BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

5. Bioanalytical Method Development and Validation

5.1. Bioanalytical Method Development and Optimization

A bioanalytical method altogether comprises the following procedures: the collection, addition of anti-coagulant, processing, storing, and analysis of biological samples for an analyte. Bioanalytical methods used for quantification of drugs and their respective metabolites in biological matrices are the key factors in generating vigorous, reproducible and consistent data that is used for the understanding of pharmacokinetic parameters [1,2].

Method development includes optimization of the various stages of sample processing, chromatographic resolution and detection. An elaborative literature survey on the bioanalytical methods on the same or similar analytes is of key importance. Some of the parameters that are frequently evaluated during bioanalytical method development and optimization are selection of internal standard, detector, column, mobile phase, organic modifier and sample preparation techniques. These are discussed in details as follows:

5.1.1. Selection of Internal Standard (IS)

Internal standards are commonly used for the quantitation of analytes in biological matrices. Selecting the appropriate internal standard is a vital aspect to attain acceptable method performance, especially with LC-MS/MS, where the matrix effect can lead to ambiguous results. Ideally, stable isotopically labeled internal standards for all analytes should be used, but often these are costly and not commercially available. Therefore, commercially available IS are opted generally [3]. One of the major advantages of using internal standard is that it reduces errors resulting from partial recovery of the analyte during the sample processing and hence reliability on the quantitative analysis is enhanced [4].

5.1.2. Detector Selection

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is typically the preferred technique for the rapid and ultra-sensitive quantification of small molecules, oligonucleotides, peptides, and proteins in different biological matrices including but not limited to plasma, serum, blood, urine, faeces, and tissues. Although MS (or MS/MS) is the typical detector for bioanalytical assays, due to its high cost, it is difficult for small laboratories to obtain and maintain it [5]. For analytes with reasonable UV absorbance, the UV/Vis detector (or the PDA detector) is a preferred choice. UV/Vis detectors are consistent, sensitive, easy-to-use, and very precise. To improve sensitivity, the maximum absorbance wavelength of the analyte is commonly used for the analysis. For the analytes without any chromophore, the choice is limited to charged aerosol detector (CAD), evaporative light scattering detector (ELSD) or

refractive index (RI) detector. While RI is a very sensitive detector, it has limited utility when it comes to gradient programming [6].

5.1.3. Selection of Column

The core of an LC system is the column. The analyte of interest and the aim of the analysis are primary determinants for the selection of columns [7]. The resolution of analytes will be primarily dependent on the selection of the column. Silica is the most common matrix for LC columns [8]. Silica matrices are easily derivatizable, chemically stable, robust, have a consistent sphere size and do not tend to compress under pressure. The particle size of the silica determines separation. In reverse phase chromatography, the non-polar stationary phase and the polar mobile phase cause the polar peaks to usually elute earlier than non-polar peaks. Due to steric hindrance, only ~ 1/3rd of the surface silanols are derivatized. Peak tailing can be observed due to the interaction of the analyte with the remaining free silanols. Selecting the right column chemistry can help with expediting the overall method development process.

For very polar compounds, that are not amenable to be retained on traditional reverse stationary phases, alternate column chemistries, *viz.*, HILIC, phenyl or cyano offer better selectivity and resolution [7,8].

5.1.4. Mobile Phase Selection

Water is typically the most abundant mobile phase used in the reverse phase chromatography. For ionizable analytes, the mobile-phase pH must be chosen based on the analyte pKa so the target analyte elutes in single dominant ionization states, either ionized or neutral [7,8]. Optimum buffering capacity occurs at a pH equivalent to the pK_a of the buffer. However, for LC-MS analysis, one has to use volatile buffers (ammonium acetate, formate, bicarbonate salts) and non-volatile buffers like phosphate, carbonate, etc cannot be used.

5.1.5. Organic Modifier

When the sample components are more hydrophobic, then they are retained in the column for relatively long periods and then the separation occurs. The normally used mobile phases are the combinations of water and organic polar solvents, mostly acetonitrile or methanol. The additives help to enhance efficiency and/or selectivity of the separation, mostly due to control of their retention. The selectivity of the developed method is also affected by changing the solvent from methanol (protic solvent) to acetonitrile (aprotic solvent). Acetonitrile/water mixtures show around 2.5 times lower viscosity than equivalent methanol/water combination; this suggests that 2.5 faster flow rates with acetonitrile as an organic modifier can be used and which in turn help to develop faster separation methods [7].

5.1.6. Selection of the Sample Preparation Technique

Sample preparation is an important technique used to clean up the sample before carrying out chromatographic analysis to improve its detection. The quantification of an analyte in biological matrices yields the data used to comprehend the time course of drug action, or pharmacokinetic parameters in animals and humans, and hence is a vital module of the drug discovery and development process [9]. Most of the bioanalytical methods have a sample preparation to remove the proteins from the sample. Protein precipitation, solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are routinely used practices in bioanalytical laboratories [10].

Protein Precipitation

In protein precipitation, water-miscible organic solvents or acid are used to eliminate the plasma proteins from the sample by denaturation and precipitation. Acids, such as trichloroacetic acid (TCA) and perchloric acid, are very effective at protein precipitants [11]. The proteins at low pH (in the presence of acids) precipitate out. Organic solvents, such as acetonitrile, methanol, acetone and ethanol are also used for precipitating the proteins and have been extensively used in bioanalysis.

Solid Phase Extraction (SPE)

In SPE, the analyte of interest is retained on the solid phase while the sample passes through, followed by elution of the analyte with a suitable solvent. SPE sorbent typically consists of a 40-60 μm silica particle with a bonded hydrocarbon (e.g. C18) phase. Owing to the many diverse available choices of sorbents, SPE is a very powerful technique [11]. SPE consists of five steps, which include conditioning, equilibration, loading, washing and elution. The SPE sorbent is conditioned by passing a solvent to wet the packing material and solvate the functional groups of the sorbent. An aqueous buffer is used for equilibration. Variable recoveries can be found if SPE sorbent dries out, before loading the sample. To reduce the viscosity, samples are diluted with an aqueous buffer. Appropriate solvents are used to remove interferences.

Liquid-Liquid Extraction (LLE)

Liquid-liquid extraction (LLE) is an extraction of the biological matrix with a water-immiscible solvent. Analytes are separated according to their differential solubilities in various liquids. The analyte is partitioned between the organic and aqueous phases. The analyte should be distributed in the organic phase under the selected conditions. For effective LLE following considerations should be taken:

- The solubility of the analyte in the extracting solvent.

- The viscosity of the extracting solvent should be low enough for ease of mixing with the sample matrix.
- The boiling point of the extraction solvent should be low to facilitate easy removal at the end of the extraction.
- pH in the sample matrix
- Thorough mixing either mechanical or manual shaking or vortexing [12].

Extraction with an individual or mixture of solvents can help produce a spectroscopically clean sample and to avoid the introduction of non-volatile components onto the MS system. Clean samples are important for minimizing ion suppression or ion enhancement and matrix effect in LC-MS/MS analyses.

An important issue that can affect the quantitative performance of a mass detector is ion suppression. Sample matrix with acceptable results, co-eluting components and cross-talk can contribute to ion suppression. Ionization suppression is the consequence of high concentrations of non-volatile ingredients present in the spray along with the analyte. The effect is more usually related to the non-volatile solute, including analyte. The precise mechanism through which the non-volatile components hinder the release of analyte into the gas phase has not been established. A plausible mechanism could be the attractive force that the drop together, which accounts for the ionization suppression (matrix effect) observed with ESI. In addition to non-volatile materials, other mechanisms such as impairing agents (e.g. TFA, trifluoroacetic acid) may also show a significant role in ionization suppression. Typically, liquid-liquid extraction produces the matrix effect, followed by solid-phase extraction. The protein precipitation method has the maximum chances of ESI response suppression. On the other hand, in ESI, signal suppression or enhancement might occur due to the co-eluting of the endogenous components from the sample matrix [13].

5.1.7. Type of Bioanalytical Method Validations

Full Validation

For any NCE under investigation, a bioanalytical method is typically developed for the very first time. Full validation should be performed to support pharmacokinetic, bioavailability, and drug interaction studies for any NCE [2].

Partial Validation

Partial validations are conducted when amendments of already validated bioanalytical methods are made. Partial validation can range from one intra-assay and precision determination to closely full validation. Some of the typical bioanalytical method amendments

include but are not limited to change of matrix within species, change in analytical methodology, method transfer between laboratories or analyst, change of species within matrix [1,2].

Cross-Validation

Cross-validation is an assessment of validation limits when two or more bioanalytical methods are used. For example, data generated using diverse bioanalytical techniques like LC-MS-MS *Vs.* ELISA are included in a regulatory submission for different studies [1,2].

Currently, LC-MS/MS is the most widely used method for bioanalysis of small molecules [14-17]. The selective multiple reactions monitoring (MRM) method provides specificity and selectivity. Nowadays, ultra-high-pressure liquid chromatography (UPLC) is utilized to reduce the run time and increase sensitivity [18]. For the determination of HCA in human plasma, a gas chromatography-mass spectrometry (GC-MS) method has been previously reported [19]. However, the described procedure used a complex sample preparation including derivatization [20-22]. This is a complex multi-step procedure, which might affect the reproducibility of the method. Moreover, not all bioanalytical laboratories have access to GC-MS, making the alternative UPLC-MS/MS the preferable choice for quantitative bioanalysis. Challenges to establishing a sensitive UPLC-MS/MS-based method for the measurement of HCA included its low molecular weight (206 Da), which generally results in high background noise in MS detector, leading to poor detection limits. Also, the presence of three carboxylic acid groups makes HCA very polar and hence difficult to use commonly used stationary phases, like C-18 or C-8 for the separation. No practical UPLC-MS/MS method has been reported for the determination of HCA in a biological matrix. Part (A) of this chapter presents the efforts made to develop and validate a fast, accurate, sensitive, specific, selective, and robust UPLC-MS/MS method for the measurement of HCA in rat plasma.

The bioanalytical methods reported so far for the quantification of QCN, GLZ, GA, and QTE deal with these compounds individually [23-25]. It was going to be inefficient (time and resource consuming) to run multiple methods for our proposed studies. So far, no analytical method has been reported for the simultaneous quantification of QCN, GLZ, GA, and QTE in rat plasma. Hence, our objective was to develop and validate a simple, rapid, and sensitive UPLC-MS/MS bioanalytical method for the simultaneous determination of QCN, GLZ, GA, and QTE. Furthermore, the method was applied to study the pharmacokinetics of these compounds. The same is discussed in Part (B) of this chapter.

5.2. Mass Spectrometry and Optimization of Assay for Quantitation of HCA in Biological Matrices: Part (A)

5.2.1. Selection of Internal Standard

An internal standard is commonly used during quantitative bioanalysis to compensate for differences in recovery, ionization efficiency and liquid handling and hence minimize variability [8]. To find a suitable internal standard, compounds having carboxylic acid groups and similar sizes, such as fumaric, succinic, FA and salicylic acids were evaluated. Based on the appropriate response, reproducibility of recovery, and detection stability, FA was finalized. Additionally, no interference in the MRM channels at the relevant retention times facilitated the application of FA as the internal standard for further studies.

5.2.2. Optimization of LC and MS Parameters

As HCA is a very polar compound (clogP; -3.6), phenyl and cyano LC columns were tried, however, HCA was either not retained or showed a noticeable peak tailing (>1.1). Hydrophilic interaction liquid chromatography (HILIC) columns have been reported to retain polar compounds through electrostatic interactions, hydrophilic partitioning and hydrogen bonding, etc [20]. Further trials were hence conducted with a HILIC column. Due to MS compatibility, different ammonium buffer salts, such as formate, acetate and bicarbonate, were screened as volatile aqueous mobile phases. On a HILIC column, these additives can alter the peak shape and retention of the analyte molecule. Combinations of the three aqueous buffers (5 mM to 20 mM) with acetonitrile and methanol as organic modifiers were tested along with the altered flow-rates (in the range of 0.6-1.0 mL/min) to optimize the resolution of HCA and FA (data not shown). Factors like peak asymmetry, peak tailing, peak response and HETP for the column were evaluated, whereupon the mobile phase consisting of 10 mM ammonium bicarbonate (mobile phase A) and 100% acetonitrile (mobile phase B) was found most suitable. Further, the selected ratio of mobile phases A and B corresponded to 20:80 *v/v*, and elution was performed in isocratic mode by pumping the mobile phases at the rate of 0.5 mL/min. HCA and FA (internal standard, IS), (both 100 ng/mL dissolved in 1:1 *v/v* combination of water:acetonitrile) were infused directly in the mass spectrometer for tuning and optimization of MS parameters. Having three carboxylic acid groups and hence the ability to easily lose one proton, HCA gave a better signal in the negative mode of ionization and hence further MS parameters were optimized in this setup. Quantitation of HCA and IS were carried out by using m/z 206.8→127.0 and m/z 280→236 transitions, respectively.

5.2.3. Method Validation

Method validation was performed as described in Section 3.10.

System Suitability

Six blank samples were injected to equilibrate the system followed by an assessment of the suitability of the system for the analysis of samples. Suitability of UPLC-MS/MS for the analysis of samples was assessed by injecting six samples of 2500 ng/mL of HCA containing 500 ng/mL of FA (MQC level). Representative system suitability data are provided in Table 5.1.

Table 5.1. Representative system suitability data of HCA obtained before injecting the second Precision and Accuracy batch.

Sample Name	Peak Area of HCA	Peak Area of IS	Area Ratio
SST-1	845947	47038	17.98
SST-2	858156	49531	17.32
SST-3	877453	49265	17.81
SST-4	819486	49182	16.66
SST-5	826123	47263	17.47
SST-6	809067	47945	16.87
Average	**839372**	**48370.67**	**17.36**
SD	**25828.72**	**1094.4**	**0.52**
% CV	**3.08**	**2.26**	**3.00**

Selectivity and Carryover Effect

Selectivity and specificity of the developed method were determined as mentioned in Section 3.10.2. The plasma obtained from six different animals did not have any significant interference at the retention time of HCA or FA. Moreover, drug-free plasma samples spiked with concentrations equivalent to the LLOQ of HCA were within the span of ±10% of nominal concentration. Also, the IS response in the blank was much less than the upper limit of 5%, suggested for the average IS response of the calibrators and QCs. These results indicated acceptable selectivity of the developed method.

Blank injection after injecting ULOQ standard did not show significant interference confirming a lack of carryover effect. Representative chromatograms of blank injected after ULOQ standard, LLOQ of HCA are depicted in Figure 5.1.

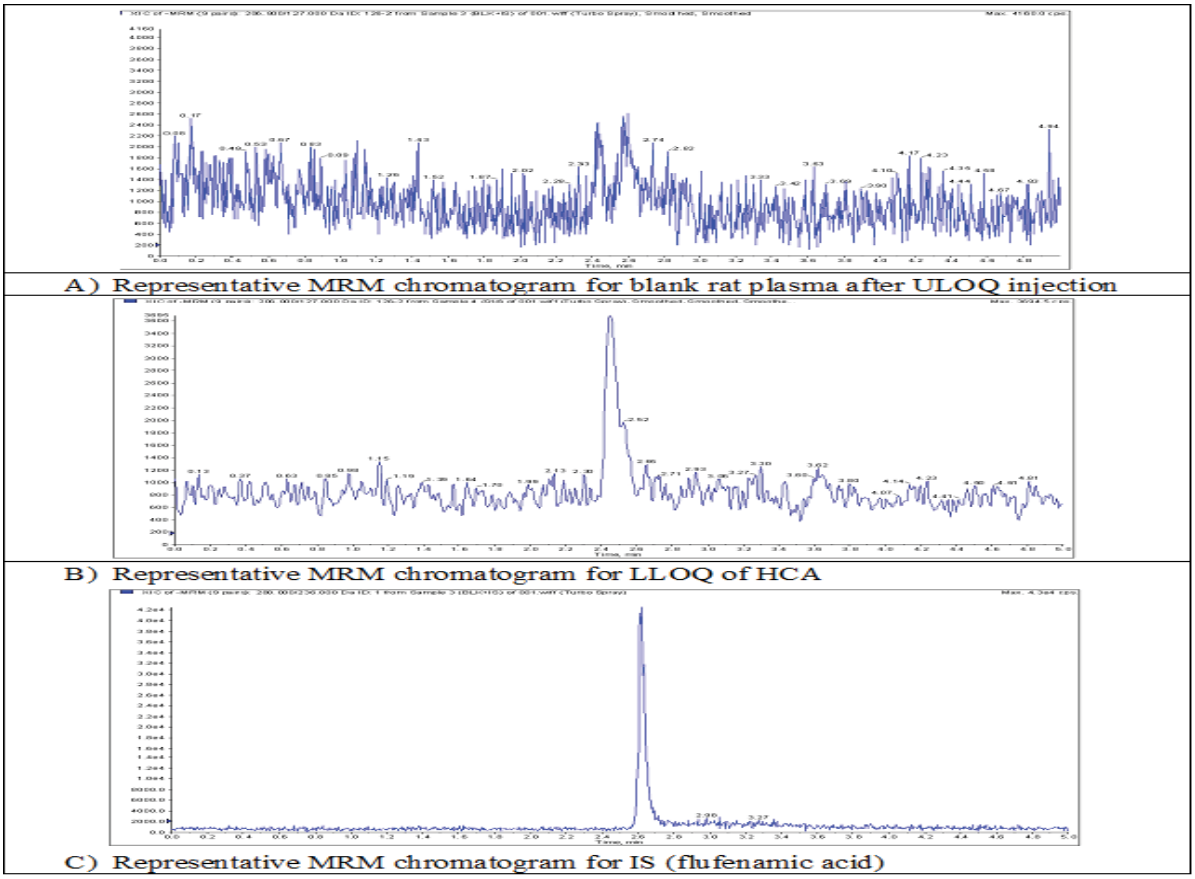

Figure 5.1. Representative chromatograms of A) Blank, B) LLOQ, and C) Internal standard.

Linearity

The linearity regression analysis for HCA indicated a linear relationship between peak area ratio and concentration in plasma over the range 10.5-10000 ng/mL with a weighted regression equation as shown Table 5.2.

Table 5.2. Calibration curve data for HCA run on five different occasions fitted to linear regression with $1/x^2$ weighing.

Calibration curve	Slope	Correlation coefficient (r^2)
1	0.00167	0.9992
2	0.00153	0.9985
3	0.00187	0.9987
4	0.00159	0.9974
5	0.00157	0.9972
Average	**0.001646**	**0.9982**
SD	**0.00013**	**0.0009**
% CV	**8.21**	**0.086**

Precision and Accuracy

The calibration curve for HCA was linear with a weighting factor of $1/x^2$ (10.5-10000 ng/mL) and average (n=5) correlation coefficient of > 0.99 (calibration curve data for HCA run on five different occasions fitted to linear regression with $1/x^2$ weighing is presented in Table 5.2). LLOQ was established as the lowest concentration in the linearity range with S/N ratio $\geq$ 10, i.e., 10.5 ng/mL.

The intra- and inter-day percent relative error ranged from -2.38 to 10.45 % and -6.57 to 11.38%, and % CV for the same was in the range of 6.51-12.00% and 4.04-13.97%, respectively (Table 5.3). These results demonstrated that both intra-and inter-day accuracy and precision were well within the acceptable limits.

Table 5.3. Precision and accuracy determination of HCA quality control samples in rat plasma.

Quality controls	Concentration Spiked (ng/mL)	Mean (ng/mL)	SD	Precision (% CV)	Accuracy (% RE)
Intra-batch					
LLOQQC	10.5	10.25	1.23	12.00	-2.38
LQC	31.25	33.04	2.15	6.51	5.73
MQC	2500.00	2761.26	204.55	7.41	10.45
HQC	3750.00	3941.25	315.62	8.01	5.1
Inter-batch					
LLOQQC	10.5	9.81	1.37	13.97	-6.57
LQC	31.25	32.42	2.16	6.66	3.74
MQC	2500.00	2784.52	112.51	4.04	11.38
HQC	3750.00	4067.95	251.64	6.19	8.48

% CV: Percent coefficient of variation (SD $\times$100/Mean)

HQC: High quality control; LLOQ: Lower limit of quantification quality control; LQC: Low quality control; MQC: Mid-quality control.

% RE: Percent Relative error ((measured value-actual value)*100/actual value); SD: Standard deviation

Recovery and Matrix Effect

An easy and fast method of sample preparation is protein precipitation through solvents (e.g., acetonitrile, methanol) or inorganic acid (e.g., TCA, 10% *v/v*), followed by centrifugation and/or filtration to remove precipitated proteins. Solvents such as methanol, acetonitrile, TCA, either alone or as mixtures in different combinations were evaluated. 10% *v/v* TCA in water provided the best extraction for both HCA and IS, and hence was selected. TCA acts primarily through two mechanisms: 1) disruption of the hydration shells around the protein and 2) anionic TCA may trigger partial protein unfolding through disruption of the electrostatic and hydrogen interactions between amino acids [12]. The results from recovery experiments are shown in Table 5.4. Mean absolute recovery for HCA (at LQC, MQC and HQC levels) was ranging

between ~82, 85 and 81%, respectively, indicating similar extraction efficiency across the calibration range. Also, variability for 6 different samples was less than 10%, providing confidence in the efficiency of the extraction procedure.

Endogenous components of plasma or metabolites in extracted bio-matrices may lead to a change in the intensity of the analyte signal. The matrix effect (IS normalized) was calculated as the peak area of the analyte in the presence of plasma compared with that in the absence of plasma (in pure solution). With the developed method, matrix effect for HCA was determined as ~97%, 96% and 95% at LQC, MQC and HQC level, respectively (Table 5.4). This suggested no significant interference from the different plasma constituents with the ionization of HCA.

Table 5.4. Recovery and matrix effect for HCA in rat plasma at LQC, MQC and HQC levels.

Concentration (ng/mL)	Recovery (%)	Matrix effect (%)
31.25	82.15 ± 10.41	97.29 ± 7.06
2500	85.87 ± 8.25	96.33 ± 9.72
3750	81.26 ± 9.53	94.61 ± 6.48

Dilution Integrity

This test was performed to verify whether plasma dilution has any adverse effect on the measurement when concentrations higher than the upper limit of quantification (ULOQ) are analyzed. For these samples, HCA concentrations of up to 50000 ng/mL could be quantified accurately after diluting with blank plasma (up to 10-fold) and accuracy of QC samples after dilution ranged from 91-109%, with precision ranging from 5 to 11% (% CV).

Stability

The stability of HCA in plasma under different anticipated storage conditions was tested with six replicates of each QC and the results are presented in Table 5.5. The accuracy values within ± 10% of nominal concentrations indicated that HCA did not undergo any significant degradation in rat plasma samples after storage for 6 h at room temperature, after three FT-C & 60 days of storage at FC. The samples after extraction and storage in auto-sampler for 24 h gave a very similar accuracy, suggesting that the samples could be run overnight. Percent stability of the drug and IS stocks stored at 5 °C for 60 days were found to be >95%.

Table 5.5. The stability data of HCA under different storage conditions.

Room temperature (23 °C) for 6 h				
Quality controls	**Concentration Spiked (ng/mL)**	**Mean ± SD**	**Precision (% CV)**	**Accuracy (% RE)**
LLOQ	10.5	9.94± 1.83	18.41	-5.33
LQC	31.25	32.56 ± 4.52	13.88	4.19
MQC	2500	2415.94 ± 104.15	4.31	-3.36
HQC	3750	3451.58 ± 175.81	5.09	-7.96
Autosampler (15 °C) for 24 h				
LLOQ	10.5	11.62 ± 1.92	16.53	10.67
LQC	31.25	33.51 ± 3.37	10.06	7.23
MQC	2500	2620.45 ± 105.26	4.02	4.82
HQC	3750	3519.55 ± 141.61	3.94	-6.15
Three freeze-thaw cycles (FT-C)				
LLOQ	10.5	12.47 ± 2.03	16.28	18.76
LQC	31.25	29.51 ± 2.19	7.42	-5.57
MQC	2500	2743.21 ± 81.48	2.97	9.73
HQC	3750	3841.52 ± 201.64	5.25	2.44
Long-term (-20 °C) for 60 days				
LLOQ	10.5	10.03 ± 1.43	14.26	-4.47
LQC	31.25	30.42 ± 3.06	10.06	-2.66
MQC	2500	2413.62 ± 195.03	8.08	-3.46
HQC	3750	3899.51 ± 124.62	3.20	3.99

% CV: Percent coefficient of variation (SD ×100/Mean); % RE: Percent Relative error {(measured value -actual value)*100/actual value}; SD: Standard deviation

5.2.4. Pharmacokinetic Study

A pharmacokinetic study was conducted as mentioned in Section 3.11.1. Plasma concentration profiles of HCA over time after *i.v.* and oral administration in Wistar rats are shown in Figure 5.2. The non-compartmental analysis was performed on the data to understand the PK parameters of HCA (Table 5.6). After *i.v.* administration at a dose of 1 mg/kg, the half-life ($t_{1/2}$) and the apparent volume of distribution (V_{ss}) of HCA were 2.1 ± 0.4 h and 0.6 ± 0.15 L/Kg, respectively. The V_{ss} was equal to the total body water (0.67 L/Kg) of the rat. Clearance (CL) value of 6.3 ± 2.1 mL/min/kg, being significantly lower than the liver blood flow of rat (55 mL/min/kg), indicated HCA to be a low extraction compound [22].

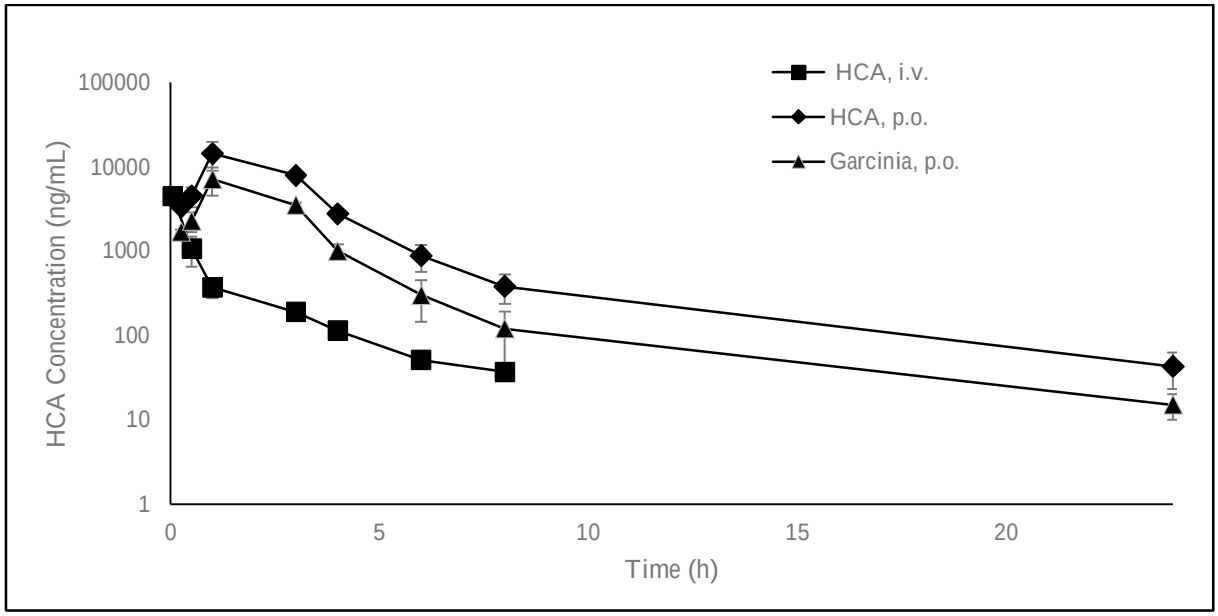

Figure 5.2. Plasma concentration-time profile of HCA after administration of HCA (*i.v.* and *p.o.*) and *Garcinia* (*p.o.*) in Wistar rats (n=3).

After oral administration of 20 mg/kg, HCA was quickly absorbed and the maximum plasma concentration, C_{max} = 14.38 ± 5.41 μg/mL was reached at 1 h. The absolute bioavailability of HCA was found to be 82.73%. The maximal attainable plasma concentration of HCA after peroral administration (C_{max}) of commercial *Garcinia* preparations (containing 60% HCA of the total weight) dosed at 20 mg/kg was found to be 7.20 ± 2.70 μg/mL. The absolute bioavailability of HCA after administration of commercial preparation was 61.31%, after correcting the dose for the actual content of HCA, *i.e.,* 12 mg/kg).

Table 5.6. Pharmacokinetic parameters of HCA after administration of HCA (*i.v.* and *p.o.*) and *Garcinia* (*p.o.*) in Wistar rats (n=3).

Pharmacokinetics parameters	**HCA *i.v.* (1 mg/kg)**	**HCA *p.o.* (20 mg/kg)**	**Garcinia *p.o.* (20 mg/kg)***
C_{max} (ng/mL)	-	14380.41 ± 5406.26	7201.11 ± 2703.84
T_{max} (h)	-	1.00 ± 0.00	2.03 ± 0.00
AUC_{last} (ng*h/mL)	2555.25 ± 704.32	42276.20 ± 10518.51	18798.91 ± 5349.23
Half life; t_{half} (h)	2.14 ± 0.42	4.40 ± 0.49	5.43 ± 0.72
MRT (h)	1.73 ± 0.25	3.20 ± 0.41	3.61 ± 0.19
Clearance (mL/min/kg)	6.37 ± 2.12	8.31 ± 3.24	10.34 ± 3.14
V_{ss} (L/kg)	0.64 ± 0.15	0.63 ± 0.20	0.61 ± 0.17
F (%)	-	82.73	61.31[a]

*: *Garcinia* was dosed at 20 mg/kg, HCA dose (12 mg/kg) was calculated based on the labelled claim, *i.e.*, 60% of the total content ; [a:] Dose normalized AUC obtained after *Garcinia* preparation considering 12 mg/kg HCA/Dose normalized AUC obtained after *i.v.* administration of HCA*100)

The incurred samples' re-analysis met the acceptance criteria (results within ± 20% of the original mean concentration), indicating that the method is rugged and reproducible. Data is provided in Table 5.7.

Table 5.7. Incurred sample reanalysis of selected samples for HCA.

Group I (HCA: *i.v.*, 1 mg/kg)			
Time Point	**Initial Conc (ng/mL)**	**Re-analyzed Conc (ng/mL)**	**% Difference**
1 h			
Animal-1	5642.74	6266.52	11.05
Animal-2	3928.01	4651.98	18.43
Animal-3	3868.30	4361.08	12.73
8 h			
Animal-1	34.15	32.05	-6.16
Animal-2	33.74	38.91	15.32
Animal-3	44.08	42.19	-4.29
Group II (HCA: *p.o.*, 20 mg/kg)			
Time Point	**Initial Conc (ng/mL)**	**Re-analyzed Conc (ng/mL)**	**% Difference**
1 h			
Animal-1	10312.15	10718.32	3.938
Animal-2	20515.01	22139.84	7.920
Animal-3	12315.12	10847.59	-11.91
24 h			
Animal-1	42.41	49.62	16.99
Animal-2	43.12	49.51	14.80
Animal-3	44.78	35.79	-20.06
Group III (Garcinia: *p.o.*, 20 mg/kg)			
Time Point	**Initial Conc (ng/mL)**	**Re-analyzed Conc (ng/mL)**	**% Difference**
1 h			
Animal-1	5156.95	4652.63	-9.77
Animal-2	10257.50	8872.48	-13.50
Animal-3	6157.57	7206.21	17.03
24 h			
Animal-1	271.78	301.94	11.09
Animal-2	127.41	150.62	18.21
Animal-3	175.58	203.06	15.64

5.3. Mass Spectrometry and Optimization of Assay for Quantitation of QCN, GLZ, GA and QTE in Biological Matrices: Part (B)

5.3.1. Selection of Internal Standard

Based on the appropriate response, reproducibility of recovery and detection stability, FA (negative mode) and propranolol (positive mode) were selected as internal standards. Additionally, no interference in the MRM channels at the relevant retention times facilitated the

application of these analytes as the internal standards for further studies with QCN, GLZ, GA and QTE.

5.3.2. UPLC-MS/MS Optimization

Multiple analytical methods including HPLC, UPLC, and LC-MS/MS for the discrete quantification of QCN, GLZ, GA and QTE have been described previously [23-25]. Most of these UPLC-MS/MS methods used gradient elution for chromatographic resolution with an analytical time of over 6 minutes for each analyte. In the present study, simple and convenient gradient elution was used with a short analytical time of 2.5 minutes. Having a short run time is an efficient way of analysis and is amenable for high-throughput. An easy and fast method of sample preparation is protein precipitation, also referred to as 'dilute and shoot [26]. Solvents such as methanol, acetonitrile, TCA, either alone or as mixtures in different combinations were used for optimization of protein precipitation method. Acetonitrile provided the best extraction for all the analytes (QCN, GLZ, GA and QTE) and hence was selected as a preferred solvent. Thus, protein precipitation method using a mixture of acetonitrile and plasma (5: 1, *v/v*) was finally used.

Both the positive and negative ionization modes were evaluated using the response of all the analytes (QCN, GLZ, GA and QTE). The results indicated that the responses of GLZ, QTE in the positive ionization mode were higher than those in the negative ionization mode, whereas the response of GA and QCN in the negative ionization mode was better than that of the positive ionization mode.

The chromatographic conditions were finalized to improve the signal response, peak shape, and shorten the run time for the simultaneous analysis of the four analytes and two internal standards. Because the ionization in ESI mode happens in the liquid state, the mobile phase additives may have a significant influence on the response of the analytes. Different aqueous phases including formic acid (from 0.05% to 0.3% *v/v*) and ammonium formate (5 mM and 10 mM) were tested to select the optimal one. The results indicated that the best peak shape and ionization were achieved using a combination of 0.1% *v/v* formic acid in 10 mM ammonium formate.

5.3.3. Method Validation

System Suitability

Suitability of UPLC-MS/MS for the analysis of samples was assessed by injecting six samples of 50 ng/mL of QCN, GLZ and GA and 5 ng/mL of QTE containing 200 ng/mL of internal standard (MQC level) as mentioned in Section 3.10.1. Precision (%

CV) for the peak area response of QCN, GLZ, GA and QTE and their respective internal standard was calculated and the system suitability was assessed. Representative system suitability data are provided in Table 5.8.

Table 5.8. Representative system suitability of QCN, GLZ, GA, and QTE data obtained before injecting first precision and accuracy batch.

Sample Name	Peak Area of QCN	Peak Area of GLZ	Peak Area of GA	Peak Area of QTE	Peak Area of FA	Peak Area of Propranolol
SST-1	380524	2524382	140644	845947	154708	282925
SST-2	374578	2359923	136210	858151	149837	312057
SST-3	370377	2202407	123652	877453	136023	349583
SST-4	342488	2032321	121973	819488	151912	330438
SST-5	354832	2263577	129501	826123	142451	301505
SST-6	386704	2297759	136164	809062	149788	284883
Average	**368250.50**	**2280894.83**	**131357.33**	**839370.67**	**147453.17**	**310231.83**
SD	**16610.19**	**165242.2**	**7533.64**	**25828.86**	**6918.36**	**26170.67**
% CV	**4.51**	**7.24**	**5.74**	**3.08**	**4.69**	**8.44**

Selectivity, Specificity and Carry-Over

Selectivity and specificity of the developed method were determined as mentioned in Section 3.10.2. No interfering peak present at the retention time of QCN, GLZ, GA, QTE, FA and Propranolol was observed in the chromatograms of six blank plasma in their respective MRM channels. The selectivity of the method from endogenous substances was confirmed by a absence of response in the blank biological matrix. Blank injection after injecting ULOQ standard did not show significant interference confirming a lack of carryover effect. Representative chromatograms of blank injected after ULOQ standard are depicted in Figure 5.3.

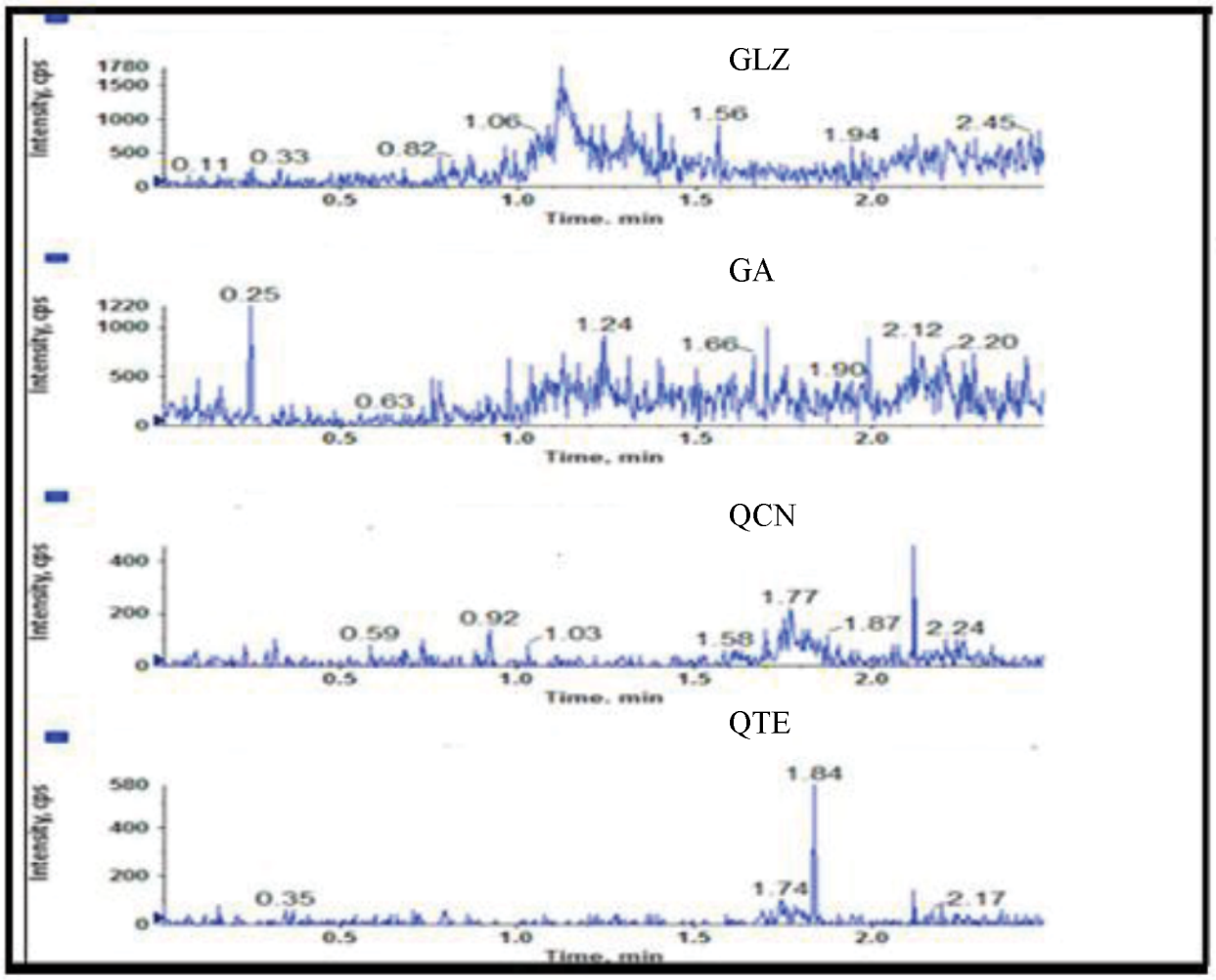

Figure 5.3. Representative chromatograms of blank injected after ULOQ standard

Linearity

Over the concentration range of 1-1000 ng/mL of QCN, GLZ and GA, and 0.1-100 ng/mL for QTE, the calibration graphs for QCN, GLZ, GA, QTE were linear with a weighting factor of $1/x^2$ and average (n=5) correlation coefficient of ≥ 0.99 (calibration curve data for QCN, GLZ, GA, QTE run on five different occasions fitted to linear regression with $1/x^2$ weighing is presented in Table 5.9).

Table 5.9. Calibration curve data for QCN, GLZ, GA and QTE run on five different occasions fitted to linear regression with $1/x^2$ weighing.

Calibration curve	Slope	Correlation coefficient (r^2)
QCN		
1	0.00348	0.9975
2	0.00384	0.9961
3	0.00317	0.9994
4	0.00394	0.9983
5	0.00389	0.9985
Average	**0.00366**	**0.99796**
SD	**0.0003**	**0.0012**
% CV	**8.197**	**0.12**
GLZ		
1	0.00275	0.9995
2	0.00286	0.9975
3	0.00224	0.9982
4	0.00265	0.9977
5	0.00271	0.9996
Average	**0.00264**	**0.9985**
SD	**0.0002**	**0.001**
% CV	**7.576**	**0.1**
GA		
1	0.00767	0.9967
2	0.00777	0.9984
3	0.00781	0.9991
4	0.00712	0.9963
5	0.00707	0.997
Average	**0.00749**	**0.9975**
SD	**0.0004**	**0.0012**
% CV	**5.34**	**0.12**
QTE		
1	0.00517	0.9969
2	0.00495	0.9988
3	0.00439	0.9987
4	0.00427	0.9975
5	0.00495	0.9961
Average	**0.00475**	**0.9976**
SD	**0.0004**	**0.0012**
% CV	**8.421**	**0.12**

Precision and Accuracy

The intra and inter-day % RE (Accuracy) and % CV (Precision) for all the analytes were within acceptable limits and indicated that the method was accurate and precise (Table 5.10).

This result demonstrated that both intra-and inter-day accuracy and precision were well within the acceptable limits mentioned in Section 3.10.5.

Table 5.10. Precision and accuracy determination of QCN, GLZ, GA and QTE quality control samples in rat plasma.

	Conc. Spiked	Intra-batch				Inter-batch			
	(ng/mL)	Mean (ng/mL)	SD	(% CV)	(% RE)	Mean (ng/mL)	SD	(% CV)	(% RE)
QCN									
LLOQQC	1	0.94	0.03	3.19	-6.00	0.99	0.06	6.06	-1.00
LQC	2	1.85	0.33	17.84	-7.50	2.03	0.19	9.36	1.50
MQC	50	55.49	6.51	11.73	10.98	53.94	3.52	6.53	7.88
HQC	750	769.84	42.09	5.47	2.65	764.2	84.59	11.07	1.89
GLZ									
LLOQQC	1	1.06	0.05	4.72	6.00	0.95	0.05	5.26	-5.00
LQC	2	1.95	0.21	10.77	-2.50	2.09	0.15	7.18	4.50
MQC	50	53.84	4.52	8.4	7.68	55.84	2.62	4.69	11.68
HQC	750	762.48	37.95	4.98	1.66	771.95	79.84	10.34	2.93
GA									
LLOQQC	1	1.15	0.06	5.22	15.00	1.19	0.07	5.88	19.00
LQC	2	2.15	0.13	6.05	7.50	2.13	0.16	7.51	6.50
MQC	50	45.85	2.95	6.43	-8.30	49.62	3.59	7.23	-0.76
HQC	750	784.62	42.99	5.48	4.62	781.44	81.3	10.4	4.19
QTE									
LLOQQC	0.1	0.109	0.006	5.5	9.00	0.103	0.009	8.74	3.00
LQC	0.2	0.216	0.019	8.8	8.00	0.194	0.016	8.25	-3.00
MQC	5	4.751	0.516	10.86	-4.98	5.162	0.637	12.34	3.24
HQC	75	76.306	4.262	5.59	1.74	74.562	2.048	2.75	-0.58

% CV: Percent coefficient of variation (SD ×100/Mean);

HQC: High quality control; LLOQ: Lower limit of quantification quality control; LQC: Low quality control; MQC: Mid-quality control;

% RE: Percent Relative error {(measured value-actual value)*100/actual value};

SD: Standard deviation.

Recovery and Matrix Effect

The results from recovery experiments are shown in Table 5.11. Also, variability for 6 different samples was less than 10%, providing confidence in the efficiency of the extraction procedure. Endogenous components of plasma or metabolites in extracted bio-matrices may lead to a change in the intensity of the analyte signal. The matrix effect (IS normalized) was calculated as the peak area of the analyte in the presence of plasma compared with that in the absence of

plasma (in pure solution) as mentioned in Section 3.10.7. The results suggested no significant interference from different plasma constituents with ionization of QCN, GLZ, GA and QTE. No significant matrix effect was observed. This indicates that the effect of ionic inhibition or enhancement on this method was negligible.

Table 5.11. Recovery and matrix effect for QCN, GLZ, GA and QTE in rat plasma at LQC MQC and HQC levels.

Analyte	Spiked concentration (ng/mL)	Matrix effect (%)	Recovery (%)
QCN	2	98.15 ± 7.64	71.73 ± 16.85
	50	93.84 ± 1.93	86.51 ± 7.23
	750	98.37 ± 0.66	79.20 ± 11.46
GLZ	2	96.31 ± 1.12	80.24 ± 8.41
	50	94.70 ± 0.38	102.99 ± 10.62
	750	98.63 ± 7.94	88.33 ± 12.83
GA	2	95.57 ± 2.17	75.07 ± 5.08
	50	99.13 ± 5.09	70.89 ± 6.47
	750	96.42 ± 4.14	78.32 ± 11.75
QTE	0.2	97.69 ± 6.07	96.47 ± 1.74
	5	90.81 ± 1.12	90.72 ± 4.02
	75	90.87 ± 1.99	92.53 ± 2.97

Dilution integrity

This test was performed to verify whether plasma dilution has any adverse effect on the quantitation of the analytes when concentrations higher than the upper limit of quantification (ULOQ) are analyzed. For these samples, the concentration of all analytes (QCN, GLZ, GA, QTE**)** up to ten times the ULOQ could be quantified accurately after diluting with blank plasma (up to 10-fold) and accuracy of QC samples after dilution ranged from 92-106%, with precision ranging from 6 to 12% (% CV).

Stability

The stability experimentations for the QC samples (LQC, MQC and HQC) comprised of the following sets: a) storing the samples at -20 °C for 60 days; b) at room temperature (23 °C) for 6 h; c) three freeze-thaw cycles. The results indicated that QCN, GLZ, GA, QTE were stable under these storage conditions. Also, these analytes were stable in the post-extracted samples kept in the auto-sampler at 15 °C for 24 h. The results show that QCN, GLZ, GA and QTE had no significant degradation in Wistar rat plasma under these experimental conditions and the

method for their simultaneous estimation in rat plasma can be applied in a pharmacokinetic study. The data are shown in Table 5.12.

The stock solution stability was evaluated at 5 °C for 60 days. The peak area ratio of the analytes to that of the respective IS, acquired from the freshly prepared and the stored stock solutions, was compared. The accuracy, expressed in % RE, was between -9.42 to 12.51%, which met the pre-set acceptance criteria. The results showed that all the stock solutions were stable under storage conditions (5 °C).

Table 5.12. The stability of QCN, GLZ, GA and QTE under storage conditions.

Storage conditions	Analyte	Nominal conc. (ng/mL)	Calculated conc. Mean ± SD (ng/mL)	% CV	% RE
Room temperature (23 °C) for 6 h	QCN	2	2.19 ± 0.25	11.42	9.5
		50	52.61 ± 5.07	9.64	5.22
		750	714.72 ± 29.53	4.13	-4.7
	GLZ	2	2.22 ± 0.15	6.76	11
		50	47.23 ± 3.72	7.88	-5.54
		750	693.22 ± 45.82	6.61	-7.57
	GA	2	1.94 ± 0.23	11.86	-3
		50	44.21 ± 3.83	8.66	-11.58
		750	675.23 ± 31.15	4.61	-9.97
	QTE	0.2	0.21 ± 0.02	9.52	5.00
		5	5.167 ± 0.206	3.99	3.34
		75	84.72 ± 1.24	1.46	12.96
Autosampler (15 °C) for 24 h	QCN	2	2.14 ± 0.17	7.94	7.00
		50	55.19 ± 4.9	8.88	10.38
		750	802.82 ± 20.84	2.6	7.04
	GLZ	2	2.15 ± 0.1	4.65	7.50
		50	54.67 ± 0.99	1.81	9.34
		750	820.59 ± 17.67	2.15	9.41
	GA	2	2.07 ± 0.1	4.83	3.50
		50	52.86 ± 4.02	7.6	5.72
		750	798.32 ± 8.53	1.07	6.44
	QTE	0.2	0.209 ± 0.015	7.18	4.50
		5	4.829 ± 0.207	4.29	-3.42
		75	78.91 ± 3.87	4.9	5.21

Table 5.12. The stability of QCN, GLZ, GA and QTE under storage conditions *….Continued*

Storage conditions	Analyte	Nominal conc. (ng/mL)	Calculated conc. Mean ± SD (ng/mL)	CV (%)	RE (%)
Three freeze-thaw cycles	QCN	2	2.18 ± 0.13	5.96	9.00
		50	54.71 ± 5.88	10.75	9.42
		750	743.32 ± 14.44	1.94	-0.89
	GLZ	2	2.1 ± 0.27	12.86	5.00
		50	52.5 ± 2.88	5.49	5.00
		750	762.89 ± 12.28	1.61	1.72
	GA	2	2.16 ± 0.16	7.41	8.00
		50	45.47 ± 3.26	7.17	-9.06
		750	739.23 ± 30.84	4.17	-1.44
	QTE	0.2	0.222 ± 0.04	18.02	11.00
		5	4.284 ± 0.274	6.4	-14.32
		75	82.25 ± 2.63	3.2	9.67
Long-term (-20 ◦C) for 60 days	QCN	2	1.85 ± 0.24	12.97	-7.50
		50	55.78 ± 0.78	1.4	11.56
		750	705.63 ± 69.13	9.8	-5.92
	GLZ	2	1.92 ± 0.37	19.27	-4.00
		50	55.27 ± 0.98	1.77	10.54
		750	749.96 ± 48.98	6.53	-0.01
	GA	2	2.18 ± 0.17	7.8	9.00
		50	45.73 ± 3.64	7.96	-8.54
		750	830.99 ± 25.9	3.12	10.80
	QTE	0.2	0.194 ± 0.013	6.7	-3.00
		5	5.24 ± 0.751	14.33	4.80
		75	71.65 ± 6.22	8.68	-4.47

5.3.4. Pharmacokinetic study

Pharmacokinetic study of QCN

A pharmacokinetic study was conducted as mentioned in Section 3.11.2. Plasma concentration profiles of QCN after dosing QCN (10 mg/kg) over time after *p.o.* administration in Wistar rats are shown in Figure 5.4. The exposures of QCN are in concordance with Pangeni *et al.* [27]. The authors have reported C_{max} to be 336 ng/mL and AUC_{inf} as 2.60 μg*h/mL at a dose of 40 mg/kg. There were many phase II metabolites of QCN detected in the plasma.

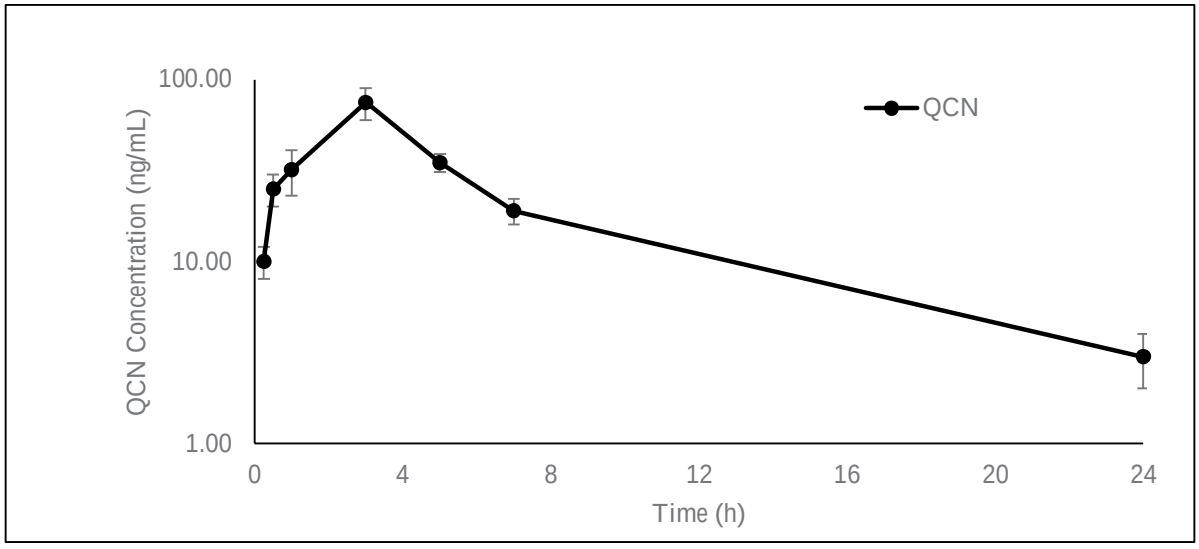

Figure 5.4. Plasma concentration-time profile of QCN after administration of QCN (*p.o.*) in Wistar rats (n=3).

The non-compartmental analysis was performed on the data to understand the pharmacokinetic parameters of QCN (Table 5.13). Very low exposures were observed in the systemic circulation. C_{max} of only 75.73 ng/mL was observed after 10 mg/kg of QCN dosed. An LLOQ was 1 ng/mL and all the samples were quantified till 24 h.

Table 5.13. Pharmacokinetic parameters of QCN after administration of QCN (*p.o.*) in Wistar rats (n=3).

Parameter	PK parameters after dosing QCN (10 mg/kg)
C_{max} (ng/mL)	75.73 ± 3.16
T_{max} (h)	3.00 ± 0.00
AUC_{inf} (ng*h/mL)	512.20 ± 75.62
$t_{1/2}$ (h)	3.53 ± 0.51

The incurred samples re-analysis met the acceptance criteria (results within ± 20% of the original mean concentration), indicating that the method is rugged and reproducible (Table 5.14).

Table 5.14. Incurred sample reanalysis of selected samples for QCN.

QCN, *p.o.*, 10 mg/kg			
Time Point	**Initial Conc (ng/mL)**	**Re-analyzed Conc (ng/mL)**	**% Difference**
1 h			
Animal-1	73.62	74.29	0.91
Animal-2	79.37	85.61	7.86
Animal-3	74.20	79.09	6.59
24 h			
Animal-1	3.02	3.16	4.64
Animal-2	3.91	3.75	-4.09
Animal-3	2.51	2.93	16.73

Pharmacokinetic Study of GLZ

Plasma concentration profiles of GA after dosing GLZ over time after *p.o.* administration in Wistar rats are shown in Figure 5.5. No quantifiable concentration of GLZ was found in plasma after dosing GLZ (10 mg/kg) as it breaks down to GA due to intestinal enzymes [24]. The exposures of GLZ and GA are in concordance with Takeda *et al.* [28]. The authors have reported C_{max} to be 606 ng/mL and AUC_{inf} as 11.7 μg*h/mL at a dose of 40 mg/kg.

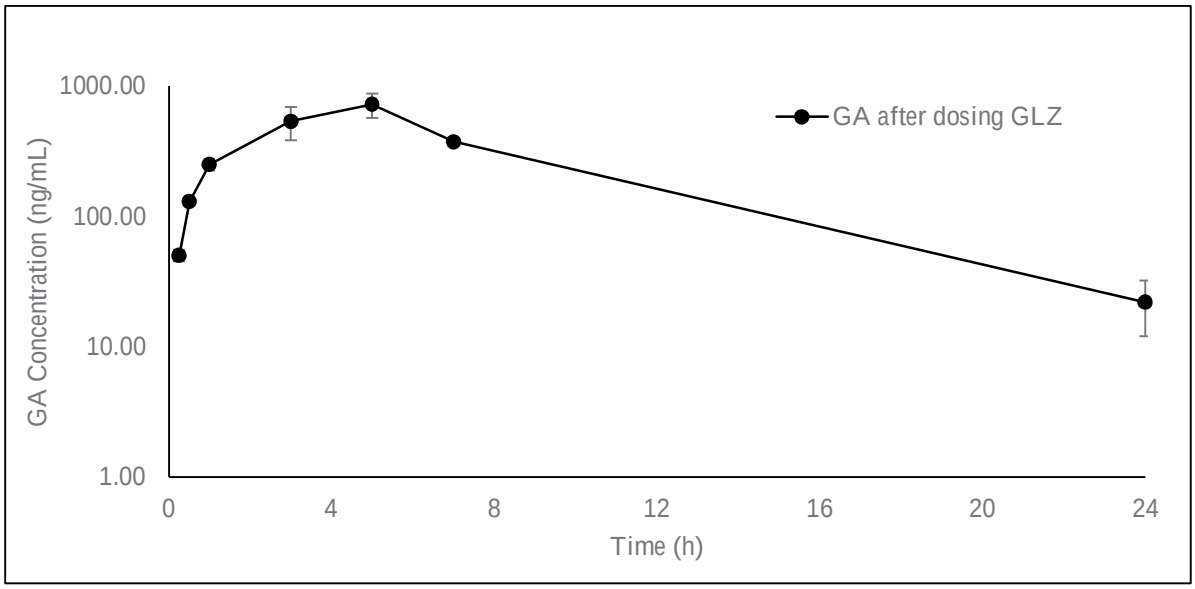

Figure 5.5. Plasma concentration-time profile of GA after administration of GLZ (*p.o.*) in Wistar rats (n=3).

The non-compartmental analysis was performed on the data to understand the pharmacokinetic parameters of GA after dosing GLZ (Table 5.15). C_{max} of 746.16 ng/mL for GA was observed after 10 mg/kg of GLZ dosed. An LLOQ for GA was 1 ng/mL and all the samples were quantified till 24 h.

Table 5.15. Pharmacokinetic parameters of GA after administration of GLZ (*p.o.*) in Wistar rats (n=3)

Parameters	PK Parameters for GA after dosing GLZ (10 mg/kg)*
C_{max} (ng/mL)	746.16 ± 17.98
T_{max} (h)	5.61 ± 1.25
AUC_{last} (ng*h/mL)	7006.43 ± 669.57
$T_{1/2}$ (h)	7.58 ± 1.34

The incurred samples' re-analysis met the acceptance criteria (results within ± 20% of the original mean concentration), indicating that the method is rugged and reproducible. (Table 5.16).

Table 5.16. Incurred sample reanalysis of selected samples for GA after dosing GLZ

Concentration of GA after dosing GLZ: *p.o.*, 10 mg/kg			
Time Point	**Initial Conc (ng/mL)**	**Re-analyzed Conc (ng/mL)**	**% Difference**
1 h			
Animal-1	750.62	725.61	-3.33
Animal-2	761.48	740.97	-2.69
Animal-3	726.37	730.09	0.51
24 h			
Animal-1	12.06	12.75	5.72
Animal-2	12.94	13.94	7.73
Animal-3	13.63	12.33	-9.54

5.4. Conclusion

Herein we report the first UPLC-MS/MS method for analysis of HCA from rat plasma. The developed bioanalytical method is fast (run-time: 5 minutes), sensitive (LLOQ, 10.5 ng/mL) and highly selective. Moreover, the developed extraction procedure through protein precipitation is simple (one-step), cost-effective and reproducible. Also, the performance of the method was validated according to USFDA bioanalytical method validation guideline. The method was successfully applied to determine *in vivo* PK of HCA, which suggested that HCA is rapidly absorbed with a moderate apparent volume of distribution (0.6 L/kg) and has a good bioavailability (~82%). Similar bioavailability of HCA was observed after the administration of *Garcinia*. (~61%) The method reported herein can be used routinely in any bioanalytical laboratory for analysis of HCA and could be helpful for further toxicological evaluation of *Garcinia* products. This method will also be used for further herb-drug interaction studies involving HCA.

Similarly, a selective, sensitive (LLOQ for GLZ, GA and QCN:1 ng/mL and for QTE: 0.1 ng/mL), rapid (run-time: 2.5 minutes), and efficient UPLC-MS/MS method has been developed and validated for the simultaneous determination of the concentrations of QCN, GLZ, GA and QTE in rat plasma. Simple and cost-effective protein precipitation using acetonitrile for sample pre-treatment and shorter acquisition time (2.5 minutes) of chromatography are some of the advantages of this method. Besides, this method was successfully applied to a pharmacokinetic study of QCN, GLZ, and GA following *p.o*. administration of QCN and GLZ to rats (Dose: 10 mg/kg). The results of this study provide the basis for further evaluation of the pharmacokinetic interaction of QCN, GLZ, GA and QTE, and the developed UPLC-MS/MS method can be applied for understanding herb-drug interactions involving QCN, GLZ, and GA

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