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Bioanalytical Method Development and Validation for Herbal Quercetin in Nano Formulation by RP-UFLC in Rabbit Plasma

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Abstract

A sensitive reverse phase ultra fast liquid chromatography (RP-UFLC) has been developed and validated for the quantification of Quercetin in nano formulation in rabbit plasma using solid phase extraction method (SPE). Best chromatographic resolution was achieved on a reverse phase Hibar®C_{18} (250×4.6 mm i.d., 5 µ) column with the mobile phase consisted of acetonitrile: pottasium dihydrogen ortho phosphate (pH 3.5) in the ratio of 65:35 (v/v) with a flow rate of 0.8 mL/min. The retention time for the drugs was found to be 8.6 min and 10.0 min for internal standard (fluticasone propionate). The analyte was detected by using a Photo-diode array (PDA) detector with LC solution software. Linearity was obtained in the concentration range of 10 to 400.0 ng/mL (r^2= 0.989). Lower limit of quantification (LLOQ) was found to be 10 ng/mL. Middle limit of quantification (MQC) was found to be 200 ng/mL and higher limit of quantification (HQC) was found to be 380 ng/mL. The average recovery of the analyte was found to ranging from 95.91 to 98.59%. The developed method isapplicablefor estimation of Quercetin in nano-formulation, routine quality control analysis, pharmacokinetic and bioequivalence studies.

Keywords: Quercetin; RP-UFLC; Rabbit plasma; Validation; Photo diode array detector; Pharmacokinetics; Nano-formulation

Introduction

Quercetin is a polyphenolic compound chemically it is 3,3',4',5,7-Pentahydroxyflavone is a common constituent found in a wide variety of fruits and vegetables [1]. Quercetin is attracting much of the researcher's attention due to its wide availability and potent biologically active agent for preventing various conditions such as for neoplastism [2], cardiovascular diseases [3], anti-inflammatory [4], bronchial hyper reactivity [5], and neurodegenerative disorders [6]. The mechanistic pathway in which it acts is mostly by direct radical scavenging of oxygen either in the free form or in the excited form.

Several studies were performed for estimation of the drug in various biological fluids by HPLC [7-19] but still these methods were not having proper chromatographic resolution where in some broad peak and the others the gap between the retention times of the drugs was not adequate. Though advanced methods such as LC-MS [10,20,21] and LC-ES-MS [22] were also developed but still there is a gap in the normal labs where the drug estimation cannot be performed on sophisticated instruments hence a simple HPLC method is suitable for analysis.

The aim of the study was to develop simple, rapid and sensitive RP-UFLC method for the determination of Quercetin in rabbit plasma which is useful for the estimation of Quercetin in developed formulations, food product analysis, quality control, pharmacokinetics and bioequivalence profiling.

Materials and Methods

Reagents and chemicals

Quercetin standard (purity>99%), Acetonitrile of HPLC grade and all other chemicals for HPLC were purchased from S.D Fine Chem Ltd. (Mumbai, India). The chemicals required for the nano formulation were purchased from Rankem Ltd. (Mumbai, India).

Instrumentation

The UFLC system comprised of Shimadzu ultra fast liquid chromatography with a binary gradient pump of LC-20AD consisting of photodiode array detector(PDA) with a 20 µL loop volume injection(Shimadzu, Japan) connected with LC solution software.

Chromatographic conditions

Chromatographic separation was achieved using Hibar®C_{18} (250×4.6 mm i.d., 5 µ) stationary phase using a mobile phase of Acetonitrile: Phosphate buffer pH 3.5 (65:35, v/v) at a flow rate of 0.8 mL/min. Quercetin was detected at 369 nm using a PDA detectorwith a retention time of 8.6 min and Fluticasone propionate as an internal standard with a retention time of 10.01 minutes.

Statistical methods

The standard calibration curves were fitted into a linear regression analysis and the graph was plotted for response factor against concentration of the standard solutions of drug and IS. The graph was used for estimating mean, standard deviation (SD) and percentage coefficient of variation (% CV).

Preparation of standard solution for plotting calibration curve

Quercetin stock solutions were prepared freshly on each day

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of analysis by dissolving 10 mg of pure Quercetin in HPLC grade acetonitrile to make the volume up to 10 mL. From the prepared stock solution serial dilutions in the range of 10 - 400 ng/mL were prepared and each solution was injected for about 20 µL in sextuplicate and the method was validated for the various parameters such as accuracy, linearity, precision, limit of quantification and detection (LOD and LOQ). Internal standard used was also prepared in same method as that of the standard.

Preparation of sample solution

The Nano Formulation (NF) sample solution was prepared by accurately weighing 10 mg of the NF and dissolved in HPLC grade acetonitrile, sonicated for 15 minutes, filtered and the volume was made up to 10 mL to obtain 1 mg/mL of solution keeping it as stock solution and further dilutions were prepared form the above prepared stock solution.

Pharmacokinetic study design

A single dose complete cross over method was employed for the study of Quercetin pharmacokinetics innanoformulation. Healthy male albino rabbits (1.5-2.0 kg) divided into three groups where group 1 (n=3) received pure drug, group 2 (n=3) received nano formulation and group 3 (n=2) was considered as a control (administered with saline solution). Blood sample of about 0.5 ml was withdrawn in heparinised tubes during different time points (0, 0.5, 1, 2, 3, 5, 7, 8, 10, 12 and 24 hrs respectively) from the marginal ear vein of the rabbit. The collected samples were centrifuged at 4000 rpm for 10 minutes and the plasma samples were stored at -20°C until analysed. Pk software was used for calculating pharmacokinetic parameters.

Method for determining linearity and accuracy

Serial dilutions (10-400 ng/mL) of standard solutions were analysed and the peak areas were recorded and the standard concentrations were plotted against response factor. Regression line was recorded for each set of analysis where accuracy was determined by quantification of the standard solution in sextuplicate for the estimation of mean and % nominal.

Method for determining precision, LOD and LOQ

Precision of the study was calculated as per ICH guidelines (International Conference on Harmonization) (ICH, 2004) [23] for determination of intermediate precision and repeatability. Both inter and intraday repeatability wasestimated by injecting standard and sample solutions in sextuplicate and thereby mean and % CV was calculated. Whereas LOD and LOQ were obtained by the formula

$LOD = 3.3\ \sigma/m$

$LOQ = 10\ \sigma/m$

Where σ is the standard deviation of intercept of regression line and m is slope of calibration curve.

Ruggedness

Ruggedness of the developed method was assessed by checking the reproducibility and retention times of the standard and sample solutions. It was performed by analysing under variations in test conditions such as laboratory, analyst, reagents, different days and instruments.

Preparation of plasma samples

A 0.5 mL of the rabbit plasma samples were spiked with 0.5 mL of internal standard solution (400 ng/mL). The above solution was vortexed for 1-3 minutes and resulting solution was extracted with Solid Phase Extraction method (SPE). The drug was extracted from the solid phase extraction cartridge using 0.5 mL of acetonitrile and analysed in UFLC.

Results and Discussion

Method development

By studying various ratios of mobile phases, pH, flow rate optimized chromatographic separation with a symmetric peak was obtained with a mobile phase of acetonitrile: phosphate buffer pH 3.5 (65:35 v/v) at a flow rate of 0.8 mL/min on a Hibar® C_{18} column stationary phase. The method suitability was checked by the analysis of the sample in the spiked plasma and the retention time of the standard Quercetin was approximately 8.6 minutes (Figure 1) and no other peaks were observed at the same retention time of the standard drug.

Specificity

Specificity of the developed method shows good separation and no additional peak was observed near the drug retention time due to the endogenous materials. No endogenous substances were interfered with the detection of the drug. Representative typical chromatograph is shown in the Figure 1 and 2.

Linearity

The linearity curve was plotted in the concentration range of 10-400 ng/mL and the calibration curve was found to be linear with a correlation coefficient of r^2=0.9892 which indicates a significant linear relationship among the amount and peak area in the tested range of concentration. There was not much variation in the inter day differences and in the standard deviation and standard error of slope values which indicates that the method was linear giving no scope for variation in the developed linearity curves and the results are given in the Figure 3 and Table 1.

Response factor against each concentration was calculated and each concentration was analysed in triplicate. The response factor was plotted in concentration range of 10-400 ng/mL.

Accuracy

The method developed was found to be accurate, the % nominal mean was found to be 95.91-98.59% which proves that the method was accurate. Table 2 indicates the % CV, % nominal and standard deviation of the standard curves of Quercetin.

Precision

Precision studies were carried out to check the inter day variation (60, 200 and 400 ng/mL) for intermediate precision studies and intraday precision (60, 200 and 400 ng/mL) for repeatability. The study was performed using a concentration range of 60-400 ng/mL where each concentration injected six times on three consecutive days. Mean and Relative Standard Deviations (RSD) were calculated and the low values of RSD indicate the method is precise and repeatable and the results are shown in Table 3.

LOD and LOQ

LOD and LOQ are calculated as given in the above formula and found to be 5 ng/mL and 10 ng/mL respectively which are within ± 15% of accuracy and precision. These are achieved with a 20 µL injection volume.

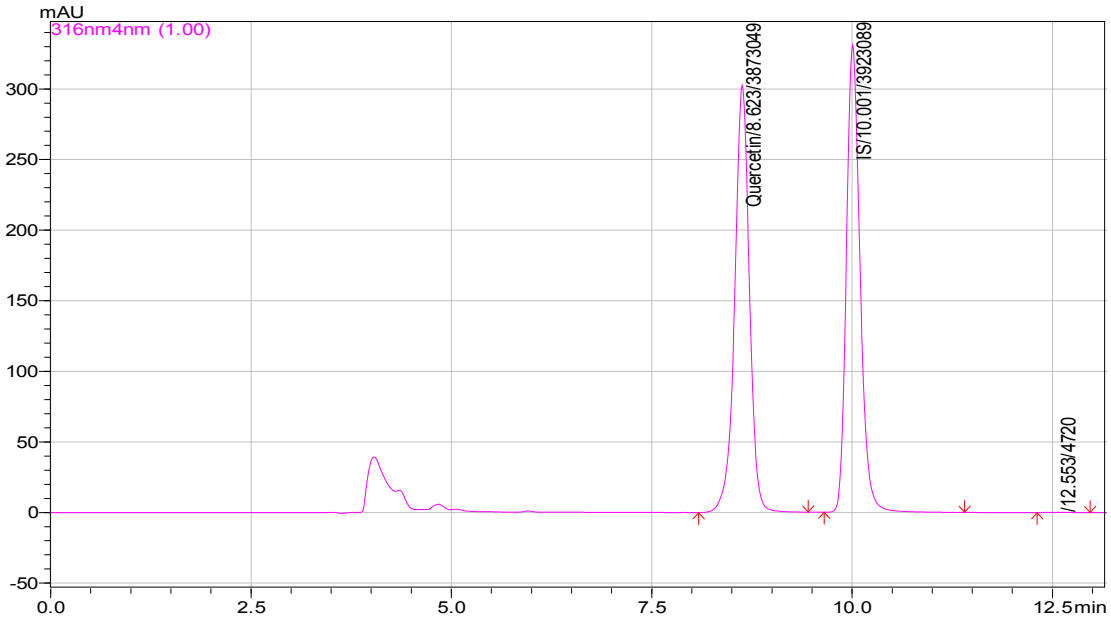

Figure 1: Typical chromatograph of the standard drug and internal standard.

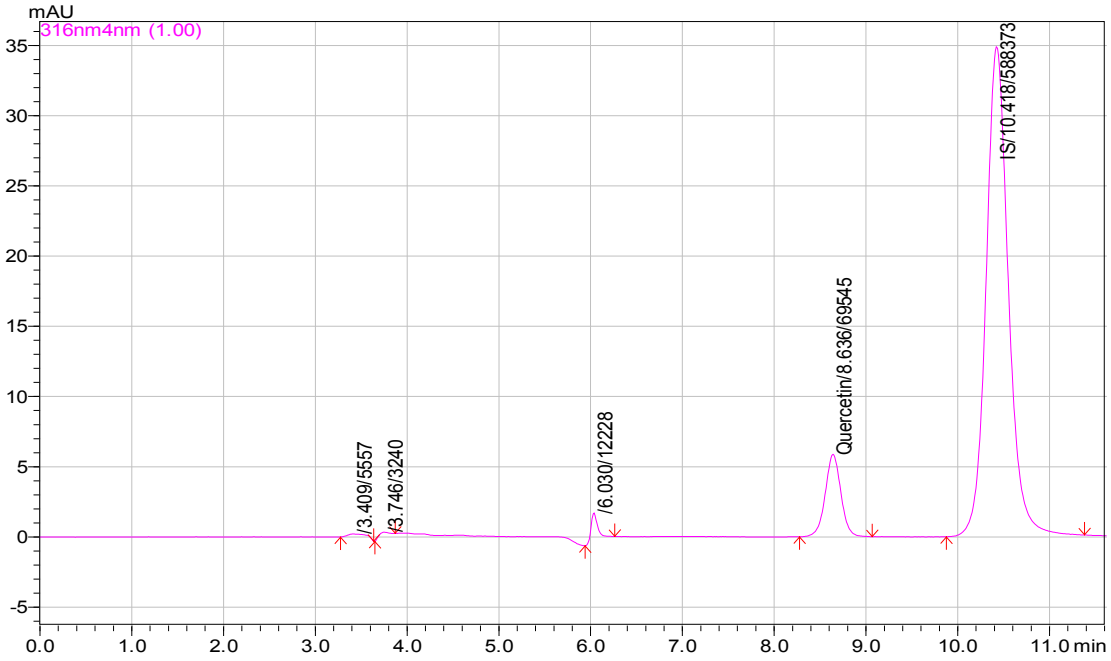

Figure 2: Typical chromatograph of the sample quercetin.

Ruggedness

Development of the method in various test conditions also led to the resolution at a retention time of 8.6 minutes for Quercetin which shows that the method is rugged.

Recovery

The mean recoveries of Quercetin at concentrations of 150 ng/mL (n=6) were 95.82, 97.57% respectively in the spiked rabbit plasma and the deviation of the study was within 15% of the limits and results are shown in the Table 2.

Pharmacokinetic studies

The concentration of Quercetin in rabbit's plasma was evaluated by UFLC method and the pharmacokinetic parameters were as given in Table 4 for both pure drug and nano formulation. The mean plasma concentration of the pure drug and the nano formulation were as shown in Figure 4.

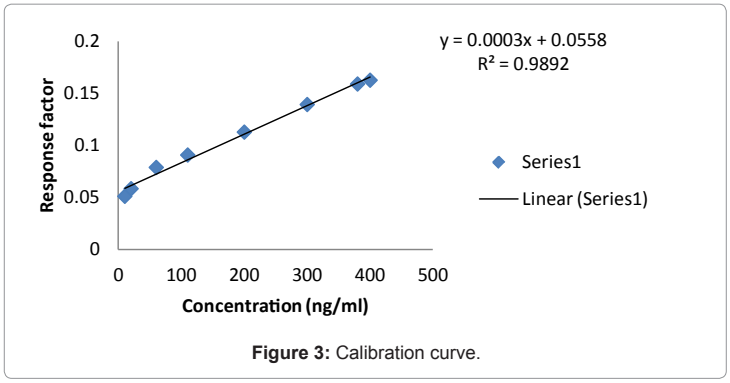

Figure 3: Calibration curve.

S.NO	Concentration (ng/mL)	Response factor
1	10	0.050926
2	20	0.058256
3	60	0.078693
4	110	0.090631
5	200	0.112637
6	300	0.139181
7	380	0.158887
8	400	0.162548

Table 1: Concentration- Response factor for Quercetin.

S.No	Actual concentration (ng/mL)	Amount spiked (ng)	Measured concentration (ng/mL)	% Recovery (mean ± SD)
1	200	150	147.89	98.59333
2	200	150	145.64	97.09333
3	200	150	146.81	97.87333
4	200	150	148.13	98.75333
5	200	150	145.83	97.22
6	200	150	143.87	95.91333
Mean			146.3617	97.57444
SD			1.592211	1.061474
CV (%)			1.087861	0.940476

Table 2: Accuracy and recovery studies.

S.No	Interday studies			Intraday studies		
	60(ng/mL)	200(ng/mL)	400(ng/mL)	60(ng/mL)	200(ng/mL)	400(ng/mL)
1	4668	15563	31104	4742	15742	31523
2	4683	15321	30984	4318	16732	31842
3	4532	15832	30627	4261	15372	31537
4	4583	15381	31832	4739	15731	31735
5	4628	15931	30999	4783	15824	31934
6	4599	15752	31873	4700	15732	31562
Mean	4615.5	15630	31236.5	4590.5	15855.5	31688.8
SD	56.159594	248.298	503.778	235.32	457.526	174.55
RSD	1.21676078	1.5886	1.61279	5.12624	2.8856	0.55083

Table 3: Precision studies.

S.NO	Time(hours) and Pharmacokinetic parameters	Nano formulation	Pure drug
		Concentration (ng/mL)	Concentration (ng/mL)
1	0	0	0
2	0.5	10.83635	9.54953
3	1	22.85125	10.3611
4	2	31.7934	15.07157
5	3	83.7119	106.6077
6	5	198.72836	125.37766
7	7	236.41532	169.30415
8	8	219.10241	195.4973
9	10	159.83774	165.18968
10	12	130.5835	120.38341
11	18	124.9776	109.0875
12	24	114.9085	98.0976
13	C_{max}	236.41532	195.4973
14	T_{max}	7	7
15	AUC_{0-t}	3197.25176	2746.21701
16	keli (h^{-1})	0.03953183	0.03829696
17	$t_{½}$ (h)	17.5339	18.0992749
18	$AUC_{0-\alpha}$	9177.63037	7850.99052

Table 4: Individual plasma concentrations (ng/mL) and pharmacokinetic parameters for developed nano formulation and pure drug.

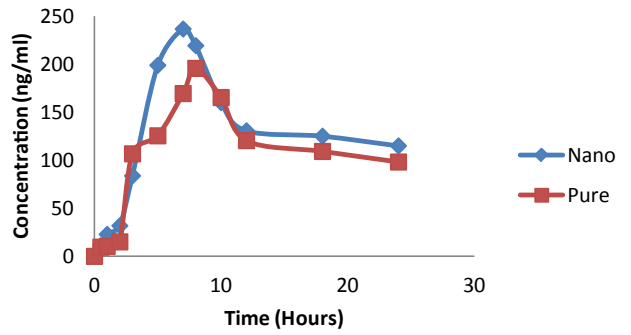

Figure 4: Mean plasma concentrations (ng/mL) curve.

Pharmacokinetic parameters were reported C_{max}– Mean plasma concentration; T_{max}– Mean time concentration; $AUC_{0-t\,,\,0-\alpha}$- Area under curve; K_{eli} – Elimination constant; $t_{1/2}$– Half-life.

Conclusion

A validated reverse phase UFLC method was developed for the estimation of Quercetin in nano formulation in rabbit plasma. The method was simple, rapid, precise, accurate and rugged. The method finds its applicability in food product analysis, pharmacokinetics and bioequivalence profiling either as an individual component or in combination with other.

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