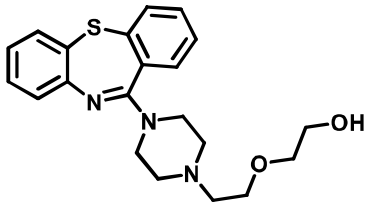
MATERIALS AND METHODS

3. Materials and Methods

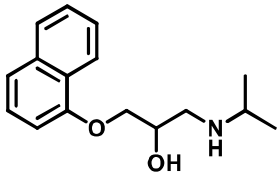
3.1. Materials

3.1.1. Chemicals

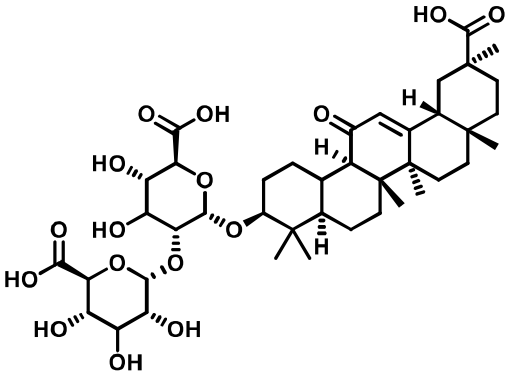
QTE [A] was procured from Micro Labs, Bangalore. Propranolol [B], GLZ [C], GA [D], QCN [E], Flufenamic acid (FA) [F], HCA[G], and Trichloroacetic acid (TCA) were purchased from Merck (Bangalore, India). The structures of all the chemicals (analytes & internal standards) are shown in Figure 3.1.



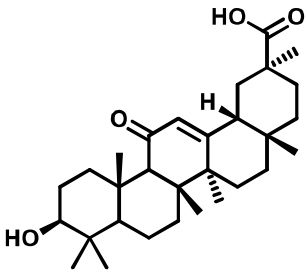
QTE (A)



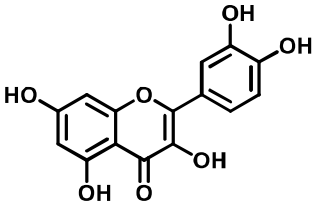
Propranolol (B)



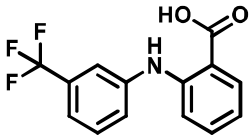
GLZ (C)



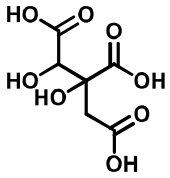
GA (D)



QCN (E)



FA (F)



HCA(G)

Figure 3.1. Chemical structures of all the analytes (A,C,D,E,G) and internal standards (B,F)

HPLC grade acetonitrile was sourced from Merck (Bangalore, India). Tablets containing *Garcinia cambogia* were purchased from Novus Life Sciences Private Limited, Mumbai, India. Ultrapure water was obtained from Millipore Elix system (Millipore, Molsheim, France). Anti-coagulating

agent heparin, 1000 IU/mL (Declot®, Zydus Cadila, India) was procured from a local pharmacy (Bangalore, India). β-Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), Itraconazole, Testosterone were procured from Sigma-Aldrich Co., St. Louis, MO, USA. All the other chemicals were of analytical grade. Blank plasma (devoid of the analyte) and blank brain homogenate was collected from healthy, adult, male Wistar rats at Invivo Biosciences and KLE College of Pharmacy, Bangalore, India.

3.1.2. Animals

Male Wistar rats weighing in the range of 200-230 g were provided by Invivo Biosciences and animal center of KLE College of Pharmacy, Bangalore, India. The rats were lodged in a controlled environment with a relative humidity of 50 ± 10% and a temperature of 25 ± 2°C on a 12 h dark light shift. The animals were acclimatized for at least four days before accepting for the experiment. The institutional animal ethics committee (IAEC) reviewed and approved the protocol for all the animal studies (Protocol no. Invivo/073 & KLE/01RU2017).

3.1.3. Instruments

An Acquity UPLCTM system (Waters Corporation, Milford, USA) was used for chromatographic separations. For quantitation of HCA, the mass analyzer was API 4000 MS/MS (Applied Biosystems, MDS Sciex, Canada) and quantitation of QCN, GLZ, GA and QTE were performed on API 5500 MS/MS (Applied Biosystems, MDS Sciex, Canada). For metabolite identification studies, Q-Exactive-OrbitrapTM (Thermo Scientific, USA), high-resolution mass spectrometer (HRMS) was used.

3.1.4. Other Instruments Used in the Study

- Analytical Balance (Sartorious)
- Ultra Sonicator (Branson)
- Vortex Shaker (Spinix)
- Micropipettes and Multipettes (Eppendorf)
- Refrigerated Centrifuge (Eppendorf)
- Biomedical Freezer (-20ºC) (Sanyo)
- Syringe (Hamilton) and Magnetic Stirrer (Remi)

3.1.5. Software

- Analyst® version 1.6
- XcaliburTM version 4.3

- Kinetica™ edition version 7.0 for calculation of PK parameters
- ADMET Predictor ® version 9.0 from GastroPlus®
- Microsoft Excel

3.1.6. LC Columns

- Acquity HILIC (150 x 2.1 mm, 1.7 μm), Waters Corporation, USA
- Kinetex C18 (50 × 2.1 mm, 1.7 μm), Phenomenex, USA.
- Acquity UPLC HSS T3 column (100 x 2.1 mm, 1.7 μm), Waters Corporation, USA.

3.2. Preparation of Rat Liver S9 Fraction

Three male Wistar rats (control) weighing 200-230 g were used. All rats were sacrificed by decapitation under anesthesia. Livers were removed quickly and placed in ice-cold saline. The livers were immediately homogenized in three volumes (3X) of the ice-cold buffer of pH 7.4 containing 50 mM Tris hydrochloric acid, 150 mM potassium chloride, and 1 mM ethylenediaminetetraacetic acid. All subsequent steps were performed under refrigerated conditions of 2-5 °C. The homogenate from rat liver was centrifuged at 9000 × g for 30 minutes at 4°C and the supernatant fraction (RLS9) was obtained. The protein concentration in the homogenate was determined by the Bradford method using bovine serum albumin (BSA) as the standard. The isolated RLS9 fraction was stored in a freezer (-20 °C) until further use [1].

3.3. Preparation of Standard Solutions, Calibration Standards (CS) and Quality Control (QC) Samples

3.3.1. HCA

Normal saline (0.154 M sodium chloride in water) and dimethyl sulphoxide (DMSO) were used to prepare stock solutions of HCA (10 mg/mL) and FA (IS, 1 mg/mL), respectively. 1 μL of HCA stock solution was diluted further with 99 μL of the normal saline to yield 100000 ng/mL ULOQWS (upper limit of quantitation of working solution). Serial dilutions of ULOQWS with normal saline yielded working standard solutions (40000, 20000, 10000, 5000, 2500, 1250, 625 and 210 ng/mL). 2.5 μL aliquot of each working standard solution was spiked in 47.5 μL of blank rat plasma to prepare the calibration standards. The resulted concentrations were 5000, 2000, 1000, 500, 250, 125, 62.5, 31.25 and 10.5 ng/mL of HCA in rat plasma. A separate stock solution of HCA (2 mg/mL) in normal saline was used for the preparation of working quality control (QC) samples, which were further diluted with blank rat plasma to generate lower limit of quantification (LLOQQC), low (LQC), medium (MQC) and high (HQC) quality control samples and

concentrations were 10.5, 31.25, 2500 and 3750 ng/mL, respectively. For every batch submitted for analysis, individual blank and mixture of IS and blank were also included. The stock of FA (IS) was diluted in 10% *v/v* TCA in water to obtain a concentration of 500 ng/mL.

3.3.2. QCN, GLZ, GA and QTE

The stock solutions of the standards of QCN, GLZ, GA, and QTE were prepared by weighing an accurate amount and dissolving in methanol to achieve a final concentration of 10 mg/mL. Stock solutions of propranolol and FA (IS, 100 μg/mL) separately were prepared in acetonitrile. The working solutions of IS (200 ng/mL of FA and propranolol, each) were obtained after dilution of the stock solutions for IS. The standard stock solutions were further diluted to prepare working solutions. The working solutions of the standards at concentrations of 10000, 5000, 2500, 500, 250, 50, 20, and 10 ng/mL for QCN, GLZ and GA; 1000, 500, 250, 50, 25, 5, 2, and 1 ng/mL for QTE, were obtained by serially diluting the stock solutions with a mixture of methanol and water (90:10, *v/v*) solution. All the stock solutions were stored in the refrigerator at 5 ± 3°C. The calibration solutions (CS), in the range of 1.0-1000.0 ng/mL for QCN, GLZ and GA and 0.1-100 ng/mL for QTE, were made by mixing 10 μL of working solution with 90 μL of rat blank plasma. A separate stock solution of QCN, GLZ, GA and QTE (10 mg/mL) in methanol was used for the preparation of working quality control (QC) samples, which were further diluted with blank rat plasma to generate lower limit of quantification (LLOQQC), low (LQC), medium (MQC) and high (HQC) quality control samples and the respective concentrations were 1.0, 2.0, 50.0, and 750.0 ng/mL for QCN, GLZ and GA, each and 0.1, 0.2, 5 and 75 ng/mL for QTE. For every batch submitted for analysis, individual blank and mixture of IS and blank were also included. All working standard/quality control solutions were stored at 5 ± 3 °C and all plasma samples were stored at -20 ± 5 °C until analysis.

3.4. Incubation of HCA, QCN and GLZ in RLS9 for Metabolite Identification

All the incubations were performed at 37 °C in an incubator and the total volume of incubation was 500 μL. Stock solutions of HCA, QCN and GLZ (10 mg/mL) were prepared as described in Section 3.3. Organic solvent concentration in the incubation solution was maintained at less than 0.5% (*v/v*). RLS9 was thawed to 37 °C before using for the experiment. Metabolism of HCA, QCN and GLZ was performed by using RLS9 in 100 mM potassium phosphate buffer of pH 7.4. The protein concentration was 1 mg/mL, and the HCA, QCN and GLZ concentration was 10 μg/mL. The control incubations were executed without HCA, QCN, GLZ, or NADPH. After pre-

incubation for 5 minutes, the metabolic reaction was kick-started by adding the NADPH cofactor solution (2.0 mM). After 60 minutes, the reactions were quenched *via* the addition of acetonitrile (1 mL) and the samples were centrifuged at 10,000 rpm for 10 minutes subsequently before injecting on to the mass spectrometer [2].

3.5. Incubation of QTE in RLS9 for Half-Life Determination

The stock solution of QTE (1 mg/mL) was prepared as described in Section 3.3 and the concentration of the organic solvent was maintained at less than 0.5% *v/v*. The incubation volume was ~ 500 μL. RLS9 was thawed to 37 °C before initiating the experiment. Metabolism of QTE was executed using RLS9 in 100 mM potassium phosphate buffer of pH 7.4. The protein concentration was 1 mg/mL, and the QTE concentration was 1 μg/mL. After pre-incubation for 5 minutes at 37 °C, the reaction was initiated through the addition of the NADPH cofactor solution (2.0 mM). Samples at different time points were taken (0, 5, 10, 20, 30, 60 minutes) and the reactions were quenched by adding 150 μL acetonitrile, and subsequently, the samples were centrifuged at 10,000 rpm for 10 minutes [3,4].

3.6. HRMS Method for Metabolite Identification of HCA, QCN and GLZ

The incubation samples were chromatographically separated on an Acquity HILIC (150 x 2.1 mm, 1.7 μm) column for HCA and on an Acquity HSS T3 column (100 x 2.1mm, 1.8 μm) for GLZ and QCN. The mobile phase contained water (A) and acetonitrile (B). Isocratic elution was followed for metabolite samples of HCA with 70% *v/v* aqueous phase. The gradient program was as follows for GLZ and QCN: 5% B for 0-1 minute, 5%-35% B for 1-5 minutes, 35%-70% B for 5-11 minutes, 70-95% B for 11-13 minutes, 95% B for 14-16 minutes, 5% B for 16-18 minutes. The flow rate of the mobile phase was set at 0.6 mL/minute and the temperature of the auto-sampler was kept at 15 °C. High-resolution mass spectrometry was performed on a Q-Exactive quadrupole-Orbitrap tandem mass spectrometer *via* an electrospray ionization (ESI) interface operated in the negative ion mode (HCA, GA and QCN) and positive ion mode (GLZ). The ESI source parameters were optimized as follows: capillary voltage, 3 kV; capillary temperature, 300°C; sheath gas flow rate, 45 arb; auxiliary gas flow rate, 15 arb; sweep gas flow rate, 5 arb; and sheath gas heater temperature, 200 °C. The data were acquired in the centroid mode from 100 to 1000 Da. The ramp collision energy was kept at 15, 30, and 40 eV.

3.7. Mass Spectrometric Method Development

Stock solutions of all the analytes were diluted in methanol: water mixture (80:20 *v/v)*, for tuning

on the mass spectrometer to get tuning solutions (100 ng/mL).

3.7.1. Tuning of Molecules on Triple Quadrupole

To achieve the maximum MS/MS response, tuning of the analytes was performed using the following procedure.

- The syringe pump was set at 10 μL/minute flow rate and following the manual tuning, the positive or negative parameters were set depending upon the structure of the molecule.

3.7.2. Identifying, Optimizing and Confirmation of Parent Ion (Q1 MS)

- The Q1 scan was selected and the corresponding center m/z value along with scan width was provided as an input. Declustering potential (30-200V, step size of 10) and entrance potential (5-15V, step size of 3) were ramped.

3.7.3. Identifying, Optimizing and Confirmation of Product/Daughter Ion (Q3 MS)

- The selected product-ion scan was run in the scan mode with the product of respective m/z of analytes. Collision energy and exit potential were subsequently adjusted to attain the optimum response of the fragment ions.

3.8. UPLC-MS/MS conditions for HCA

The chromatographic separations were performed on an Acquity UPLC and the mass analyzer was API-4000 MS/MS. The stationary phase was Acquity HILIC (150 mm x 2.1 mm, 1.7 μm) column, maintained at 40 °C. Processed samples were placed into an auto-sampler, wherein the temperature was set at 15 ± 3 °C. Mobile phase A consisted of 10 mM ammonium bicarbonate prepared in Milli-Q water, and mobile phase B consisted of 100% acetonitrile. The sample injection volume was 2 μL with a total run time of 5 minutes. The turbo gas temperature and ion spray needle voltage were 500 °C and -4500 V, respectively. Multiple reaction monitoring (MRM) mode was selected to capture transitions of HCA: Q1 206.8 (m/z) to Q3 127 (m/z), and FA (internal standard): Q1 280 (m/z) to Q3 236 (m/z). The mass parameters for HCA were adjusted at declustering potential (DP): -50V, entrance potential (EP): -15V, collision exit potential (CXP): -20V, collision energy (CE): -15V, dwell time: 100 ms; and for the IS, corresponding DP, EP and CE values were -90V, -10V and -25V, respectively. CXP and dwell time were the same as that for HCA. Quadrupole Q1 and Q3 were set on the unit resolution. For acquisition and chromatogram data processing, the Analyst® software was used.

3.8.1. Sample Processing for HCA

The sample extraction for HCA was accomplished with single-step protein precipitation using

10% *v/v* TCA in Milli-Q water (containing 500 ng/mL of FA). In brief, 30 μL of either of the study/CS/QC samples were mixed with 150 μL of 10% *v/v* TCA in Milli-Q water and vortex mixed for 10 minutes followed by centrifugation (Eppendorf Centrifuge 5804 R, Germany) at 2755 ×g for 5 minutes at 4 °C. 2 μL of the supernatant solution was injected into the UPLC-MS/MS.

3.9. UPLC-MS/MS Conditions for QCN, GLZ, GA and QTE

Acquity UPLC™ and API-5500 MS/MS mass analyzer were used to perform UPLC-MS/MS analysis. Kinetex C18 column (50 mm × 2.1 mm, 1.7 μm) maintained at 40 °C was used as the stationary phase to carry out the separations. The processed samples were retained in an auto-sampler (maintained at 15 ± 3 °C) and 2 μL solution was injected onto the system with a run time of 2.5 minutes. The mobile phase consisted of 10 mM ammonium formate with 0.1% *v/v* formic acid in water (A) and acetonitrile (B) in binary gradient ratio with a flow rate of 0.5 mL/minute. The gradient proportion was as follows: started with 90% of A held up to 0.4 minutes; decreased to 70% by 0.6 minutes; held at 70% up to 1.6 minutes; further decreased to 20% by 1.9 minutes; maintained at the same ratio till 2.2 minutes; the composition was brought back to 90% at 2.2 minutes and maintained constant up to 2.5 minutes. The detailed mass spectrometry operating conditions are listed in Table 3.1. For acquisition and data processing, Analyst® software was used.

Table 3.1. Major working parameters for tandem mass-spectrometer method.

Parameter	Analyte					
	GLZ	GA	QCN	QTE	Propranolol	FA
Mode of analysis*	Positive	Negative	Negative	Positive	Positive	Negative
Q1, m/z	823.2	469.2	301.2	384.5	260	280.1
Q3, m/z	453.2	425.2	150.9	253.5	183.1	236.1
Source temperature, °C	550	550	550	550	550	550
Dwell time, ms	50	50	50	50	50	50
GS1, psi	40	40	40	40	40	40
GS2, psi	45	45	45	45	45	45
Ion spray voltage, V	5000	-4500	-4500	5000	5000	-4500
Entrance potential, V	10	-10	-10	10	10	-10
Declustering potential, V	120	-100	-75	120	120	-100
Collision energy, V	25	-30	-25	30	28	-15
Collision cell exit potential, V	20	-15	-10	20	20	-15

3.9.1. Sample Processing for QCN, GLZ, GA and QTE

Sample extraction for all the four analytes was performed through single step precipitation of protein using acetonitrile (containing internal standard, i.e., 200 ng/mL of FA and propranolol each). In brief, 25 μL of each study/CS/QC sample was mixed with 150 μL of acetonitrile containing IS and vortex mixed for 10 minutes followed by centrifugation (Eppendorf Centrifuge 5804 R, Germany) at 2755 ×g for 5 minutes at 4 °C. From the supernatant solution, 2 μL was injected into the UPLC-MS/MS.

3.10. Method Validation

The developed methods for the estimation of HCA, GLZ, GA, QCN and QTE were validated as per recommendations of USFDA guidelines for bioanalytical method validation [5,6].

3.10.1. System Suitability

The aqueous standards were processed at MQC level and six injections were made on the UPLC-MS/MS consecutively. The mean, standard deviation (SD) and coefficient of variation (CV) for the retention time and response of the analyte and IS were calculated.

Before evaluating system suitability on the day of analysis, at least six blank samples were used to equilibrate the system. Suitability of the UPLC-MS/MS system for the analysis of HCA containing samples was evaluated by injecting six samples containing 2500 ng/mL of HCA and 500 ng/mL of FA (MQC level). To assess system suitability for the estimation of QCN, GLZ, GA and QTE, six samples containing 50 ng/mL of QCN, GLZ, GA and 5 ng/mL of QTE and 200 ng/mL of internal standard (MQC level) were injected.

Precision (CV%) of the peak area response of HCA, QCN, GLZ, GA, QTE and their respective internal standards was calculated and the system was considered suitable for the analysis, only if the CV% was $\leq 10\%$

3.10.2. Selectivity & Specificity

Six blank matrix and six LLOQ standards prepared from six different lots of plasma were processed. The screened and accepted batches of the biological matrix were used to make spiked calibration curve standards (CS) and quality control (QC) samples from six rats. The samples were analyzed and evaluated for any potential interferences with the endogenous components at the retention time of analyte and internal standard (IS) by comparing the response in the blank matrix against the mean response of the extracted LLOQ standard. For HCA, the samples were injected in the order of extracted blank (BLK), ULOQ (5000 ng/mL), extracted blank and LLOQ

(10.5 ng/mL). For QCN, GLZ, GA and QTE, the order was: extracted blank (BLK), ULOQ (1000 ng/mL for QCN, GLZ, GA and 100 ng/mL for QTE), extracted blank and LLOQ (1 ng/mL for QCN, GLZ, GA and 0.1 ng/mL for QTE). The methods were accepted, if:

i. The response of the interfering peak(s) at the retention time(s) of the analyte(s) was $<$ 20% of the mean LOQ response.
ii. The response of the interfering peak(s) at the retention time(s) of IS was $<$ 5% of the mean of IS response.
iii. At least 80% of the screened matrix lots met the above acceptance criteria (i & ii).
iv. The lowest standard (LLOQ) with S/N ratio $\geq$ 10, had $\leq$ 20% variance (% CV; precision) and accuracy within ± 20% of the nominal spiked concentration.

3.10.3. Sensitivity

Six LLOQ standards were processed as per the method and analyzed with an accuracy and precision batch. An extracted standard of 10.5 ng/mL for HCA, 1 ng/mL for QCN, GLZ, GA and 0.1 ng/mL for QTE was used to establish the LLOQ of the methods.

The limit of quantification was acceptable, if both the criteria of precision (CV $\leq$ 20%), and accuracy (± 20%) were met.

3.10.4. Linearity

Linearity was assessed for at least five calibration curves generated using spiked samples. A minimum of eight standard points excluding blanks were considered for each matrix based standard curve.

The methods were considered linear in the evaluated range if at least 75% of the non-zero standards met the following criteria (including the LLOQ & ULOQ samples).

i. No more than ± 20% deviation of the LLOQ standard from nominal concentration.
ii. No more than ± 15% deviation of the standards other than LLOQ from nominal concentration.

3.10.5. Accuracy & Precision

For accuracy and precision determination, calibration standards (CS) and quality control (QC) samples were prepared and processed as per the method. The mean concentrations, standard deviation, accuracy and precision at each LLOQQC, LQC, MQC and HQC concentration level were determined. Both, intra (within)- and inter (between)- batch accuracy and precision were calculated by analyzing five validation batches.

Intra-day accuracy and precision were assessed through the analysis of QC samples at four diverse levels (n = 6 for each level) on the same day. The different levels were selected to establish the performance of the method and to define the LLOQ. The ULOQ was determined through the highest level on the calibration curve. Samples with a concentration above the established ULOQ were diluted before re-analysis. To confirm the inter-day accuracy and precision, the samples from the intra-day accuracy study were analyzed on five different days. For HCA, the calibration curve was established through spiking plasma (blank) samples with HCA to get nine concentrations in the range of 10.5 - 5000 ng/mL. The accuracy and precision were determined by analyzing six replicates of the QC samples (LLOQQC, LQC, MQC and HQC) in rat plasma and read against the calibration curve on the same day. Further, calibration curves were prepared by spiking the blank plasma samples to achieve eight different concentrations in the range of 1-1000 ng/mL for QCN, GLZ, GA and 1-100 ng/mL for QTE. The accuracy and precision of the method were established by analyzing six replicates of QC samples (LLOQQC: 1 ng/mL; LQC: 2 ng/mL; MQC: 50 ng/mL and HQC: 750 ng/mL for QCN, GLZ, GA and LLOQQC: 0.1 ng/mL; LQC: 0.2 ng/mL; MQC: 5 ng/mL and HQC: 75 ng/mL for QTE) in rat plasma and read against the calibration curve on the same day to obtain intra-day precision and accuracy.

The linearity of the developed methods was deemed suitable for the intended purpose of analysis if the following acceptance criteria were met for five validation batches including ruggedness batch.

Precision: The CVs of $\leq 15\%$ for LQC, MQA and HQC and CV of $\leq 20\%$ for LLOQQC were considered acceptable.

Accuracy: For between (inter)- and within (intra)- batch mean concentrations, a deviation within ±15% of the nominal value at LQC, MQC and HQC and within ± 20% at the LLOQ QC concentration was accepted.

3.10.6. Recovery

Six aliquots of spiked quality control samples at three different concentrations corresponding to LQC, MQC and HQC from precision and accuracy batches were withdrawn from the freezer for recovery experiments. The samples were treated as per the method, injected on to the UPLC-MS/MS system and the absolute peak responses were recorded. Simultaneously, aqueous spiked standards of concentrations corresponding to LQC, MQC and HQC were analyzed for their chromatographic peak responses. The mean response at each concentration level of the extracted

standard(s) and the aqueous spiked standard was calculated and compared to mean response of the spiked standards (LQC, MQC and HQC). The percentage recovery for each batch of LQC, MQC and HQC was calculated by using the formula given below:

$$\% \text{ Recovery} = \frac{\text{Mean Peak Response of Extracted Samples}}{\text{Mean Peak Response of Aqueous Samples}} \times 100 \quad \textbf{Formula 3.1}$$

The mean % recovery, standard deviation (SD) and coefficient of variation (CV) for each concentration of LQC, MQC and HQC were calculated.

3.10.7. Matrix Effect

Four different lots of biological matrices at a concentration equivalent to LQC, MQC and HQC in six replicates were prepared and processed. The samples were analyzed along with one set of calibration curve standards prepared from a different lot of screened plasma. The mean concentration of QC samples was then calculated against calibration curve for the individual lots. The accuracy within ±15% of the nominal value(s) at LQC, MQC and HQC levels from the six different lots and less than 15% CV (precision) were criteria to establish no significant matrix effect during the analyses.

3.10.8. Stability

Stock Solution Stability

The stock solutions of the analyte(s) and internal standard(s) were prepared and stored in the refrigerator (5 ± 3 ºC) for at least 60 days. The stability samples, i.e., analyte(s) and IS were withdrawn and allowed to reach room temperature before preparing their appropriate dilutions. Consecutively, fresh stock of analyte(s) and internal standard(s) were prepared (comparison sample). Six consecutive injections for stability and comparison samples were made on UPLC-MS/MS and their peak responses were recorded.

The stability of the stock was evaluated by comparing the mean response of freshly prepared stock solution after applying the correction factor of concentration of analyte(s) or IS (comparison samples) to that of the stored stocks (stability samples) as per the formula given below.

$$\% \text{ Stability} = \frac{\text{Mean Area response of stability stock X Conc. of comparison stock}}{\text{Area response of comparison stock X Conc. of stability stock}} \times 100 \quad \textbf{Formula 3.2}$$

Percent stability of the analyte and IS stocks was considered appropriate if it was within the range of 90-110%.

Long Term Sample Stability in Matrix

The long-term sample stability in the matrix was performed at -20 ºC. Following an appropriate storage period, six replicates of LQC, MQC & HQC samples were removed from the freezer and thawed to room temperature. The samples were then processed and analyzed against freshly spiked calibration standards and QC samples (comparison samples).

The long-term sample stability was evaluated by comparing the mean of back-calculated concentration of the stability samples to the mean of back calculated concentration of the comparison samples. The stability duration in days was established as the difference between the date of analysis of QC samples (stability samples) and the date of preparation of spiked stability QC samples (i.e., date of spiking).

The stability of the analyte(s) in the matrix for the duration of the storage was accepted if it was within the range of 85%-115%.

Auto-Injector Stability

The stability duration and temperature for auto-injector stability was selected based on the characteristics of the analyte and anticipated run-time for the batch be used for the sample analysis. For the assessment, 6 QC samples were retrieved from the freezer at each of LQC, MQC & HQC concentrations and processed. The processed samples were then placed in an auto-sampler (stability samples).

Following the required stability period, the samples (freshly processed comparison sample) along with stability samples stored in auto-sampler were prepared and injected. The auto-sampler stability was evaluated by comparing the mean of back-calculated concentration of stability samples against the mean back-calculated concentration of the comparison sample.

The duration of auto-sampler stability was calculated from the time of keeping QC sample in the auto-sampler to the last injection of stability QC samples. The temperature of the storage of samples in the auto-sampler was accordingly defined.

If the concentration of the analyte from the matrix was within the range of ± 15%, it was deemed stable for the stated duration at the particular temperature in the auto-sampler.

Bench Top Stability in Matrix

Each of LQC, MQC & HQC samples (n=6) were retrieved from the freezer and thawed at room temperature for 6 h. After 6 h, another set of QC and calibration curve samples were taken out from the freezer and thawed. All the sets of low, mid and high QC samples were processed and

analyzed using a freshly processed calibration curve along with QC samples. The stability was evaluated by comparing the mean of back-calculated concentrations of stability samples against the mean of back-calculated concentrations of freshly processed comparison samples.

The bench-top stability of the analyte(s) in the matrix was considered appropriate if the concentration was within the range of 85%-115% of the nominal value.

Freeze-Thaw Stability in Matrix

At least 3 cycles of freeze-thaw (FT-C1, FT-C2 & FT-C3) were evaluated for stability on three identified sets of samples, containing six replicates of each LQC, MQC & HQC samples. All the sets of the freeze-thaw stability samples were stored at the recommended temperature in the freezer. After a minimum of 24 h freezing, all three sets from the freezer were retrieved and kept at room temperature up to complete thawing and subsequently vortexed. After the completion of the FT-C1, the samples were restored at the same freezing temperature. After a minimum of 12 h freezing, FT C-2 and FT C-3 sets were retrieved from the freezer and kept at room temperature up to complete thawing and vortexed. This was again repeated for FT C-3 samples and all the samples were treated as per the method with a set of freshly spiked calibration curve standards and quality control samples. The freeze-thaw stability was evaluated by comparing the mean of back-calculated concentrations of stability samples against the mean of back-calculated concentrations of freshly spiked comparison samples.

Similar to other stability evaluations, acceptance criteria for freeze-thaw stability was to have a concentration within the range of 85- 115% of the nominal value at LQC, MQC & HQC levels.

3.10.9. Dilution Integrity

Spiked dilution integrity standard with a concentration equivalent to 2 times of the highest calibration curve standard was prepared and aliquot into different pre-labeled polypropylene tubes and stored in the freezer below -20ºC. Six samples were prepared by diluting this standard 10 times with the screened biological matrix and identified as 1/10 dilution integrity samples. An appropriate volume was aliquot into pre-labeled sample tubes. The dilution integrity was conducted through six replicates of 1/10 dilution integrity samples. The samples were analyzed along with calibration curve standards by using the proposed sample extraction and chromatographic/mass spectrometry conditions.

The dilution integrity was deemed acceptable if the mean % nominal was within ±15% and CV for the replicates was $\leq$ 15%.

3.10.10. Incurred Sample Re-analysis

At least two samples from each animal at C_{max} and elimination phase were analyzed for incurred sample re-analysis(ISR). The following formula was used to calculate the difference in the results from the original analysis and re-analyzed samples.

$$ISR = \left\{ \frac{\text{Re-analyzed concentration - Original concentration)}}{\text{Original concentration}} \right\} X\ 100 \ldots\ldots\ldots \textbf{Formula 3.3}$$

The results were considered acceptable if, for at least 2/3rd of the selected samples, the percent difference was within 20%.

3.11. Pharmacokinetic (PK) Studies

For each PK study, healthy male Wistar (n = 6) rats, weighing in the range of 200-230 g were used.

3.11.1. HCA

The experiments were conducted in group I (HCA, 1 mg/kg dissolved in normal saline administered via *i.v.* bolus); group II (HCA, 20 mg/kg dissolved in normal saline; administered *p.o.*); and group III (*Garcinia* commercial formulation, 20 mg/kg suspended in 0.5% *w/v* carboxymethyl cellulose; administered *p.o*). Animals were fasted for at least 12 h before the dosing with *ad libitum* access to water for groups II and III. Animals were allowed to access chow after 4 h of dosing. The blood samples were taken from retro-orbital sinus at 0.08, 0.5, 1, 3, 4, 6, 8 and 24 h after dosing (group I) and 0.25, 0.5, 1, 3, 4, 6, 8, and 24 h after dosing (group II and III). The overall study design is summarized in Table 3.2.

Table 3.2. Study design for the estimation of pharmacokinetic parameters of HCA in Wistar rats.

Group	Chemical Dosed	Route of Administration/12 h fasting before dosing (Yes/No)	Dose (mg/kg)	Sampling Time Points (h)
I	HCA	*i.v.*/No	1	0.08, 0.5, 1, 3, 4, 6, 8 and 24
II	HCA	*p.o.*/Yes	20	0.25, 0.5, 1, 3, 4, 6, 8 and 24
III	*Garcinia*	*p.o.*/Yes	20	0.25, 0.5, 1, 3, 4, 6, 8 and 24

3.11.2. QCN and GLZ

The experiments were conducted in group I each for QCN and GLZ. For both the groups, a dose of 10 mg/kg suspended in 0.5% *w/v* carboxy methylcellulose was separately prepared and administered *via* oral gavage. Animals were fasted for at least 12 h before the dosing with *ad libitum* access to water for both the groups. Animals were allowed to access chow after 4 h of

dosing. The study design of the pharmacokinetic study for QCN and GLZ is summarized in Table 3.3.

Table 3.3. Study design for the estimation of pharmacokinetic parameters of QCN and GLZ in Wistar rats.

Group	Chemical Dosed	Route of Administration/12 h fasting before dosing (Yes/No)	Dose (mg/kg)	Sampling Time Points (h)
I	QCN	*p.o.*/Yes	10	0.25, 0.5, 1, 3, 4, 6, 8 and 24
I	GLZ	*p.o.*/Yes	10	0.25, 0.5, 1, 3, 4, 6, 8 and 24

The heparin containing blood (22 IU heparin/mL of blood) was centrifuged at 4000 rpm for 8 minutes at 5 °C to obtain the plasma and the same was stored at -20 °C prior to analysis. Samples collected at different time points were treated as per the method and read against the calibration curve(s) prepared in the blank rat plasma. The plasma concentration profiles with respect to time were studied by Kinetica™ edition (version 7.0) software (San Francisco, CA, USA) using non-compartmental analysis. The absolute bioavailability (% F) was calculated using the following formula:

$$F\ (\%) = ([AUC_{0\text{-last}}\ (\text{oral}) \times \text{Dose}\ (i.v.)) / [AUC_{0-\text{last}}\ (i.v.) \times \text{Dose}\ (p.o.)]) \times 100\% \quad \textbf{Formula 3.4}$$

3.12. HCA-QTE Pharmacokinetic Interaction Study

To evaluate the effects of HCA on the pharmacokinetics of QTE, animals were divided into four groups (n=6 for each group). The animals were treated with HCA at a dose of 20 mg/kg/day, *p.o.* (group I and III) or vehicle, *p.o.* (group II and IV) for 7 days. On the seventh day, after 1 h of HCA and vehicle administration, QTE (10 mg/kg, *p.o.*) was administered to rats (group I and II). For group III and IV, on the seventh day, after 1 h of HCA and vehicle administration, QTE (1 mg/kg, *i.v.* bolus) was given to the animals through a saphenous vein. Briefly, each animal from all the groups was anesthetized on the evening before QTE administration for insertion of a polyethylene cannula in a tail vein to be used for serial blood sampling. Anesthesia was achieved by the use of pentobarbital (60 mg/kg, *i.p.*). Animals (groups I and II) were submitted to an overnight fasting period, with free access to water, before QTE administration. To avoid the food effect on the pharmacokinetics of QTE, the fasting period was maintained for at least 4 h after its administration. Animals in groups III and IV were not fasted, as they were dosed through an intravenous route.

Approximately 0.25 mL of the blood sample was collected into heparin containing tube (22 IU heparin/mL of blood) at 0.25, 0.5, 1, 3, 5, 7, and 24 h after *p.o.* administration of QTE and 5 minutes, 0.25, 0.5, 1, 3, 5, 7, and 24 h after *i.v.* administration of QTE. The study design is summarized in Table 3.4.

Table 3.4. Study design for understanding HCA-QTE pharmacokinetic interaction.

Group	Perpetrator (Dose (mg/kg)/Route of administration/Days of administration)	Victim Studied (Dose (mg/kg)/Route of Administration/Fasting 12 hr before dosing (Yes/No))	Sampling Time Points (h)
I	HCA (20 mg/kg/*p.o.*/7)	QTE (10 mg/kg/ *p.o.*/Yes)	0.25, 0.5, 1, 3, 4, 6, 8 and 24
II	Vehicle (*p.o.*/7)	QTE (10 mg/kg/ *p.o.*/Yes)	0.25, 0.5, 1, 3, 4, 6, 8 and 24
III	HCA (20 mg/kg/*p.o.*/7)	QTE (1 mg/kg/*i.v.*/No)	0.08, 0.5, 1, 3, 4, 6, 8 and 24
IV	Vehicle (*p.o.*/7)	QTE (1 mg/kg/*i.v.*/No)	0.08, 0.5, 1, 3, 4, 6, 8 and 24

To obtain plasma, all the blood samples were centrifuged at 4000 rpm at 5 °C for 8 minutes. The animals were euthanized and the brains were collected from all four groups after terminal sampling. All the brain samples were homogenized in phosphate buffer saline (PBS) buffer. The plasma samples and brain homogenates were stored separately at -20 °C until analysis.

3.13. QCN-QTE Pharmacokinetic Interaction Study

To evaluate the effects of QCN on the pharmacokinetics of QTE, animals were divided into four groups (n=6 for each group). The animals were pre-treated with QCN at a dose of 10 mg/kg/day (group I and III) or vehicle (group II and IV) for 7 days. On the seventh day, after 1 h of QCN and vehicle administration QTE (10 mg/kg, *p.o.*) was administered to rats (group I and II) through oral gavage. On the seventh day, after 1 h QCN and vehicle administration, QTE (1 mg/kg, *i.v.* bolus) was administered to rats (group III and IV) through a saphenous vein. Briefly, each animal of all groups was anesthetized on the evening before QTE administration for insertion of a polyethylene cannula in a tail vein to be used for serial blood sampling. Anesthesia was performed by pentobarbital (60 mg/kg, *i.p.*). Animals (groups I and II) were submitted to an overnight fasting period, with free access to water, before QTE administration. To avoid the effect of food on QTE pharmacokinetics, the fasting period was maintained for at least 4 h after its administration. Animals in groups III and IV were not fasted, as they were dosed through an *i.v.* route. The procedure for serial blood sampling was similar to the one explained above for the HCA-QTE pharmacokinetic interaction study (Section 3.12). No blood sample could be collected from

animals belonging to group III (pre-treated with QCN) as after *i.v.* administration of QTE (1 mg/kg), all the animals succumbed to death within 3-5 minutes of dosing. The study design is summarized in Table 3.5.

Table 3.5. Study design for understanding QCN-QTE pharmacokinetic interaction.

Group	Perpetrator (Dose (mg/kg)/Route of administration/Days)	Victim Studied (Dose (mg/kg)/Route of Administration/12 h Fasting before dosing (Yes/No))	Sampling Time Points (h)
I	QCN (10 mg/kg/*p.o.*/7)	QTE (10 mg/kg/ *p.o.*/Yes)	0.25, 0.5, 1, 3, 4, 6, 8 and 24
II	Vehicle (*p.o.*/7)	QTE (10 mg/kg/ *p.o.*/Yes)	0.25, 0.5, 1, 3, 4, 6, 8 and 24
III	QCN (10 mg/kg/*p.o.*/7)	QTE (1 mg/kg/*i.v.*/No)	--
IV	Vehicle (*p.o.*/7)	QTE (1 mg/kg/*i.v.*/No)	0.08, 0.5, 1, 3, 4, 6, 8 and 24

The processing of the study samples was similar to that of the ones from HCA-QTE pharmacokinetic interaction study (Section 3.12).

3.14. GLZ-QTE Pharmacokinetic Interaction Study

Similar to the pharmacokinetic interaction studies of HCA-QTE, to evaluate the effects of GLZ on QTE pharmacokinetics, the rats were divided into four groups (n=6 for each group). The animals were pre-treated with GLZ at a dose of 100 mg/kg/day (group I and III) for 7 days. The vehicle groups (group II and IV) were the same as that of the ones used in QCN-QTE interaction study, (see Section 3.13), as the formulation vehicle was same for both the studies. On the seventh day, after 1 h of GLZ or vehicle administration, QTE (10 mg/kg, *p.o.*) was administered to rats (group I and II). On the seventh day, after 1 h GLZ or vehicle administration, QTE (1 mg/kg, *i.v.* bolus) was administered to the animals (group III and IV) through a saphenous vein. Each animal from all the groups was anesthetized on the evening before QTE administration for insertion of a polyethylene cannula in a tail vein that was later used for serial blood sampling. Anesthesia was performed by using pentobarbital (60 mg/kg, *i.p.*). Animals (groups I and II) were kept on overnight fasting, with free access to water, before QTE administration. To avoid the effect of food on QTE pharmacokinetics, the fasting period was maintained till 4 h after its administration. Animals in groups III and IV were not fasted. The procedure for serial blood sampling was similar to the one explained above for HCA-QTE pharmacokinetic interaction study (Section 3.12). The overall study design to understand this interaction potential is summarized in Table 3.6.

Table 3.6. Study design for understanding GLZ-QTE pharmacokinetic interaction.

Group	Perpetrator (Dose (mg/kg)/Route of administration/Days)	Victim Studied (Dose (mg/kg)/Route of Administration/Fasting 12 hr before dosing (Yes/No))	Sampling Time Points (h)
I	GLZ (100 mg/kg/*p.o.*/7)	QTE (10 mg/kg/ *p.o.*/Yes)	0.25, 0.5, 1, 3, 4, 6, 8 and 24
II	Vehicle (*p.o.*/7)*	QTE (10 mg/kg/ *p.o.*/Yes)	0.25, 0.5, 1, 3, 4, 6, 8 and 24
III	GLZ (100 mg/kg/*p.o.*/7)	QTE (1 mg/kg/*i.v.*/No)	0.08, 0.5, 1, 3, 4, 6, 8 and 24
IV	Vehicle (*p.o.*/7)*	QTE (1 mg/kg/*i.v.*/No)	0.08, 0.5, 1, 3, 4, 6, 8 and 24

* Same groups as that of the ones used in QCN-QTE interaction study (Table 3.5)

Sample processing for the study was similar to that of HCA-QTE pharmacokinetic interaction study (as explained in Section 3.12).

3.15. Pharmacokinetic (PK) Data analysis

The peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were obtained from the experimental data. The plasma concentration profile was analyzed by Kinetica™ edition software version 7.0 (San Francisco, CA, USA) using the non-compartmental analysis. $AUC_{0-\infty}$, [the AUC from time zero to infinity] was calculated from equation $AUC_{0\text{-last}} + (C_{last}/k_{el})$, where C_{last} is the quantifiable concentration and k_{el} is the apparent elimination rate constant calculated by log-linear regression of the elimination segment of the concentration-time profile. The concentrations below the lower limit of quantification of the assay were taken as zero for all calculations. All the pharmacokinetic parameters are expressed as the $\text{mean} \pm \text{SD}$. Statistical analyses of main pharmacokinetic parameters were analyzed for significance using the independent sample Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

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