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GREEN SYNTHESIS AND ANALYTICAL TECHNIQUE FOR THE SEPARATION OF SUBSTITUTED CHLOROPHENYL HYDRAZINE ISOMERS BY REVERSE PHASE HPLC METHOD

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ABSTRACT
4-Chlorophenylhydrazine hydrochloride (4-CPH) is a crucial intermediate in organic synthesis, utilized in various applications including the production of Carprofen and wastewater treatment. This study focuses on the synthesis and impurity analysis of 4-CPH on green approach, with particular emphasis on the detection and quantification of positional isomers 2-Chlorophenylhydrazine hydrochloride (2-CPH), 3-Chlorophenylhydrazine hydrochloride (3-CPH), and 4-Chloroaniline. A high-performance liquid chromatography (HPLC) method was developed using a Waters X-Bridge C18 column and optimized for resolution and sensitivity. The method demonstrated effective separation, with detection limits for 2-CPH, 3-CPH and 4-Chloroaniline at 0.02%, 0.04%, and 0.02% respectively. The validated HPLC method provides reliable analysis for the quality control of 4-CPH, ensuring low impurity levels in the final product.
Keywords: Green Approach, 4-Chlorophenylhydrazine, RP HPLC, Positional Isomer Separation, Genotoxic Impurity.

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INTRODUCTION

4-Chlorophenylhydrazine hydrochloride (4-CPH) (**2a)** is an important substituted phenylhydrazine[1] in organic synthetic chemistry, specifically used as an intermediate and raw material. It is often referred to as a non-heterocyclic building block in synthetic chemistry. This compound is a colorless to light yellow crystalline solid that is soluble in water and methanol. 4-CPH is synthesized from 4-chloroaniline.[1] 4-CPH is used as an intermediate in the synthesis of Carprofen[2], a well-known nonsteroidal anti-inflammatory analgesic with antipyretic activity.[3,4] Also, it is an important intermediate of Edaravone, which is used to treat stroke and amyotrophic lateral sclerosis (ALS).[5] Additionally, 4-Chlorophenylhydrazine hydrochloride has been shown to react with acidolysis, which can be utilized for wastewater treatment.[6] Substituted phenylhydrazines serve as key starting materials in Fischer indole synthesis, as indole ring moieties are important structural components in diverse natural pharmaceutical agents.[7] Among various methods to synthesize and functionalize indole moieties, the Fischer indole synthesis (FIS) is the most well-established and practical strategy. One example is demonstrated by Sperry *et al.,* who used 4-Chlorophenylhydrazine as a precursor to carry out FIS, generating a pentacyclic spirobisindole.[8] 2-CPH (**2b**), 3-CPH (**2c**), and 4-Chloroaniline (**1**) are impurities generated during the chemical synthesis of 4-CPH (**2a**). Process impurities are identified through various chromatographic and spectroscopic techniques, either independently or in conjunction with other methods. High-performance liquid chromatography (HPLC) has been extensively utilized in impurity profiling due to its diverse applications facilitated by a broad selection of detectors and stationary phases. Its sensitivity and cost-effective separation capabilities contribute to its widespread use.[9]

Detecting and quantifying 2-CPH and 3-CPH during the production of 4-CPH is challenging, as these are aromatic positional isomers. 2-CPH, 3-CPH and 4-Chloroaniline impurities significantly impact the purity and effectiveness of 4-CPH. Completely removing these impurities from the product is strenuous. Therefore, it is crucial to achieve a substantial reduction in the levels of 2-CPH, 3-CPH and 4-Chloroaniline

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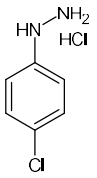
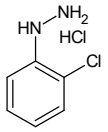
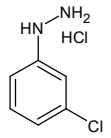
impurities to the minimum feasible extent in 4-CPH. A novel and valid approach for detecting and quantifying trace impurities in 4-CPH must be established. In this study, we have prepared a substituted phenylhydrazine green approach using water as a solvent, also we developed a Green analytical method to control the positional isomers including 4-chloroaniline[10] (genotoxic impurity) in a single method itself to avoid further derivatives formation of API molecules. Control strategy needs to be established at final stages, to maintain the ICH quality of active pharmaceutical ingredients.



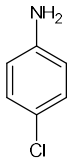
4-Chlorophenylhydrazine hydrochloride **(2a)**



2-Chlorophenylhydrazine hydrochloride **(2b)**



3-Chlorophenylhydrazine hydrochloride **(2c)**



4-Chloroaniline **(1)**

Fig.-1: Structure of 2-CPH, 3-CPH, 4-CPH and 4-Chloro Aniline

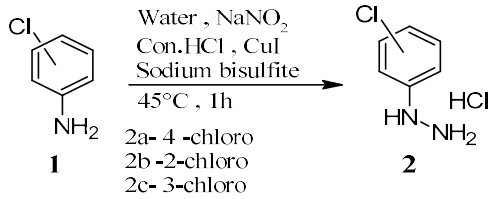
EXPERIMENTAL

Materials and Chemicals

All the solvents and chemicals used were analytical grade and purchased from Spectrochem and Sigma-Aldrich. Additionally, the standard impurities 4-CPH, 2-CPH and 3-CPH samples with a purity of 99+% were synthesized. The purity of the standard and impurities 4-CPH, 2-CPH, 3-CPH and 4-Chloroaniline were verified by HPLC. Throughout the investigation, Milli-Q water (Bedford, USA) was used. Disodium hydrogen phosphate of analytical reagent (AR) grade, along with HPLC-grade acetonitrile, methanol and orthophosphoric acid were obtained from Merck (Darmstadt, Germany).

Synthesis of Substituted Chlorophenylhydrazine

Add 12.5g of substituted Chloroaniline slowly added into 50g of 30% hydrochloric acid, stir vigorously, add dropwise a 20% aqueous solution containing 7.5g of sodium nitrite at -5-0°C, and keep it warm for half an hour after the addition is complete, Add catalyst of Copper(I) iodide, add 12g of sodium bisulfite in lot wise under control of the temperature, after the addition, slowly increase the temperature to 45°C, keep it for one hour, cool to 0-5°C to crystallize and isolate the solid.



Scheme-I: Synthetic Route of Substituted Phenyl hydrazine

Structural Characterization

4-Chlorophenylhydrazine hydrochloride (2a)

^{1}H NMR (400 MHz, DMSO-d6) δ 7.00 – 7.02, (d, 2H), 7.32 – 7.34 (d, 2H), 8.46 (s, broad, 1H), 10.34 (d, broad, 3H). ESI-mass: calcd. (found): 142.5 (143) (M+1)+; IR (KBr disc, νmax, cm^{-1}): 3205.40, 2988.5, 2688.4, 1584.0, 1496.4, 1098.6, 873.2, 818.6, 649.1, 482.53.

2-Chlorophenylhydrazine hydrochloride (2b)

^{1}H NMR (400 MHz, DMSO-d6) δ 6.96 – 7.0, (t, 1H), 7.18 – 7.20 (d, 1H), 7.29 – 7.31, (t, 1H), 7.40 – 7.42 (d, 1H), 8.01 (s, broad, 1H), 10.47 (d, broad, 3H). ESI-mass: calcd. (found): 142.5 (143.3) (M+1)+; IR (KBr disc, νmax, cm^{-1}): 3142.2, 3024.4, 2662.6, 1586.9, 1507.9, 1074.3, 887.5, 768.3, 685.0, 489.7.

3-Chlorophenylhydrazine hydrochloride (2c)

^{1}H NMR (400 MHz, DMSO-d6) δ 6.94 – 6.96, (2, 2H), 7.09 (d, 1H), 7.29 – 7.31, (t, 1H), 7.40 – 7.42 (d, 1H), 8.01 (s, broad, 1H), 10.47 (d, broad, 3H). ESI-mass: calcd. (found): 142.5 (143.3) (M+1)+ ; IR (KBr disc, νmax, cm-1): 3142.2, 3024.4, 2662.6, 1586.9, 1507.9, 1074.3, 887.5, 768.3, 685.0, 489.7.

Analytical Methods

High-Performance Liquid Chromatography Analysis

The analysis of 2-CPH, 3-CPH and 4-Chloroaniline impurities was conducted using a Shimadzu LC-PDA system model LC 2050 (Japan). The separation was performed on a Waters X-bridge C18 column (250 mm × 4.6 mm, 3.5 µm). Data acquisition and processing were managed using Lab Solutions software (Japan). Additionally, a Shimadzu analytical balance (Japan) was utilized for precise measurements. The detection wavelength was established by scanning the UV spectrum of 4-CPH and its impurities over a range of 200 to 400 nm. Although the maximum absorbance was observed at 210 nm, an irregular baseline at this wavelength necessitated the selection of 239 nm for the analysis. (Fig.-2, 3, 4, 5). For Detailed analytical standards and sample preparation refer to supplementary data.

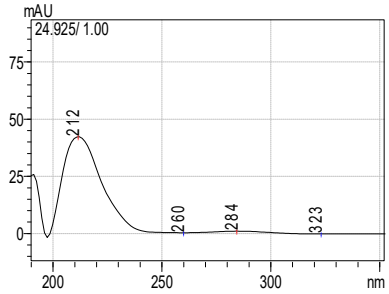


Fig.-2: Spectral data of 4-CPH

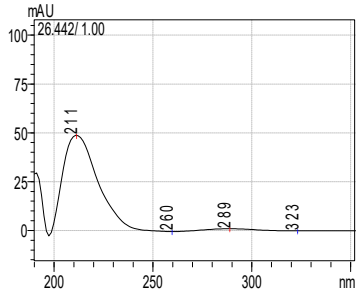


Fig.-3: Spectral data of 3-CPH

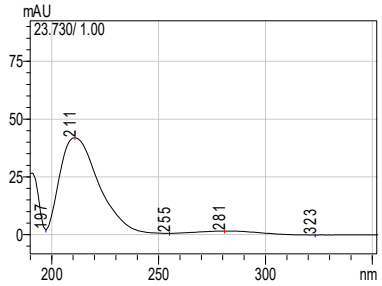


Fig.-4: Spectral data of 2-CPH

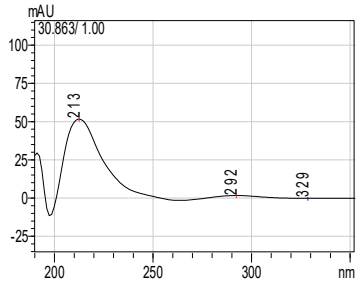


Fig.-5: Spectral data of 4-Chloroaniline

Conditions and Development Approach

The pKa value for 4-CPH is approximately 5.2. Initial trials were conducted using a Shimpack C18 column (250 mm length, 4.6 mm ID, 5 µm particle size) with a 10 mM dipotassium hydrogen phosphate buffer with different pH and acetonitrile as the organic modifier. These trials revealed that the 2-CPH isomer co-eluted with 4-CPH. Further trials were conducted using an X-Terra RP C18 column (250 mm length, 4.6 mm ID, 5 µm particle size) with the same buffer and organic modifiers, but the 2-CPH isomer still co-eluted with 4-CPH. Some separation was observed with a Waters X-Terra RP C18 column (250 mm length, 4.6 mm ID, 3.5 µm particle size). However, subsequent trials using the same chromatographic conditions but varying the organic modifier combination (Eluent B) resulted in poor resolution between 3-chlorophehyl hydrazine (3-CPH) and 4-CPH.

Adjusting the buffer pH to 7.5 with orthophosphoric acid and using a premixed acetonitrile and methanol solvent at different ratios as organic modifiers with an X-Terra RP C18 column (250 mm length, 4.6 mm ID, 3.5 µm particle size) not led to the separation of 3-CPH and 2-CPH isomers from 4-Cl-Phenyl hydrazine (4-CPH) including an unknown peak co-eluted with 4-CPH. Adjusting the buffer pH to 8.5 with orthophosphoric acid and using the same solvent mix with an X-Terra RP C18 column (250 mm length, 4.6 mm ID, 3.5 µm particle size) resulted in 2-CPH eluting earlier than 4-CPH due to pH sensitivity, and 3-CPH eluting after 4-CPH, achieving a resolution greater than 2.0 between the isomers and 4-CPH, though the recovery was poor. Trials using an X-Bridge C18 column (250 mm length, 4.6 mm ID, 3.5 µm particle size) also achieved a resolution greater than 2.0 between the isomers and 4-CPH, but recovery remained poor. A subsequent trial with the same X-Bridge column, using a buffer pH of 8.7, found that the 2-CPH peak tailed the 4-CPH peak due to pH sensitivity, followed by 3-CPH. The resolution remained over 2.0,

but recovery issues persisted. Optimization involved adjusting the gradient, flow rate, buffer strength and column oven temperature to improve the resolution between 4-CPH, 2-CPH, 3-CPH and 4-chloroaniline. The goal was to achieve a USP resolution greater than 1.5 between 4-CPH and its related impurities, specifically 3-CPH and 2-CPH, to resolve recovery issues caused by close elution of the 3-CPH peak with 4-CPH. With a higher sample concentration to enhance sensitivity, the column temperature was reduced to 20°C for better resolution between 3-CPH and 4-CPH. The Waters X-Bridge C18 column (250 mm length, 4.6 mm ID, 3.5 µm particle size) and the autosampler port were maintained at 5°C for separation and stability. Eluent A and B were 20 mM disodium hydrogen phosphate (pH 8.7, adjusted with orthophosphoric acid) and a 1:1 premixed acetonitrile and methanol solution, respectively.

The gradient elution procedure was as follows:

0-10 min: 70% Eluent A, 30% Eluent B
10.0-20.0 min: 70% Eluent A, 30% Eluent B
20.0-30.0 min: 60% Eluent A, 40% Eluent B
30.0-48.0 min: 40% Eluent A, 60% Eluent B
48.0-50.0 min: 40% Eluent A, 60% Eluent B
50.0-65.0 min: 70% Eluent A, 30% Eluent B

A Ghostbuster column (50 x 4.6 mm) from Welch was introduced to improve the baseline and eliminate blank interference. The flow rate was set at 0.6 mL/min, with a total analysis time of 65 minutes and an injection volume of 5 µL. The rinse volume was maintained at 1000 µL, and the needle was washed with water and acetonitrile (20:80, v/v). For sample preparations, 0.2% OPA in a premixed water and acetonitrile solution (75:25, v/v) was used as the diluent. Sample concentration was kept 0.5mg/ml.

RESULTS AND DISCUSSION

Validation

The proposed HPLC method was validated in accordance with ICH guidelines to ensure the reliability, consistency and accuracy of the analytical results.[11]

Specificity

Specificity was assessed by separately injecting 5 µL of individual working solutions of 2-CPH (0.50%), 3-CPH (0.50%), 4-chloroaniline (0.50%) and 4-CPH (0.30%). Additionally, a solution containing 0.50% of each impurity (2-CPH, 3-CPH, and 4-chloroaniline) spiked with the 4-CPH sample was analyzed. These samples, along with a diluent blank, were injected into a Waters X-Bridge C18 column and analyzed under the proposed HPLC conditions. No interference was observed with diluent components at the retention times of 2-CPH (23.1 min), 3-CPH (25.8 min), 4-chloroaniline (30.3 min) and 4-CPH (24.3 min) (Fig.-6). This confirmed the specificity of the LC-PDA method for analyzing 2-CPH, 3-CPH and 4-chloroaniline. Peak purity data for the analytes and impurities are presented in Fig.-6.

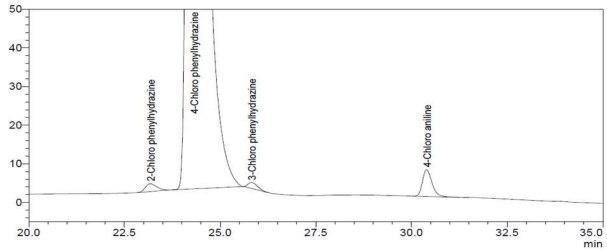


Fig.-6: Spike Sample Specificity Reference Chromatogram

Limit of Quantification (LOQ) and Limit of Detection (LOD)

The solutions of 2-CPH, 3-CPH, 4-CPH and 4-chloroaniline were quantitatively diluted stepwise with the diluent. These diluted solutions were then separately injected into a Waters X-Bridge C18 column and analyzed under the proposed HPLC conditions. The limits of quantification (LOQ) and detection (LOD) were determined for 2-CPH, 3-CPH, 4-CPH and 4-chloroaniline, with the LOQ and LOD defined as the concentrations (%) that produced signal-to-noise ratios of ≥10 and ≥3, respectively. The LOQ and LOD values (Table 1) and chromatogram in Fig.-7 and 8 demonstrated the sensitivity of the HPLC method for analyzing 2-CPH, 3-CPH, 4-CPH and 4-chloroaniline at low levels.

Table-1: HPLC methodology sensitivity results

Content	LOQ		LOD	
	S/N Proportion	(%)	S/N Proportion	(%)
2-CPH	34.3	0.07	11.7	0.02
3-CPH	50.4	0.13	17.5	0.04
4-CPH	14.0	0.07	5.0	0.02
4-Chloro aniline	102.1	0.07	36.3	0.02

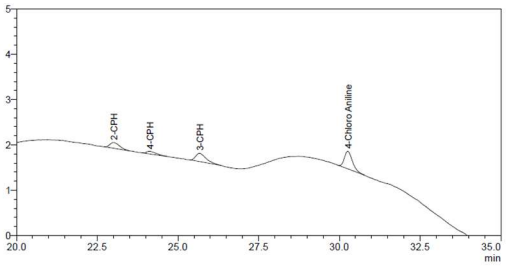


Fig.-7: LOD Chromatogram

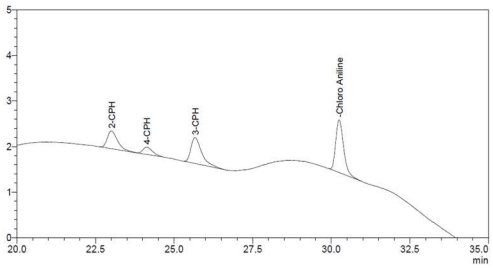


Fig.-8: LOQ Chromatogram

Linearity

Five calibration solutions containing 2-CPH, 3-CPH and 4-Chloro aniline impurities, along with 4-CPH, were prepared in concentration ranges of 0.07-1.83%, 0.13-1.70%, 0.07-1.87% and 0.07-3.68% respectively. These solutions were analyzed twice. Calibration curves for 2-CPH, 3-CPH, 4-chloroaniline and 4-CPH were developed by plotting the mean area obtained against the concentrations. The regression equations and linearity correlation coefficients for 2-CPH, 3-CPH, 4-chloroaniline and 4-CPH are shown in Table-2. The correlation coefficients, all greater than 0.99, confirmed the linearity of the HPLC method (Fig.-9, 10, 11, and 12).

Table-2: HPLC Methodology Linearity and Line Equation Results

Parameter	2-CPH	3-CPH	4-Chloro Aniline	4-CPH
Linearity	0.07 to 1.83 %	0.13 to 1.70 %	0.07 to 1.87 %	0.07 to 3.68 %
Y-Intercept	-307.937	-261.654	414.666	-1476.167
Slope	93980.359	101988.923	202454.774	115627.112
% Y-Intercept	-0.732	-0.618	0.435	-1.423
Correlation Coefficient(R)	1.000	1.000	1.000	1.000
Regression Coefficient(R^2)	1.000	1.000	1.000	1.000

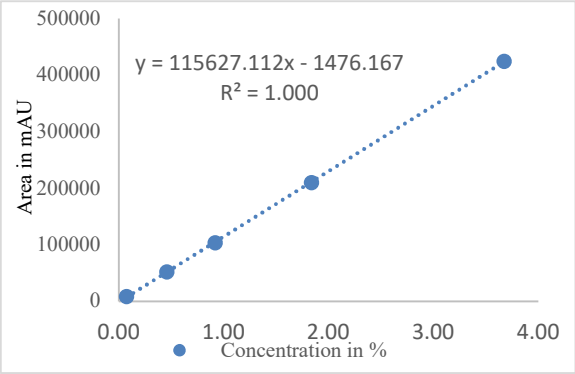


Fig.-9: Linearity graph of 4-CPH

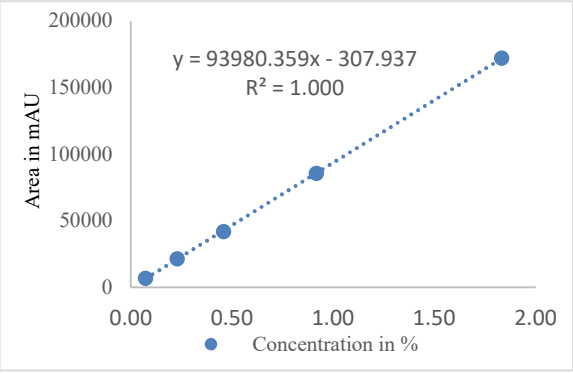


Fig.-10: Linearity graph oh 2-CPH

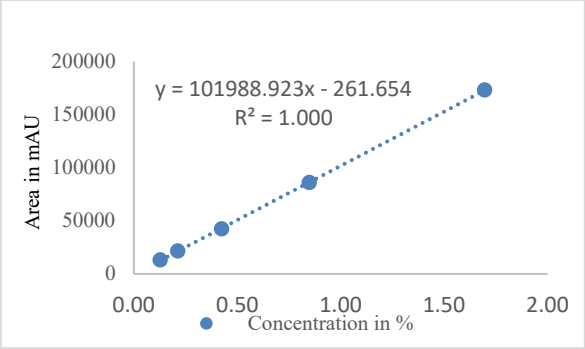


Fig.-11: Linearity graph oh 3-CPH

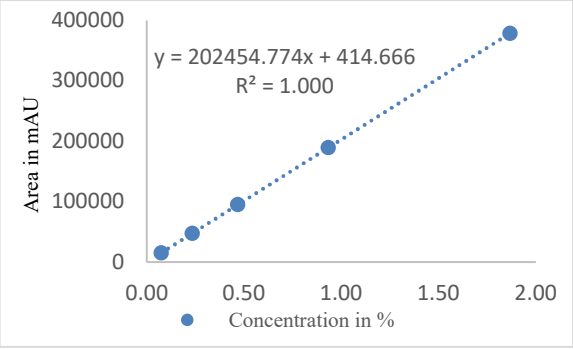


Fig.-12: Linearity graph oh 4-Chloroaniline

System Precision

Working solutions of 2-CPH (0.50%), 3-CPH (0.50%), 4-chloroaniline (0.50%) and 4-CPH (0.3%) were separately injected (5 µL) into a Waters X-Bridge C18 column and analyzed under the proposed HPLC conditions in six replicates.

The peak areas for 2-CPH, 3-CPH, 4-chloroaniline and 4-CPH were recorded. The percent relative standard deviations (RSD) between the peak areas from the six replicates were 0.63%, 1.98%, 3.92%, and 2.43% respectively (Table-3). These results demonstrate the system's precision in analyzing 2-CPH, 3-CPH, 4-chloroaniline and 4-CPH.

Method Precision

Working solutions of 4-CPH, both as a pure sample and as a sample spiked with 2-CPH (0.50%), 3-CPH (0.50%) and 4-chloroaniline (0.50%) were separately injected (5 µL) into a Waters X-Bridge C18 column and analyzed under the proposed HPLC conditions in six replicates. The concentrations of 2-CPH, 3-CPH and 4-chloroaniline were determined. The percent relative standard deviations (RSD) between the concentrations of 2-CPH, 3-CPH, 4-chloroaniline and the single maximum unknown impurity from the six replicates were 0.83%, 0.70%, 0.60% and 7.73% respectively (Table-3). These results demonstrate the HPLC method's precision in analyzing 2-CPH, 3-CPH, 4-chloroaniline and the single maximum unknown impurity in the 4-CPH sample.

Table-3: HPLC Methodology Precision Results

Samples	2-CPH		4-chloro aniline		4-CPH		3-CPH	
	System precision	Method precision	System precision	Method precision	System precision	Method precision	System precision	Method precision
	Peak area	Amount obtained (%)	Peak area	Amount obtained (%)	Peak area	Single Max.Unknown Impurity (%)	Peak area	Amount obtained (%)
1	40907	0.5265	102324	0.4935	33369	0.176	32503	0.4205
2	40748	0.5274	110166	0.4958	33440	0.219	33843	0.4222
3	40903	0.5295	102409	0.4928	32805	0.184	32273	0.4224
4	40600	0.5193	102029	0.4871	31990	0.194	32329	0.4150
5	40466	0.5196	102614	0.4927	32464	0.204	32394	0.4220
6	40260	0.5218	110439	0.4909	31412	0.198	32051	0.4228
Average	40647	0.5240	104997	0.4921	32580	0.196	32566	0.4208
SD	256.15	0.00	4114.96	0.00	792.28	0.015	643.67	0.00
RSD	0.63	0.83	3.92	0.60	2.43	7.73	1.98	0.70

Recovery

Accuracy was evaluated through a recovery analysis following ICH guidelines. Known concentrations of 2-CPH, 3-CPH and 4-chloroaniline standard solutions corresponding to the LOQ level, 100% and 200% of the specification limit were added to the 4-CPH sample solution. Accuracy was assessed by injecting the sample solutions three times, and the results are presented in Table-4. The recoveries of 2-CPH, 3-CPH and

4-chloroaniline were satisfactory, demonstrating the HPLC method's accuracy in analyzing 2-CPH, 3-CPH, and 4-chloroaniline in the 4-CPH sample.

Table-4: HPLC Methodology Accuracy Results

Accuracy level	Amount Add up (%)	Amount obtained (%)	Recovery (%)	Average of Recovery (%)
2-CPH recovery				
LOQ	0.0832	0.0799	96.1	94.6
	0.0832	0.0772	92.8	
	0.0832	0.0788	94.8	
100%	0.5244	0.5265	100.4	100.6
	0.5244	0.5274	100.6	
	0.5244	0.5295	101.0	
200%	1.0488	1.0516	100.3	100.5
	1.0488	1.0630	101.4	
	1.0488	1.0486	100.0	
3-CPH recovery				
LOQ	0.1356	0.1277	94.2	94.9
	0.1356	0.1321	97.4	
	0.1356	0.1263	93.2	
100%	0.5060	0.4205	83.1	83.3
	0.5060	0.4222	83.4	
	0.5060	0.4224	83.5	
200%	1.0120	0.9979	98.6	99.3
	1.0120	1.0147	100.3	
	1.0120	1.0021	99.0	
4-Chloroaniline recovery				
LOQ	0.0891	0.1024	115.0	114.7
	0.0891	0.1037	116.4	
	0.0891	0.1005	112.8	
100%	0.5072	0.4935	97.3	97.4
	0.5072	0.4958	97.8	
	0.5072	0.4928	97.2	
200%	1.0144	0.9936	97.9	98.8
	1.0144	1.0118	99.7	
	1.0144	0.9998	98.6	

Solution Stability

The stability of 2-CPH, 3-CPH and 4-chloroaniline solutions was assessed by analyzing samples at concentrations of 0.50%. The stability of these compounds was evaluated over 18 hours when stored at 2-8°C, by comparing them to freshly prepared spike solutions of 2-CPH, 3-CPH and 4-chloroaniline, as well as a sample containing a single maximum unknown impurity.

The percent relative differences in the content of 2-CPH, 3-CPH, 4-chloroaniline and the single maximum unknown impurity between the initial and approximately 18-hour intervals are provided in Tables-5a, 5b, and 5c. These results confirm the stability of 2-CPH, 3-CPH and 4-chloroaniline for up to 18 hours and the single maximum unknown impurity for up to 12 hours.

Table-5a: Stability of 2-CPH in solution

Time	2-CPH	
	Mean amount obtained (%)	Relative difference (%)
0 hr	0.5265	0.51
18 hr	0.5292	

Table-5b: Stability of single maximum unknown impurity in solution

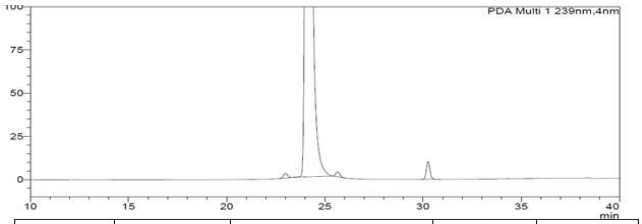
Time	Single maximum Unknown impurity in solution	
	Mean amount obtained (%)	Relative difference (%)
0 hr	0.176	1.13
12 hr	0.178	

Table-5c: Stability of 3-CPH and 4-chloroaniline in Solution

Time	3-CPH		4-chloro aniline	
	The mean amount obtained (%)	Relative difference (%)	The mean amount obtained (%)	Relative difference (%)
0 hr	0.3369	1.36	0.4935	0.06
18 hr	0.3415		0.4938	

System Suitability

System suitability testing is conducted to assess the adequacy and performance of the HPLC system during the analysis of 2-CPH, 3-CPH, 4-CPH and 4-chloroaniline. System suitability was evaluated by examining the resolution between 3-CPH and 4-CPH and by calculating the percent relative standard deviations of 2-CPH, 3-CPH, 4-CPH and 4-chloroaniline throughout the analysis. Peak areas were obtained from six replicate injections of a working solution at 0.50% for 2-CPH, 3-CPH and 4-chloroaniline, at 0.30% for 4-CPH. As shown in Table 6, the resolution ranged from 2.48 to 2.50 and the percent relative standard deviations ranged from 0.63% to 6.02% for 2-CPH, 3-CPH, 4-CPH and 4-chloroaniline. These results confirm the suitability of the HPLC system for analyzing 2-CPH, 3-CPH, 4-CPH and 4-chloroaniline in Table-6.



Name	Ret.Time	Area	Area %	Resolution	RRT
2-CPH	23.77	38559	0.31	-	0.95
4-CPH	24.96	12143014	98.41	1.83	1.00
3-CPH	26.44	22584	0.18	2.48	1.06
4-Chloro aniline	30.88	116224	0.94	8.67	1.24

Fig.-13: System Suitability Typical Reference Chromatogram

Table-6: HPLC Device Suitability Results

Parameter	2-CPH		3-CPH	
	Standard area	Bracketing Standard area	Standard area	Bracketing Standard area
System suitability evaluation throughout the analysis				
Average*	40647	40403	32566	31838
SD	256.15	343.59	643.67	891.85
%RSD	0.63	0.72	1.98	2.80
	4-CPH		4-Chloroaniline	
Parameter	Standard area	Bracketing Standard area	Standard area	Bracketing Standard area
System suitability evaluation throughout the analysis				
Average*	32580	30807	104997	103777
SD	792.28	1965.91	4114.96	3069.14
%RSD	2.43	6.02	3.92	3.19
Resolution between 3-CPH and 4-CPH	System Suitability		Bracketing System Suitability	
	2.50		2.48	

Application of HPLC Methodology Developed

The developed and validated HPLC methodology was used to detect and quantify 2-CPH, 3-CPH, single max unknown impurity and 4-chloroaniline in three batches of 4-CPH samples. The result shows that the content of 2-CPH and 3-CPH was below the detection limit (0.02% and 0.04%, respectively) in all batches of 4-CPH. Similarly, the content of 4-chloroaniline was below the quantification limit of 0.07% in all batches. However, the content of a single maximum unknown impurity was above the quantification limit (0.07%) in all batches of 4-CPH.

Analysis of the Green Graph Character of Method using the AGREE Software

AGREE[12] is a green analytical software that can be used with any analytical technique to assess the environmental friendliness of the developed analytical procedures. The software supports green analytical chemistry regarding sample handling, derivatization, mechanization, simplification, solvent safety study, quality of sample used for analyzing, various components quantified in one analysis and energy consumption per run. It is established based on these 12 important elements of relating analysis with methodology. The developed HPLC method was estimated for Green graph using this software and was observed 0.69 score which shows that the developed technique is environmentally benign.

The AGREE results were captured in Fig.-14 and 15.

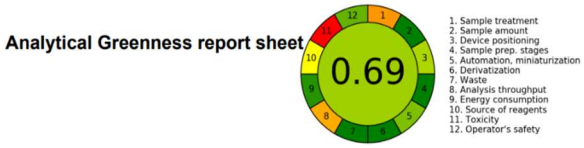


Fig.-14: Green Graph for HPLC Method Applying the AGREE Software

Criteria	Score
1. Direct analytical techniques should be applied to avoid sample treatment.	0.3
2. Minimal sample size and minimal number of samples are goals.	1.0
3. If possible, measurements should be performed in situ.	0.66
4. Integration of analytical processes and operations saves energy and reduces the use of reagents.	1.0
5. Automated and miniaturized methods should be selected.	0.75
6. Derivatization should be avoided.	1.0
7. Generation of a large volume of analytical waste should be avoided, and proper management of analytical waste should be provided.	1.0
8. Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time.	0.34
9. The use of energy should be minimized.	0.93
10. Reagents obtained from renewable sources should be preferred.	0.5
11. Toxic reagents should be eliminated or replaced.	0.0
12. Operator's safety should be increased.	0.8

Fig.-15: Based on 12 Analytical Principles we Observed 0.69 of 1.0 Scale Indicates the Green Analytical Technique of This Method

The method for analyzing impurities in 2-CPH, 3-CPH and 4-chloroaniline was developed using a Waters X-Bridge C18 column (250 × 4.6 mm, 3.5 µm) with gradient elution. The mobile phases consisted of 20 mM disodium hydrogen phosphate, adjusted to pH 8.7 (Eluent A), and a 50:50 v/v mixture of acetonitrile and methanol (Eluent B). The diluent used was 0.2% orthophosphoric acid (OPA) in a 75:25 v/v mixture of water and acetonitrile. The analysis was performed using an HPLC system with a PDA detector in gradient mode. The proposed HPLC methodology demonstrated good linearity across the concentration ranges: 0.07% to 1.83% for 2-CPH, 0.13% to 1.70% for 3-CPH, 0.07% to 1.87% for 4-chloroaniline and 0.07% to 3.68% for unknown impurities. System precision was found to be satisfactory, with relative standard deviations (RSD) of 0.63% for 2-CPH, 1.98% for 3-CPH, 3.92% for 4-chloroaniline and 2.43% for unknown impurities. Method precision was also acceptable, with RSD values of 0.83% for 2-CPH, 0.70% for 3-CPH, 0.60% for 4-chloroaniline and 7.73% for unknown impurities. Accuracy was confirmed with recovery rates of 100.6% for 2-CPH, 84% for 3-CPH and 97.0% for 4-chloroaniline. The method also achieved low detection limits of 0.02% for 2-CPH, 0.04% for 3-CPH, 0.02% for 4-chloroaniline and 0.02% for unknown impurities, along with low quantitation limits of 0.07% for 2-CPH, 0.13% for 3-CPH, 0.07% for 4-chloroaniline and 0.07% for unknown impurities. This HPLC methodology is suitable for evaluating the quality of 4-CPH samples with respect to the presence of 2-CPH, 3-CPH and 4-chloroaniline impurities.

CONCLUSION

The developed HPLC methodology offers accurate, sensitive, specific and precise analysis for the simultaneous quantitation of three impurities 2-CPH, 3-CPH, and 4-chloroaniline as well as a single maximum unknown impurity in 4-CPH samples with a greener approach. The method features reduced limits of detection (LOD) and quantitation (LOQ) for 2-CPH, 3-CPH, 4-chloroaniline and the single maximum unknown impurity. This HPLC method has proven effective for the quality assessment of 4-CPH samples concerning the presence of 2-CPH, 3-CPH, 4-chloroaniline and the single maximum unknown impurity. Therefore, it can be considered in the quality control analysis and also can be used in pharmaceutical drug substances quantification.

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CONFLICT OF INTERESTS

The authors have no conflicts of interest regarding this investigation.

AUTHOR CONTRIBUTIONS

All the authors contributed considerably to this manuscript, participated in analytical development and validation, synthesis, structure characterization, result interpretation, reviewing, editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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