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Box–Behnken Design Based Development of UV-Reversed Phase High Performance Liquid Chromatographic Method for Determination of Ascorbic Acid in Tablet Formulations

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Citation: Azmi, S.N.H.; Al Hoqani, U.; Al Mamari, J.O.S.; Al Mamari, B.M.S.; Al Jassasi, B.S.A.R.; Al Rubaiai, A.S.S.; Rahman, N.; Nasir, M.; Haque, S.M.; Khan, S.A.; et al. Box–Behnken Design Based Development of UV-Reversed Phase High Performance Liquid Chromatographic Method for Determination of Ascorbic Acid in Tablet Formulations. *Separations* **2022**, *9*, 361. https://doi.org/10.3390/separations9110361

Academic Editors: Salvatore Barreca and Barbara Bojko

Received: 10 September 2022
Accepted: 1 November 2022
Published: 9 November 2022

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Abstract: A simple, sensitive, accurate and inexpensive UV-reversed-phase high-performance liquid chromatographic method was developed for the determination of ascorbic acid in tablet formulations. The method was based on the separation of ascorbic acid using a mobile phase of an acetonitrile-NaH_2PO_4-H_3PO_4 buffer solution (pH = 3) (5:95 *v*/*v*) with a UV detection wavelength of 245 nm and a flow rate of 0.8 mL min^{-1} at ambient column temperature. The variables of the proposed method, such as acetonitrile fraction (%), flow rate (mL min^{-1}) and column temperature (°C), were optimized on the peak area by response surface methodology via the Box–Behnken design. The mobile phase was passed isocratically, and the separation of ascorbic acid was performed at the retention time of 4.1 min. A calibration graph was obtained and found to be linear in the concentration range of 10–180 µg mL^{-1}. The method suitability was assessed and an asymmetry factor of 1.15 was obtained. The proposed method was successfully applied for the determination of ascorbic acid in tablet formulations and statistically compared with the results of the reference method. The performance of the proposed method was excellent and in agreement with the reference method. The recovery percentage of the proposed and reference methods was in the range of 99.98–100.04% and showed compliance (100 ± 2%) with regulatory guidelines.

Keywords: ascorbic acid; Box–Behnken design; HPLC; ICH; validation

1. Introduction

Ascorbic acid (vitamin C) is chemically known as (5*R*)-5-[(1*S*)-1,2-Dihydroxyethyl]-3,4-dihydroxyfuran-2(5H)-one. It is a white, crystalline powder. It is easily oxidized when exposed to high temperatures, oxygen, alkaline pH, traces of metal ions and some enzymes [1]. The drug is freely soluble in distilled water and sparingly soluble in ethanol. The drug is listed in the British Pharmacopoeia [2], United States Pharmacopoeia [3], European pharmacopoeia [4] and British National Formulary 2021 [5]. The drug is used to

prevent scurvy. The clinical signs of scurvy disease appear in men when the whole-body content of ascorbic acid is less than 300 mg [6] or at intakes lower than 10 mg of ascorbic acid per day [7]. Such intakes are recognized with a plasma ascorbic acid concentration less than 11 μmol L^{-1} or leukocyte levels below 2 nmol per 10^8 cells [8]. In recent years, health agencies have recommended the use of ascorbic acid for the treatment of patients with moderate to severe COVID-19 disease [9,10]. Ascorbic acid is one of the most important essential micronutrients used for maintenance, growth and development of all body tissues. In addition, ascorbic acid is involved in many biochemical functions and utilized for the neutralization of free radicals and absorption of iron at the gastrointestinal level. It leads to synthesis and protection from oxidation of collagen, catecholamines, cholesterol, amino acids and some peptide hormones as an essential enzyme cofactor [11]. Ascorbic acid plays a major role in the initiation and progression of diverse chronic and acute diseases related to oxidative damage and inflammation [12]. The increasing interest in ascorbic acid as a micronutrient makes this entity the subject of significant investigation. Ascorbic acid can be supplemented through pharmaceutical tablets. Hence, the preparation of tablets with active ascorbic acid in pharmaceutical formulations is a very essential part of the pharmaceutical industry to provide safety and efficacy to patients. Therefore, it is important to quantitate active ascorbic acid in pharmaceutical formulations by accurate and validated analytical methods. The British Pharmacopeia and European Pharmacopoeia assayed ascorbic acid by titrimetry. In literature, some research studies have focused on the analysis of drugs and their impurities [13–17]. Ascorbic acid with inactive ingredients in tablet formulations could be given orally to patients with great ease. The important inactive ingredients of ascorbic acid tablets are microcrystalline cellulose, macrogol 6000, lactose monohydrate, magnesium stearate, sodium starch glycolate (Type A) and colloidal anhydrous silica.

Various other analytical methods, such as spectrophotometry [18–23], thin layer chromatography [24–28], capillary electrophoresis [29] and voltammetry [30], have been published for the determination of ascorbic acid in pharmaceutical formulations and biological fluids. UV-HPLC methods render more advantages over spectrophotometry, thin layer chromatography, capillary electrophoresis and voltammetry in terms of the multicomponent analysis of analytes (even more than two components), sensitivity, wide linear dynamic range, selectivity and specificity via separation. Therefore, the UV-HPLC method is frequently used for drug analysis and is considered much better than UV spectrophotometry alone. Samples may be contaminated to give a falsely high concentration of the desired analyte in a UV protocol. UV-HPLC provides the possibility of separating out these interfering compounds.

Analytical methods based on HPLC are also popular because of high accuracy, specificity, sensitivity, separation capability and short analysis time [31,32]. Several HPLC methods [33–52] have been reported for the determination of ascorbic acid in fruits, vegetables, indigenous spice plants, drug formulations and biological fluids. Moreover, several factors, such as composition of the mobile phase, pH, column temperature and flow rate, affect the performance of HPLC methods. Therefore, a response surface methodology (RSM) was utilized for optimizing the variables of the analytical methods to achieve the best performance [53–56]. The Box–Behnken design was selected because it provides a fewer number of experimental runs to optimize the variables. Recently, RSM via Box–Behnken design (BBD) was applied to optimize the flow rate, mobile phase composition and pH for separation and quantitation of domperidone and lansoprazole by HPLC method [57].

Keeping in view the quality and quantitative aspects of ascorbic acid in pharmaceutical formulations and biological fluids, we planned to develop a rapid, effective and sensitive UV-reversed phase high performance liquid chromatographic method for the determination of ascorbic acid in tablet formulations. The separation conditions of ascorbic acid through the column were optimized by response surface methodology using a Box–Behnken design. The proposed method was validated as per the International Council for Harmonization guidelines [58].

2. Materials and Methods

2.1. Apparatus

An HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific, Sunnyvale, CA, USA) with an autosampler (ASI-100 automated sample injector, Sunnyvale, CA, USA) of 20 µL and a UV-visible diode array detector (Dionex Ultimate 3000, Thermo Fisher Scientific, Sunnyvale, CA, USA) were used. The separation was performed with a chromatographic column Acclaim^{TM} 120 C18 5 µm 120A^{0} (4.6 × 250 mm, Dionex bonded silica products, Sunnyvale, CA, USA). A Chromeleon 6 Service Pack product (Version 2000, Thermo Fisher, Sunnyvale, CA, USA) was applied for peak integration and sample analysis to quantify ascorbic acid. An LDV-A12 Diaphragm vacuum pump (Labtron, Camberley, UK) was used for suction operation to drive liquid movement and accelerate filtration of the filtered solutions.

A UV-visible spectrophotometer (UV 1900 Shimadzu, Kyoto, Japan) equipped with 1-cm quartz cells was used to record the spectrum of ascorbic acid. pH values of the test solutions were measured using a Hanna benchtop pH meter (HI5222, Woonsocket, RI, USA). A Milli-Q Direct 8 Ultrapure Water System (Millipore, Molsheim, France) was used to purify demineralized water. A Branson Sonicator 2800 (Branson Ultra Sonics Corporation, Danbury, CT, USA) was used to dissolve ascorbic acid. The tablet solutions were filtered using a 0.45µm polyethersulfone membrane filter (ISOLAB, Wertheim, Germany). Design-Expert software (Version 13, StatEase, Minneapolis, MN, USA) was utilized to generate the Box–Behnken design (BBD) matrix for optimizing variables involved in developing HPLC method. OriginPro 2020b software (OriginLab Corporation, Northampton, MA, USA) was used to generate a linear regression equation to assay ascorbic acid in tablets.

2.2. Reagents and Standards

All solvents and mobile phases were degassed in an ultrasonic water bath and filtered in an HPLC filtration flask through a 0.45 µm polyethersulfone membrane filter (ISOLAB, Wertheim, Germany) using a vacuum pump. Acetonitrile, sodium dihydrogen phosphate, potassium dihydrogen phosphate, orthophosphoric acid (HPLC grade) and ascorbic acid were obtained from Sigma Aldrich, USA. Tablets of ascorbic acid, such as Redoxon (1000 mg, Bayer, Basel, Switzerland) and C-Tamin (500 mg, Vital-Health, Las Vegas, NV, USA), were purchased from a local Omani pharmacy shop.

2.2.1. Preparation of Buffer Solutions for HPLC Method Development

Phosphate buffer solutions of varying pH (pH 3.0, 3.5, 4.0 and 4.65) were prepared by mixing a fixed volume of aqueous sodium dihydrogen phosphate solution (0.4%) with varied volumes of orthophosphoric acid. The pH of the buffer solutions was measured using a Hanna pH meter.

2.2.2. Preparation of Mobile Phases for HPLC Method Development

The mobile phases of 2.5, 5.0 and 7.5% acetonitrile fraction were prepared by mixing 2.5, 5.0 and 7.5 mL of acetonitrile (Sigma Aldrich, St. Louis, MO, USA) with 97.5, 95.0 and 92.5 mL of phosphate buffer solution with a pH of 3. The mobile phases were degassed in an ultrasonic water bath and filtered through a 0.45µm filter using a vacuum filtration for HPLC studies.

2.2.3. Preparation of Standard Solutions of Ascorbic Acid

Both 0.02% and 0.05% ascorbic acid (200 & 500 µg mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA) standard solutions were prepared for UV spectra and HPLC method development, respectively. The said respective solutions of 0.02% and 0.05% ascorbic acid were prepared by dissolving 0.02 g and 0.05 g ascorbic acid in 150 mL Erlenmeyer stoppered conical flasks with 75 mL of distilled water. The ascorbic acid solutions were sonicated using the sonicator at 30 ± 1 °C for half an hour. The solutions were mixed thoroughly and transferred into

100 mL standard volumetric flasks. The solutions were diluted up to the mark with distilled water.

2.3. Procedure to Determine λ_{max} of Ascorbic Acid by UV Specrophotometry

A total of 1 mL of ascorbic acid (200 μg mL^{-1}) solution was put into a series of 10 mL standard volumetric flasks and diluted with respective mobile phases of different pH: 3.0, 3.5, 4.0 and 4.65 corresponding to 20 μg mL^{-1} ascorbic acid (after dilution). The UV-visible spectrum of the drug solution at different pH levels was scanned in the wavelength range of 190 to 340 nm against the reagent blank prepared similarly without ascorbic acid solution. The absorbance at λ_{max} of ascorbic acid was recorded and compared to finalize the UV detection wavelength for HPLC studies.

2.4. Optimization of Variables for HPLC Method Development

A response surface methodology (RSM) via a Box–Behnken design (BBD) was applied to optimize the variables of the proposed HPLC method. In this study, the range and levels of each variable were investigated by preliminary experiments and taken as a fraction of acetonitrile (2.5–7.5%), flow rate (0.7–0.9 mL min^{-1}) and column temperature (20–30 °C). Design-Expert software was utilized to generate the BBD matrix, which consisted of 17 experimental runs. The experiments were executed according to the BBD matrix and the coefficients of the response model were determined by fitting the measured data into a second-degree polynomial equation. The relationship between the predicted response and the variables involving linear, quadratic and interaction terms is expressed as [59,60]:

$$A = \beta_0 + \sum_{i=1}^{f} \beta_i X_i + \sum_{i=1}^{f} \sum_{j=1}^{f} \beta_{ij} X_i X_j + \sum_{i=1}^{f} \beta_{ii} X_i^2 + \varepsilon \quad (1)$$

$$i \neq j$$

where A is the peak area (predicted); β_0, β_i, β_{ii} and β_{ij} are the constant, linear, quadratic and interaction coefficients of the model, respectively; X_i and X_j represent coded variables; and ε is the random error. The validity of the model was verified by analysis of variance (ANOVA).

2.5. Procedure for the Determination of Ascorbic Acid by Proposed Method

Aliquots (0.2–3.6 mL) of ascorbic acid (500 μg mL^{-1}) were pipetted into a series of 10 mL standard volumetric flasks and diluted up to the mark with mobile phase: acetonitrile-NaH_2PO_4-H_3PO_4 buffer solution (pH = 3) (5:95 *v*/*v*) equivalent to 10–180 μg mL^{-1} ascorbic acid (after dilution). The mobile phase was passed isocratically and delivered thoroughly to clean the HPLC column. Then, 20 μL of each ascorbic acid test solution was injected into the sample port with a flow rate of 0.8 mL min^{-1} and the column temperature was maintained at 25 ± 1 °C. The detection was carried out at 245 nm. The HPLC chromatogram was partitioned for retention time and peak area. The calibration graph was plotted between the peak area and the initial concentration of ascorbic acid. The linear equation was generated using calibration data points of ascorbic acid concentration and the peak area through OriginPro 2020b software and utilized for assay of active ascorbic acid in tablets.

2.6. Procedure for the Determination of Ascorbic Acid by Reference Method [61]

Different aliquots (0.3–2.4 mL) of ascorbic acid (500 μg mL^{-1}) were pipetted into a series of 10 mL standard volumetric flasks and diluted up to the mark with mobile phase: methanol-KH_2PO_4-H_3PO_4 buffer solution (pH = 3) (5:95 *v*/*v*) corresponding to 15–120 μg mL^{-1} ascorbic acid (after dilution). The said mobile phase was passed thoroughly to clean the HPLC column. A total of 20 μL of each ascorbic acid solution was injected into the sample port with a flow rate of 1.0 mL min^{-1} and the column temperature was maintained at 25 ± 1 °C. An isocratic elution procedure was followed with UV detection at 248 nm.

Separation was performed and the HPLC chromatogram was partitioned for retention time and peak area. In addition, theoretical plates and asymmetry factors were recorded. The calibration curve was plotted between the peak area and the initial concentration of ascorbic acid. The linear regression equation was obtained using calibration data by OriginPro 2020b software. The amount of ascorbic acid was estimated in tablets using a linear regression equation.

2.7. Procedure for the Assay of Ascorbic Acid in Tablets

Two tablets of Redoxon and C-Tamin were taken from the container and weighed at 8.277 g and 1.155 g, respectively. The contents of said tablets were finely powdered in agate mortar and pestle separately. The Redoxon tablet fine powder (0.207 g) and C-Tamin tablet fine powder (0.058 g) equivalent to 50 mg of active ascorbic acid were weighed and transferred into 150 mL Erlenmeyer conical flasks. To each flask, 60 mL of distilled water was added and the solid residue was dissolved. The contents of the flasks were sonicated using the sonicator at 30 ± 1 °C for 30 min. The solutions were filtered through a filter funnel equipped with Whatmann No. 42-filter paper into a 100 mL standard volumetric flask. The solid residue was washed well with 3 × 10 mL portions of doubly distilled water and the filtrate was diluted up to the mark with distilled water. The tablet solutions of ascorbic acid were passed through a 0.45 µm polyethersulfone membrane filter in the syringe and the filtrates were assayed for active ascorbic acid content in tablet formulations following the procedures given in Sections 2.5 and 2.6.

3. Results and Discussion

The development of the HPLC method was based mainly on mobile phase selection and UV detection wavelength. Therefore, prior to development of the HPLC method, ascorbic acid solution was scanned spectrophotometrically in various solvents to finalize the UV detection wavelength. The UV visible absorption spectra of 20 µg mL^{-1} ascorbic acid in distilled water and the mobile phase consisting of acetonitrile (5 mL) and phosphate buffer solution (95 mL of varying pH 3.0, 3.5, 4.0 or 4.65) were scanned in the wavelength range of 190–340 nm and the results are summarized in Table 1. It is clear from Table 1 that the ascorbic acid in the mobile phases made up of acetonitrile (5 mL) and phosphate buffer solution of pH 3 and 3.5 (95 mL each) provided absorption peaks at 245 & 247 nm, respectively. Therefore, the absorption peak at 245 nm due to ascorbic acid and the mobile phase (acetonitrile, 5 mL: phosphate buffer pH 3.0; 95 mL) were selected for the HPLC method development.

Table 1. Absorbance and peak of 20 µg mL^{-1} ascorbic acid in distilled water and mobile phases of different pH.

S.No.	Solvent/Mobile Phase	Peak (nm)	Absorbance
1	Distilled water (pH 7)	260	1.106
2	0.4% NaH_2PO_4 (pH 3 maintained with H_3PO_4)-Acetonitrile (90:10 *v*/*v*)	245	1.226
3	0.4% NaH_2PO_4 (pH 3.5 maintained with H_3PO_4)-Acetonitrile (90:10 *v*/*v*)	247	1.277
4	0.4% NaH_2PO_4 (pH 4.0 maintained with H_3PO_4)-Acetonitrile (90:10 *v*/*v*)	254	1.230
5	0.4% NaH_2PO_4 (pH 4.65)-Acetonitrile (90:10 *v*/*v*)	261	1.742

The UV-visible spectra of 20 µg mL^{-1} ascorbic acid in distilled water and mobile phase with different pH are shown in Figure 1.

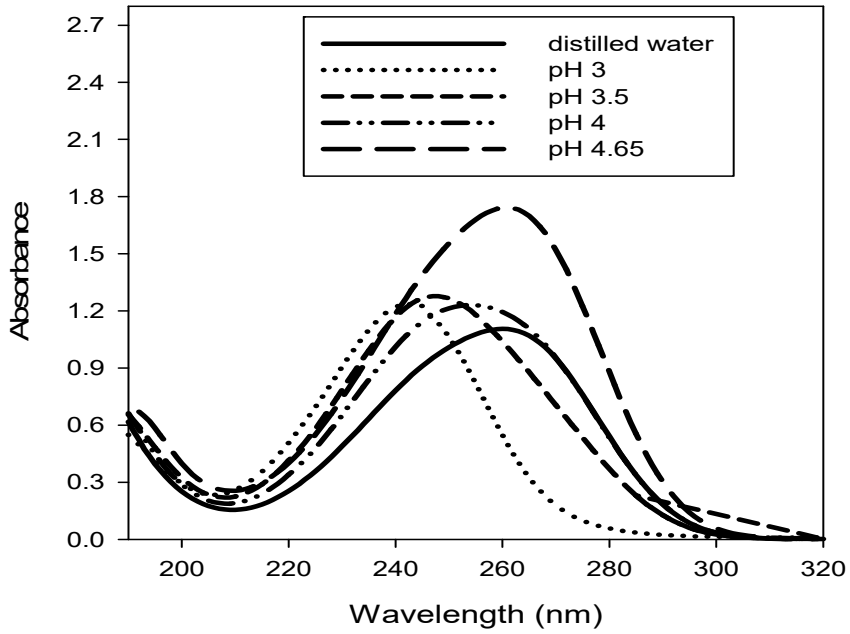

Figure 1. UV- visible spectra of 20 µg mL^{-1} ascorbic acid in distilled and mobile phase of different pH.

3.1. BBD Design for HPLC Optimization

For optimization of the HPLC method, 17 experimental runs involving different combinations of selected variables were conducted to measure the response (peak area) and the results are reported in Table 2.

Table 2. Box–Behnken design matrix with experimental and predicted peak area.

Std	Run	Factor A Acetonitrile Fraction %	Factor B Flow Rate mL min^{-1}	Factor C Column Temp. °C	Experimental Peak Area mAU	Predicted Peak Area mAU
3	1	2.5	0.9	25	35.50	35.40
14	2	5.0	0.8	25	53.42	53.42
12	3	5.0	0.9	30	52.09	52.07
10	4	5.0	0.9	20	52.96	53.04
7	5	2.5	0.8	30	35.10	35.19
4	6	7.5	0.9	25	36.74	36.75
2	7	7.5	0.7	25	35.34	35.41
9	8	5.0	0.7	20	50.10	50.12
8	9	7.5	0.8	30	35.34	35.41
1	10	2.5	0.7	25	33.90	33.89
15	11	5.0	0.8	25	53.42	53.42
13	12	5.0	0.8	25	53.42	53.42
5	13	2.5	0.8	20	33.50	33.49
11	14	5.0	0.7	30	52.27	52.20
16	15	5.0	0.8	25	53.42	53.42
17	16	5.0	0.8	25	53.42	53.42
6	17	7.5	0.8	20	36.20	36.11

The experimental data were fitted to linear, interactive and quadratic models. The statistical parameters are given in Table 3.

Table 3. Summary statistics of the BBD model for development of the HPLC method.

S. No.	Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
1	Linear	1.00×10^{1}	0.0067	−0.2225	−0.7869	2.35×10^{3}	
2	2FI	1.14×10^{3}	0.0095	−0.5849	−2.8738	5.10×10^{3}	
3	Quadratic	7.46×10^{-2}	1.0000	0.9999	0.9995	6.22×10^{-1}	Suggested

The predicted R^2 is in reasonable agreement with adjusted R^2 if the difference between them is 0.2. In this study, the quadratic model was selected based on (i) the difference of 0.0004 (less than 0.2) between predicted R^2 (0.9995) and adjusted R^2 (0.9999), (ii) high value of R^2 (1.0000) and (iii) low value of standard deviation (7.46×10^{-2}) and PRESS (6.22×10^{-1}). The multi-regression analysis of measured data gave a relationship between the peak area and the selected variables. The predicted response can be computed using the following Equation (2) in terms of coded factors:

$$\text{Peak area} = 53.42 + 0.7349A + 0.6974B + 0.275C - 0.0252AB - 0.575AC - 0.76BC - 17.43A^2 - 0.6471B^2 - 0.9169C^2 \quad (2)$$

In the selected model, the main interaction and quadratic effects were evaluated. For the sake of finding the most important effects and interactions, the analysis of variance (ANOVA) of the quadratic model equation was performed. The results are given in Table 4.

Table 4. Analysis of variance for the quadratic model related with the optimization of variables for the development of the HPLC method.

S. No.	Source	Sum of Squares	Degree of Freedom	Mean Square	F-Value	*p*-Value	
1	Model	1.32×10^{3}	9	1.46×10^{2}	2.64×10^{4}	<0.0001	Significant
2	A-Acetonitrile fraction	4.32	1	4.32	7.78×10^{2}	<0.0001	Significant
3	B-Flow rate	3.89	1	3.89	7.01×10^{2}	<0.0001	Significant
4	C-Column Temp.	0.605	1	0.605	1.09×10^{2}	<0.0001	Significant
5	AB	2.6×10^{-3}	1	2.6×10^{-3}	0.4592	0.5198	Insignificant
6	AC	1.32	1	1.32	2.38×10^{2}	<0.0001	Significant
7	BC	2.31	1	2.31	4.16×10^{2}	<0.0001	Significant
8	A^2	1.28×10^{3}	1	1.28×10^{3}	2.30×10^{5}	<0.0001	Significant
9	B^2	1.76	1	1.76	3.18×10^{2}	<0.0001	Significant
10	C^2	3.54	1	3.54	6.37×10^{2}	<0.0001	Significant
	Residual	3.89×10^{-2}	7	5.6×10^{-3}			
	Lack of Fit	3.89×10^{-2}	3	1.3×10^{-2}			
	Pure Error	0	4	0			
	Cor Total	1.32×10^{3}	16				

The quadratic model terms are significant when $p < 0.05$. In this study, the *p*-value for the quadratic model terms was very low (<0.0001). This demonstrates that the quadratic model was highly significant for the observed results. Additionally, all model terms (AC, BC, A^2, B^2, C^2) except AB were significant because *p*-values were less than 0.0001. The *p*-value of model term AB was 0.5198, indicating that the model term AB was insignificant. Therefore, Equation (2) was modified and expressed as:

$$\text{Peak area} = 53.42 + 0.7349A + 0.6974B + 0.275C - 0.575AC - 0.76BC - 17.43A^2 - 0.6471B^2 - 0.9169C^2 \quad (3)$$

Furthermore, the F-value of the quadratic model was much higher (26,352.64) than the critical F-value at 95% confidence level for 9 degrees of freedom (3.179), which suggests that the selected model was significant. The adequacy of the quadratic model was also confirmed from the plot of the predicted response versus the actual response (Figure 2).

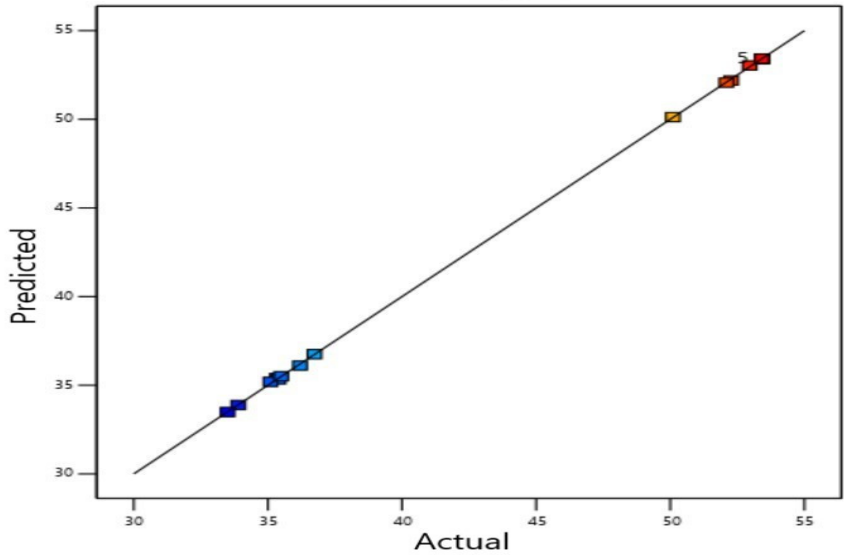

Figure 2. Plot of experimental versus predicted peak area.

3.2. Response Surface Plots

The response surface plots are the graphical representation of the quadratic equation to assess the interactive effect of variables on the response (peak area). Figure 3(BC) shows the response surface plot as a function of flow rate (mL min^{-1}) and column temperature (°C) and their mutual interaction on the peak area at a fixed concentration of vitamin C (50 µg mL^{-1}). The peak area increased with increase in flow rate (mL min^{-1}) and column temperature (°C) and a maximum peak area of 53.42 was obtained at a flow rate of 0.8 mL min^{-1} and column temperature of 25 °C. The combined effect of column temperature (°C) and acetonitrile fraction (%) on the peak area is shown in Figure 3(AC). As can be seen in Figure 3(AC), the peak area was found to increase with an increase in temperature and acetonitrile fraction (%). The highest peak area was observed at a column temperature of 25 °C and 5% acetonitrile. The response surface plots of quadratic model terms (BC and AC) were significant. The optimum conditions for independent variables were: flow rate 0.8 mL min^{-1}, 5.0% acetonitrile and 25 °C column temperature.

3.3. Method Validation

Method validation is the process of ensuring that the proposed method is accurate, reproducible and sensitive within the selected analysis range for the intended application, such as the assay of ascorbic acid in tablets. The data elements required for method validation [58] are (i) system suitability, (ii) robustness, (iii) linearity and range, (iv) limits of detection and quantitation, (v) precision, (vi) specificity and (vii) accuracy.

3.3.1. System Suitability

The optimization process for HPLC method development was performed (n = 6) using a UV detection wavelength of 245 nm, acetonitrile fraction (%), flow rate (mL min^{-1}) and column temperature (°C) at 50 µg mL^{-1} of vitamin C on the peak area. The system suitability parameters were assessed and the results are summarized in Table 5.

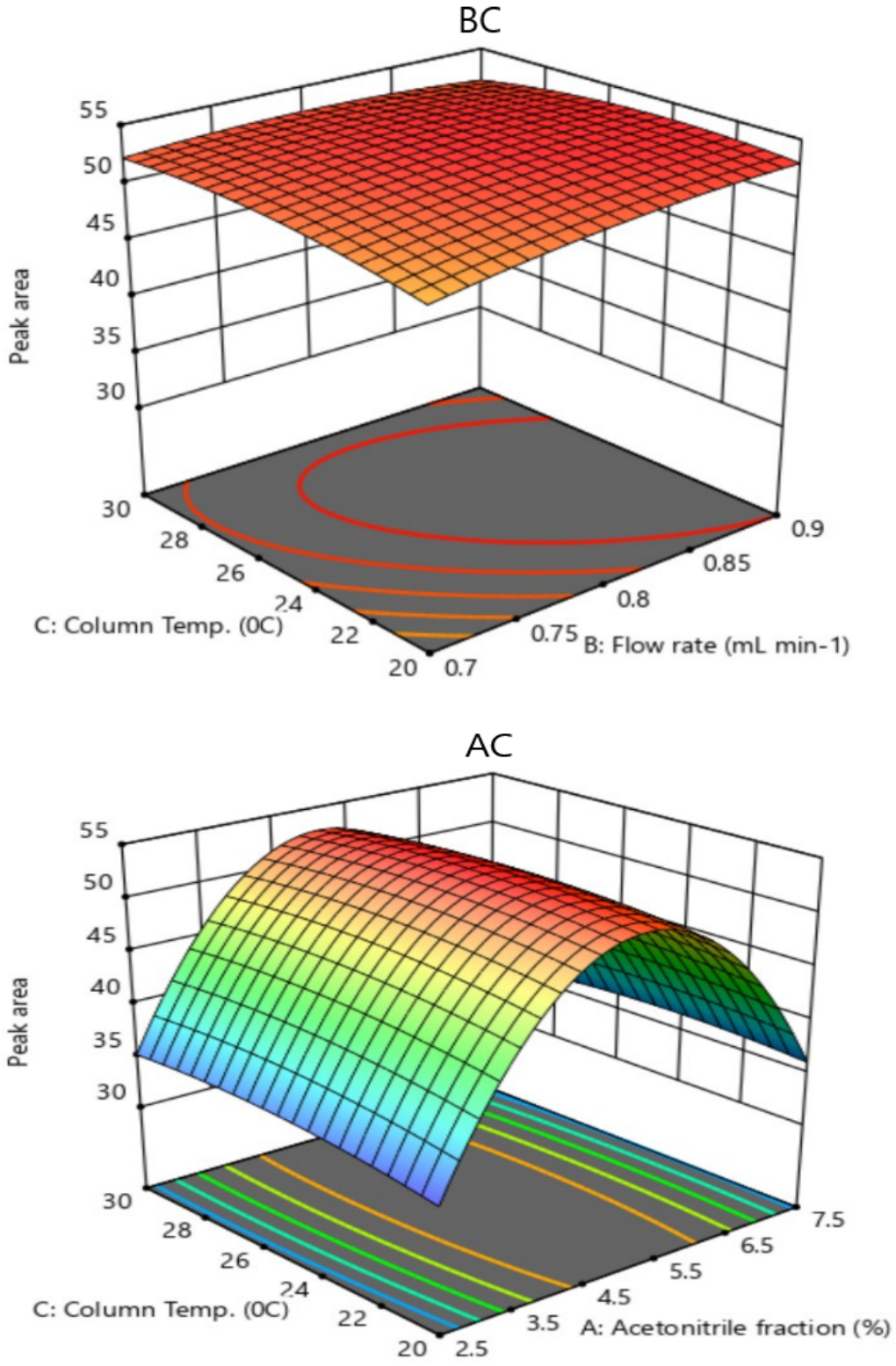

Figure 3. Three-dimensional response surface plots as a function of (BC): flow rate (mL min^{-1}) and column temperature (°C) and (AC): acetonitrile fraction (%) and column temperature (°C) at 50 µg mL^{-1} concentration of ascorbic acid on the peak area.

Table 5. HPLC responses at varied acetonitrile fractions with a constant flow rate and column temperature at 50 μg mL^{-1} ascorbic acid.

Parameters	Unit	Range & Level		
		Low	**Medium**	**High**
Variables				
Acetonitrile: buffer	%	2.5:97.5 (*v*/*v*)	5.0:95.0 (*v*/*v*)	7.5:92.5 (*v*/*v*)
Flow rate	mL min^{-1}	0.8	0.8	0.8
Column temperature	°C	25	25	25
Ascorbic acid	μg mL^{-1}	50	50	50
Responses				
Peak area	mAU	34.51	53.42	37.10
Retention time	min	4.5	4.1	2.43
Theoretical plate		7000	13,785	9000
Asymmetry		1.15	1.15	1.15

As can be seen from the table, at the optimized parameters through BBD, system suitability parameters were at the highest rank (level) and yielded theoretical plates of 13,785, a retention time of 4.1 min and an asymmetry factor of 1.15. The HPLC chromatogram at 50 μg mL^{-1} of ascorbic acid is shown in Figure 4.

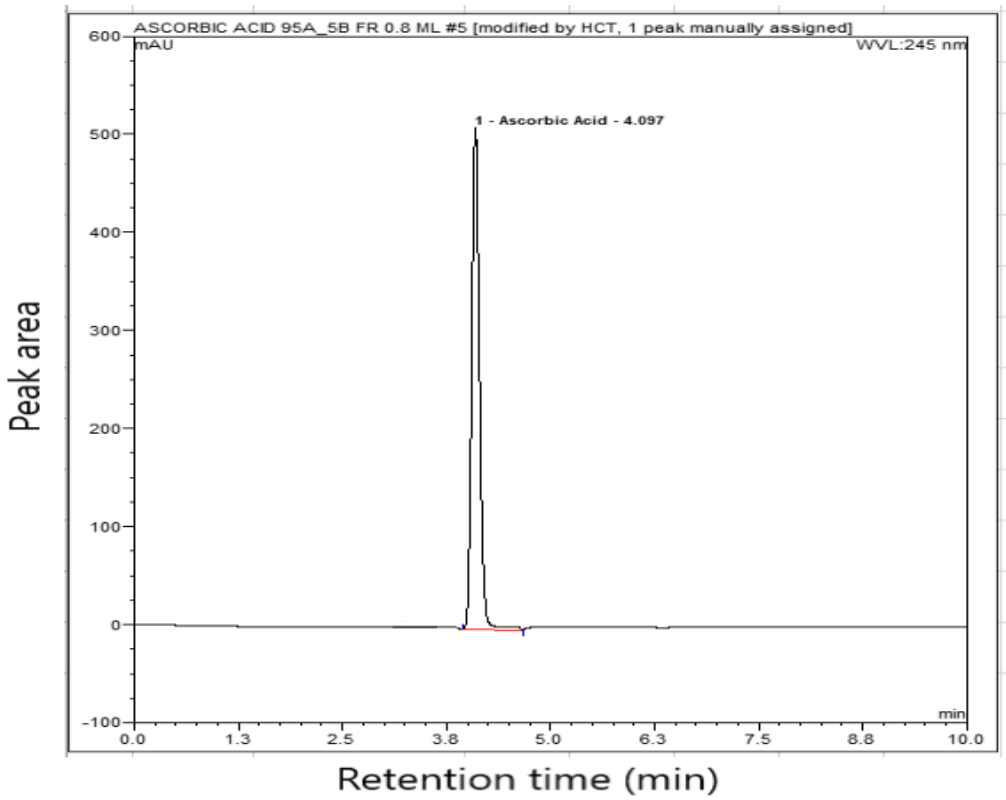

Figure 4. HPLC chromatogram of ascorbic acid at 50 μg mL^{-1} (mobile phase-acetonitrile: NaH_2PO_4-H_3PO_4 buffer solution (pH = 3) (5:95 *v*/*v*).

3.3.2. Robustness

Robustness is a measure of the performance of the proposed method in terms of the peak area when small and deliberate changes are made to the optimized method parameters. Robustness evaluation was performed by change in the Acclaim^{TM} 120 C18 column, 4.6 (id) × 250 mm, 5μm particle size (Dionex, Sunnyvale, CA, USA) with the Lunar C18 column, 4.6 (id) × 250 mm, 5μm particle size (Phenomenex, Torrance, CA, USA) and a small change in the phosphate buffer pH (±0.2) did not affect the peak area.

3.3.3. Linearity and Range

Under the optimized experimental conditions, different volumes of 0.05% ascorbic acid corresponding to 10, 50, 80, 100, 130, 150 and 180 μg mL^{-1} ascorbic acid were loaded into the sample port at a flow rate of 0.8 mL min^{-1} with the mobile phase of acetonitrileNaH_2PO_4-H_3PO_4 buffer solution (pH = 3) (5:95 *v*/*v*) and a UV detection wavelength of 245 nm at ambient column temperature (25 °C). The calibration graph was constructed by plotting the peak area against the initial concentration of ascorbic acid and found to be linear in the range of 10–180 μg mL^{-1} with a correlation coefficient (r) of 0.999. The high value of r demonstrates the good linearity of the calibration graph and the significant relationship between the peak area and the concentration of ascorbic acid. The parameters for analytical performance of the proposed and reference methods are summarized in Table 6.

Table 6. Analytical and regression characteristics of the proposed and reference HPLC methods.

Parameters	HPLC Method	
	Proposed	**Reference**
UV detection wavelength (nm)	245	248
Retention time (min)	4.1	4.1
No. of theoretical plates	13,785	13,781
Asymmetry factor	1.15	0.96
Beer's law range (μg mL^{-1})	10–180	15–120
Linear regression equation	PA = 2.57×10^{-3} + 1.069 C	PA = 0.1046 + 1.064 C
Standard deviation of intercept, S_a	0.124	8.23×10^{-2}
Confidence limit of the intercept, $\pm tS_a$	0.319	0.212
Standard deviation of the slope, S_b	1.09×10^{-3}	1.13×10^{-3}
Confidence limit of the slope, $\pm tS_b$	2.80×10^{-3}	2.91×10^{-3}
Standard deviation of the calibration line (S_o)	0.143	9.71×10^{-2}
Variance (S_o^2)	2.05×10^{-2}	9.43×10^{-3}
Correlation coefficient ®	0.999	0.999
Limit of detection, LOD (μg mL^{-1})	0.44	0.30
Limit of quantification, LOQ (μg mL^{-1})	1.34	0.91
Number of concentration levels (*n*)	7	7

3.3.4. Limits of Detection and Quantitation

The limits of detection (LOD) and quantitation (LOQ) of the proposed and reference methods were calculated as per the ICH guidelines [49] for the validation of analytical procedures using the following formula:

$$LOD = (3.3S_0)/b \quad (4)$$

$$LOQ = (10S_0)/b \quad (5)$$

where S_0 is the standard deviation of the calibration line and b is the slope of the regression equation. Both LOD and LOQ were found to be 0.44 and 1.34 μg mL^{-1} for the proposed method and 0.30 and 0.91 μg mL^{-1} for the reference method. The limits of detection and quantitation are in agreement if both values are less than the lowest concentration level of the linear dynamic range. In this study, both methods provided LOD and LOQ, which are less than the lowest concentration level of the proposed (10 μg mL^{-1}) and reference methods (15 μg mL^{-1}). Hence, the proposed method was acceptable and in agreement with the reference method. The proposed method was superior in terms of higher linear dynamic range with lower intercept and higher slope values as compared to the reference method.

The statistical analysis of the calibration data was evaluated in terms of error (S_c) encountered in the determination of the initial concentration of ascorbic acid using the following expression [62,63]:

$$S_C = \frac{S_0}{b}\left[1 + \frac{1}{n} + \frac{(Y - \bar{y})^2}{b^2 \sum (C - \overline{C})^2}\right]^{\frac{1}{2}} \quad (6)$$

where $\overline{C}$ and $\bar{y}$ are the average concentration of ascorbic acid and peak area, respectively at 7 different concentrations of ascorbic acid in 10–180 μg mL^{-1}; and b and S_0 are the slope and standard deviation of the calibration line, respectively. The uncertainty in the determination of ascorbic acid at 95% confidence level with 5 degrees of freedom was calculated and is shown in Figure 5. As can be seen from the figure, the least error of 0.143 μg mL^{-1} was achieved at 100 μg mL^{-1} of ascorbic acid, which demonstrates the acceptable precision of the proposed method.

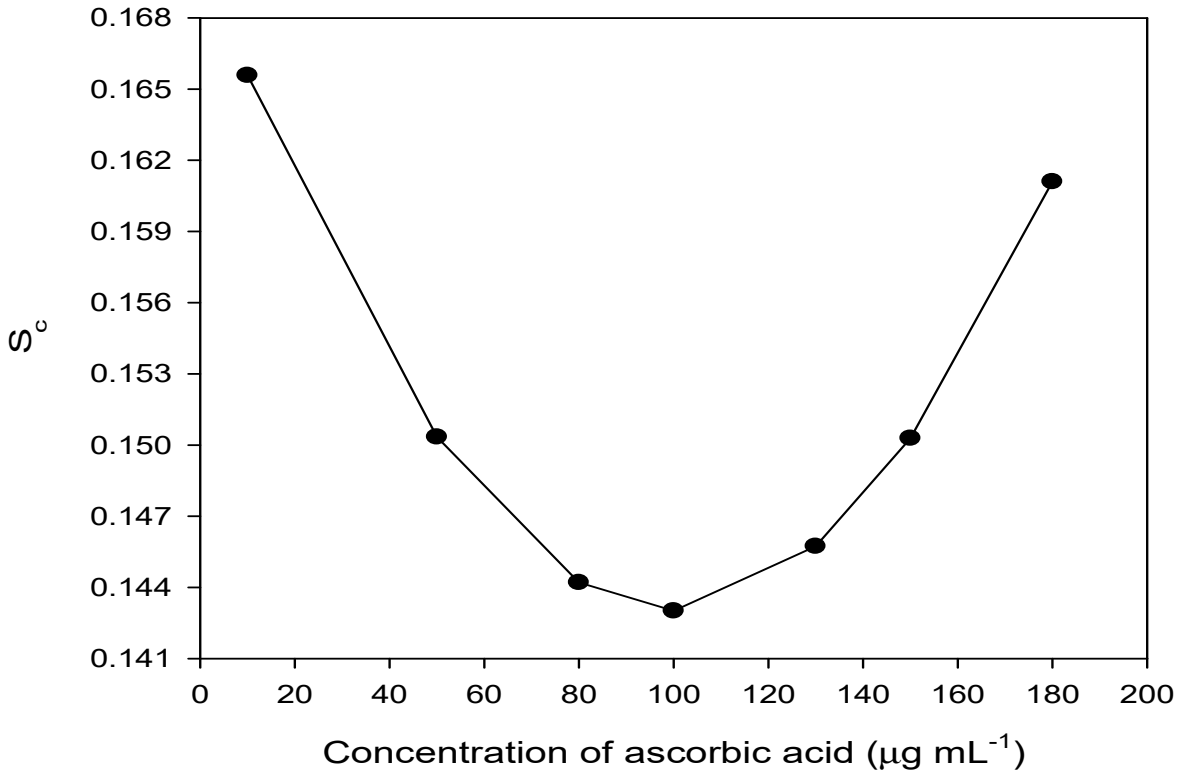

Figure 5. Uncertainty error (S_c) in the determination of ascorbic acid.

The value of S_c was considered to determine the % uncertainty confidence limit (UCL) at 95% confidence level with 5 degrees of freedom using the following the equation:

$$\%\ \mathrm{UCL} = \frac{t_p\, S_c}{C} \times 100 \quad (7)$$

The % uncertainty confidence limit was calculated and the results are shown in Figure 6 by plotting % uncertainty confidence limit against the concentration of ascorbic acid (μg mL^{-1}).

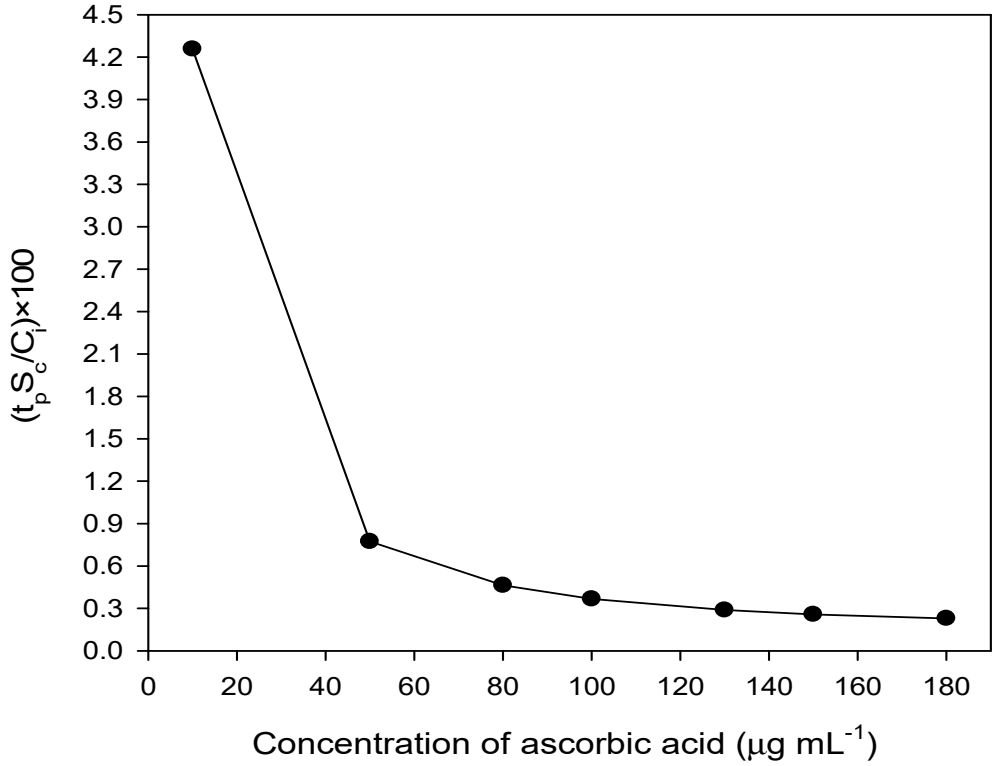

Figure 6. % Uncertainty confidence limit in the determination of ascorbic acid at 95% confidence level.

3.3.5. Precision

The intra-day and inter-day precision of the proposed HPLC method were investigated by performing 5 replicate independent analyses at 3 different concentration levels (low, medium and high) of ascorbic acid (40, 120 and 160 µg mL^{-1}) within the linear dynamic range on the same day (intra-day) and on 5 consecutive days (inter-day). The results of the analyses are summarized in Table 7. The % relative standard deviation and % recovery for intra-day and inter-day assays were in the range of 0.059–0.122% and 99.92–100.08%; and 0.074–0.162% and 99.94–100.11, respectively. The results reveal that the % recovery of ascorbic acid was in the permissible range of 100 ± 2% [64], indicating that the proposed method is precise within the acceptable limit. Furthermore, the RSD values were less than 2%, hence the proposed method is precise within the acceptable limits.

Table 7. Intra-day and inter-day precisions of the proposed HPLC method.

Initial Concentration of Imipramine HCl (µg mL^{-1})	Intra-day Assay and Inter-day Precisions					
	Measured Concentration ± SD (µg mL^{-1})		RSD [a] (%)		Recovery [a] (%)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
40.0	39.97 ± 0.122	40.04 ± 0.131	0.305	0.326	99.92	100.11
120.0	119.96 ± 0.059	120.16 ± 0.074	0.050	0.62	99.97	100.11
160.0	160.13 ± 0.095	159.91 ± 0.162	0.059	0.102	100.08	99.94

[a] 5 independent analyses.

3.3.6. Specificity

The selectivity and specificity of the proposed HPLC method for quantitation of ascorbic acid in tablet formulations of Redoxon 1 g (Bayer, Basel, Switzerland) and C-Tamin 0.5 g (Vital Health, Las Vegas, NV, USA) was assessed. The excipients found in the tablets were mostly eliminated during tablet solution preparations through filtration via Whatmann filter paper, as well as by filtration through a 0.45 µm polyethersulfone

membrane with the help of a syringe. The same drug solutions were assayed at 100 μg mL^{-1} of ascorbic acid concentration by the proposed and reference methods and did not show any significant change in peak area nor retention time. Hence, the proposed method is specific and selective in the presence of excipients (microcrystalline cellulose, macrogol 6000, lactose monohydrate, magnesium stearate, sodium starch glycolate and colloidal anhydrous silica), even when encountered in tablet formulations.

3.3.7. Accuracy

The accuracy of the proposed method was evaluated and the % recovery of ascorbic acid was compared with the reference method. The amount of active ascorbic acid in tablets of ascorbic acid, such as Redoxon 1 g (Bayer, Basel, Switzerland) and C-Tamin 0.5 g (Vital Health, Las Vegas, NV, USA), was assayed by the proposed HPLC and reference methods. Five independent replicated analyses at 100 μg mL^{-1} of active ascorbic acid from tablet solutions were performed. The amount of active ascorbic acid in both tablets was determined by the proposed and reference methods using the following regression equations:

$$C = (\text{Peak area} - 2.57 \times 10^{-3})/1.069 \text{ (Proposed method)} \quad (8)$$

$$C = (\text{Peak area} - 0.1046)/1.064 \text{ (Reference method)} \quad (9)$$

The obtained peak area at 100 μg mL^{-1} of active ascorbic acid was substituted in the said regression equation and the amount of ascorbic acid was calculated. The % recovery of active ascorbic acid in both tablets by the proposed and reference methods was in the range of 99.98 to 100.04%. The increase or decrease in % recovery results were less than ±2% [64], hence the proposed method is accurate and comparable to the reference method.

The significance of the proposed and reference methods was evaluated by an interval hypothesis test at the same concentration of ascorbic acid (100 μg mL^{-1}). The results are summarized in Table 8. Paired t- and F-values for both tablets by the proposed and reference methods were calculated [65] and found to be less than the tabulated t (ν = 0 8, 2.306) and F-values (ν = 4, 4; 6.39) at 95% confidence level. Hence, it is evidenced through calculated t- and F-values that that there is no significant difference between the proposed HPLC and reference methods.

Table 8. Significance of testing between the proposed HPLC and reference methods at 100 μg mL^{-1} of active ascorbic acid in tablet formulations at 95% confidence level.

Tablet Formulations	Concentration Found ± SD (μg mL^{-1}) [a]					
	Method		t-Value [b]	F-Value [b]	θ_L [c]	θ_U [c]
	Proposed	Reference				
Redoxon 1000 mg (Bayer, Switzwerland)	99.98 ± 0.11	100.04 ± 0.07	1.04	2.10	0.999	1.002
C-Tamin 500 mg (Vital Health, USA)	99.98 ± 0.11	100.04 ± 0.07	1.12	2.28	0.999	1.002

[a] Five independent replicate analyses. [b] Theoretical t (ν = 0 8) and F-values (ν = 4, 4) at 95% confidence level are 2.306 and 6.39, respectively. [c] A bias, based on recovery experiments, of ±2% is acceptable.

The % recovery of ascorbic acid in Redaxon and C-Tamin by the proposed and reference methods were in the range of 99.98–100.04. The bias of ±2% in % recovery is allowed [64], hence the results of both methods were in agreement and accurate. The bias in recovery results by the proposed and reference methods was calculated by an interval hypothesis test using the following quadratic equation [66,67]:

$$\theta^2\left(\bar{x}_1^2 - \frac{S_p^2\, t^2}{n_1}\right) - 2\theta\bar{x}_1\bar{x}_2 + \left(\bar{x}_2^2 - \frac{S_p^2\, t^2}{n_2}\right) = 0 \quad (10)$$

The 2 roots (lower limit, θ_L and upper limit, θ_U) of the quadratic equation were calculated and found to be in the range of 0.999 (θ_L between 0.98–1.00) to 1.002 (θ_U between

1.00–1.02) for Redaxon and C-Tamin. As evidenced, the results are acceptable and in compliance with regulatory Canadian Health Protection Branch guidelines [64].

3.4. Comparison of the Proposed Method with Other Published HPLC Methods

The performances of the proposed HPLC method and other published HPLC methods are presented in Table 9.

Table 9. Performances of the proposed HPLC method and other published HPLC methods for determination of ascorbic acid.

S. No.	Mobile Phase	Column	Temperature (°C) and UV Detection Wavelength (nm)	Linear Range (μg mL^{-1}) & Retention Time (min)	Flow Rate (mL min^{-1})	EcoScale Greenness Point	References
1	Methanol−0.05 M sodium dihydrogen phosphate (35:65 *v*/*v*), pH 2.5 adjusted with orthophosphoric acid.	Promosil C18, LC column, 4.6 (id) × 250 mm, 5 μm particle size (Agela Technologies Inc., Torrance, CA, USA)	37 & 220	3.0–60 & 2.4	1.0	61	[33]
2	Methanol−0.03 M sodium dihydrogen phosphate (55:45 *v*/*v*), pH 4 adjusted with orthophosphoric acid.	CLC Shim-pack C8 column, 4.6 (id) × 250 mm, 5 μm particle size (Shimadzu Corporation, Kyoto, Japan)	25 & 255	0.5–10 & 3.1	1.0	71	[34]
3	1 mM sodium pentane sulphonate in (0.4 mL of formic acid + 25 mL of methanol + 75 mL distilled water).	LiChrosorb C18, 4.6 (id) × 250 mm, 5 μm particle size, (Merck, Darmstadt, Germany)	25 & 254	5–40 & 3.53	1.0	61	[35]
4	5 mM cetyl trimethyl ammonium bromide + 50 mM KH_2PO_4	Symmetry C18 column, 4.6 (id) × 280 mm, particle size 5 μm (Waters, Milford, MA, USA).	25 & 254	1–100 & 8.1	1.2	56	[36]
5	5 mM Tetrabutyl ammonium hydroxide (pH 6, adjusted with H_3PO_4)-methanol (80:20 *v*/*v*)	Venusil XBP C18 column, 4.6 (id) × 250 mm, 5 μm particle size (Agela Technologies Inc., USA)	25 & 245	10–100 & 3.1	0.9	71	[37]
6	Acetonitrile-dichloromethane-0.25% KH_2PO_4 (5:5:95 *v*/*v*/*v*)	Cosmosil 5C18-MS-II column, 4.6 (id) ×250 mm, 5 μm particle size (Nacalai Tesque Inc., Kyoto, Japan)	25 & 246	0.05–30 & 2.54	1.0	66	[38]

Table 9. *Cont.*

S. No.	Mobile Phase	Column	Temperature (°C) and UV Detection Wavelength (nm)	Linear Range (μg mL^{-1}) & Retention Time (min)	Flow Rate (mL min^{-1})	EcoScale Greenness Point	References
7	A: Phosphate buffer (pH 2.7, adjusted with H_3PO_4)-B: Methanol (A 90–80% for 5 min: B 10–20% for 5 min *v*/*v*) (gradient mode)	Alltima C18 column, 3 (id) × 100 mm, 3 μm particle size (Grace company, Salt Lake City, UT, USA)	30 & 243	0.3–1.0 & 1.85	0.4	62	[39]
8	0.01 M dihydrogen ammonium phosphate (pH 2.6, adjusted with H_3PO_4)	Spherisorb C18 column, 4.6 (id) × 150 mm, 3 μm particle size, (Waters, USA)	25 & 254	1.9–125 & 2.81	1.0	51	[40]
9	Octylamine + salicylic acid (0.005 M)	LiChrospher RP-18 HPLC column, 4(id) × 250 mm, 5 μm particle size, (Merck, Germany)	25 & 254	0.2–10.0 & 5.97	1.0	51	[41]
10	0.2 M NaH_2PO_4 (pH 2.14 adjusted with 0.5 MHCl	PLRP − S 100 A°, 4.6 (id) × 250 mm, 5 μm particle size (Agilent, Santa Clara, CA, USA)	40 & 220	0.02–10.0 & 7.1	0.5	61	[42]
11	Methanol-phosphate buffer (0.01 M KH_2PO_4, pH 2.0) 35:65 (*v*/*v*)	Gemini C18 LC column, 4.6 (id) × 250 mm, 5 μm particle size (Phenomenex, USA)	25 & 245	12.2–21.4 & 2.5	1.5	61	[43]
12	Metaphosphoric acid (0.2%)-methanol-acetonitrile (90:8:2, *v*/*v*/*v*)	LiChrospher® 100–RP18, 4.6 (id) × 250 mm, 5 μm particle size (Phenomenex, USA)	254 & 24.0 ± 2.0	1.0–12.0 & 3.4	1.0	71	[44]
13	Acetonitrile and 50 mM ammnonium acetate buffer pH 6.8 (78:22 *v*/*v*)	SeQuant ZIC HILIC, 2.1 (id) × 150 mm, 3.5 μm particle size (Merck, Germany)	268 & 23	0.1–100 & 4.66	0.3	61	[45]
14	5% methanol in 25 mmolL^{-1} sodium dihydrogen phosphate pH 4.8	Discovery C18, 4 (id) × 250 mm, 5 μm particle size (Merck, Germany)	265 & 25	0.35–44.03 & 4.2	0.5	61	[46]
15	Water is brought to pH 2.2 with sulphuric acid:methanol (80:20)	Haisil C18 100 Å column (25 cm × 4.6 mm (Higgins Analytical, Mountain View, CA, USA)	243 & 25	1.0–80.0 & 3.18	1.0	61	[47]

Table 9. *Cont.*

S. No.	Mobile Phase	Column	Temperature (°C) and UV Detection Wavelength (nm)	Linear Range (µg mL^{-1}) & Retention Time (min)	Flow Rate (mL min^{-1})	EcoScale Greenness Point	References
16	1.5 g of 1-hexanesulfonic acid sodium salt dissolved in 500 mL acetic acid (pH 2.6)	Superspher C18 column, 4 (id) ×125 mm, 4µm particle size (Merck, Germany)	20 & 280	150–250 & 3.5	0.7	56	[48]
17	Methanol−0.05 M sodium dihydrogen phosphate buffer (gradient mode)	Shim-pack C18 VP-ODS-2 RP column, 4.6 (id) × 150 mm, 5 µm particle size (Shimadzu, Japan)	35 & 254	29.8–476.9 & 3.0	0.8	61	[49]
18	Methanol—KH_2PO_4 (5 $mmolL^{-1}$), pH 2.65 (gradient mode)	LiChrospher® 100–RP18, 4.6 (id) × 250 mm, 5 µm particle size (Phenomenex, USA)	25 & 245	0–300 & 4.01	0.8	61	[50]
19	Ion-pair solution-acetonitrile (98:2 *v*/*v*)	Hypersil C18 BDS column. 4 (id) × 250 mm, 5 µm particle size (Thermo Fisher Scientific, USA)	25 & 275	0–200 & 2.2	1.0	76	[51]
20	Acetonitrile + phosphate buffer (pH 6.5) (10:90 *v*/*v*)	Onyx Monolithic C18, LC column, 4.6 (id) × 100 mm, (Phenomenex, USA)	25 & 235	150–450 & 1.6	1.0	60	[52]
21	Methanol + 0.4% KH_2PO_4 (pH 3.0); (5.0:95 *v*/*v*)	Lunar C18 column, 4.6 (id) × 250 mm, 5µm particle size (Phenomenex, USA)	25 & 248	15–120 & 4.05	1.0	61	Reference method [61]
22	Acetonitrile + 0.4% NaH_2PO_4 (pH 3.0); (5.0:95 *v*/*v*)	Acclaim™ 120 C18 column, 4.6 (id) × 250 mm, 5µm particle size (Dionex, USA).	25 & 245	10–180 & 4.1	0.8	66	Proposed work

It is clear from the table that most of the published high performance liquid chromatographic methods [33–47,60] have a linear dynamic range between 0.05–125 µg mL^{-1}. These methods used more typical mobile phases with typical buffer solutions, such as sodium pentane sulphonate, in the presence of formic acid along with methanol [35], hexadecyltrimethyl ammonium bromide along with KH_2PO_4 [36], tetrabutyl ammonium hydroxide in the presence of H_3PO_4 along with methanol [37], a combination of acetonitrile and dichloromethane along with an acidic buffer solution [38] and a 15mM phosphate buffer (90–80%, pH 2.7) with methanol (10–20%) gradient mode [39] for elution of ascorbic acid. Hence, these methods involve more steps prior to analysis with a lower linear dynamic range, which makes these methods tedious and expensive. The proposed method utilizes a mobile phase of acetonitrile along with a phosphate buffer solution with a pH of 3, providing a higher linear dynamic range (10–180 µg mL^{-1}), a retention time of 4.1 min, an asymmetry factor of 1.15 and a theoretical plate of 13,785 at room temperature. Hence,

the overall performance of the proposed method is satisfactory and within the permissible limits, and can therefore be used as alternate method for routine quality control analysis of active ascorbic acid in tablet formulations.

3.5. Greenness Profile of the Proposed Method

The analytical ecoscale [68] was utilized to evaluate the greenness of the proposed method for determining ascorbic acid in tablet formulations. The analytical ecoscale penalty points of 34 was assigned to the proposed method on using the link (http://ecoscale.cheminfo.org/calculator) with parameters in software in the order/sequence of 24.358 mM of sodium dihydrogen phosphate dihydrate (CAS No. 13472-35-0), 3.515 mM of phosphoric acid (CAS No. 7664-38-2), 4.756 mM of acetonitrile (CAS No. 75-05-8), an instrument for controlled addition of chemicals, room temperature < 1 h and adding solvent.

The greenness point can be achieved by subtracting the penalty points from 100. The greenness of the adopted method can be (i) excellent (if greenness point > 75), (ii) acceptable (if greenness point > 50) or not up to the mark (if greenness point < 50). In this study, the greenness point of the proposed method was 66, hence the proposed method was acceptable to assay ascorbic acid in tablet formulations. In addition, the greenness points of the reported HPLC methods were calculated and are given in Table 9. It is evident from the table that the proposed HPLC method was found to be greener (66 greenness points) than the majority of the reported HPLC methods [33,35,36,39–43,45–50,52,61], or similar to the reported HPLC method [38] but less green than four reported HPLC methods [34,37,44,51]. In all three reported methods [34,37,44] where the greenness points of 71 were obtained, each has a lower linear dynamic range than the proposed HPLC method. The reported HPLC method [51] where the highest greenness point of 75 was achieved has a comparable linear dynamic range (0–200 μg mL^{-1}) to the proposed HPLC method, but involves more reagents and solvent (1-hexane sulfonic acid, acetic acid, triethylamine and acetonitrile), which makes this reported HPLC method tedious and cumbersome. Hence, due to the merits listed above, a greener quality approach and successful application of the proposed HPLC method in assaying ascorbic acid in tablets renders this method novel and superior.

4. Conclusions

The proposed HPLC method is simple, sensitive, accurate and inexpensive. The proposed HPLC method was not impeded by common excipients present in tablet formulations. The results of the proposed HPLC method are in agreement with those of the reference method and show compliance with regulatory guidelines in terms of % recovery of ascorbic acid in tablet formulations. The proposed method follows system suitability parameters, such as a asymmetry factor of 1.15 (less than 1.8) and a higher theoretical plate of 13,785. These merits, high reproducibility, ease of operation and sensitivity make the proposed method more suitable for routine quality control analysis of ascorbic acid in pharmaceutical industries, hospitals and academic institutions.

Author Contributions: The conceptualization, writing of the original draft and methodology development were contributed by S.N.H.A., U.A.H. and N.R. The investigations, formal analysis and data curation were performed by J.O.S.A.M., B.M.S.A.M., B.S.A.R.A.J. and A.S.S.A.R. The data validation using Design Expert software was performed by S.N.H.A., M.N. and N.R. The format editing and review of the manuscript were performed by S.M.H., S.A.K., Q.U.A. and Z.A.Z. Article processing charges were paid by Z.A.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data is contained within the article. The analytical Eco scale weblink of (http://ecoscale.cheminfo.org/calculator) was accessed on 26 October 2022 for assessing greenness points. The pH was monitored through the weblink of (http://www.omnicalculator.com/chemistry/henderson-hasselbalch) (accessed on accessed on 26 October 2022).

Acknowledgments: The authors thank the higher-ups and the Vice Chancellor of the University of Technology and Applied Sciences (Higher College of Technology Muscat) Sultanate of Oman, for support in carrying out this work. The authors are grateful to all technical staff for their help and unwavering support through the entire research work. In addition to this, all authors thank the University Malaysia Sabah for its generous support and financial assistance in order to pay the article processing charges of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Valente, A.; Albuquerque, T.G.; Sanches-Silva, A.; Costa, H.S. Ascorbic acid content in exotic fruits: A contribution to produce quality data for food composition databases. *Food Res. Int.* **2011**, *44*, 2237–2242. [CrossRef]
2. British Pharmacopoeia Commission. *British Pharmacopoeia, Vol. I*; Her Majesty Stationary Office: London, UK, 2018; pp. 203–204.
3. USP 42. In *United States Pharmacopoeia*; The United States Pharmacopeial Convention: Rockville, MD, USA, 2018; pp. 377–378.
4. European Pharmacopoeia. *European Pharmacopoeia*, 9th ed.; European Directorate for the Quality of Medicines & HealthCare: Strasbourg, France, 2017; pp. 1760–1761.
5. NHS. *British National Formulary 81, Chapter 9: Blood and Nutrition*; Royal Pharmaceutical Society: London, UK, 2021; p. 1132.
6. Baker, E.M.; Hodges, R.E.; Hood, J.; Sauberlich, H.E.; March, S.C. Metabolism of ascorbic-1–14C acid in experimental human scurvy. *Am. J. Clin. Nutr.* **1969**, *22*, 549–558. [CrossRef]
7. Krebs, N.A.; Peters, R.A.; Coward, K.H. Vitamin C requirement of human adults: Experimental study of vitamin C deprivation in man. *Lancet* **1948**, *254*, 853–858.
8. World Health Organization and Food and Agriculture Organization of the United Nations. Vitamin C. In *Vitamin and Mineral Requirements in Human Nutrition*, 2nd ed.; World Health Organization and Food and Agriculture Organization of the United Nations: Geneva, Switzerland, 2004; p. 132.
9. Rossetti, C.A.; Real, J.P.; Palma, S.D. High dose of ascorbic acid used in Sars COVID-19 treatment: Scientific and clinical support for is therapeutic implementation. *Ars Pharm.* **2020**, *61*, 145–148.
10. Hiedra, R.; Lo, K.B.; Elbashabsheh, M.; Gul, F.; Wright, R.M.; Albano, J.; Azmaiparashvili, Z.; Patarroyo Aponte, G. The use of IV vitamin C for patients with COVID-19: A case series. *Expert Rev. Anti Ther.* **2020**, *18*, 1259–1261. [CrossRef] [PubMed]
11. Dasgupta, A.; Klein, K. Chapter 15—Antioxidant Vitamins and Minerals, in Antioxidants. In *Food, Vitamins and Supplements*; Elsevier: San Diego, CA, USA, 2014; pp. 277–294.
12. Grosso, G.; Bei, R.; Mistretta, A.; Marventano, S.; Calabrese, G.; Masuelli, L.; Giganti, M.G.; Modesti, A.; Galvano, F.; Gazzolo, D. Effects of vitamin C on health: A review of evidence. *Front. Biosci.* **2013**, *18*, 1017–1209.
13. Azmi, S.N.H.; Iqbal, B.; Al Humaimi, N.S.H.; Al Salmani, I.R.S.; Al Ghafri, N.A.S.; Rahman, N. Quantitative analysis of cefixime via complexation with palladium (II) in pharmaceutical formulations by spectrophotometry. *J. Pharm. Anal.* **2013**, *3*, 248–256. [CrossRef]
14. Husain, A.; Iram, F.; Siddiqui, A.A.; Almutairi, S.M.; Mohammed, O.B.; Khan, S.A.; Azmi, S.N.H.; Rahman, N. Identification of metabolic pathways involved in the biotransformation of eslicarbazepine acetate using UPLC-MS/MS, human microsomal enzymes and in silico studies. *J. King Saud Univ. Sci.* **2021**, *33*, 101281. [CrossRef]
15. Siddiqui, M.R.; Al-Othman, Z.A.; Rahman, N. Analytical techniques in pharmaceutical analysis: A review. *Arab. J. Chem.* **2017**, *10*, S1409–S1421. [CrossRef]
16. Al Othman, Z.A.; Rahman, N.; Siddiqui, M.R. Review on pharmaceutical impurities, stability studies and degradation products. *Rev. Adv. Sci. Eng.* **2013**, *2*, 155–166. [CrossRef]
17. Rahman, N.; Azmi, S.N.H.; Wu, H.F. The importance of impurity analysis in pharmaceutical products: An integrated approach. *Accredit. Qual. Assur.* **2006**, *11*, 69–74. [CrossRef]
18. Qureshi, S.Z.; Saeed, A.; Haque, S.; Rahman, N. Spectrophotometric method for the estimation of Vitamin C in drug formulations. *Anal. Lett.* **1990**, *23*, 995–1003. [CrossRef]
19. Salkić, M.; Selimović, A. Spectrophotometric determination of L-Ascorbic Acid in pharmaceuticals based on its oxidation by potassium peroxymonosulfate and hydrogen peroxide. *Croat. Chem. Acta* **2015**, *88*, 73–79. [CrossRef]
20. Shishehbore, M.R.; Aghamiri, Z. A highly sensitive kinetic spectrophotometric method for the determination of ascorbic acid in pharmaceutical samples. *Iran. J. Pharm. Res.* **2014**, *13*, 373–382.
21. Hussein, A.Z.M. Spectrophotometric determination of ascorbic acid in aqueous solutions and in pharmaceuticals formulations. *J. Al-Nahrain Univ.* **2013**, *16*, 65–71. [CrossRef]
22. Mowry, S.; Ogren, P.J. Kinetics of methylene blue reduction by ascorbic acid. *J. Chem. Educ.* **1999**, *76*, 970–973. [CrossRef]
23. Hasegawa, Y.; Sugawara, T. Thermodynamic parameters of complexation of lanthanoid (III) with ascorbic acid. *Inorg. Chim. Acta* **1988**, *143*, 277–280. [CrossRef]
24. Pyka-Pajak, A.; Dołowy, M.; Parys, W.; Bober, K.; Janikowska, G. A simple and cost-effective TLC-densitometric method for the quantitative determination of acetylsalicylic acid and ascorbic acid in combined effervescent tablets. *Molecules* **2018**, *23*, 3115. [CrossRef]

25. Akasaka, K. Simple determination of L-ascorbic acid on TLC by visual detection using autocatalytic reaction. *Anal. Sci.* **2013**, *29*, 505–509. [CrossRef]
26. Polak, B.; Pajurek, E. Separation of some vitamins in reversed-phase thin-layer chromatography and pressurized planar electrochromatography with eluent containing surfactant. *Sci. Rep.* **2021**, *11*, 21851. [CrossRef]
27. Alam, P.; Kamal, Y.T.; Alqasoumi, S.I.; Foudah, A.I.; Alqarni, M.H.; Yusufoglu, H.S. HPTLC method for simultaneous determination of ascorbic acid and gallic acid biomarker from freeze dry pomegranate juice and herbal formulation. *Saudi Pharm. J.* **2019**, *27*, 975–980. [CrossRef] [PubMed]
28. Peter, E.L.; Kaligirwa, A.; Lukwago, T. Stability indicating high-performance thin-layer chromatography method for estimation of ascorbic acid in *Hibiscus sabdariffa* L. aqueous extract. *J. Complement. Med. Res.* **2019**, *10*, 50–57. [CrossRef]
29. Koh, E.V.; Bissell, M.G.; Ito, R.K. Measurement of vitamin C by capillary. electrophoresis in biological fluids and fruit beverages using a stereoisomer as an internal standard. *J. Chromatogr.* **1993**, *633*, 245–250. [CrossRef]
30. Amayreh, M.; Hourani, W.; Hourani, M.K. Voltammetric determination of ascorbic acid in pharmaceutical formulations using modified iodine-coated platinum electrode. *J. Vitae* **2021**, *28*, 346228. [CrossRef]
31. Nojavan, S.; Khalilian, F.; Kiaie, F.M.; Rahimi, A.; Arabanian, A.; Chalavi, S. Extraction and quantitative determination of ascorbic acid during different maturity stages of *Rosa canina* L. fruit. *J. Food Compos. Anal.* **2008**, *21*, 300–305. [CrossRef]
32. Russel, L.F. Food Analysis by HPLC. In *Quantitative Determination of Water-Soluble Vitamins*, 2nd ed.; Nollet, L.M.L., Ed.; Marcel Dekker: New York, NY, USA, 2000; pp. 403–476.
33. Ibrahim, F.; El-Enany, N.; El-Shaheny, R.N.; Mikhail, I.E. Development and validation of a new HPLC method for the simultaneous determination of paracetamol, ascorbic acid, and pseudoephedrine HCl in their co-formulated tablets. Application to in vitro dissolution testing. *Anal. Sci.* **2015**, *31*, 943–947. [CrossRef] [PubMed]
34. El-Din, M.S.; Eid, M.; Zeid, A.M. Development and validation of RP-HPLC method for simultaneous determination of ascorbic acid and salicylamide in their binary mixtures: Application to combined tablets. *J. Chromatogr. Sep. Tech.* **2012**, *3*, 1000137. [CrossRef]
35. Tsvetkova, B.; Pencheva, I.; Zlatkov, A.; Peikov, P. Simultaneous high-performance liquid chromatography determination of paracetamol and ascorbic acid in tablet dosage forms. *Afr. J. Pharm. Pharmacol.* **2012**, *6*, 1332–1336. [CrossRef]
36. Kim, Y.; Kim, M.G. HPLC-UV method for the simultaneous determinations of ascorbic acid and dehydroascorbic acid in human plasma. *Transl. Clin. Pharmacol.* **2016**, *24*, 37–42. [CrossRef]
37. Hu, L.; Li, L.; Luo, Z.; Yang, J.; Liu, W. Determination of trace vitamin C by ion-pair HPLC with UV detection in calcium gluconate and vitamin C compound oral solution. *J. Chromatogr. Sci.* **2012**, *50*, 102–107. [CrossRef]
38. Mahmood, H.S.; Ahmad, R.R. High performance liquid chromatographic determination of ascorbic acid in pharmaceutical preparations, fruit juices and human Serum. *Rafidain J. Sci.* **2018**, *27*, 79–92.
39. Stana, M.; Sorana, M.L.; Marutoiub, C. Extraction and HPLC Determination of the ascorbic acid content of three indigenous spice plants. *J. Anal. Chem.* **2014**, *69*, 998–1002. [CrossRef]
40. Chebrolu, K.K.; Jayaprakasha, G.K.; Yoo, K.S.; Jifon, J.L.; Patil, B.S. An improved sample preparation method for quantification of ascorbic acid and dehydroascorbic acid by HPLC. *Food Sci. Technol.* **2012**, *47*, 443–449. [CrossRef]
41. Gennaro, M.C.; Bertolo, P.L. L-Ascorbic acid determination in fruits and medical formulations by ion interaction reagent reverse phase HPLC technique. *J. Liq. Chromatogr.* **1990**, *13*, 1419–1434. [CrossRef]
42. Lloyd, L.L.; Warner, F.P.; White, C.A.; Kennedy, J.F. Quantitative reversed phase HPLC analysis of L-ascorbic acid (vitamin C) and identification of its degradation products. *Chromatographia* **1987**, *24*, 371–376. [CrossRef]
43. Garnero, C.; Longhi, M. Development of HPLC and UV spectrophotometric methods for the determination of ascorbic acid using hydroxypropyl-β-cyclodextrin and triethanolamine as photostabilizing agents. *Anal. Chim. Acta* **2010**, *659*, 159–166. [CrossRef]
44. Maia, A.M.; Baby, A.R.; Yasaka, W.J.; Suenaga, E.; Kaneko, T.M.; Velasco, M.V.R. Validation of HPLC stability-indicating method for Vitamin C in semisolid pharmaceutical/cosmetic preparations with glutathione and sodium metabisulfite, as antioxidants. *Talanta* **2007**, *71*, 639–643. [CrossRef]
45. Novakova, L.; Solichova, D.; Pavlovicova, S.; Solich, P. Hydrophilic interaction liquid chromatography method for the determination of ascorbic acid. *J. Sep. Sci.* **2008**, *31*, 1634–1644. [CrossRef]
46. Kand'ar, R.; Zakova, P. Determination of ascorbic acid in human plasma with a view to stability using HPLC with UV detection. *J. Sep. Sci.* **2008**, *31*, 3503–3508. [CrossRef]
47. Ahmida, M.H.S. Determination of ascorbic acid in vitamin C (tablets) by high-performance liquid chromatography. *Asian J. Chem.* **2009**, *21*, 6463–6467.
48. Mitić, S.S.; Kostić, D.A.; Nasković-Dokić, D.C.; Mitic, M.N. Rapid and reliable HPLC method for the determination of vitamin C in pharmaceutical samples. *Trop. J. Pharm. Res.* **2011**, *10*, 105–111. [CrossRef]
49. Abe-Matsumoto, L.T.; Sampaio, G.R.; Bastos, D.H.M. Is titration as accurate as HPLC for determination of vitamin C in supplements? *Am. J. Anal. Chem.* **2020**, *11*, 269–279. [CrossRef]
50. Klimczak, I.; Gliszczyńska-Świgło, A. Comparison of UPLC and HPLC methods for determination of vitamin C. *Food Chem.* **2015**, *175*, 100–105. [CrossRef] [PubMed]
51. Muller, L.D. Improved extraction methods for avoiding the interference of copper in the LC determination of ascorbic acid in multivitamin-mineral tablets. *J. Pharm. Biomed. Anal.* **2001**, *25*, 985–994. [CrossRef]

52. Koblová, P.; Sklenářová, H.; Brabcová, I.; Solich, P. Development and validation of a rapid HPLC method for the determination of ascorbic acid, phenylephrine, paracetamol and caffeine using a monolithic column. *Anal. Methods* **2012**, *4*, 1588–1591. [CrossRef]
53. Rahman, N.; Sameen, S.; Kashif, M. Application of Box–Behnken design and desirability function in the optimization of spectrophotometric method for the quantification of WADA banned drug: Acetazolamide. *J. Mol. Liq.* **2019**, *274*, 270–277. [CrossRef]
54. Rahman, N.; Khan, S. Experimental design approach in the optimization of potentiometric method for lansoprazole determination using lansoprazole-tungstate based ion-selective electrode. *Ind. Eng. Chem. Res.* **2018**, *57*, 9351–9361. [CrossRef]
55. Azmi, S.N.H.; Al-Jassasi, B.M.H.; Al-Sawafi, H.M.S.; Al-Shukaili, S.H.G.; Rahman, N.; Nasir, M. Optimization for synthesis of silver nanoparticles through response surface methodology using leaf extract of *Boswellia sacra* and its application in antimicrobial activity. *Environ. Monit. Assess.* **2021**, *193*, 497. [CrossRef]
56. Azmi, S.N.H.; Al-Masrouri, Z.N.; Al-Lamki, I.R.; Al-Jabri, A.K.; Rahman, N.; Nasir, M.; Abdelrahman, K.; Fnais, M.S.; Alam, M. Development and validation of spectrophotometric method for determination of imipramine hydrochloride in tablets (solid materials). *J. King Saud Univ.-Sci.* **2022**, *34*, 101823. [CrossRef]
57. Afzal, M.; Muddassir, M.; Alarifi, A.; Ansari, M.T. Box-Behnken assisted validation and optimization of an RP-HPLC method for simultaneous determination of domperidone and lansoprazole. *Separations* **2021**, *8*, 8010005. [CrossRef]
58. International Council for Harmonization. *ICH Q14, Analytical Procedure Development and Revision of Q2(R1) Analytical Validation (Concept Paper)*; ICH: Geneva, Switzerland, 2018.
59. Demirel, C.; Kabutey, A.; Herák, D.; Sedlacek, A.; Mizera, C.; Dajbych, O. Using Box–Behnken design coupled with response surface methodology for optimizing rapeseed oil expression parameters under heating and freezing conditions. *Processes* **2022**, *10*, 490. [CrossRef]
60. Azmi, S.N.H.; Al Lawati, W.M.; Al Hoqani, U.H.A.; Al Aufi, E.; Al Hatmi, K.; Al Zadjali, J.S.; Rahman, N.; Nasir, M.; Rahman, H.; Khan, S.A. Development of a Citric-Acid-Modified Cellulose Adsorbent Derived from *Moringa peregrina* Leaf for Adsorptive Removal of Citalopram HBr in Aqueous Solutions. *Pharmaceuticals* **2022**, *15*, 760. [CrossRef] [PubMed]
61. Thao, N.M.P.; Vi, N.T.T.; Van, N.T.N. Determination of vitamin C in multivitamin effervescent tablets by high-performance liquid chromatography. *Can Tho J. Med. Pharm.* **2019**, *5*, 108–117.
62. Rahman, N.; Ahmad, Y.; Azmi, S.N.H. Kinetic spectrophotometric method for the determination of norfloxacin in pharmaceutical formulations. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 359–367. [CrossRef]
63. Miller, J.C.; Miller, J.N. Errors in instrumental analysis; regression and correlation. In *Statistics for Analytical Chemistry*, 3rd ed.; Ellis Horwood: Chichester, UK; Prentice Hall: Hoboken, NJ, USA, 1993; p. 119.
64. Canada Health Protection Branch. *Drugs Directorate guidelines, Acceptable Methods*; Draft; Ministry of National Health and Welfare: Ottawa, ON, Canada, 1994.
65. Rahman, N.; Haque, S.M.; Azmi, S.N.H.; Rahman, H. Optimized and validated spectrophotometric methods for the determination of amiodrone hydrochloride in commercial dosage forms using *N*-bromosuccinimide and bromothymol blue. *J. Saudi Chem. Soc.* **2017**, *21*, 25–34. [CrossRef]
66. Rahman, N.; Sameen, S.; Kashif, M. Spectroscopic study on the interaction of haloperidol and 2,4-dinitrophenylhydrazine and its application for the quantitation in drug formulations. *Anal. Chem. Lett.* **2016**, *6*, 874–885. [CrossRef]
67. Hartmann, C.; Smeyers-Verbeke, J.; Pinninckx, W.; Heyden, Y.V.; Vankeerberghen, P.; Massart, D.L. Reappraisal of hypothesis testing for method validation: Detection of systematic error by comparing the means of two methods or of two laboratories. *Anal. Chem.* **1995**, *67*, 4491–4499. [CrossRef]
68. Płotka-Wasylka, J. A new tool for the evaluation of the analytical procedure: Green analytical procedure index. *Talanta* **2018**, *181*, 204–209. [CrossRef]