Journal of Applied Pharmaceutical Research
Volume 13 Issue 3, Year of Publication 2025, Page 119 – 128
DOI: https://doi.org/10.69857/joapr.v13i3.834

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Research Article

JOURNAL OF APPLIED PHARMACEUTICAL RESEARCH | ***JOAPR***
www.japtronline.com ***ISSN: 2348 – 0335***

A BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF GLYCOPYRROLATE AND NEOSTIGMINE IN RAT PLASMA BY LC-MS AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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Article Information
Received: 25th April 2025
Revised: 5th June 2025
Accepted: 19th June 2025
Published: 30th June 2025

Keywords
LC-MS, Glycopyrrolate, Neostigmine and Pharmacokinetics Studies

ABSTRACT

Background: After surgery, non-depolarizing neuromuscular blocking medications include neostigmine (NEO) and glycopyrrolate (GLY). Numerous traditional approaches, such as HPLC and UPLC procedures, are established for the quantification of GLY and NEO; nevertheless, they lack sensitive and specific analysis, especially in complex matrices. Using the LC/MS approach, this work develops a bioanalytical method for quantifying both drugs in rat plasma and applies it to pharmacokinetic studies. **Methodology:** The plasma was extracted using acetonitrile, and Rivastigmine was employed as an internal standard. An MRM method with positive ions was used for multiple reactions. A C18 column and a mobile phase - 70:30 mixture of acetonitrile and buffer was utilised at a flow rate of 1 ml/min. Plasma vortex for 10 minutes and centrifuged at 4000 rpm at 20°C. Validation and stability studies are conducted according to the ICH guidelines. The pharmacokinetic study by WinNonlin (Version 5.2) software. **Results and Discussion:** Rt for Glycopyrrolate and Neostigmine at 1.838 and 2.800min. GLY has a precision (%CV) of 0.45 at HQC and 3.57 at LQC. NEO had a precision (%CV) of 1.13 at HQC and 2.79 at LQC. From 2 to 40 ng/mL of GLY and 10 to 200 ng/mL of NEO, the standard curves showed a linear relationship. LOD and LOQ for both drugs were 3pg/mL and 10pg/mL. **Conclusion:** A simple, affordable, reliable, and sensitive approach for quantifying GLY and NEO in rat plasma using LC-MS, with Rivastigmine serving as the internal standard, was developed, validated, and successfully applied in the pharmacokinetic study of rat plasma.

INTRODUCTION

A crucial aspect of developing new pharmaceutical products is accurately measuring the amount of drug in biological matrices. The purpose of the bioanalytical method is to precisely determine the concentration of the drug, its metabolite, or both, in the biological matrix. Bioanalytical data were used to inform pharmacokinetic evaluations, including bioavailability (BA) and bioequivalence (BE), as well as human clinical pharmacology

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studies. Preclinical investigations in non-human pharmacology and toxicology also employ the bioanalytical approach [1].

Over the past few decades, numerous investigations have been conducted to identify quaternary ammonium compounds. Due to their cationic nature, they are challenging to detect. Some enzyme-linked immunosorbent assay methods have been developed for estimating quaternary ammonium compounds, but cross-reactions have been observed [2]. For the detection of QA drugs, a wide variety of different chromatographic and spectrometric approaches have been published. The lack of specificity in the majority of these analytical techniques is a significant drawback that causes problems with identification and quantification in complex matrices. By using mass spectrometers, we can identify quaternary compounds. The QA compounds can be identified by using mass spectrometry methods [3]. Recently, the LC–MS approach has emerged as the preferred methodology for quantifying QA in bioanalytical studies.

In liquid chromatography/mass spectrometry (LC/MS), the ability to separate things is combined with the ability to detect things using mass spectrometry. LC-MS has become essential for the pharmaceutical industry to generate accurate pharmacokinetic, pharmacodynamic, toxicokinetic, and bioequivalence data during the drug development process [4].

Glycopyrronium bromide (GLY) is a synthetic anticholinergic agent with a quaternary ammonium structure. It was recently approved for treatment in patients with chronic obstructive pulmonary disease as a long-acting muscarinic antagonist [6]. Because quaternary ammonium is a strongly ionised compound at physiological pH, Glycopyrronium bromide has poor penetration of the placental and blood-brain barriers [3]. The chemical structure of Glycopyrronium bromide is shown in Figure 1 [6].

Neostigmine methyl sulphate (NEO) is classified as a quaternary ammonium anticholinesterase. Due to its impact on skeletal muscle, neostigmine is primarily utilised in the treatment of myasthenia gravis [6]. This substance is additionally employed in anaesthesia to mitigate the effects of other neuromuscular blocking agents. The chemical structure of Neostigmine methyl sulphate is shown in Figure 2 [6]. In comparison to the combination of atropine and neostigmine metilsulfate, the Glycopyrrolate-neostigmine injection demonstrates a reduction in initial tachycardia. It offers enhanced protection against the cholinergic effects associated with neostigmine methylsulfate [7]. The combination of Glycopyrronium bromide and neostigmine methylsulfate demonstrates superior cardio stability compared to the individual effects of each substance [8].

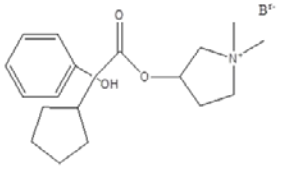

Figure 1: Chemical structure of Glycopyrronium bromide

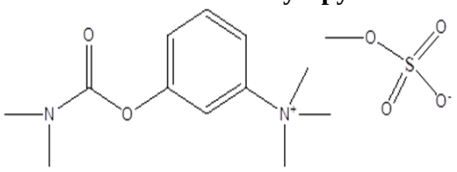

Figure 2: Chemical structure of Neostigmine methylsulfate

Rivastigmine was used as an internal standard. Rivastigmine and neostigmine both function similarly by attaching to the acetylcholinesterase enzyme, which is why they are both categorised as cholinesterase inhibitors. According to the literature research, there is currently no well-established LC-MS approach for estimating GLY and NEO [5]. The objective of this study is to develop and validate a bioanalytical method for measuring the quantitative levels of Neostigmine and Glycopyrrolate in rat plasma using LC-MS, with the eventual goal of using this method in a pharmacokinetic investigation [9]. The pharmacokinetics of GLY and NEO were studied by collecting plasma from rats following oral administration using the procedures that had been developed.

MATERIALS AND METHODS

Reagents and chemicals

The reference samples, Glycopyrrolate and Neostigmine, were procured from Biocon in Bangalore. The Merck Chemical Division in Mumbai provided all of the chemicals, including acetonitrile and methanol (graded using LC/MS). Throughout the investigation, HPLC-grade water from the Milli-Q water purification system was regularly used.

Instrumentation

The Waters 2695 HPLC was used to perform chromatography. It is interfaced with the SCIEX QTRAP 5500 mass spectrometer, which is capable of reliably transferring the separated components from the LC Column to the MS ion Source. The data interpretation is performed using ABSCIEX software.

Chromatography conditions

In this procedure, a Symmetry C18 column measuring 150 x 4.6 x 3.5 µm was selected, and a mobile phase consisting of a 70:30 mixture of acetonitrile and buffer (0.1% formic acid) was utilised at a flow rate of 1 ml/min.

Mass spectrometry conditions

The complete analysis was conducted using the SCIEX QTRAP 5500 mass spectroscopy instrument, and data acquisition was performed with the assistance of ABSCIEX software. The ionisation technique employed for this method was the Opti Flow Turbo V ionisation in positive ion mode. The MS recordings were carried out in multiple reaction monitoring (MRM) mode using Positive Polarity. Mass spectra of the Product ions of Glycopyrrolate and Neostigmine drugs are shown in Figures 3 and 4.

Calibration standard and Quality control samples preparation

A Standard Stock Solution was prepared with a concentration of 20 ng/ml of Glycopyrrolate and 100 ng/ml of Neostigmine. The internal standard, Rivastigmine Standard Stock Solution, was prepared at a concentration of 400 ng/mL. A volume of 500 µL from the standard stock solution was carefully transferred into a 2 mL centrifuge tube. Combine 200µl of plasma, 500µl of internal standard, 300µl of Acetonitrile, and 500µl of diluent into the mixture. Rotate it for 20 minutes. Systematically filter the supernatant liquid and transfer it into an HPLC vial.

Preparation of Linearity Solutions: The linearity solutions with concentrations ranging from 2 nanograms to 40 nanograms per milliliter of Glycopyrrolate and 10 nanograms to 200 nanograms per milliliter of Neostigmine were prepared. This was centrifuged at 4000 RPM for 15–20 minutes. The supernatant solution in an LC vial was collected and injected into the chromatograph. During method optimization, a blank chromatogram is developed, as shown in Figure 5.

Extraction procedure

Assign labels to the centrifuged and treated plasma samples based on their respective time intervals. Add 500 µL of diluent to approximately 200 µL of plasma and ensure thorough mixing. Next, incorporate 300 µL of Acetonitrile to precipitate all proteins and thoroughly mix using a vortex cyclo mixer. Spin the sample in the centrifuge at 4000 RPM for a duration of 15 to 20 minutes. Transfer the supernatant solution into an HPLC vial and proceed to inject it into the chromatograph.

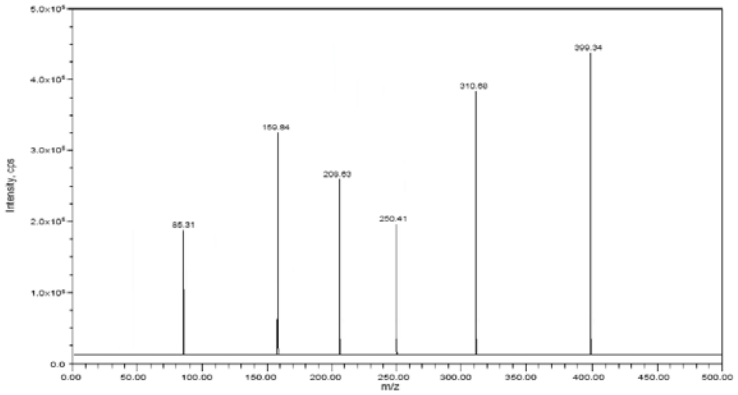

Figure 3: Mass spectra of Product ion of Glycopyrrolate

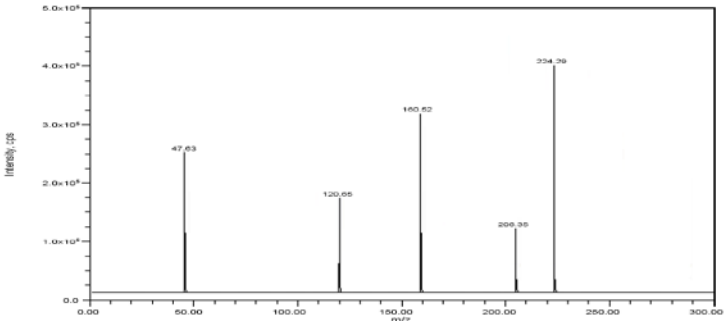

Figure 4: Mass spectra of Product ion of Neostigmine

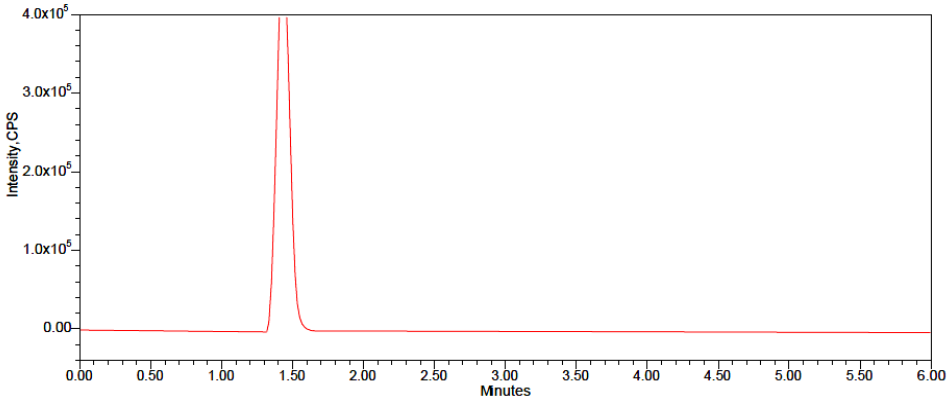

Figure 5: Chromatogram of Blank

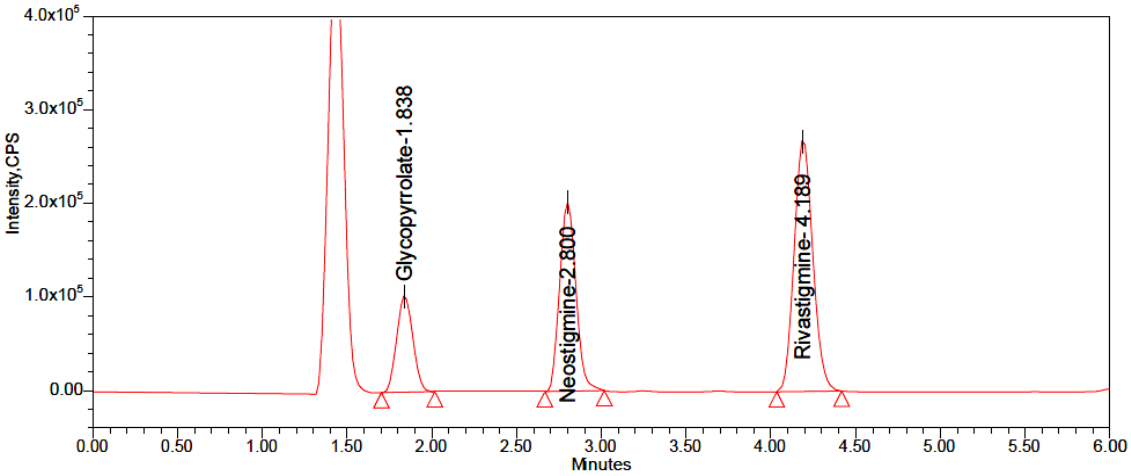

Figure 6: Chromatogram of System suitability

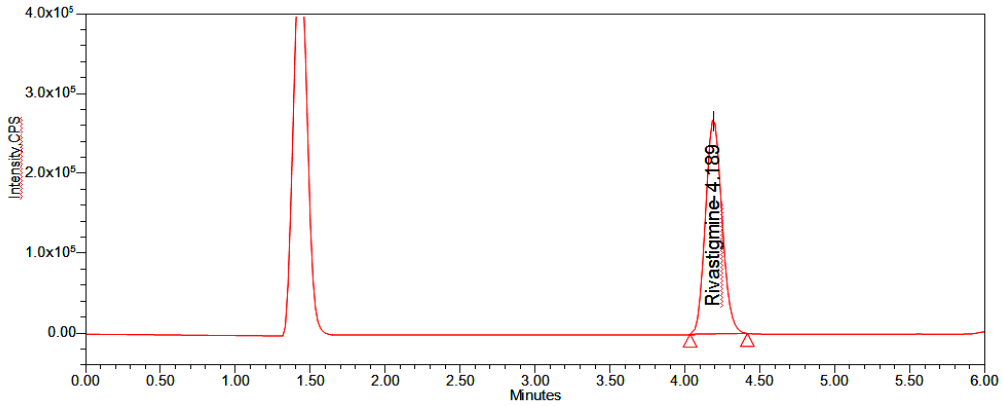

Figure 7: Specificity Chromatogram of Internal Standard

Methodology for Analysis

Blank (Plasma solution), Linearity solutions, and sample solutions were injected into the chromatograph, and the chromatograms were obtained. The peak area counts for Glycopyrrolate and Neostigmine were measured. The concentration of Glycopyrrolate and Neostigmine present in the plasma sample was determined from the equation obtained from the Linearity Curve.

RESULTS AND DISCUSSIONS

System suitability

Evaluation of instrument effectiveness by analysing a set of reference standards conducted before the analytical process [10]. The calculated coefficients of variation for Neostigmine and Rivastigmine were determined to be 0.26 and 0.14, respectively. On the other hand, the area ratios of Glycopyrrolate and Rivastigmine exhibited coefficients of variation of 0.19 and 0.34, respectively. As a result, it satisfied the system suitability requirements. Chromatogram of System suitability during optimization of a method for quantification of Glycopyrrolate and Neostigmine with Rivastigmine as internal standard is shown in figure 6.

Auto sampler carryover

The response in the carryover area for subsequent injections is determined to be less than 20% [10]. Thus, the carryover effect was passed by the method.

Specificity and screening of biological matrix

It was checked in six different blank rat plasma samples for overlapping peaks. None were found at the retention times of either Glycopyrrolate and Neostigmine or ISTD. Specificity Chromatogram of Internal Standard during method optimization is shown in figure 7.

Matrix effect

The matrix of plasma constituents during the ionisation of the analyte was established by analysing the response of post-

extracted plasma standard MQC samples (20 ng/ml of Glycopyrrolate and 100 ng/ml of Neostigmine) (n = 6) in comparison to the response of the analyte from neat samples at corresponding concentrations [11]. The intended method for assessing the matrix effect was evaluated using chromatographically screened rat plasma. The precision (%CV) values are recorded at 0.45 for Glycopyrrolate at HQC and 3.57 at LQC. The precision (%CV) values are 1.13 for Neostigmine at high-quality control (HQC) and 2.79 at low-quality control (LQC). The matrix effect of the HQC sample chromatogram is shown in Figure 8. The chromatogram of the LQC sample matrix effect is shown in Figure 9.

Sensitivity: The %CV for Glycopyrrolate and Neostigmine was found to be 2.89% and 0.43%. Hence, it passed the sensitivity.

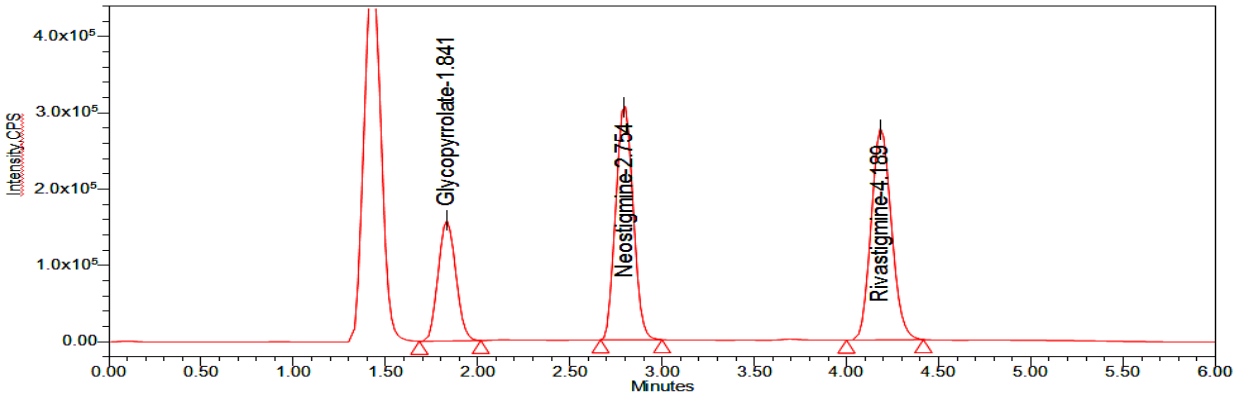

Figure 8: Matrix Effect Chromatogram of Higher Quality Control sample

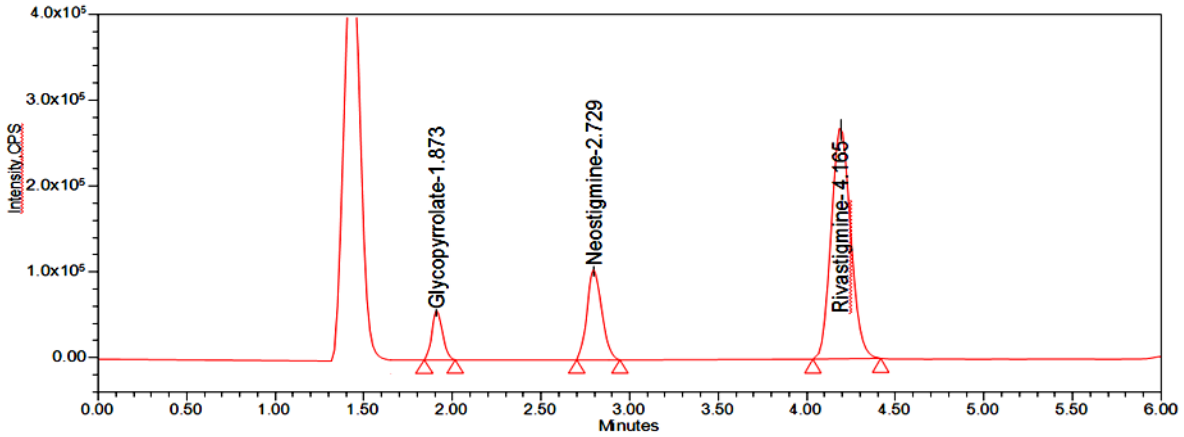

Figure 9: Matrix Effect Chromatogram of Lower Quality Control Sample

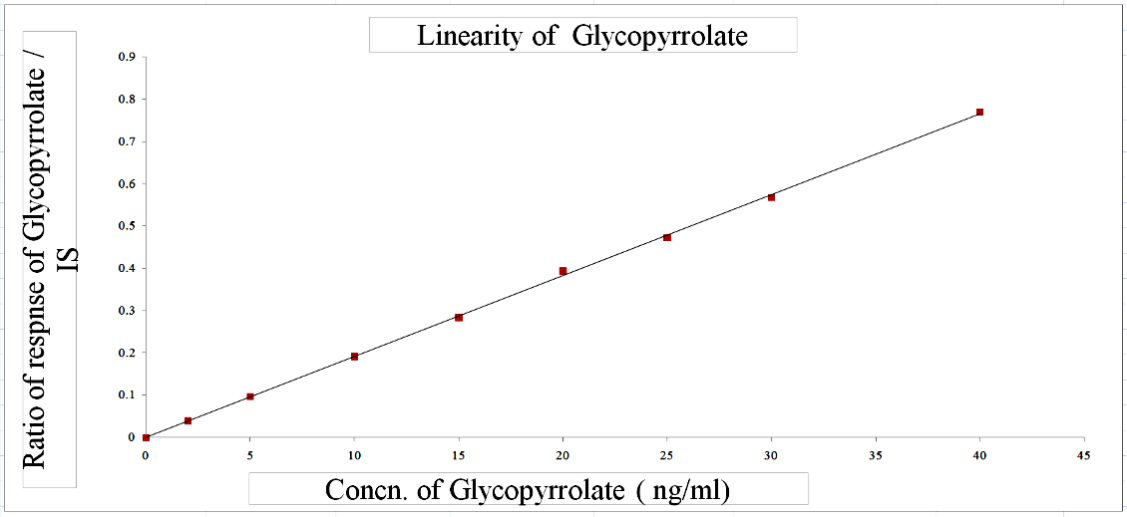

Figure 10: Calibration plot for concentration v/s Area ratio of Glycopyrrolate

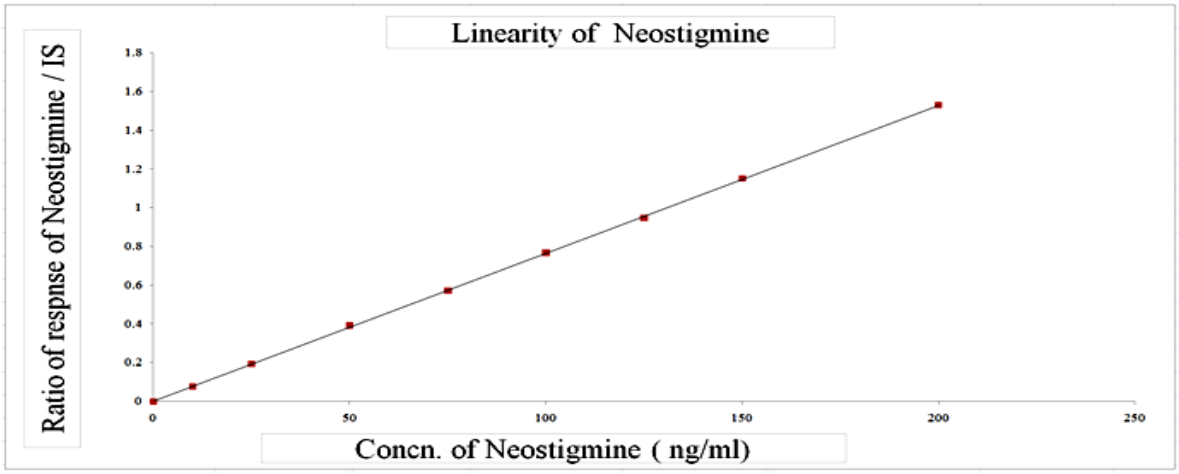

Figure 11: Calibration plot for concentration v/s Area ratio of Neostigmine

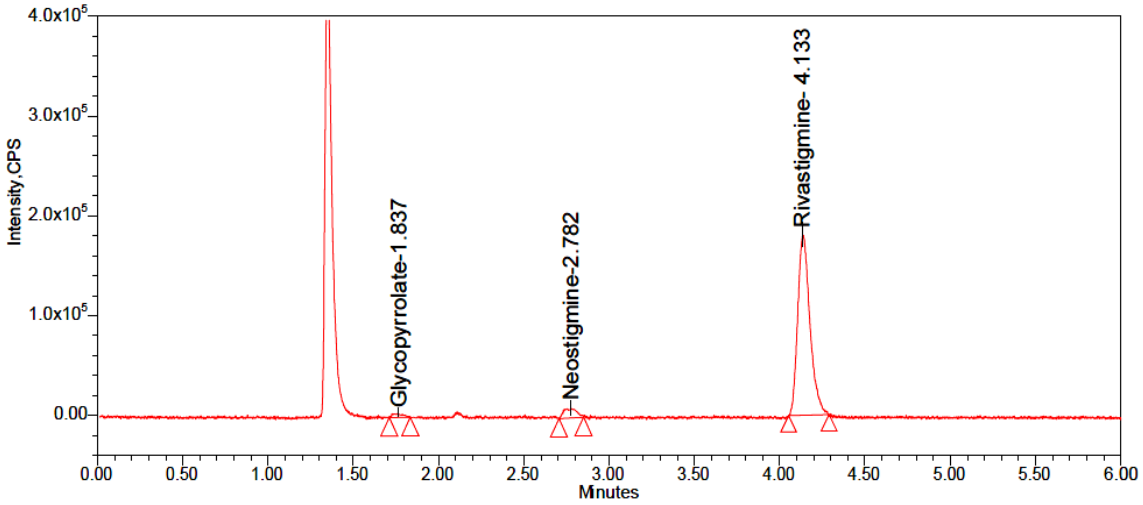

Figure 12: Chromatogram showing Limit of Detection results

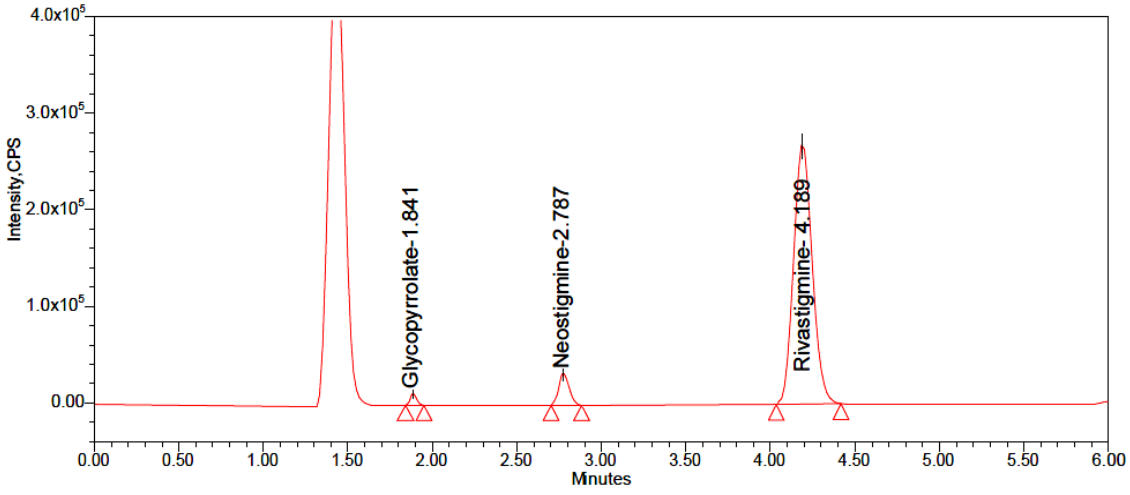

Figure 13: Chromatogram showing Limit of Quantification results

Linearity

For concentrations ranging from 2 to 40 ng/mL of Glycopyrrolate and 10 to 200 ng/mL of Neostigmine, the standard curves were linear. The correlation value was 0.999. The samples were measured by comparing the analyte peak area to the IS peak area [11]. Peak area ratios were plotted against plasma concentrations. Calibration plots for concentration v/s Area ratio of Glycopyrrolate and Neostigmine are shown in Figures 10 and 11.

LOD and LOQ

The limits of detection and quantification are calculated based on the signal-to-noise ratio. Results for LOD and LOQ are shown in Table 1. Chromatograms showing the LOD and LOQ results are shown in Figures 12 and 13.

Precision and Accuracy

Six replicates containing Glycopyrrolate and Neostigmine at four distinct QC levels were examined to assess intra-assay precision and accuracy. By examining the four levels of QC samples on four separate runs, the inter-assay precision was determined [11]. Acceptable data must be accurate within 85–115% of the actual values and precise within ±15% of the relative standard deviation (RSD), except LLQC, which requires accuracy between 80–120% and RSD <20%.

Glycopyrrolate HQC, MQC, LQC, and LLQC samples had respective mean accuracy percentages of 94.42%, 98.76%, 96.65%, and 99.52%. Neostigmine HQC, MQC, LQC, and LLQC samples had respective mean accuracy percentages of 100.13%, 99.41%, 100.98%, and 101.08%.

Table 1: Results showing the Limit of Detection and Limit of Quantification Results

Drug Name	LOD (S/B) Value	LOQ (S/B) Value
Glycopyrrolate	3	10
Neostigmine	3	10

Recovery of analyte

The recovery of drug and IS was evaluated at three concentration levels, namely low, medium, and high quality control. Recovery was calculated by comparing its response in replicate samples with that of the neat standard solution responses [5]. Analyte recovery from a sample matrix (extraction efficiency) is a comparison of the analytical response from an amount of analyte added to that determined from the sample matrix. Because of the fundamental properties of Glycopyrrolate and Neostigmine, extraction was carried out using methanol as a solvent.

The Glycopyrrolate HQC (30 ng/mL) sample's mean recoveries for the extracted and unextracted responses were 95.76% and 95.95%, respectively. Neostigmine HQC (150 conc in ng/ml) sample rate of recovery for extracted response and unextracted response were 99.35% and 99.44%, respectively. The average recovery of the internal standard (100 ng/ml) was 100.41%.

Stability

By comparing the observed concentrations of low, medium, and high QC samples with the spiking concentration under seven storage settings, the stability of Glycopyrrolate and neostigmine was examined [12]. Bench top stability was assessed to determine the stability of the analyte in plasma by placing the QC samples at room temperature for a full day. Both drugs successfully meet the criteria of the Bench Top stability test. Assessment of Auto Sampler Stability: To evaluate the stability of the treated plasma sample, high-quality samples were meticulously prepared and subsequently stored in the auto sampler at 4°C for 24 hours. [12]. For Glycopyrrolate and Neostigmine, the percentage CV of HQC, MQC, and LQC, as well as the mean accuracy, pass the freeze-thaw stability (-20°C), auto sampler stability, wet extract stability, dry extract stability for 12 hours, short-term stability, and long-term stability for 28 days.

Pharmacokinetic Studies

The isolation of glycopyrrolate and neostigmine from rat plasma was achieved using the liquid extraction method. To achieve this, 300 µL of acetonitrile was added to the polypropylene tubes. The plasma was vortexed for 10 minutes, followed by centrifugation at 4000 rpm and a temperature of 20°C. The supernatant fluid from each tube was transferred to a separate tube and subjected to evaporation at 40°C until dry. A 500 µL diluent was incorporated into the sample and vortexed for 5 minutes, followed by the transfer of the sample into auto-sampler vials for injection.

Samples were injected into the bodies of six rats, and subsequent samples were collected at various time intervals: 5, 10, 20, 30, 40, and 60 minutes. Subsequently, the samples were diluted according to the test method, and these samples were placed in the autosampler. Record their respective values from the chromatogram, and the values for Glycopyrrolate and Neostigmine at various time intervals are given in Table 2.

Both drugs, comprising 0.5 mg of Glycopyrrolate and 2.5 mg of Neostigmine, were administered via injection into the rat's body. Blood samples were subsequently collected at 5, 10, 20, 30, 40, and 60 minutes post-dose using K2 EDTA Vacutainer tubes. To examine potential interferences, plasma pre-dose samples were collected and analysed. The samples were subjected to centrifugation at 70rpm to isolate the plasma, which was

subsequently stored at -10 °C. In addition to the quality control samples, four spiked plasma samples were prepared. All these samples undergo analysis to obtain the results. The pharmacokinetic characteristics for both Neostigmine and Glycopyrrolate samples were calculated using WinNonlin (Version 5.2) software. Recovery plots for Glycopyrrolate and Neostigmine in Rat plasma are shown in Figures 14 and 15. The stability of samples was assessed using the incurred sample reanalysis (ISR) technique. Two samples were chosen for ISR from each patient based on the pharmacokinetic profile's elimination phase and Cmax. The percentage difference should not exceed 20%, as the samples were considered stable [13].

Table 2: Data showing the Pharmacokinetic studies for Glycopyrrolate and Neostigmine

Time Intervals (min)	Glycopyrrolate (ng/ml)	Neostigmine (ng/ml)
5	6.776	89.836
10	12.404	59.248
20	15.658	33.246
30	18.733	21.014
40	11.013	12.175
60	0	0

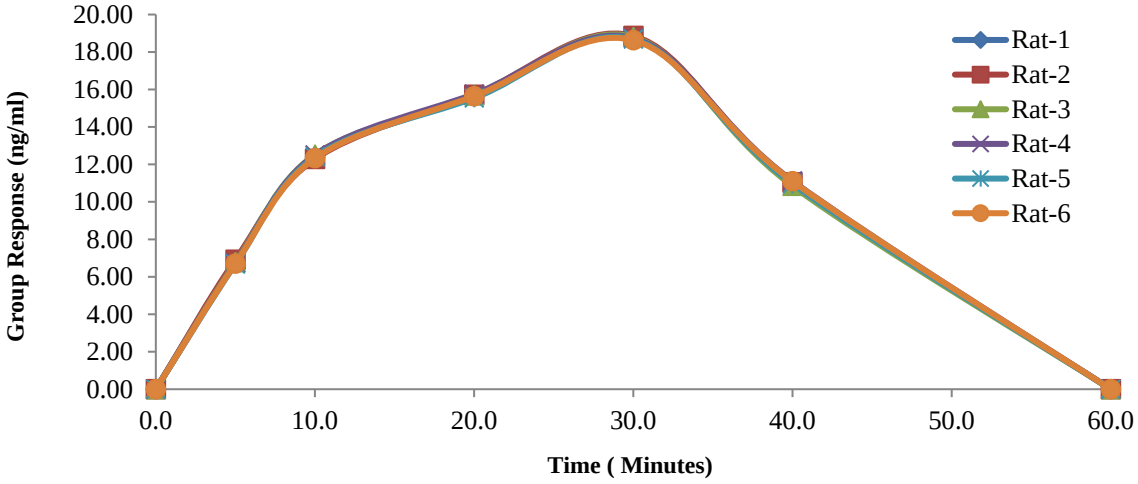

Figure 14: Recovery plot for Glycopyrrolate in Rat plasma

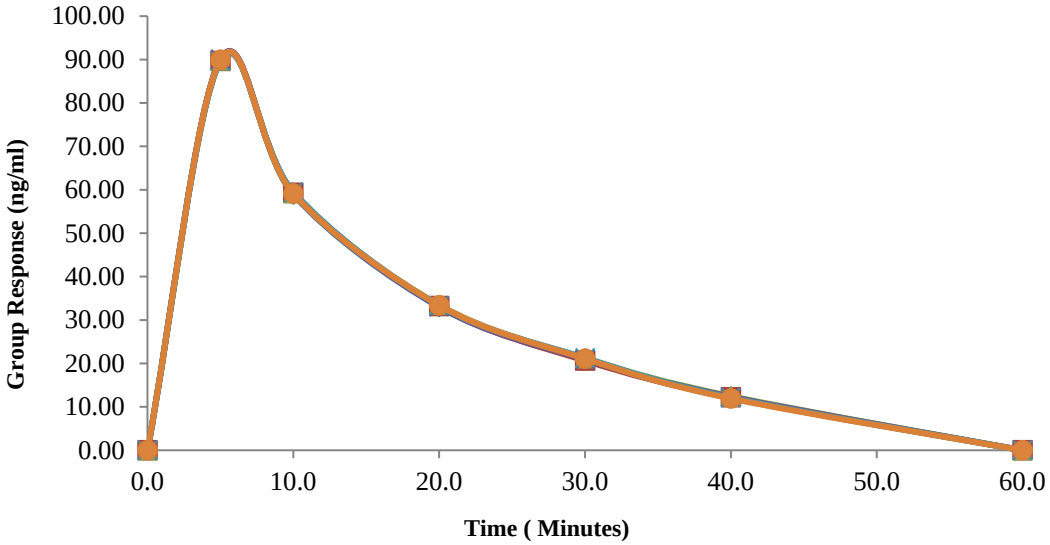

Figure 15: Recovery plot for Neostigmine in Rat plasma

CONCLUSION

This study aimed to develop a simple, affordable, reliable, and sensitive approach for quantifying Glycopyrrolate and Neostigmine using LC-MS, with Rivastigmine serving as the internal standard. The work shows less run time compared to other work articles. The retention time for Glycopyrrolate and

Neostigmine is 1.838 and 2.800 minutes, respectively, with a total chromatographic duration of 6.0 minutes. The linearity achieved was within the range of 20-600 ng/mL for Glycopyrrolate and 0.25-7.5 ng/mL for Neostigmine, showing a correlation coefficient of 0.999. The intra-batch and inter-batch precision (% CV) values across all five levels (LLOQ, LQC, MQC, HQC, and ULOQ) are less than 11.15.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

D. Akiladevi and Vasu Babu wrote the initial draft of the article. Vasu Babu worked on the literature and the method development. D Akila Devi helped oversee the MS recording and extraction. Vasu Babu analyzed the validation parameters. Vasu Babu conducted the pharmacokinetics study under the guidance of D. Akila Devi.

ABBREVIATIONS

LC–MS: Liquid chromatography–Mass spectrometry; m/z: mass to charge ratio; GLY: Glycopyrrolate; NEO: Neostigmine; QA: Quaternary Ammonium; %CV: Coefficient of Variation; HQC: High Quality Control; LQC: Low Quality Control; MQC: Middle Quality Control; Low; LLOQ: Lower Limit of Quantification; ULOQ: Upper Limit of Quantification; ISR: Internal Standard Reference; mg: Milligram; mL: Milliliter; µL: Microliter; µm: Micrometer; ACN: Acetonitrile; HPLC: High-Performance Liquid Chromatography; API: Active Pharmaceutical Ingredient;

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