

Robustness Study and Superior Method Development and Validation for Analytical Assay Method of Atropine Sulfate in Pharmaceutical Ophthalmic Solution

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How to cite this paper: Chowdhury, Md.N.S., Dalal, S.N., Islam, Md.A., Hossain, Md.A., Das, P.K., Hossain, S. and Das, P. (2024) Robustness Study and Superior Method Development and Validation for Analytical Assay Method of Atropine Sulfate in Pharmaceutical Ophthalmic Solution. *American Journal of Analytical Chemistry*, 15, 151-164.

<https://doi.org/10.4236/ajac.2024.155010>

Received: April 4, 2024

Accepted: May 14, 2024

Published: May 17, 2024

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Abstract

Background: The robustness is a measurement of an analytical chemical method and its ability to contain unaffected by little with deliberate variation of analytical chemical method parameters. The analytical chemical method variation parameters are based on pH variability of buffer solution of mobile phase, organic ratio composition changes, stationary phase (column) manufacture, brand name and lot number variation; flow rate variation and temperature variation of chromatographic system. The analytical chemical method for assay of Atropine Sulfate conducted for robustness evaluation. The typical variation considered for mobile phase organic ratio change, change of pH, change of temperature, change of flow rate, change of column etc. **Purpose:** The aim of this study is to develop a cost effective, short run time and robust analytical chemical method for the assay quantification of Atropine in Pharmaceutical Ophthalmic Solution. This will help to make analytical decisions quickly for research and development scientists as well as will help with quality control product release for patient consumption. This analytical method will help to meet the market demand through quick quality control test of Atropine Ophthalmic Solution and it is very easy for maintaining (GDP) good documentation practices within the shortest period of time. **Method:** HPLC method has been selected for developing superior method to Compen-

dial method. Both the compendial HPLC method and developed HPLC method was run into the same HPLC system to prove the superiority of developed method. Sensitivity, precision, reproducibility, accuracy parameters were considered for superiority of method. Mobile phase ratio change, pH of buffer solution, change of stationary phase temperature, change of flow rate and change of column were taken into consideration for robustness study of the developed method. **Results:** The limit of quantitation (LOQ) of developed method was much lower than the compendial method. The % RSD for the six sample assay of developed method was 0.4% where the % RSD of the compendial method was 1.2%. The reproducibility between two analysts was 100.4% for developed method on the contrary the compendial method was 98.4%.

Keywords

Robustness, Method Validation, HPLC, Compendial Method, Method Development, GDP, LOQ

1. Introduction

Atropine Sulfate is a FDA approved drug and its available as Atropine Sulfate Ophthalmic solution form [1]. The market demand of Atropine Sulfate is tremendously increasing all over the world. It is expected that the compound annual growth rate (CAGR) of Atropine Sulfate will be remarkable during the prognosis of year 2023-2030 [2]. So, the pharmaceutical manufacturing company are producing bulk amount of the Atropine Sulfate ophthalmic preparation to meet the customer demand as well as to serve people. Atropine Sulfate Ophthalmic solution is prescribed for immediate therapy for the myopia progression [3]. However, Atropine Sulfate has various ophthalmic strength like 1% w/v, 0.5% w/v, 0.05% w/v, 0.025% w/v, 0.01% w/v. Low concentration strength like 0.05% w/v, 0.025% w/v, 0.01% w/v are the most suitable for the treatment of myopia progression relative to the high dose strength [4].

Atropine Sulfate ophthalmic solution is a compendial HPLC method where two types of buffer solution containing sodium acetate, triethylamine and organic solvent methanol has been used [5]. The main drawback of this method is time consuming and expensive. The purpose of this study is to develop a cost effective, robust stability indicating validated and reproducible method for Atropine Sulfate Ophthalmic solution.

Atropine is an alkaloids originated from natural plant *Atropa belladonna* and Atropine sulfate is the polymorphism of Atropine [6]. Alkaloids are basic molecules containing nitrogen groups. Whereas, some of alkaloids also contain acidic molecule [7]. In order to develop the cost effective method pH of the molecule, buffer selection, mobile phase and column has taken into consideration.

2. Literature Review

Chowdhury reported on his research about superior HPLC method development

and validation of Rifampicin on pharmaceutical solid dosage form to compendial method. He changed the mobile phase ratio and pH for developing his method. He found accurate, precise and reproducible method for assay value determination. However, he did not evaluate the robustness study of his method to prove the method acceptability [8].

Kathrin Koll *et al.* described in their article about method validation of herbal products by high performance thin layer chromatography. They considered solution stability of analyte, selectivity/specificity, robustness and method reproducibility [9].

In order to prove the stability indicating and validation of analytical method LOQ, LOD, accuracy, repeatability, linearity, robustness, specificity/selectivity need to perform [10].

Velusamy B *et al.* narrated about their research. They designed for the analytical method for the quantification of nine impurities of apixaban and they successfully completed the validation. They also ensured the robustness of the analytical method [11].

O. Szerkus *et al.* developed and validated a HPLC-MS method for the quantification of levofloxacin and ciprofloxacin in human prostate biotates and completed the method robustness study [12].

3. Purpose

The main goal of this study is to develop a cost effective, robust HPLC method development by QbD approach for Atropine Sulfate Ophthalmic Solution by maintaining good documentation practices. It will be helpful for both the researcher and also useful for quality control scientist of pharmaceutical industry. We will develop the cost effective robust method appropriately considering analytical target profile as per method development guidelines [13].

4. Materials and Methods

Analytical assay method for Atropine Sulfate Ophthalmic solution of Compendial method was used for understanding the analytical target profile (ATP). On the other hand, superior HPLC method was developed based on pH, pKa, molecular weight, pH dependent solubility of Atropine Sulfate molecule. Atropine Sulfate Ophthalmic solution 0.05% w/v and placebo were taken as a gift from local pharmaceutical company of Bangladesh.

Study Design

Initial Assessment

pH of Atropine Sulfate

pH of 0.0015 molar solution 10.0 [14].

Solubility of Atropine Sulfate

Atropine Sulfate is very soluble in water, freely soluble in alcohol and even more in boiling alcohol; freely soluble in glycerin [15].

Molecular Weight of Atropine Sulfate

289.4 g/mol [16]

pKa of Atropine Sulfate

pKa = 9.43 [17]

Selection of Buffer Solution pH

The buffer solution is most effective within ± 1 pH. However, the buffer solution provides much more buffering capacity within ± 2 pH units from the pKa [18]. Based on the buffering capacity the pH of buffer solution for mobile phase may be 7 or 11 ($\text{pH} = 9.43 \pm 2$). Based feasibility of stationary phase pH 7.0 has been selected for pH of buffer solution of mobile phase.

Selection of Buffer [19]

Phosphate buffer has a buffering capacity from pH 6.2 to pH 8.2 (Figure 1). So, phosphate buffer has been selected for mobile phase preparation. 10 mM to 50 mM is the usual concentration for phosphate buffer solution. However, 50 mM will provide sharp peak with good resolving capacity. So, 50 mM concentration has been selected for buffer solution preparation [18].

Selection of Organic Solvent [18]

The solubility of phosphate buffer salt is poorer in acetonitrile and worst in tetrahydrofuran. But the solubility is relatively high in methanol. At 80% of organic solvent of acetonitrile, only 5 mM of phosphate buffer is soluble where 15 mM of phosphate buffer is soluble at methanol. For this reason, methanol has been selected for organic solvent.

Column Characterization [20]**Selection of Stationary Phase**

Less than 5000 Molecular Weight is considered solubility. C18 suits water-soluble compounds; other options exist for organic-soluble or chiral molecules.

Higher than 5000 Molecular Weight is Chosen based on solubility; reverse phase chromatography remains a top choice. The molecular weight of Atropine sulfate is 289.4 and the mobile phase is polar. So, reverse phase c18 column has been selected for its suitability to the intended purpose.

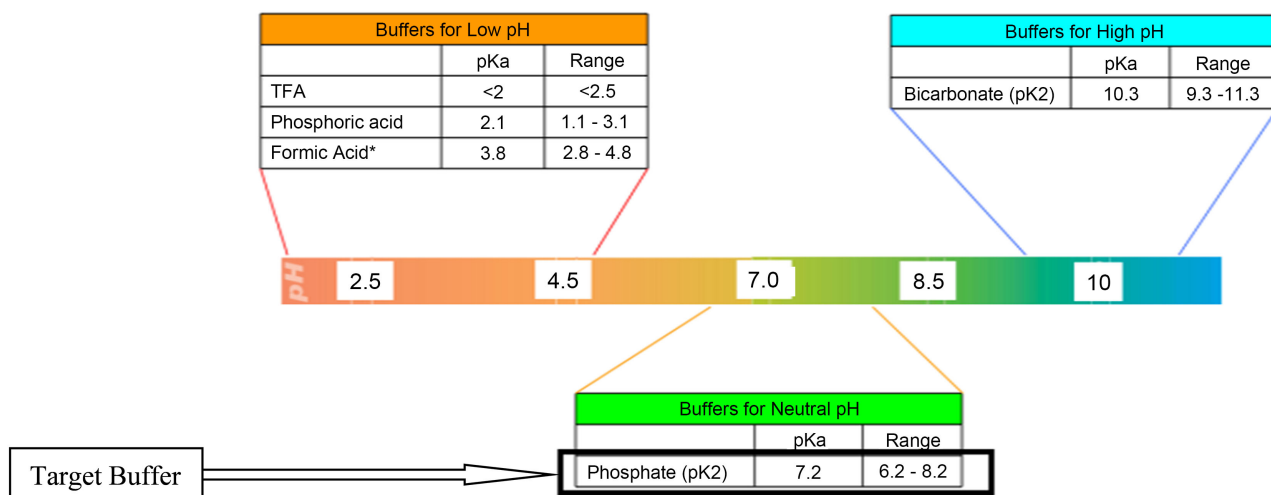


Figure 1. Buffering capacity of phosphate buffer.

Selection of Particle Size

Lower particle size provides higher efficiency. However, 5 - 10 μm particle size is the common use for analytical chemistry. So, 5 μm particle size has been selected for column.

Selection of Column Dimension and Length

Larger internal diameter columns (≥ 4 mm) is excelled in high sample loading capacity and standard HPLC compatibility but consume more solvent, offer lower sensitivity, and lack LC-MS compatibility. For this reason, 3 mm inner dimension has been selected for column. Lower length of column helps to elute the target molecule quickly. So, 150 mm length of column has been selected.

Selection of Wavelength

Compensial wavelength 225 nm was selected

Method Validation [21]

Instrument Information

Laboratory instruments such as Analytical Balance (Sartorius, Switzerland), Semi micro Balance (Metler Toledo, Switzerland), Micro Balance (Metler Toledo, Switzerland), High Performance Liquid Chromatography with PDA detector (Waters Alliance, USA, pH meter (Metler Toledo, Switzerland)), were used for this study.

Chemical Reagents and Standard

Potassium dihydrogen phosphate (Sharlau, Spain), Sodium hydroxide pellets (Merck, Germany), Sodium acetate trihydrate (Sharlau, Spain), Acetonitrile (Sharlau, Spain), Methanol (Sharlau, Spain), Distilled water (Ultrapure) were used. All the reagents were used analytical grade. The reference standard of Atropine Sulfate was obtained from a local pharmaceuticals as a gift sample for research. The purity of Atropine sulfate reference standard was 98.9% on as is basis.

Chromatographic Condition

HPLC column (3.0-mm \times 150 mm; 5- μm packing C18), injection volume (10 μL), wavelength (225 nm), flow rate (1.0 mL/min) were attributed as instrument parameter for operation.

Methodology

Preparation of Buffer Solution

Dissolved 6.812 g of Potassium dihydrogen phosphate into 1000 mL of purified water. Adjusted pH 7.02 by diluted sodium hydroxide solution.

Note: Buffer solution prepared at room temperature

Preparation of Optimized Mobile Phase

Mixed 400 mL of buffer solution and 600 mL of methanol to make 1000 mL of mobile phase. Degassed mobile phase by ultrasonic water bath. Filtered the mobile phase using 0.2 μm membrane filter.

Preparation of Standard Stock Solution

Taken 50.12 mg of Atropine Sulfate standard into 50 mL volumetric flask. Added 30 mL of purified water and sonicated for 5 minutes with intermittent shaking. Cooled to room temperature. Diluted up to the mark with purified water.

Preparation of Standard Solution

Taken 10.0 mL of the solution into 100 mL volumetric flask and diluted up to the mark with purified water. Used 0.45 μ m PTFE syringe filter for transferring the standard solution into HPLC system.

Concentration: 0.1 mg/mL as Atropine sulfate

Note: Standard solution prepared at room temperature

Preparation of Selectivity/Specificity sample**Placebo Preparation**

Taken 2.0 mL of placebo for Atropine sulfate 0.05% w/v into 100 mL volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking. Used 0.45 μ m PTFE syringe filter for transferring the placebo solution into HPLC system.

Spiked Sample Preparation

Taken 2.0 mL of placebo for Atropine sulfate 0.05% w/v and 1.0 mL of standard stock solution into 10 mL volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking. Used 0.45 μ m PTFE syringe filter for transferring the spiked sample solution into HPLC system.

Concentration: 0.1 mg/mL as Atropine sulfate

Preparation of Repeatability (Precision) Sample

Taken 2.0 mL of Atropine sulfate 0.05% w/v into 10 mL volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking. Used 0.45 μ m PTFE syringe filter for transferring the sample solution into HPLC system. Prepared another five sample solution following precision sample.

Concentration: 0.1 mg/mL as Atropine sulfate

Preparation of Linearity Sample**50% Linearity Solution**

Taken 1.0 mL of standard stock solution into 10 volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking.

Concentration: 0.05 mg/mL as Atropine sulfate

80% Linearity Solution

Taken 0.8 mL of standard stock solution into 10 volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking.

Concentration: 0.08 mg/mL as Atropine sulfate

100% Linearity Solution

Taken 1.0 mL of standard stock solution into 10 volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking.

Concentration: 0.1 mg/mL as Atropine sulfate

120% Linearity Solution

Taken 1.2 mL of standard stock solution into 10 volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking.

Concentration: 0.12 mg/mL as Atropine sulfate

150% Linearity Solution

Taken 1.5 mL of standard stock solution into 10 volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking.

Concentration: 0.15 mg/mL as Atropine sulfate

Used 0.45 μ m PTFE syringe filter for transferring the Linearity sample solution into HPLC system.

Preparation of Accuracy Sample

80% Accuracy Sample

Taken 1.6 mL of placebo for Atropine sulfate 0.05% w/v and 0.8 mL of standard stock solution into 10 mL volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking. Prepared another two sample solution as per 80% accuracy sample.

Concentration: 0.8 mg/mL as Atropine sulfate

100% Accuracy Sample

Taken 2.0 mL of placebo for Atropine sulfate 0.05% w/v and 1.0 mL of standard stock solution into 10 mL volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking. Prepared another two sample solution as per 100% accuracy sample.

Concentration: 0.1 mg/mL as Atropine Sulfate

120% Accuracy Sample

Taken 2.4 mL of placebo for Atropine sulfate 0.05% w/v and 1.2 mL of standard stock solution into 10 mL volumetric flask. Dilute up to the mark with purified water. Mixed well by handshaking. Prepared another two sample solution as per 120% accuracy sample.

Concentration: 0.12 mg/mL as Atropine Sulfate

Robustness Study Using Youden's Test [22]

The robustness evaluation of the chromatographic method for the Atropine Sulfate determination in Pharmaceutical Ophthalmic solution was performed using the method proposed by Youden e Steiner in 1975. Five analytical parameters were identified and small variations were included in the nominal values of the method. Then, ten runs were conducted targeting to determine the influence of each parameter in the final result. **Table 1** represents the identified parameters for robustness, actual condition and typical variation condition of the parameters for Atropine Sulfate and **Table 2** narrates the design for the factorial combination of identified parameters for 10 run into HPLC system.

Table 1. Analytical parameters and variations for the robustness evaluation of atropine sulfate.

Parameters		Nominal Condition		Typical Variation	
A/a	Methanol concentration in mobile phase	60%	A	55%	a
A/a'				65%	a'
B/b	Buffer solution pH	7.00	B	6.95	b
B/b'				7.05	b'
C/c	Column temperature	25°C	C	20°C	c
C/c'				30°C	c'
D/d	Mobile Phase Flow rate	1.0 mL/min	D	0.95 mL/min	d
D/d'				1.05 mL/min	d'
E/e	Methanol supplier	Sharlau	E	RCI labscan	e

Table 2. Factorial combination for robustness evaluation of atropine sulfate.

Parameters	1	2	3	4	5	6	7	8	9	10
Methanol concentration in mobile phase	A	A	a	a'	A	A	A	A	A	A
Buffer solution pH	B	b	B	B	b'	B	B	B	B	B
Column temperature	C	C	C	C	C	c	C	C	C	c'
Mobile Phase Flow rate	D	D	D	D	D	D	d	D	d'	D
Methanol supplier	E	E	E	E	E	E	E	e	E	E

5. Results and Discussion

It was observed that no peak was eluted at the elution zone of Atropine Sulfate for blank and placebo solution and the purity angle of Atropine sulfate was lower than the purity threshold. So, the peak is pure and the method is specific for Atropine sulfate identification (**Table 3**). The developed method was precise enough to provide reproducible results. The % RSD for the six sample solutions is 1.3 (**Table 4**). It is very important to ensure the accuracy of an analytical method to confirm the method's accuracy for target molecule quantification. The accuracy results of nine sample solutions were satisfactory and the 95% confidence interval of the nine sample solution was between 98.97% and 101.10% (**Table 5**). It ensures that whatever the number of analysis for the assay quantification used, the method's accuracy would be contained between this confidence interval limits. Linearity is another parameters for a method analytical target profile (ATP). It disclosed the minimum and maximum range of concentration for an analytical method for a molecule. If the concentration result out of limit of both the minimum and maximum concentration then the outcome will be unacceptable. So, linearity study is vital part for an analytical method. The linearity study of Atropine Sulfate developed method was performed from 50% to 150% of working concentration and found satisfactory results. The correlation coefficient value for linearity study of Atropine Sulfate was 0.999 (**Table 6**). Both compendial method and developed method was used for Atropine Sulfate repeatability and other analytical target profile (ATP) parameters. It was found satisfactory for both method but the developed method was superior (**Table 7**). However, the retention time of developed method is 2.345 minutes and the retention of compendial method is 11.175 minutes. Though, both method provides satisfactory results, the developed method is superior to compendial method due to least amount reagent in mobile phase (cost effective) and short time for operation. **Figure 2** and **Figure 3** represents the specimen chromatogram for developed method and compendial method.

Robustness is the integral part of method development and validation. Five parameters were taken into consideration for robustness study and ten analysis were performed with the variation of five parameters. All of the ten runs is found satisfactory. But the assay value for condition number 8 found low relative

to others (97.4%) (Table 8). Table 9 disclosed that the average value of typical variation for the robustness study was subtracted from the original condition for the assay, retention time, tailing factor and theoretical plate of Atropine Sulfate. The typical maximum difference value for % assay for the supplier variation of methanol was high 1.67%. Moreover, the maximum typical difference value for retention time, tailing factor and theoretical plate was for 0.67%, 0.03 and 150. So, the developed method is robust in terms of mentioned design study.

Table 3. Specificity results.

Sample Name	RT in minutes (Atropine Sulfate)	Purity Angle	Purity Threshold
Blank	-	-	-
Placebo	-	-	-
Spiked Solution	2.345	0.101	0.235

Table 4. Repeatability results of atropine sulfate in ophthalmic solution.

Sample Name	% Assay (Atropine Sulfate)	Average (%)	% RSD
1	98.912	99.529	1.3
2	97.421		
3	101.234		
4	100.245		
5	99.234		
6	100.128		

Table 5. Accuracy results of atropine sulfate in ophthalmic solution.

Sample Name	% Recovery	Average (%)	95% Confidence Interval	
			Lower limit	Upper Limit
80% Accuracy-1	99.51	98.94	98.97	100.10
80% Accuracy-2	98.43			
80% Accuracy-3	98.89			
100% Accuracy-1	99.41	99.15	98.97	100.10
100% Accuracy-2	99.31			
100% Accuracy-3	98.74			
120% Accuracy-1	101.21	100.51	98.97	100.10
120% Accuracy-2	100.44			
120% Accuracy-3	99.87			

Table 6. Linearity results of atropine sulfate in ophthalmic solution.

Sample Name	Concentration (mg/mL)	Concentration (%)	Area (AU)	Co relation coefficient, R
1	0.05	50	500,240	0.999
2	0.08	80	815,123	
3	0.1	100	1,001,240	
4	0.12	120	1,204,532	
5	0.15	150	1,545,623	

Table 7. Comparison between compendial method and USP Results.

Parameters	Compendial Method	Developed Method
Specificity by RT	11.175 minutes	2.345 minutes
Repeatability (average assay)	98.461	99.529
Linearity (R)	0.999	0.999
Accuracy (average)	99.812	99.534
Accuracy (% RSD)	1.2	0.9

Table 8. Results for 10 analyses performed for robustness evaluation of Atropine Sulfate.

Condition	Area		Retention Time		Tailing Factor		Theoretical Plate		% Assay
	Sam	Std	Sam	Std	Sam	Std	Sam	Std	
1	1,011,240	1,031,445	2.34	2.32	1.01	1.02	6123	6012	99.8
2	1,023,241	1,071,049	2.11	2.10	1.01	1.03	6250	6125	99.5
3	1,001,241	1,091,247	3.12	3.33	1.01	1.00	5124	5945	99.2
4	1,061,242	1,011,543	1.94	1.99	1.06	1.04	5874	5812	98.5
5	1,091,242	1,051,741	4.16	4.25	1.05	1.01	5324	5463	98.6
6	1,071,245	1,041,440	2.41	2.38	1.08	1.02	6124	5945	99.1
7	1,061,277	10,918,242	3.42	3.40	1.09	1.03	5712	5842	99.9
8	1,041,210	1,071,544	2.37	2.35	1.06	1.02	5924	5621	97.4
9	1,071,211	1,081,346	2.01	2.00	1.08	1.04	5824	6231	98.7
10	1,031,288	1,021,848	2.27	2.25	1.05	1.02	5128	5765	98.3

Table 9. Effect of analytical parameters in content for the robustness evaluation of atropine sulfate considering RT, N, assay value.

Parameters	% Assay*	RT*	Tailing factor*	N*
Methanol concentration A = 60%; a = 55%, a' = 65%	98.91 – 99.2 = 0.2	2.63 – 3.23 = –0.6	1.04 – 1.03 = 0.01	5838 – 5688 = 150
Buffer solution pH	98.86 – 99.05 = –0.19	2.49 – 3.16 = –0.67	1.04 – 1.03 = 0.01	5813 – 5791 = 22
Column temperature	98.95 – 98.70 = 0.25	2.70 – 2.33 = 0.37	1.04 – 1.04 = 0.00	5825 – 5740 = 85
Mobile Phase Flow rate	98.80 – 99.30 = –0.5	2.61 – 2.71 = –0.10	1.03 – 1.06 = – 0.03	5785 – 5775 = 10
Methanol supplier	99.07 – 97.4 = 1.67	2.66 – 2.36 = 0.3	1.04 – 1.04 = 0.00	5812 – 5773 = 39

*Average values of the nominal condition—average value of typical variation.

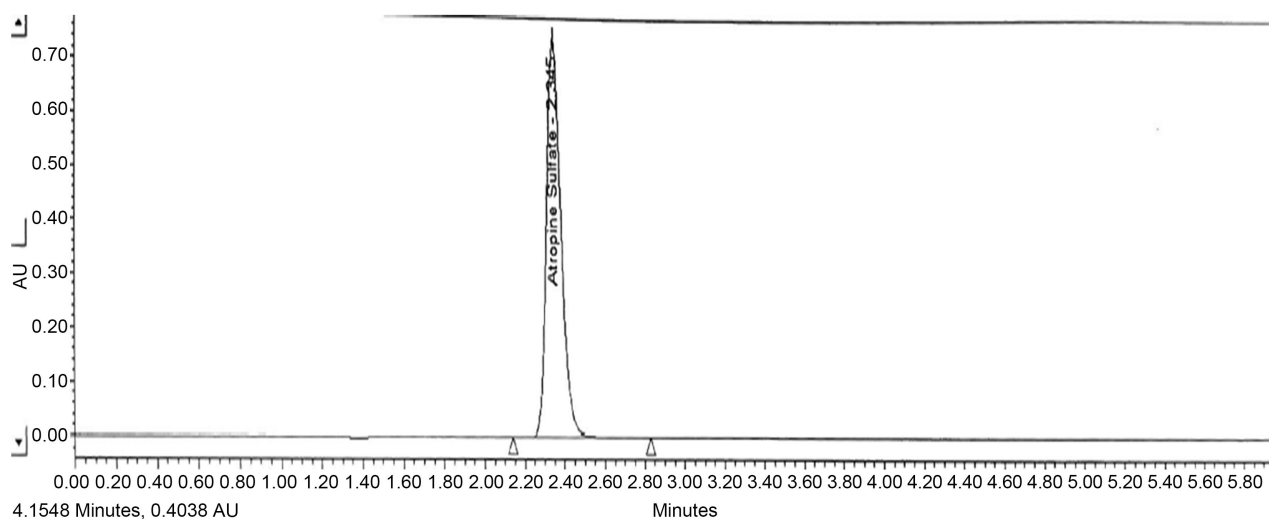


Figure 2. Specimen chromatogram of developed method.

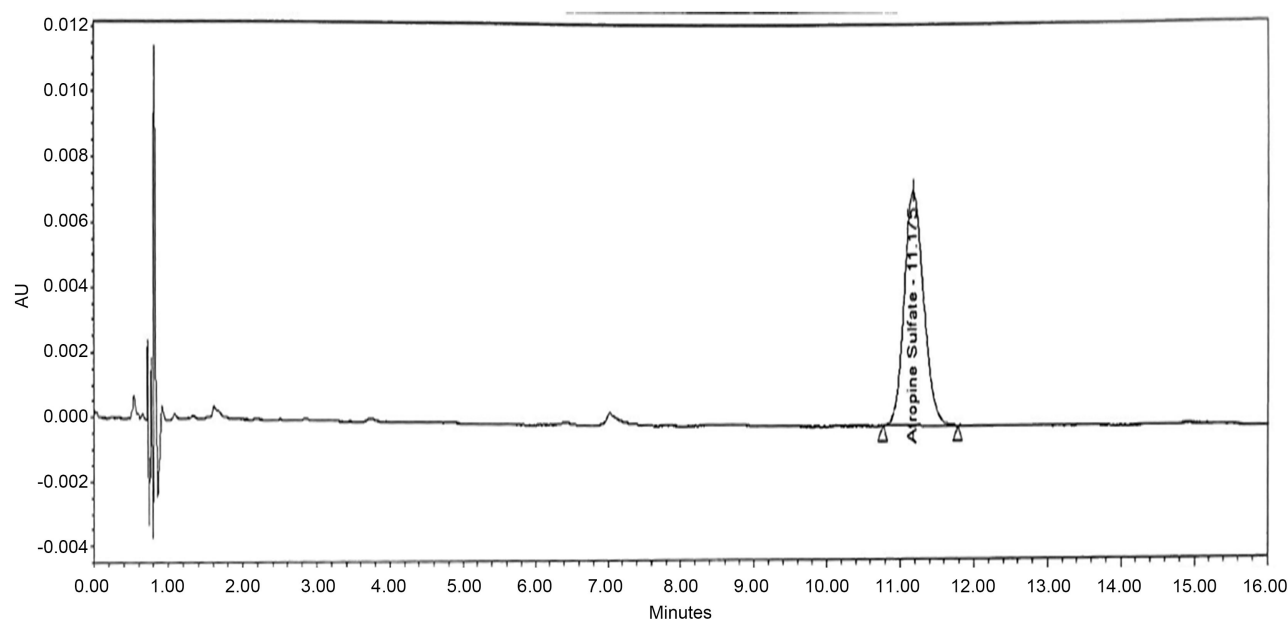


Figure 3. Specimen chromatogram of compendial method.

6. Conclusions

In order to provide a cost effective, least time, robust and superior method development and validation, this research was performed. This developed robust method will be helpful for the pharmaceutical manufacturing company, quality control scientist and researchers. It can also be used as a study material for method development for the students.

The data summarizes that the developed method is superior to the compendial method based on time consuming and cost. This information will also help for drug control authority about the nature of this developed method. This method is developed only for Atropine Sulfate. It will be better if the method was developed for combined product with Atropine Sulfate.

Funding

This research was conducted from the self fund for purchasing and collecting sample, reagent and standard.

Acknowledgements

The research proposal and plan was initiated by Md Nazmus Sakib Chowdhury and the maximum amount of publication fees were provided by Sreekanta Nath Dalal, Md Ariful Islam, Anwar Hossain, Pranab Kumar Das, Shakawat Hossain and Parajit Das.

Authors' Contribution

This research was designed by Md Nazmus Sakib Chowdhury and performed by Md Nazmus Sakib Chowdhury, Md Ariful Islam, Anwar Hossain, Pranab Kumar Das, Shakawat Hossain and Parajit Das. Sreekanta Nath Dalal was observed the analysis and reviewed the article. All authors drafted the article.

Conflicts of Interest

The authors disclose no conflicts of interest regarding the publication of this paper.

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Abbreviation

ATP: Analytical target profile

HPLC: High performance liquid chromatography

RT: Retention time

N: Theoretical plate

RSD: Relative standard deviation

mg/mL: Milligram per milliliter

g: Gram

PTFE: Ploy tetrafuluro ethylene

µm: Micro metre

LOD: Limit of detection

LOQ: Limit of quantitation

nm: Nano metre

Sam: Sample

Std: Standard