Research Article

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Robust In Vitro HPLC-DAD Method for Accurate Quantification of N-Carbamylglutamate in Drug Formulations

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ABSTRACT

A comprehensive in vitro testing method has been developed and validated for accurately quantifying N-Carbamylglutamate (Carglumic acid) using a high-performance liquid chromatography instrument with a diode array detector. A method specifically developed for pharmaceutical quality control utilizes a reverse-phase C18 column for precise and reliable measurement of Carglumic acid. The chromatographic parameters were refined for peak resolution, employing a phosphate buffer and acetonitrile as the mobile phase, with detection setup at 205 nm. The method was validated under ICH Q2(R2) requirements. The approach exhibited a significant level of specificity, with no interference from excipients or other possible impurities. Linearity was achieved across the entire concentration range, yielding an R^2 value of 0.999. Precision (RSD $\leq$ 2) was demonstrated by intra- and inter-day variability being within acceptable limits. Recovery tests validated accuracy (98 – 102%). Determining the limit of detection (LOD, 0.1 ppm) and the limit of quantitation (LOQ, 0.5 ppm) was conducted. This in vitro test method was valid and effective for the routine quantification of Carglumic acidin pharmaceutical formulations, serving as a crucial tool for ensuring product quality and regulatory compliance. The methods' elevated sensitivity and endurance make it appropriate for bioanalytical applications, such as dissolution testing and stability analysis.

Keywords: Carglumic Acid, N-Carbamylglutamate, *in vitro,* N-carbamylglutamate dissolution

1. Introduction

The development of reliable, accurate, and precise analytical methods is essential in the pharmaceutical sector to ensure the safety and efficacy of such medications. N-Carbamylglutamate, a synthetic derivative of Carglumic Acid, has a very significant role in the management of inherited urea cycle disorders [1–10]. The rising need for precise quantification of Carglumic acid highlights the necessity for a reliable analytical approach. Present techniques are either unsuitable or not fully set for pharmaceutical applications [11].

N-carbamyl-L-glutamic acid (Carglumic acid) is a white to off-white crystalline powder. The molecular weight is 190.17 g/mol, and it exhibits polymorphism, which influences its physical stability and solubility under various conditions. Although it is highly soluble in water, it has low solubility in organic solvents such as methanol and ethanol, making aqueous solutions the preferred medium for its production. Carglumic acid's hygroscopic characteristics need storage in moisture-controlled conditions to prevent deterioration and maintain its therapeutic integrity over time [12,13].

This substance's pKa value is approximately 3.1, indicating its acidic properties and optimal stability in slightly acidic to neutral pH circumstances. It is prone to degradation in highly acidic or basic settings, which can reduce its potency if not properly prepared and stored. As a result, understanding its physicochemical properties is critical for developing reliable analytical processes and ensuring consistency in pharmaceutical manufacturing [14].

Because of their sensitivity, high-performance liquid chromatography (HPLC) methods are ideal for precisely measuring carglumic acid. These characteristics also influence its dissolving properties, particularly in formulations designed for oral administration. Enhancing dissolution profiles, which commonly involve using phosphate buffer solutions at pH 6.8, ensures bioavailability and therapeutic efficacy. These characteristics underline the importance of methodically designing analytical and stability testing to maintain the purity and efficacy of carglumic acid in pharmaceutical applications [15–19].

This study aims to provide a validated *in vitro* assay utilizing use of high-performance liquid chromatography via a diode array detector (HPLC-DAD) for the precise quantification of Carglumic acid in pharmaceutical formulations. The methodology prioritizes the optimization of chromatographic parameters, including mobile phase composition and detection wavelength, to ensure the precise separation and quantification of Carglumic acid [20–23]. A reverse-phase C18 column was eluted by a buffer and acetonitrile-containing mobile phase, and DAD was set to 205 nm [24]. Validation was performed under The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH Q2(R2)) recommendations, addressing essential parameters like specificity, linearity, precision, accuracy, and sensitivity. This *in vitro* assay method offers accurate quantification and enables key quality control protocols in pharmaceutical production [25–29]. This assay method signifies a major enhancement in the analytical testing of Carglumic acid, providing increased sensitivity, accuracy, and robustness relative to current methodologies. The validated method is also appropriate for extensive bioanalytical applications, such as dissolution and stability testing, rendering it a significant asset in pharmaceutical quality control [30].

Besides the HPLC-DAD method, an *in vitro* dissolving system was utilized to assess the release profile of Carglumic acid (Figure 1) from pharmaceutical formulations [14, 23, 24]. Dissolution tests were conducted with a phosphate buffer (pH 6.8) as *in-vitro* system media, effectively simulating the intestinal environment and rendering it appropriate for orally administered medicines [22, 25]. The dissolving method guarantees precise measurement of the drug's release kinetics, yielding critical data for bioavailability and therapeutic efficacy [34–37]. The pH 6.8 buffer was chosen for its applicability in mimicking the physiological setting of the small intestine, the primary site for medication absorption [38–41]. This configuration facilitates the examination of dissolving rates and guarantees that the formulations comply with regulatory standards for dissolution testing [42–48].

This study highlights the necessity of establishing an efficient *in vitro* test method for quantifying Carglumic acid, given its significance in treating genetic urea cycle diseases [49]. Contemporary analytical techniques, especially those derived from established pharmacopeial standards, may exhibit insufficient sensitivity and robustness for regular pharmaceutical testing. The novel HPLC-DAD test method intro-

duced here seeks to address these flaws by providing improved precision, sensitivity, and reproducibility [19, 42–51]. Moreover, the verified method could significantly improve laboratory efficiency, facilitating quality control and regulatory compliance in the pharmaceutical industry [21, 52–55].

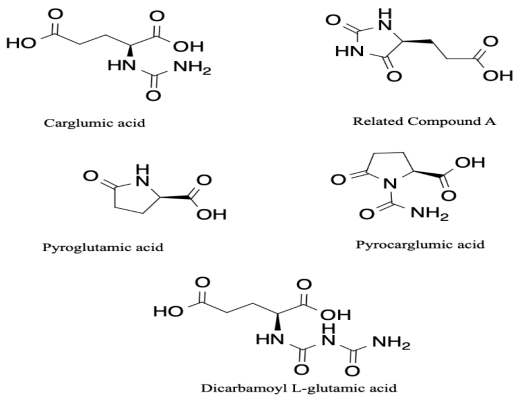

Figure 1. Chemical Structure of Carglumic Acid

2. Material and Methods

Carglumic acid, which was procured from Nuray Chemicals, India, had an assay of 99.6% w/w on an as-is basis. In addition, HPLC-grade LiChrosolv acetonitrile, as well as 85% (LiChropur, Germany) ortho-phosphoric acid and potassium dihydrogen phosphate (LiChropur, Germany), were sourced, through Supelco (Merck, Darmstadt). Furthermore, other reagents, including hydrochloric acid (ACS Reagent, Germany), sodium chloride (Emsure, Germany), sodium acetate (Emsure, Germany), glacial acetic acid (Emsure, Germany), and sodium hydroxide (Emsure, Germany), were also obtained from the same supplier (Merck, France). Moreover, Milli-Q water, generated by the Millipore purification system, was consistently used as the dissolving medium, diluent, and for the preparation of mobile phases.

2.1. Instrumentation

This study utilized an Agilent Infinity II HPLC (1260) system (USA), which included a 1260 Quat Pump (G7111B), a 1260 MCT (G7116B), a 1260 Vial Sampler with a column oven (G7129A), and a 1260 DAD WR detector (G7115A) for analytical purposes. In addition, the dissolution testing was performed using a Distek Dissolution (USA) system that featured the Eclipse 5300 and Dissolution System 2500, alongside Mettler Toledo (XPR205, Switzerland) analytical balances and SevenExcellence pH meters (S475-K, Switzerland). Furthermore, the Waters (USA) HSS T3; Xselect Peptide column (186008870, 250 × 4.6 mm, 5.0 µm particle size) was chosen for the chromatographic separation.

2.2. Preparation of the Dissolution Media

pH 6.8 media: A 0.2 M solution of potassium dihydrogen phosphate was prepared by first weighing 27.22 g of KH_2PO_4, dissolving it in a minimal amount of water, and then diluting it to a total volume of 1000 mL with filtered water. Additionally, a 0.2 M sodium hydroxide solution was created by weighing 8.4 g of NaOH, dissolving it in a small amount of water, and subsequently adjusting the final volume to 1000 mL using distilled water. To formulate a pH 6.8 phosphate buffer, 250.0 mL of the 0.2 M potassium dihydrogen phosphate solution was mixed with 112.0 mL of the 0.2 M sodium hydroxide solution, and this mixture was further diluted to a final volume of 1000 mL with distilled water.

pH 4.5 media: An acetate buffer solution with a target pH of 4.5 was prepared by first dissolving 17.94 g of sodium acetate trihydrate into 6000 mL of water. After ensuring the complete dissolution of the sodium acetate, 9.96 mL of glacial acetic acid was gradually added to the solution, followed by thorough mixing to ensure homogeneity. The solution's pH was then carefully monitored and adjusted to 4.5 ± 0.05 using a calibrated pH meter, adding small amounts of either acetic acid or sodium acetate as needed to achieve the desired pH range. Once the pH was confirmed to be within the specified range, the solution underwent filtration through a 0.45 µm hydrophilic membrane filter to remove any particulate matter and ensure clarity.

pH 1.2 media: A 0.2 M sodium chloride solution was prepared by accurately weighing 23.38 g of sodium chloride (NaCl), which was then transferred into a 2000 mL volumetric flask. The salt was initially dissolved in 1000 mL of distilled water with constant stirring until fully dissolved. The solution was then diluted to a total volume of 2000 mL by adding more distilled water. Similarly, a 0.2 M hydrochloric acid solution was prepared by carefully adding 50.0 mL of concentrated hydrochloric acid (HCl) to a 3000 mL volumetric flask partially filled with distilled water. The mixture was stirred thoroughly to ensure

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even distribution, and then additional distilled water was added to bring the final volume up to 3000 mL. For the preparation of the pH 1.2 acid solution, a precise combination of 2550 mL of 0.2 N hydrochloric acid, 1500 mL of 0.2 M sodium chloride, and 1950 mL of distilled water were mixed. This mixture was thoroughly stirred to ensure uniformity and achieve the intended pH level. [56, 57].

2.3. Preparation of the Mobile Phase

To prepare the solution, 27.2 g of potassium dihydrogen phosphate was carefully weighed and transferred into a suitable container. It was then dissolved in 1000 mL of water, ensuring complete dissolution by stirring thoroughly. Following this, the pH of the solution was carefully adjusted to 2.5 ± 0.05 using small increments of phosphoric acid, with constant pH monitoring to achieve the desired range. After reaching the target pH, the solution was combined with acetonitrile in a 98:2 volume-to-volume ratio, ensuring thorough mixing to achieve a homogeneous mixture. The final step involved filtering the solution through a 0.45 μm hydrophilic PTFE membrane filter to remove any particulate matter, thereby ensuring a clear and purified solution.

2.4. Preparation of Standard Solution

To prepare the standard solution, 20 mg of carglumic acid reference standard was accurately weighed and then transferred into a 50 mL volumetric flask. The reference standard was initially dissolved with a small amount of pH 6.8 phosphate buffer solution, followed by further dilution up to the 50.0 mL mark using the same buffer, ensuring complete dissolution. The solution was then agitated for 30 minutes using a magnetic stirrer to ensure uniformity. Next, 2.0 mL of this primary solution was carefully pipetted and transferred into a separate 20 mL volumetric flask. It was further diluted to 20.0 mL with a diluent containing 40 μg/mL of carglumic acid, achieving the required concentration. The resulting standard solution was found to be stable for up to 74 hours when stored at a temperature of 5 °C, ensuring its suitability for subsequent analytical procedures.

2.5. Preparation of the Test Solution

The process begins by placing a commercially available tablet (which contains 200 mg of carglumic acid as the active ingredient) into a weighing container to record its initial mass accurately. The tablet is then dissolved by adding 2.5 mL of water, ensuring it begins to disintegrate. The partially dissolved tablet is transferred into a dissolution vessel containing 700 mL of phosphate buffer solution, maintained at pH 6.8 and a constant temperature of 37 °C. To ensure complete transfer, the container is rinsed with 50 mL of pre-heated phosphate buffer solution, matching the same pH and temperature. After the dissolution period, a sample is collected from the medium for analysis. Specifically, 3.0 mL of this sample is withdrawn and mixed with 10.6 mL of pH 6.8 phosphate buffer solution, followed by further dilution to a total volume of 20.0 mL using a diluent containing 40 μg/mL of carglumic acid. The prepared standard solution demonstrates stability for up to 60 hours when stored at 5 °C, ensuring reliable results over this duration.

2.6. Method Development Studies

Initially, a suitable dissolution medium was chosen for the method development studies, and the stability and solubility of Carglumic acid in this medium were evaluated. This investigation utilized a 750 mL phosphate buffer at pH 6.8. In the profile tests performed in pH 1.2 and pH 4.5 conditions, samples obtained from the dissolving system were diluted with pH 6.8 buffer to preserve a consistent pH and assure stability throughout the testing process. In the HPLC system, the introduction of organic solvent resulted in peak shifting or distortion over time, possibly due to the elevated buffer concentration. Consequently, a completely aqueous mobile phase was selected and the Waters HSS T3 column was chosen. The total analysis duration was 12 minutes, with a column temperature of 30 *°C. A sample temperature of 5 °C was maintained to guarantee sustained stability. A wavelength of 205 nm was selected for this procedure, informed by the UV spectra from previously established impurity methods. The* analytical technique parameters are detailed in Table 1, while the parameters of the dissolution system are enumerated in Table 2.

2.7. Validation Parameters

ICH, plays a crucial role in establishing the accuracy and reliability of analytical procedures used within the pharmaceutical industry. This methodology was developed in accordance with both ICH Q2(R2) guidelines and the European Pharmacopoeia (EP)

Table 1. Method Parameters of HPLC Instrument

Parameters	
Column	C18; HSS T3; Xselect Peptide; 250 x 4.6 mm, 5 µm (Waters)
Wavelength (nm)	205 nm
Mobil Phase	200 mM, pH 2.5 phosphate buffer: Acetonitrile 98:2 (v/v)
Flow Rate (mL/min)	1.1 mL/min
Column Temperature (°C)	30 °C
Sampler Temperature (°C)	5 °C
Injection Volume (µL)	100 µL
Run Time (min)	12 min

Table 2. Method Parameters of Dissolution System

Parameters	
Apparatus	Apparatus II (Paddle)
Volume	750 mL
Rate	100 rpm
Temperature	37 °C
Medium	pH 6.8 Phosphate buffer
Time	15 min

standards, ensuring compliance with global requirements. The validation process included a thorough evaluation of several critical parameters, such as stress testing to assess method stability, specificity to confirm the method's ability to identify the analyte accurately, system compatibility to verify the instrument's performance, as well as precision, linearity, and range to determine the method's consistency and scope. Additionally, robustness testing was performed to evaluate the method's reliability under varied conditions, while limits of quantification (LOQ) and detection (LOD) were established to ensure the method's sensitivity. This rigorous validation confirmed that the analytical procedure was well-suited for its intended purpose. Furthermore, stability assessments were carried out across different storage conditions to verify the method's durability over time.

2.7.1. System Suitability

The implementation of this test is crucial for successful method development, ensuring that both the LC equipment and the analytical method consistently deliver accurate and reproducible results. To assess system suitability, six consecutive injections of a standardized solution were performed. During this assessment, key parameters such as capacity factor (k'), retention time (tR), theoretical plates, peak area, and the tailing factor of carglumic acid were carefully measured at a wavelength of 205 nm. The relative standard deviation (RSD) percentage was calculated for the peak area, retention time (tR), theoretical plates, and tailing factor to confirm the system's performance. According to the standards established by the European Directorate for the Quality of Medicines & HealthCare (EDQM, EP 2.2.46), it is mandated that the RSD for the peak area should not

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exceed 0.85% across the six injections. Additionally, the tailing factor is required to be within an acceptable range of 0.8 to 2.0, while the number of theoretical plates must be greater than 2000 to demonstrate adequate column efficiency and separation quality. This comprehensive evaluation ensures that the system and method meet stringent criteria, thereby affirming their reliability for analytical applications.

2.7.2. Specificity

Specificity is an essential parameter in liquid chromatography, as it demonstrates the method's ability to distinctly separate the analyte from complex sample matrices, ensuring that no interference is present from solvents in the chromatograms of carglumic acid and its related impurities at the specified wavelength. To confirm the specificity of the method, a thorough analysis was conducted on chromatograms obtained from blank solutions, including the mobile phase and solvents, along with placebo formulations and their mixtures. These evaluations were performed rigorously at various quality control levels to ensure the method's ability to accurately identify and quantify carglumic acid without interference from other components. This comprehensive approach verifies that the method is highly selective for the analyte, thereby supporting its suitability for analytical purposes.

2.7.3. Linearity and Range

To evaluate the linearity of the method, five calibration solutions with varying concentrations were prepared, covering a range of the limit of quantification up to 60 μg/mL. A calibration curve was constructed by plotting the concentration of carglumic acid on the x-axis and the corresponding peak area on the y-axis, which enabled a clear visualization of the relationship between concentration and detector response. From this plot, a regression equation was derived, providing a mathematical expression of the linear relationship. For each calibration solution, the response ratio was calculated by dividing the observed peak area by the respective concentration. This step ensured a consistent measure of detector response across the different concentrations, confirming the method's linearity within the specified range.

2.7.4. Accuracy

This study involved the preparation of three samples at the limit of quantification, specification value, and 150% concentration levels. The objective was to assess the alignment between anticipated and actual outcomes. The assessment criteria established that the relative standard deviation (RSD) for each result must remain below 2%, with a recovery range of 95–105%.

2.7.5. Precision

Precision evaluates the consistency and reliability of an analytical method. This is established by modifying several factors and verifying that these alterations do not affect the method or its efficacy. Considered factors include

- intraday variability.
- employment of an alternative analytical column (same brand, differing lot number)
- utilization of distinct instruments

2.7.6. Limit of Quantification and Limit of Detection

The method's sensitivity was assessed by determining the limit of quantification (LOQ) and limit of detection (LOD), both essential for identifying samples at low concentrations, which is significant for in vitro analysis. The LOQ denotes the minimum concentration for accurate quantification, whereas the LOD signifies the lowest detectable level of impurities. The calculation method for LOQ and LOD was based on the signal-to-noise ratio (*S*/N) method described in EP 2.2.46, and the calculations were performed using HPLC software (Openlab CDS). The method consistently measures carglumic acid at low concentrations, enhancing its applicability in pharmaceutical quality control and ensuring accurate detection of trace dissolution during dissolution/in vitro testing.

2.7.7. Solution Stability

To evaluate the practical application of the proposed analytical method, an extensive study was conducted to assess the stability of carglumic acid solutions over time. Solution stability is a vital aspect, as it directly influences the integrity of the impurity profile, ensuring that it accurately reflects real-world conditions. This study involved monitoring carglumic acid solutions under the specified storage conditions and duration to determine whether any degradation occurred. The findings indicated that carglumic acid solutions maintained their stability throughout the entire storage period, with no significant changes in composition. This result not only validates the ro-

bustness and reliability of the method but also demonstrates its suitability for routine analysis, ensuring consistent and accurate impurity profiling during real-world applications.

2.7.8. Filter Selection

Selecting an appropriate filter is a crucial step in impurity analysis, as it impacts both the accuracy and precision of the results. Extensive research and testing were conducted to identify the optimal filter for sample preparation, ensuring the effective removal of potential interferences while preserving the target impurities. Among the various filters tested (0.45 µm hydrophilic PTFE, 0.45 µm Nylon, 0.45 µm Cellulose acetate), the hydrophilic PTFE filter showed superior performance by efficiently retaining impurities while minimizing sample loss. This not only enhanced the overall efficiency of the sample preparation process but also improved the method's reliability. To validate the performance of the filters, samples prepared using different types of filters were analyzed via HPLC over six consecutive injections, with results compared to non-filtered samples obtained through centrifugation. This comparative analysis confirmed that the hydrophilic PTFE filter consistently provided accurate and precise results, making it the preferred choice for the analytical method.

2.7.9. Robustness (Design of Experiment)

A robustness study employing the Plackett–Burman design was conducted to systematically evaluate the influence of multiple factors on the performance of the analytical method, particularly for impurity analysis in carglumic acid. The primary objective of this study was to identify key factors that could potentially affect the method's robustness and to assess its sensitivity to variations in these parameters.

3. Results and Discussion

3.1. Method Development for HPLC and Dissolution System

The method development efforts were advanced primarily for two systems: the HPLC component and the simultaneous development of the dissolution method. The decisions regarding the dissolution system, such as the selection of the appropriate mobile phase and working range, were interdependent and progressed concurrently.

One of the key aspects of method development in the HPLC system was ensuring an appropriate separation and peak shape. The differing pH levels of the dissolution media resulted in alterations to the pH of the mobile phase, leading to complications such as peak shifts and distortions in peak shape, notably manifested as tailing factors. To address this, a 200 mM phosphate buffer was selected as the mobile phase. Previous studies have shown that the optimal pH for obtaining a suitable peak shape for Carglumic acid was 2.5 or lower. Consequently, this pH was adopted based on experimental results, and an aqueous mobile with a low amount of organic (acetonitrile) solvent was preferred. In routine analyses, to preserve the longevity of the column, a guard column may be utilized, or the column may need periodic washing with water.

3.2. Validation Results of HPLC Analysis

The result of the developed methods' specificity is shown in Figure 2, which demonstrated no interference to Carglumic acid in HPLC analysis due to placebo and blank injections. The specificity results from the validation study are presented below in Figure 2. All validation results are summarized in a tabulated form in Table 3.

The method's robustness was further validated through the Plackett-Burman experimental design. The findings of this experimental design are presented in Tables 5 and 6. This robustness study indicated that the method remained unaffected by minor variations, and the validation of the method was finalized. The method yielded linear results from 0.5 ppm (LOQ) to 60 ppm.

During the evaluation of chromatographic parameters using the Plackett-Burman experimental design, the statistical analysis revealed that the model lacked statistical significance and displayed a low explanatory coefficient, indicating that the model did not adequately explain the variations observed. Despite this outcome, the analytical method itself proved to be robust, as it maintained consistent performance even when subjected to minor variations in chromatographic conditions. This robustness suggests that the method is reliable and capable of producing accurate results, despite potential fluctuations in operational parameters, thus confirming its suitability for routine analytical use. To achieve robustness, key chromatographic parameters were carefully op-

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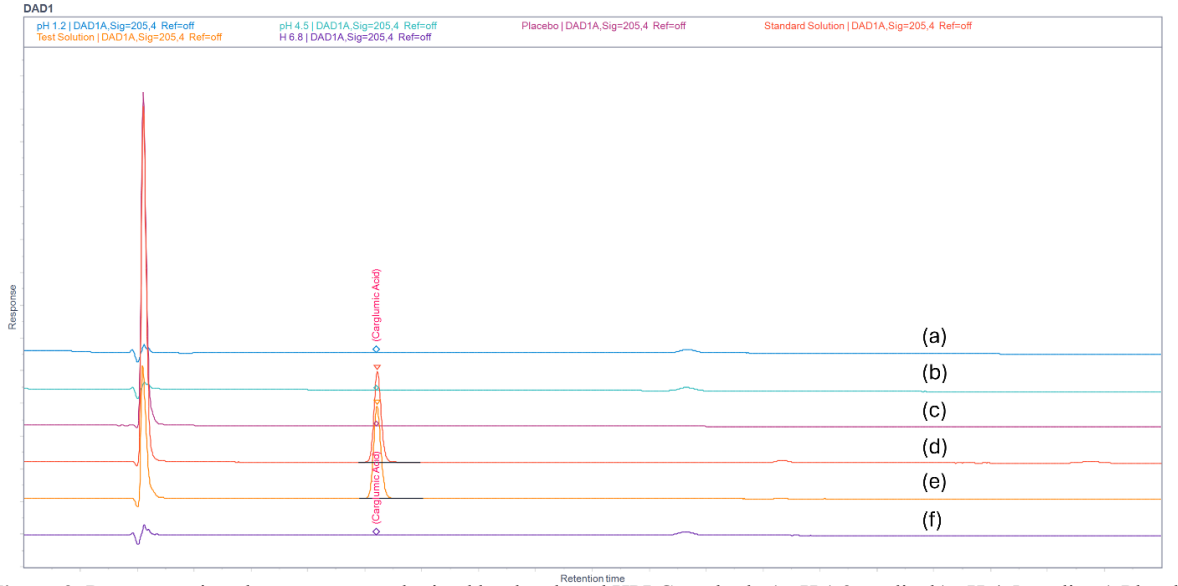

Figure 2. Representative chromatograms obtained by developed HPLC method: a) pH 1.2 media, b) pH 4.5 media, c) Placebo solution, d) Standard solution, e) Test solution and f) pH 6.8 media.

Table 3. Summary of validation results of each parameter

Validation Parameters	Acceptance Criteria		Results
System Suitability	%RSD ≤ 0.85 for N = 6 injections		%RSD=0.17 N = 6
Specificity	No interference from blank, placebo between carglumic acid peak		No interference observed
Linearity and Range	$R^2 \geq 0.99$		Complies ($R^2 \geq 0.9997$ in the range of 0.5 ppm to 60 ppm)
Accuracy	0.5 µg/mL (1.0%), 40 µg/mL (100%), 60µg/mL (150%)	Should be within the limit of 98%−102% for each sample	Complying, all accuracy results are within the limit of 98-102%, Table 4.
Precision	%RSD ≤ 2 N =6 each day, intra days		%RSD of precision and intermediate precision complies with the limit. Precision=99.25 ± 0.45 %RSD= 0.18 (N=6) Intermediate Precision=98.94 ± 0.38 %RSD= 0.15 (N=6) Intra days= 99.10 ± 0.12 %RSD=0.18 (N=12)
LOD/LOQ	LOQ s/N ≥ 10 (RSD % ≤ 10) N = 6 LOD 2 ≤ s/N ≤ 3		$\bar{x}$ s/N = 12.9 (RSD=3.2, N=6) 0.5 ppm $\bar{x}$ s/N= 2.5 (N=2) 0.1 ppm

timized to ensure that minor fluctuations would not impact analytical outcomes. This optimization confirmed the method's resilience, supporting its reliability for routine analytical applications.

A filtration study was necessary for the samples taken from the dissolution medium and transferred to the HPLC system. The results of this study are presented below in Table 7. The suitable filter was determined to be a 0.45 µm hydrophilic PTFE syringe filter.

Table 4. Results of accuracy parameter.

Accuracy Solution	Concentration (µg/mL)	Recovery (%)	Mean (%)
Accuracy Solution (a)	0.5 µg/mL	101.55% 101.51% 101.09%	101.38%
Accuracy Solution (b)	40 µg/mL	100.71% 100.67% 100.72%	100.70%
Accuracy Solution (c)	60 µg/mL	100.78% 101.15% 100.40%	100.78%

Table 5. Plackett - Burman Experimental Design run order

Run Order	pH of Mobile Phase	Column Temperature (°C)	Flow Rate (mL/min)	Wavelength (nm)
1	2.4	28	1.15	203
2	2.4	32	1.15	207
3	2.6	28	1.15	207
4	2.4	28	1.05	203
5	2.6	32	1.05	207
6	2.6	32	1.15	203
7	2.4	32	1.15	203
8	2.4	28	1.05	207
9	2.4	32	1.05	207
10	2.6	28	1.15	207
11	2.6	32	1.05	203
12	2.6	28	1.05	203

Table 6. Results of Plackett – Burman Experimental Design Analysis

Results	pH of Mobile Phase	Column Temperature (°C)	Flow Rate (mL/min)	Wavelength (nm)	Model	R^2 Value
						Model
%Carglumic Acid	0.112	0.226	0.762	0.412	0.305	0.4581
Tailing Factor	0.485	0.811	0.655	0.354	0.581	0.8894

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Dissolution studies were conducted in three different in vitro environments, which will be utilized during the product development. The specification for the product's dissolution medium is Q ≥ 90% within 15 minutes. This dissolution criterion was achieved in all environments tested. Results of dissolution analysis is shown in Figure 3.

4. Conclusions

The present study efficiently developed and validated a reliable in vitro method for quantifying Carglumic acid, combining high-performance liquid chromatography with a diode array detector (HPLC-DAD). The method's efficacy is attributed to its reliability,

Table 7. Filter Selection Results

Run Order	Unfiltered Results %	0.45 µm Hydrophilic PTFE	0.45 µm Nylon	0.45 µm Cellulose Acetate
1	500.1	500.2	495.1	495.4
2	500.8	500.7	497.5	493.3
3	500.4	501.1	494.2	491.1
4	501.5	500.9	496.2	492.5
5	500.4	500.1	495.8	492.7
6	500.2	500.4	494.3	494.8
Mean	500.6	500.6	495.5	493.3
RSD	0.10	0.08	0.25	0.32
%Consistency	-	100.0	98.9	98.6

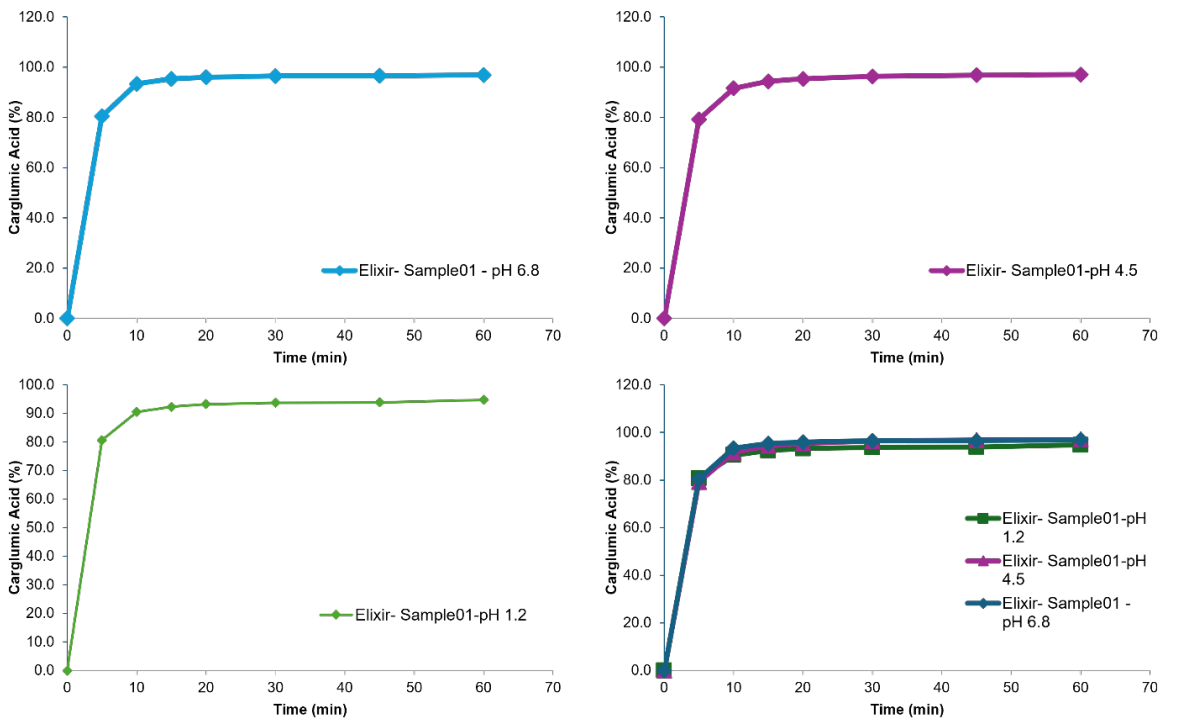

Figure 3. Dissolution profiles of in-house carglumic acid tablet in pH 1.2, pH 4.5, and pH 6.8 dissolution media

precision, and sensitivity, according to ICH Q2(R2) guidelines and conforming to pharmaceutical industry requirements. The approach demonstrated excellent specificity, precision, and linearity across a broad concentration range, achieving a correlation coefficient (R^2) of 0.99. This high level of performance was attained by utilizing a reverse-phase C18 column, which effectively separated the analyte from potential impurities. The chromatographic parameters were carefully optimized, including adjustments to the mobile phase composition and setting the detection wavelength to 205 nm, which enhanced the sensitivity and selectivity of the analysis. This optimization ensured that the method could consistently deliver accurate and reproducible results over a wide concentration range, confirming its reliability for analytical applications. The findings demonstrated negligible interference from excipients and contaminants, rendering it exceptionally appropriate for standard quality control applications within the pharmaceutical sector.

Additionally, the study validated the method's robustness to slight variations in chromatographic conditions, as confirmed by employing the Plackett-Burman experimental design. Despite the model created to evaluate the impact of chromatographic conditions lacking statistical significance, the approach consistently shows durability and precision. This analytical method has also demonstrated applicability for extensive bioanalytical applications, such as dissolution and stability testing, rendering it a significant instrument in evaluating product quality and bioavailability.

The *in vitro* dissolution method, developed to simulate the conditions of the intestinal environment (pH 6.8), employs a medium specifically chosen based on its inclusion in the reference product's patent specifications. [66] This choice underscores the relevance of pH 6.8 as a critical parameter for assessing the release profile of orally administered pharmaceuticals. By providing significant insights into the drug release kinetics, this method proves essential for understanding the dissolution behaviour of Carglumic acid formulations. Furthermore, the study validates a reliable analytical approach for quantifying Carglumic acid, thereby contributing to enhanced pharmaceutical quality control, improved therapeutic outcomes, and compliance with stringent regulatory standards for products containing this active pharmaceutical ingredient. This study provides a validated method for quantifying Carglumic acid, thereby improving pharmaceutical quality control and assuring enhanced therapeutic efficacy and regulatory compliance for formulations containing this active pharmaceutical ingredient.

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Conflict of Interest

The author/editor has no conflicts of interest, financial or otherwise, to declare.

Statement of Contribution of Researchers (Bold, First Letters are Capital Letters)

Example: Concept – B.A., N.E.B.A.; Design – B.A., N.E.B.A.; Supervision – N.E.B.A.; Resources B.A.; Materials – B.A.; Data Collection and/or Processing – B.A.; Analysis and/or Interpretation – B.A.; Literature Search – B.A.,N.E.B.A.; Writing – B.A.,N.E.B.A.; Critical Reviews –N.E.B.A.)

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