Chapter 2

Bioanalytical method development and validation for osteoprotective candidate K058

2.1. Experimental methods

2.1.1. Chemicals and reagents

(2S, 3S)-(+)-3'4'5,7-tetrahydroxydihydroflavonol-6-C- β -D-glucopyranoside (K058) (Fig-2.1) was isolated at MPC division, Central Drug Research Institute (C.D.R.I). The internal standard (IS) ononin (Fig-2.1) was obtained from Indofine Chemicals, New Jersey, U.S.A. HPLC grade methanol and glacial acetic acid were obtained from SRL, Mumbai, India and S.D. Finechem limited, Mumbai, India respectively. Heparin sodium injection I.P (Beparine, 1000 IU/mL) was procured from Biological E Ltd, Hyderabad, India. Purified water was prepared in our division from Millipore Milli-Q system. Female S.D. rats were procured from Laboratory animals division, C.D.R.I. Drug-free heparinised plasma was obtained from different young, healthy female S.D rats in the Laboratory Animal Division of the institute. Plasma was stored at -20° C till further use.

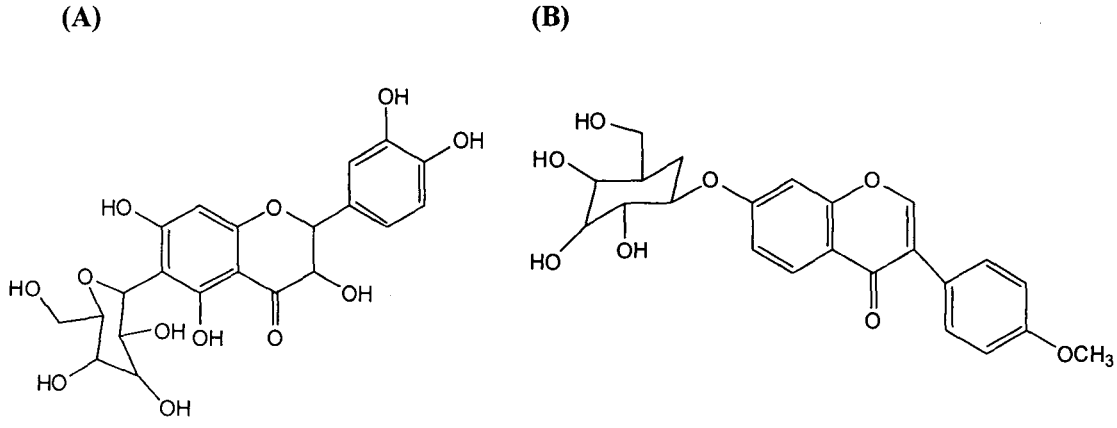

Fig-2.1: Structures of (A) K058, (B) Ononin

2.1.2. Preparation of stock and working solutions

The stock solution of K058 (1mg) was prepared in 1mL of methanol (1mg/mL) and it was diluted in methanol to get 10μg/mL. This solution was serially diluted in methanol to get the working solutions (WS) of concentration 5, 2.5, 1.25, 0.625, 0.3125, 0.156 and 0.078μg/mL. These WS of K058 were used to prepare calibration standards (CS) in blank female rat plasma. The working solutions of K058 at the concentration of 8, 1 and 0.1 μg/mL were also prepared from the stock solution of K058 by serial dilution. These WS were used to prepare quality control (QC) samples. The stock solution of IS, ononin

(1mg/mL) was also prepared in methanol and the working solution of IS (5µg/mL) was prepared from it in methanol.

2.1.3. Preparation of calibration standards and quality control samples

5µl of K058 WS (10µg/mL to 0.078µg/mL) was added to 95µl of blank female rat plasma, and vortexed to get the final concentration of 500, 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9ng/mL for calibration curve. Quality control (QC) samples at the concentration of 400ng/mL, 50ng/mL and 5ng/mL were prepared separately in blank plasma as high, medium and low QC respectively from the corresponding WS. To each CS and QC, 5µl of IS (5µg/mL) working solution was added to get the concentration of 250ng/mL (Table-2.1).

Table-2.1: Preparation of calibration standards (CS) and quality controls (QC)

Concentration of WS[a] (µg/mL)	**Volume of WS + Plasma**	**CS/QC concentration (µg/mL)**
0.078	5µL + 95µL	CS 3.9
0.156	5µL + 95µL	CS 7.8
3.125	5µL + 95µL	CS 15.6
6.25	5µL + 95µL	CS 31.25
12.5	5µL + 95µL	CS 62.5
25	5µL + 95µL	CS 125
50	5µL + 95µL	CS 250
0.1	5µL + 95µL	QC 5
1	5µL + 95µL	QC 50
8	5µL + 95µL	QC 400
To each AS and QC, 5 µL of IS (5µg/mL) was added		

Note: a-Working Solution of K058

2.1.4. Sample preparation

The extraction of K058 and IS from rat plasma was done by solid phase extraction (SPE) method using 3CC oasis cartridge in automated RapidTrace (RT) SPE work station, Caliper Lifesciences, Hopkinton, MA, USA. 5µl of K058 WS and 5µl IS solution was added to 95 µl of female rat plasma and vortexed. Spiked plasma was diluted to 0.5mL with water in a glass test tube and placed in RT processing system for SPE. The SPE cartridges were conditioned initially with 6mL of methanol, followed by conditioning with 6mL of 0.1% acetic acid. After conditioning of cartridges, spiked plasma (0.5mL) was loaded in the cartridge. Then the cartridges were rinsed with 6ml of 10% methanol in 0.1% acetic acid. Finally, K058 and IS was eluted from cartridges by 2ml of methanol and water (8:2) in two steps (2x1mL). The organic layer obtained after extraction was evaporated to dryness and the residue was reconstituted in 200 µl of mobile phase and 20 µl was used for injection in LC-MS-MS.

2.1.5. Chromatographic and mass spectrometric conditions

Perkin Elmer pump with autosampler and controller connected to API 4000, Applied Biosystems mass spectrometry with ESI and triple quadrupole was used for HPLC-MS/MS analysis of K058. The mobile phase consisted of methanol and 0.1% acetic acid in water (v/v) in isocratic conditions of 80:20 at the flow rate of 0.5ml/min. For the elution of K058 and IS, reverse phase Zorbax C18, 100 x 4.6 mm id and 5µm column with C18 guard cartridge was used. The run time was 5 minutes and the injection volume was 20µL

Mass spectrometric analysis was performed in negative ion mode of ESI source. The source temperature was set at 400°C and the ionisation voltage at -4500 V. The multiple reactions monitoring (MRM) conditions used for the quantitative analysis of K058 was 465/345 at declustering potential (DP) and collision energy (CE) of -130 and -20 units respectively. The MRM 489.2/266.6 at DP of -50 units and CE of -20 units was used for ononin (IS). Data acquisition and analysis were performed in Analyst software ver 1.4.2.

2.1.6. Validation of LC-MS/MS method

The LC-MS/MS method for K058 was validated for selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, dilution integrity and stability in female rat plasma. The selectivity and specificity of the LC-MS/MS method were evaluated by comparing the response obtained for blank plasma with that of LLOQ of calibration standard. The linearity of the method was determined from the calibration standards prepared for the range of concentrations prepared in female rat plasma. The ratio of peak area of test compound to IS of calibration standards was used for the construction of calibration curve to check linearity.

The intra-day and inter-day accuracy (% bias), precision (% C.V.), recovery, matrix effect and stability were determined from quality control samples (N=5) prepared on three days. The quality control samples at low, medium and high concentration were used for the method validation. The stability of K058 in female rat plasma was determined at various storage conditions for the quality control samples (N=3 for each QC). The dilution integrity was determined to evaluate the effect of dilution of plasma samples on the actual concentration of compound. This is required when the level of compound in plasma exceeds the linearity range of the developed LC-MS/MS method. To determine dilution integrity, WS of concentration 8µg/mL was used for spiking in plasma. The spiked plasma (400ng/mL) was diluted by 2, 5 and 10 times to get the concentration of 200, 80 and 40ng/mL respectively. These dilutions were prepared in triplicate and tested for dilution integrity.

2.2. Results and discussions

2.2.1. Optimization of chromatography and mass spectrometric conditions

To obtain selective and sensitive MRM condition, the ionisation of K058 was checked by infusion experiment. K058 (Mol. Wt= 466) gave abundant base peak $(M-H)^-$ 465 of high intensity. MS/MS spectra analysis by infusion experiment showed 345 as the prominent product ion of 465 (Fig-2.2.1). The DP -130 and CE -20 units were found to be optimum parameters for the MRM 465/345. The IS ononin is also a glycoside and its ionisation was better in negative mode and it gave the base peak $(M+CH_3COO)^-$ of mol.wt 489. The

ion 266.6 was obtained as the prominent product ion for IS at the DP of -50 units and CE of -20 units (Fig-2.2.2). Acetonitrile and methanol were tried for liquid chromatographic (LC) elution on Zorbax C18 (100 x 4.6, 5µm) column. The peak shape was better when methanol was used as organic modifier. Hence, methanol and 0.1% acetic acid was selected as the suitable mobile phase at the flow rate of 0.5mL/min to get a shorter run time of 5min.

As K058 is a glucoside, which is a polar compound, protein precipitation and solid phase extraction methods were tested for extraction from female rat plasma. The recovery was very low and ionic suppression was high in protein precipitation method. So SPE method was tried for the optimization of extraction process. 3mL oasis cartridge was found to provide better recovery than 1mL cartridge. The recovery was best when 6mL of solvents were used for conditioning and rinsing the cartridges. SPE procedure was found to be the most suitable and best for the extraction of both K058 and IS (ononin) from rat plasma and therefore optimized SPE method was applied in further studies.

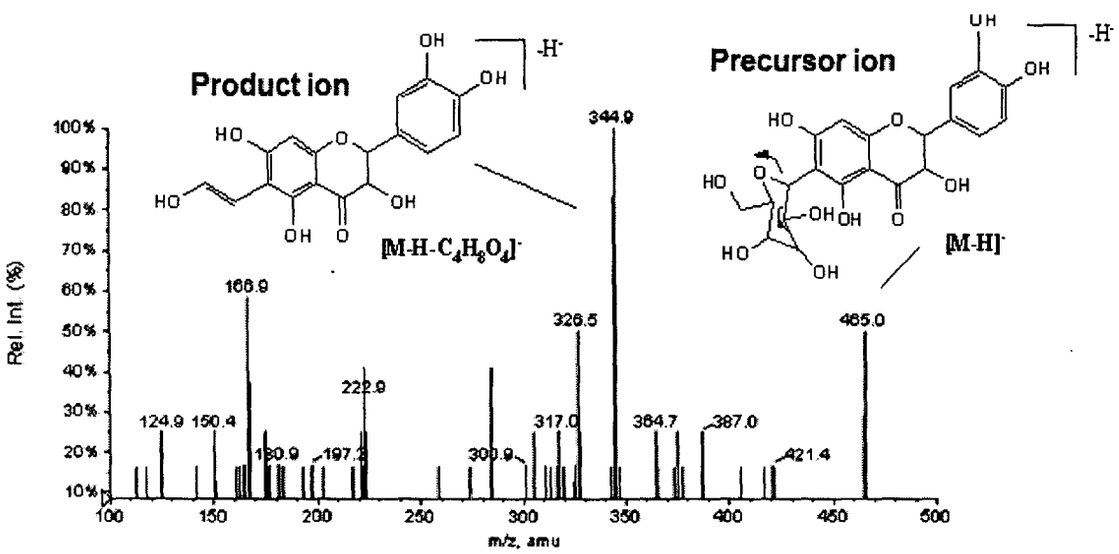

Fig-2.2.1: MS/MS spectrum of K058

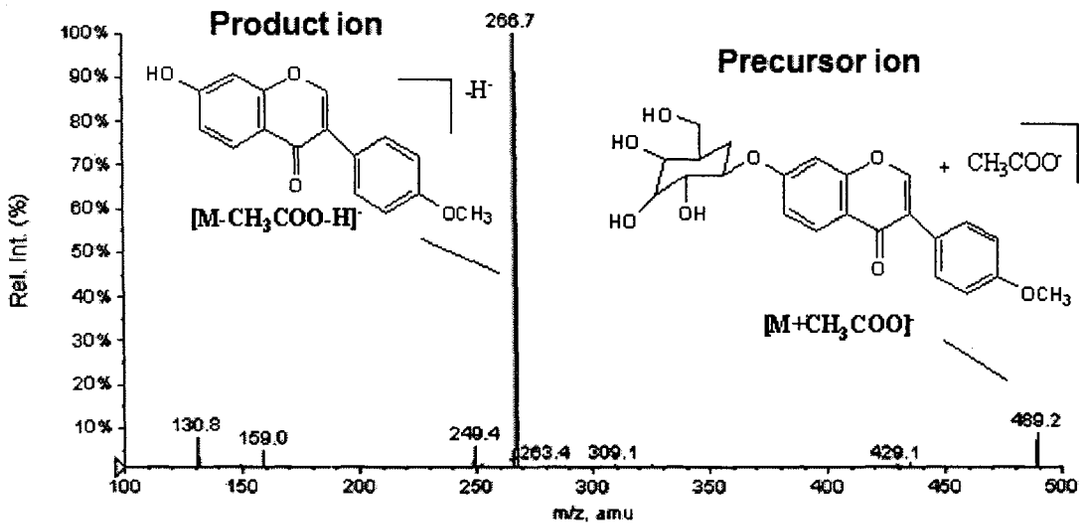

Fig-2.2.2: MS/MS spectrum of ononin

2.2.2. Validation of LC-MS/MS method

Selectivity, Sensitivity and Linearity

The LC-MS/MS method developed for K058 and the IS (ononin) was selective and specific. No interference was observed in the chromatogram of the blank plasma for the MRMs selected when compared with the chromatogram of rat plasma spiked with K058 and IS (Fig. 2.3). 1.95ng/mL was the limit of detection (LOD) and 3.9ng/mLwas the lower limit of quantitaion (LLOQ) of this method. Linearity was obtained for the concentration ranging from 3.9ng/mL to 500ng/mL. The regression coefficient (r) was greater 0.9993±0.0005 for the calibration curve by applying the weighting scheme of 1/x.

Recovery and Matrix effect

The extraction of K058 and IS by SPE method was high, with low matrix effect. The recovery was greater than 80% for K058 and greater than 50% for IS (Table-2.2). The matrix effect calculated for post extracted samples of K058 were in the range of -3.98% to -8.97 %, which indicates the absence of significant ionic suppression or enhancement.

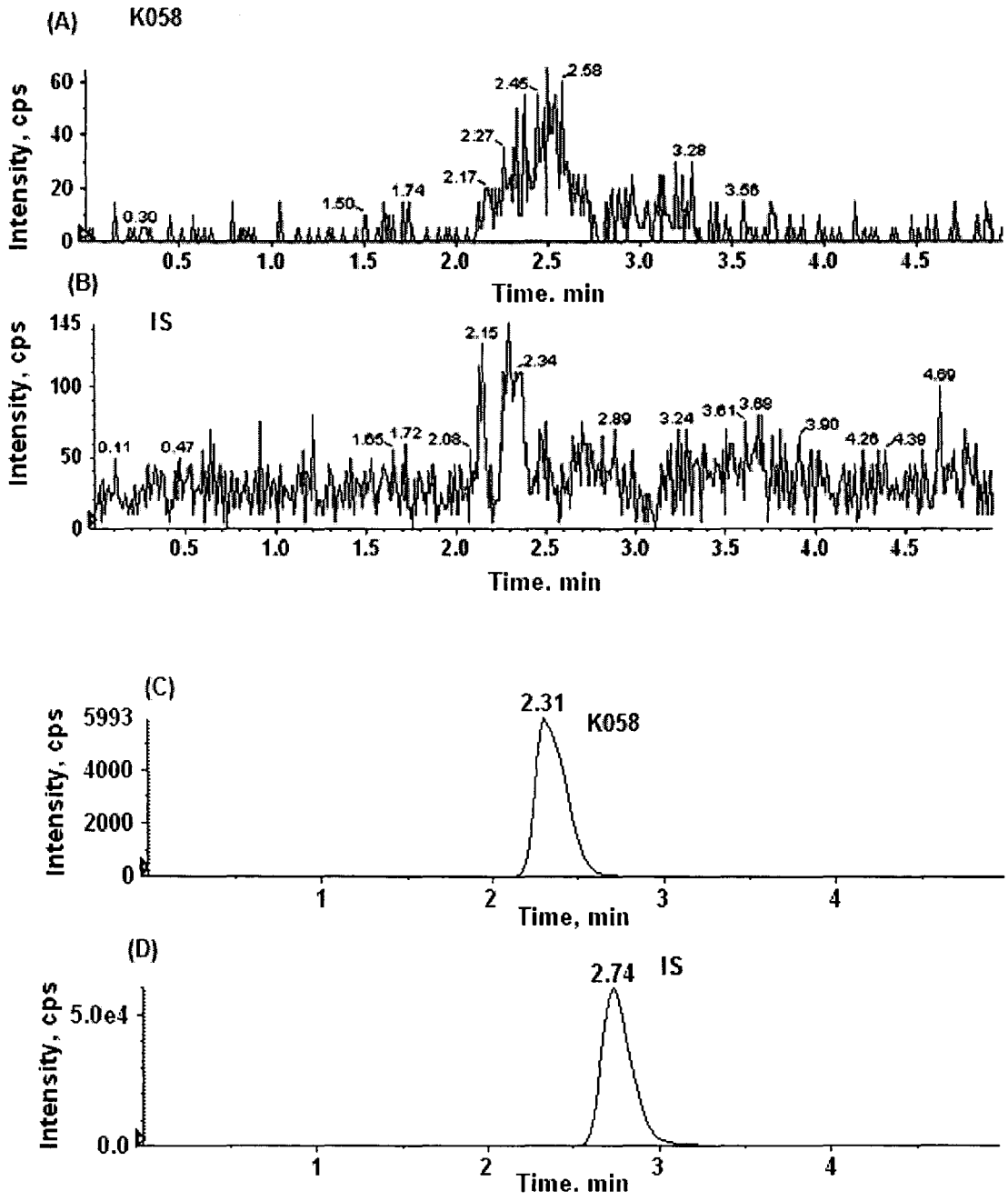

Fig-2.3: LC-MS/MS chromatogram of blank plasma for MRM of (A) K058 (B) IS; rat plasma spiked with (C) K058 and (D) IS

Accuracy and Precision

The intra-day and inter-day accuracy (% bias) and precision (% C.V.) of the LC-MS/MS method was calculated for quality control samples at low, medium and high concentrations. These values are presented in Table-2.2. The results showed that the method was accurate and precise with values of % bias and % C.V. lying between -0.18 and 9.90. These values were within the acceptable limit of ±15%.

Table-2.2: Intra-day and Inter-day accuracy and precision of K058 in rat plasma

Quality control (ng/mL)	**Accuracy (% Bias)**		**Precision (% C.V.)**		**% Recovery**	
	Intra-day	Inter-day	Intra-day	Inter-day	K058	IS*
5	-0.19	1.82	7.12	5.96	92.53±11.2	
50	3.89	3.17	9.90	0.88	88.94±2.88	56.46±3.27
400	8.98	8.80	9.41	3.51	110.20±7.99	

Note: Recovery of IS calculated at 250ng/mL

Stability in plasma

The stability of K058 in plasma samples was evaluated for quality control samples in triplicate under various storage conditions. The calculated concentrations of K058 in stability samples were compared with those of freshly prepared quality control samples (t=0) to determine the % bias. The results show that % bias values of three freeze thaw cycles were in the range of 3.56 to 11.45 (Table-2.3, Fig-2.4). The % bias values of bench top stability, long term stability, auto sampler and dry residue stability studies were less than 8% (Table-2.4). These values indicate that the K058 was stable in plasma after three freeze-thaw cycles, short-term, long-term storage and in processed samples.

Table-2.3: Freeze thaw stability of K058 in rat plasma

Quality control (ng/mL)	**Cycle-1 (% Bias)**	**Cycle-2 (% Bias)**	**Cycle-3 (% Bias)**
5	-3.66	3.56	5.51
50	4.12	3.64	11.45
400	4.73	6.92	-4.89

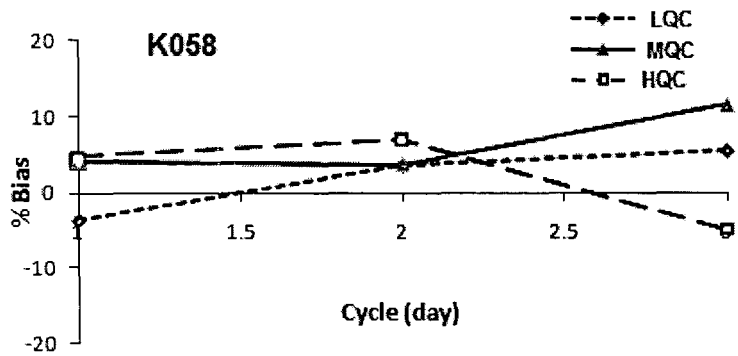

Fig-2.4: Graph of freeze thaw stability of K058 in rat plasma

Table-2.4: Stability of K058 at different conditions

Quality control (ng/mL)	Benchtop stability (% Bias)	Long term stability (% Bias)	Auto sampler stability (% Bias)	Dry residue Stability (% Bias)
5	-1.57	4.18	-1.20	3.70
50	3.54	5.62	0.10	5.96
400	-5.31	4.44	4.20	-7.48

Dilution integrity

The results of dilution integrity are presented in Table-2.5. The % bias and % C.V. values calculated for the diluted samples were within the acceptable limit of ±15%. This indicates that developed LC-MS/MS procedure can be applied for diluted samples of plasma, if observed concentration of compound in samples is greater than the upper limit of the linearity range.

Table-2.5: Accuracy and precision values of K058 for dilution integrity

Nominal concentration (ng/mL)	Calculated concentration (ng/mL)	% Bias	% C.V.
40	36.06±1.91	9.83	5.29
80	78.80±3.04	1.5	3.85
200	206.33±21.21	-3.17	10.28

2.3. Conclusion:

A selective, sensitive and rapid LC-MS/MS method has been developed and validated for quantitative analysis of K058 in female rat plasma. The method was sensitive with LOD and LLOQ of 1.95ng/mL and 3.9ng/mL respectively. The validated LC-MS/MS method was linear for the concentration range from 3.9ng/mL to 500ng/mL. The method was accurate and precise with % bias and %C.V. values for intra-day and inter-day batch not greater than 10. The recovery of K058 and IS by SPE procedure was greater than 85% and 50% respectively, with absence of significant ionic suppression or enhancement. K058 was stable in plasma and processed samples at various storage conditions. The validated parameters of LC-MS/MS method for K058 show that it is suitable to apply for the pharmacokinetic study of K058 in female rats.