

Development and validation of Stability indicating RP-HPLC method for the estimation of Glycopyrrolate and Neostigmine in bulk and tablet dosage form

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Abstract:

The aim of the proposed research work was to develop and validate a simple, selective and sensitive method for the analysis of Glycopyrrolate and Neostigmine in bulk and tablet dosage form. In this paper, a simple and reliable HPLC method was developed and validated for the evaluation of Glycopyrrolate and Neostigmine. A Luna C-18 analytical column, 4.6 mm × 250 mm packed with 5 μm particle size containing mixture of Water: Acetonitrile (70:30, v/v) was used. The flow rate was 1.0 mL min⁻¹ and the UV detection was performed at 255 nm. The retention times of Glycopyrrolate and Neostigmine were 2.90min and 4.71min respectively. Linearity range of Glycopyrrolate and Neostigmine were in the range of 0.20 - 3.03 mg mL⁻¹ and 1.0 – 15.02 mg mL⁻¹ respectively. The proposed method was validated for linearity, accuracy, precision, robustness of Glycopyrrolate and Neostigmine. The RSD values for all parameters were found to be less than 2, which indicates the validity of method and results obtained by this method are in fair agreement. Finally this method can be used for better analysis and pharmaceutical formulations Glycopyrrolate and Neostigmine drug.

Key words: Glycopyrrolate and Neostigmine, Validation, Evaluation and formulation.

1. Introduction

Glycopyrronium Bromide is a quaternary ammonium anticholinergic agent. The quaternary ammonium moiety renders Glycopyrronium Bromide highly ionised at physiological pH and it thus penetrates the blood brain and placental barriers poorly. Glycopyrronium Bromide has a more gradual onset and longer duration of action than atropine.

Neostigmine Metilsulfate is a quaternary ammonium anticholinesterase. Glycopyrronium Bromide and Neostigmine Metilsulfate Injection is associated with less initial tachycardia and better protection against the subsequent cholinergic effects of Neostigmine Metilsulfate than a mixture Of Atropine and Neostigmine Metilsulfate. Neostigmine is used mainly for its effects on skeletal muscle in myasthenia gravis and in anesthesia

for termination of the effects of competitive neuromuscular blocking drugs.

In addition, residual central anticholinergic effects are minimised due to the limited penetration of Glycopyrronium Bromide into the central nervous system. Administration of Glycopyrronium Bromide with Neostigmine Metilsulfate is associated with greater cardiostability than administration of Glycopyrronium Bromide and Neostigmine Metilsulfate separately.

Glycopyrronium Bromide and Neostigmine Metilsulfate are routinely administered simultaneously to reverse residual non-depolarising (competitive) neuromuscular block. Numerous clinical studies, which demonstrate this to be a safe and effective combination, have been published.

Over 90% of the Glycopyrronium Bromide disappears from serum within 5 minutes following intravenous administration. The drug is rapidly excreted into bile with highest concentrations being found 30 to 60 minutes after dosing with some product being detected up to 48 hours after administration.

Glycopyrronium Bromide is also rapidly excreted into urine with the highest concentrations being found within 3 hours of administration. Over 85% of product is excreted within 48 hours. It has subsequently been confirmed in a single dose pharmacokinetic study using radio immunological assay procedures that Glycopyrronium Bromide was rapidly distributed and/or

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excreted after intravenous administration. The terminal elimination phase was relatively slow with quantifiable plasma levels remaining up to 8 hours after administration. The elimination half-life was 1.7 hours.

Neostigmine Metilsulfate is extensively hydrolyzed in the blood. In one study, following intravenous administration, the plasma concentration declined to about 8% of its initial value after 5 minutes with a distribution half-life of less than one minute.

Elimination half-life ranged from about 15-30 minutes. Trace amounts of Neostigmine Metilsulfate could be detected in the plasma after one hour. In a study in non-myasthenic patients, the plasma half-life was 0.89 hours.

Glycopyrrolate chemically known as (3S)-3-[[2-(2R)-cyclopentyl-2-hydroxy-2-phenylacetyl]oxy]-1,1-dimethylpyrrolidin-1-ium (Figure:1). Molecular formula $C_{19}H_{28}NO_3$

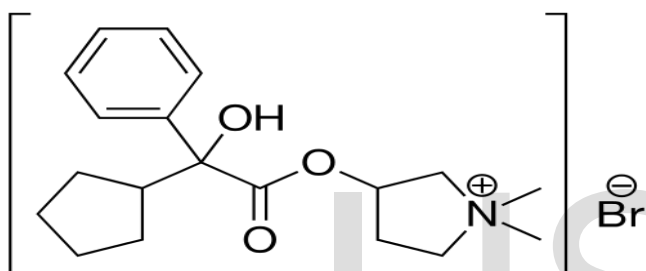


Figure 1: Chemical structure of Glycopyrrolate

Neostigmine chemically known as 3-[[dimethylcarbamoyl]oxy]-N,N,N-trimethylanilinium (Figure:2). Molecular formula $C_{12}H_{19}N_2O_2$

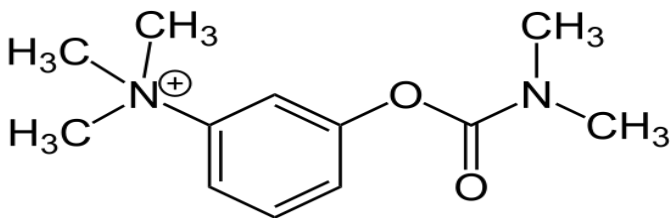


Figure 2: Chemical structure of Neostigmine

2. Experimental

2.1 Chemicals and reagents

All the reagents used in the experimental work were of analytical grade. HPLC grade water was prepared by Milli-Q reverse osmosis (Millipore, Bedford, USA) and meets European Pharmacopoeia requirements. Acetonitrile (Sigma-Aldrich, Merck and Rankem) were used for preparing the mobile phase. Mobile Phase was used as solvent.

Working standards of Glycopyrrolate and Neostigmine were provided by Glenmark Pharmaceuticals (Mahape, Navi Mumbai). Glycopyrrolate and Neostigmine was checked by comparison with European Pharmacopoeia CRS standards. Formulation was obtained from.

2.2 Chromatographic conditions (instrumentation and analytical conditions)

An Alliance 2695 (Waters, USA) chromatographic system was used, equipped with a Quaternary pump, and waters 2996 photo diode array detector, Luna C18 column, auto sampler thermostat and degasser. Chromatographic software Empower was used for data collection and processing. Separations were performed using Luna C-18 analytical column, 4.6 mm × 250 mm packed with 5 μm particle size. A 1m long steel capillary with 0.25 mm internal diameter, was inserted between the injection system and the entrance of the column, and injection volume was 10 μL. Separations and simultaneous determination of Glycopyrrolate and Neostigmine were performed using the mixture of Water : Acetonitrile (70:30, v/v) as a mobile phase. Mobile phase was filtered through a 0.45 μm Millipore filter. The flow rate was 1.0 mL min⁻¹ and the UV detection was performed at 255 nm.

3. Preparation of solutions

3.1 Standard solution preparation

Solution-A : Weigh accurately about 20 mg of Glycopyrrolate working standard are taken into a 100 mL volumetric flask. Add 70 mL of diluent, sonicate to dissolve and dilute to volume with diluent.

Solution-B : Weigh accurately about 100 mg of Neostigmine working standard are taken into a 100 mL volumetric flask. Add 70 mL of diluent, sonicate to dissolve and dilute to volume with diluent. Further dilute each 5mL of solution-A&B to 50 mL with the diluent. Further dilute 5mL of standard stock solution- to 50 mL with the diluent.

3.2 Assay sample preparation

Weigh about 15 mg of sample taken into a 100 mL volumetric flask. Add 70 mL of diluent, sonicate to dissolve and dilute to volume diluent. Further dilute 5 mL to 50 mL with the diluent. Filter through 0.45μ Nylon syringe filter.

4. Validation procedure

Chromatographic separation was optimized in the aim to obtain a resolution above 1.5 between all components, with the respect of stationary and mobile phase compositions, flow rate, sample volume, detection wavelength and temperature.

The method was validated for linearity, range, precision (repeatability and intermeditated precision), specificity, limit

of quantization, limit of detection, robustness and forced degradation.

4.1 Linearity and range

Standard calibration curves were prepared with five calibrators over a concentration range of 0.20–3.03 mg mL⁻¹ for Glycopyrrolate and 1-15.02 mg mL⁻¹ for neostigmine. The data of peak area versus drug concentration were treated by linear least square regression analysis. The standard curves were evaluated for linearity.

4.2 Precision

The precision of the assay was studied with respect to both repeatability and intermediated precision. Repeatability was calculated from six replicate injections of freshly prepared solution in the same equipment on the same day. Repeatability for Glycopyrrolate and Neostigmine was realized with a 20 and 100 µg mL⁻¹ solution. The experiment was repeated by assaying freshly prepared solution at the same concentration on 2 additionally consecutive days to determine intermediate precision. Precision was expressed by the % of the relative standard deviation (R.S.D.) of the analyte peaks.

4.3 Specificity

Specificity of a method can be defined as absence of any interference at retention times of peaks of interest, and was evaluated by observing the chromatograms of blank samples and samples spiked with Glycopyrrolate and Neostigmine. The variable number of excipient used in generic versions of Glycopyrrolate and Neostigmine, as well as the lack of information in the composition of some generic formulations makes it difficult to assess selectivity by traditional analysis comparison with a placebo solution.

4.4 Limits of detection and quantization

Limits of detection (LOD) and limits of quantization (LOQ) were provided and calculation was made with the following equations:

$$LOD = 3.3 \sigma / S$$

$$LOQ = 10 \sigma / S$$

When σ was the standard deviation of the response (estimated from the standard deviation of y - intercepts or regression lines) and S was the slope of the standard curve.

4.5 Sensitivity

The sensitivity (6σ) of an analytical method is defined by the minimum variation that requires to be applied to the magnitude measured in order to obtain a significant variation in the signal measured.

4.6 Robustness

Robustness of method was investigated by varying the

chromatographic conditions such as change of flow rate ($\pm 20\%$), organic content in mobile phase ($\pm 2\%$), wavelength of detection ($\pm 5\%$). Robustness of the developed method was indicated by the overall %RSD between the data at each variable condition.

4.7. Forced degradation

Forced degradation should be no interference between the peaks obtained for the chromatogram of forced degradation preparations. The degradation peaks should be well separated from each other and the resolution between the peaks should be at least 1.0 and the peak purity of the principal peaks shall pass.

4.8 Stability

Stability by preparing the analytical solution and injecting at periodic intervals of 24 hours to 48 hours at 3 to 4 hour intervals depending on the instrument utilization and sequence of injection.

5. Results and discussion

In this paper, we developed the reverse phased column procedure for a suitable method for the pharmaceutical analysis of Glycopyrrolate and Neostigmine drug and tablets. A typical chromatogram obtained by using the mobile phase. The precision, accuracy and forced degradation of the method was determined from Glycopyrrolate and Neostigmine dosage form and obtained. Inter and intra- day studies were performed in three concentrations of the drug was reported on three consecutive days.

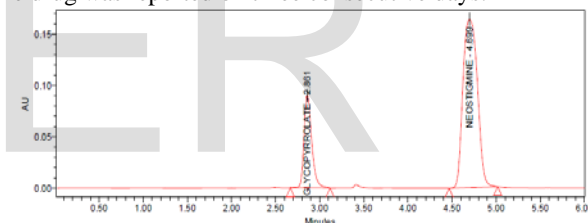


Figure 3: Typical Chromatogram

6. METHOD VALIDATION

The method was validated for linearity, precision, accuracy, robustness, ruggedness, forced degradation and stability of the method was studied by the Glycopyrrolate and Neostigmine.

Linearity was prepared in the range of 0.20-3.03 µg/ml and 1-15.02 µg/ml solutions are analyzed through the high pressure liquid chromatographic technique. The peak area were plotted against concentration was subjected to linear plots (Figure:4 and 5).

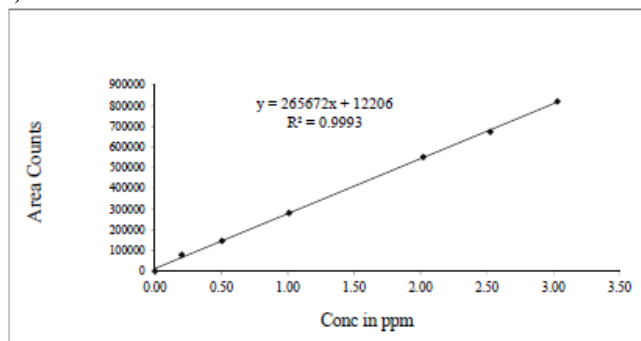


Figure 4: Linearity plot for Glycopyrrolate

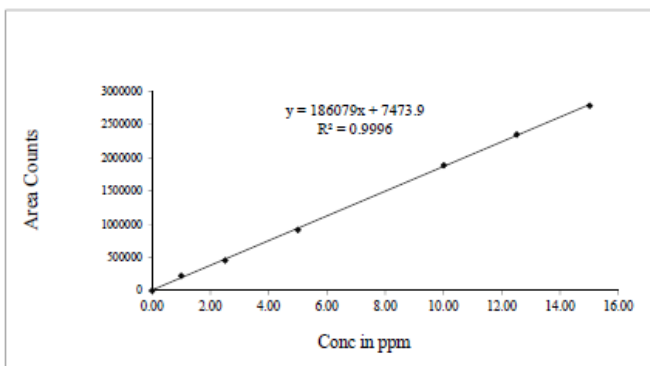


Figure 5: Linearity plot for Neostigmine

Precision of this method was studied in inter day and intra day variation. The precision of intraday studies of six different concentration of the drug was repeated thrice in a day and in the inter day variation studies of six different concentration of the drug was repeated on three consecutive days. The developed method was found to be precise as the percentage of RSD values for inter-day and intra-day precision studies were found to be less than 2%. Good recoveries (98 - 100%) of the drug were obtained at each added concentration, indicating that the method was accurate.

Table 1: Recovery of Glycopyrrolate drug

Amount of Glycopyrrolate	Recovery Solution (area)	% drug recovery
0.958	254967	100.2
2.068	547450	100.1
2.958	790401	100.7

Table 2: Recovery of Neostigmine drug

Amount of Neostigmine drug mg/ml	Recovery Solution (area) mAU	% drug recovery
4.68	874559	100.6
10.1	1871153	99.8
15.01	2785542	99.9

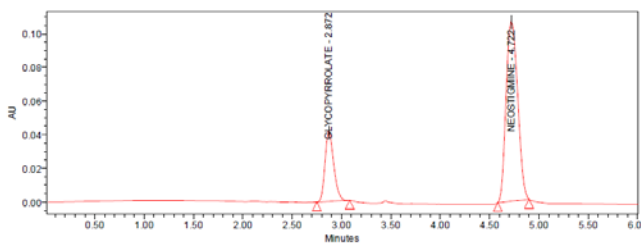


Figure 6: Chromatogram for Accuracy 50%

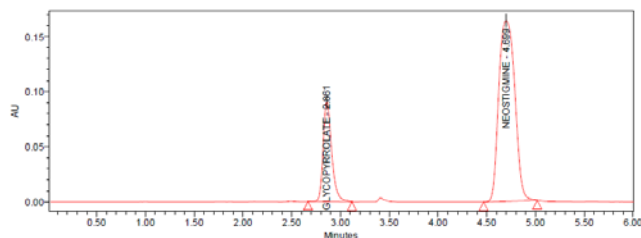


Figure 7: Chromatogram for Accuracy 100%

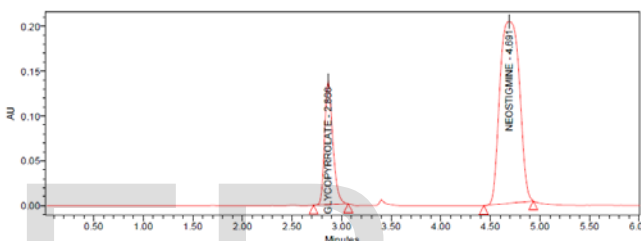


Figure 8: Chromatogram for Accuracy 150%

The limit of detection (LOD) for Glycopyrrolate and Neostigmine were found to be 0.055 and 0.2525 $\mu\text{g/mL}$ calculated from related equation ($S/N = 3$). The similar study claimed that a narrow working range (LOQ) such as 0.11– 0.50 $\mu\text{g/mL}$ for Glycopyrrolate and Neostigmine were obtained at the excitation wavelength of 255 nm.

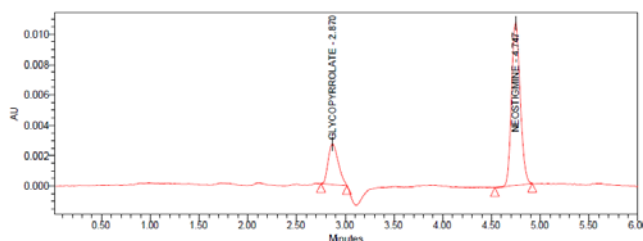


Figure 9: Chromatogram for LOD

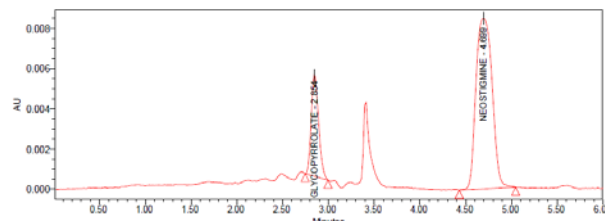


Figure 10: Chromatogram for LOQ

Forced degradation study was observed that upon treatment of Glycopyrrolate and Neostigmine with different strengths of base

(0.05 N and 0.5 N NaOH), acid (0.05 N, 0.5 N and 1 N HCl) and hydrogen peroxide and Thermal and Photolytic (20 %) the degradation was observed in (Table 3). Further it is important to note that from the chromatograms (Figure 11 to 17), it is evident that although the degraded peaks are observed. The Glycopyrrolate and Neostigmine were stable under the applied stress conditions like Thermal, acid and alkaline and oxidative degradation states.

Table 3 : Results of force degradation studies

Stress Condition/duration/solution	Degradation
Acid degradation (0.5 N HCl, 1 hr)	21%
Alkaline degradation (0.5 N NaOH, 1 hr)	24%
Oxidative degradation (30 % H ₂ O ₂ , 80°C for 10 min)	21.1%
Reduction Degrdaton (10% Sod.Bisul, 1hr)	24.3%
Thermal degradation (Solid sample, 80°C, 3 hr)	25.3%
Photolytic Degradation (sample expose sun light 6 hr)	22.6%
Hydralysis Degradation	25.3%

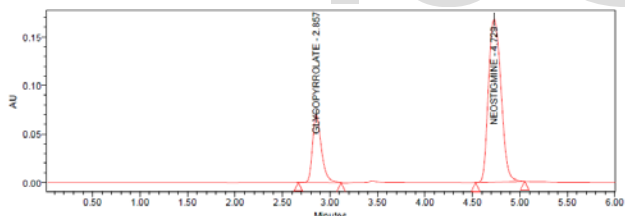


Figure 11: Chromatogram for Acid Degradation

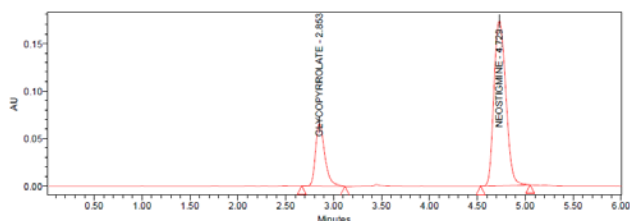


Figure 12: Chromatogram for Alkali Degradation

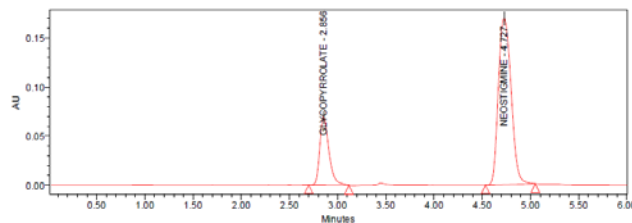


Figure 13: Chromatogram for Peroxide Degradation

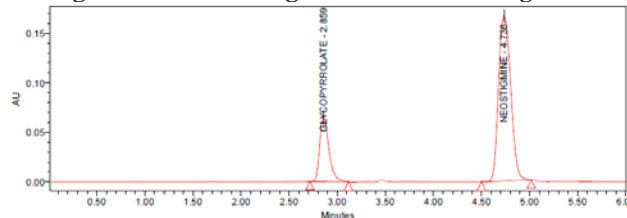


Figure 14: Chromatogram for Reduction Degradation

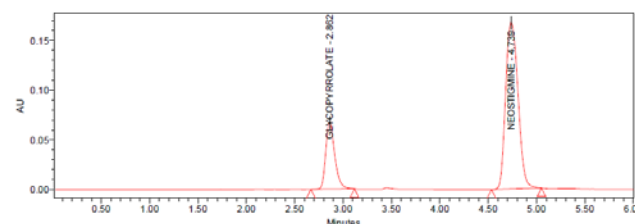


Figure 15: Chromatogram for Thermal Degradation

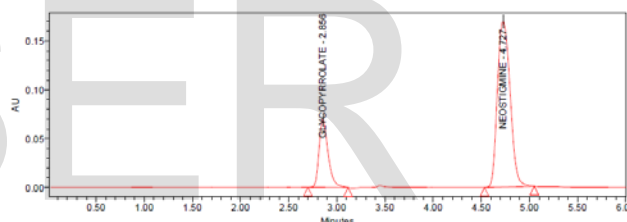


Figure 16: Chromatogram for Photolytic Degradation

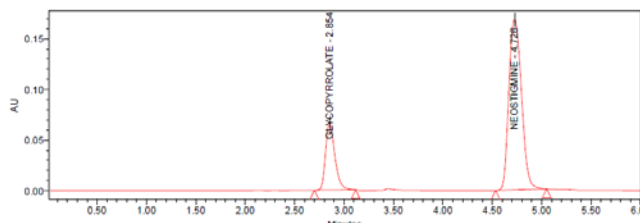


Figure 17: Chromatogram for Hydralysis Degradation

Robustness of the method small changes in chromatographic conditions such as change in flow rate ($\pm 20\%$), organic content in mobile phase ($\pm 2\%$), pH (± 0.2) and wavelength of detection ($\pm 5\%$) studied to determine the robustness method for the analysis of Glycopyrrolate and Neostigmine.

The influence of changes in chromatographic parameters are shown in table 4. The chromatographic data figure no's 18 to 23.

Table 4: Results for Robustness study

Change in parameter	% RSD
Flow (0.8 ml/min)	0.17
Flow (1.2 ml/min)	0.25
Wavelength (260 nm)	0.30

Wavelength (250 nm)	0.15
Organic phase composition (+2%)	0.11
Organic phase composition (-2%)	0.26

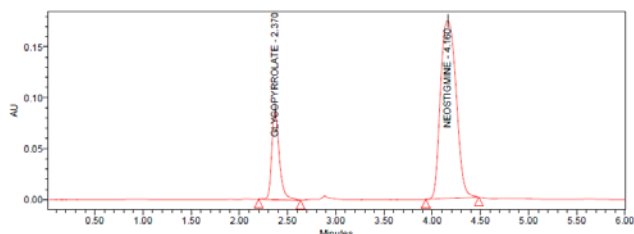


Figure 18: Chromatogram for Flow Plus

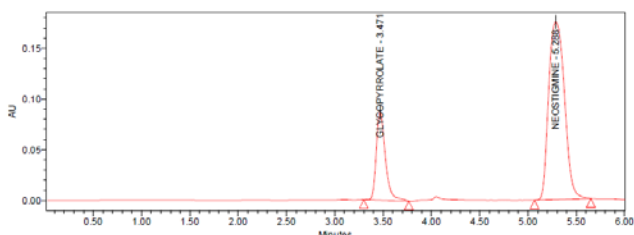


Figure 19: Chromatogram for Flow Minus

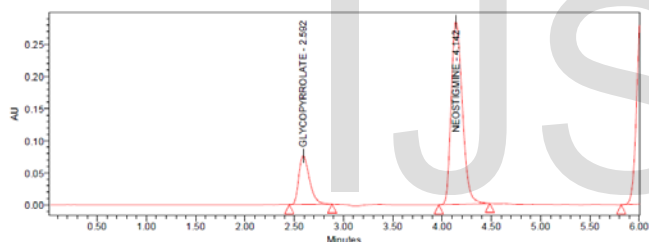


Figure 20: Chromatogram for Org Plus

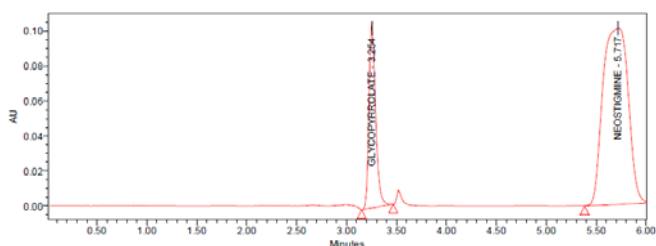


Figure 21: Chromatogram for Org Minus

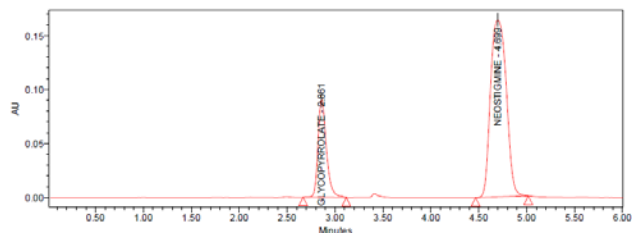


Figure 22: Chromatogram for Wave Plus

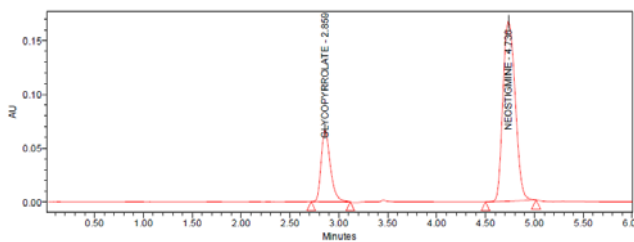


Figure 23: Chromatogram for Wave Minus

CONCLUSIONS

The developed method is accurate, precise and reliable for the analysis of Glycopyrrolate and Neostigmine in pharmaceutical formulations. This method was validated for linearity, accuracy, precision, robustness of Glycopyrrolate and Neostigmine drug. The RSD values for all parameters were found to be less 2, which indicates the validity of method and results obtained by this method are in fair agreement. Finally this method can be used for better analysis and pharmaceutical formulations Glycopyrrolate and Neostigmine drug.

References

1. [http\\Wikipedia.org/wiki/ Glycopyrrolate](http://Wikipedia.org/wiki/ Glycopyrrolate).
2. www.rxlist.com
3. Indian Pharmacopoeia, Volume-II. Government of India. Ministry of health and Family welfare New Delhi, The controller of publication 2007; p.1278-1279.
4. Beckett AH, Stenlake JB. Practical Pharmaceutical Chemistry. 4th ed. part II, New Delhi: CBS Publisher and Distributor; 1997. P. P. 158-164.277.
5. ICH, Q2R1 Validation of Analytical Procedures: Text and Methodology; International Conference on Harmonization, Geneva; 1996.
6. ICH. Q1A (R2), Stability testing of new drug substances and products, International conference on Harmonization. Geneva, 2003.
7. Hansel TT, Neighbour H, Erin EM, et al. (October 2005). Glycopyrrolate causes prolonged bronchoprotection and bronchodilatation in patients with asthma. *Chest*. 128 (4): 1974-9. doi:10.1378/chest.128.4.1974 Budawari S. The Merck Index. Whitehouse Station, NJ: Merck and Co. Inc; 2006. pp. 927-1573.
8. Rumpler, M.J.; Colahan, P.; Sams, R.A. (2014). The pharmacokinetics of glycopyrrolate in Standardbred horses. *J. Vet Pharmacol Ther*. 3 (37): 260-8. Martindale – The complete drug reference. London: Pharmaceutical Press; 2005. pp. 635-42.
9. Kim WO, Kil HK, Yoon KB, Yoon DM (May 2008). Topical glycopyrrolate for patients with facial hyperhidrosis. *Br. J. Dermatol*. 158 (5): 1094-7. Rang HP, Dale MM, Pitter JM, Moore PK. Pharmacology. New Delhi: Elsevier; 2005. pp. 657-63.
10. Bajaj V, Langtry JA (July 2007). Use of oral glycopyrrolate bromide in hyperhidrosis. *Br. J. Dermatol*. 157 (1): 118-21. Government of India. 2, 3. New Delhi: The Controller of Publications; 2010. Indian Pharmacopoeia; pp. 372-374. (646-650, 1552-1564, 1780-4).

11. Neostigmine Methylsulfate. International Drug Price Indicator Guide. Retrieved 8 December 2016.
12. Trifilieff A, Ethell BT, Sykes DA, Watson KJ, Collingwood S, Charlton SJ, Kent TC. Comparing the cardiovascular therapeutic indices of glycopyrronium and tiotropium in an integrated rat pharmacokinetic, pharmacodynamic and safety model. *Toxicol Appl Pharmacol.* 2015 287(1):9-16.
13. Glossop PA, Watson CA, Price DA, Bunnage ME, Middleton DS, Wood A, James K, Roberts D, Strang RS, Yeadon M, Perros-Huguet C, Clarke NP, Trevethick MA, Machin I, Stuart EF, Evans SM, Harrison AC, Fairman DA, Agoram B, Burrows JL, Feeder N, Fulton CK, Dillon BR, Entwistle DA, Spence FJ. Inhalation by design: novel tertiary amine muscarinic M₂ receptor antagonists with slow off-rate binding kinetics for inhaled once-daily treatment of chronic obstructive pulmonary disease. *J Med Chem.* 2011 54(19):6888-904.
14. Baraka A. Neostigmine Versus Sugammadex for Reversal of Neuromuscular Block. *Middle East J Anaesthesiol.* 2016 Jun;23(5):507-8.
15. Iwasai S, Kinoshita Y, Asagoe Y, Matsuzaki T, Arai M, Sato T. Anaphylactoid Reactions Suspected to Be Caused by Neostigmine in Pediatric Patients under General Anesthesia. *Masui.* 2016 Apr;65(4):377-9. Japanese.
16. Dodds MM, Frazer CD, Lipman J, Reade M. Use of neostigmine for acute colonic pseudo-obstruction in a patient receiving dexmedetomidine. *Crit Care Resusc.* 2016 Mar;18(1):59-61. No abstract available.
17. Choi ES, Oh AY, Seo KS, Hwang JW, Ryu JH, Koo BW, Kim BG. Optimum dose of neostigmine to reverse shallow neuromuscular blockade with rocuronium and cisatracurium. *Anaesthesia.* 2016 Apr;71(4):443-9.
