**RESEARCH ARTICLE**

# A Stability Demonstrating HPLC Method Development and Validation for Related Substances in Oxtriphylline using Quality by Design

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**ABSTRACT**

A new reverse phase HPLC procedure, stability demonstrating for estimating related substances in oxtriphylline, was developed and validated according to ICH guidelines by applying Analytical Quality by Design (AQbD) principles. The three critical method parameters identified were mobile phase flow rate, column temperature, and mobile phase pH. Based on these factors, the resolution between oxtriphylline and theophylline related compound F and the tailing of oxtriphylline peak were selected as critical method attributes. XBridge BEH C18 with measurements 150 x 4.6 mm, 5 µ was used to achieve peak separation. The mobile phase flow rate was at 1.0 mL/min and 40°C was the column temperature. The injection volume was 10 µL, the autosampler temperature was 25°C, and UV detection was done at 270 nm. The elution was a gradient. Analytical method validation parameters like precision, accuracy, specificity, linearity, and solution stability of solution were proved. The linearity of the method was established from 0.05 to 1% of sample concentration. The correlation coefficient was more than 0.999. The percentage recoveries for all impurities were from 100.71 to 102.45%, and the relative standard deviation (RSD) was as less than 1% proving the procedure is precise. The recoveries of all impurities were proven from LoQ to 150% level of sample concentration. The recoveries ranged from 98.40 to 101.70% at LoQ, 101.56 to 101.88 at 100% level, and 101.28 to 102.54 at 150% level of spiking. Robustness studies proved that small and deliberate changes to flow (± 0.2 mL/min), column temperature (± 5°), and mobile phase pH variation (± 0.2 units) does not impact the method. Also, the stability-indicating feature of the method was demonstrated by performing forced degradation studies. The method developed by AQbD approach was accurate, precise, linear specific, robust, and stability-demonstrating to quantify oxtriphylline and its related substances.

**Keywords:** Design space, Forced degradation studies, HPLC, Method development, Method validation, Oxtriphylline, Quality by Design, Related substances

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## INTRODUCTION

Oxtriphylline is a choline salt of theophylline or choline theophylline[1] (Figure 1). It has anti-asthmatic nature. As soon as oxtriphylline enters the body, it converts to theophylline.[2] It shows its pharmacological action by inhibiting the production of phosphodiesterase and prostaglandin. It also regulates calcium distribution and its flux and antagonizes adenosine. This, in turn, causes the relaxation of smooth muscles in the bronchial area and causes vasodilation.[1] Since oxtriphylline is a theophylline choline salt, there is a possibility that theophylline-reported impurities like theophylline-related substances B, C, D, F, and caffeine will be present in the drug as an impurity.[3] Also, caffeine is similar in structure to theophylline.[4]

An extensive literature survey was done to check for any reported methods for oxtriphylline and its related impurities. The united states pharmacopeia does not have any reported method for related impurities.[5] There was only a single reported method for estimating oxtriphylline in combination with salbutamol and bromhexine hydrochloride in pharmaceutical combination dosage form by RP-HPLC.[6] Several high-pressure liquid chromatography methods are reported to estimate theophylline in biological fluids and food drinks.[7-17]

In pharmaceutical analysis, quantifying a drug substance and its related substances is often the most challenging task during analytical method development. This can be attributed to structural and physicochemical properties similarities between the API and their related substances.

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Traditional development of the analytical method approach is time-consuming as it follows the approach of one-factor-at-a-time (OFAT). Traditionally, at any given instance, only one variable is altered sequentially until a desired result is obtained, which is time-consuming.[18]

Quality-by-Design (QbD) assisted systematic methodology is essential to overcome this challenge. When the principles of QbD are applied to analytical methods, it can be referred a Analytical-Quality-by-Design (AQbD). AQbD is a logical approach to developing new ideas that begin with specified objectives and emphasizes product and process awareness based on sound research and high-quality risk management. It is advised by ICH guideline Q8 (R2) and is frequently demanded in pharmaceutical product development.[19]

In this research work, an AQbD methodology was used to design and evaluate an HPLC method for related compounds in oxtriphylline that could demonstrate stability. The current work was challenging as related substances have similar molecular structures and co-elute closely.

## MATERIALS

### Material and Reagents

Oxtriphylline and theophylline-related substances B, C, D, F, and caffeine were obtained as gift samples from SS Pharma, Guntur, Andhra Pradesh (state), India. Glacial acidic acid was acquired from spectrochem. Methanol (HPLC grade) was bought from Rankem Laboratories Pvt Ltd. Concentrated hydrochloric acid, sodium hydroxide pellets and hydrogen peroxide were purchased from Merck.

### Instruments and Equipment

Analyte separation was achieved on the HPLC waters-acquity model system fitted out with an autosampler and PDA detector. Empower 3 software was used as a processing and reporting software. A hot-air laboratory-grade oven, was used for thermal degradation. UV crosslinker with a UV lamp and UV fluorescence lamp was used for photolytic degradation. Digital pH meter (Sartorius - PP-15), ultrasonic bath (Toshcon by Toshniwal) was used in this study.

ACD labs software– Physchem Suite 2020.1.1 was used to better understand the physicochemical properties of all the analytes while Design Expert 8.0.7.1 was used for designing space for the experiment.

### Chromatographic Conditions of HPLC

XBridge BEH C18 with measurements 150 x 4.6 mm, 5 µ was used to achieve peak separation. The temperature of the column was fixed to 40°C, and the flow rate was kept constant at 1-mL/min. The injection volume was 10 µL, the autosampler temperature was 25°C, and UV detection was done at 270 nm. The elution was a gradient, and the program was as follows (Table 1).

**Table 1:** HPLC gradient elution program

| *Time (mins)* | *Mobile phase A* | *Mobile phase B* |
|---|---|---|
| 0 | 95 | 5 |
| 30 | 50 | 50 |
| 35 | 50 | 50 |
| 35.1 | 95 | 5 |
| 40 | 95 | 5 |

### Preparation of Mobile Phase, and Diluent

Peaks were eluted using mobile phase-A (10 mM ammonium acetate solution whose pH was modified to 5.5 by glacial acetic acid). The diluent was HPLC water and the mobile phase-B was HPLC methanol.

### Preparing Standards and Stock Solutions for Impurities

Impurity stock solutions of 0.5 mg/mL each of oxtriphylline standard, theophylline related compound RC-B, theophylline related compound RC-C, theophylline related compound RC-D, theophylline related compound F, and caffeine were prepared by taking the weight of 5 mg of respective material in a 10 mL flask and diluting to volume with diluent. Next, the diluent was used to dilute 2 mL of each stock to 20 mL.

### Preparation of Working Standard Solutions

Working standard stock was properly diluted to a concentration of 0.001 mg/mL or 0.1% in 50 mL of diluent to create the standard working solution.

### Sample Solution

Oxtriphylline was precisely weighed out to be roughly 25 mg and then deposited into a 25 mL volumetric flask. A 10 mL of diluent was then added, sonicated to dissolve, then made up to the correct amount with a diluent before being combined. (Concentration 1-mg/mL of oxtriphylline).

### Analytical Validation of Method

Several metrics, including specificity, linearity, method precision, accuracy, detection limit determination, and quantitation limit determination, were used to verify the newly devised technique.

### System Suitability Solution

The suitability of the chromatographic system was tested by infusing 1.0 g/mL of theophylline-related chemical F and 1.0 mg/mL of oxtriphylline into the HPLC system. Before starting the sequencing, the resolution between theophylline-related compound F and oxtriphylline was tested.

### Specificity

The method's specificity was validated by injecting a solution containing 0.0005 mg/mL of oxtriphylline, theophylline RC B, theophylline RC C, theophylline RC D, theophylline RC F, and caffeine solutions. All contaminants were added to the sample solution, and interferences with recognized peaks were checked. The method's specificity was further tested by looking for interferences in force-damaged samples.

### Linearity

Oxtriphylline and its RCs solutions were prepared from 0.05 to 1.0% of the sample concentration. Five different concentrations ranging from 0.0005, 0.001, 0.002, 0.005, and 0.01 mg/mL

were prepared individually. A calibration curve was created for oxtriphylline and its RCs to show the peak regions against the relevant concentration. Each analyte should have a correlation coefficient (r) of not less than 0.99. Normalized intercept/slope was also reported.

### Method Precision

Six duplicate preparations with known concentrations (0.005 mg/mL of all impurities) were injected on the same day. The peak area of each of the six injections' standard deviations was determined as a percentage (%RSD).

### Accuracy

Accuracy was proven by the addition of standard technique at different spiking levels of 150, 100%, and limit of quantitation level. The sample was prepared in duplicate at each level, and a known quantity of each impurity was added. At each level, the mean recovery was computed and reported.

### Detection Limit and Quantitation Limit

The limit of detection for oxtriphylline and related compounds was estimated from the linearity calibration curve. The limit of quantitation level solution for oxtriphylline and related compounds was estimated by preparing a solution of a concentration of 0.00025 mg/mL.

### Robustness

The method was evaluated with minor, deliberate variations to flow (0.8 to 1.2 mL/min), column temperature (35 to 45°), and mobile phase pH 5.3 to 5.7. The method's robustness is checked by monitoring the resolution between oxtriphylline and theophylline-related compound F. Also, the tailing of the oxtriphylline peak was verified.

### Solution Stability

The solution stability parameter was performed at the limit of the quantitation (LoQ) level solutions. Standard and sample solutions spiked with related compounds at LoQ level were injected every 2 until 24 hours. The percentage area difference between the original injection and injection at various time intervals was calculated and reported.

### Forced-degradation Studies

Forced-degradation studies are performed to develop a stability-demonstrating method for drug substance analysis. The drug material was subjected to challenging environmental conditions during forced degradation trials- physical stress and chemical stress to get optimum degradation (5–15%). Physical stress conditions/solid-state analysis includes photolytic degradation (exposure to UV/visible light), thermal (105°C), and humidity (85°/85%RH). In contrast, chemical stress/solution state analysis includes acid (0.1 N HCl in water), base (0.1 N NaOH in water), and 3% aqueous hydrogen peroxide (Table 2).

## RESULT AND DISCUSSION

### Analytical Method Development

According to the AQbD strategy, which comprises establishing the analytical target profile (ATP) and selecting the crucial method features, the development of analytical methods was started (CMA). This was followed by the selection of critical method parameters (CMPs) based on risk assessment of quality. Subsequently, using simulations from the Design Expert program, experiments (DoE) were designed to create the MODR (method operable domain region).

Analytical target profiling establishes the goal and range of the technique. For the quantification, a robust approach was created for oxtriphylline-related compounds. RP-HPLC was selected as the separation and quantifying technique.

Critical method attributes were identified, selected, and measured to reach the analytical target profiling requirements. Using ACD labs software–Physchem Suite, a number of predictions in modules were made based on structure-based calculations like partition coefficient (logD), pH, pKa, %dominant ionic forms. Based on the comparison of logD vs pH (Figure 2) and %dominant ionic forms *vs* mobile phase pH (Figure 3), mobile phase, flow rate pH range, column temperature and suitable column type was predicted. Based on these factors, the resolution between oxtriphylline and theophylline RCF and tailing of oxtriphylline peak were selected as critical method attributes. These two critical method attributes ensure optimum resolution between the critical pair and optimum peak symmetry. The minimum peak resolution of atleast 1.5 between oxtriphylline and theophylline RCF and tailing of not more than 2.0 for oxtriphylline peak was finalized as the limits.

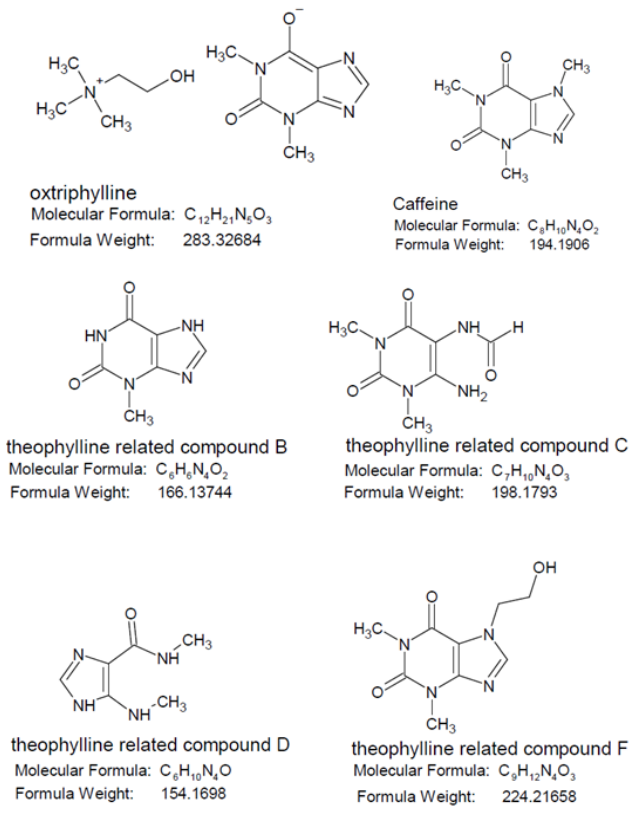



**Figure 1:** Structure of oxtriphylline, caffeine and theophylline related compound B,C,D,F.

**Table 2:** Forced degradation sample treatment procedure details

| *S. No.* | *Condition* | *Sample quantity (mg)* | *Volume of solution added* | *Neutralisation volume (mL)* | *Total volume (mL)* |
|---|---|---|---|---|---|
| 1 | As such | 20 | - | - | 100 |
| 2 | Acid degradation/0.1N HCl/3 hours at 60°C | 20 | 10 mL 0.1N aqueous HCl | 10 mL 0.1 N Aqueous NaOH | 100 |
| 3 | Base degradation/0.1N NaOH/3 hours at 60°C | 20 | 10 mL 0.1N Aqueous NaOH | 10 mL 0.1 N aqueous HCl | 100 |
| 4 | Oxidative or peroxide degradation/3%H2O2/3 hours at 60°C | 20 | 10 mL Aqueous 6% $H_2O_2$ | 100 | 100 |
| 5 | Photolytic degradation | 20 | - | 100 | 100 |
| 6 | Thermal degradation/105°/3 Days | 20 | - | 100 | 100 |
| 7 | Humidity degradation/85°/85%RH-3 Days | 20 | - | 100 | 100 |

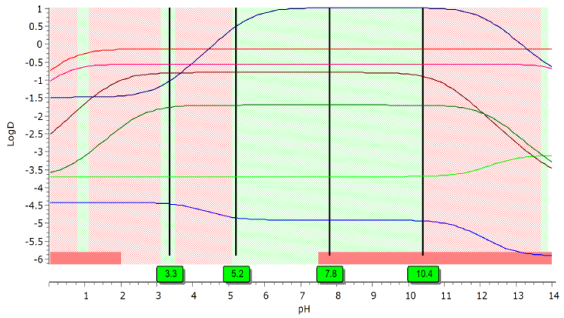



**Figure 2:** Design space prediction as a function of mobile phase pH vs partition coefficient (logD).

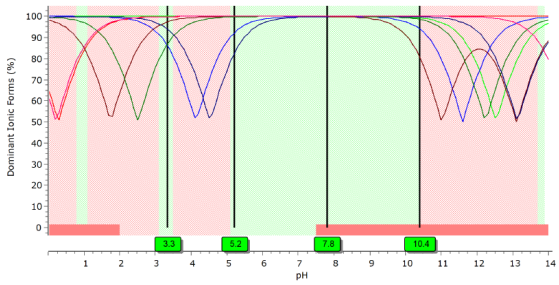



**Figure 3:** Design space prediction as a function of mobile phase pH vs dominant ionic forms (in%) of analytes.

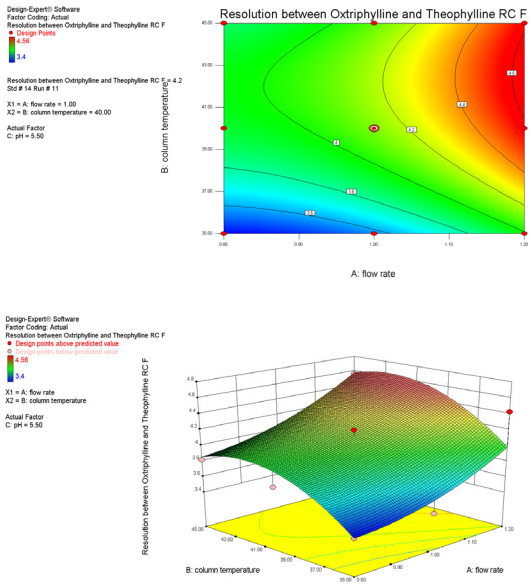



**Figure 4:** Overlap plot showing optimized HPLC condition as a point with the design space for selected as critical method attribute-resolution between oxtriphylline and theophylline RC F.

Critical method parameters are the variables that impact the critical method attributes in one or more ways and henceforth must be controlled and monitored to confirm that quality in work design is met. The three critical method parameters identified were mobile phase flow rate, column temperature, and mobile phase pH.

Based on these factors, the resolution between oxtriphylline and theophylline RC F and the tailing of the oxtriphylline peak were selected as critical method attributes. The matrix of the experiment is detailed in Table 3.

The three levels of critical method parameters are the flow rate of mobile phase 0.8, 1.0, 1.2 mL/min, oven column temperatures 35, 40, 45°C, while pH of the mobile phase was 5.3, 5.5, 5.7. The pH range was selected for good peak shapes of the analysts while mobile phase flow rate and oven column temperature ensure proper resolution between the critical pairs of peaks.

The relevance of the design model was assessed using the statistical approach known as analysis of variance (ANOVA). The findings showed that all models had significant *p-values* of 0.0001 or above and the correlation coefficient was between 0.8873 to 0.9071 for all critical method parameters, showing that a large percentage of the diversity in the answers explained by the models, demonstrating the models' high level of competence.

A novel HPLC method was developed that estimates oxtriphylline-related compounds like theophylline RCB, theophylline RCC, theophylline RCD, and theophylline RCF and caffeine the newly developed exhibits stability-indicating capability.

**Table 3:** The conditions and results of design space experiments.

| *Run* | *Factor 1 A: flow rate ml/min* | *Factor 2 B: column temperature °C* | *Factor 3 C: pH* | *Response 1 Resolution between Oxtriphylline and Theophylline RC F* | *Response 2 Tailing for Oxtriphylline* |
|---|---|---|---|---|---|
| 1 | 1.00 | 45.00 | 5.30 | 4.02 | 1.08 |
| 2 | 0.80 | 45.00 | 5.70 | 3.80 | 1.04 |
| 3 | 1.20 | 40.00 | 5.30 | 4.45 | 1.03 |
| 4 | 1.00 | 35.00 | 5.70 | 3.45 | 1.10 |
| 5 | 1.20 | 35.00 | 5.50 | 4.43 | 1.04 |
| 6 | 1.00 | 40.00 | 5.30 | 4.19 | 1.11 |
| 7 | 1.00 | 40.00 | 5.70 | 4.18 | 1.11 |
| 8 | 1.20 | 35.00 | 5.70 | 4.01 | 1.04 |
| 9 | 1.20 | 40.00 | 5.70 | 4.54 | 1.05 |
| 10 | 1.20 | 35.00 | 5.30 | 3.43 | 1.04 |
| 11 | 1.00 | 40.00 | 5.50 | 4.20 | 1.04 |
| 12 | 1.00 | 45.00 | 5.70 | 4.02 | 1.08 |
| 13 | 0.80 | 40.00 | 5.30 | 3.81 | 1.09 |
| 14 | 1.20 | 45.00 | 5.30 | 4.43 | 1.07 |
| 15 | 0.80 | 35.00 | 5.30 | 3.43 | 1.05 |
| 16 | 0.80 | 35.00 | 5.70 | 3.45 | 1.05 |
| 17 | 0.80 | 40.00 | 5.70 | 3.75 | 1.04 |
| 18 | 0.80 | 45.00 | 5.50 | 3.82 | 1.05 |
| 19 | 0.80 | 40.00 | 5.50 | 3.73 | 1.04 |
| 20 | 1.20 | 45.00 | 5.50 | 4.56 | 1.11 |
| 21 | 1.20 | 40.00 | 5.50 | 4.55 | 1.03 |
| 22 | 1.20 | 45.00 | 5.70 | 4.54 | 1.04 |
| 23 | 0.80 | 45.00 | 5.30 | 3.91 | 1.04 |
| 24 | 0.80 | 35.00 | 5.50 | 3.40 | 1.02 |
| 25 | 1.00 | 35.00 | 5.50 | 3.40 | 1.12 |
| 26 | 1.00 | 45.00 | 5.50 | 4.01 | 1.12 |
| 27 | 1.00 | 35.00 | 5.30 | 3.40 | 1.11 |

**Table 4:** Linearity results showing correlation coefficient and normalized intercept/slope ratio parameter

| *Level (%)* | *Concentration (mg/mL)* | *Peak area* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Theophylline RCC* | *Theophylline RCB* | *Theophylline RCD* | *Oxtriphylline* | *Theophylline RCF* | *Caffeine* |
| 0.05 | 0.0005 | 23329 | 17540 | 8237 | 10300 | 12003 | 13672 |
| 0.1 | 0.001 | 46251 | 34829 | 21477 | 20722 | 23828 | 27453 |
| 0.2 | 0.002 | 91457 | 69586 | 40226 | 42277 | 47747 | 54995 |
| 0.5 | 0.005 | 218948 | 172628 | 74234 | 110393 | 118004 | 136058 |
| 1.0 | 0.01 | 420676 | 343486 | 147339 | 230248 | 235212 | 271089 |
| R2 | | 0.9997 | 0.9999 | 0.9973 | 0.9998 | 0.9999 | 0.9999 |
| Normalized intercept/slope | | 0.0259 | 0.0039 | 0.0823 | -0.0248 | 0.0038 | 0.0033 |

XBridge BEH C18 was preferred over other columns as it has a wide usable range of pH and very low column bleeding compared to other C18 columns. This column, combined with optimized gradient condition with methanol in mobile phase B, helps achieve sufficient resolution between all impurities. Since oxtriphylline is a basic molecule, the pH of the buffer was selected to be slightly acidic pH.

## Analytical Method Validation

### *Suitability of system parameter*

The suitability of the chromatographic system is an essential element of verifying the HPLC system as it checks if the chromatographic system is adequately working well as per the set conditions. The resolution between theophylline-related

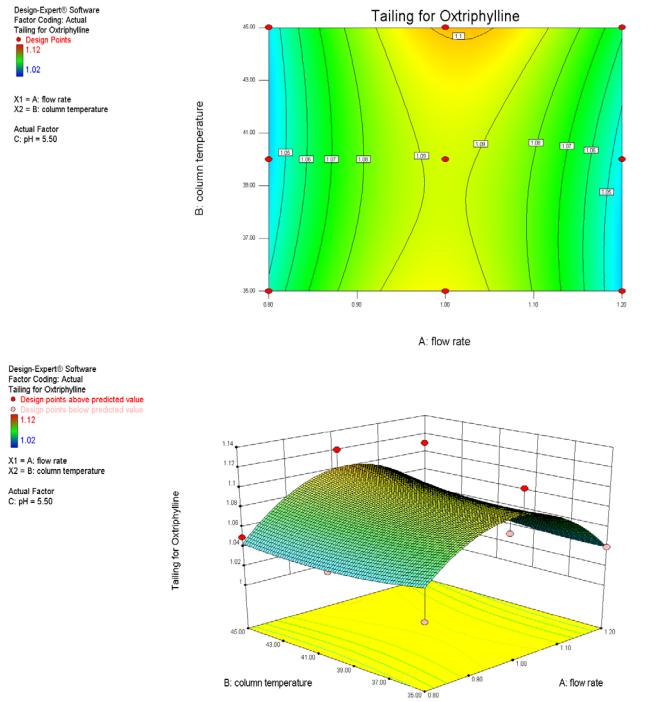
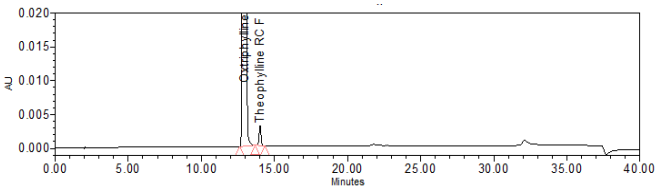


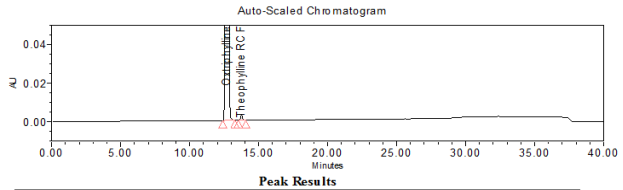

**Figure 5:** Overlap plot showing optimized HPLC condition as a point with the design space for selected as critical method attribute - tailing of oxtriphylline peak.




**Peak Results**

| | Name | RT | Area | USP Resolution |
|---|---|---|---|---|
| 1 | Oxtriphylline | 12.92 | 19623179 | |
| 2 | Theophylline RC F | 14.01 | 26862 | 4.6 |

**Figure 6a:** System suitability solution presenting resolution between theophylline related compound F and oxtriphylline.




**Peak Results**

| | Name | RT | Purity1 Angle | Purity1 Threshold | Area | % Area | RT Ratio | USP Resolution | USP Tailing |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Oxtriphylline | 12.68 | 1.025 | 1.290 | 20179786 | 99.88 | 1.00 | | 1.12 |
| 2 | Theophylline RC F | 13.75 | 0.542 | 0.683 | 23896 | 0.12 | 1.08 | 4.36 | 1.04 |

**Figure 6b:** System suitability solution showing tailing of oxtriphylline peak.

compound F and oxtriphylline was 4.6. Generally, peak resolution should be more than 1.5 and oxtriphylline peak tailing 1.12 refer to Figures 6a and b.

*Specificity*

The specificity parameter is validated to ensure whether there are any inferences of unknown peaks to impurity peaks and main compound peaks. The specificity parameter was evaluated by checking for interferences from unknown peaks with known peaks. The optimized chromatogram is presented in Figure 7. The chromatogram demonstrates the capability of the procedure to resolve and separate known peaks from unknown peaks.

*Linearity*

Linearity for the main analyte and all the related compounds was demonstrated by selecting a range from 0.0005 to 0.01 mg/mL. The concentration level ranges from 0.05 to 1% of the sample concentration. The correlation coefficient for oxtriphylline and all of its impurities was more than 0.99. The normalized intercept/slope was illustrated in Table 4.

*Method Precision*

The precision reflects how closely a set of measurements taken from several samples agree. For precision parameter, six replicate preparations were prepared and spiked with all impurities at 0.005 mg/mL concentration or 0.5% level to sample concentration. The average percentage recovery and percentage relative standard deviation (%RSD) for all impurities were determined through six injections of precision. The %RSD for all impurities was less than 2. The precision results were given in Table 5.

*Accuracy*

By injecting three replicate preparations of samples that had been spiked with known quantities of impurities at three levels—limit of quantitation, 100, and 150% corresponding to limited concentrations of known impurities, the accuracy technique was established. The mean recovery for each impurity was computed at each of the three levels.

The average recovery of impurities at all three levels was from 98.40 to 101.70% at LoQ, 101.56 to 101.88 at 100% level, and 101.28 to 102.54 at 150%. The percentage relative standard deviation for all the impurities at all levels was less than 2. The results are elucidated in Table 6. This proves that the method was accurate for oxtriphylline and its related compounds for a range of LoQ to 150%.

*Detection Limit and Quantitation Limit*

The detection limit indicates the least analyte concentration the method is capable of detecting correctly. The quantitation limit depicts the most negligible analyte concentration the method shall quantify accurately. Together, the detection limit and quantitation limit exhibit the method's sensitivity. The detection and quantitation limits were assessed by performing a serial dilution of all stock solutions of all the analytes to obtain the signal-to-noise ratio of about 3:1 for the detection limit and about 10:1 for the quantitation limit.[20,21] Values were tabulated in Table 7.

*Robustness*

Minor, deliberate variations to chromatographic conditions were made, and system suitability parameters like resolution between oxtriphylline and theophylline-related compound

**Table 5:** Percentage relative standard deviation showing precision of the method

| | *Theophylline RC C* | *Theophylline RC B* | *Theophylline RC D* | *Theophylline RC F* | *Caffeine* |
|---|---|---|---|---|---|
| Average peak area | 216544 | 176544 | 80125 | 123745 | 137645 |
| Average %recovery | 101.89 | 101.56 | 102.14 | 102.45 | 100.71 |
| %RSD | 0.14 | 0.25 | 0.91 | 0.14 | 0.35 |

**Table 6:** Mean recoveries of impurities at different spiking levels

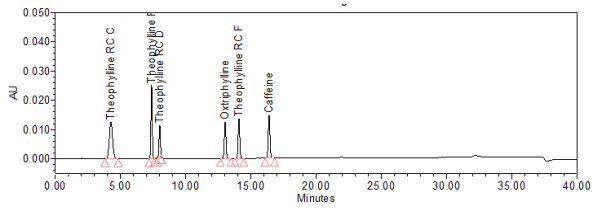
| *Spiking level* | *Parameter* | *Theophylline RC C* | *Theophylline RC B* | *Theophylline RC D* | *Theophylline RC F* | *Caffeine* |
|---|---|---|---|---|---|---|
| Limit of quantitation | Average peak area | 23425 | 18452 | 12665 | 12809 | 14951 |
| | Average % recovery | 98.23 | 101.25 | 98.40 | 101.65 | 101.70 |
| | % RSD | 0.87 | 0.45 | 0.16 | 1.25 | 1.25 |
| 100% level | Average peak area | 224862 | 175456 | 80147 | 122624 | 137370 |
| | Average % recovery | 101.88 | 101.56 | 101.74 | 101.56 | 101.71 |
| | % RSD | 0.17 | 0.49 | 0.48 | 0.17 | 0.56 |
| 150% level | Average peak area | 459785 | 354561 | 156485 | 245675 | 274777 |
| | Average % recovery | 101.54 | 101.45 | 101.23 | 101.28 | 101.45 |
| | % RSD | 0.52 | 0.14 | 0.45 | 0.15 | 0.18 |

**Table 7:** Detection limit and quantitation limit values

| | *Theophylline RC C* | *Theophylline RC B* | *Theophylline RC D* | *Theophylline RC F* | *Caffeine* |
|---|---|---|---|---|---|
| Limit of detection | 0.0456 µg/mL | 0.0098 µg/mL | 0.0132 µg/mL | 0.0479 µg/mL | 0.0078 µg/mL |
| Limit of quantitation | 0.1505 µg/mL | 0.0323 µg/mL | 0.0436 µg/mL | 0.1581 µg/mL | 0.0257 µg/mL |

**Table 8:** Robustness data for variations in mobile phase pH, flow and oven column temperature

| *Robustness conditions* | *Resolution between oxtriphylline and theophylline related compound F* | *Tailing of oxtriphylline peak* |
|---|---|---|
| Flow Variation | | |
| 0.8mL/min | 3.73 | 1.11 |
| 1.0mL/min | 4.20 | 1.12 |
| 1.2mL/min | 4.55 | 1.11 |
| Column temperature | | |
| 35° | 3.40 | 1.11 |
| 40° | 4.01 | 1.12 |
| 45° | 4.20 | 1.12 |
| Mobile phase pH | | |
| 5.3 | 4.18 | 1.11 |
| 5.5 | 4.19 | 1.11 |
| 5.7 | 4.20 | 1.11 |




**Peak Results**

| | Name | RT | Area | % Area | Purity1 Angle | Purity1 Threshold | Purity1 Flag |
|---|---|---|---|---|---|---|---|
| 1 | Theophylline RC C | 4.261 | 218948 | 26.37 | 10.817 | 37.928 | No |
| 2 | Theophylline RC B | 7.381 | 172628 | 20.79 | 6.175 | 17.825 | No |
| 3 | Theophylline RC D | 8.006 | 74234 | 8.94 | 2.989 | 11.455 | No |
| 4 | Oxtriphylline | 13.020 | 110393 | 13.30 | 11.630 | 45.369 | No |
| 5 | Theophylline RC F | 14.078 | 118004 | 14.21 | 7.572 | 30.445 | No |
| 6 | Caffeine | 16.388 | 136058 | 16.39 | 8.012 | 28.587 | No |

**Figure 7:** Typical chromatogram showing the specificity of oxtriphylline and all related compounds

F and tailing of oxtriphylline peak was verified. The data from Table 8 indicate no significant differences in results. Thus, this method is suitably reproducible and precise for slight variations in mobile phase flow, column temperature, and mobile phase pH.

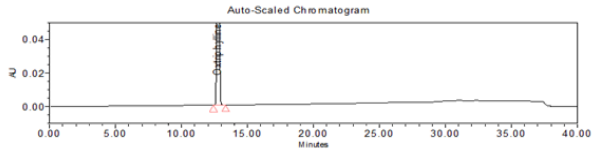
*Solution Stability*

Solution stability helps determine the stability of all impurities in the prepared sample, which helps in planning analysis. Freshly prepared standard solution with LoQ level impurities and spiked sample with LoQ level impurities were analyzed. The stability period with less than 10% changes in the peak was calculated for all impurities from the initial time point. The stability period for all impurities was stable for 24 hours at room temperature.

*Forced Degradation Studies*

Acid degradation: When oxtriphylline was treated with 0.1 N aqueous HCl degradation solutions for three days at room temperature, the drug was found to be degraded by approximately 0.56%.

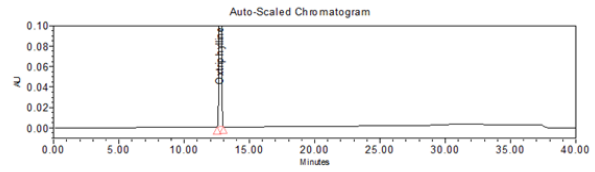
a) *Chromatogram of Acid degradation sample (0.2 mg/mL)*




Peak Results

| | Name | RT | Purity1 Angle | Purity1 Threshold | Area | % Area | RT Ratio | USP Tailing |
|---|---|---|---|---|---|---|---|---|
| 1 | Oxtriphylline | 12.76 | 0.124 | 0.259 | 3853135 | 100.00 | 1.00 | 1.05 |

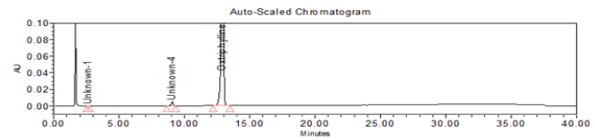
b) *Chromatogram of Base degradation sample (0.2 mg/mL)*




Peak Results

| | Name | RT | Purity1 Angle | Purity1 Threshold | Area | % Area | RT Ratio | USP Tailing |
|---|---|---|---|---|---|---|---|---|
| 1 | Oxtriphylline | 12.76 | 0.144 | 0.257 | 3893644 | 100.00 | 1.00 | 1.05 |

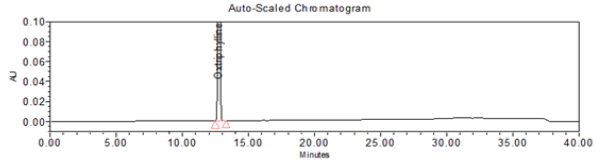
c) *Chromatogram of Peroxide degradation sample (0.2 mg/mL)*




Peak Results

| | Name | RT | Purity1 Angle | Purity1 Threshold | Area | % Area | RT Ratio | USP Resolution | USP Tailing |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Unknown-1 | 2.62 | 5.488 | 9.790 | 1615 | 0.04 | 0.20 | | 1.03 |
| 2 | Unknown-4 | 9.07 | 0.745 | 1.039 | 45161 | 1.23 | 0.70 | 35.49 | 0.86 |
| 3 | Oxtriphylline | 12.90 | 0.150 | 0.279 | 3623940 | 98.73 | 1.00 | 15.28 | 0.88 |

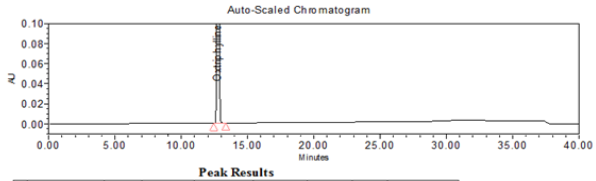
d) *Chromatogram of Humidity degradation sample (0.2 mg/mL)*




Peak Results

| | Name | RT | Purity1 Angle | Purity1 Threshold | Area | % Area | RT Ratio | USP Tailing |
|---|---|---|---|---|---|---|---|---|
| 1 | Oxtriphylline | 12.76 | 0.123 | 0.257 | 3799009 | 100.00 | 1.00 | 1.05 |

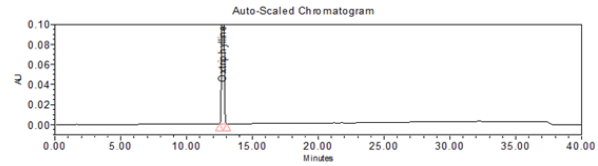
e) *Chromatogram of Photolytic degradation sample (0.2 mg/mL)*




Peak Results

| | Name | RT | Purity1 Angle | Purity1 Threshold | Area | % Area | RT Ratio | USP Tailing |
|---|---|---|---|---|---|---|---|---|
| 1 | Oxtriphylline | 12.77 | 0.101 | 0.259 | 3862214 | 100.00 | 1.00 | 1.05 |

f) *Chromatogram of Thermal degradation sample (0.2 mg/mL)*




Peak Results

| | Name | RT | Purity1 Angle | Purity1 Threshold | Area | % Area | RT Ratio | USP Tailing |
|---|---|---|---|---|---|---|---|---|
| 1 | Oxtriphylline | 12.74 | 0.131 | 0.258 | 3942410 | 100.00 | 1.00 | 1.05 |

**Figure 8:** Forced degradation chromatograms a) acidic degradation; b) basic degradation; c) oxidative degradation; d) Humidity degradation; e) Photolytic degradation; f) Thermal degradation.

Base degradation: Oxtriphylline was treated under base degradation at 0.1N aqueous NaOH for three days; at room temperature, the drug was found to be degraded by approximately 0.53%.

Peroxide degradation: Oxtriphylline was treated under peroxide degradation at 3% $H_2O_2$ degradation solutions for 4 hours at room temperature; the drug was found to be degraded by approximately 8.32%.

Thermal degradation: Oxtriphylline was treated under thermal conditions (105$^o$) for three days in an oven; the drug was found to be degraded by approximately 1.84%.

UV-visible light (ICH guideline): Oxtriphylline was treated for 22 hrs under photolytic conditions, and the drug was found to be degraded by approximately 0.52%.

Humidity (85°/85%RH-3 Day): Oxtriphylline was treated under humidity conditions; the drug was found to be degraded by approximately 1.28%.

The forced degradation study was completed, and stressed samples were analyzed. The chromatograms were processed at 270 nm to detect the degradation impurities of oxtriphylline. The max plot showed an absorbance of about 0.8AU at 0.2 mg/mL oxtriphylline concentration. The PDA peak purity and spectral library match angles met the target threshold criteria for the aforementioned stressed conditions. All the degradation impurities are separated as per the acceptance criteria. For forced degradation chromatograms, refer to Figure 8.

## CONCLUSION

The novel developed reverse-phase high-performance liquid chromatography procedure demonstrates the potential to resolve and quantify all the related compounds of oxtriphylline. Further, the forced degradation studies data shows that the unknown formed degradants are well resolved within the same method without interference with the known peaks. The proposed procedure is a sensitive, specific, precise, accurate, robust, and stable indication method and can be used to estimate related compounds of oxtriphylline.

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