Research Article

Development and Validation of A LC-MS/MS Method for the Determination of Gallic Acid in Rabbit Serum

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ABSTRACT

A novel bio-analytical method was developed and validated for the quantitative determination of Gallic acid in Rabbit serum by using liquid-liquid extraction chromatography and tandem mass spectrometric detection (HPLC-MS/MS). Extraction of Gallic acid from the endogenous substances is succeeded after liquid-liquid extraction by using HPLC-MS/MS system. Gallic acid was eluted in isocratic mode with methanol and 0.1% formic acid in water (60: 40v/v) at a flow-rate of 0.8 mL/min on Phenomenex Luna C18, 150*3.9 mm, 5µm particle size column. Galli acid-D2 was used as the internal standard (IS). The liquid-liquid extraction recovery was found 67% designates good recovery. The validation results demonstrated that the present method was found to be precise and accurate. The stability tests indicated that the Gallic acid in Rabbit serum is stable for three freeze-thaw cycles at both -20 °C and -70 °C, 18-h ambient storage, 15-day frozen storage at both -20 °C and -70 °C. The results also showed no significant matrix effect (<0.28%). The present method was found to be sensitive and selective at very low levels of linearity range 5-1000 ng/mL, based on a sample volume of 100 µL, with a linear correlation coefficient of ≥ 0.99. The method was strictly validated according to the USFDA guidelines. Acquired results demonstrated that proposed method can be applied for evaluation of pharmacokinetic data of Gallic acid in Rabbit serum samples.

Keywords: Gallic acid; Bioanalytical; HPLC-MS/MS; Liquid -Liquid extraction (LLE); Quantification; Rabbit serum

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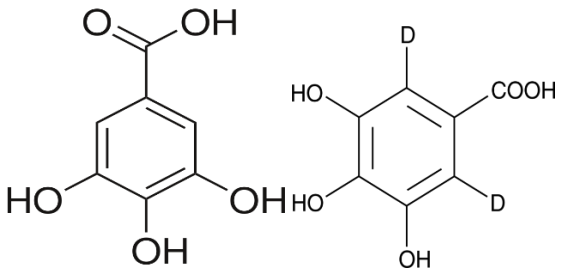
INTRODUCTION

Gallic acid (GA), a class of phenolic compounds, also known as 3,4,5- trihydroxybenzoic acid, is a naturally occurring secondary metabolite found in various plants, vegetables, nuts and fruits.[1–3] Among many polyphenols, Gallic acid is a low molecular weight tri-phenolic compound with excellent anti-inflammatory and anti-oxidative activities. [4] In addition, Gallic acid also has several evident pharmacological effects including anti-tumor, anti-bacterial, anti-diabetes, anti-obesity, anti-microbial and anti-myocardial ischemia. [5–10]

The anti-inflammatory mechanisms of Gallic acid mainly involved MAPK (mitogen-activated protein kinase) and NF-κB (nuclear factor kappa B) signaling pathways. It thus weakens the inflammatory response by reducing the release of inflammatory cytokines, chemokines, adhesion molecule and cell infiltration. Due to its excellent pharmacological activities, Gallic acid is expected to be a potential candidate for the treatment of various inflammation-related diseases.[11] The structural formulae of Gallic acid and Gallic acid D_2 are shown in **Figure 1.**



Gallic acid Gallic acid-D_2

Figure 1: Chemical structure of Gallic acid and Gallic acid D_2

Analytical methods for determination of gallic acid, quercitrin, and quercetin in biological samples, including high-performance liquid chromatography (HPLC) with MS detection, have been described in several studies.[12,13,14] However currently no established highly sensitive bioanalytical method in biological fluids using LC-MS/MS.

Therefore, the objective of this study is to develop highly sensitive, accurate and precise bioanalytical method procedures for determining Gallic acid in Rabbit serum using liquid chromatography coupled with mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Chemicals and reagents

Methanol, Ethyl acetate grade of HPLC, Formic acid analytical reagent grade and hydrochloric acid concentrated grade were purchased from Scharlab S.L., Eurolab and Fisher chemicals respectively. The reference standard Gallic acid and internal standard (IS) Gallic acid D_2 were purchased from Dr. Ehrenstorfer –LGC.

Normal Rabbit serum was collected from Ajman veterinary and agriculture extension center, Ministry of Climate Change and Environment (MOCCAE), Ajman, UAE.

Preparation of standard solutions, calibration standards, and quality control (QC) samples

Standards and Quality control samples were prepared from two separate stock solutions. Weighed accurately 5.00 mg of Gallic acid standard and transferred into a 5 mL of volumetric flask, dissolved in 2 mL of methanol and the volume was made up to the mark with the same to obtain a concentration of 1.00 mg/mL. This stock solution was used for preparation of calibration standards and quality control spiking working samples in 40% methanol.

The IS working solution (100.00 ng/mL) was prepared in 40 % methanol in water from IS stock solution (1 mg/ml). Stored the analyte stock solutions, IS stock solution, and working solutions in polypropylene vials at 2-8 °C in refrigerator. Prepared the calibration standards were at concentrations of 5,10, 25, 100, 250, 500, 800 and 1000 ng/mL and quality control sample i.e., 5.00 (LLOQ), 15.00 (low), 500.00 (mid), 800.00 (high), and 8000 (10-fold dilution) ng/mL were prepared on each day of analysis by spiking in rabbit serum using calibration standard and quality control spiking\ working samples. The lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) of this method were 5 ng/mL and 1000 ng/mL, respectively. For evaluation of stability of Gallic acid in rabbit serum, the QC serum samples at low and high-QC level concentrations were prepared in pre-labeled polypropylene vials and then stored in a freezer maintained at approximately -70 °C and -20 °C for testing sample storage stability under this temperature conditions.

Sample extraction

Added 100 µl serum sample to a vial containing the 25 µl of IS (100 ng/ml), 200 µl of 0.1 N Hcl and mixed well. Added 2 ml of ethyl acetate and vortexed for 5 min and then centrifuged at 2,500 rpm for 5 min. The upper layer was separated and evaporated dry. The dried material was then reconstituted with 200 µL of reconstitution solution (Buffer 90: Methanol 10) and 20 µL was injected into the API 5500+ LC-MS/MS.

LC-MS/MS conditions

MS/MS detection was performed using a Sciex API 5500+ triple quadrupole mass spectrometer with a Turbo Ionspray® ionization source operated in the Negative ion mode. The mass spectrometry parameters such as analyte fragmentation pattern and collision energy, etc. were optimized by infusing the analyte and the IS solutions. The LC-MS interface conditions such as gas flows, source temperature, etc. were optimized via tee-mixing the analyte standard solution with the mobile phase at a flow-rate of 0.800 mL/min. All optimized parameters for this method are presented in **Table 1.**

Table 1: Tandem mass spectrometric parameters for the LC-MS/MS assay.

Source temperature (ºC)	-500
Dwell time per transition (ms)	-150
GS1	-55
GS2	-50
Curtain gas setting CAD gas	-25 -50
Ion Spray voltage (V)	-3500
Declustering potential (V)	-40
Collision energy (eV)	-45
Collision Cell Exit Potential (V)	-5
Resolution for Q1 and Q3 Unit	Unit
Mode of analysis Positive	Negative
Ion transition for Gallic acid	m/z 169.0/125.0
Ion transition for IS, Gallic acid D_2	m/z 171.0/126.9

Quantification and assay validation

The peak areas of the analyte and the IS were integrated by using *Analyst®* software version 1.7.2. For each analytical run, a calibration curve was derived from analyte/IS peak area ratio against the analyte concentration using a weighed ($1/x^2$) linear least-squares regression. The regression equation was then used to calculate the concentration of rabbit serum samples. During the method validation, intra-day and inter-day precision (CV %) and accuracy (RE% or percent Bias of theoretical value) were calculated. The presented method was validated following the FDA guidance for bioanalytical method validation [15] for intra-day and inter-day precision and accuracy, linearity, selectivity, sensitivity, dilution integrity, and short-term and long-term sample stability.

RESULTS AND DISCUSSION

Chromatography

Separation of the analyte from potential interferences in the matrix was achieved on a Phenomenex Luna C18,

150*3.9 mm, 5µm particle size column at 25 °C column oven temperature. An isocratic mobile phase mixture of a) 0.1% Formic acid in water b) Methanol (40:80 v/v) was used at a flow-rate of 0.800 mL/min. A 1:1 methanol-water solution was used as the injector washing solvent for the prevention of potential carryover from the needle. No carryover from the injection needle was observed during the method validation. Retention times for both Gallic acid and IS were approximately 1.55 min and 1.55 min, respectively. The representative chromatograms of matrix blank, LLOQ and ULOQ samples are shown in Figure 2A, 2B & 2C respectively. The clean chromatogram of the matrix blank obtained from the ULOQ injection of a matrix blank extract immediately after an ULOQ sample demonstrated that this method had neither injector carryover nor analytical column carryover.

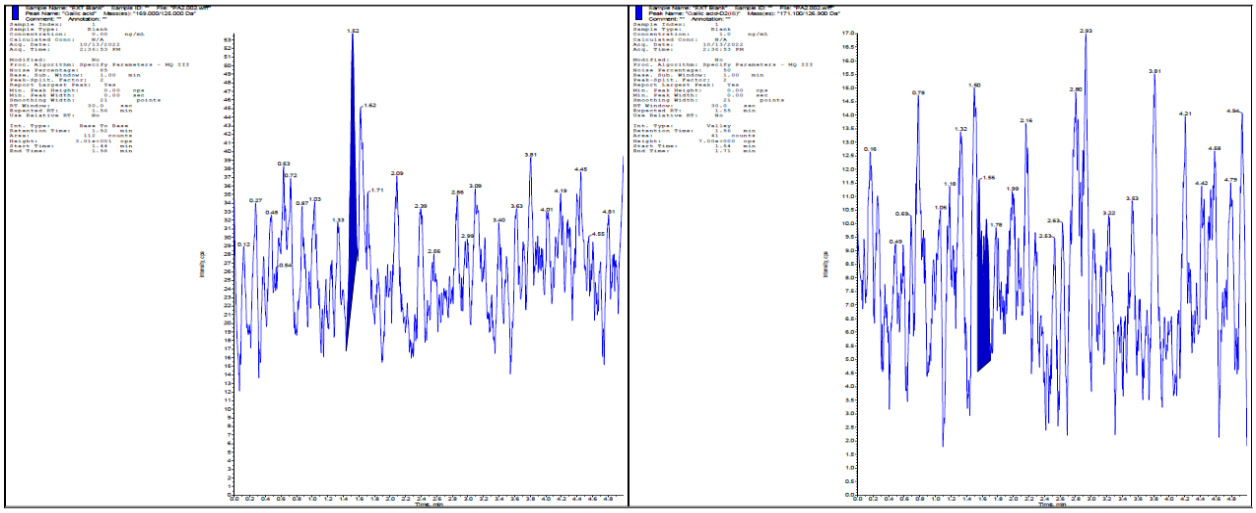


Figure 2A: Typical chromatogram of rabbit blank serum sample

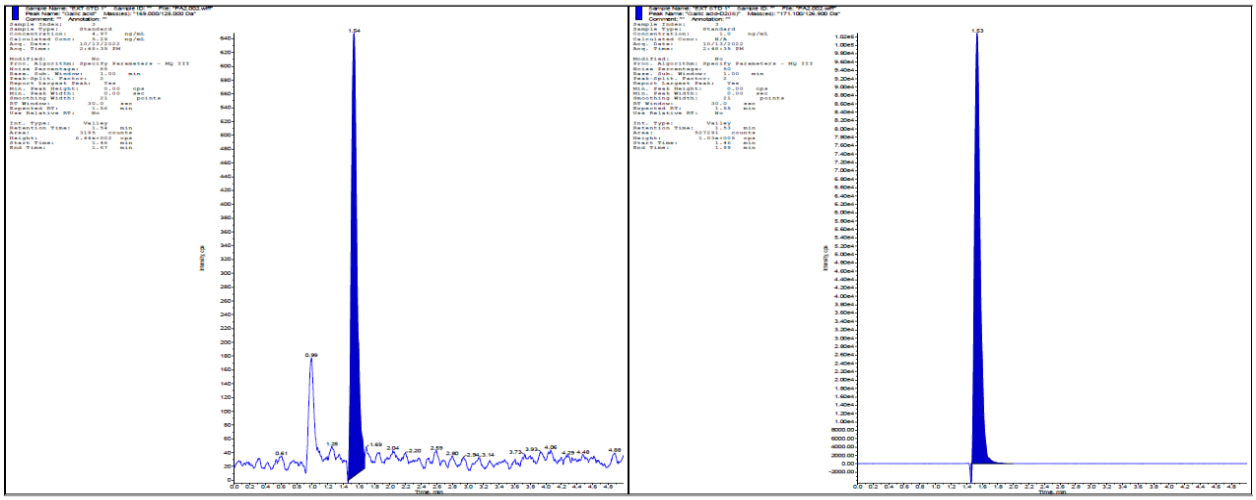


Figure 2B: Typical chromatogram of LLOQ (4.97 ng/ml)

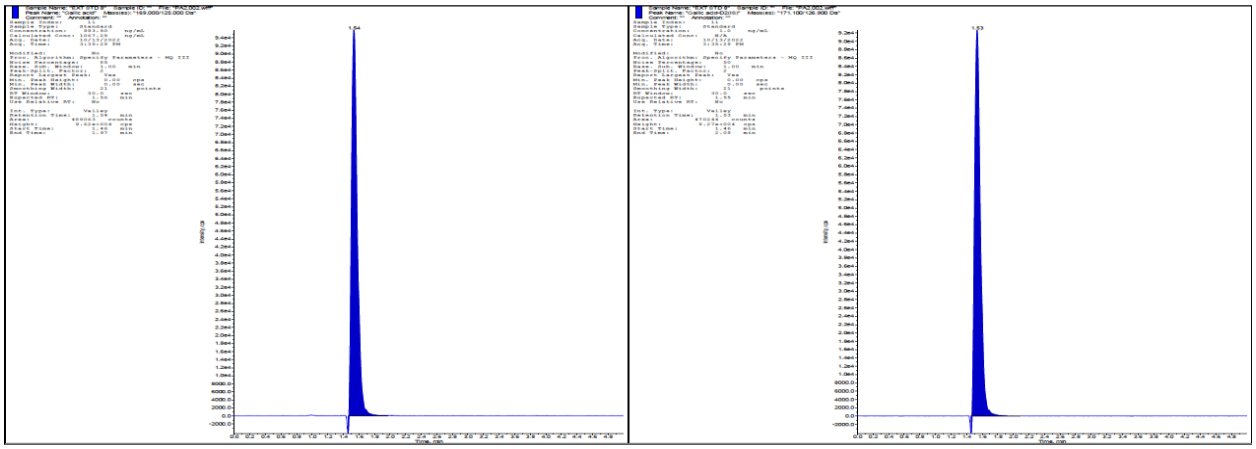


Figure 2C: Typical chromatogram of ULOQ (993.50 ng/ml)

Linear curve range and assay sensitivity (LLOQ)

The linearity of the calibration curve was evaluated from three individual batches on three different days. The linear dynamic range for Gallic acid was from 5.00 to 1000 ng/mL based on a 100 µL serum. The coefficient of determination (r^2) of the calibration curve was between 0.9970 and 0.9997 and the %bias of mean back-calculated concentrations of standards ranged from -7.44 to 4.03 of the theoretical values presented in **Table 2**. Eighteen replicates (six replicates in each of three batches) of the lower limit of quantification (LLOQ) samples were used to evaluate the inter-day precision and accuracy at the lower end of the assay range in three separate runs. The inter-day CV% was 13.92% (n = 18) and the accuracy, expressed as percent bias, was 0.52 presented in **Table 3**. The representative LC-MS/MS calibration curve standards is shown in **Figure 3.**

Precision, accuracy, dilution integrity and recovery

Six replicates of QC samples for each of three consecutive runs were used to evaluate the intra-day and inter-day precision and accuracy at low-, mid-, and high-QC concentration levels. As shown in **Table 3**, the intra-day CV (n = 6) was ≤10.65% and the inter-day CV (n = 18) was ≤13.92%. The % bias from the nominal of mean values of intra-day and inter-day assays was between -9.56 to 9.85 and 0.52 to 6.32, respectively. To validate partial-volume assay, a dilution factor of 10 with blank serum was processed with the dilution QC sample (8,000 ng/mL). A 10 µL of the dilution QC was mixed with 90 µL of blank matrix in in pre-labelled polypropylene tubes. Results for the dilution QC (DiQC) samples showed a % bias from nominal of mean value 0.97 and the corresponding CV was 2.02% (n = 6). These results indicate that the present method has satisfactory precision, accuracy and dilution integrity.

Table 2: Back-calculated Gallic acid calibration standards in Rabbit serum

Nominal Concentrations (ng/mL)	Back calculated mean concentration (ng/mL)	Standard deviation	Precision (CV%, N = 3)	% Bias from nominal
4.97	4.96	0.363	7.32	-0.27
9.94	8.40	1.532	18.24	-7.44
24.84	25.22	1.043	4.14	1.53
99.35	96.11	1.327	1.38	-3.26
248.38	246.07	5.480	2.23	-0.93
496.75	497.38	12.298	2.47	0.13
794.80	803.84	7.234	0.90	1.14
993.50	1033.55	43.451	4.20	4.03

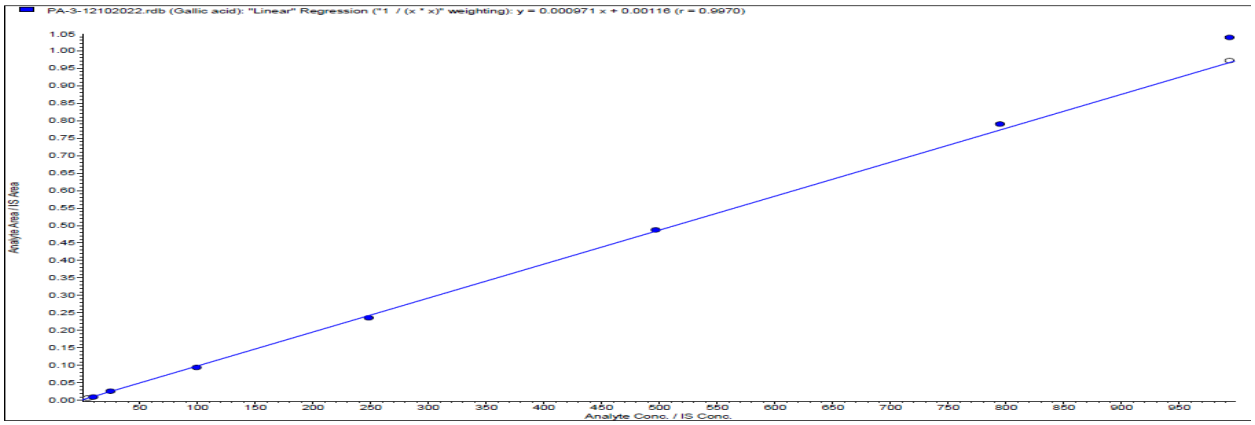


Figure 3: Calibration curve standard

Table 3. Intra-day and inter-day precision and accuracy studies of quality control samples including LLOQ and the diluted QC* samples.

	Intra - day					Inter-day			
QC sample nominal concentration (ng/mL)	LLOQ	LQC	MQC	HQC	DIQC	LLOQ	LQC	MQC	HQC
	5.5	14.9	496.75	819.64	8000	5.5	14.9	496.75	819.64
N	6	6	6	6	6	18	18	18	18
Mean	4.97	15.52	519.54	900.37	8077.87	5.53	15.84	510.02	871.46
%CV	10.65	4.88	1.51	2.55	2.02	13.92	5.65	1.76	4.13
%Bias	-9.56	4.15	4.59	9.85	0.97	0.52	6.29	2.67	6.32

* 10-fold dilution with the control matrix applied to the DiQC (8,000 ng/mL).

Recovery of Gallic acid was evaluated by comparison of mean analyte response in processed LQC, MQC and HQC samples in Rabbit serum with mean analyte response from neat solutions at respective levels. Mean recovery values for Gallic acid were 63.47, 69.64 and 69.98 % at low, medium and high quality control levels, respectively. The results are presented in **Table 4** (recovery of IS not shown).

Table 4: Recovery studies of Gallic acid in Rabbit serum

QC sample	Precision (CV%, N = 6)	% Recovery
LQC: 14.9 ng/mL	3.65	63.47
MQC: 496.75 ng/mL	0.87	64.64
HQC: 819.64 ng/mL	1.28	69.98

Selectivity and matrix effect

Selectivity was evaluated by extracting blank matrix from six different lots and comparing the MS/MS response at the retention times of the analyte to the responses of the LLOQ. No significant peak was observed in any of these 6 lots of blank serum samples for analyte and IS. The matrix effect on the responses for both Gallic acid and IS were investigated by preparing a LQC and HQC level with six different individual lots of serum. The matrix effects were evaluated by comparing the peak area obtained from the post-extraction spiked sample to a pure solution at the same nominal concentration. Matrix effect was calculated as ME (%) = [1 – peak area of post-extraction spiked sample/peak area of pure solution] × 100. With this LLE procedure and chromatographic conditions, the matrix effects for Gallic acid and Gallic acid D_2 were -0.60% and 0.28%, respectively.

Sample stability

Stability tests were conducted to evaluate the analyte stability serum samples under different conditions. The ambient storage and freeze-thaw, long-term frozen storage stability at two different temperatures were tested and the results are presented in **Table 5**. As shown in Table 5, mean % change from nominal values for ambient storage at LQC and HQC level was 5.13 and 3.13, respectively. Mean % change from nominal values for freeze-thaw stability (3cycles) and long term stability for 15 days at -70°C for LQC and HQC level was 2.51 and 3.06, respectively and at -20°C for LQC and HQC level was 0.98 and 3.13, respectively.

Re-injection of the accepted precision and accuracy batch stored in auto sampler at 4°C was performed to evaluate the stability of analyte in processed samples. The samples of the accepted precision and accuracy batch were re-analyzed after 48 hours and the results were within acceptance criteria. The % CV of the quality control samples ranged between 1.12 to 5.20 %. The percentage change (%Bias) of the nominal concentration across quality control samples ranged in between -9.47% to 14.16%. The results are presented in **Table 6.**

Table 5: Stability studies of Gallic acid in Rabbit serum

Sample Concentration	Mean conc. found (ng/mL)	Precision (CV%, n = 6)	Mean % Change from nominal
	Plasma sample ambient storage (18 h)		
LQC: 14.90 ng/mL	15.67	3.38	5.13
HQC: 819.64 ng/mL	845.33	0.98	3.13
	Long-term stability for 15 days and Freeze-thaw cycles (n = 3) at -70°C		
LQC: 14.90 ng/mL	15.27	4.20	2.51
HQC: 819.64 ng/mL	844.69	1.07	3.06
	Long-term stability for 15 days and Freeze-thaw cycles (n = 3) at -20°C		
LQC: 14.90 ng/mL	3.10	3.38	0.98
HQC: 819.64 ng/mL	803.25	5.13	3.13

Table 6. Re-injection reproducibility for 48 hours at 4°C

	Intra - day			
QC sample nominal concentration (ng/mL)	LLOQ	LQC	MQC	HQC
	505	14.9	496.75	819.64
N	4.59	17.01	513.42	796.09
Mean	0.239	0.423	6.086	8.928
%CV	5.20	2.49	1.19	1.12
%Bias	-9.47	14.16	3.36	-2.87

CONCLUSIONS

A new LC-MS/ MS method for the quantification of Gallic acid in Rabbit serum was developed. The method was found to be superior in LLOQ compared with other methods.[16] The specificity, sensitivity, accuracy, precision, recovery and stability met the requirements of USFDA guidance. The validity of proposed method supports its use for routine assay in pharmacokinetic studies or bioequivalence studies and will be useful in Therapeutic drug monitoring.

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