



Method development and validation of Bio Flavanoid-Morin Hydrate by RP-HPLC in Human Plasma

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ABSTRACT

Objective: To develop a new, rapid and sensitive RP-HPLC method for the estimation of morin in human plasma by protein precipitation method (PPT) because of more recovery. **Method:** Chromatographic separation was carried out on a reverse phase C₁₈ column using mixture of 10 mM potassium dihydrogen phosphate (pH 5.0) and acetonitrile (60:40, v/v) at a flow rate of 1.0 ml/min with UV detection at 260 nm. The method was validated and found to be linear in the range of 100 -500 ng/ml. **Results:** The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operator has proven that the method is robust and rugged. The present method is suitable in terms of precise, accurate and specific during the study. **Conclusion:** The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments which are complicated, costly and time consuming rather than a simple HPLC–UV method. The present method was successfully applied for bioavailability and bioequivalence studies and pharmacokinetic profile studies.

Key words: Application to PK profile studies, Method development, Morin and validation.

INTRODUCTION

Flavonoids are a large class of compounds consisted of A and C rings of benzo-1-pyran-4-quinone and a B ring, and further sub classified as flavones (basic structure), flavonols (having a hydroxyl group at the 3-position), isoflavones (B ring binds to the 3-position), flavanones (2–3 bond is saturated), and catechins (C-ring is 1-pyran), chalcones

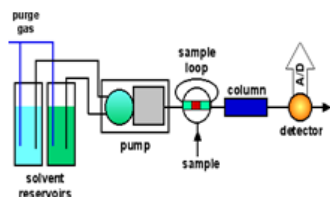
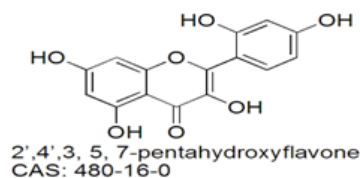
(C-ring is opened), and anthocyanidins (C-ring is 1-pyran, and 1–2 and 3–4 bonds are unsaturated). It has many substituents such as hydroxyl and/or methoxyl groups. Flavones can prevent many diseases including cancers through antioxidative action and/or the modulation of several enzyme functions. Example, they may reduce coronary heart disease mortality¹ by suppressing the oxidation of low-density lipoprotein.² Anticarcinogenic activity of flavones is expressed by their agonism and/or antagonism of carcinogenesis-related receptors such as epidermal growth factor,³ arylhydrocarbon receptor⁴ and estrogen receptor β .⁵ The secretion of cytokines,^{6–8} and expression of protein kinases in tumor cell proliferation^{9,10} could be modulated by polyphenols.

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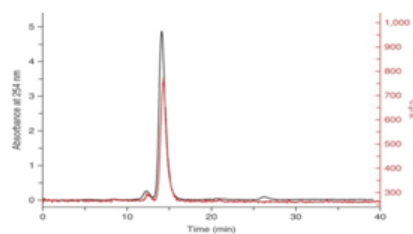
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Mobile phase: 10 mM potassium dihydrogen phosphate (pH 5.0) and acetonitrile (60:40, v/v) at a flow rate of 1.0 ml/min with UV detection at 260 nm. Run time 5 mins.



Graphical Abstract

Morin is typical representative of flavonoids which bears most of their physiological functions. Hence, it is of interest to develop simple, accurate and precise method for the determination of morin in human plasma. So far, only limited analytical methods such as thin-layer chromatography,¹¹ gas chromatography,¹² capillary electrophoresis,¹³⁻¹⁷ electrochemical measurements,¹⁸ high-performance liquid chromatography (HPLC). HPLC method was most commonly used to separate and analyse flavonoids because of its more accurate and precision. As a reference method we used modified RP-HPLC determination of morin based on extraction of morin from human plasma samples with trichloroacetic acid. Literature survey reveals that no simple, rapid and reproducible methods so far reported for the development of morin in human plasma. The objective of the present investigation was to develop a new, rapid and sensitive RP-HPLC method for the estimation of morin in human plasma. In the present study, a more sensitive, and precise method was to be developed and applied to the pharmacokinetic study.

EXPERIMENTAL

Materials and reagents

Morin Hydrate (99.65%) reference standard was procured from Sigma Aldrich Pvt Ltd. (Mumbai, India). Acetonitrile

(HPLC grade) was obtained from Merck, Germany. trichloro acetic acid was obtained from System Malaysia. Methanol obtained from QREC and HPLC grade water was used throughout the study.

Equipment

HPLC chromatographic separation was performed on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector. LC solution version 1.25 was applied for data collecting and processing (Shimadzu, Japan).

Preparation of the calibration standards and quality control (QC) samples

The stock solution of morin was prepared in methanol at a concentration of 1.0 mg/ml. The working solution of 100.0 ng/ml was prepared by appropriately diluting the stock solution of morin. Morin working solution was used to prepare the spiking stock solutions for construction of six-point calibration curve (100.0 - 500.0 ng/ml) and QC samples at three different levels (100.0, 300.0, 500.0 ng/ml). All the stock solutions were refrigerated (2-8°C) when not in use. Calibration standards and QC samples were prepared in bulk by spiking 250.0 µl of respective spiking stock solutions to 250.0 µl of human plasma and then

aliquoted. These were stored at -70°C until analysis.

Sample preparation for analysis

At the time of analysis, the plasma samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. A volume of 500 μl of sample was pipetted into 2.0 ml centrifuge tube and 250 μl of precipitating agent (10% trichloroacetic acid) was added. The resulting solution was vortexed for 2 minutes and centrifuged at 5000 RPM for 5 min. 20.0 μl of the sample was injected to the HPLC system for analysis. All the procedures were performed at room temperature.

Chromatographic conditions

The samples were chromatographed on a Princeton SPHER C_{18} (250 x 4.6 mm i.d., 5 m) column with a flow rate of 0.9 ml/min. The mobile phase used was acetonitrile – potassium dihydrogen phosphate (60:40 % v/v). potassium dihydrogen phosphate used was 20 mM solution in water with pH being adjusted to 5.0 with orthophosphoric acid solution. The injection volume was 20.0 μl . The UV-visible detector was set at 260 nm.

Validation

The method has been validated¹⁹ for selectivity, sensitivity, recovery, linearity, precision, accuracy and stability.

Selectivity

The selectivity of the method was evaluated by comparing the chromatograms obtained from the sample containing morin standard with those obtained from blank samples.

Sensitivity

Sensitivity was determined in terms of LLOQ (Lower Limit of Quantification) where the response of LLOQ was at least five times greater than the response of interference in blank matrix at the retention time or mass transitions of the analyte.

Linearity

For linearity, different concentrations of standard solution were prepared to contain 100.0 ng mL^{-1} to 500.0 ng/ml of morin. These solutions were analyzed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor vs. concentration of the standard solutions. Standard curve fitting was determined by applying the simplest model that adequately describes

the concentration-response relationship using appropriate weighting and statistical tests for goodness of fit.

Precision and Accuracy

Intraday and interday precision method was used to determine the precision. The intra-assay precision and accuracy was calculated for five replicates at each Lower Limit of Quantification (LLOQ), Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC) levels, each on the same analytical run, and inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs.

Stability Studies

Stability study was carried out at room temperature stock solution stability, refrigerated stock solution stability, freeze thaw stability, short term stability and long term stability were determined. Room temperature stock solution stability was carried out at 0, 3 and 8 hours by injecting four replicates of prepared stock dilutions of morin equivalent to middle quality control sample concentration and the stock dilution equivalent to the working concentration. Refrigerated stock solution stability was determined at 7, 14 and 21 days by injecting 4 replicates of prepared stock dilutions of the analyze equivalent to the middle quality control sample concentration. The stability studies of plasma samples spiked with morin were subjected to three freeze - thaw cycles, short term stability at room temperature for 3 h and long term stability at -70°C over four weeks. Stability of standard solutions was performed at room temperature over 6 h and after freezing for four weeks has also been carried out. The stability of triplicate spiked human plasma samples following three freeze thaw cycles was analyzed. At room temperature for 1.00 to 3.00 h before extraction the stability of triplicate short term samples spiked with morin was investigated. The plasma samples for long term stability were stored in the freezer at 70°C until the time of study.

RESULTS AND DISCUSSION

Selectivity

No interfering endogenous compound peak was observed at the retention time of analyte. Under chromatographic conditions, the retention times of morin was 5.12 min. Representative chromatograms of Lower Limit of Quantization (LLOQ) and one study sample containing morin are shown in Figure. 1 and 2 respectively.

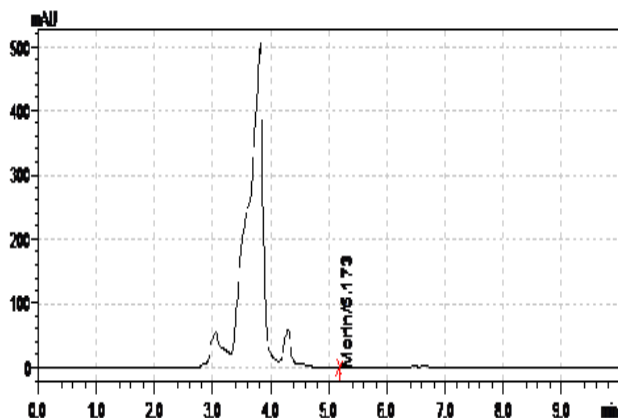


Figure 1: Typical chromatogram of blank plasma

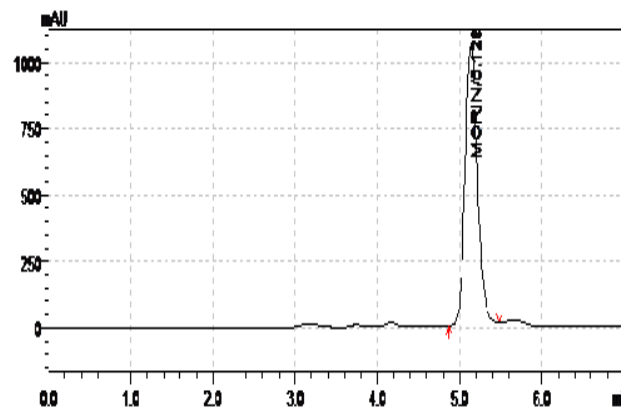


Figure 2: Typical chromatogram of standard solution

Table 1: Calibration curve of Morin

Morin	
Concentration (ng/ml)	Peak area
100	75126
150	112889
200	148252
250	179815
300	223378
500	377630

Table 2: Results of analysis of recovery studies in human plasma

Drug		
	Prepared Concentration (ng/ml)	% Recovery
Morin	100	89.56
	300	90.52
	500	91.02

Sensitivity

The sensitivity of the experiment was carried out at LLOQ level. The average percentage deviation from the nominal concentration was less than 10%.

Linearity and Recovery studies

The calibration curves were linear over the range of 100.0-500.0 ng mL⁻¹. The correlation coefficient was > 0.9996. Calibration curve data of morin result shown in Table 1. Recovery studies presented in Table 2 Figure. 3.

Precision and Accuracy

Both intra-day and inter-day accuracy and precision of the method were determined by analysis of the control human

plasma spiked with morin at LLOQ, LQC, MQC and HQC. All QCs concentration was calculated using the calibration curve. The accuracy and precision of the method were described as a percentage bias and the percentage relative standard deviation, respectively Table 3.

Speed of Analysis

The analysis is performed within 11 min in which 5 min for extraction of plasma and 6 min for chromatography Figure 4.

System suitability

System suitability test was performed daily before the run of analytical batch to check detector response to the analyze. Both the formulations were well tolerated by all

Table 3: Intraday and interday precision studies of Morin in human plasma

	Intraday studies			Interday studies		
	100	300	500	100	300	500
	(ng/ml)					
Mean	98.7268	298.8793	499.0173	98.8039	298.7657	499.3013
SD	0.811	0.346	0.263	0.691	0.786	0.33
%CV	0.822	0.116	0.052	0.699	0.263	0.066
%Accuracy	98.7	99.6	99.8	98.8	99.5	99.8

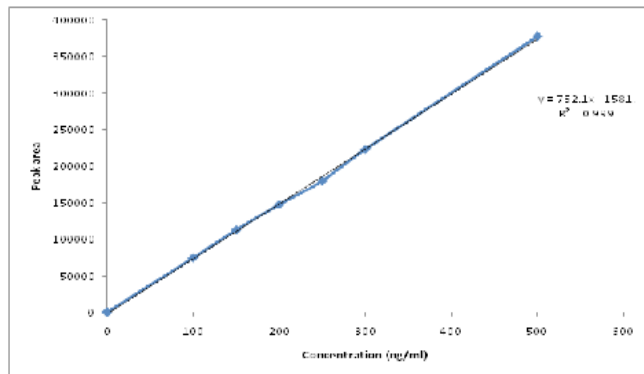


Figure 3: Calibration curve of Morin in blank human plasma

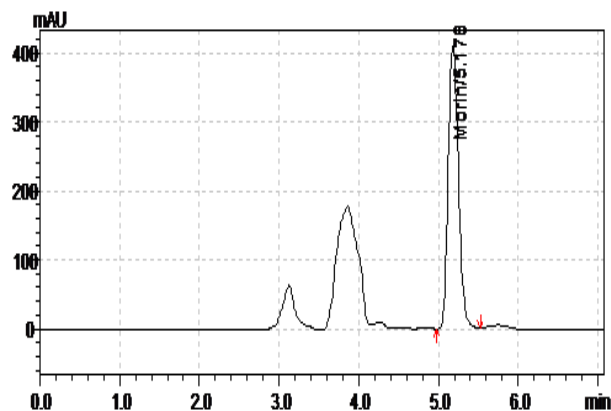


Figure 4: Typical chromatogram of spiked solution

Table 4: System suitability studies

Parameters	Morin
Theoretical plate/meter	4985
Asymmetric factor	0.91
LOD (ng/ml)	50
LOQ (ng/ml)	100

the volunteers in both the phases of study Table 4.

Stability

Analysis of the stock solution was performed at 100.0 ng/ml. After storage for 15 days at 2-8°C and at room temperature for 6h, more than 97.5% of morin remained unchanged, based on peak areas in comparison with freshly prepared solution of morin (100.0 ng/ml) This suggests that the morin in standard solution is stable for at least 15 days when stored at 2-8°C and for 6 h at room temperature.

Bench top stability of morin in plasma was investigated at LQC and HQC levels. This revealed that the morin in plasma was stable for at least 6 h at room temperature. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with morin at LQC and HQC level did not affect the stability of morin Long term stability of the morin in plasma at -70°C was also performed after 30 days of storage at LQC, HQC levels. The results of the stability studies are shown in Table 5. The above results indicated that the morin was stable in the studied conditions.

CONCLUSION

A simple and sensitive method for morin in human plasma

Table 5: Stability Study of Morin in Human Plasma

Concentration (ng/ml) (n = 6)	Concentration found (mean ± S.D.) (ng/ml)	% CV
Short-term stability (1, 2, 3 h)		
100	99.85±0.43	3.10
500	498.63±1.25	1.95
Long-term stability (4 weeks)		
100	98.96±1.04	4.17
500	498.75±1.15	1.96
Stock Solution Stability		
100	99.54±0.97	2.96
500	499.63±1.24	1.09
Freeze thaw Stability (3 Cycle)		
100	98.99±1.27	3.87
500	499.02±0.59	2.12
Auto Sampler Stability		
100	99.52±0.85	2.89
500	499.97±0.49	1.856
Stability of Morin in Plasma (20°C)		
100	99.63±0.75	3.19
500	499.67±1.07	1.27

was developed and validated as per FDA guidelines. This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, faster run time (6 min) and lower sample requirements. Hence, this method may be useful for single and multiple ascending dose studies in human subjects. The developed method has excellent sensitivity, reproducibility and specificity. The method has been successfully used to provide the bioavailability and bioequivalent study of morin in animal and human plasma. The developed assay showed acceptable precision, accuracy, linearity, stability, and specificity so applicable to clinical tool.

Highlights of Paper

- Method was developed and validated as per FDA guidelines.
- The developed method has sensitivity and selectivity, faster run time (6 min) and lower sample requirements.

Author Profile



- Dr.S.Muralidharan has been completed M.Pharm and Ph.D in the Department of Pharmaceutical Analysis in Tamil Nadu Dr.MGR Medical University, India. Presently working as a Senior Lecturer in Faculty of Pharmacy, AIMST University, Malaysia.



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