

ICH M10-Based Validation of a Novel Chromogenic Bioanalytical Method for Quantifying Hesperidin in Biological Samples

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ABSTRACT

Aim: A Chromogenic bioanalytical approach had been developed as well validated for the assessment of specific compound hesperidin in accordance with the ICH M10 guidelines. 2,6-Dichloro quinone 4-chloro imide chromogenic reagent was used to develop the chromogenic method in the visible region. **Materials and Methods:** When the phenolic group in hesperidin reacts with Gibb's reagent at alkaline medium NaOH solution pH 9, a nucleophilic aromatic substitution reaction takes place, resulting in a blue-coloured product. For this, an ELICO SL-210 UV-visible spectrophotometer was used. A protein precipitation technique using methanol as a precipitating agent was utilized for extracting the compound from human blood plasma. The performance of the method was evaluated by calculating a number of validation parameters including matrix effect, calibration curve, accuracy, precision, Re-injection reproducibility and benchtop stability studies. **Results:** Both Chromogenic Visible region and UV region bioanalytical methods for hesperidin were validated with $r^2 > 0.999$ within the range of concentrations 5-35 $\mu\text{g}/\text{mL}$ at 613 nm and 20-140 $\mu\text{g}/\text{mL}$ at 285 nm was linearly well-demonstrated for both methods, within and between-run precisions were found to be in limits $< 15\%$. These analytes had extraction recoveries of 98.91% and 98.14%. **Conclusion:** Results showed that the approach was exact, accurate, and is convenient to use for therapeutic drug monitoring and pharmacokinetic studies because it is very quick and accurate in determining the amount of hesperidin in human plasma.

Keywords: 2,6-Dichloro quinone 4-chloro imide, M10 ICH guidelines, Method validation, Protein Precipitation.

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INTRODUCTION

Hesperidin occurs naturally as bioflavonoid glycoside mostly present in citrus fruits such as oranges and lemons. In 2019, the USFDA approved it. It is a class of flavonoids, known for their antioxidant and health-promoting properties. Structurally, hesperidin consists of a flavanone backbone with a rutinose sugar moiety attached, contributing to its distinct chemical properties. It has antioxidants, anti-inflammatory, vasoprotective, neuroprotective, and anticancer mechanisms.¹ It works by modulating various biochemical pathways such as NF- κ B, oxidative stress, blood pressure regulation, and inflammation. These mechanisms contribute to their therapeutic potential in cardiovascular, metabolic, neurological, and inflammatory disorders. Hesperidin exhibits better solubility within organic solvents such as ethanol, methanol, as well as dimethyl sulfoxide,

commonly used for its extraction and preparation. HSP has low solubility in water. Hesperidin stability during sample storage and analysis must be carefully assessed to ensure data reliability. Biological samples often contain complex components that necessitate specific extraction and cleanup procedures. Protein precipitation using methanol is a commonly used technique for the extraction and isolation of hesperidin from biological samples, particularly plasma and serum. This method efficiently removes proteins that interfere with analytical measurements.²⁻⁸

General mechanism of 2,6-dichloroquinone4-chloroimide reagent with Hesperidin

In the reaction between hesperidin and 2,6-dichloroquinone-4-chloroimide under basic conditions (NaOH pH 9 diluted with HCl), the mechanism proceeds as Deprotonation of the phenolic group on hesperidin to form a phenolate ion (O^-). The phenolate ion nucleophilically attacks the electrophilic carbonyl group of the quinone. This forms a tetrahedral intermediate, which is resonance stabilized. Oxidation leads to the formation of a conjugated charge-transfer complex. The extended conjugation



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absorbs visible light, producing a blue colour.⁹⁻¹¹ General mechanism was illustrated in Figure 1.

MATERIALS AND METHODS

Instruments

Double beam Ultraviolet-Visible spectrophotometer, make: ELICO, model: SL 210, Photo Diode Array Detector, Glass cuvette, The output signal was monitored in spectra treats software, Weighing balance, pH meter.^{12,13}

Chemicals

All the reagents as well chemicals utilized were analytical grade and were prepared freshly. Hesperidin API was gifted by Laurel pharma Gibb's Reagent, Ethanol, Dilute HCl, Sodium Hydroxide, Distilled water, plasma was required.

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

10 mg of HSP pure drug was weighed in 10 mL of vol. flask and dissolved, filled up to mark using ethanol resultant solution is 1000 µg/mL. From 1000 µg/mL, pipetted out 1 mL in 10 mL of volumetric flask and make up with ethanol resultant solution is 100 µg/mL. From 100 µg/mL serial dilutions were prepared.

Preparation of Reagents

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

Weighed 0.2 g of Gibbs Reagent in 100 mL vol. flask and filled up to mark using ethanol.

Preparation of pH 9 Sodium Hydroxide Solution 0.01 M

Weighed 0.04 g of Sodium Hydroxide transferred into 100 mL volume flask and filled up to mark using distilled water and adjust to pH 9 with dilute HCl.

Preparation of Blank plasma

1 mL of plasma was taken in centrifugation tube to it added 5 mL methanol and vortexed for 3 min after centrifugation was carried out at 1000 rpm for 30 min. Two layers that get separated supernatant liquid was collected in 10 mL volumetric flask to it 1 mL of Gibbs reagent and 1 mL of 0.01 M sodium hydroxide solution (pH 9 adjusted with diluted HCl) were added to the supernatant liquid and filled up to mark using distilled water for chromogenic method at 613 nm. For 285 nm UV region bioanalytical method blank plasma was extracted in same way without adding chromogenic reagents.

Procedure for plasma extraction

Extraction procedure for chromogenic method and UV spectroscopy method

The bioanalytical method was performed using Protein precipitation extraction procedure. Plasma samples were taken out of the refrigerator on the day of analysis and allowed to thaw at room temperature. Two centrifugation tubes contain 1 mL of the plasma extracted from human blood. 0.2 mL and 0.5 mL stock solutions were added from 1000 ppm stock solution in two different centrifugation tubes. The mixture was vortexed for 2 min. 5 mL of methanol as extracting solvent was then added. The mixture was vortexed for 3-4 min once more. Ultimately, Centrifuging the mixture for 30 min at 1000 rpm, as extracted sample was separated, 5 mL of the clear supernatant liquid was quantitatively transferred into 10 mL volumetric flask then 1 mL of Gibbs reagent and 1 mL of 0.01 M sodium hydroxide solution (pH 9 adjusted with diluted HCl) were added to the supernatant liquid, and then distilled water was added to the final volume 10 mL. At specified wavelength of 613 nm the solution's absorbance has been measured against corresponding reagent blank. Other 5 mL clear supernatant solvent was withdrawn into another 10 mL vol. flask and filled to mark using distilled water and scanned in 200-400 nm against blank plasma. At specified wavelength 285 nm maximum absorption was shown. % Recovery of hesperidin content in human plasma by chromogenic bioanalytical method at 613 nm and UV region method at 285 nm was found to be 98.91% and 98.14%.¹⁴⁻¹⁹

Procedure for preparation of LLOQ, LQC, MQC, HQC samples for chromogenic bioanalytical method

Taken 1 mL of plasma in four different centrifugation tubes then added 0.5 mL, 1.5 mL, 2.5 mL, 3.5 mL from 100 ppm standard stock solution in 4 centrifugation tubes then resultant solutions will be 5 ppm, 15 ppm, 25 ppm, 35 ppm. Then added 5 mL of methanol in four concentrations to precipitate proteins. Centrifugation was carried out for 30 min at 1000 rpm after the mixture had been vortexed for 2 min. After that, 5 mL of the clear supernatant liquid was quantitatively transferred to 10 mL volumetric flasks then 1 mL of Gibbs reagent and 1 mL of 0.01 mL sodium hydroxide solution (pH 9 adjusted with diluted HCl) were added to the supernatant liquid, and volume was then adjusted by adding distilled water to 10 mL. At specified wavelength of 613 nm solution's absorbance has been measured against corresponding reagent blank.

Preparation of LLOQ 20 ppm, LQC 60 ppm, MQC 100 ppm, HQC 140 ppm for UV bioanalytical method

Taken 1 mL of plasma in four different centrifugation tubes to it added 0.2 mL, 0.6 mL, 1 mL, 1.4 mL from 1000 ppm stock solution and added 5 mL of methanol, vortexed for 5 min and kept for centrifugation at 1000 rpm for 30 min. Two layers were

separated supernatant liquid was collected in 10 mL vol. flask and adjusted to mark using distilled water. The resulting solutions will be 20 ppm, 60 ppm, 100 ppm, 140 ppm.

Validation parameters

Selectivity

The ability of analytical techniques to distinguish and quantify an analytical compound in the existence of possibility of substances that interfere in a biological matrix that is blank is known as selectivity. Both the lipaemic and hemolyzed matrix ($n \geq 1$ source each) and the blank matrix consisting of ($n \geq 6$ sources) should show no discernible response or interference within the analyte and internal standard.²⁰⁻²⁴

Limits: As per ICH M10 guidelines limits for each matrix, the percentage of responses related to interfering components shouldn't exceed 20% of analytical substance response within the LLOQ and 5% of the IS response within the LLOQ sample.

Specificity

A bioanalytical technique's specificity is its capacity to identify and then distinguish a specific analyte from all other compounds, that includes related chemical substances.^{25,26}

Limits: As per ICH M10 guidelines limits must not exceed 20% of the analytical response within LLOQ and 5 per cent of IS response within LLOQ sample.

Matrix Effect

Changes in analytical substance response resulted on by interfering compounds and frequently unidentified substances within the sample matrix are known as matrix effects.^{27,28}

Limits: As per ICH M10 guidelines precision (percent coefficient of variation, or CV) shouldn't exceed 15%, and accuracy must fall within $\pm 15\%$ of nominal measurement.

Calibration curve and range

The relationship between an analytical platform's response to analyte as well as nominal concentrations of analyte is demonstrated. Lowest value of prepared standards represents LLOQ, and highest value of prepared standards in calibration defines the bioanalytical calibration range.

Samples of blank, zero samples in addition to at least six prepared standards for calibration, which include LLOQ as well as ULOQ, are all presented in a calibration curve.

Limits: As per ICH M10 guidelines each calibration standard's back-calculated concentrations should be accurate to within $\pm 20\%$ of nominal measurement concentration within LLOQ and within $\pm 15\%$ at all other levels.

Accuracy and Precision

Analysis of quality control samples in both differentiated runs that is (between-run) along with (within-run) refers to each single run should be used to determine accuracy as well precision. Both accuracies as well precision are analyzed utilizing similar runs as well as data.

QCs over accuracy parameter along with precision sample runs have been prepared at least four different levels of concentration for the parameter calibrating curve to be generated during method validation: its LLOQ, among three times the Lower limit quantification for (low analytical QC), between 30% as well 50% from the range of calibrating values that is (for medium analytical QC), and at least 75 percent for the ULOQ (for highest analysing QC) samples.^{29,30}

Limits: As per ICH M10 guidelines except for LLOQ that do not exceed 20%, accuracy and precision should be within 15%.

Reinjection Reproducibility

If test samples were reinjected It should be analysed to determine viability for samples that were previously processed as well as promoting the storage before being reinjected.^{31,32}

Limits: As per ICH M10 guidelines reinjection reproducibility % CV should be within 15%.

Stability

Analyte stability in the matrix was determined. It is necessary to assess the following stability tests: bench-top (short-term) as well as long-term and freeze-thaw stability.

Bench-top stabilities within matrix

Experiments on benchtop stability of the matrix can be planned, carried out to account for conditions of handling research samples in the lab. Both low as well high-quality control samples had been thawed similarly about research study samples and maintained on the benchtop for the same period of time and at similar temperature for 8 hr and were determined in triplicates by measuring absorbances.³³⁻³⁹

Limits: As per ICH M10 guidelines the mean concentrations for each Quality control sample level must be within $\pm 15\%$ of nominal concentration.

RESULTS AND DISCUSSION

Bioanalytical Method Validation as Per ICH M10 Guidelines Using 2,6-Dichloro Quinone 4-Chloroimide.

Validation parameters

The following validation parameters are covered in accordance with M10 ICH guidelines:

Table 1: Matrix effect, With in run precision, Reinjection reproducibility data of QC samples at 613nm by chromogenic bioanalytical method.

Matrix effect of 15, 35 µg/mL HSP				
QC Concentration Levels	Matrices	Mean	S D	%CV
LQC 15 µg/mL	Matrix 1	0.659233333	0.075943268	11.51993755
	Matrix 2	0.6612	0.090156115	13.63522604
	Matrix 3	0.658766667	0.089378297	13.56751968
HQC 35 µg/mL	Matrix 1	1.662233333	0.235064126	14.14146385
	Matrix 2	1.6458	0.216021828	13.1256427
	Matrix 3	1.6087	0.178452711	11.09297637
With in run precision of concentrations 5, 15, 25, 35 µg/mL				
QC Levels	Concentration	Average	S D	%CV
LLOQ	5 µg/mL	0.24634	0.045778903	18.583625
LQC	15 µg/mL	0.61208	0.075854446	12.39289736
MQC	25 µg/mL	1.14196	0.123427217	10.80836609
HQC	35 µg/mL	1.44416	0.176224255	12.20254368
Reinjection reproducibility of 15, 25, 35 QC samples				
LQC	15 µg/mL	0.67306	0.076764627	11.4053171
MQC	25 µg/mL	1.09926	0.116811699	10.62639402
HQC	35 µg/mL	1.44456	0.175782957	12.16861585

Table 2: Calibration concentration ranges at 613 nm and 285 nm.

Calibration concentration ranges for standard chromogenic HSP at 613 nm		
Sl. No.	Concentration (µg/mL)	Absorbance
1	5 µg/mL	0.2021
2	10 µg/mL	0.4185
3	15 µg/mL	0.6158
4	20 µg/mL	0.8110
5	25 µg/mL	1.0145
6	30 µg/mL	1.2125
7	35 µg/mL	1.4108
Calibration concentration ranges for standard HSP at 285 nm		
Sl. No.	Concentration (µg/mL)	Absorbance
1	20	0.1897
2	40	0.3582
3	60	0.5324
4	80	0.7195
5	100	0.8991
6	120	1.0788
7	140	1.2578

Specificity

Procedure: The blank scanning was carried out in visible region from 400 to 800 nm.

Matrix Effect

Procedure: To assess the matrix effect, at least three replicates of both low QC samples of 15 ppm and High QC samples of 35 ppm. That were each made using 3 different individual matrices, from a minimum of six different sources or lots should be examined by adding chromogenic reagents and scanned against reagent blank at 613nm in visible region. Standard deviation and %CV were calculated for 15 ppm and 35 ppm as tabulated in Table 1. % CV for matrix effect at LQC was found to be 11.51%, 13.63%, 13.56% and HQC was found to be 14.14%, 13.12%, 11.09% for matrices 1, 2, 3.

Calibration curve and Range

Procedure for preparation of calibration standards

The concentration range that is used to establish the calibration curve is 5 ppm to 35 ppm. From 100 µg/mL standard solution (prepared from human plasma) a series of dilutions were prepared by adding methanol as precipitating agent and 1 mL of Gibbs reagent and 1 mL of 0.01M Sodium Hydroxide solution (pH 9 adjusted with Dilute HCl) added, For every concentration, absorbances were measured in visible region spectrophotometer at the maximum wavelength 613 nm against reagent blank using chromogenic reagents and plotting of calibration graph involved taking absorbance and concentration within the X and Y axes

and then results obtained were tabulated as shown in Table 2 and Figure 2.

Accuracy and Precision

Procedure for within-run accuracy and precision

At least 5 sample replicates should be analysed at each sample of QC concentration range for each analytical run to determine within-run bioanalysis accuracy and precision. LLOQ 5 ppm, LOQ 15 ppm, MQC 25 ppm, HQC 35 ppm were prepared in individual 3 sets extraction procedures were carried out and reagents were added and scanned against reagent blank at 613nm. Absorbance was measured, Standard deviation and % CV were calculated as tabulated in Table 1. % CV was found to be within limits for LLQC, LQC, MQC, HQC samples.

Procedure for running accuracy and precision

QC samples of each concentration level must be analysed over a minimum of 2 consecutive days in a minimal of 3 analytic runs to assess between-run accuracy along precision. LLOQ 5 ppm, LQC 15 ppm, MQC 25 ppm, HQC 35 ppm were prepared in individual 3 sets and scanned against reagent blank at 613 nm over 2 days. Average, standard deviation and %CV were calculated as

tabulated in Table 3. % CV for samples was found to be within limits.

Reinjection reproducibility

Procedure

The evaluation of reproducibility of reinjection involves injecting a run consisting of standard calibrations and at least five replicates containing 15 ppm low QC, 25 ppm middle QC and 35 ppm high QCs were prepared and scanned against reagent blank at 613 nm. For these three levels standard deviation and percent coefficient of variance was calculated as tabulated in Table 1. % CV for the LQC, MQC, and HQC samples has been found to be 11.40%, 10.62%, 12.16%.

Stability

Bench-top stabilities within matrix

Procedure: 15 ppm LQC and 35 ppm HQC samples were prepared in three replicates by following extraction procedure and to it added chromogen reagents and scanned against reagent blank at 613 nm. Analyses at time intervals starting from 0, 2 hr as well 4 hr and 6 hr, up to 8 hr. For these standard deviation and percent coefficient of variance was calculated as tabulated in

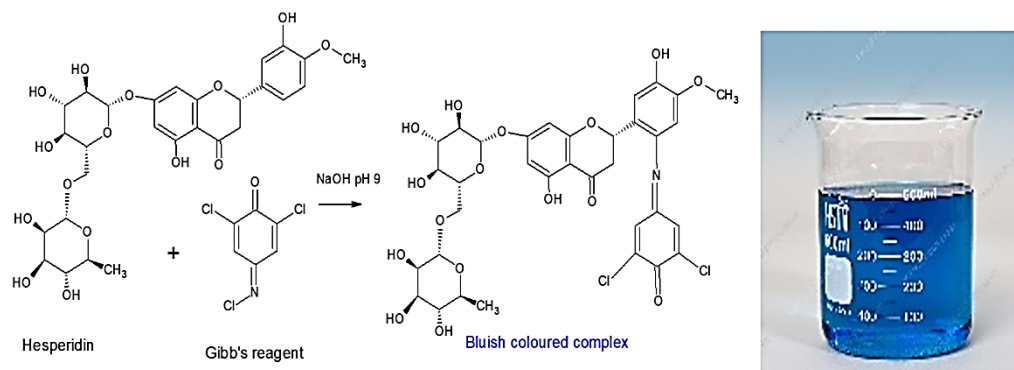


Figure 1: Reaction of Gibbs Reagent with Hesperidin.

Table 3: Between run precision of concentrations over two days at 613 nm and 285 nm.

Between run precision of concentrations over two days at 613 nm							
QC Levels	Concentration (µg/mL)	Average		S D		%CV	
		Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
LLOQ	5 µg/mL	0.244866	0.2238666	0.0425495	0.0380769	17.37661	17.008758
LQC	15 µg/mL	0.6862	0.6630	0.0745488	0.0860983	10.86401	12.986171
MQC	25 µg/mL	1.191533333	1.1156	0.1596289	0.1656788	13.39693	14.851095
HQC	35 µg/mL	1.4632	1.41633333	0.17093378	0.1930124	11.68218	13.627617
Between run precision of concentrations over two days at 285nm							
LLOQ	20 µg/mL	0.1397	0.15536666	0.0266	0.0291220	19.04080	18.744073
LQC	60 µg/mL	0.580766667	0.5722	0.06065816	0.0584941	10.44449	10.222682
MQC	100 µg/mL	0.834766667	0.82476666	0.09496153	0.0950425	11.37581	11.523566
HQC	140 µg/mL	1.247133	1.2328	0.13842446	0.1332328	11.09941	10.807339

Table 4: Bench top stability studies of LQC and HQC samples at 613 nm and 285 nm.

Bench top stability studies of LQC 15 µg/mL and HQC 35 µg/mL samples at 613nm				
QC Concentration Levels	Duration	Mean	S D	%CV
LQC 15 µg/mL	0 hr	0.6559	0.0688722	10.50041172
	2 hr	0.6457	0.070376985	10.89933171
	4 hr	0.6192	0.064486975	10.41456322
	6 hr	0.547166667	0.057806689	10.56473153
	8 hr	0.504166667	0.06386551	12.66753912
HQC 35 µg/mL	0 hr	1.662233333	0.235064126	14.14146385
	2 hr	1.555933333	0.170664154	10.96860322
	4 hr	1.3062	0.157127591	12.02936698
	6 hr	1.1173	0.114106442	10.21269503
	8 hr	0.894333333	0.124569833	13.92879238
Bench top stability studies of LQC 60 µg/mL and HQC 140 µg/mL samples at 285 nm				
QC Concentration Levels	Duration	Mean	Standard Deviation	%CV
LQC 60 µg/mL	0 hr	0.539333333	0.056922096	10.55415869
	2 hr	0.522335667	0.056922096	10.89760848
	4 hr	0.445866667	0.052962298	11.87850581
	6 hr	0.340633333	0.043274935	12.70425727
	8 hr	0.241933333	0.03301641	13.64690413
HQC 140 µg/mL	0 hr	1.250366667	0.136550223	10.92081438
	2 hr	1.191666667	0.159832954	13.41255562
	4 hr	1.072866667	0.121829444	11.35550646
	6 hr	1.030633333	0.150972889	14.64855488
	8 hr	0.904333333	0.109738978	12.13479302

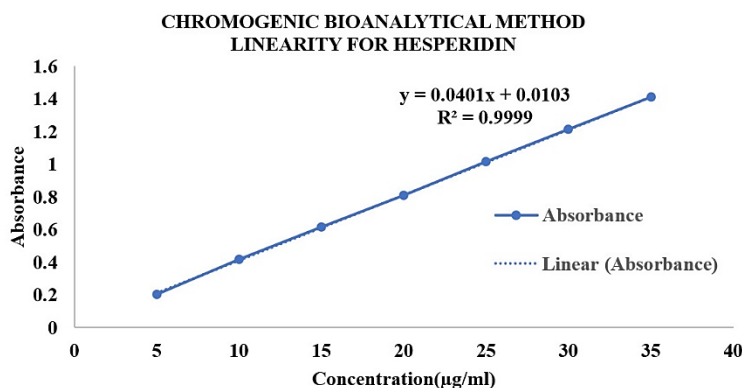
**Figure 2:** Calibration plot of chromogenic bioanalytical method.

Table 4. % CV at LQC 15 µg/mL and HQC 35 µg/mL was found to be within limits from 0-8 hr indicates stability of HSP.

Bioanalytical Method Validation by UV Spectrophotometer as Per ICH M10 Guidelines

Selectivity

Procedure: At 285 nm selective HSP peak shown absorbance.

Specificity

Procedure: The blank scanning was carried out in visible regions from 200 to 400 nm.

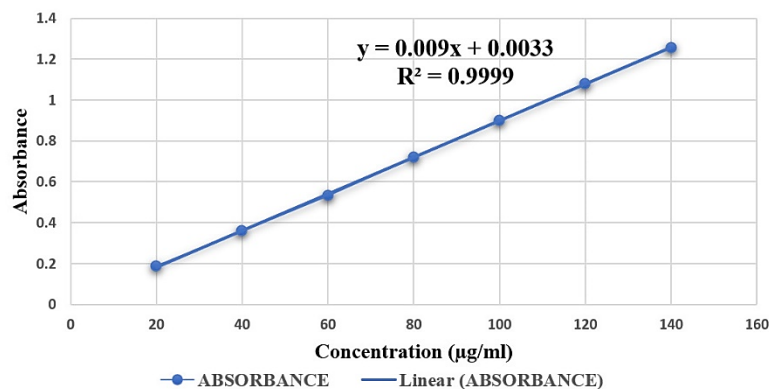
Matrix effect

Procedure: To assess the matrix effect, at least 3 different replicates of both LQC samples of 60 ppm along with HQC samples of 140 ppm that were prepared as 3 individual sets of matrices from a

Table 5: Matrix effect, with in run precision, Reinjection reproducibility data of QC samples at 285 nm by UV region bioanalytical method.

Matrix effect of 60 µg/mL and 140 µg/mL HSP				
QC Concentration Levels	Matrices	Mean	S D	%CV
LQC 60 µg/mL	Matrix 1	0.580766667	0.059962655	10.32474115
	Matrix 2	0.582666667	0.061164737	10.49738042
	Matrix 3	0.570266667	0.063498189	11.13482387
HQC 140 µg/mL	Matrix 1	1.246833333	0.138228808	11.08639015
	Matrix 2	1.245833333	0.138430933	11.11151305
	Matrix 3	1.242833333	0.14179035	11.40863752
Within run precision of concentrations 20, 60, 100, 140 µg/mL				
QC Levels	Concentration	Average	S D	%CV
LLOQ	20 µg/mL	0.19346	0.033241661	17.18270475
LQC	60 µg/mL	0.5539	0.056656288	10.22861315
MQC	100 µg/mL	0.80388	0.08467055	10.53273496
HQC	140 µg/mL	1.22488	0.141385402	11.54279622
Reinjection reproducibility of 60, 100, 140 QC samples				
QC Levels	Concentration	Average	S D	%CV
LQC	60 µg/mL	0.55378	0.056396073	10.18384062
MQC	100 µg/mL	0.8051	0.083113868	10.32342165
HQC	140 µg/mL	1.23478	0.141657164	11.47225932

BIOANALYTICAL METHOD FOR HESPERIDIN AT 285nm

**Figure 3:** Calibration plot of Hesperidin.

minimum of six different sources or lots and should be examined in UV-visible spectroscopy against the reagent blank at 285 nm. Standard deviation and % CV were calculated as tabulated in Table 5. % CV for matrix effect at LQC sample was found to be 10.32%, 10.49%, 11.13% and HQC sample was found to be 11.08%, 11.11% and 11.40% for matrices 1, 2, 3.

Calibration curve and Range

Procedure for preparation of calibration standards

The concentration range that is used to establish the calibration curve is 20 µg/mL-140 µg/mL. From 1000 µg/mL standard

solution a series of dilutions were prepared by adding methanol as precipitating agent, vortexed for 5 min and kept for centrifugation at 1000 rpm for 30 min. Two layers were separated, supernatant liquid was collected in 10 mL vol. flask and adjusted to mark using distilled water.⁴⁰⁻⁴² For every analytical sample absorbances had been measured at the maximum wavelength (λ_{max}), 285 nm against reagent blank and plotting of calibration graph involved taking absorbance and concentration within the X and Y axes and then results obtained were tabulated as shown in Table 2 and Figure 3.

Accuracy and Precision

Procedure for within run accuracy and precision

Within run precision was determined for 5 replicates at proportions of concentrations that include LLOQ 20 ppm, LQC 60 ppm, MQC 100 ppm, HQC 140 ppm analysed on the same day were prepared in individual 3 sets extraction procedures were carried out and scanned against reagent blank at 285 nm. Absorbance was measured, Standard deviation and % CV was calculated as tabulated in Table 5. % CV for samples of LLQC, LQC, MQC, and HQC have been found to be within acceptable limits.

Procedure for between run accuracy as well precision

Between run precision measurements made over an interval of 2 days for 3 different replicates of the concentrations of plasma QC samples. LLOQ 20 ppm, LQC 60 ppm, MQC 100 ppm, HQC 140 ppm were prepared in 3 replicates and scanned against reagent blank at 285 nm over 2 days. Average, standard deviation and %CV were calculated as tabulated in Table 3. % CV for samples was found to be within limits.

Reinjection Reproducibility

Procedure: The evaluation of reinjection reproducibility involves injecting a run consisting of standard calibrations and at least five replicates containing LQC 60 ppm, MQC 100 ppm, HQC 140 ppm were prepared and scanned against reagent blank at 285 nm for these three levels standard deviation and percent coefficient of variance was calculated as tabulated in Table 5. % CV for the LQC, MQC, and HQC samples has been found to be 10.18%, 10.32%, 11.47%.

Benchtop stability in matrix

Procedure: Both samples low QCs 60 µg/mL and high QCs 140 µg/mL should be thawed similarly to the research study samples and maintained on the benchtop for the same period of time and at similar temperature for 8 hr and was determined in triplicates. LQC 60 ppm, HQC 140 ppm were prepared and scanned against reagent blank at 285 nm. Standard deviation and percent coefficient of variance was calculated and tabulated in Table 4. % CV at LQC 60 µg/mL and HQC 140 µg/mL was found to be within limits from 0-8 hr indicates stability of HSP.

CONCLUSION

A sensitive and quick chromogenic UV visible spectrophotometric bioanalytical method was developed in this study to quantify the amount of hesperidin in human plasma. This approach provides precise and accurate eligible recovery and stability, making them suitable for routine quality control and product standardization. Techniques like HPLC or using LC-MS/MS is possible for higher specificity as well trace-level quantification. This method can be used effectively in the pharmacokinetic investigation. As a result,

we anticipate that these results will contribute to the establishment of a scientific basis along with guidance for proper, safe clinical application of HSP.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

UV: Ultraviolet; **ICH:** International Council on Harmonization; **PPM:** Parts per million; **µg:** Microgram; **LLOQ:** Lower limit of quantification; **ULOQ:** Upper limit of quantification; **CV:** Coefficient of variance.

ETHICAL APPROVAL

The present research work involves human plasma collected from blood banks.

SUMMARY

Validated chromogenic and UV methods were developed for measuring hesperidin in human plasma following ICH M10 guidelines. Method validation included tests for matrix effect, calibration curve linearity, precision, accuracy, reinjection reproducibility, and stability. Linearity was observed between 5-35 µg/mL at 613 nm and 20-140 µg/mL at 285 nm, with $r^2 > 0.999$. Precision values %CV for LLOQ was obtained within 15-20% and LQC, MQC as well HQC samples remained within acceptable limits (<15%), and extraction recoveries were accurate. Hesperidin can be quickly and affordably quantified using chromogenic bioanalytical methods in biological research, dietary supplements, and regular quality assessment and preliminary screening, making a substantial contribution to natural product chemistry research and the manufacturing of products based on hesperidin.

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