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Original Article

A Reverse Phase Stability Indicating HPLC Method Development for Estimation Assay of Benzyl Alcohol and Glycopyrrolate in Glycopyrrolate Injection

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ABSTRACT

Background: The present work is focused on developing a simple stability indicating method for the estimation of the assay of benzyl alcohol and glycopyrrolate in glycopyrrolate injection using reverse high performance liquid chromatography. **Methods:** A simple, selective, rapid, precise, and gradient reversed-phase high-pressure liquid chromatography procedure has been proposed to estimate the assay of benzyl alcohol and glycopyrrolate for glycopyrrolate injection. It was developed on a Kromasil 100-5, C_8 250 mm x 4.6 mm, 5 µm column using mobile phase-A, containing pH 2.5 potassium dihydrogen phosphate buffer and methanol in a ratio of 80:20 v/v. and mobile phase-B, containing pH 2.5 potassium dihydrogen phosphate buffer and methanol in a ratio of 10:90 v/v at a flow rate of 1.5 mL/min. The detection of glycopyrrolate and benzyl alcohol was carried out at 222 nm and 254 nm. A sharp peak was obtained for glycopyrrolate and benzyl alcohol at a retention time of about 8.4 and 4.8 min. **Results:** The method was validated for specificity (blank, system suitability solution, standard, and sample and stress samples for any interference), precision (six replicate sample preparations), robustness (by slightly varying critical method parameters), and solution stability, and the results were found satisfactory. **Conclusion:** The method is specific, precise, accurate, and robust for estimation of the assay of benzyl alcohol of glycopyrrolate in glycopyrrolate injection.

Key words: Glycopyrrolate, High-pressure liquid chromatography, Validation, Buffer solution, Benzyl alcohol.

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INTRODUCTION

Glycopyrrolate injection is a synthetic anticholinergic agent. Each 1 mL vial of injection contains 0.2 mg of glycopyrrolate active substance and benzyl alcohol, a sufficient quantity of water for injection, and hydrochloric acid or sodium hydroxide for pH adjustment, when necessary. Glycopyrrolate (GLY) is a quaternary ammonium salt that shows analogue activity to that of atropine and scopolamine. Its chemical formula is $C_{19}H_{28}BrNO_3$ and its mass is 398.33.[1-5] The chemical structure of GLY is shown in Figure 1.

GLY is a crystalline powder that is white in colour and odourless. It is soluble in water and alcohol, and practically insoluble in chloroform and ether.

Benzyl alcohol (Figure 2) is an organic compound and its chemical formula is $C_6H_5CH_2OH$. The benzyl group is frequently represented as "Bn," and so benzyl alcohol is represented as BnOH in chemical notation. BnOH is a colourless liquid that has a moderate, aromatic fragrance. It is a good solvent due to its polarity, minimal toxicity, and low vapour pressure. BnOH has a moderate water solubility and is miscible with alcohols and diethyl ether. The anion produced by the deprotonation of the alcohol group is known as benzylate or benzyloxide.[6] BnOH is used in a wide range of cosmetic products as an aroma ingredient, a preservative, a solvent, and as a component that helps reduce viscosity.[7] Low concentrations of BnOH are used in intravenous treatments, cosmetics, and topical therapeutic approaches.[7] When employing high concentrations of BnOH as a preservative solution, considerable precaution should be taken because benzaldehyde is generated when the solution is utilized as a preservative.[7,8]

The United States Food and Drug Administration (FDA) approved benzyl alcohol, marketed under the trade name Ulesfia. It was approved for the treatment of head lice in 2009 for children 6 months of age and older.[9] It obstructs the louse from closing its spiracles. These are subsequently clogged with water, mineral oil, or other substances, resulting in the insect's death by asphyxiation.[9] BnOH is a main component in shampoo lotion with 5% benzyl alcohol because it works well to get rid of lice.[9-11]

During the literature search, all the available articles for GLY[1–5] and for benzyl alcohol[7–12] were reviewed to know the physicochemical properties of glycopyrrolate and benzyl alcohol. In addition, all the articles related to the estimation of glycopyrrolate and benzyl alcohol were reviewed and found that all the available methods were focused on estimating a single component, i.e., either glycopyrrolate or benzyl alcohol, and no single HPLC method was found for estimation of both. Furthermore, none of the available articles were clear about the method's stability indicating power, i.e., how degradation impurities and known impurities interference were avoided for the estimation of glycopyrrolate and benzyl alcohol.[13-21] Hence, this research work is focused on developing a single RP-HPLC stability indicating method for estimation of both glycopyrrolate and benzyl alcohol in both drug products and drug substances. It is also focused on developing a fast and cost-effective

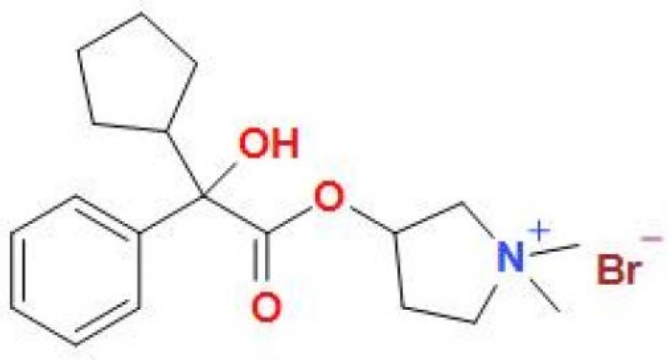

Figure 1: Chemical Structure of or Glycopyrrolate.

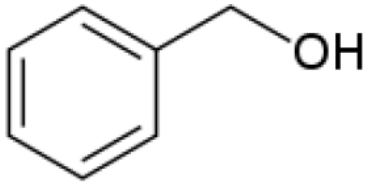

Figure 2: Chemical Structure of benzyl alcohol.

method of analysis to save precious analytical time. Easily available and cheap chemicals have been used for this research work.

MATERIALS AND METHODS

Materials

Glycopyrrolate injection, glycopyrrolate working standard and benzyl alcohol were purchased from market. Potassium dihydrogen phosphate, orthophosphoric acid (88%) were used as analytical grade of Merck Ltd. Methanol and acetonitrile used were of HPLC grade solvents of Merck and Rankem Ltd. Purified water used was from Milli-Q water purification system.

Instruments and Equipment

HPLC of the make-Water e2695 series, with UV/PDA detector was used in the experiment. Analytical balance (Sartorinus), pH meter (Thermo scientific), Ultra sonic bath (PCI analytics) was used during sample preparation.

Method of Analysis

Preparation of Mobile Phase

Preparation of 10% orthophosphoric acid: Transfer 1 mL of orthophosphoric acid in to a 10mL of water.

Preparation of pH 2.5 Potassium Dihydrogen Phosphate Buffer

Weigh and transfer about 3.4 g of potassium dihydrogen phosphate into 1000 mL of water and mix well to dissolve. Adjust the pH to 2.50 ± 0.05 with orthophosphoric acid. Filter the solution through a 0.45µm membrane filter and degas it.

Preparation of Mobile Phase A

Mix pH 2.5 potassium dihydrogen phosphate buffer and methanol the ratio 80:20% v/v and sonicate for 5 min.

Preparation of Mobile phase B

Mix pH 2.5 potassium dihydrogen phosphate buffer and methanol the ratio 10:90% v/v and sonicate for 5 min.

Preparation of Diluent

Use diluent as pH 2.5 potassium dihydrogen phosphate buffer

Preparation of Glycopyrrolate Standard Stock Solution

Weigh and transfer about 50 mg Glycopyrrolate working standard into a 50 mL volumetric flask, add about 30 mL of diluent and sonicate to dissolve. Dilute to volume with diluent and mix well.

Preparation of Benzyl Alcohol Standard Stock Solution

Weigh and transfer about 110 mg Benzyl alcohol standard into a 50 mL volumetric flask, add about 30 mL of diluent and sonicate to dissolve. Dilute to volume with diluent and mix well.

Preparation of Standard Solution

5mL of 1 mg/mL glycopyrrolate standard solution and 5 mL of 2.2 mg/mL benzyl alcohol standard stock solution is pipetted out into a 100 mL volumetric flask, then made up to volume with diluent.

Preparation of Glycopyrrolate Sample Solution

Transfer 5 mL of sample solution into a 20 mL volumetric flask, add about 10 mL of diluent and dilute to volume with diluent.

Preparation of Benzyl Alcohol Sample Solution

Transfer 5 mL of glycopyrrolate sample solution into a 100 mL volumetric flask, add about 60 mL of diluent and dilute to volume with diluent.

HPLC Method

Inject 35µL of blank, standard and sample solution on waters HPLC system (e2695) with UV/PDA detector setup with 1.0mL/min flow rate. A chromatographic column of Acquity Kromasil 100, C8, 250 mm × 4.6 mm, 5 µm particle size was used. Column temperature was 30°C, gradient elution mode i.e.: at 0 min 40% mobile phase B, at 12 min 40%, at 20 min 85%, at 23 min 40%, at 30 min 40%, and wavelength of detection was 222 nm for glycopyrrolate and 254 nm for benzyl alcohol.

RESULTS

Method Development and Optimization of Chromatographic Conditions

Optimization of Buffer

Based on the available literature multiple development trails were conducted to get good peak shape and better resolution between benzyl alcohol and glycopyrrolate using different combination of buffers with organic modifiers like methanol and acetonitrile. Finally, from multiple optimization trails pH 2.5 phosphate buffer with combination of methanol was found suitable to separate benzyl alcohol and glycopyrrolate with gradient elution.[22,23]

Optimization of Stationary Phase

The optimization of method was started with normal C18, C8 stationary phases, multiple trails were conducted with different brands of C18, C8 columns, upon multiple experiments in Kromasil 100, C8, 250 mm × 4.6 mm, 5 µm column was finalized based on the separation and retention of benzyl alcohol and glycopyrrolate peaks with column temperature 30°C.[24]

Optimization of Wavelength

Since the both benzyl alcohol and glycopyrrolate are having chromophores, based on available literature UV detection mode was

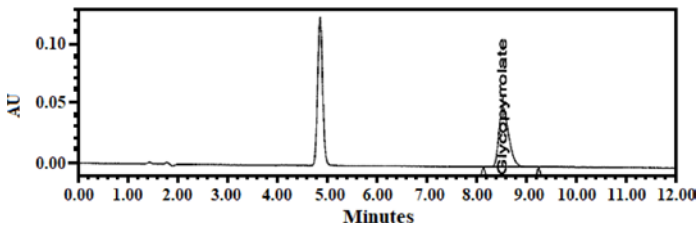

Figure 3: Representative chromatogram of glycopyrrolate standard at 222 nm.

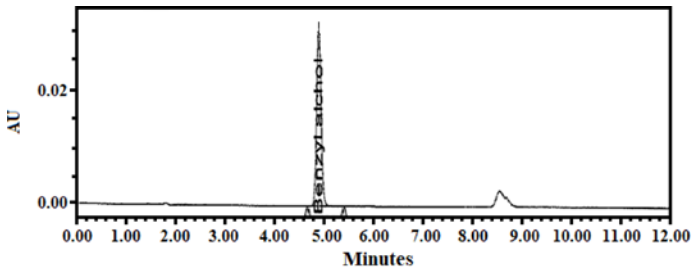

Figure 4: Representative chromatogram of benzyl alcohol 254 nm.

chosen with dual wavelength i.e.: 222 nm for glycopyrrolate (Figure 3) and 254 nm for benzyl alcohol (Figure 4).[22-24]

Optimization of sample Preparation and Diluent

To achieve good peak shape and desired recovery different combination of diluents were evaluated at different concentration levels. Finally, with pH 2.5 potassium dihydrogen phosphate buffer good peak shape and desired recovery was achieved with the sample concentration of 0.110 mg/mL for benzyl alcohol and 0.05 mg/mL for glycopyrrolate with the injection load of 35 µL.

Method Validation Results and Discussion

To prove the suitability of optimized chromatographic conditions, the optimized chromatographic conditions was validated as per of ICH Q2 (R1) guidelines. The parameters evaluated were system suitability, specificity, linearity, precision, accuracy and robustness.[25-27]

System Suitability

The suitability of HPLC system was proved by injecting standard solution (five times) and by assessing the tailing factor (NMT 2.0), theoretical plate count (NLT 2000) and % RSD (NMT 2.0%) of both benzyl alcohol and glycopyrrolate, The injected standard solution was assessed for tailing factor, theoretical plate count and % RSD of both benzyl alcohol and glycopyrrolate, found to be met with system suitability requirement.

Specificity

The specificity of the test method was proved by preparing the samples like placebo, standard, sample, spiked samples as per methodology and the prepared samples were injected into HPLC system which is equipped with PDA detector, in addition to above the glycopyrrolate injection samples were exposed to harsh stress conditions and the exposed samples were injected into HPLC system with PDA detector and evaluated the resultant chromatograms for peak purity, the peak purity results were found to be met with acceptance criteria i.e.: the purity angle of both glycopyrrolate and benzyl alcohol was found to be less than that of purity threshold [Table 1 and 2].

Method Precision

The precision of the of the test method was proved in preparing the six sample preparations from same homogeneous samples as per methodology and the prepared samples were injected into HPLC system and calculated the %RSD for % assay results of both glycopyrrolate and benzyl alcohol. The % RSD results of both glycopyrrolate and benzyl alcohol was found to be less than 2.0% [Table 3].

Accuracy

The accuracy of the test method was proved by spiking the known amount of drug substance to the placebo solution. Accuracy study solutions were prepared in triplicate preparations by spiking the known

Table 1: Degradation study results of benzyl alcohol.

S. No	Name of stress and condition	Purity angle	Purity threshold
	Benzyl alcohol		
01	As such solution	0.183	2.087
02	Acid stress/1N-60°C/1hr	0.201	1.752
03	Base Stress/0.1N-RT/1min	0.203	1.724
04	30% Peroxide stress- RT/5hr	0.185	4.093
05	Water stress-60°C/1hr	0.176	2.191
06	Thermal (80°C for 4hr)	0.182	2.198
07	Humidity-1day	0.180	2.240
08	Photo stability	0.196	1.680

Table 2: Degradation study results of glycopyrrolate.

S. No	Name of stress and condition	Purity angle	Purity threshold
	Glycopyrrolate		
01	As such solution	0.072	1.001
02	Acid stress/1N-60°C/1hr	0.052	0.263
03	Base stress/0.1N-RT/1min	0.050	0.275
04	30% Peroxide stress- RT/5hr	0.060	5.596
05	Water stress-60°C/1hr	0.070	1.565
06	Thermal (80°C for 4hr)	0.074	1.651
07	Humidity-1day	0.078	1.686
08	Photo stability	0.063	0.415

Table 3: Method precision study results.

S. No	Name of component	% Assay of glycopyrrolate	% Assay of benzyl alcohol
01	Precision sample-01	102.1	104.0
02	Precision sample-02	102.3	104.2
03	Precision sample-03	102.3	104.0
04	Precision sample-04	102.3	104.2
05	Precision sample-05	102.3	104.2
06	Precision sample-06	102.2	103.8
	Average % assay	102.3	104.1
	% RSD	0.1	0.2

Table 4: Accuracy study results.

S. No	Accuracy level	% Recovery of glycopyrrolate	% Recovery of benzyl alcohol
01		100.2	101.7
02	50%	100.6	99.4
03		100.9	101.1
04		100.8	100.9
05	*100%*	100.4	98.5
06		101.0	101.4
07		99.8	99.6
08	150%	100.0	98.5
09		100.1	100.8

amount of benzyl alcohol and glycopyrrolate at 50 %, 100 % and 150% levels of target concentration as per methodology and the prepared samples were injected into HPLC system. Calculated the % recovery against "mg found verses mg added" for both glycopyrrolate and benzyl alcohol. The % recovery results of both glycopyrrolate and benzyl alcohol was found to be within the limits i.e.: between 98.0% to 102.0% [Table 4].

Solution Stability

The solution stability for standard and sample solutions were established for the period of 48 hr at both room temperature and refrigerated conditions. The standard and sample solutions were prepared as per the methodology and injected into HPLC system, calculated the % difference in the % assay of respective time point against initial time point and found that the solution stability results are well within the acceptance criteria up to 48 hr for glycopyrrolate and up to 24 hr for benzyl alcohol as the observed % difference in the % assay of respective time was found less than 2.0%.

Robustness

The robustness method was proved by deliberately varying the flow rate, column temperature and mobile phase buffer pH as mentioned in the Table 4 from the optimized method conditions. Standard solution was prepared and injected into HPLC system; the resultant chromatograms were evaluated for system suitability parameters. The system suitability results were found to be met with acceptance criteria for all variable robustness parameters.

DISCUSSION

Based on multiple experiments, chromatographic conditions were finalized like pH 2.5 phosphate buffer with methanol combination as mobile phase, Kromasil 100-5 C8, 250 mm x 4.6 mm, 5 µm as stationary phase with a detection wavelength of 222 nm for glycopyrrolate and 254 nm for benzyl alcohol, and also optimized the standard and sample concentration for both benzyl alcohol and glycopyrrolate as 0.11 mg/ml and 0.05 mg/mL, respectively. Upon optimization, 35 µL of standard and sample solutions were injected into on waters HPLC system (e2695) with a UV/PDA detector setup with a 1.0 mL/min flow rate and confirmed the suitability of standard and sample concentration by evaluating peak symmetry and column efficiency.

The optimized chromatographic conditions were further subjected to analytical method validation to prove that the optimized conditions are suitable for their intended use, i.e., for quantification of benzyl alcohol and glycopyrrolate in glycopyrrolate injection. In view of method validation, the optimized chromatographic conditions were assessed for system suitability, specificity (blank interference, placebo interference, and forced degradation study), method precision, recovery, solution stability, and robustness.

System suitability was proved by injecting five replicate injections of standard solution into the HPLC system with the assessment of tailing factor (NMT 2.0), theoretical plate count (NLT 2000) and % RSD (NMT 2.0%) for both benzyl alcohol and glycopyrrolate peaks, and finding all the system suitability results were met with predefined acceptance criteria.

The specificity of the test method was proved by preparing and injecting the samples like placebo solution and spiked samples as per the methodology into the HPLC system, which is equipped with a PDA detector. In order to prove the stability-indicating power of the optimized HPLC method, the glycopyrrolate injection samples were exposed to stress conditions like acid hydrolysis, basehydrolysis, oxidative, thermal, photolytic, and humidity conditions. The exposed samples were injected into the HPLC system with a PDA detector and the resultant chromatograms of placebo and spiked samples were assessed. There was no interference at the retention of both benzyl alcohol and glycopyrrolate and the exposed sample chromatograms were assessed for peak purity. The peak purity results were found to be met with acceptance criteria, i.e., the purity angle of both glycopyrrolate and benzyl alcohol was found to be less than the purity threshold. Hence, from the specificity results, it was concluded that the method was found to be specific for estimation of benzyl alcohol and glycopyrrolate in glycopyrrolate injection.

Method precision was assessed by preparing six sample preparations of glycopyrrolate injection as per optimized sample preparation procedure and injecting them into the HPLC system, calculated the % assay of benzyl alcohol and glycopyrrolate from six sample preparations and also calculated the %RSD for both benzyl alcohol and glycopyrrolate. From the % RSD results, it was concluded that the method is precise enough to estimate both benzyl alcohol and glycopyrrolate in glycopyrrolate injection.

The accuracy of the test method was proved by calculating the % recovery against the amount of benzyl alcohol and glycopyrrolate recovered versus the known amount of benzyl alcohol and glycopyrrolate added. To prove the accuracy, solutions were prepared in triplicate preparations by adding the known amount of benzyl alcohol and glycopyrrolate at 50%, 100%, and 150% levels of test concentration, and the prepared accuracy samples were injected into the HPLC system. Calculated the % recovery against "mg recovered versus mg added" for both glycopyrrolate and benzyl alcohol. The % recovery results of both glycopyrrolate and benzyl alcohol were found to be between 98.0% and 102.0%. Therefore, the method was found to be accurate for estimation of both benzyl alcohol and glycopyrrolate. In addition, the stability of standard and sample solutions was established at both room temperature and refrigerated conditions for a period of 48 hr. To establish solution stability, the standard and sample solutions were prepared as per the methodology and injected into the HPLC system at initial, after 24 hr, and after 48 hr. The % difference in the % assay of benzyl alcohol and glycopyrrolate was calculated between the initial and respective time points. From the results, it was found that the benzyl alcohol is stable for up to 24 hr and glycopyrrolate is stable for up to 48 hr.

Robustness of the test method was established by injecting the standard solution five replicate injections and by deliberately varying the flow rate (±10%), column temperature (± 5°C) and mobile phase buffer pH (± 0.2 units) from the optimized method conditions, the resultant chromatograms were assessed for system suitability parameters like

%RSD of peak area, tailing factor and USP plate count and found to be within the acceptance criteria, hence the method was found to be robust for estimation of both benzyl alcohol and glycopyrrolate in glycopyrrolate injection.

CONCLUSION

A simple, specific, precise, accurate and robust method has been developed for the estimation of assay of benzyl alcohol and glycopyrrolate in glycopyrrolate injection with Kromasil 100-5 C_8, 250 mm × 4.6 mm, 5 µm using mobile phase-A pH 2.5 ± 0.05 potassium dihydrogen phosphate buffer and methanol the ratio 80:20% v/v and mobile phase-B pH 2.5±0.05 potassium dihydrogen phosphate buffer and methanol the ratio 10:90% v/v at a flow rate of 1.5 mL/min at a detection wavelength of 222 nm for glycopyrrolate and 254 nm for benzyl alcohol, the gradient RP-HPLC method was developed and validated for specificity, accuracy, precision and robustness parameters. Based on the results it was concluded that the method is suitable for estimation of assay of benzyl alcohol and glycopyrrolate in glycopyrrolate injection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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