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# DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ATROPINE SULPHATE AND DIPHENOXYLATE HYDROCHLORIDE IN BULK DRUG AND TABLET DOSAGE FORM

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## ABSTRACT
The aim of the study is to develop a stability indicating method for the concomitant determination of Atropine and Diphenoxylate in bulk and pharmaceutical dosage forms by RP-HPLC method. The method employed for the determination includes KROMASIL C18 column with the solvent system being Na2HPO4: Methanol (50:50), maintaining ambient temperature, flow rate of 1.0 ml/min and the run time was 6 minutes. Detection was carried out with a PDA detector at 225 nm and the pH was adjusted to 4.5. validation of the method was performed and the effect of Forced degradation of the sample under various conditions were evaluated. The outcomes of the method with the above selected criterion met the specifications of the regulatory guidelines such as ICH. Accuracy and recovery of the method was deduced in the range of 99-101%, linearity was perceived in the range of 50-150 µg/mLfor both Atropine and diphenoxylate. The regression coefficient values for atropine and diphenoxylate were found to be 0.997 and 1 respectively. Precision of the method was found to be less than 2 for both the drugs. Robustness of the method was evaluated and the results met the acceptance limits. Outcomes of the forced degradation studies were within the limit i.e., 5-20%, indicative of stability of drugs under various conditions. All the outcomes of the method development, validation and degradation studies proved that the method is reliable for the simultaneous estimation of above said drugs in bulk and pharmaceutical dosage forms.

**KEYWORDS:** RP-HPLC, Atropine, Diphenoxylate, ICH guidelines, stability-indicating.

## INTRODUCTION
Diarrhoea is generally caused due to increase in the peristaltic movements in the gut, which results in increased defecations and excesive loss of water. Atropine and diphenoxylate are two medications used in combination for management of diarrhoea. In combination, it is marked under the brand name called Lomotil containing a fixed dose of Diphenoxylate and atropine, the former being in a highest dose than the latter. The combination belongs to the class of anti-motility agents, where diphenoxylate is used to decrease the movements in the intestines. Atropine is used to prevent the exploited utilization of diphenoxylate by patients.

## MATERIALS AND METHODS
### Equipment Kit
- Waters 2695 module in the HPLC system
- Empower 2 Waters software
- Waters 2699 module Photodiode array (PDA) detector
- KROMASIL C-18 column; 250 mm × 4.6 mm; 5 µm dimension particle size

### Chemical Substances
- Na2HPO4
- Ortho-Phosphoric Acid

### Solvents
- Methanol

### Drug
- Atropine sulphate (ATP)
- Diphenoxylate (DPH)

### Tablet dose
- Brand name: Lomotil®
- Name: Atropine Sulphate (ATP) and Diphenoxylate (DPH)
- Claimed strength: ATP - 0.025 ppm and DPH - 2.50 ppm

- Company manufacturing: Pfizer

**PREPARATION OF MOBILE PHASE**

$Na_2HPO_4$ is weighed and transferred into a 1000 ml volumetric flask. Add a small amount of HPLC graded water to dissolve it. The volume is made up to 1000 ml with HPLC grade water. Sonicate the solution to remove any air particles present.

To prepare the desired mobile phase 500 ml of the prepared buffer solution is mixed with 500 ml of methanol and sonicated to eliminate the air particles.

**PREPARATION OF ATROPINE & DIPHENOXYLATE STOCK SOLUTION**

Precisely weigh and transfer 0.025 mg Atropine sulphate and 2.50 mg diphenoxylate into 100 ml of volumetric flask. Add 10 ml of Methanol to dissolve the substances. Shake the solution or sonicate it for 20 minutes to eliminate the entrapped air particles and is used as a stock solution.

Dilute 1 ml of the above formulated solution to 10 ml with water to obtain standard solution for analysis.

**PREPARATION OF ATROPINE & DIPHENOXYLATE SAMPLE SOLUTION**

Accurately weigh and transfer 94 mg of Lomotil® crushed tablet powder equivalent to 0.025 mg Atropine Sulphate and 2.5 mg Diphenoxylate into a 100 ml of volumetric flask and add 10 ml Methanol and sonicate it for 20 min (or) shake 10 min and makeup with Methanol.

Transfer above solution (1ml) into 10 ml flask. Dilute the volume with Water. Filter the solution through a 0.45µm filter, inject into HPLC system.

**WORKING LINEARITY CALIBRATED SOLUTIONS OF ATROPINE &DIPHENOXYLATE**

Working concentrations solutions of ATP (0.01-0.03 µg/ml) and DPH (1-3 µg/ml) are prepared according to linearity concentration range by diluting stock solutions with suitable amount of methanol.

1. **Solution 1:** Pipette 0.5 ml of stock solution into a 100 ml volumetric flask, dilute with methanol to obtain concentrations of 0.0125 µg/ml of ATP and 1.25 µg/ml of DPH respectively.
2. **Solution 2:** Pipette 0.7 ml of stock solution into a 100 ml volumetric flask, dilute with methanol to obtain concentrations of 0.018 µg/ml of ATP and 1.875 µg/ml of DPH respectively.
3. **Solution 3:** Pipette 1 ml of stock solution into a 100 ml volumetric flask, dilute with methanol to obtain concentrations of 0.025 µg/ml of ATP and 2.50 µg/ml of DPH respectively.
4. **Solution 4:** Pipette 1.2 ml of stock solution into a 100 ml volumetric flask, dilute with methanol to obtain concentrations of 0.0312 µg/ml of ATP and 3.125 µg/ml of DPH respectively.
5. **Solution 5:** Pipette 1.5 ml of stock solution into a 100 ml volumetric flask, dilute with methanol to obtain concentrations of 0.0375 µg/ml of ATP and 3.75 µg/ml of DPH respectively.

**RESULTS AND DISCUSSIONS**

**ATP AND DPH HPLC METHOD ASSESMENT PREREQUISITES:**

Mobile Phase : $Na_2HPO_4$: Methanol (50:50)
Column : KROMASIL, C18, 150 × 4.6mm, 5µm
Flow Rate : 1.0 ml/Min
Temperature : 25˚C
Volume : 10µl
Run time : 6 min
Detector : PDA, 225 nm
pH : 4.5

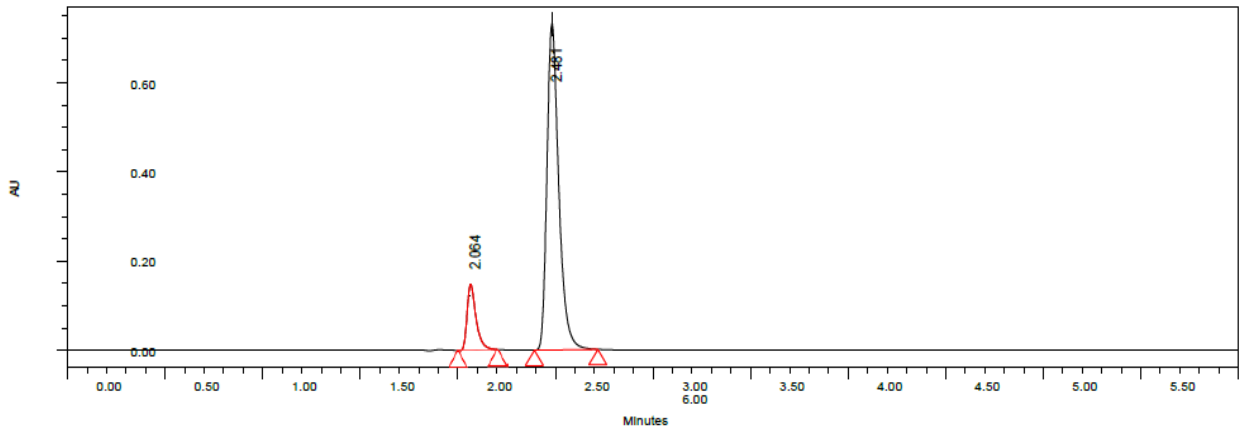



**Fig 1: Results of Trial.**

| | Name | Retention Time | Area | % Area | Height | USP Resolution | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|---|---|---|
| 1 | | 2.064 | 478585 | 13.48 | 148786 | | 1.50 | 10142 |
| 2 | | 2.481 | 3072146 | 86.52 | 732656 | 4.24 | 1.38 | 8446 |

**Observation:** Both the components are eluted and all the system suitability parameters are within the limit.

## METHOD VALIDATION

### System suitability tests

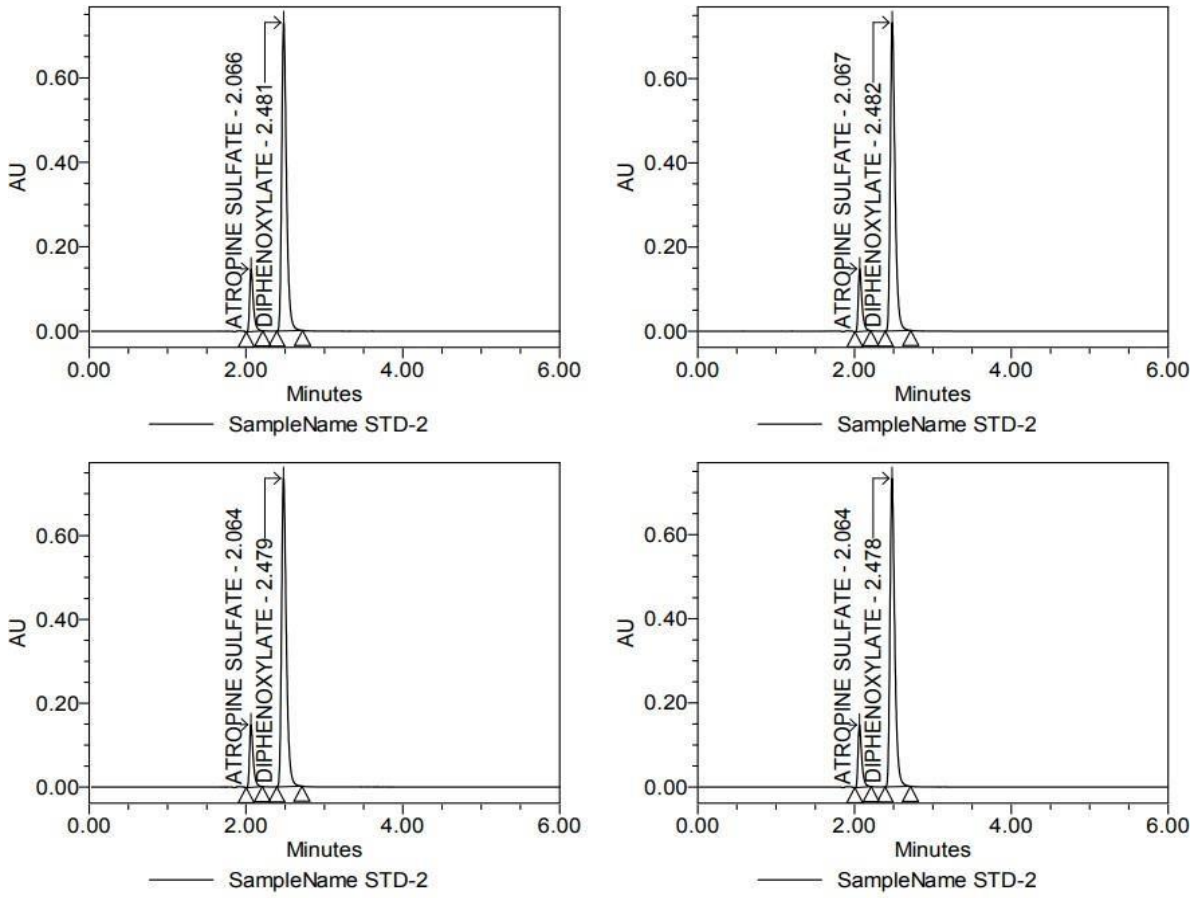
System suitability tests are done to evaluate the parameters as number of theoretical plates, peak tailing, retention time, resolution, plate count, etc. Sample solution of ATP and DPH are instilled six times in to the HPLC system with a KROMASIL C18, 150 × 4.6mm, 5µm column, with the solvent system $Na_2HPO_4$: Methanol (50:50). The outcomes of the tests are given in the following table.

**Table 1: System suitability results for ATP.**

| S.no | Peak Name | Rt | Area | USP Plate Count | USP Tailing |
|---|---|---|---|---|---|
| 1 | Atropine sulphate | 2.064 | 481122 | 10173 | 1.51 |
| 2 | Atropine sulphate | 2.064 | 480568 | 10055 | 1.52 |
| 3 | Atropine sulphate | 2.065 | 485629 | 9659 | 1.50 |
| 4 | Atropine sulphate | 2.066 | 481253 | 10068 | 1.49 |
| 5 | Atropine sulphate | 2.067 | 480171 | 10150 | 1.53 |
| Mean | | | 481748.7 | | |
| % RSD | | | 0.5 | | |

**Table 2: System suitability results for DPH.**

| S.no | Peak Name | Rt | Area | USP Resolution | USP Plate Count | USP Tailing |
|---|---|---|---|---|---|---|
| 1 | Diphenoxylate | 2.478 | 3077153 | 4.22 | 8450 | 1.39 |
| 2 | Diphenoxylate | 2.479 | 3078638 | 4.24 | 8492 | 1.39 |
| 3 | Diphenoxylate | 2.480 | 3085138 | 4.17 | 8287 | 1.38 |
| 4 | Diphenoxylate | 2.481 | 3075589 | 4.22 | 8419 | 1.38 |
| 5 | Diphenoxylate | 2.482 | 3072692 | 4.22 | 8461 | 1.39 |
| Mean | | | 3077841.8 | | | |
| % RSD | | | 0.2 | | | |



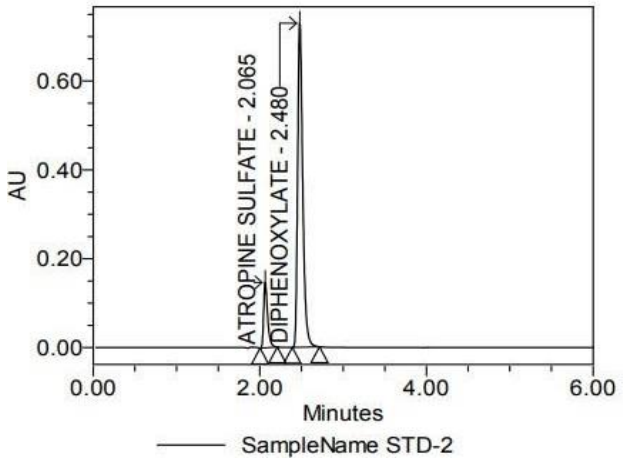



**Fig 2: System suitability results of ATP & DPH.**

**Selectivity**

Selectivity of the method is evaluated by instilling blank and working concentration of sample solutions in to the HPLC system equipped with a KROMASIL C18, 150 × 4.6mm, 5µm and solvent system $Na_2HPO_4$: Methanol (50:50). The criterion is adjusted as per the specifications of optimization conditions. The selectivity of the designed method is tested by comparing the chromatograms of the blank with the sample/placebo.

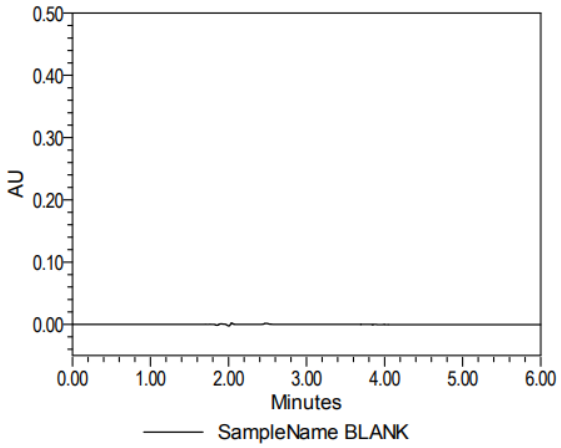



**Fig 3: Chromatogram of Blank.**

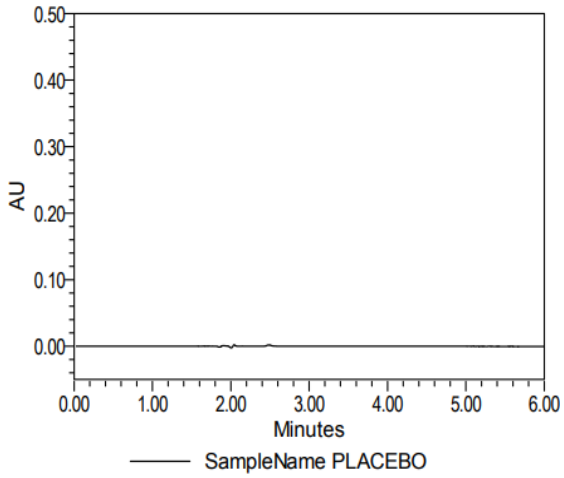



**Fig 4: Chromatogram of Placebo.**

**Linearity**

Working concentrations of ATP & DPH are prepared in the level of upper interval and lower interval of 0.01-0.03 µg/ml and 1-3 µg/ml respectively and are instilled into the HPLC systemwith KROMASIL C18, 150 × 4.6mm, 5µm. The peaks area for the respective concentrations were recorded and a plot of concentration vs peak areas is plotted, and the $R^2$ values were 0.9978 and 1 respectively, which proves that the said method is linear in the abovesaid range.

**Table 3: Results of Linearity and range of ATP & DPH.**

| ATP | | | DPH | | |
|---|---|---|---|---|---|
| **Concentration (%)** | **Concentration (µg/ml)** | **Peak area** | **Concentration (%)** | **Concentration (µg/ml)** | **Peak area** |
| | | | | | |
| 50 | 0.0125 | 541083 | 50 | 1.25 | 1105140 |
| 75 | 0.018 | 813669 | 75 | 1.875 | 1675423 |
| 100 | 0.0250 | 1061247 | 100 | 2.50 | 2233693 |
| 125 | 0.0312 | 1330576 | 125 | 3.125 | 2802591 |
| 150 | 0.0375 | 1611742 | 150 | 3.75 | 3368096 |

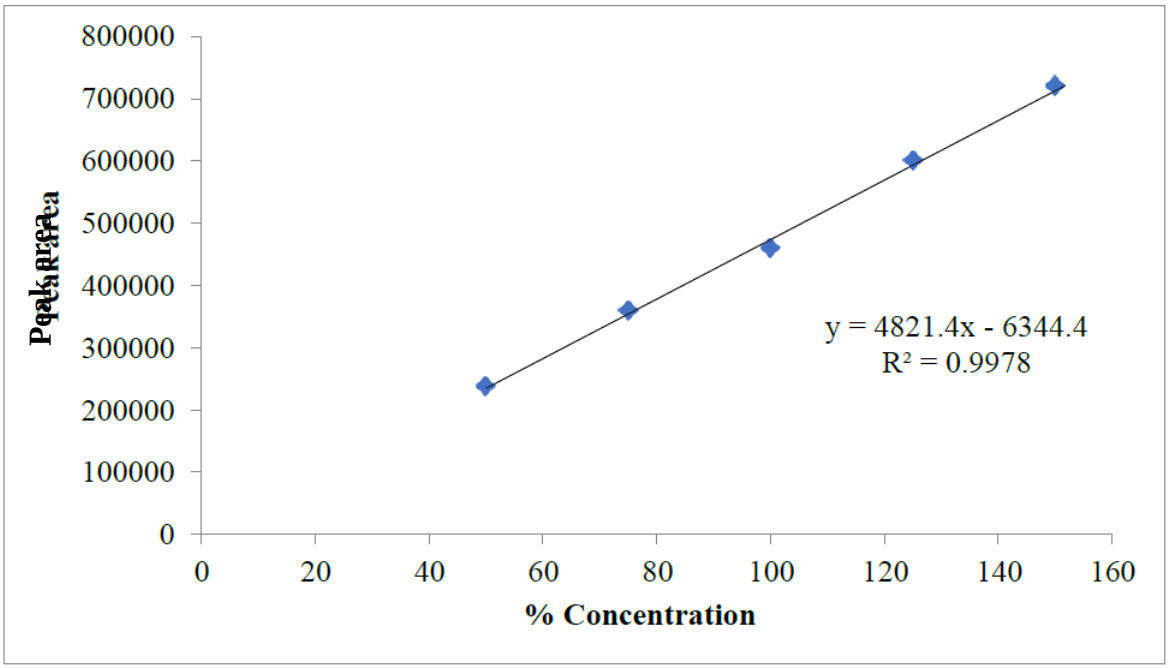



**Fig 5: Calibration curve for ATP.**

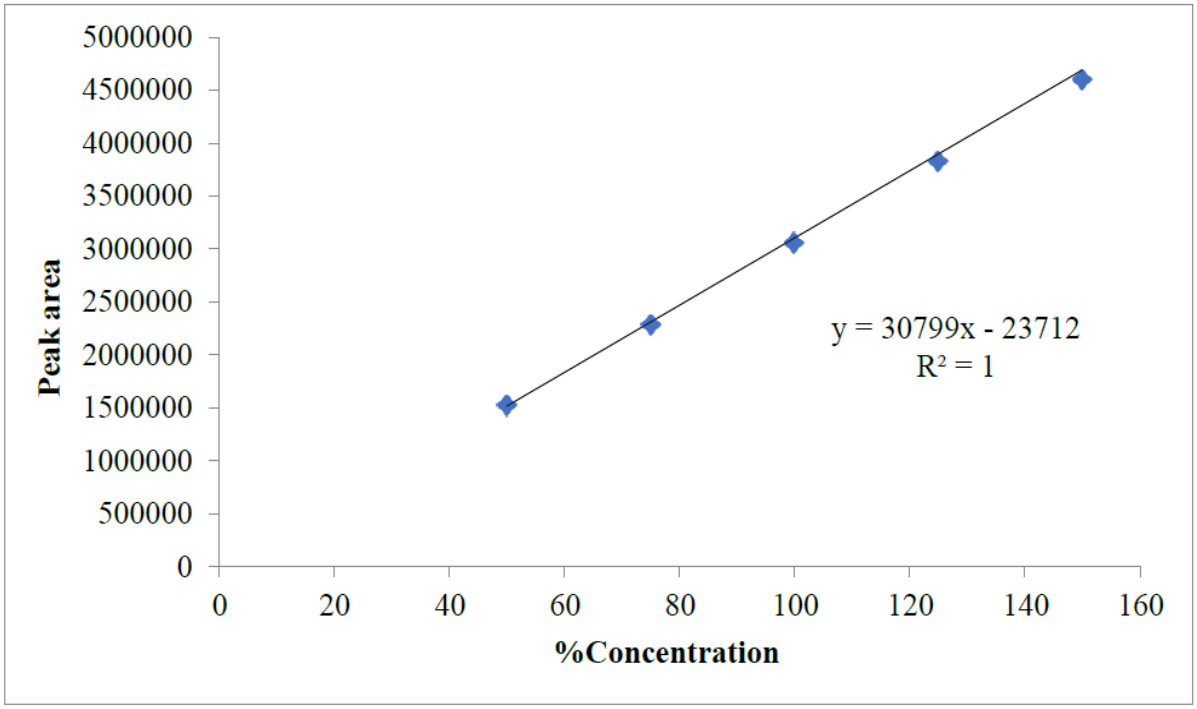



**Fig 6: Calibration curve for DPH.**

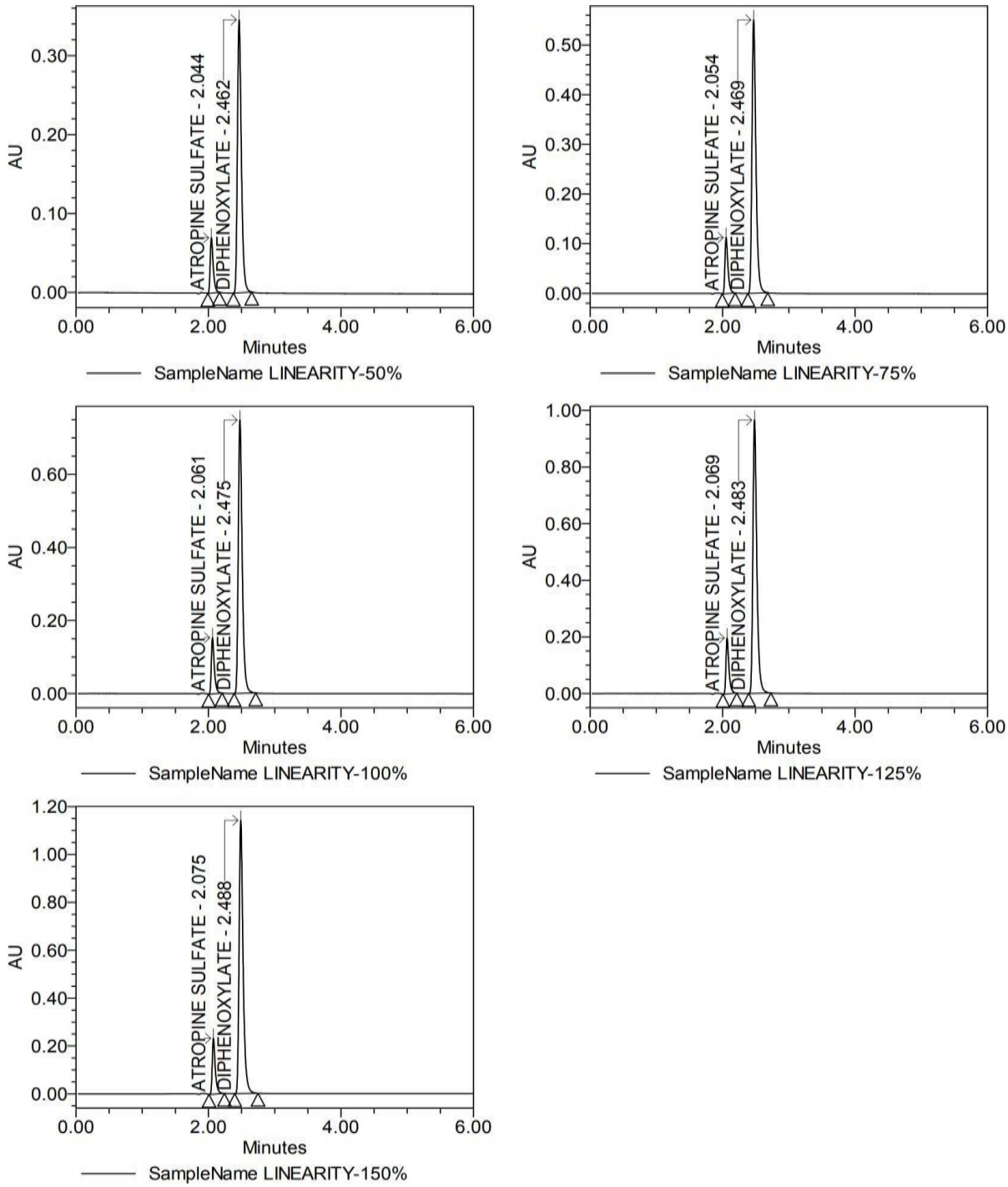



**Fig 7: Chromatograms for linearity and range of ATP & DPH.**

**Limit of detection**

Sample solution of ATP & DPH were instilled into the HPLC system on a KROMASIL C18, 150 × 4.6mm, 5µm, modifying the settings according to the optimized parameters selected for the method. Evaluation of LOD was done based on signal to noise ratio. The formula for calculation of LOD is 3.3* σ/s and was understood to be 0.000 µg/ml and 0.001 µg/ml respectively. Thus, the strategy employed was proved to be sensitive for analyzing the above-mentioned drugs.

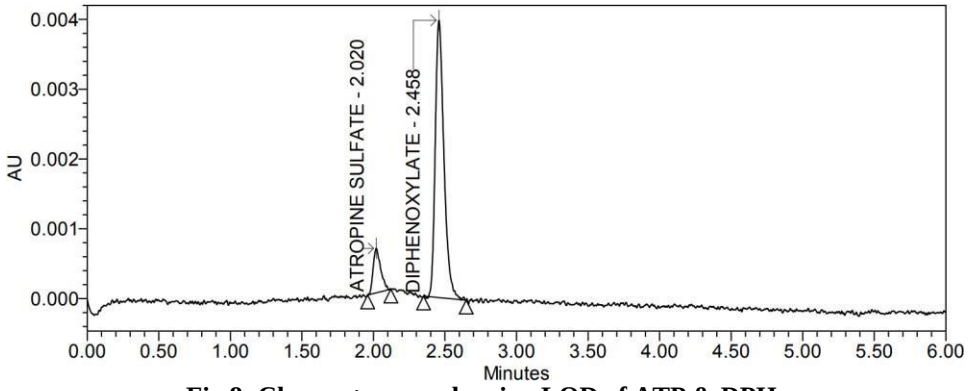



**Fig 8: Chromatogram showing LOD of ATP & DPH.**

**Limit of Quantification**

Sample solution of ATP & DPH were instilled into the HPLC system on a KROMASIL C18, 150 × 4.6mm, 5µm, modifying the settings according to the optimized parameters selected for the method. Evaluation of LOD was done based on signal to noise ratio. The formula for calculation of LOD is 10 * σ/s and was found to be 0.000 µg/ml and 0.004 µg/ml respectively.Thus, the strategy employed was proved to be sensitive for analyzing the above-mentioned drugs.

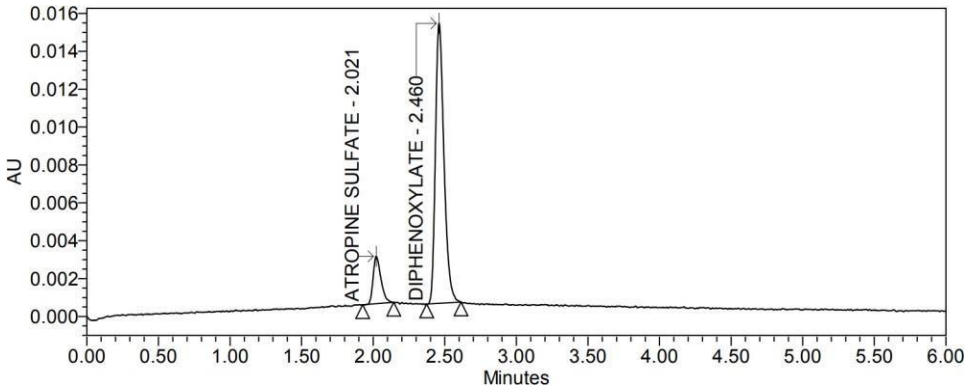



**Fig 9: Chromatogram showing LOQ of ATP & DPH.**

**Precision**

The precision of the proposed method is evaluated based on the repeatability results. Six injections of working concentrations of ATP & DPH were instilled in the HPLC system on a KROMASIL C18, 150 × 4.6mm, 5µm column and the settings were modified according to the cardinal parameters. Repeatability of the chosen method was evaluated based on the % RSD obtained from the peak areas, deduced to be 0.16 and 0.18 respectively, showing that the said method is precise for the analysis of ATP & DPH.

**Table 4: Precision results for ATP & DPH.**

| Drug name | Peak area | Average | Standard deviation | % RSD |
|---|---|---|---|---|
| **ATP** | 479163 | 479795.33 | 0.16 | 0.16 |
| | 479203 | | | |
| | 479161 | | | |
| | 480539 | | | |
| | 480921 | | | |
| | 479785 | | | |
| **DPH** | 3050646 | 3057699.5 | 0.18 | 0.18 |
| | 3051802 | | | |
| | 3058078 | | | |
| | 3059811 | | | |
| | 3061884 | | | |
| | 3063976 | | | |

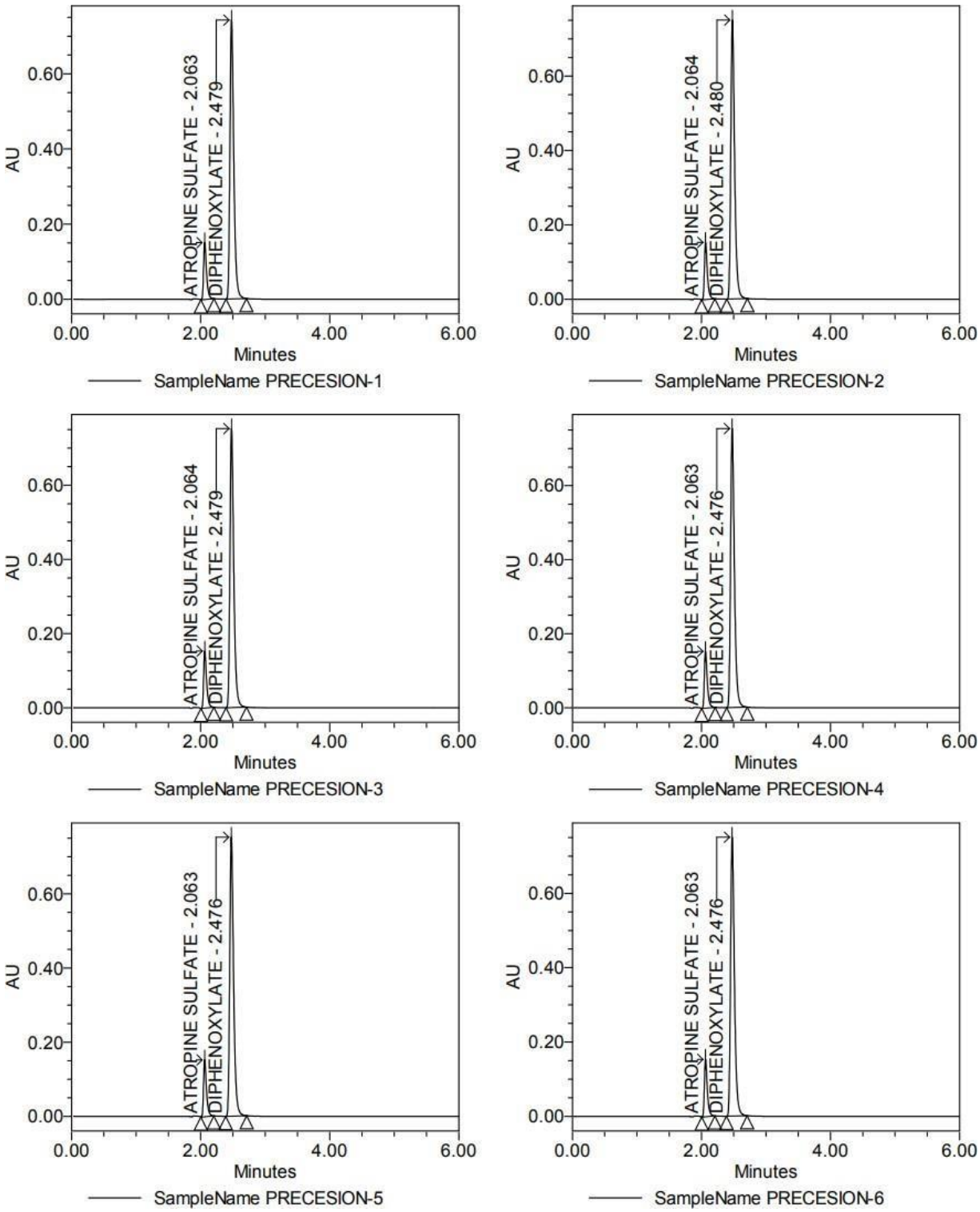



**Fig 10: Chromatograms showing precision of ATP & DPH.**

**Accuracy and recovery**

Working concentration solutions of ATP & DPH were instilled into the HPLC system on a KROMASIL C18, 150 × 4.6mm, 5µm column, the settings were modified according to the optimized criteria. The % RSD and assay of the sample are calculated from the peaks area obtained, proving that the method is accurate for estimation of ATP & DPH simultaneously.

Recovery of the method is valuated by spiking the sample solutions of ATP & DPH at a level of 50%, 100% and 150% and the % recovery after spiking was made in contrary with that of the amount of the drug content in the sample before spiking. Results obtained prove that the method is judicious for the concomitant quantification of ATP & DPH in the formulations.

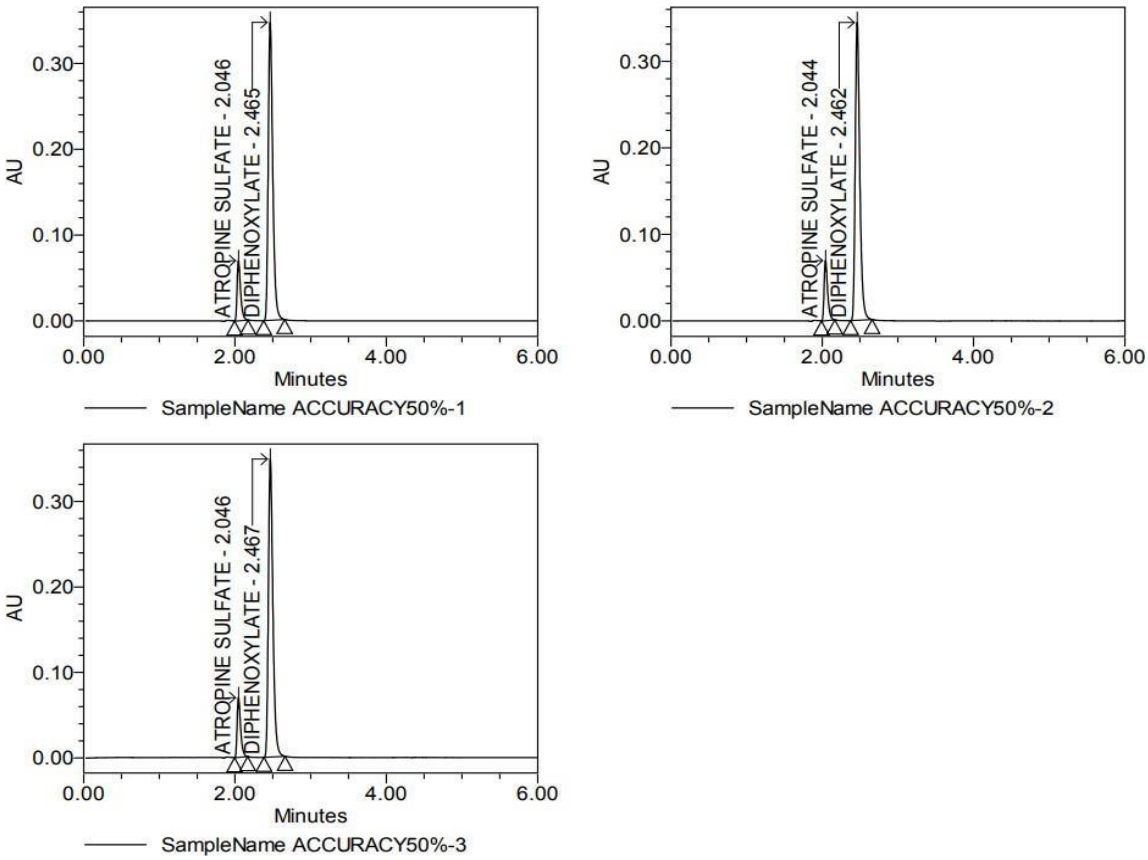



**Fig 11: Recovery of ATP & DPH at a level of 50% spiking.**

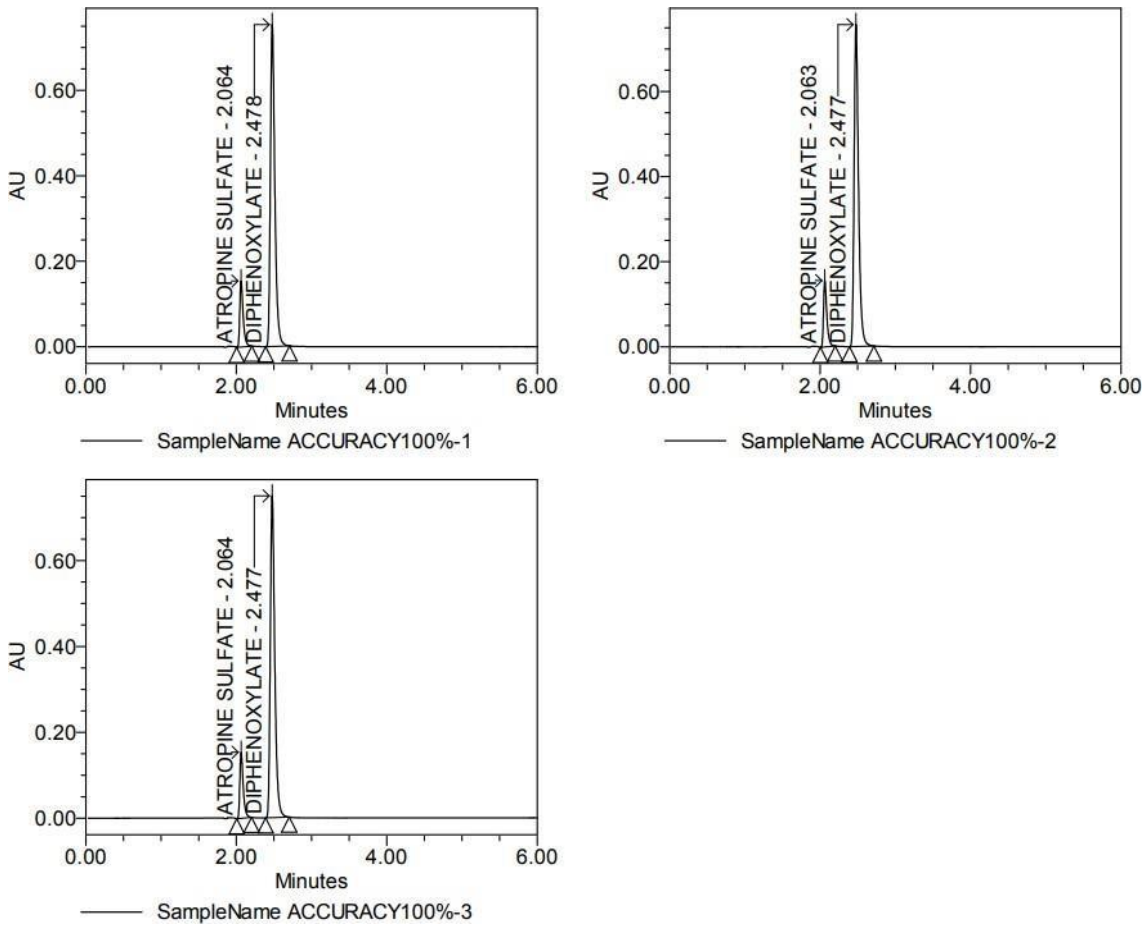



**Fig 12: Recovery of ATP & DPH at a level of 100% spiking.**

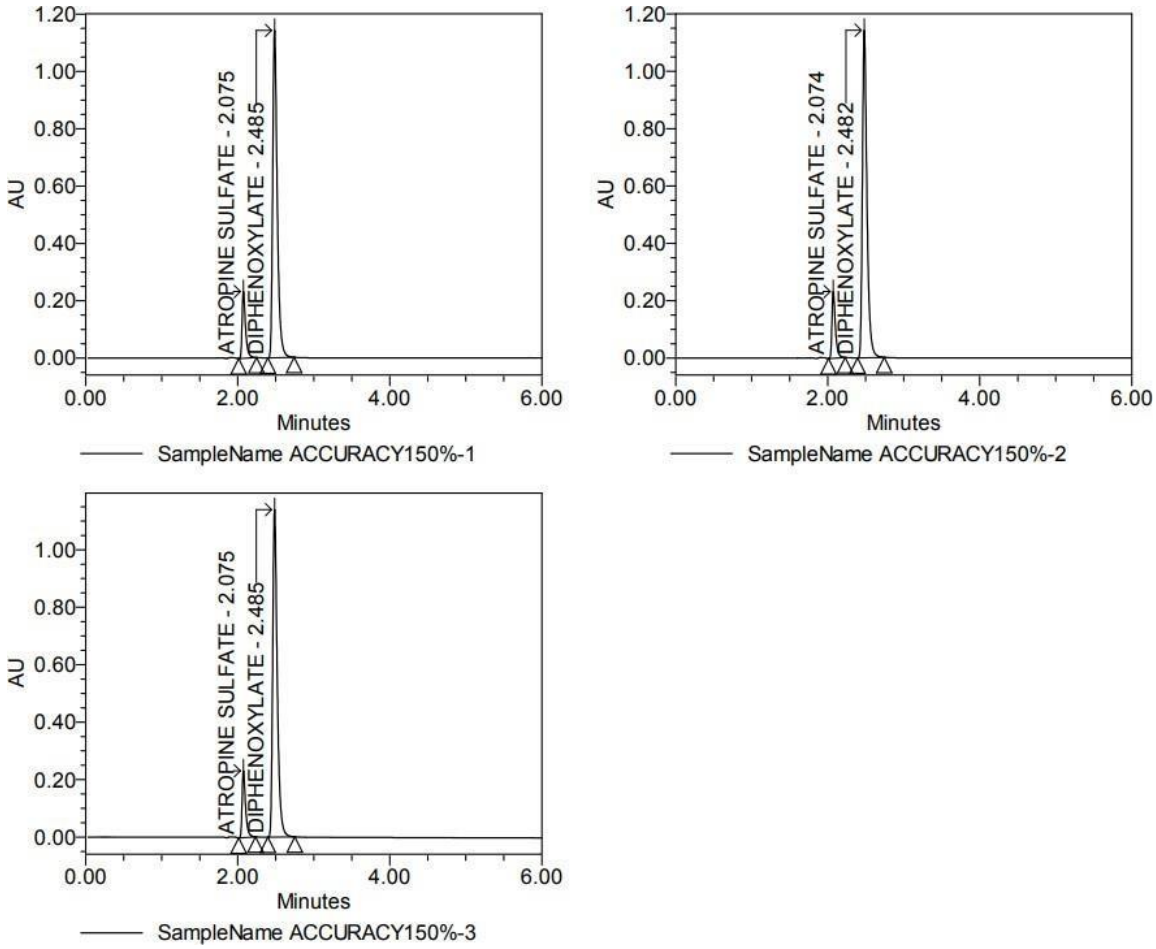



**Fig 13: Recovery of ATP & DPH at a level of 150% spiking.**

**Table 5: Recovery results for Atropine Sulphate.**

| Spiking value (%) | Peak Area | Amount of drug added | Total amount of drug found | Recovery of drug | Mean recovery value |
|---|---|---|---|---|---|
| 50 | 238901 | 0.012 | 0.01 | 100 | 100 |
| 50 | 239640 | 0.012 | 0.01 | 100 | |
| 50 | 238804 | 0.012 | 0.01 | 100 | |
| 100 | 479106 | 0.025 | 0.02 | 100 | 100 |
| 100 | 479922 | 0.025 | 0.02 | 100 | |
| 100 | 480875 | 0.025 | 0.02 | 101 | |
| 150 | 721952 | 0.037 | 0.04 | 101 | 100 |
| 150 | 720248 | 0.037 | 0.04 | 100 | |
| 150 | 720420 | 0.037 | 0.04 | 100 | |

**Table 6: Recovery results for Diphenoxylate.**

| Spiking value (%) | Peak Area | Amount of drug added | Total amount of drug found | Recoveryof drug | Mean recovery value |
|---|---|---|---|---|---|
| 50 | 1511106 | 1.238 | 1.22 | 99 | 99 |
| 50 | 1515813 | 1.238 | 1.23 | 99 | |
| 50 | 1527192 | 1.238 | 1.24 | 100 | |
| 100 | 3064532 | 2.475 | 2.48 | 100 | 100 |
| 100 | 3056285 | 2.475 | 2.48 | 100 | |
| 100 | 3059921 | 2.475 | 2.48 | 100 | |
| 150 | 4597498 | 3.713 | 3.72 | 100 | 100 |
| 150 | 4601663 | 3.713 | 3.73 | 100 | |
| 150 | 4595436 | 3.713 | 3.72 | 100 | |

**Robustness**

Working concentration solutions of ATP & DPH were passed into the HPLC system equippedwith a KROMASIL C18, 150 × 4.6mm, 5µm column, and modification of the selected parameters such as wavelength, pH, flow rate, ratio of mobile phase and temperature was done toevaluate the robustness of the method. Number of theoretical plates, peak tailing, peak resolution, retention times and peak areas of ATP & DPH after modification were calculated and the results show that the chosen method is robust for the concomitant quantification of ATP & DPH in the dosage forms.

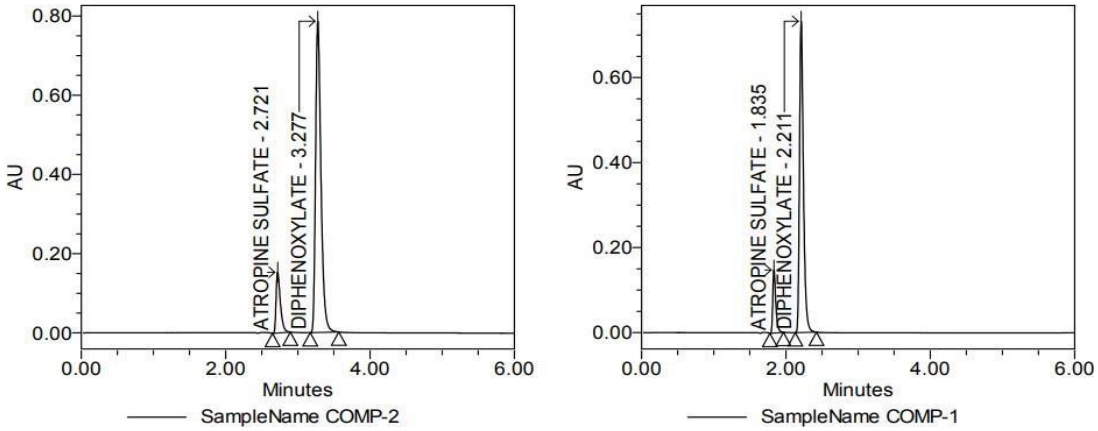



**Fig 14: Chromatograms showing robustness of ATP & DPH- Mobile Phase composition modification.**

**Table 7: Robustness results- mobile phase composition modification.**

| Mobile phase (Methanol) | Sample name | Rt | Peak area | USP resolution | USP plate count | USP tailing |
|---|---|---|---|---|---|---|
| 35% | ATP | 2.185 | 1363098 | | 8166 | 1.45 |
| 55% | ATP | 1.599 | 993706 | | 6983 | 1.47 |
| 35% | DPH | 2.829 | 2833813 | 5.82 | 9103 | 1.29 |
| 55% | DPH | 2.098 | 2067183 | 5.68 | 7931 | 1.32 |

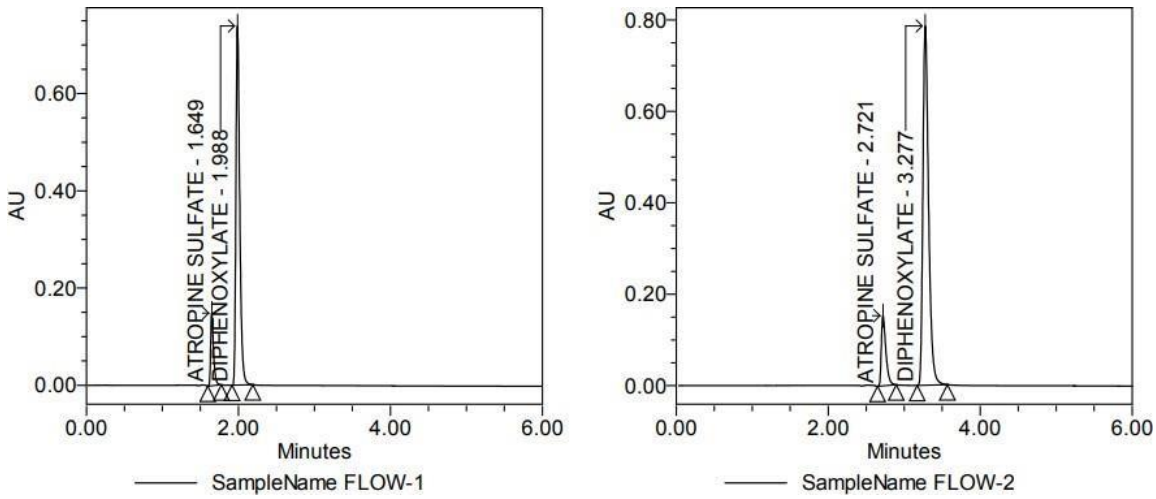



**Fig 15: Chromatograms showing robustness of ATP & DPH- Flow rate modification.**

**Table 8: Robustness results- Flow rate modification.**

| Flow rate (ml/min) | Sample name | Rt | Peak area | USP resolution | USP plate count | USP tailing |
|---|---|---|---|---|---|---|
| 0.9 | ATP | 1.463 | 912052 | | 6865 | 1.49 |
| 1.1 | ATP | 2.185 | 1363098 | | 8166 | 1.45 |
| 0.9 | DPH | 1.910 | 1885873 | 5.53 | 7769 | 1.34 |
| 1.1 | DPH | 2.829 | 2833813 | 5.82 | 9103 | 1.29 |

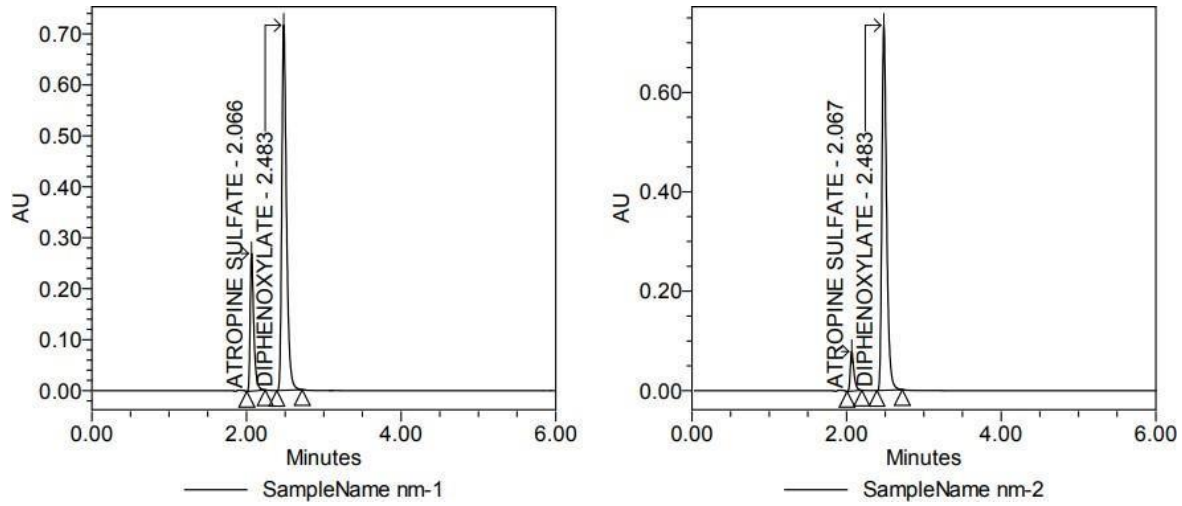



**Fig 16: Chromatograms showing robustness of ATP & DPH- Wavelength modification.**

**Table 9: Robustness results- wavelength modification.**

| Wavelength (nm) | Sample name | Rt | Peak area | USP resolution | USP plate count | USP tailing |
|---|---|---|---|---|---|---|
| 246 | ATP | 1.761 | 1584445 | | 7413 | 1.47 |
| 250 | ATP | 1.760 | 655507 | | 7446 | 1.48 |
| 246 | DPH | 2.310 | 2157770 | 5.82 | 8264 | 1.31 |
| 250 | DPH | 2.309 | 2343027 | 5.83 | 8326 | 1.32 |

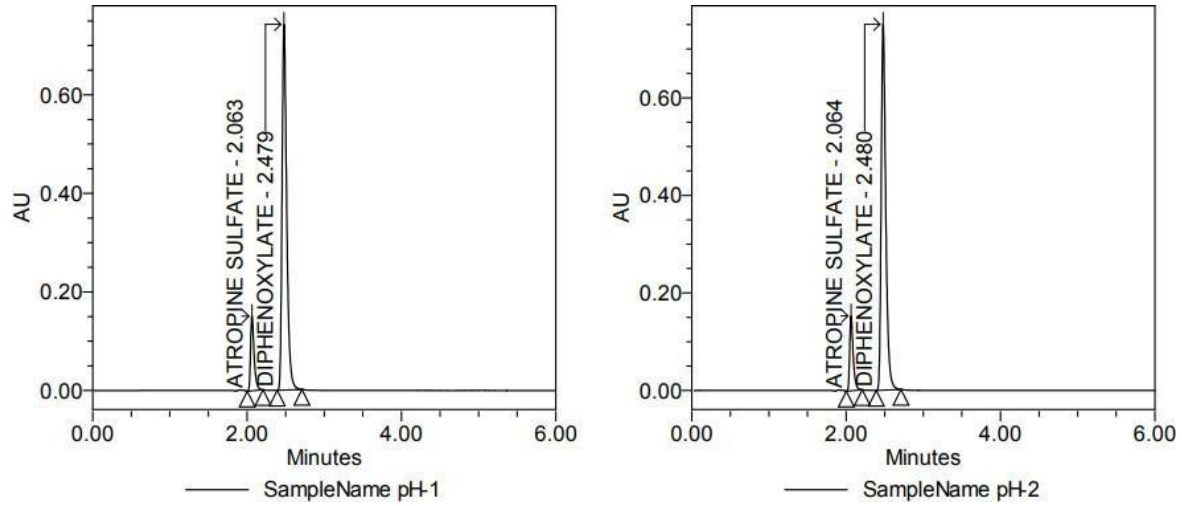



**Fig 17: Chromatograms showing robustness of ATP & DPH- pH modification.**

**Table 10: Robustness results - pH modificatio**

| pH | Sample name | Rt | Peak area | USP resolution | USP plate count | USP tailing |
|---|---|---|---|---|---|---|
| 5.3 | ATP | 1.758 | 1087453 | | 7532 | 1.48 |
| 5.7 | ATP | 1.757 | 1087998 | | 7370 | 1.48 |
| 5.3 | DPH | 2.305 | 2260741 | 5.87 | 8508 | 1.32 |
| 5.7 | DPH | 2.301 | 2257971 | 5.78 | 8343 | 1.32 |

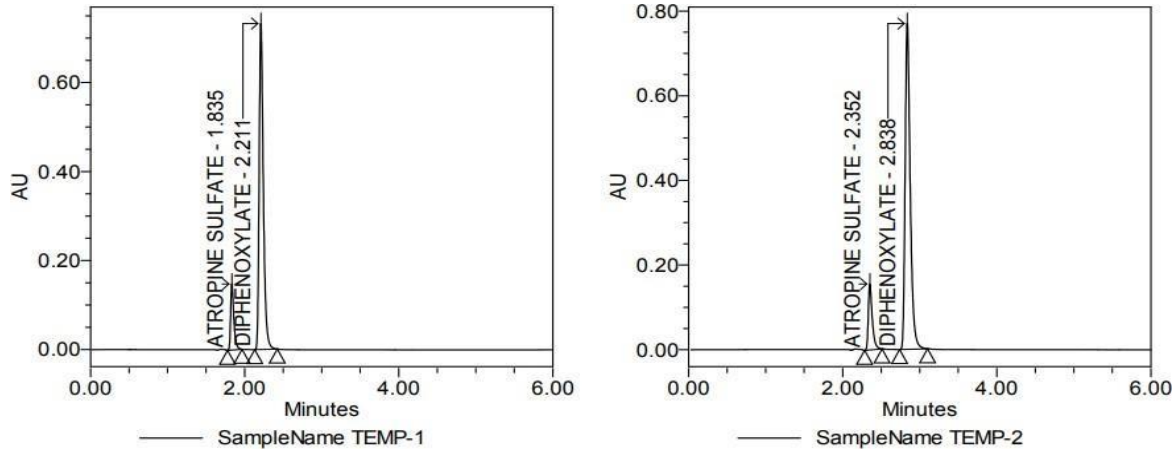



**Fig 18: Chromatograms showing robustness of ATP & DPH- Temperature modification**

**Table 11: Robustness results- Temperature modification.**

| Temperature (˚C) | Sample name | Rt | Peak area | USP resolution | USP plate count | USP tailing |
|---|---|---|---|---|---|---|
| 23 | ATP | 1.835 | 427785 | | 10059 | 1.49 |
| 27 | ATP | 2.352 | 552193 | | 10838 | 1.54 |
| 23 | DPH | 2.211 | 2755995 | 4.28 | 8346 | 1.38 |
| 27 | DPH | 2.838 | 3529827 | 4.50 | 9249 | 1.39 |

**Forced degradation studies**

Stability of the sample solutions were tested by exposing the working sample solutions of ATP& DPH to various conditions such as Acid, Base, Peroxide, Thermal (Heat), Moisture (Humidity) and Sunlight. The peak areas of ATP & DPH working Solutions before exposing to degradation and after degradation were collated and % amount degraded was evaluated. The results are enumerated in the table below.

**Table 12: Results for degradation studies of ATP & DPH Order of Stability.**

| Stress Applied | ATP | | | DPH | | |
|---|---|---|---|---|---|---|
| | Response | % Remained | % Degraded | Response | % Remained | % Degraded |
| **No stress** | 478585 | 100 | 0 | 3072146 | 100 | 0 |
| **Acid** | 429967 | 88.98 | 11.02 | 2792731 | 90.46 | 9.54 |
| **Base** | 443489 | 91.78 | 8.22 | 2858876 | 92.61 | 7.39 |
| **Peroxide** | 457409 | 94.66 | 5.34 | 2945450 | 95.41 | 4.59 |
| **Thermal** | 434899 | 90.00 | 10.00 | 2750192 | 89.09 | 10.91 |
| **Sunlight** | 445752 | 92.25 | 7.75 | 2829604 | 91.66 | 8.34 |
| **Humidity** | 477609 | 98.84 | 1.16 | 3060990 | 99.15 | 0.85 |

**ATP:** Acid > Thermal > Base > Sunlight > Peroxide > Humidity
**DPH:** Thermal > Acid > Sunlight > Base > peroxide > Humidity

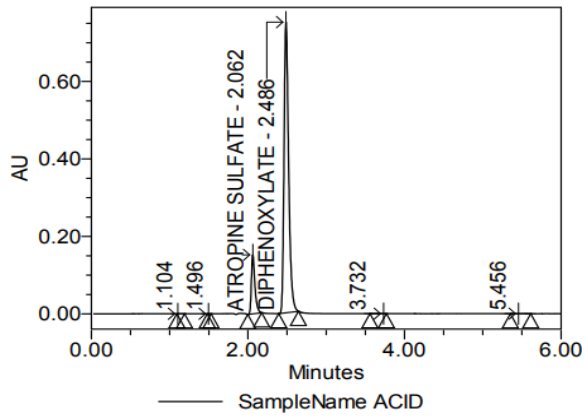



**Fig 19: Chromatogram showing acid degradation of ATP & DPH.**

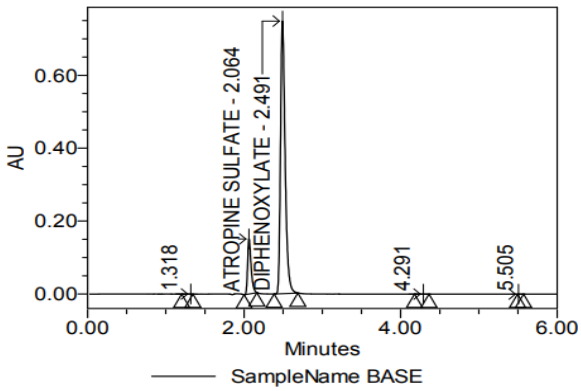



**Fig 20: Chromatogram showing base degradation of ATP & DPH.**

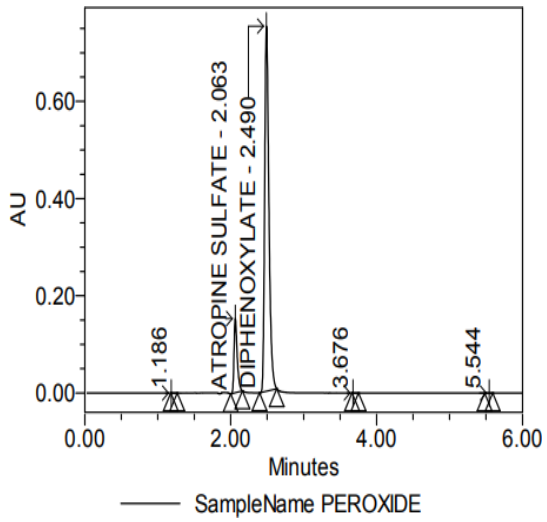



**Fig 21: Chromatogram showing peroxide degradation of ATP & DPH.**

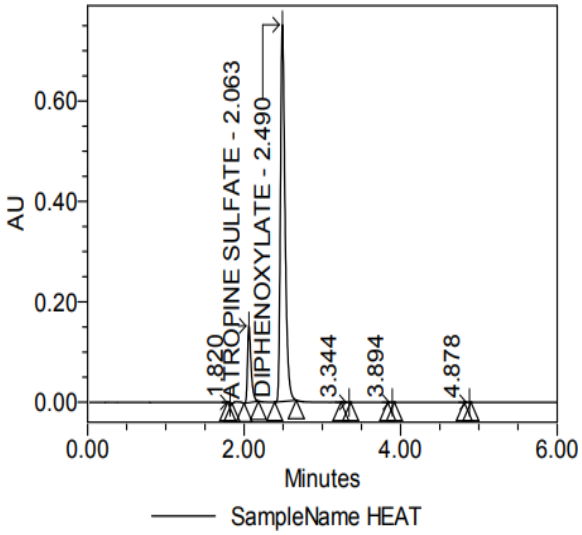



**Fig 22: Chromatogram showing thermal degradation of ATP & DPH.**

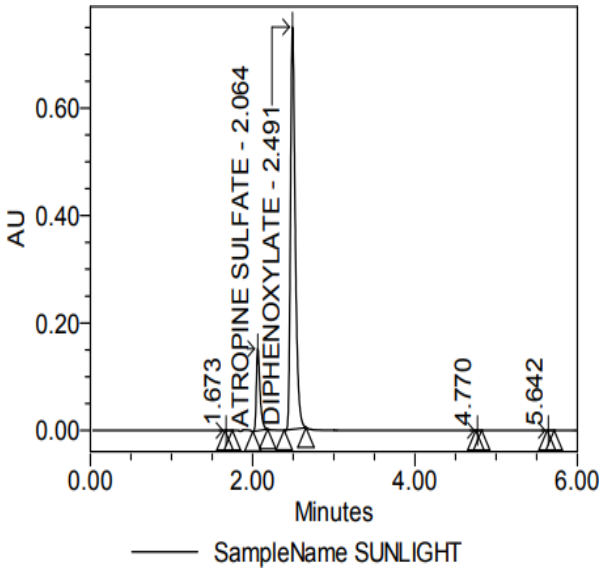



**Fig 23: Chromatogram showing light degradation of ATP & DPH.**

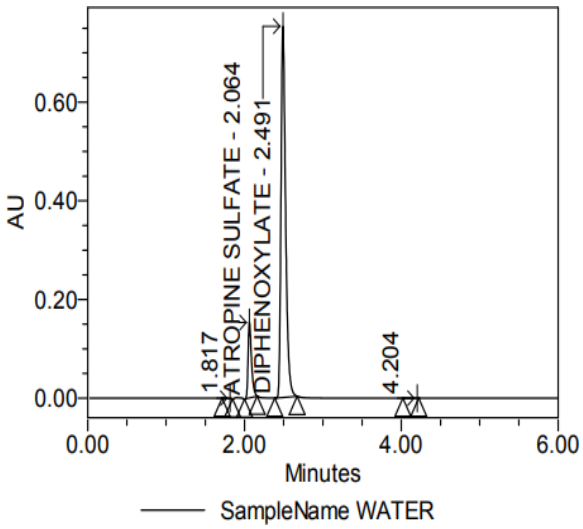



**Fig 24: Chromatogram showing moisture (water) degradation of ATP & DPH.**

## CONCLUSION

Development of method and its validation for the simultaneous estimation of Atropine and Diphenoxylate being the prime objective of the study was carried out by performing various trials using RP-HPLC method. Out of the total trials carried out, use of KROMASIL C18 column as a stationary phase and the solvent system employed being buffer and methanol in the ration of 50:50 showed all the system suitability parameters inside the acceptable criteria and hence considered as the optimized method. Validation of the developed method parameters was performed using the above optimized parameters. Forced degradation studies, indicative of stability of products on storage was carried out and the outcomes were within the limit. Hence the proposed method can be utilized for the routine simultaneous estimation of above said drugs.

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