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RESEARCH ARTICLE

Stability Indicating Assay Method Development and Validation by RP-UPLC with PDA detector for Simultaneous Estimation of Glycopyrrolate and Neostigmine in Pharmaceutical dosage form

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ABSTRACT:
The presented research aimed to create a simple, precise, and accurate isocratic reversed-phase stability indicating Ultra Performance Liquid Chromatography (UPLC) method for simultaneous quantification of Glycopyrrolate and Neostigmine in bulk and injectable dosage form. On a Symmetry-C18(150mm X 4.6mm, 3.5m) column, isocratic separation was accomplished. The mobile phase is composed of acetonitrile and 1% orthophosphoric acid buffer (70:30 v/v) flowing at a rate of 1ml/min, with detection at 255nm utilising a photo-diode array detector. To apply stress conditions, the drug was treated to acid degradation, alkali degradation, peroxide degradation, photolysis, and heat. Specificity, precision, linearity, accuracy robustness, and solution stability were all validated. Glycopyrrolate and Neostigmine showed linearity in the range of 1-15mg mL-1 and 5-75mg mL-1, respectively. Glycopyrrolate accuracy was from 98.9 to 100.2 percent, whereas Neostigmine accuracy ranged from 98.4 to 100.2 percent. The detection of Glycopyrrolate and Neostigmine test does not interfere with degradation products produced as a result of stress studies, hence this method is regarded as stability-indicating.

KEYWORDS: Glycopyrrolate; Neostigmine; RP-UPLC, Stability testing.

INTRODUCTION:
Neostigmine is primarily utilised in myasthenia gravis for its effects on skeletal muscle and in anaesthesia to stop the effects of competing neuromuscular blocking drugs[1].Glycopyrronium bromide (GLY), 3-[(cyclo pentyl hydroxy phenyl acetyl)oxy]-1,1-dimethyl-pyrrolidinium bromide, is a synthetic anticholinergic agent with a quaternary ammonium structure. It was recently approved for use as a long-acting muscarinic antagonist in patients with chronic obstructive pulmonary disease. Glycopyrronium Bromide is highly ionised at physiological pH due to the quaternary ammonium component, and hence penetrates the blood-brain and placental barriers poorly[2]. Neostigmine methyl sulfate (NEO), [3-(dimethyl-carbamoyl oxy)phenyl]-trimethylazanium; methyl sulfate is a quaternary ammonium anticholinesterase.

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In comparison to a mixture of atropine and neostigmine metilsulfate, glycopyrrolate-neostigmine injection causes less initial tachycardia and provides superior protection against the cholinergic effects of neostigmine metilsulfate. Furthermore, residual central anticholinergic effects are minimal due to Glycopyrronium Bromide's restricted penetration into the central nervous system[2]. Glycopyrronium Bromide in combination with Neostigmine Metilsulfate is accompanied with better Cardio stability than Glycopyrronium Bromide and Neostigmine Metilsulfate alone. To reverse residual non-depolarising (competitive) neuromuscular block, Glycopyrronium Bromide and Neostigmine Metilsulfate are usually given together[3].

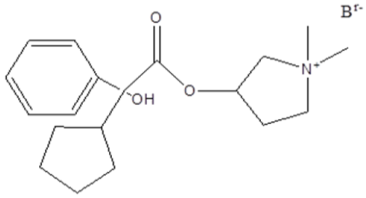

Figure 1: Chemical structure of Glycopyrronium Bromide

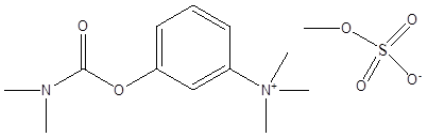

Figure 2: Chemical structure of Neostigmine Methyl Sulfate

According to the literature review, no UPLC approach for simultaneous estimate of GLY and NEO has been published. The goal of this research is to develop and validate a new stability-indicating RP-UPLC method for simultaneous estimation of GLY and NEO in pharmaceutical dosage forms. Degradation studies in alkali, acid, and oxidative degradation were carried out. Validation of the developed method's applicability in accordance with the International Conference on Harmonisation (ICH)[4].

MATERIAL AND METHODS:

Reagent and chemicals:

HPLC graded Acetonitrile, Ortho phosphoric acid., and HPLC graded water are procured from J.K. Scientific Co., (Bangalore) and pure standards are obtained of Glycopyrronium bromide and Neostigmine methyl sulfate was obtained from Louis pharmaceutical private Limited, Medak. Waters Associates, Inc. provided Waters Symmetry-C18.

Chromatographic Conditions and Instrumentation:

Waters Acquity UPLC with photodiode array (PDA) detector was used to perform the chromatogram. For chromatographic separation, a Symmetry C18 (150mm x 4.6mm, 3.5m) column was utilised. Detection was done using a PDA detector 255nm and Empower 2 software. Acetonitrile and 1% orthophosphoric acid buffer (70:30 v/v) make up the mobile phase, which is sonicated and degassed before use. The diluent was mobile phase, and the flow rate was kept constant at 1.0ml/min. The temperature of the column was kept at room temperature. The injection volume was 10 litres, with a total run time of 6 minutes. Figure 3 depicted a PDA spectrum of GLY and NEO.

Standard Solution:

In a 100ml volumetric flask, weigh 10mg of Glycopyrrolate and 50mg of Neostigmine working standard diluted to volume with diluent. 5ml of stock solution was pipetted into a 50ml volumetric flask and diluted to volume with diluent to reach a final concentration of GLY 10/ml and NEO 50/ml, which was then filtered through 0.45 membrane filter paper. Six replicate standard injections were used in a system suitability study.

Sample solution:

A dosage of 0.5mg GLY and 2.5mg NEO injection volume equivalent was correctly measured and settled in a volumetric flask (50ml) for up to 15 minutes with the use of 10ml of diluent injection. The volume is ultimately 50ml diluted and filtered with a membrane filter paper of 0.45µ. Including GLY 10µ/ml and NEO 50µ/mL in the prepared stock injection sample.

Stress degradation study:

The stress degradation investigation was conducted in accordance with ICH Q1A criteria (R2)[13]. Degradation of the sample stock solution was carried out (10ml). Degradation of acid by taking 10mL of 0.1M hydro chloric acid, basic degradation by taking 10mL of 0.1M sodium hydroxide, 10mL of 3% H2O2 for oxidative degradation, 10ml sample stock solution by exposition to UV light for 24 hours, and thermal sampling by keeping a sample at 70ºC. For 24 hours at room temperature, all samples were exposed to various stress conditions. All of the samples were diluted to 50ml with diluent after degradation, and the test sample concentrations of 10g/ml GLY and 50g/ml NEO were determined using the proposed method. In all of the stress settings, the percentage assay, degradation, and peak purity of GLY and NEO were determined[1].

RESULT AND DISCUSSION:

Method development:

The development of an ultra-high-performance liquid chromatographic method for the determination of GLY and NEO in bulk and injection dosage form was optimised through the use of different analytical columns (Water's symmetry C18, 150 4.6mm, 3.5m; Zodiac phenyl column C18, 150 4.6mm, 3.5m; Develosil C18, 150 4.6mm, 3.5m) and different rations and flow rate of mobile phase systems (Ortho Phosphoric acid and acetonitrile). GLY and NEO were eluted optimally using a chromatographic system that included a mobile phase of acetonitrile and 1% orthophosphoric acid buffer (70:30 v/v), a flow rate of 1ml/min, a column temperature of 35°C, an injection volume of 10µl and a detection wavelength of 255nm. GLY was eluted from the column with a retention time of 1.869 minutes and Neostigmine with a retention time of 2.750 minutes, respectively.

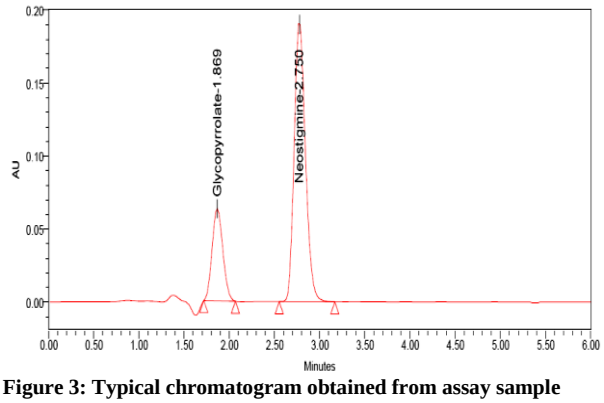

Figure 3: Typical chromatogram obtained from assay sample

Validation of the method:
This method was validated in accordance with ICH Q2 (R1) guidelines for system suitability, specificity, linearity, sensitivity, precision, robustness and selectivity[9,12]

Specificity:
The specificity of the test was determined by comparing chromatograms of the standard solution to chromatograms of the placebo[11]. In a placebo solution, Glycopyrrolate and Neostigmine showed no interaction.

Accuracy:
Multiple level recovery tests were used to verify accuracy by spiking the standard solution in placebo at three distinct levels. A standard stock solution containing 100g/ml GLY and 500g/ml NEO was prepared and spiked with levels of the standard drugs equal to 50%, 100%, and 1500% of the original solution. The average recovery ranged from 98.4 percent to 100.2 percent when these solutions were analysed for recovery studies. Table 02 shows the results of the accuracy determination[4,8,9].

Table 1: Accuracy results of Glycopyrrolate and Neostigmine

% Spike level	Glycopyrrolate			Neostigmine		
	Amount of API added (mg)	Amount Recovered (mg)	% Recovered	Amount of API added (mg)	Amount Recovered (mg)	% Recovered
50%	5	4.97	99.4	25	24.63	98.5
	5	5	100.0	25	24.61	98.4
	5	4.98	99.6	25	24.53	98.1
100%	10	10.06	100.6	50	50.06	100.1
	10	9.99	99.9	50	50.36	100.7
	10	10.01	100.1	50	49.74	99.5
150%	15	14.92	99.5	75	75.54	100.7
	15	14.76	98.4	75	75.29	100.4
	15	14.82	98.8	75	74.65	99.5

Precision:
For the quantification of GLY and NEO, the approach was confirmed to be accurate after six sample preparations. On a different day, the precision was examined. The precision and intermediate precision variation were calculated in percentage relative standard deviation, and the findings of GLY and NEO were determined to be less than 2.0 percent[9].

Linearity:
For GLY, linearity was reached in the range of 1-15 µ/ml, while for NEO, it was acquired in the range of 5-75µ/ml. Calibration curve graph were plotted and the coefficient of regression obtained was 0.9996 for GLY and 0.9990 for NEO respectively. The slope obtained 18656.22 and 46375.47 respectively for GLY and NEO

Ruggedness and Robustness:
The robustness of the method was tested using several instruments, and the robustness was determined by changing the flow rate by ±0.1 and the mobile phase organic composition by ±10. The approach was proven to be rugged and robust in these situations based on the results[8,9].

LOD and LOQ:
The lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact number is the detection limit of an individual analytical method[11]. The detection limits for GLY and NEO were found to be 0.01 and 0.05, respectively. The lowest amount of analyte in a sample that can be quantitatively measured with sufficient precision and accuracy is the quantification limit of a particular analytical procedure. The detection limits for GLY and NEO were 0.1 and 0.5, respectively[9].

Force degradation:
The fore degradation investigation was carried out according to ICH criteria (ICH, Q2B) in acid, basic, oxidation, photolytic, and thermal conditions for both placebo and formulation. Refluxing the sample and placebo into individual flasks at 60ºC for six hours with 0.1M HCl, 0.1M NaOH, and 10% Hydrogen peroxide was used in the stress studies. UV light was used to test the sample's photolytic stability[8,9]. The drug and formulation were shown to be stable under all stress situations.

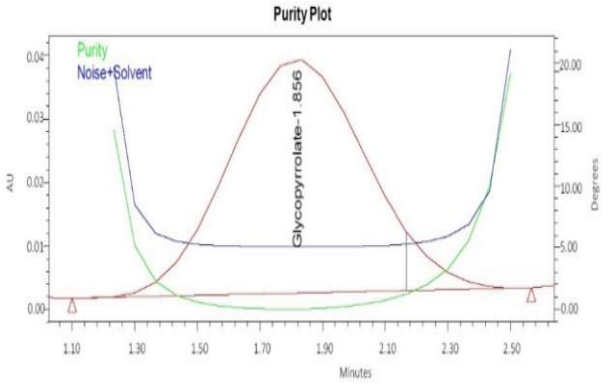

Figure 4: Acid degradation purity plot for Glycopyrrolate

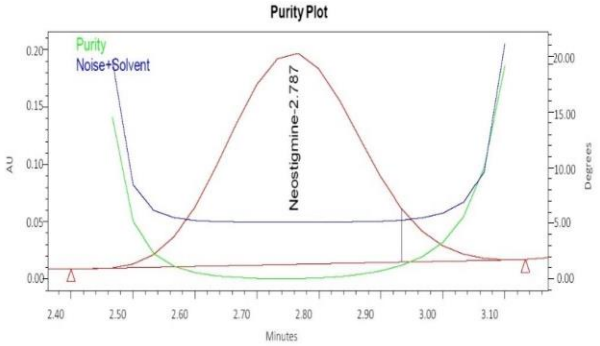

Figure 5: Acid degradation purity plot for Neostigmine

Table 2. Acid degradation results of Glycopyrrolate

S. No	Sample weight (mg)	Area Counts	% Label Claim	% Degradation	Purity Angle	Purity Threshold
1	100	191196	99.9	0.1	0.338	10.826
2	100	184642	96.5	3.5	0.34	10.827

Table 3. Acid degradation results Neostigmine

S. No	Sample weight (mg)	Area Counts	% Label Claim	% Degradation	Purity Angle	Purity Threshold
1	100	2444417	99.7	0.3	4.185	10.719
2	100	2351935	96	4	4.191	10.726

Solution stability:

Both the standard and the sample were tested at room temperature for 24 hours[9]. The results revealed that the retention time, peak area, and relative standard deviation of GLY and NEO remained unchanged for all solutions, indicating that no deterioration occurred during this time.

Table 4: Results of the method's validation and system suitability studies

Method characteristic	Glycopyrrolate	Neostigmine
Theoretical plates	3261	5089
Resolution	----	4.92
Tailing factor	1.05	1.06
Accuracy (%RSD)	0.66	1.02
Precision (%RSD)	0.172	0.502
Intermediate Precision (%RSD)	0.25	0.49
LOD	0.01	0.1
LOQ	0.05	0.5

CONCLUSION:

A Simple, precise, accurate, and reliable stability indicating RP-UPLC method was developed for simultaneous estimation of Glycopyrrolate and Neostigmine in injection formulation. As these proposed methods have the lowest retention time for both the drugs it can be used as regular basis in analysis of raw material and formulations.Linearity, accuracy, precision, ruggedness, robustness, and force degradation studies have all been validated using the established approach. The lowest LOQ value indicates the more sensitivity of the method. The % degradation for Glycopyrrolate for acid, alkali, peroxide, Thermal and photolytic was found to 3.5, 3.1, 1.188, 0.349 and 0.336. The % degradation forNeostigmine for acid, alkali, peroxide, Thermal and photolytic and thermal stress conditions were found to be 4, 4.1, 3.7, 3.3 and 3.1 these values indicates that both the drugs are resistant towards all forced degradation conditions.

CONFLICT OF INTEREST:

No

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