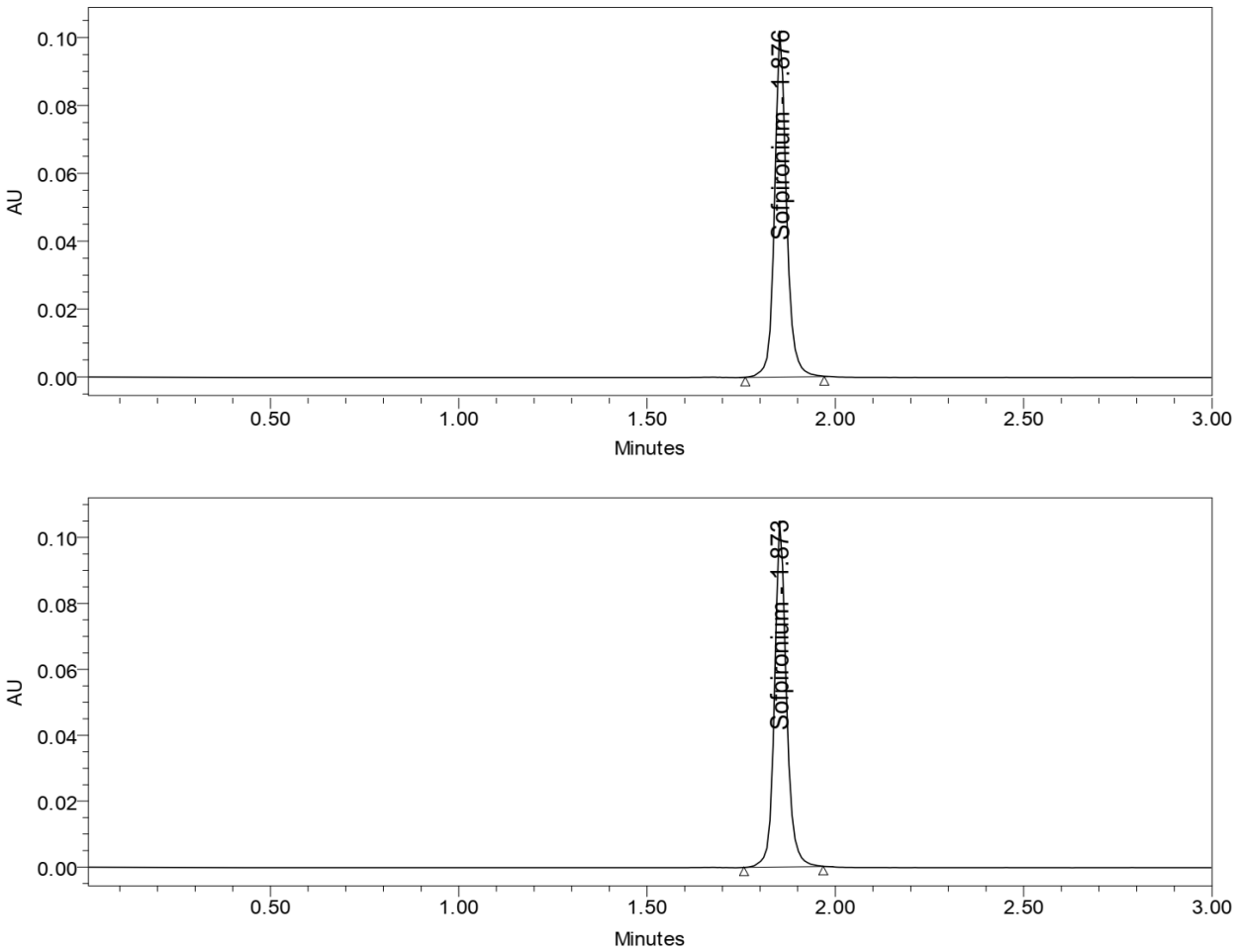


Fig 6.15 Linearity 75% Chromatogram

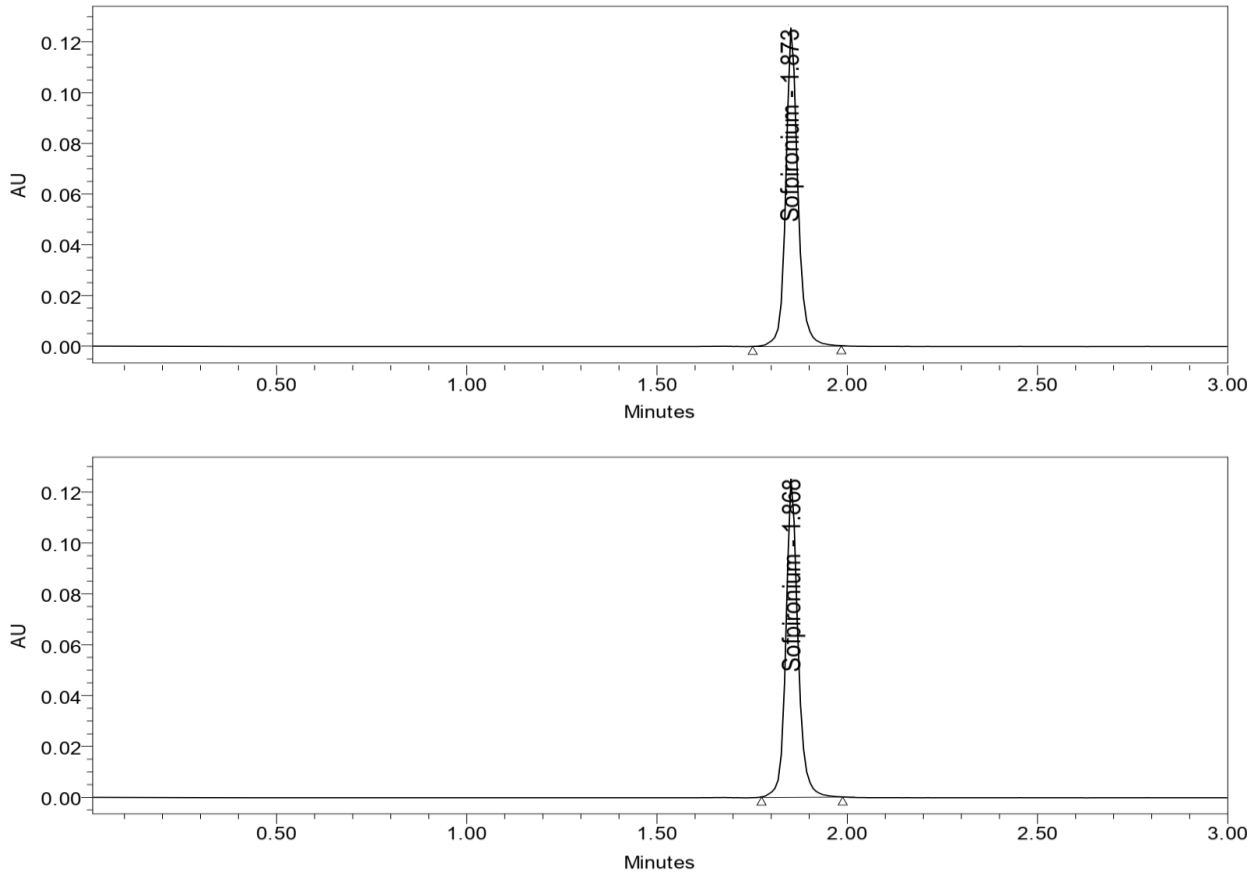


Fig 6.16 Linearity 100% Chromatogram

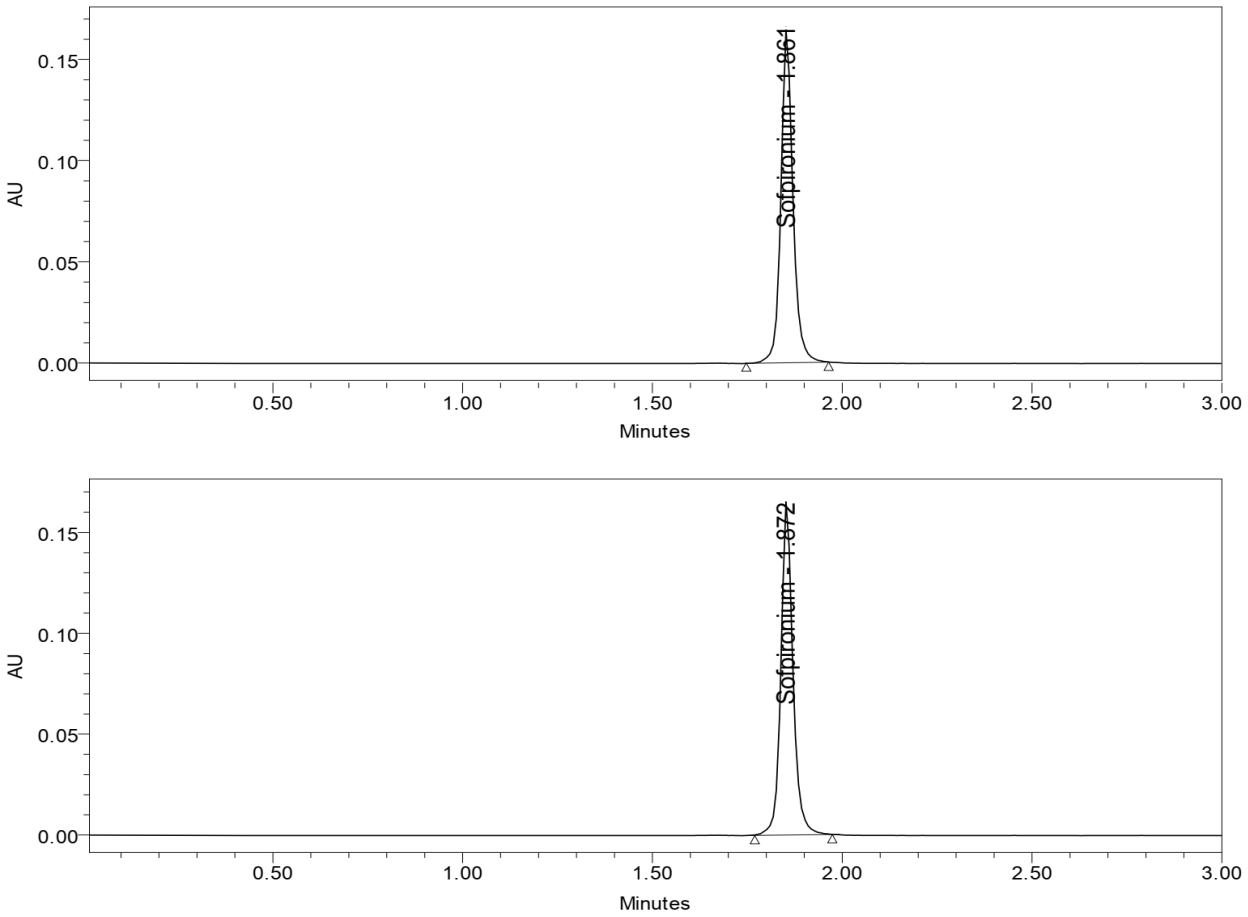


Fig 6.17 Linearity 125% Chromatogram

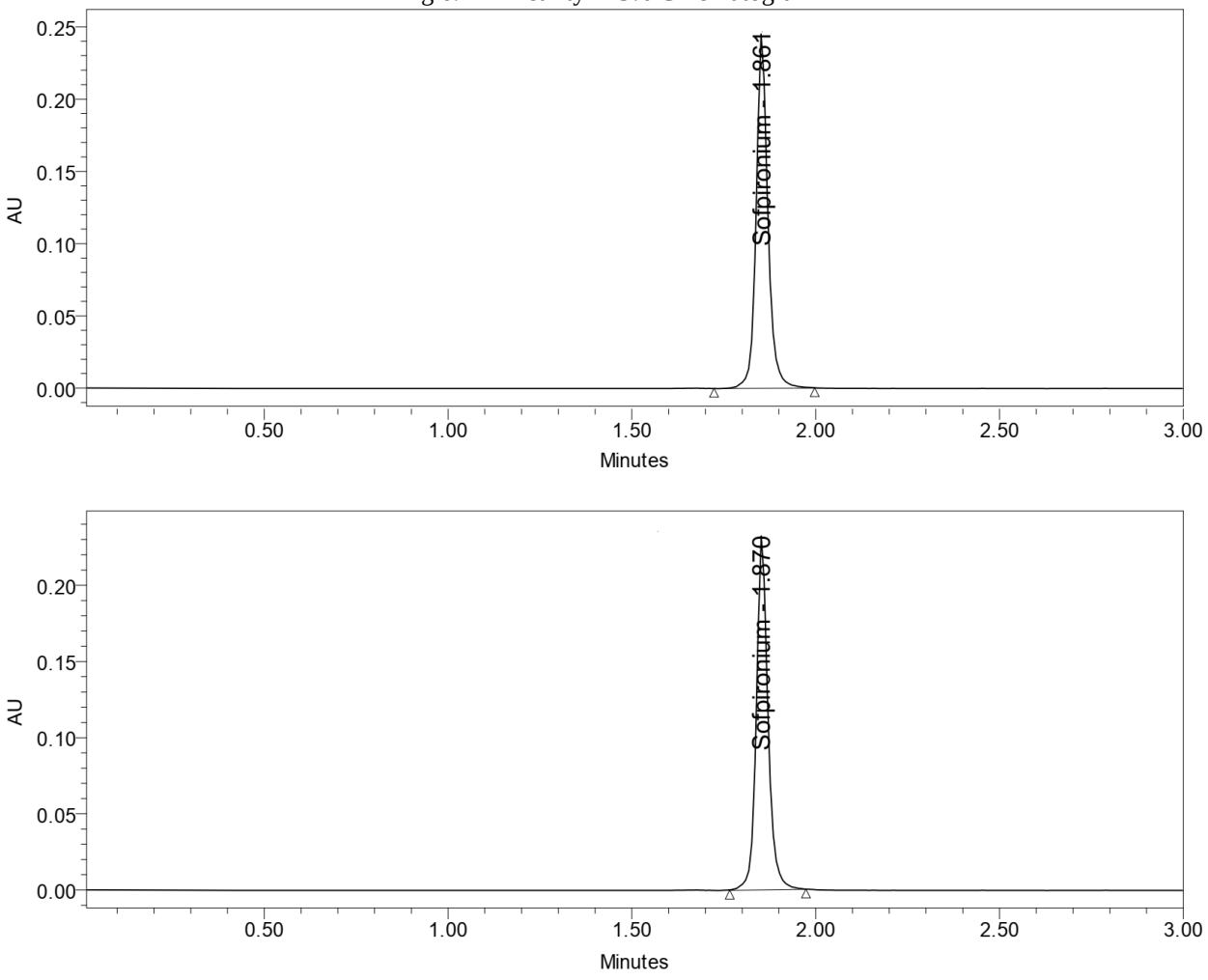


Fig 6.18 Linearity 150% Chromatogram

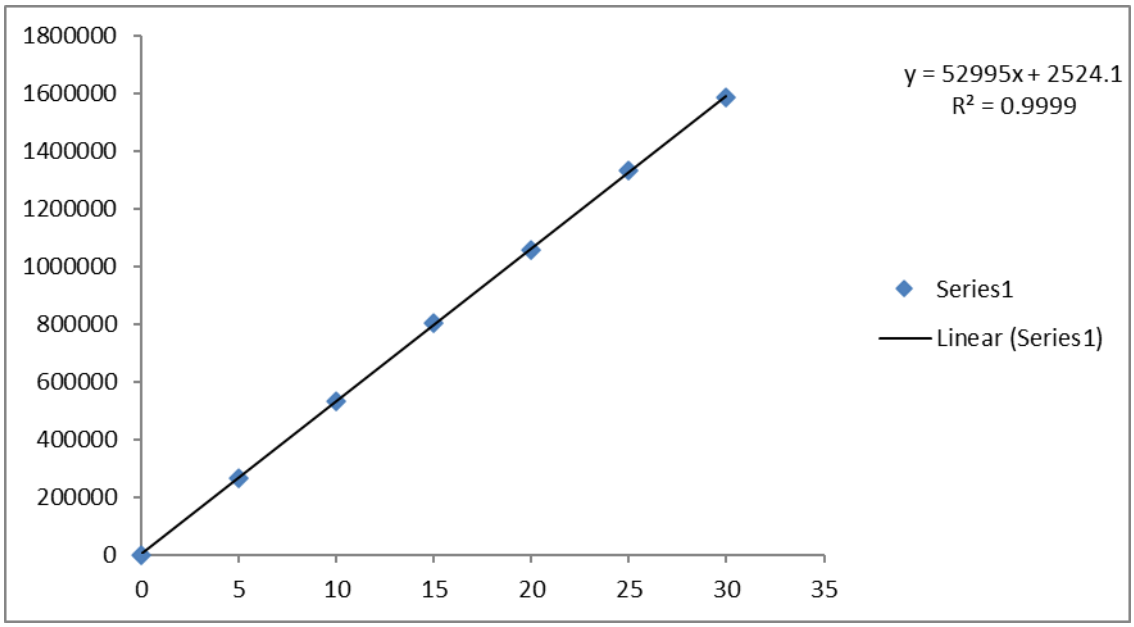


Fig 6.19 Linearity Plot

6.4 Accuracy: Three Concentrations of 50%, 100%, 150% are Injected in a triplicate manner and %Recovery was calculated as 99.94%. And chromatograms were shown in fig 6.11-6.13.

Table 6.5 Accuracy data

% Level	Amount Spiked (µg/mL)	Amount recovered(µg/mL)	% Recovery	Mean %Recovery
50%	10	9.95	99.47	100.02%
	10	9.95	99.45	
	10	9.98	99.76	
100%	20	20.23	101.13	
	20	19.87	99.33	
	20	19.97	99.83	
150%	30	30.04	100.14	
	30	29.99	99.96	
	30	30.11	100.38	

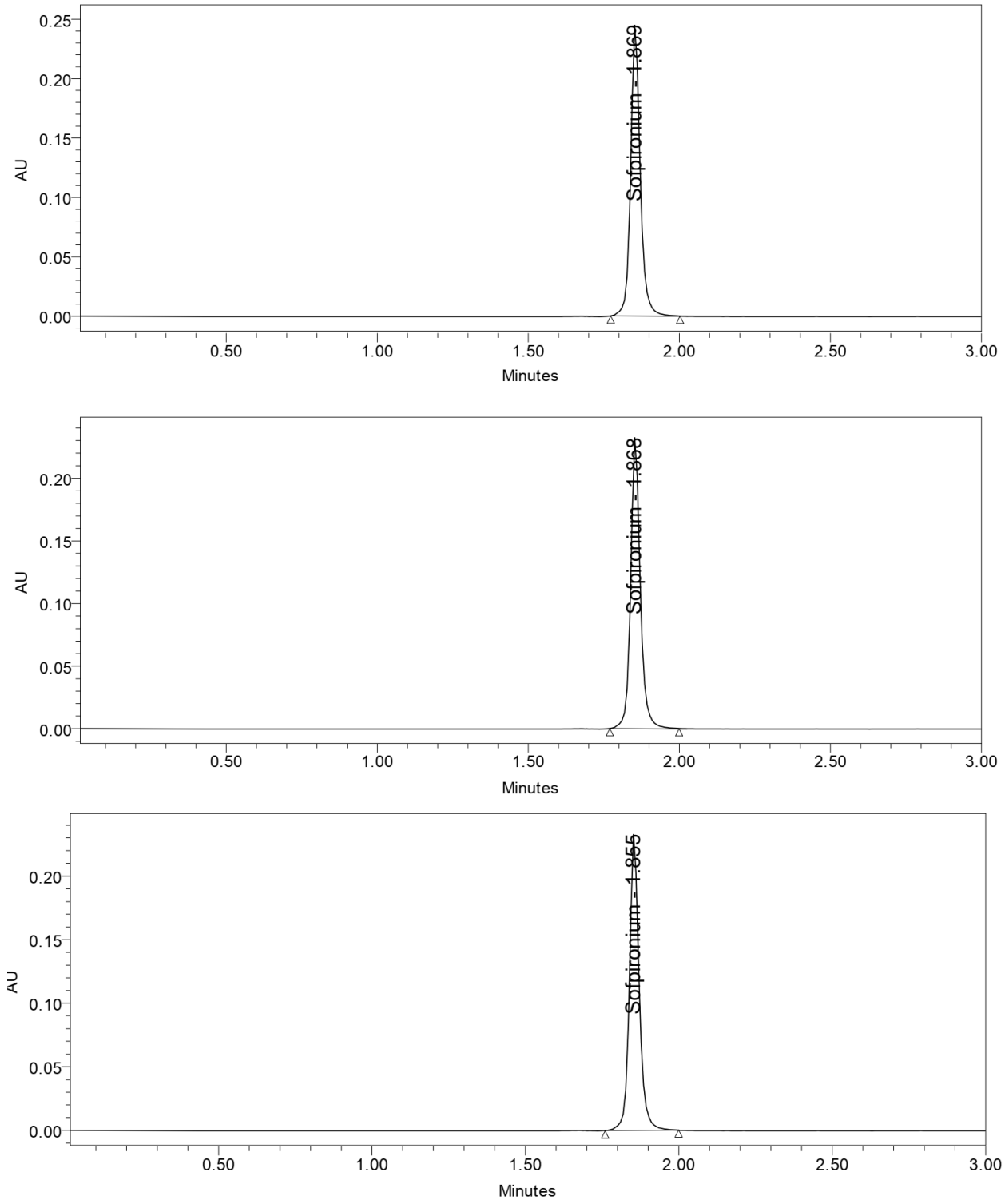


Fig 6.20 Accuracy 50% Chromatogram

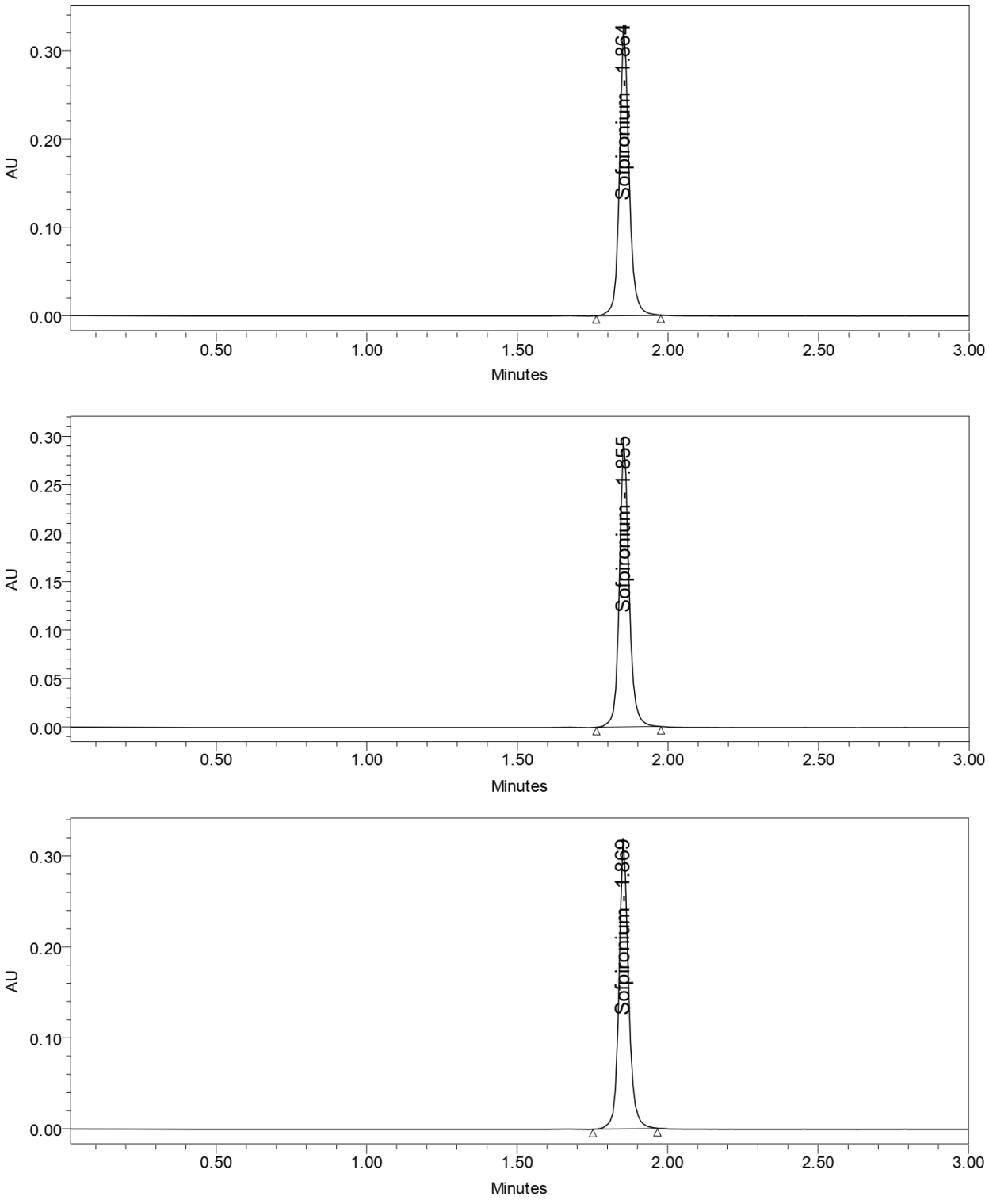


Fig 6.21 Accuracy 100% Chromatogram

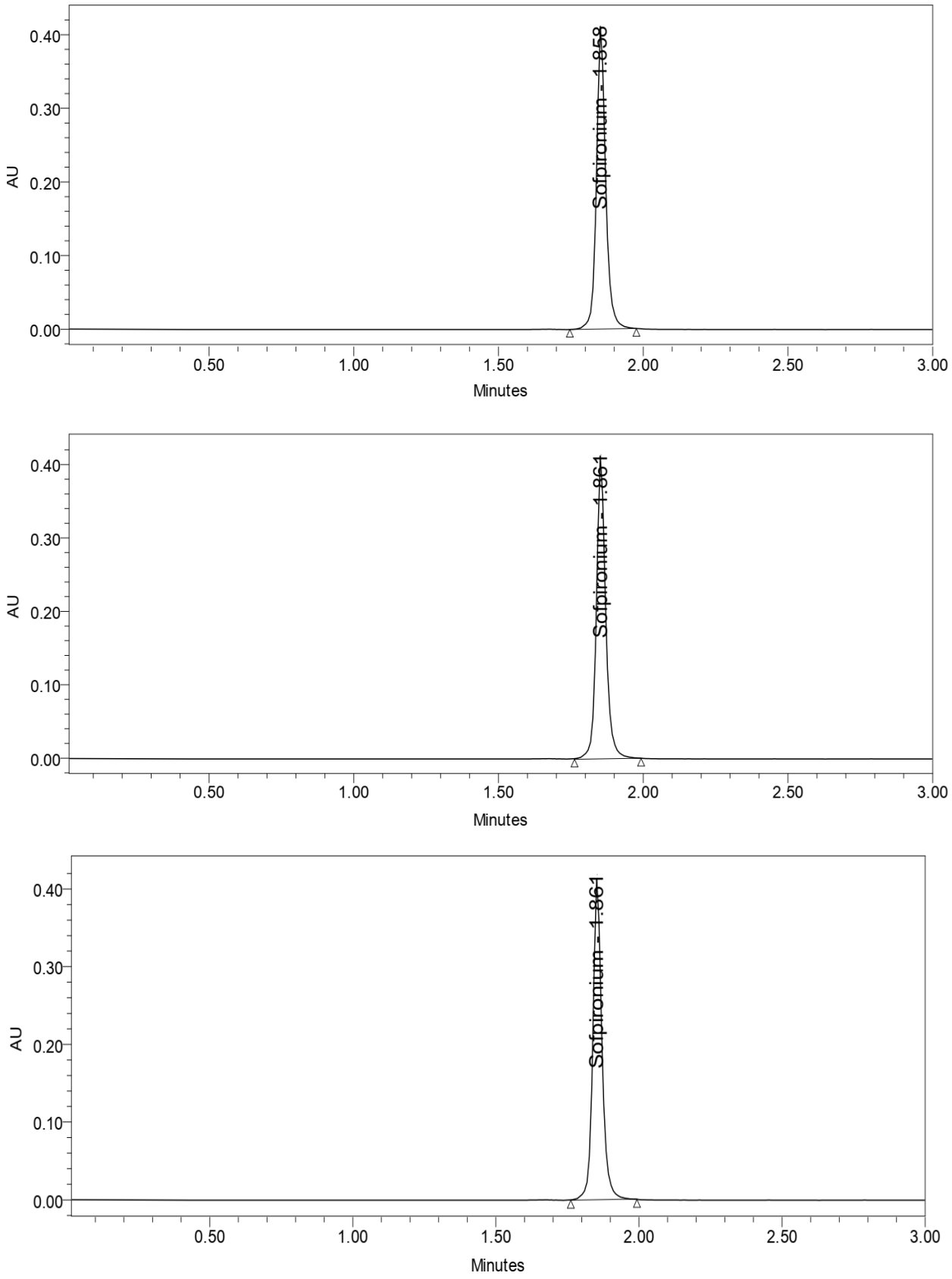


Fig 6.22 Accuracy 150% Chromatogram

6.5 LOD: Detection limit of the Sofpironium in this method was found to be 0.05µg/ml.

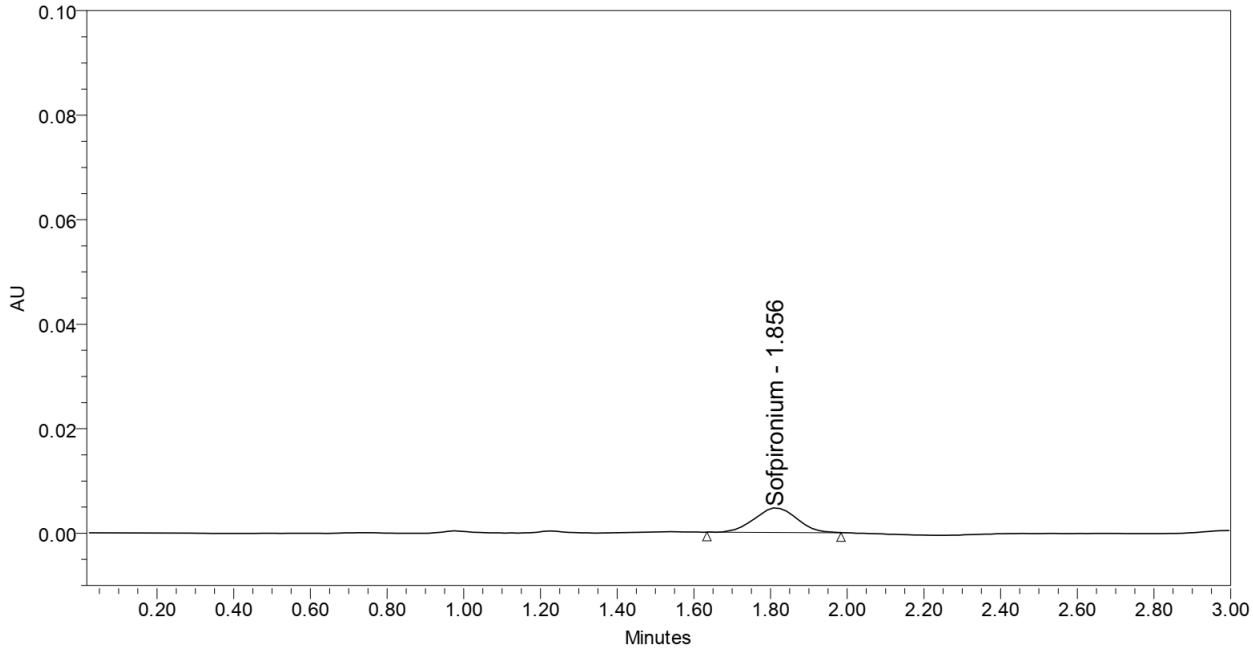


Fig 6.23 LOD Chromatogram of Sofpironium

6.6 LOQ: Quantification limit of the Sofpironium in this method was found to be 0.15µg/ml.

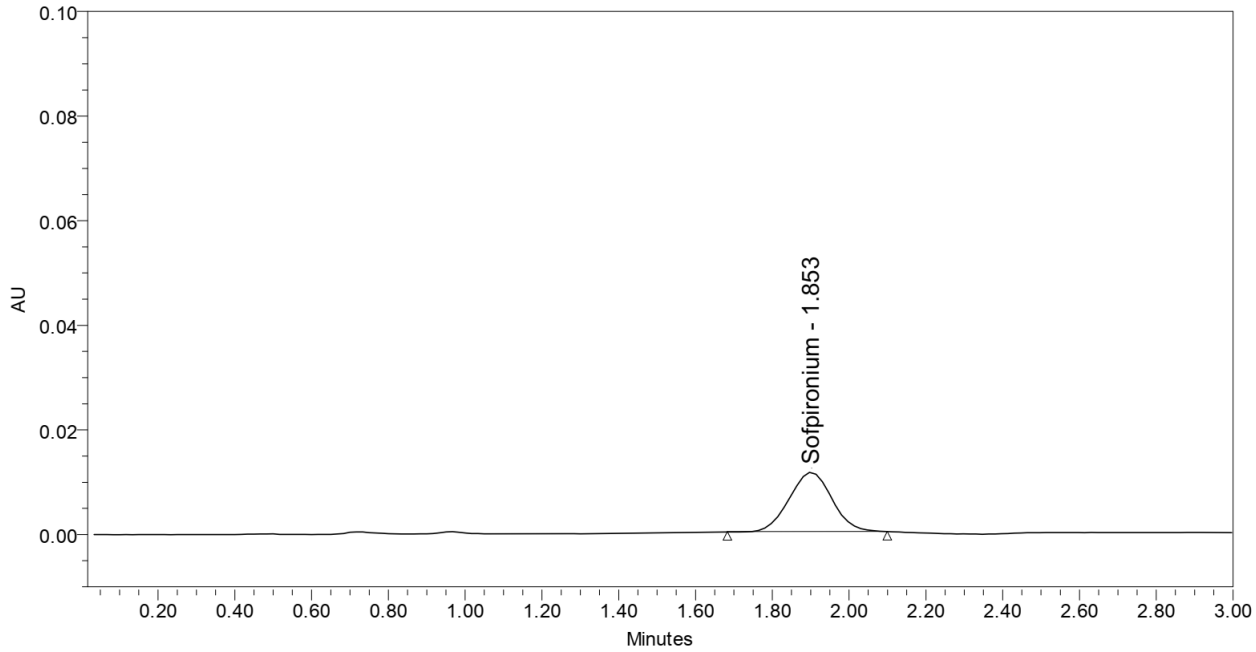


Fig 6.24 LOQ Chromatogram of Sofpironium

6.7 Robustness: Small Deliberate change in the method is made like Flow minus, flow plus, Mobile phase minus, Mobile phase plus, Temperature minus, Temperature Plus. %RSD of the above conditions is calculated.x

Table 6.6 Robustness Data

Parameter	%RSD
Flow Minus	0.5
Flow Plus	0.7
Mobile phase Minus	0.2
Mobile phase Plus	0.3
Temperature minus	0.6
Temperature plus	0.6

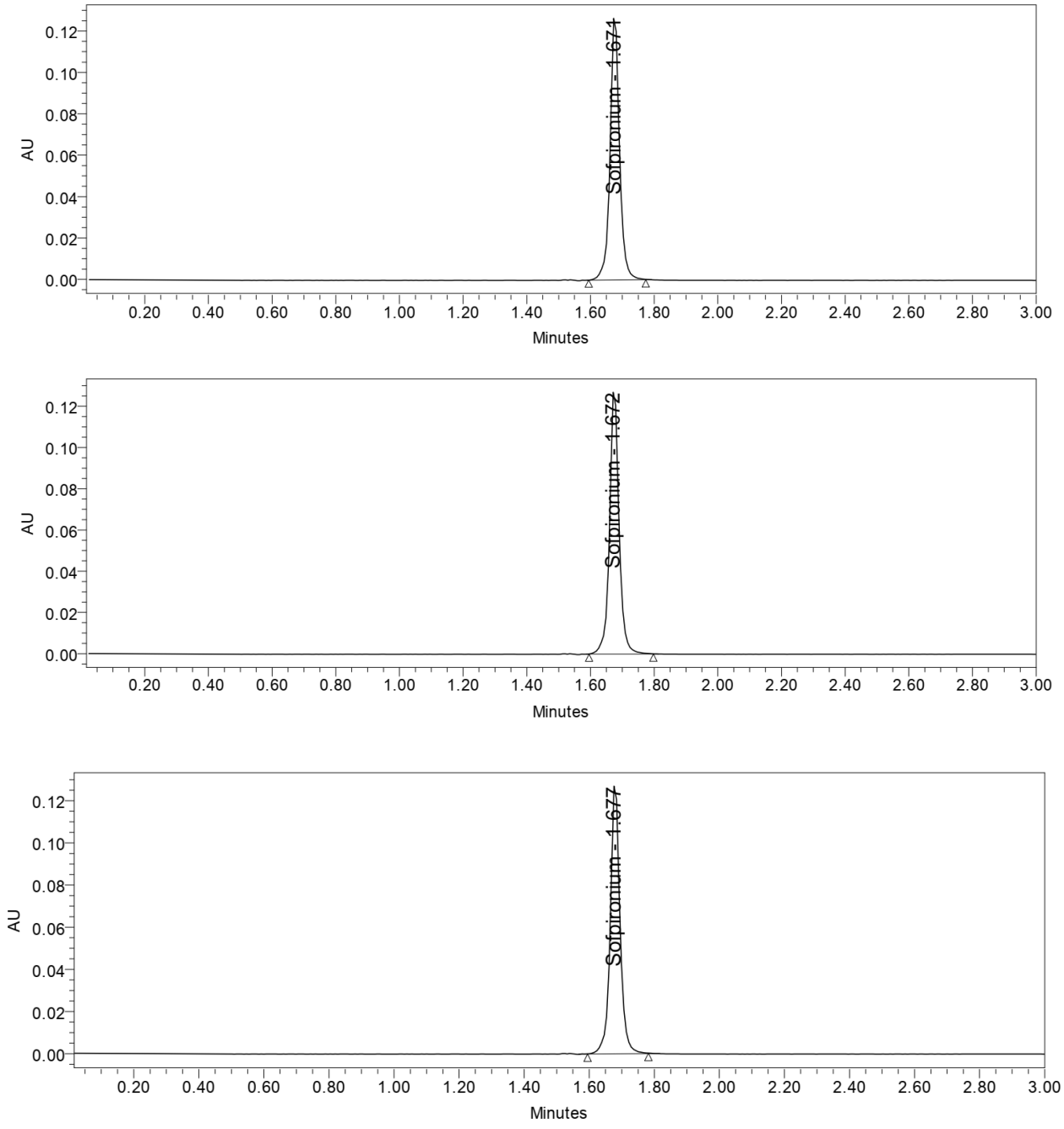


Fig 6.25 Flow minus Chromatogram of Sofpironium

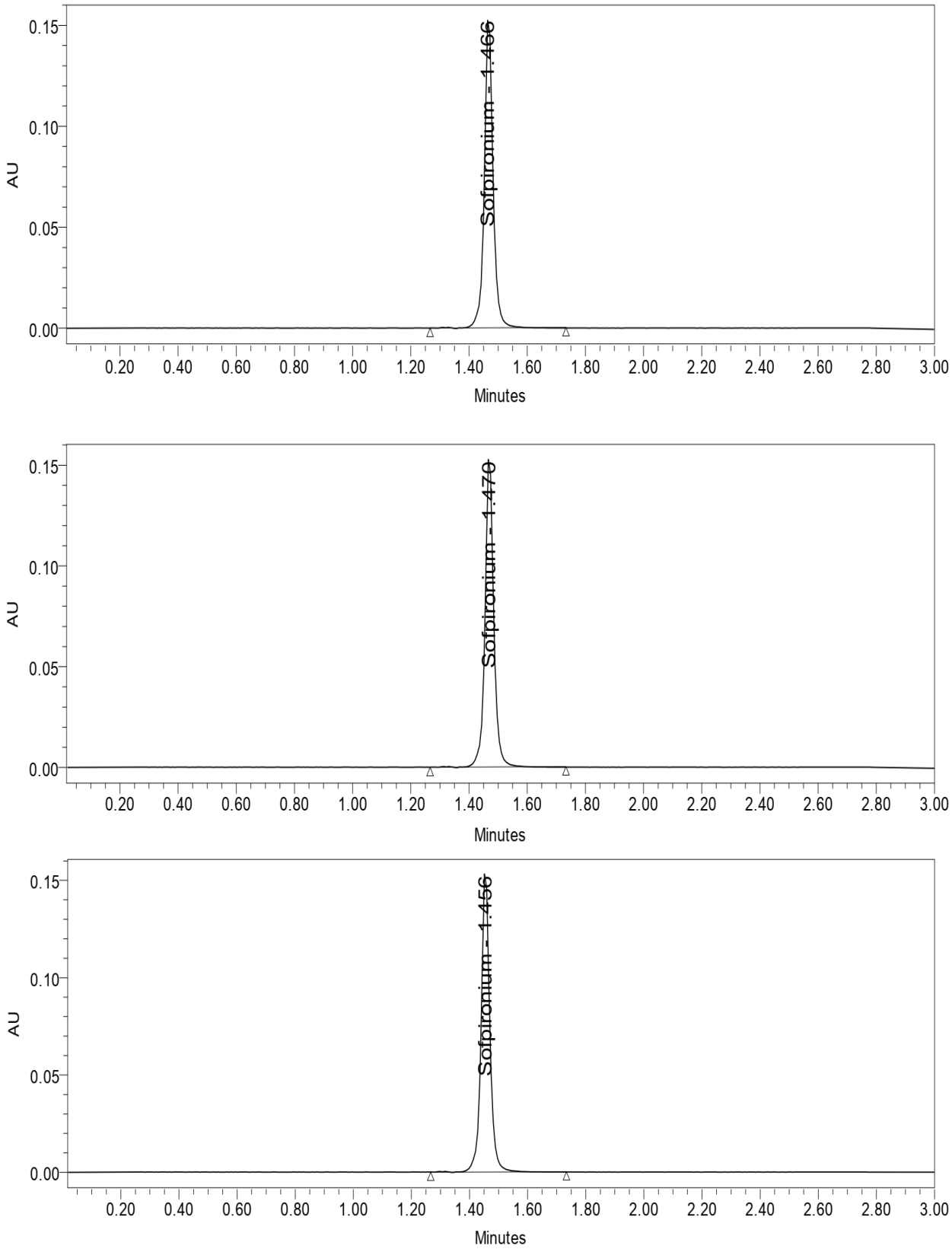


Fig 6.26 Flow plus Chromatogram of Sofpironium

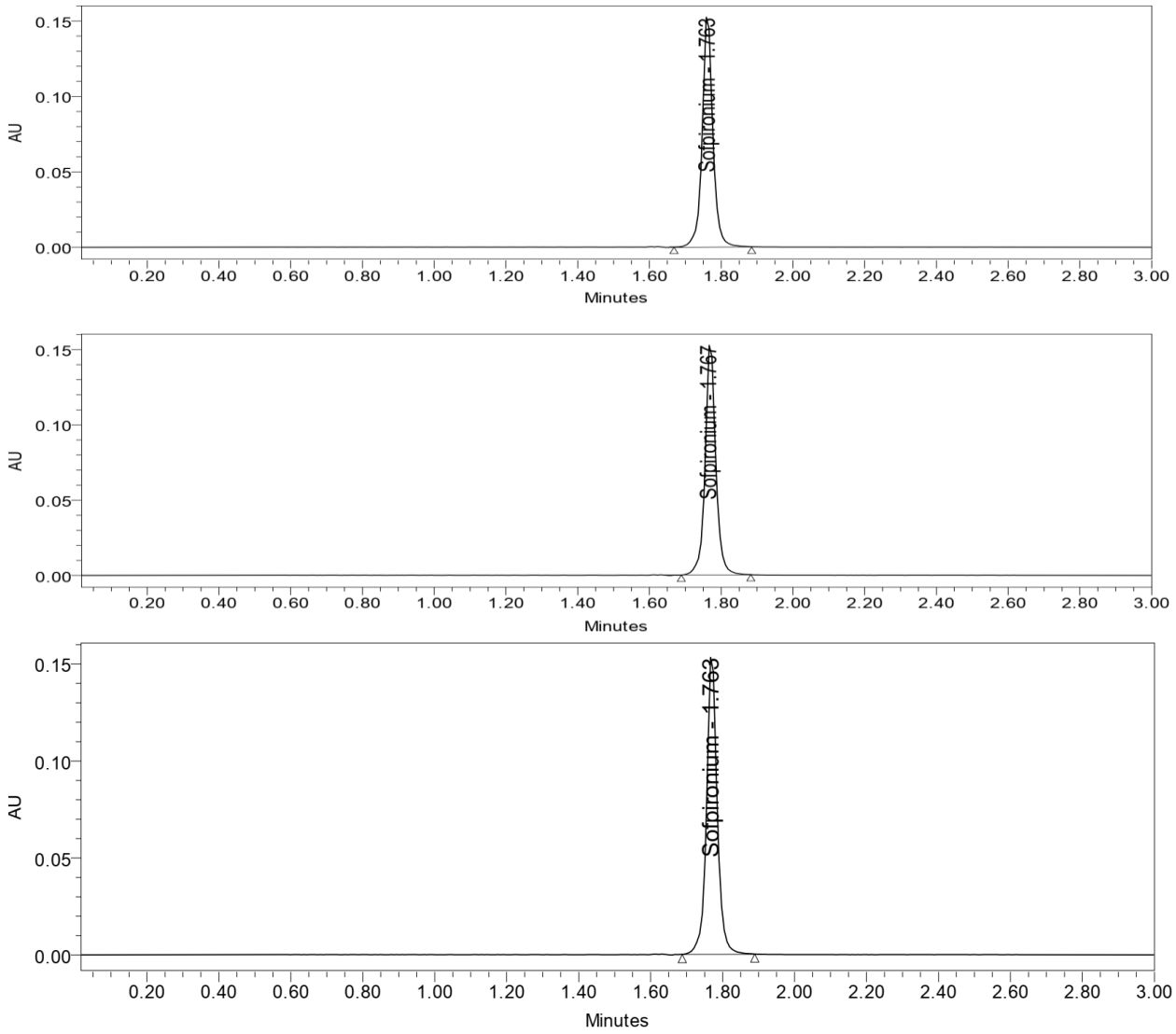
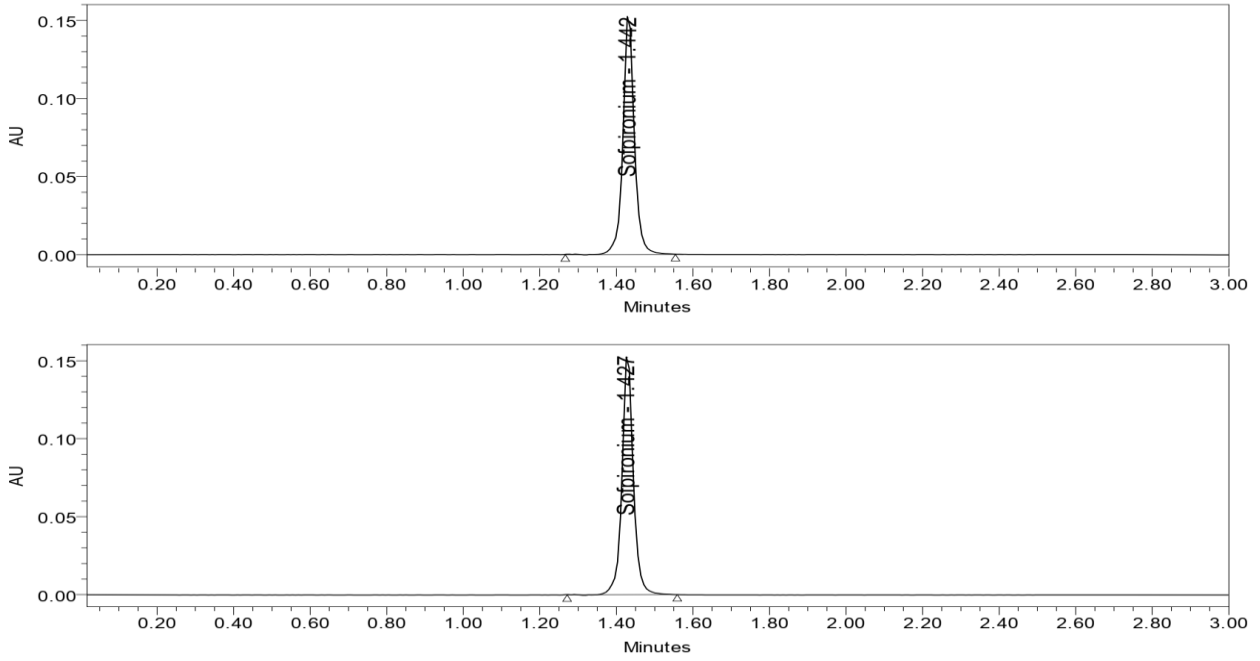


Fig 6.27 Mobile phase minus Chromatogram of Sofpironium



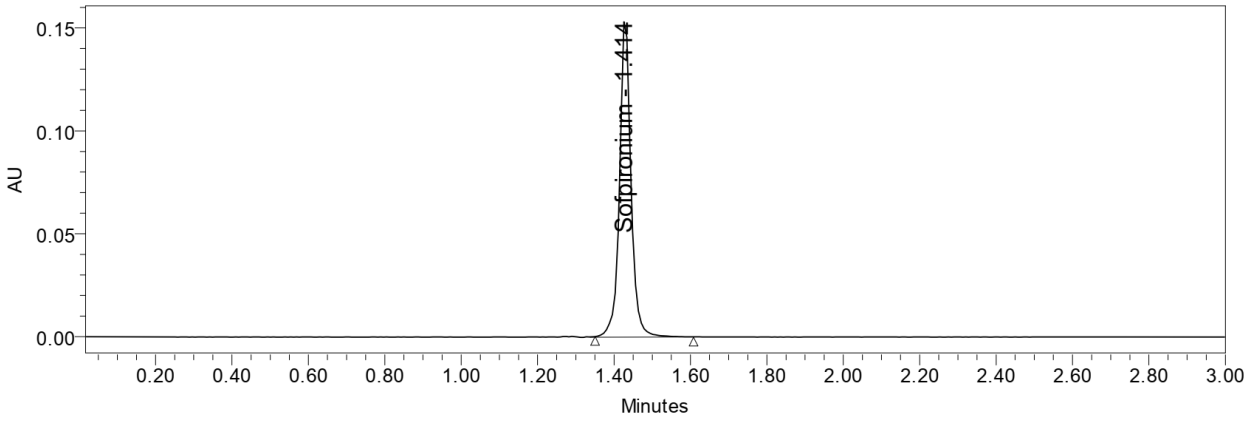
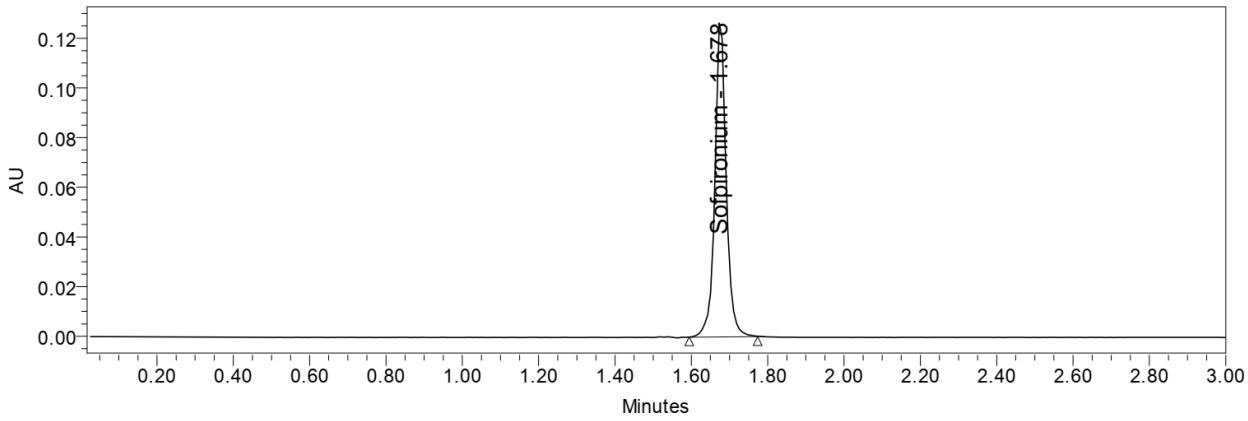
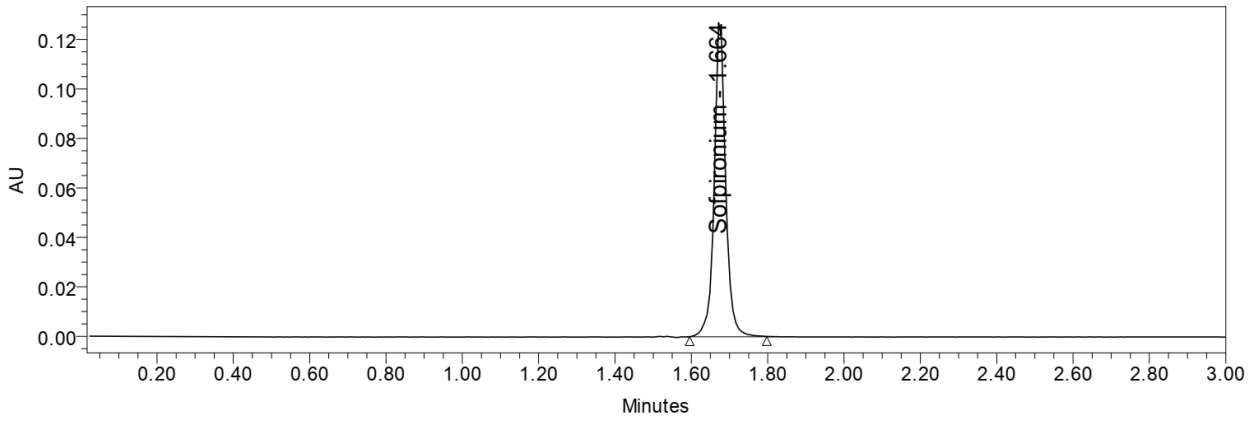


Fig 6.28 Mobile phase plus Chromatogram of Sofpironium





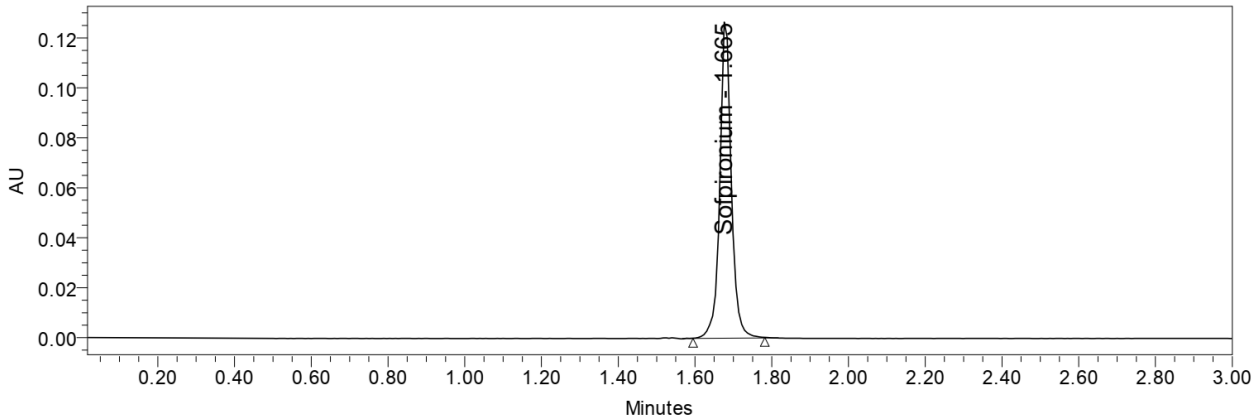


Fig 6.29 Temperature minus Chromatogram of Sofpironium

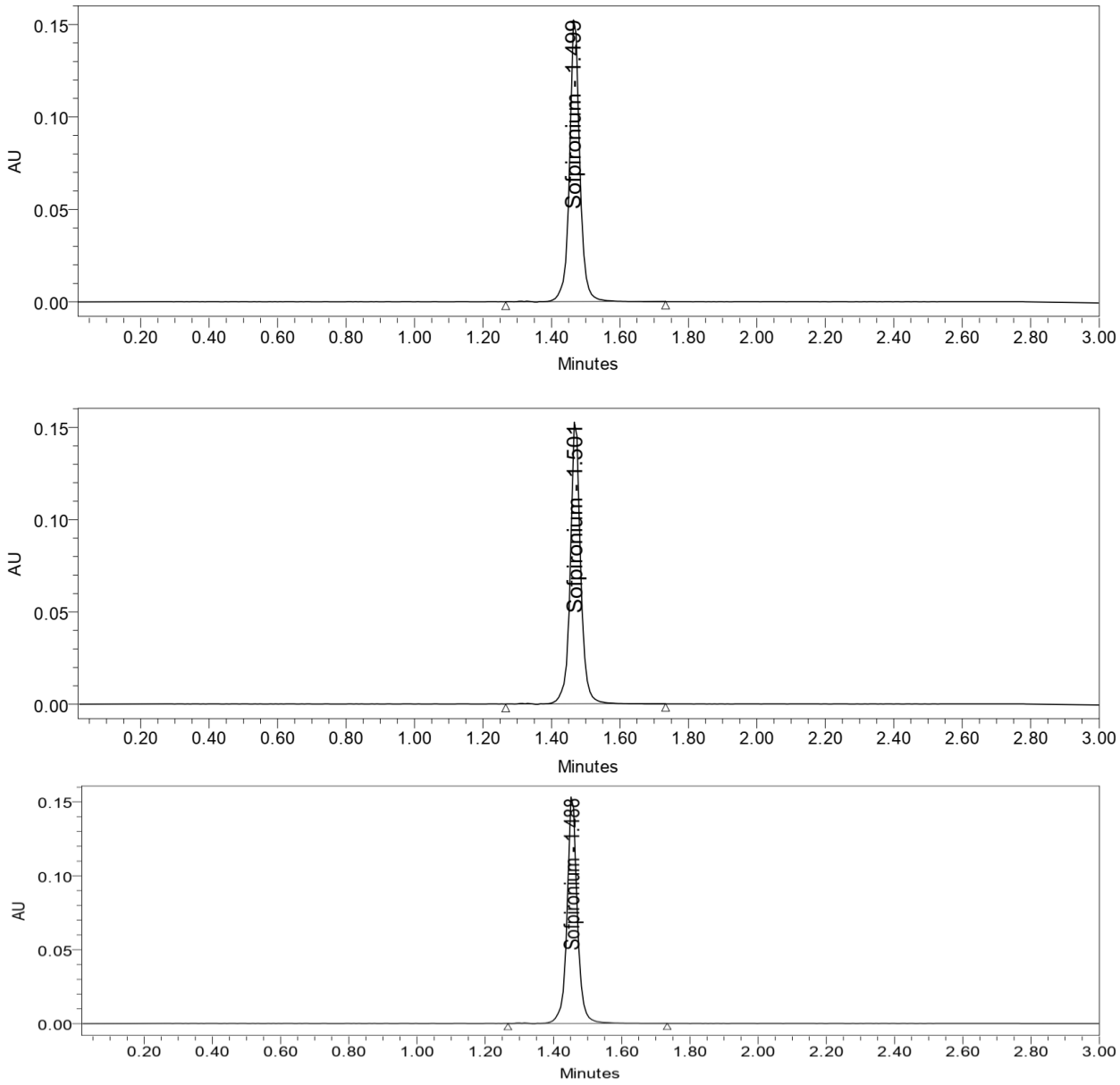


Fig 6.30 Temperature plus Chromatogram of Sofpironium

6.8 ASSAY OF MARKETED FORMULATION

Standard solution and sample solution were injected separately into the system and chromatograms were recorded and drug present in sample was calculated using before mentioned formula.

Table 6.7 Assay of Formulation

Sample No	%Assay
1	100.35
2	98.87
3.	99.50
4.	98.80
5.	99.77
6.	97.53
AVG	99.14
STDEV	0.98
%RSD	0.98

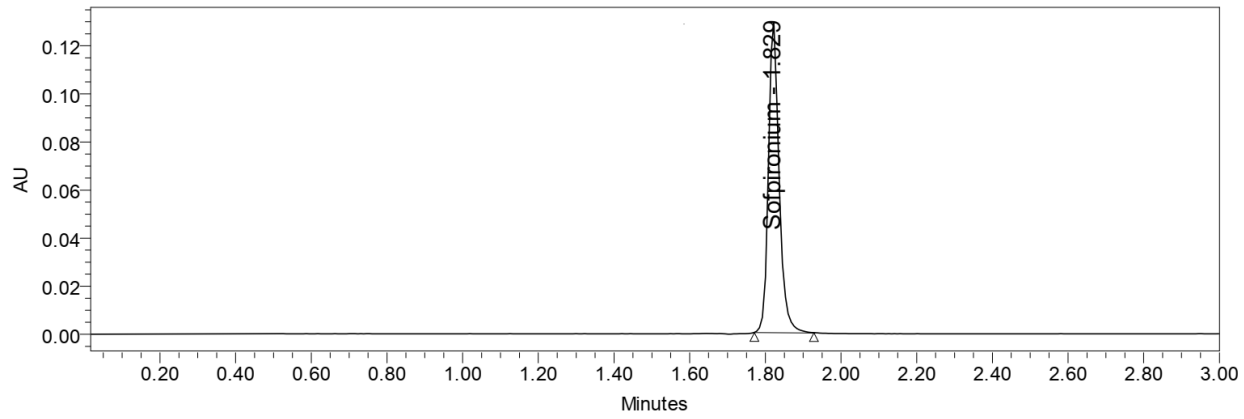


Fig 6.31 Standard chromatogram

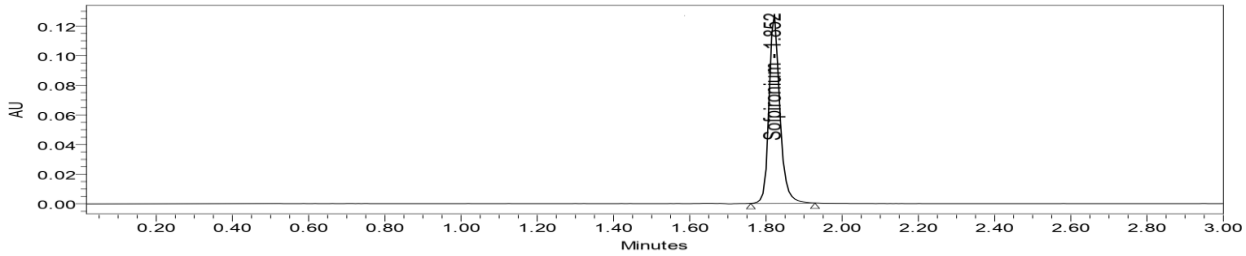


Fig 6.32 Sample chromatogram

Summary and Conclusion

Summary

Parameters		Sofpironium	LIMIT
Linearity Range (µg/ml)		5-30µg/ml	R< 1
Regression coefficient		0.999	
Slope(m)		52995	
Intercept(c)		2524.1	
Regression equation (Y=mx+c)		y = 52995x + 2524.1	
Assay (% mean assay)		99.94%	90-110%
Specificity		Specific	No interference of any peak
System precision %RSD		0.4	NMT 2.0%
Method precision %RSD		1.0	NMT 2.0%
Accuracy % recovery		99.94%	98-102%
LOD		0.05/ml	NMT 3 µg/ml
LOQ		0.15µg/ml	NMT 10µg/ml
Robustness	FM	0.5	%RSD NMT 2.0
	FP	0.7	
	MM	0.2	
	MP	0.3	
	TM	0.6	
	TP	0.6	

7. Conclusion

The present study was aimed at developing a sensitive, precise, and accurate stability indicating UPLC method for the analysis of Sofpironium in bulk drug and pharmaceutical dosage forms by using QbD approach using Design Expert® software. The central composite design experimental design describes the interrelationships of mobile phase and pH at three different level and responses to be observed were retention time, theoretical plates, and peak asymmetry with the help of the Design Expert 11.0 version. Here, a better understanding of the factors that influence chromatographic separation with greater confidence in the ability of the developed UPLC method to meet their intended purposes is done. The QbD approach to analytical method development was used for better understanding of method variables with different levels. it is a faster way of developing method, which helps choosing much better method condition during the development process through design space. The validation results confirmed the usefulness of the method. The method was found to be precise, accurate and linear, whereas the stress degradation studies identified the potential degradation products which can form during the shelf life of the product. In order to affect proper elution of the component peaks, mixtures of Acetonitrile with Phosphate Buffer in different combinations were tested as mobile phase on a non-polar ACQUITY UPLC BEH C18 Column, 1.7 µm, 2.1 mm X 50 mm. A mixture of AmmoniumFormate (73.6) (pH 3.6) and Methanol (26.3 v/v) was proved to be the most suitable of the combination since the chromatographic peaks were better defined and resolved and almost free from tailing. The retention time for Sofpironium was at 0. ± 10% min respectively injected at a flow rate of 0.28mL/min. The detection wave length was fixed at 219.0 nm as the peak areas were consistent and reproducible over a run time of 3.00 minutes. The injection volume was optimized at 0.2 µL. The method obeyed linearity in the range of 5-30 µg/mL for Sofpironium respectively as observed from the linearity curves. The regression equations for Sofpironium were found to be y = 52995x + 2524.1 (R2 = 0.999) respectively. The specificity of the method was established by studying the Sofpironium peaks in the presence of excipients. The commonly present excipients did not pose any interference at the retention time of the drug as they were not identified. The repeatability and intermediate precision were studied by analyzing the sample solutions of Sofpironium. The low coefficients of variation obtained in the intraday (1.0 %) and inter day precision (1.2 %) study are indicative of the precision of the method. High recovery values for Sofpironium (99.94%) obtained from the analysis of dosage forms by the proposed method indicates the accuracy of the method. The deliberate changes in the method (flow rate and temperature) have not much affected the peak tailing, theoretical plates, resolution, and percent assay. This indicates that the present method is robust (% RSD < 2). The limit of detection and limit of quantitation for Sofpironium were found to be 0.05 µg/mL and 0.15 µg/mL respectively. The low values of LOD and LOQ as obtained by the proposed method indicate the sensitivity of the method. The developed UPLC method was applied for the analysis of Sofpironium in marketed formulations. The marketed dosage form was found to contain an average of 99.94 ± 10 % w/v of Sofpironium as stated on the label claim. The absence of additional peaks indicates non-interference of common excipients used in the Sample preparations. The proposed UPLC method was also applied for the forced degradation studies on Sofpironium under a variety of conditions like acid and base hydrolysis, oxidation, and heat and photo stability. The drugs were found to be stable except in acidic, Basic, Peroxide stress conditions. The drug peaks in these degradations were found to be homogenous and no other peaks merged. No major degradants were found in Neutral stress, photo stability and Thermal degradation studies. As the developed method could effectively separate Sofpironium from the degradants, it can be employed as a stability indicating assay. System suitability testing was performed prior to the study of each validation parameter and the verified parameters like tailing factor (< 2.0), resolution (> 8.2), column efficiency (> 2000) and repeatability (% RSD < 2) ensured that the equipment, electronics, and analytical operations for the samples analysed could be constituted as an integral system that can be evaluated as a whole.

The developed UPLC method is specific, sensitive, precise, accurate and stability indicating. The method is linear over a wide range, economical and utilizes a mobile phase which can be easily prepared. All these factors make this method suitable for quantification of Sofpironium in bulk drugs and pharmaceutical dosage forms without any interference from degradants or excipients. This method can also be used for the regular quality control analysis of Sofpironium, Results which were obtained from the validation of developed analytical method were within limit as per ICH guidelines.

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