RESEARCH ARTICLE

Donepezil and Quercetin Simultaneous Estimation in Rat Plasma Using Developed Bioanalytical HPLC Method: Relevance in Pharmacokinetic Studies

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ABSTRACT

The objective of this investigation was to create and apply a reliable reverse phase high performance liquid chromatography (RP-HPLC) technique for the concurrent measurement of donepezil (DPZ) and quercetin (QT) in rat blood samples for pharmacokinetic research. This is the first publication that introduces a method for DPZ and QT simultaneous determination in rat plasma by high-performance liquid chromatography (HPLC). Using a mobile phase of methanol and HPLC grade water (pH 2.8; adjusted with 0.05% v/v orthophosphoric acid) 45:55 v/v with 2-3 drops of triethylamine (TEA) in an isocratic elution mode at flow rate of 1.0ml/min, DPZ and QT were successfully separated chromatographically on a Hypersil gold C-18 column (250 mm × 4.6 mm, 5µm). The retention time for DPZ and QT was observed to be 6.3 and 12.3 minutes and was detected at an isobestic wavelength of 273 nm using a UV detector. The method was shown to be precise (%RSD < 2%), accurate (96–100%), and specific for the simultaneous detection of DPZ and QT. Several freeze-thaw cycles of the treated plasma samples did not significantly affect the analyte's stability. The method's applicability was subsequently confirmed through an oral pharmacokinetic investigation in rats. Since the results were deemed trustworthy, the validated RP-HPLC method may be used to simultaneously detect and quantify both drugs. The method worked well for evaluating the pharmacokinetic characteristics in wistar rats following a single oral dose of 5 mg/kg of QT and 10 mg/kg of DPZ. It is well acknowledged that the chromatographic process is straightforward, robust, accurate, exact, and repeatable.

Keywords: Donepezil, Quercetin, RP-HPLC, Simultaneous determination, Bioanalytical method validation, Pharmacokinetics.

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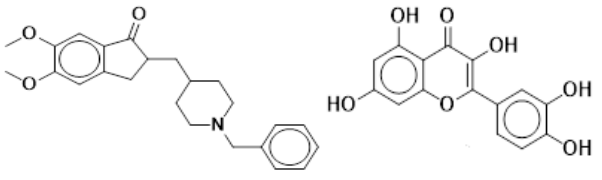
INTRODUCTION

For successful drug development, the development of selective, sensitive, and reliable bioanalytical procedures for which it is crucial to quantitatively assess drugs and their metabolites in biological matrices. For the detection and quantification of drugs and metabolites in biological matrices at low concentrations, high-pressure liquid chromatography is a commonly used analytical technique. As a result, developing and validating a bioanalytical High-performance liquid chromatography (HPLC) system for low-dose drugs is important.[1] Alzheimer's disease dementia is treated with the drug donepezil (DPZ), an acetylcholinesterase inhibitor (Figure 1a).[2] The United States Food and Drug Administration (FDA) has approved DPZ for the treatment of Alzheimer's disease.[3] It has a potential anti-Alzheimer action by suppressing acetylcholine and increasing cholinergic transmission.[4] For detecting DPZ alone, several methodologies have been published.[5-9] Memantine, galantamine, and rivastigmine have all been determined in combination with DPZ.[10-12]

Fresh green leafy vegetables, red onions, apples, tomatoes, red grapes, green tea, and black tea all contain the naturally occurring flavonoid quercetin (QT) (Figure 1b).[13] Owing to its anti-inflammatory and antioxidant effects, QT has therapeutic potential for disorders like cardiovascular disease, cancer,



a) b)

Figure 1: Chemical structure: a) Donepezil; b) Quercetin[21,22]

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and neurological disease.[14] It reduces oxidative stress and neuroinflammation, which protects neuronal cells. Several HPLC approaches for determining QT in just plasma samples[15,16] or in partnership with other drugs have been published.[17-19] Nevertheless, no suitable validated HPLC approach for simultaneously measuring and quantifying DPZ and QT.

Thus, the objective of the current study is to create and validate a straightforward, inexpensive, fast, sensitive, and stability-indicating HPLC technique for determining DPZ and QT simultaneously in rat plasma. The method was validated as per Food and Drug Administration (FDA) specifications.[20] To ascertain the applicability of the suggested and verified approach, the pharmacokinetic profile of DPZ and QT in male Wistar rats was examined.

MATERIALS AND METHODS

Materials

Analytical standard grade QT was purchased from Yarrow Pharma, Mumbai (≥ 98%). DPZ has obtained a kind gift sample from Cipla Mumbai. HPLC grade Methanol was procured from Merck Specialities Pvt. Ltd (Mumbai, India). AR grade O-phosphoric acid (OPA) and HPLC grade triethylamine (TEA) were procured from SD Fine Chemicals (India). Before use, Milli-Q filtration system (Millipore GmBH, Germany) was used to purify HPLC-grade Milli-Q water. All of the reagents and solvents used in this study were of analytical quality.

Methods

Instrumentation

Chromatographic analysis was carried out on a Jasco HPLC 4000, LC-Net II/ADC HPLC with a UV-vis detector. Analysis and separation were performed on the Hypersil gold C-18 column. A flow rate of 1.0 mL/min is used in the isocratic elution mode, the mobile phase comprised of methanol and Milli-Q water (pH 2.8; balanced with 0.05% v/v orthophosphoric acid; 45:55 v/v) and 2 to 3 drops of TEA. The injection volume was 20 µL. During 20 minutes, DPZ and QT were simultaneously examined and detected in the eluent at 273 nm.

Development and Optimization of Bioanalytical Method

Preparation of standard solutions

DPZ and QT (10 mg each) were accurately weighed and added into a 10 mL volumetric flask to make a final concentration of 1000 µg/mL in 10 ml of methanol. This was labeled as a "stock solution". Six standards with concentrations of 20, 40, 60, 80, 100, 150, and 200 µg/mL were created from DPZ and QT stock solutions for DPZ and QT calibration curves. The stock solutions of DPZ and QT were also utilized to make LQC, MQC, and HQC standards having strengths of 80, 140, and 200 µg/mL used to test the other validation parameters.

Preparation of plasma matrix

A protein precipitation method was used to prepare all of the blank rat plasma samples.

Validation of the HPLC Method[20]

The USFDA requirements for compatibility of the system, precision, specificity, accuracy, linearity, LoD, LoQ, and robustness were employed to validate the optimized method for simultaneous quantification of DPZ and QT.

System suitability

In order to guarantee system compatibility, DPZ and QT's tailing/asymmetry factor, peak retention time, peak area, and theoretical plates were used. It is ideal to have theoretical plates >2000, asymmetry factor <2, and CV <1% for system applicability. The appropriate properties of the established approach were assessed using a concentration of 100 µg/mL. To determine the system's appropriateness, samples were analyzed in triplicate.

- *Linearity*

six standard concentrations of 20, 40, 60, 80, 100, 150, and 200 µg/mL were used for linearity. To confirm the linearity of the analytical procedure, the calibration curves' slope, intercept, and correlation coefficient (peak area vs. concentration) were computed.

- *Accuracy and precision*

To evaluate the accuracy of the optimized method, six replicates of each sample were loaded into the HPLC at the concentrations of LQC (80 µg/mL), MQC (140 µg/mL), and HQC (200 µg/mL).

Three injections of LQC, MQC, and HQC samples, each produced by a different analyst, were performed under similar test settings. The %relative standard deviation was calculated using the mean data that were gathered. The linear equations were used to determine the DPZ and QT peak regions, and the actual concentrations were computed in reverse. The accuracy of the method was determined by comparing the actual concentrations with theoretical concentrations.

Determination of LoD and LoQ[23]

The efficacy of DPZ and QT for simultaneous examination was investigated. Lower limits of quantification (LLoQ) and the limit of detection (LoD) were calculated using the signal/noise ratio (S/N).

Robustness[24]

One by one, the effects of various chromatographic factors on retention duration and peak area ratio were examined, including column temperature, mobile phase flow rate, and mobile phase composition. 20 µg/mL was used as a reference concentration in this test. The mean peak area ratio for DPZ and QT, as well as the mean retention time, were calculated for each condition (n = 3).

Stability

The stability was tested using a standard with a concentration of 200 µg/mL at 24 ± 4°C. The amounts of DPZ and QT in the prepared sample were determined by analysis of concentrations at 1, 6, 12, 24, and 48 hours. At each time point, the concentrations of DPZ and QT were determined (n = 3).

Applications of the Validated Analytical Method

Study of pharmacokinetic in wistar rats

- *Animals*

The Poona College of Pharmacy's Institutional Animal Ethics Committee (IAEC) authorized an experimental protocol based on the CPCSEA's recommendations (IAEC/PCP/PCT06/2021-2022). We procured male Wistar rats from the National Institute of Biosciences that weighed 200–250 g. Before the experiment, the animals were given a week to become habituated to the temperature and humidity conditions of the experiment. Rats were given filtered water and typical pelletized food.

- *Sample collection and experimental design*

Male wistar rats (n = 6) were given pure DPZ (5mg/kg) and QT (10mg/kg) orally. From rats that had been mildly sedated with ether, 0.5 to 1.5 mL of blood were drawn and centrifuged at 4°C for approximately 20 minutes at 15,000 rpm and resultant plasma was stored at -20°C till further investigation. Using WinNonlin 4 software (a non-compartmental model), the pharmacokinetic parameters were examined.

RESULTS AND DISCUSSION

UV Wavelength Determination

The absorption maxima for DPZ are at approximately 270 nm, while QTs are around 370 nm.[25,26] According to UV data, the absorption maxima of DPZ are around 268 nm, while the absorption maxima of QT are about 370 nm. In combination, the isobestic point of both compounds is discovered to be 273 nm. Both drug methods were developed and validated at a wavelength of 273 nm.

Development and Optimization of the Method

In order to achieve the proper analyte separation, the mobile phase's composition was optimized through the use of different solvent ratios in the preliminary experiments, including water, methanol, and ACN. Several concentrations of the mobile phase were tried to optimize it. Based on the recovery of DPZ and QT in several solvents, the extraction solvent was chosen. Moreover, this method's theoretical plate values for QT and DPZ were over 2000 at 8654 and 8588, respectively. Retention times for DPZ and QT were 6.3 and 12.3 minutes respectively. Both drugs have a tailing factor of less than 2. Figures 2a and 2b show the developed method's DPZ and QT peaks.

System Suitability

For both drugs, the results of injections performed in triplicate revealed that the observed limits fall within ranges that were deemed to be acceptable. At 6.3 and 12.3 minutes, DPZ and QT were consistently kept and well distanced, indicating the integrated HPLC system's remarkable reproducibility of repeated injections with a relative standard deviation % (%RSD) of < 0.2. In every chromatographic run, the DPZ and QT peaks' tailing factors were never greater than two, good column efficacy is indicated by good peak regularity (the allowed limit is two) and a constant number of theoretical plates exceeding 2000 over the whole separation process. Table 1 displays the results.

Table 1: System suitability parameters of donepezil and quercetin

parameters	*Value*	
	Donepezil	*Quercetin*
Peak area	25501 ± 0.014	33261 ± 0.036
Retention time	6.3 ± 0.0074	12.3 ± 0.0038
Theoretical plates	8654.3 ± 0.028	8588.6 ± 0.013
Tailing factor	1.2 ± 0.246	1.20 ± 0.176

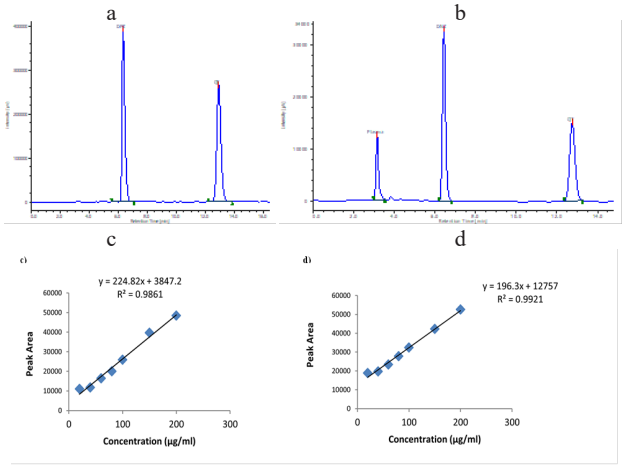

Figure 2: a) Chromatogram demonstrating retention time of peaks of donepezil and quercetin without plasma and b) with plasma, c) calibration curve of donepezil, and d) quercetin.

Linearity and range

Within the predefined ranges of 20 to 200 µg/mL, the DPZ and QT calibration curves (Figures 2c and 2d) were both linear. The equation for linear regression Y = 2248.2x + 3847.2, the correlation coefficient (r^2) for DPZ was 0.9861. Similarly, the r^2 value for QT was 0.9921, with the linear regression equation Y = 1963x + 12757. The method was linear since all of the concentrations found ranged between 85 and 115% of their respective nominal values.

Accuracy and precision

The percentage recovery was calculated to assess the accuracy of the established HPLC analytical method; the recovery was found to be in the 96 to 100% range with an RSD of less than 2%. This result demonstrates the method's effectiveness in analyzing both drugs (Table 2). LQC, MQC, and HQC injections were used to conduct intraday and between-day experiments. The peak regions identified for DPZ and QT separately over three days were consistent and exact. The outcomes for both intraday and interday illustrate the developed method's excellent repeatability and precision, with all data presented in RSD %(< 2). Table 3 shows the intraday and interday precision results for DPZ and QT.

LoD and LoQ

The LoD and LoQ for DPZ and QT were 1.0 µg/ml and 0.5 µg/ml, respectively.

Robustness

When some of the chromatographic conditions' parameters were changed, no significant changes were observed, indicating

Table 2: Accuracy study results of the donepezil and quercetin in rat plasma

Levels	*Theoretical concentration (µg/mL)*	*Recovered Actual concentration (µg/mL) (mean ± SD) (N =3)*	*RSD (%)*	*Recovery (%) (mean ± SD)*	*RSD (%)*
Donepezil					
LQC	80	67.29 ± 0.212	1.67	98.65 ± 1.66	1.68
MQC	140	126.7 ± 0.219	1.59	98.58 ± 1.87	1.90
HQC	200	173.7 ± 0.367	2.43	96.97 ± 1.9	2.00
Quercetin					
LQC	80	72.54 ± 0.128	1.02	98.03 ± 0.99	1.01
MQC	140	124.2 ± 0.188	1.38	97.13 ± 1.36	1.40
HQC	200	178.3 ± 0.208	1.42	96.42 ± 1.37	1.42

Table 3: Donepezil and quercetin intra- and inter-day precision at three distinct concentration levels (80, 140, and 200 µg/mL)

parameters	*Conc. (µg/mL)*	*mean area ± SD (N = 3) (cm^2)*	*RSD (%)*
	80	32669 ± 550.83	1.68
Repeatability (intraday precision)	140	35778.3 ± 575.96	1.60
	200	38472 ± 773.58	2.01
	80 (A1, A2, A3)	30336.6 ± 572.41	1.88
Interanalyst	140 (A1, A2, A3)	33693.3 ± 497.09	1.47
	200 (A1, A2, A3)	38945.3 ± 803.43	2.06
Day 1, day 2, day 3	80	38815.3 ± 743.42	1.91
Day 1, day 2, day 3	140	35036.3 ± 696.76	1.98
Day 1, day 2, day 3	200	38564 ± 569.46	1.47
Repeatability (intraday precision)	80	45605.3 ± 464.45	1.01
	140	49727.3 ± 697.04	1.40
	200	53877 ± 764.85	1.41
Interanalyst	80 (A1, A2, A3)	45412.6 ± 617.85	1.36
	140 (A1, A2, A3)	47602 ± 763.01	1.60
	200 (A1, A2, A3)	52571.6 ± 939.8	1.78
Day 1, day 2, day 3	80	44618.3 ± 607.93	1.36
Day 1, day 2, day 3	140	47420.3 ± 578.99	1.22
Day 1, day 2, day 3	200	51522.6 ± 734.27	1.42

Table 4: Optimization of donepezil and quercetin robustness studies

parameters	*Value*	*Conc.(µg/mL)*	*mean area ± SD (N = 3) (cm^2)*	*RSD (%)*	*Mean Rt (min) ± SD) (N = 3)*	*RSD (%)*
Donepezil						
Flow rate (mL/min)	0.8	20	25666.3 ± 787.98	3.07	8.1	1.00
	1	20	25557.6 ± 411.37	1.60	6.3	0.74
	1.2	20	25365 ± 555.54	2.19	5.4	0.86
	300	20	25246.6 ± 467.74	1.85	6.4	1.93
wavelength	273	20	25442.6 ± 477.46	1.87	6.4	0.73
	270	20	24910.3 ± 473.87	1.90	6.4	0.73
Quercetin						
Flow rate (mL/min)	0.8	20	32786.3 ± 268.15	0.81	15.4	0.30
	1	20	33103.3 ± 250.61	0.75	12.2	0.38
	1.2	20	32943.3 ± 577.93	1.75	10.4	0.45
	300	20	32854.6 ± 596.55	1.81	12.30	0.38
wavelength	273	20	33680.3 ± 289.4	0.85	12.4	1.00
	270	20	32943.6 ± 645.9	1.9	12.3	0.38

Table 5: Stability studies

Time (h)	*Concentration (µg/mL) (± SD, N = 3)*	
	Donepezil	*Quercetin*
1	174.06 ± 0.298	177.65 ± 0.20
6	169.67 ± 0.308	176.63 ± 0.23
12	179.78 ± 0.207	180.65 ± 0.115
24	177.92 ± 0.103	173.44 ± 0.164
48	180.06 ± 0.069	176.61 ± 0.156

Table 6: Pharmacokinetic characteristics of donepezil and quercetin in rat plasma following a single oral dosage

Parameters	*Donepezil*	*Quercetin*
T_{max}(h)	4	2
C_{max} (µg/mL)	3.22 ± 0.3358	5.61 ± 5.66
$AUC_{(0-t)}$(hr* µg/mL)	12.066 ± 0.499	6.945 ± 2.778
AUC $_{(0-\alpha)}$ (hr*hr* µg/mL)	12.156 ± 0.740	8.415 ± 3.366
MRT(h)	3.404 ± 0.3566	4.953 ± 0.1496

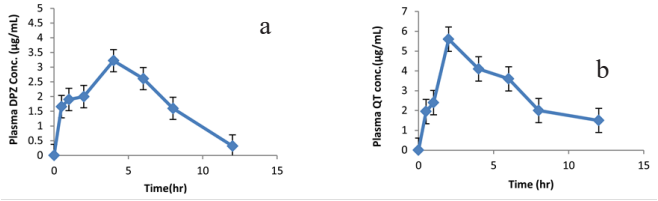

Figure 3: a) Donepezil and b) Quercetin plasma concentration-time profiles

that the method is resistant to slight purposeful changes in wavelength and flow rate. The resolution was greater than two, and the DPZ and QT peaks were symmetric (tailing factor <2). Retention times for DPZ and QT had a % RSD of <1, showing the analytical method's robustness to modest alterations (Table 4).

Stability

The samples were tested for DPZ and QT stability at 1, 6, 12, 24, and 48 hours (Table 5). At each time point, the assessment was repeated three times. Because of some methanol evaporation, the sample concentration increased slightly over time. The concentration results at different times, did not differ statistically significantly.

Applications of the Verified Analytical Method

Pharmacokinetic study

Oral dosages of DPZ (5 mg/kg) and QT (10 mg/kg) were administered to male Wistar rats, and the levels of DPZ and QT with respect to time in plasma were satisfactorily measured using the current HPLC method. Based on pharmacokinetic studies, the peak plasma concentrations of DPZ and QT eventuate at 6 and 12 minutes, respectively, and have a mean plasma drug concentration-time profile (Figure 3). The values of each important pharmacokinetic parameter are compiled in Table 6.

CONCLUSION

The current bioanalytical HPLC approach has been demonstrated to be straightforward, sensitive, exact, accurate, robust, and reproducible for the simultaneous measurement of DPZ and QT in preclinical samples (rat plasma), and it complies with FDA standards. This method separates the two drugs from the matrix in a single step; the specificity of the method is demonstrated by interference peak absence at the retention durations. As a result, the method developed can be utilized to analyze DPZ and QT in rat plasma samples regularly. To determine pharmacokinetic parameters, male wistar rats were effectively used in a single-dose oral pharmacokinetic research using DPZ and QT. The novel approach is affordable and could be utilized in the future for bioequivalence, bioavailability, dug interaction, and clinical studies. As a result of the positive findings of this study, the developed approach may be utilized in preclinical and clinical trials to assess these drugs in biological samples.

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COMPLIANCE WITH ETHICAL STANDARDS

The Animal Ethics Committee (IAEC) authorized an experimental protocol based on the CPCSEA's recommendations (IAEC/PCP/PCT06/2021-2022).

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