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Quantification of Hesperidin in Bulk, Ayurvedic Formulations Including Plant Extracts and Human Plasma Spiked Using Chromogenic Method

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To develop and validate a simple, highly sensitive UV visible spectrophotometric technique for Quantitative analysis of Hesperidin in Ayurvedic formulation & bulk including plant extracts using chromogenic compound Gibb's reagent called (2, 6-Dichloro quinone 4-Chloroimide). Chromogenic method was developed in visible region by using Gibbs reagent. When the phenolic group in hesperidin upon reaction with Gibb's reagent in presence of NaOH at pH 9, a nucleophilic aromatic substitution reaction occurs, producing a blue coloured product. Gibb's reagent is primarily utilized to identify and quantify phenols which is measured at 500-670nm. Hesperidin is a category of bioflavonoid. It is used to treat blood vessel conditions like varicose veins and Haemorrhoids. The proposed methodology was effectively used to determine quantity of hesperidin in dosage forms, bulk powder. In compliance with ICH guidelines Q2(R1), the primary maxima for spectrometric determination of this compound Hesperidin by using an ELICO SL210 an UV-Visible spectrophotometer equipped with spectral treats software has been determined to be wavelength absorbance at 613 nm, due to its optimized and

enhanced reproducibility for other dilutions at that wavelength. The approach was then validated based on ICH validation parameters including linearity, precision, accuracy, ruggedness & robustness. A linear regression coefficient value (R2) at 0.9999 was observed, this indicates that concentration series from 2-40µg/ml was linearly well-demonstrated. %RSD of precision was within limits <2%. Accuracy of visible method was within acceptable limits. A rapid and sensitive bioanalytical method was developed for determining Hesperidin in human plasma by using protein precipitation extraction method.

Keywords: Gibb's reagent, Hesperidin, UV-Visible spectrophotometer, Ayurvedic Formulations, Madiphal Rasayana.

1. Introduction

Hesperidin is one type of flavanone glycoside. Hesperetin serves as its aglycone. The term "hesperidium" refers citrus tree fruit is the origin of its name. French chemist M. Lebreton extracted hesperidin for the first time in 1828 from white innermost layer within citrus peels. In 2019, hesperidin was approved. It is also known as Hesperetin 7-rhamnoglucoside, Cirantin, Hesperetin 7-rutinoside, and Hesperidoside. Its category is Flavonoid-7-o-glycosides [1]. Chemical Formula is $C_{28}H_{34}O_{15}$. Readily soluble in organic solvents as Dimethyl formamide and Dimethyl sulfoxide. In methanol and ethanol, it dissolves slightly and insoluble with water. 610.56 g/mol is molecular weight. Physical properties include solid and light brown appearance.

Hesperidin causes apoptosis and arrest of cell cycles, which prevents the growth of cancer cells by interacting with a wide range of known cellular targets. Evidence also suggests that it could potentially effective in inhibiting angiogenesis, chemoresistance, and tumour cell metastasis. HSP could serve as a potential therapeutic substance for treating a variety of CNS conditions, including neurodegenerative conditions. Blood vessel disorders like hemorrhoids, disorders like veins that are varicose, and insufficient blood transportation (venous stasis) are conditions that can be treated when combined with different citrus bioflavonoids like Diosmin [2-8]. (Fig. 1) Shows chemical structure of Hesperidin.

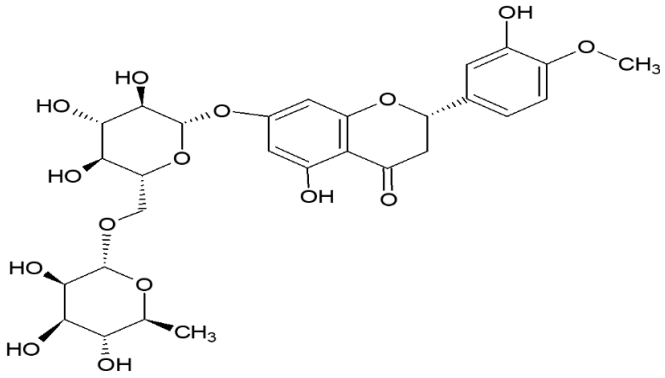

Figure 1. Chemical structure of Hesperidin

Different approaches to quantify hesperidin through various techniques, include spectrophotometry and spectrofluorimetric, RP-HPLC, HPTLC and Hyphenated techniques like LC-MS for determining hesperidin was eventually reported. In the reported methods, there are no chromogenic methods available for the estimation of Hesperidin drug. So, there is a need to develop simple, sensitive chromogenic method. We here developed a simple and precise spectrophotometric technique for quantification of HSP using chromogenic compound Gibbs reagent. The research approach utilizes novel chromogenic reagent 2,6-dichloro quinone 4-chloroimide, when combined with HSP in presence of a sodium hydroxide solution at pH 9, resulting in formation of blue product with maximum absorption at wavelength λmax (613nm) [9,10].

Mechanism of Gibb's Reagent

Imines are produced when phenol compounds and Gibb's reagent reaction takes place. Gibb's reagent is used in solvolysis of reagent to form 2, 6-dichloro quinone mono imines. Gibbs reagent coupled with phenolic substance to produce an adduct which is developed when the para position of phenolic compound is attached with 2, 6-dichloro quinone mono imine. Following adducts deprotonation, a colourful 2, 6-dichloro indophenols product is formed. Used for the detection of herbicides containing phenoxy acetic acid, aromatic hydrocarbons, primary, secondary, and tertiary aliphatic amines, as well as antioxidants and used for identifying the p-alkoxy and un-substituted phenols [11].

General Mechanism of Gibbs Reagent Reaction with Hesperidin

Gibbs reagent or 2,6-Dichloroquinone 4-chloroimide reacts with phenolic group in hesperidin through a nucleophilic aromatic substitution reaction. The phenolic hydroxyl group in hesperidin attacks the electrophilic carbon in the quinone-imide ring system of Gibbs reagent. This forms an intermediate, which then rearranges to eliminate a chlorine atom, resulting in the phenolic group attaching to the quinone-imide ring, replacing one of the chlorine atoms. The technique was successfully employed for determining the Hesperidin in ayurvedic and pharmaceutical formulations [12,13]. (Fig. 2) shows reaction of Gibbs reagent with Hesperidin standard drug solution.

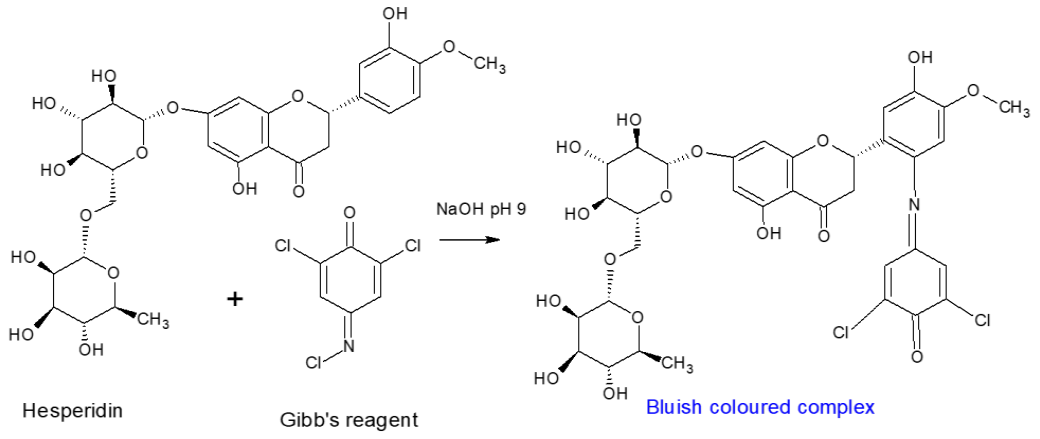

Figure 2. Reaction of Gibbs Reagent with Hesperidin

2. Materials and Methods

Aim

Aim of this method is to develop and validate accurate, simple & precise UV-visible spectrophotometric technique for quantifying hesperidin within bulk, Ayurvedic formulations and various plant extracts.

Instruments

Weighing balance, PH meter, Double beam Ultra Violet-Visible spectrophotometer, containing 1.8 nm spectral bandwidth and ± 0.5 nm wavelength accuracy to test the absorbance of the resultant solution, two matching quartz cells with a path length of 1 cm are used. Make: ELICO, Model: SL 210, Photo Diode Array Detector, Glass cuvette, The output signal was monitored in spectra treats software [14,15].

Chemicals

All analytical-grade, freshly prepared reagents and chemicals were utilized. Hesperidin API was gifted by Laural pharma and commercial dosage form Hesperidin & liquid formulation of madiphal rasayana (Baidyanath) was acquired from local pharmacies. Gibb's Reagent, Ethanol, Dilute HCL, Sodium Hydroxide, Distilled water, Ammonium chloride, Ammonia solution are required.

Preparation of stock solution (1000µg/ml) and serial dilutions

10mg of HSP pure drug was weighed in 10ml of volumetric flask & dissolved, make up to the mark with ethanol resultant solution is 1000µg/ml. From 1000 µg/ml, pipetted out 1ml in 10ml of volumetric flask and make up with ethanol resultant solution is 100µg/ml. From 100µg/ml serial dilutions were prepared. From 100µg/ml, 4ml was pipetted in 10ml volumetric flask then make up with ethanol the resultant solution is 40 µg/ml.

Preparation of Reagents

Preparation of 0.2% (w/v) Gibbs Reagent:

Weighed 0.2g of Gibbs Reagent in 100ml volumetric flask and make up with Ethanol.

Preparation of Ammonia Buffer pH 10:

Weighed 5.4 g of Ammonium chloride and transferred into 100ml volumetric flask and dissolved in distilled water. To it added 35ml of 10M Ammonia and make up with distilled water.

Preparation of pH 9, 0.01M Sodium Hydroxide Solution:

Weighed 0.04g Sodium Hydroxide transferred into 100ml volumetric flask and make up with distilled water and adjust to pH 9 with dilute HCL.

Procedure for determination of Hesperidin

Aliquots of the HSP standard drug solution in volumes of 0.2,0.4,0.6,0.8,1.4,2.0,2.6,3.2,3.8 & 4.0 ml were transferred into a series in 10 ml volumetric flasks & 0.2% Gibb's Reagent (1ml) was added, after 2 minutes 1ml of 0.01M Sodium

Hydroxide solution (pH 9 adjusted with Dilute HCL) were added. After 5 minutes persistent blue colour was produced with good intensity. Distilled water used for makeup. The absorbance of HSP as bluish chromogen measured at 613nm against corresponding reagent that is blank. The linearity of the absorbance vs. concentration calibration curve for HSP in standard solution was examined for different concentration levels of 2, 4, 6, 8, 14, 20, 26, 32, 38, and 40µg/ml. The mean ± standard deviation for a slope, intercept as well as correlation coefficient was identified for standard curves.

Analysis of pharmaceutical & Ayurvedic formulations.

Assay of HSP with dosage form

The proposed approach has been applied on commercially accessible Hesperidin tablets (50mg). 10 tablets were taken and crushed into fine powder. 10mg equivalent HSP tablets powder weighed & then dissolved with ethanol in 100ml volumetric flask up to mark with ethanol. Kept 15min for sonication, filtered after sonication, further dilution was prepared to 20 µg/ml. To get the desired concentration among the linearity range, this solution was further diluted, and the same procedure was employed with respect to standard. The blue-coloured chromogen's absorbance was analysed at a specified wave length against to corresponding blank reagent. The drug content was then determined, and its results obtained are statistically validated. Percent assay was calculated using y = mx+c and was found to be 99.8%.

Assay of ayurvedic formulation madiphal rasayana

Madiphal Rasayana is a natural ayurvedic herbal tonic high in vitamin C that helps digestive system, relieves nausea and acidity, and is optimal for those with chronic digestive disorders or morning sickness while on pregnancy. It consists of sendha namak, which relieves cramping in muscles and controls sodium levels. It eases morning sickness, constipation, gastric discomfort, and hyperacidity.

Measured volume of 2ml of madhiphal rasayana sample was transferred in 10ml of volumetric flask and to it 0.2% w/v Gibb’s Reagent (1ml) was added, after 2 minutes 1ml of 0.01M Sodium Hydroxide solution (pH 9 adjusted with Dilute HCL) added then final volume was made to 10ml with distilled water. Absorbance of solution was measured at a specified wavelength 613nm against water as blank. By applying the formula y = mx+c, the percentage purity of ayurvedic formulation Madiphal rasayana was determined to be 99.35% using the proposed approach. The amount of hesperidin found in ayurvedic formulation Madhiphal Rasayana was 45 µg/ml.

Isolation and Quantification of Hesperidin from Orange Peel

A 250 ml round-bottom flask is filled with 200 ml of petroleum ether (40–60°C). A small amount of glass wool is placed over extraction sleeve of the Soxhlet extraction and loaded with 55g of dried and powdered orange peel. The Soxhlet extractor unit is equipped with a reflux condenser, and the reaction mixture has been heated under strong reflux for 2 hours. petroleum ether extract was discarded. The contents of the extraction sleeve are spread out in a large crystallization dish for removal of adherent petroleum ether. In next step, the material is inserted into an extraction sleeve once more and extracted using 200 ml of methanol, until solvent leave the sleeve colourless till one hour. Hesperidin glycoside is extracted using

methanol, while pet ether eliminates essential oils from peels. 0.01g of HSP was taken and dissolved in 1mg/ml stock solution of ethanol. From 1000µg/ml prepared 10µg/ml to it 0.2% w/v Gibb's Reagent 1ml was added, after 2 minutes 1ml of 0.01M Sodium Hydroxide solution (pH 9 adjusted with Dilute HCL) added then final volume was made to 10ml with distilled water. By applying the formula y = mx+c & the amount of hesperidin found in orange peel extraction was 2.4 µg/ml.

Optimization of experimental conditions

A number of experiments was performed to accomplish conditions required for producing blue colour complex with maximum, well-defined intensity. From number of trials stability was not persistent. Final optimized trial was conducted then colour complex was produced with good intensity and was stable [16-19].

Optimized trial

Taken 0.4 ml of standard solution hesperidin in10ml volumetric flask. 1ml of 0.2% (w/v) Gibbs reagent was added after 2minutes added 1ml 0.01M sodium hydroxide solution pH 9 adjusted with Dilute HCL and after 5 min bluish colour was developed and make up with distilled water. Persistent blue colour complex was produced with good intensity and it was stable up to 60min. The Resultant solution was scanned against reagent blank. After the four trials the fourth trial is the optimized trial wavelength is 613nm. The optimized Solution is 0.01M Sodium Hydroxide Solution (pH 9 adjusted with Dilute HCL). Finally, the method is optimized after four trails. The colour is optimized and it was Persistent Bluish colour [20].

Determination of (λmax) wavelength of colour product

Formation of bluish-coloured product, which results from reaction of hesperidin (40 ppm) coupled with 2,6-Dichloroquinone 4-chloro imide (0.2% w/v) in presence of 0.01M sodium hydroxide solution (pH 9 adjusted with diluted HCL), depends on the combination and order of chemicals modifications. This formed blue product was scanned between 500-670 nm in order to measure absorbance. The scanning showed that an absolute maximum absorbance at 613 nm has been obtained against to distilled water as reagent .Therefore, λmax 613nm was utilized in all subsequent experiments and validation parameters [21-25].

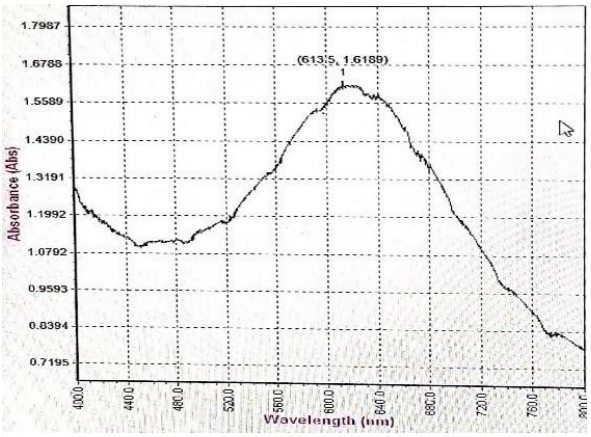

Figure 3. Determination of λmax of Hesperidin pure drug.

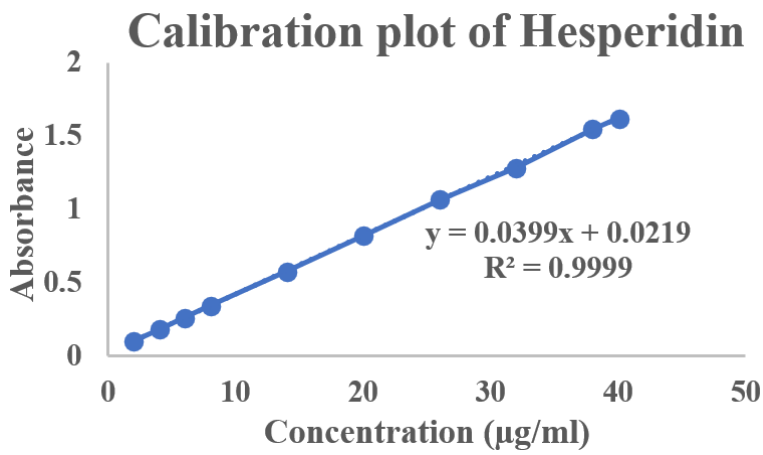

Figure 4. Linearity of Hesperidin using Gibbs reagent.

Colour Stability

Colour Stability with Respect to Time

NaOH solution at pH 9: This provides a moderately alkaline environment that might be suitable for the reaction without causing significant degradation of hesperidin. Colour stability remains constant up to 1 hour and after 60min the colour stability decreases. High pH can lead to enhanced reactivity but may also introduce instability. [26].

Colour Stability with Respect to pH

Reaction conditions at Sodium Hydroxide pH 14

At pH 14, the highly alkaline environment may cause rapid degradation of reactants and the coloured complex themselves, leading to a loss of colour or a change in colour intensity. The reaction between hesperidin and the Gibbs reagent at pH 14 with NaOH is likely to produce colour, but the stability of this colour over time can be compromised due to the highly alkaline conditions. To mitigate these effects, it is important to minimize exposure to light, oxygen, and high temperatures, and to conduct measurements promptly after reaction.

Reaction conditions at Ammonia buffer pH 10

This slightly higher pH might enhance the reactivity with Gibbs reagent but could potentially risk greater degradation of hesperidin. The reaction of hesperidin with Gibbs reagent in an ammonia buffer at pH 10 typically results in the formation of stable coloured complex. This colour is due to interaction between the phenolic groups in hesperidin and Gibbs reagent in alkaline environment provided by the buffer. The exact shade and intensity of the colour can be influenced by factors such as concentration, reaction time, and environmental conditions. NaOH solution at pH 9 would likely be better choice, as it provides a sufficiently alkaline environment to facilitate reaction while minimizing risk of degrading hesperidin [27-29].

UV Method Validation Parameters

Following ICH guidelines, the Q2R1 method had been developed and validated for evaluating the overall reliability of quantitative analytical method in considering a variety of

parameters, which comprises linearity, precision, accuracy & sensitivity. Table 1 provides a summary of the analytical data from calibration curves, which involve standard deviations to slope as well as intercept, and system suitability parameters. The developed method indicates high sensitivity [30].

Table 1. Validation parameters of proposed method

Parameters	**Hesperidin values**
Absorption Wavelength (nm)	613nm
Beers law range (µg/ml)	2 - 40 µg/ml
Limit of Detection (µg/ml)	0.21318 µg/ml
Limit of Quantification (µg/ml)	0.64601µg/ml
Correlation coefficient (r^2)	0.9999
Slope (m)	0.0399
Intercept (c)	0.0219
Regression equation (y)	Y = 0.0399x + 0.0219

3. Results and Discussion

Linearity

HSP calibration curves has been plotted from calibration data under performance of optimal experimental reaction conditions. For HSP, linearity was examined in pure solution through concentration ranges of 2–40µg/ml. For each of the standard curve slope, intercept, and correlation coefficient, mean ± standard deviation was determined [31-35]. Developed method follows according to Beer's law & concentration ranging from 2-40 µg/ml. Concentration vs absorbance was plotted with linear regression equation Y = 0.0399x + 0.0219 & correlation value 0.999 for HSP. Correlation coefficient (r2) was found to be 0.9999 and it was found to be within the limits.

Precision

Repeatability, intermediate precision, and reproducibility are the three levels at which precision may be evaluated. Precision assessed based on its intra- and inter-day precision. The samples containing 20µg/ml of HSP were analysed six times upon same day that is (intra-day precision) & 2 consecutive days was (inter-day precision) [36, 37].

After analysis, %RSD, or relative standard deviation had been determined to be within acceptable limits. The results showing proposed approach was precise. For repeatability standard deviation was found to be 0.0025775957. %RSD was found to be 0.5967%. % RSD was found to be within limits less than 2 as per ICH guidelines as shown in Table.

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \qquad \boxed{\%\,\mathrm{RSD} = \left(\frac{\mathrm{SD}}{\mathrm{mean}}\right) \times 100}$$

Accuracy

Through a recovery studies, proposed methodology was established and validated. Three differentiated concentration ranges of pure HSP were added to the drug's commercial form (Tablets) in order to analyse recovery using the standard addition method. The suggested method offers good accuracy, as evidenced by the recovery percentage value, which ranges

from 98.03% to 99.46%. Accuracy was done typically by testing for presence of sample that has been spiked with a suitable standard drug concentrations that yields accurate results (50%, 100% & 150%). Using triplicates, the mean percentage recovery has been calculated [38-40]. Table 3 represents the data relating to the spiked studies for accuracy. % Recovery for Accuracy was found to be within limits 98-99%.

Table 2. Repeatability, Intraday and Interday precision data of 20µg/ml Hesperidin drug solution.

Concentration(µg/ml)	**Repeatability Absorbance**	**Intraday (Morning) Absorbance-1**	**Intraday (Evening) Absorbance-2**	**Interday Day-1 Absorbance**	**Interday Day-2 Absorbance**
20 µg/ml	0.8199	0.8192	0.8163	0.8196	0.8164
20 µg/ml	0.8154	0.8199	0.8143	0.8146	0.8123
20 µg/ml	0.8164	0.8123	0.8263	0.8192	0.8042
20 µg/ml	0.8215	0.8146	0.8243	0.8162	0.8056
20 µg/ml	0.8165	0.8196	0.8196	0.8199	0.8123
20 µg/ml	0.8153	0.8184	0.8159	0.8162	0.8146
Mean	0.8175	0.8173	0.8194	0.8176	0.8109
Standard Deviation	0.0025775957	0.00313560	0.004889376	0.002225758	0.005262698
%RSD	0.3153022262	0.38365373	0.596702001	0.272230710	0.648994811

Table 3. Recovery studies data of Method developed indicates triplicates.

Percentage Level	**Sample Absorbance**	**Spiking Absorbance**	**Total Absorbance**	**% Recovery**	**Mean % Recovery**
50% (20ppm +2ppm	0.8185	0.1017	0.9189	98.72%	98.72%
			0.9196	99.41%	
			0.9182	98.03%	
100% (20ppm +4ppm)	0.8185	0.1815	0.9984	99.11%	99.14%
			0.9976	98.67%	
			0.9994	99.66%	
150% (20ppm + 6ppm)	0.8185	0.2613	1.0764	98.69%	99.12%
			1.0784	99.46%	
			1.0778	99.23%	

Robustness and Ruggedness

The method developed was also validated with the robustness parameter. A small but deliberate change in the method developed is assessed. Here the 20µg/ml solution of the standard drug was scanned at + & - 1 nm of the λmax 613nm. Standard deviation from robustness data & Relative standard deviation were estimated. %RSD was found to be less than 2 as per ICH guidelines. Table 4 shows that developed approach was robust, with % RSD values of 0.7275% and 0.4925% at 612nm and 614nm, respectively [41-43]. Ruggedness was assessed by utilizing the same established procedure for analyzing 20µg/ml of HSP utilizing the same instrument by two distinct individual analysts on separate days under the exact same conditions. It demonstrates the method's reliability and consistency under different conditions, ensuring that minor changes do not significantly impact the accuracy, precision, or overall performance of the analytical procedure [44]. Ruggedness of HSP drug solution with % RSD values of 0.5007% and 0.4612% by two different analysts.

Table 4. Robustness data of 20µg/ml Hesperidin drug solution.

Concentration (µg/ml)	**Absorbance at 612nm (-1nm)**	**Absorbance at 613nm**	**Absorbance at 614nm (+1nm)**
20	0.7846	0.8199	0.8212
20	0.7865	0.8154	0.8182

20	0.7892	0.8164	0.8176
20	0.7998	0.8215	0.8246
20	0.7843	0.8165	0.8198
20	0.7885	0.8153	0.8185
Mean	0.7888	0.8175	0.8199
SD	0.00573898945	0.0025775957	0.0040383164
%RSD	0.7275595144	0.3153022262	0.4925376753

Detection limit and the Quantitation limit

The lowest concentrations of analytes that may be detected and quantified are known as LOD and LOQ, and they have the signal-to-noise ratios at 3:1 & 10:1, respectively. Detection limit and the quantitation limit of the method developed were calculated from the calibration standards. Detection limit value 0.21318 µg/ml & Quantitation limit value 0.64601µg/ml, demonstrating the established UV Visible spectrophotometric approach was sensitive in determining the concentration of hesperidin [45,46].

$DL = \frac{3.3\,\sigma}{S}$ & $QL = \frac{10\,\sigma}{S}$

Where; σ = Standard deviation of response,

S = Slope value obtained from y = mx+c equation.

Bioanalytical Method Development

Bioanalytical method was performed using Protein precipitation extraction procedure [47]. Plasma samples were taken out of the refrigerator on the day of analysis, and allowed to thaw at room temperature. 1 ml of human plasma was added into the centrifugation tube along with 1ml of a standard 20 ppm hesperidin solution for spiking. Then mixed with 5ml of methanol to precipitate proteins. Centrifugation was carried out for 15 minutes at 1000 rpm after the mixture had been vortexed for 2 minutes. After that, 5ml of the clear supernatant liquid was quantitatively transferred to 10ml volumetric flask then 1ml of Gibbs reagent and 1ml of 0.01 ml sodium hydroxide solution (pH 9 adjusted with diluted HCL) were added to the supernatant liquid, and final volume was adjusted to 10ml using distilled water. At specified wavelength of 613 nm, the absorbance of solution was measured against water as blank. % Recovery of the Bioanalytical method of hesperidin content in human plasma was found to be 98.91%.

4. Conclusion

A simple, rapid and highly sensitive chromogen approach has been developed for quantification of hesperidin. Validated method is extended to bio samples, pharmaceutical formulations and different Ayurvedic formulations, Plant extracts to Quantify Hesperidin. The Chromogenic method is practical and affordable. By employing Gibbs reagent, precise measurements are achieved, ensuring accurate determination of hesperidin content in both raw material and formulated products. Method validation confirms the accuracy, precision, specificity, and sensitivity of the analytical procedure, establishing its suitability for routine analysis and regular quality control examination in the bulk, pharmaceutical, ayurvedic formulation (Madiphal Rasayana) and herbal industries.

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CONFLICTS OF INTEREST

The authors have no financial or other conflicts of interest to disclose.

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