Chapter 4

Bioanalytical method development and validation for osteogenic candidates K095, K054 and S006-1709

4.1. Experimental methods

4.1.1. Chemicals and reagents

Medicarpin (3-hydroxy, 9-methoxy pterocarpan, K095), Cladrin (7-hydroxy, 3',4'-dimethoxyisoflavone,, K054) and S006 1709 (3-hydroxy pterocarpan) (Fig-4.1) were synthesized at MPC division, Central Drug Research Institute (C.D.R.I). The internal standards (IS) 7-hydroxy isoflavone and biochanin (Fig-4.1) were obtained from Indofine Chemicals, New Jersey, U.S.A. HPLC grade acetonitrile 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.

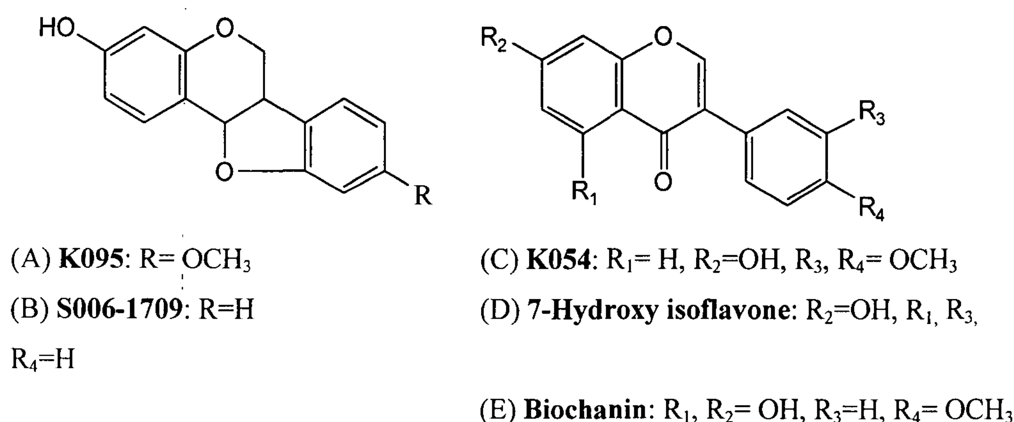


Fig-4.1: Structures of (A) K095, (B) S006-1709, (C) K054, (D) 7-hydroxy isoflavone and (E) Biochanin

4.1.2. Preparation of stock and working solutions

Medicarpin (K095)

The stock solution of K095 (1mg) was prepared in 5 mL of acetonitrile (200μg/mL) and was diluted in acetonitrile to get 10μg/mL. This solution was serially diluted in

acetonitrile to get the working solutions of concentration 5, 2.5, 1.25, 0.625, 0.3125, 0.156 and 0.078μg/mL. These working solutions (WS) of K095 were used to prepare calibration standards (CS) and quality control (QC) samples in blank female rat plasma. The stock solution of IS, 7-hydroxy isoflavone (1mg/mL) was also prepared in acetonitrile and the working solution of IS (2.5μg/mL) was prepared from it in acetonitrile.

Cladrin (K054)

The stock solution of K054 (1mg) was also prepared in 5 mL of acetonitrile (200μg/mL). The WS of cladrin (20μg/mL) was prepared by spiking 100μl of the stock solution in acetonitrile. This solution was serially diluted in acetonitrile to get WS at the concentration of 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156 and 0.078μg/mL. The CS and QC samples in blank female rat plasma were prepared from these WS of K054. The WS of IS (1μg/mL) was prepared in acetonitrile from the stock solution (1mg/mL) of 7-hydroxy isoflavone.

S006-1709

The stock solution was prepared by dissolving 1mg of S006-1709 in 5 ml of acetonitrile to get the concentration of 200μg /mL. This stock solution was diluted stepwise in acetonitrile to get the WS of concentration 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156 and 0.078μg/mL. These WS were used to prepare calibration standards (CS). Further the working solutions of S006-1709 at the concentration of 16μg/mL, 2μg/mL, and 0.2μg/mL were also prepared in acetonitrile for preparation of quality control (QC) samples. The stock solution of biochanin (IS) was also prepared in acetonitrile (200μg /mL) and was diluted in acetonitrile to get working solution of 1μg/mL.

4.1.3. Preparation of calibration standards and quality control samples

Medicarpin (K095)

5μl of working solution (5μg/mL to 0.078μg/mL) of K095 was added to 95μl of blank female rat plasma, and vortexed to get the final concentration of 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9ng/mL for calibration curve. QC samples at the concentration of

250ng/mL, 31.25ng/mL and 3.9ng/mL were prepared separately in blank plasma as high QC (HQC), medium QC (MQC) and low QC (LQC) respectively. To each CS and QC, 5µl of IS (7-hydroxy isoflavone) working solution (2.5µg/mL) was added to get the concentration of 125ng/mL (Table-4.1).

Table-4.1: Preparation of calibration standards (CS) and quality controls (QC) of K095

Concentration of WS[a] (µg/mL)	Volume of WS + Plasma	CS/QC concentration (ng/mL)
0.078	5µL + 95µL	CS 3.9
0.156	5µL + 95µL	CS 7.8
0.3125	5µL + 95µL	CS 15.6
0.625	5µL + 95µL	CS 31.25
1.25	5µL + 95µL	CS 62.5
2.5	5µL + 95µL	CS 125
5	5µL + 95µL	CS 250
0.078	5µL + 95µL	QC 3.9
0.625	5µL + 95µL	QC 31.25
2.5	5µL + 95µL	QC 250
To each CS and QC, 5µL of IS (2.5 µg/mL) was added		

Note: a - Working Solution of K095

Cladrin (K054)

5µl of working solution (20µg/mL to 0.078µg/mL) of K054 was added to 95µl of blank female rat plasma, and vortexed to get the final concentration of 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9ng/mL for calibration curve of cladrin. Quality control (QC) samples at the concentration of 1000ng/mL (quality control high-QCH), 62.5ng/mL (quality control medium-QCM) and 7.8ng/mL (quality control low-QCL) were prepared in blank plasma by spiking 5µl of working solutions of cladrin at 20, 1.25 and 0.156µg/mL respectively in 95µl of blank female rat plasma. To each CS and QC, 5µl of IS (7-hydroxy isoflavone) working solution (1µg/mL) was added to get the concentration of 50ng/mL (Table-4.2).

Table-4.2: Preparation of calibration standards (CS) and quality controls (QC) of K054

Concentration of WS[a] (μg/mL)	Volume of WS + Plasma	CS/QC concentration (ng/mL)
0.078	5μL + 95μL	CS 3.9
0.156	5μL + 95μL	CS 7.8
0.3125	5μL + 95μL	CS 15.6
0.625	5μL + 95μL	CS 31.25
1.25	5μL + 95μL	CS 62.5
2.5	5μL + 95μL	CS 125
5	5μL + 95μL	CS 250
10	5μL + 95μL	CS 500
20	5μL + 95μL	CS 1000
0.156	5μL + 95μL	QC 7.8
1.25	5μL + 95μL	QC 62.5
20	5μL + 95μL	QC 1000
To each CS and QC, 5μL of IS (1μg/mL) was added		

Note: a - Working Solution of K054

S006-1709

5μl of working solution (20μg/mL to 0.078μg/mL) was added to 95μl of blank female rat plasma, and vortexed to get the final concentration of 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9ng/mL for calibration curve of S006 1709. Quality control (QC) samples at the concentration of 800ng/mL (quality control high-QCH), 100ng/mL (quality control medium-QCM) and 10ng/mL (quality control low-QCL) were prepared in blank plasma by spiking 5μl of working solutions of S006 1709 at 16, 2 and 0.2μg/mL respectively in 95μl of blank female rat plasma. To each CS and QC, 5μl of IS (biochanin) working solution (1μg/mL) was added to get the concentration of 50ng/mL (Table-4.3).

Table-4.3: Preparation of calibration standards (CS) and quality controls (QC) of S006-1709

Concentration of WS[a] (µg/mL)	Volume of WS + Plasma	CS/QC concentration (ng/mL)
0.078	5µL + 95µL	CS 3.9
0.156	5µL + 95µL	CS 7.8
0.3125	5µL + 95µL	CS 15.6
0.625	5µL + 95µL	CS 31.25
1.25	5µL + 95µL	CS 62.5
2.5	5µL + 95µL	CS 125
5	5µL + 95µL	CS 250
10	5µL + 95µL	CS 500
20	5µL + 95µL	CS 1000
0.2	5µL + 95µL	QC 10
2	5µL + 95µL	QC 100
16	5µL + 95µL	QC 800
To each CS and QC, 5µL of IS (1µg/mL) was added		

Note: a- Working Solution of S006-1709

4.1.4. Sample preparation

Liquid-liquid extraction (LLE) method was used for processing of calibration standards, quality control and test samples of K095, K054 and S006-1709 using diethyl ether. The processing volume of plasma sample was 100µL. To each standard and sample, 5µL of IS working solution was added and vortexed for 15 s. After addition of IS, 1.5mL of diethyl ether was added, vortexed for 5 min and centrifuged for 15 min at 3500 rpm. Then the aqueous layer was frozen in liquid nitrogen, the upper organic layer was transferred to a test tube and evaporated to dryness in speed vac evaporator. The plasma in the aqueous layer was reprocessed in the same way and the dry residue obtained after double extraction was reconstituted in 200µL of mobile phase, vortexed and 20µl of it was injected for analysis.

4.1.5. Chromatographic and Mass spectrometric conditions

Medicarpin (K095)

Perkin Elmer pump with autosampler and controller connected to API 4000 (Applied Biosystems, Toronto, Canada) with electrospray ionization (ESI) and triple quadrupole mass spectrometer was used for HPLC-MS/MS analysis of K095. The mobile phase consisted of acetonitrile and 0.1% acetic acid in water (v/v) in isocratic conditions of 60:40 at the flow rate of 0.5ml/min. For the separation of K095 and IS, reverse phase C18 Phenomenex (Ultramex) (150x4.6mm, 5µm) column with Phenomenex guard cartridge was used (Table-4.4). The run time was 10 minutes and the injection volume was 20µL

Mass spectrometric analysis was performed in positive ion mode of ESI source. The source temperature was set at 500°C and the ionisation voltage at 5500 V. Multiple reactions monitoring (MRM) selected for quantitative analysis of K095 and the other parameters optimized for MS/MS are given in Table-4.5. Data acquisition and analysis were performed in Analyst software ver 1.4.1.

Cladrin (K054) and S006-1709

Shimadzu UFLC pump (LC-20AD) with autosampler (SIL-HTc) and degasser (DGU-20A3) connected to API 4000 Q trap, (Applied Biosystems, Toronto, Canada) mass spectrometer were used for HPLC-MS/MS analysis of K054 and S006-1709. The mobile phase consisted of acetonitrile and 0.1% acetic acid in water (v/v) in isocratic condition of 6:4 and 8:2 for K054 and S006-1709 respectively at the flow rate of 0.5ml/min. Reverse phase C18 Phenomenex (Ultramex), 150x4.6mm, 5µm column with Phenomenex guard cartridge was used for the separation of K054 and IS. The elution of S006-1709 and biochanin (IS) were carried out on Discovery C18 column (100x4.6mm, 5µm) (Table-4.4). The run time of the LC-MS/MS method for K054 and S006-1709 was 7 and 5 minutes respectively. The injection volume was 20µL for both the methods.

Mass spectrometric analysis was performed in positive ion mode of ESI for K054 whereas S006-1709 was analyzed in negative ionisation mode of ESI. The MRM and

other mass parameters selected for quantitative analysis of K054, S006-1709 and the IS are summarized in Table-4.5. The mass spectrometer was operated in unit resolution at dwell time of 200ms for each MRM transition. Data acquisition and analysis were performed in Analyst software ver 1.4.2.

4.1.6. Validation of LC-MS/MS method

The LC-MS/MS methods for K095, K054 and S006-1709 were validated for selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect and stability in female rat plasma. The selectivity and specificity of the LC-MS/MS method for each compound 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 CS prepared for the range of concentrations prepared in female rat plasma. The ratio of peak area of test compound to IS of CS 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 QC samples (QCL, QCM and QCH) prepared on three days (N=5 for each QC). The stability of each compound in female rat plasma was determined at various storage conditions. QCL, QCM and QCH were used for the evaluation of stability.

Table-4.4: Chromatographic conditions for K095, K054 and S006-1709

Markers	Column	Mobile Phase (%)		Flow rate (mL/min)
		A	B	
Medicarpin (K095)	Phenomenex	60	40	0.5
Cladrin (K054)	Phenomenex	60	40	0.5
S006-1709	Discovery	80	20	0.5

Note: A- acetonitrile, B- 0.1% acetic acid

Table-4.5: Mass spectrometric conditions for K095, K054 and S006-1709

Markers	Molecular weight	MRM transitions (Ionization mode)	D.P.[a]	C.E.[b]
Medicarpin (K095)	270	271/137 (+)	60	25
Cladrin (K054)	298	299/283 (+)	140	30
S006-1709	240	239.1/169.2 (-)	-70	-35
7-Hydroxy isoflavone[c]	238	239/137(+)	81	44
Biochanin[d]	284	283/267.4 (-)	-90	-47

Note: a - Declustering Potential, b- Collision Energy, c - Internal Standard for K095 and K054, d- Internal standard for S006-1709

4.2. Results and discussions

4.2.1. Optimization of chromatographic and mass spectrometric conditions

Medicarpin (K095)

The mobile phase acetonitrile (A) and 0.1% acetic acid (B) in MlliQ water in isocratic condition (60:40) was found to be optimal for the chromatographic elution of K095 and IS on Phenomenex RP18 (150x4.6 mm, 5µm) column. The method was short with the retention time of 8.1±0.2 minute for K095 and 6.3±0.2 minute for IS. In LC-MS/MS method for bioactive markers of F147 by gradient elution (Part-I, Chapter-1), negative mode of ionisation was used for quantitative analysis of K095. But, in isocratic mode, interference was observed when negative mode was used. So for quantitative analysis of K095 in female rat plasma by isocratic elution, positive mode was preferred because of better selectivity. 7-Hydroxy isoflavone was used as an IS for K095. Though 7-Hydroxy isoflavone is not a pterocarpan, it shares structural similarity with K095 because of its isoflavone structure (Fig. 4.1) and sensitivity in positive ion mode for quantitative analysis. As for as fragmentation is concerned, Retro-Diel's-Alder (RDA) fragmentation is one of the important pathways for isoflavones. In the MRMs of both K095 and IS, the RDA fragment was found to be prominent product ion and hence it was selected for their quantitative analysis (Fig - 4.2.1, 4.2.2).

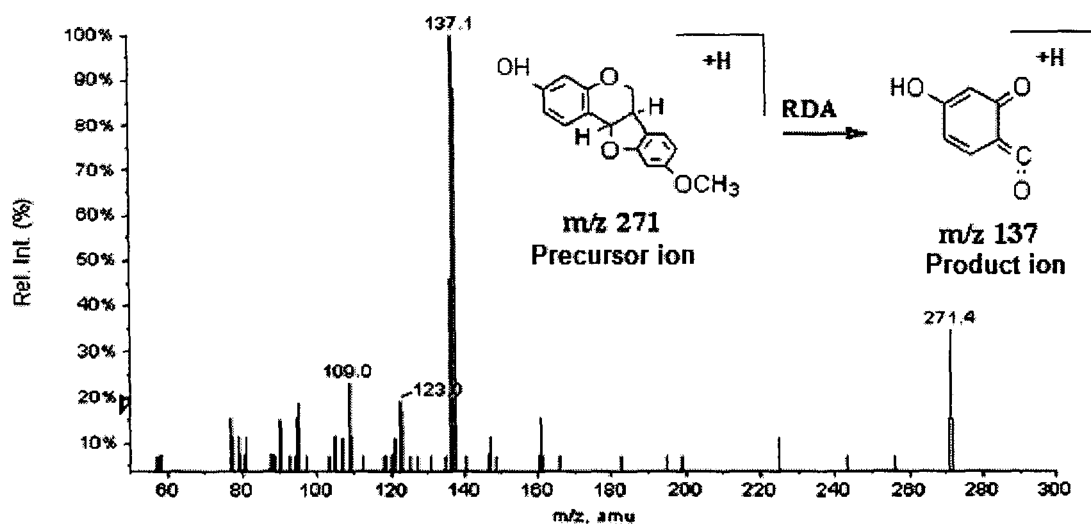
Cladrin (K054)

The LC method optimized for K054 was same as that of K095 (60:40 of acetonitrile and 0.1% acetic acid in MlliQ water). The retention time of K054 and IS was 4.2±0.1and 4.8±0.1 minute respectively. For K054, interfence was observed when negative mode was used in isocratic condition. Therefore, positive mode of ionisation was selected for quantitative analysis. 7-hydroxy isoflavone, being structurally similar to K054 and sensitive in positive ion mode, was selected as IS for quantitative analysis of K054. There was a difference in the retention time of IS (7-hydroxy isoflavone) between the LC-MS/MS method for K095 and K054, inspite of similar chromatographic conditions. This difference was due to the change in the HPLC instrument used for these two methods.

S006-1709

Ionisation of S006-1709 was checked in both positive and negative modes of electro spray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) by infusion pump (Harvard Apparatus, Inc., USA). The sensitivity was high in negative mode and the most abundant ions observed were $(M-H)^-$ molecular ion. Product ion spectra analysis of $(M-H)^-$ was performed at various collision energy to select appropriate product ions. Two prominent product ions m/z 169 and m/z 116.9 were observed in the MS/MS spectrum of S006-1709(Fig-4.2.2). Finally the transition m/z 239.1 to 169.2 was selected for the quantitative analysis of S006-1709 because of its high sensitivity. Discovery RP18 column with solvents acetonitrile (A) and 0.1% acetic acid in water (B) were selected for liquid chromatography to get short run time with proper elution and good peak shape. The mobile phase composition was optimised to 80:20 v/v of A: B at the flow rate of 0.5mL/min. The run time was short and the retention time (t_R) of S006-1709 was 3.6 min. Biochanin was selected as an internal standard for S006-1709. The MS/MS spectrum of biochanin in negative ionization mode is given in Fig-4.2.3

(A) Medicarpin (K095)



(B) Cladrin (K054)

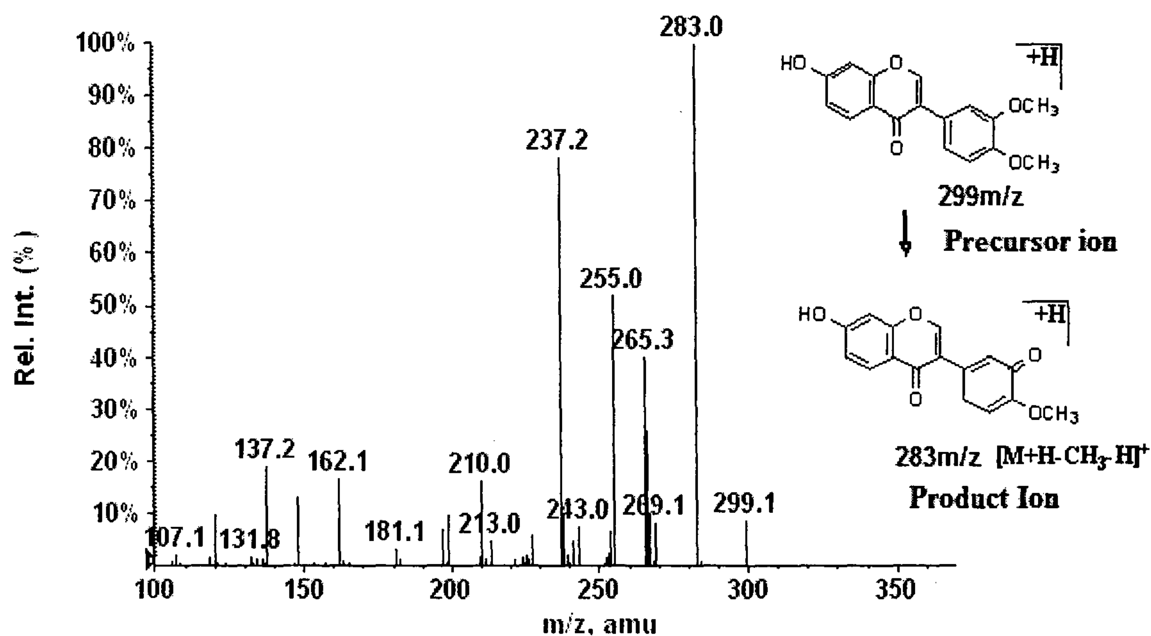
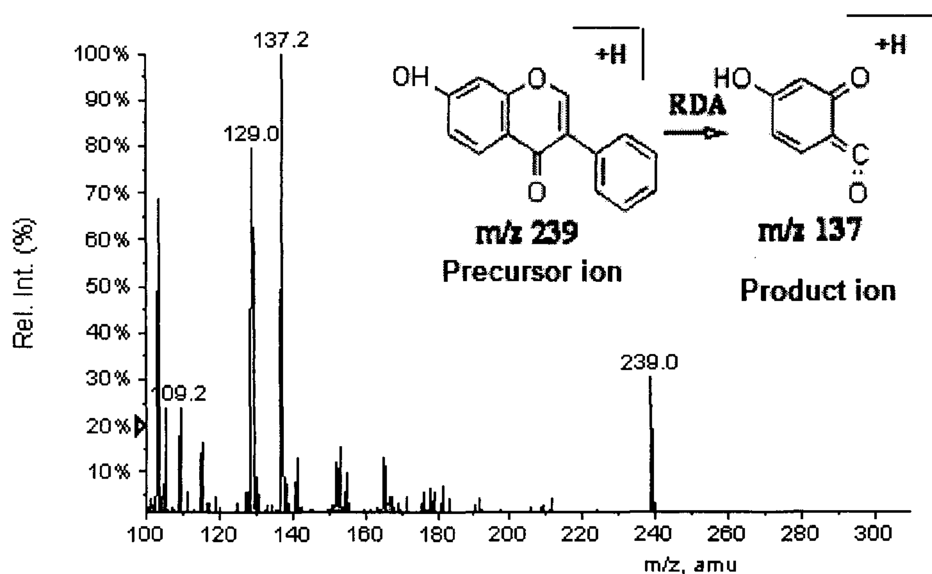


Fig-4.2.1: MS/MS spectra of (A) Medicarpin and (B) Cladrin

(A) 7-Hydroxy isoflaovne



(B) S006-1709

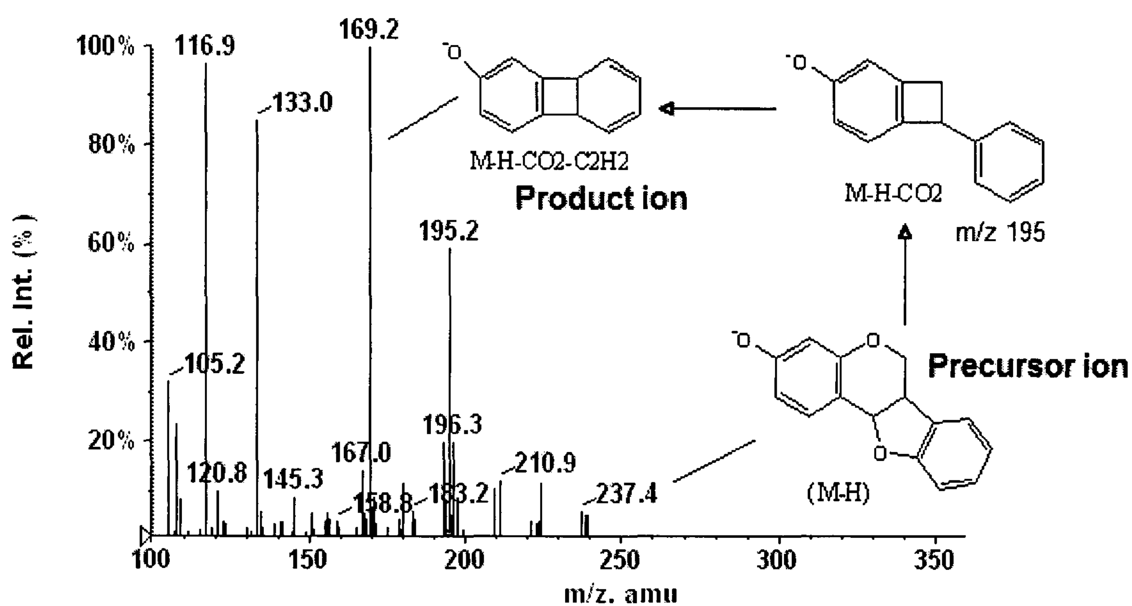


Fig-4.2.2: MS/MS Spectra of (A) 7-hydroxy isoflavone and (B) S006-1709

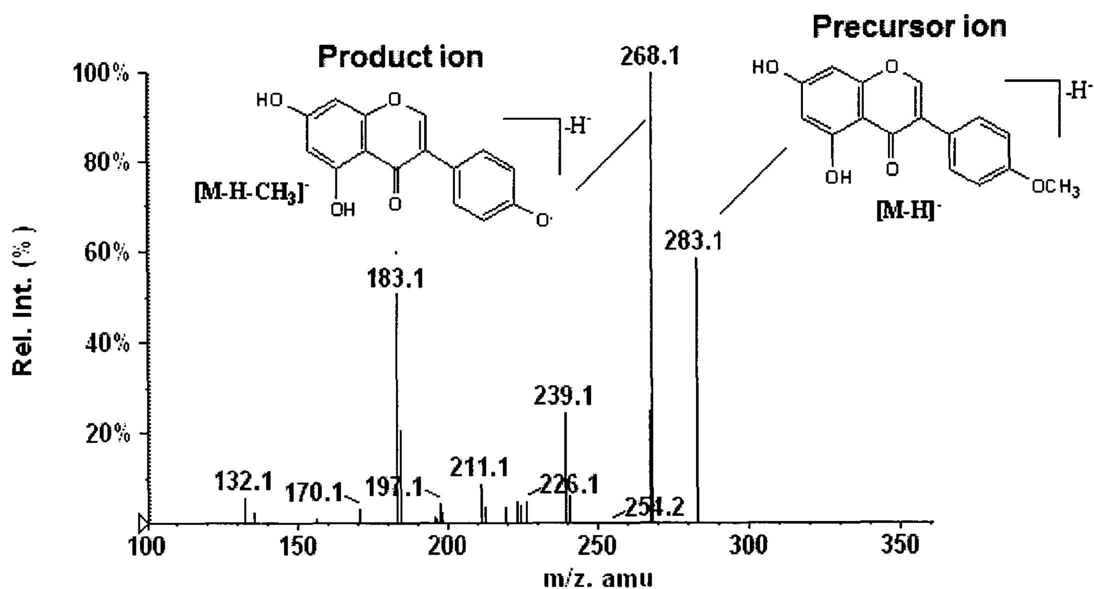


Fig-4.2.3: MS/MS spectrum of biochanin

4.2.2. Validation of LC-MS/MS method

Selectivity, Sensitivity and Linearity

The HPLC-MS/MS method developed for each test compound (K095, K054 or S006-1709) and the IS (7-Hydroxy isoflavone) was selective and specific. There were no interferences observed in the chromatogram of the blank plasma for the MRMs selected when compared with the chromatogram of rat plasma spiked with corresponding test compound and IS (Fig- 4.3.1 - Fig-4.5.2).

The method for K095 was sensitive with 1.95ng/mL as the limit of detection (LOD) with signal to noise ratio greater than 3 and 3.9ng/mL as the lower limit of quantitaion (LLOQ). The method was linear for the concentration ranging from 3.9 to 250ng/mL with the regression coefficient (r) greater than 0.99 applying the weighting scheme of 1/x (Table-4.6).

The LC-MS/MS method for K054 was sensitive with 3.9ng/mL as the limit of detection (LOD) and linear over the concentration range of 7.8ng/mL to 1000ng/mL (Table-4.6). The LOD of the LC-MS/MS method for S006-1709 was 1.95ng/mL at which the signal

to noise ratio was greater than three. This method was linear for the concentration ranging from 3.9ng/mL to 1000ng/mL (Table-4.6).

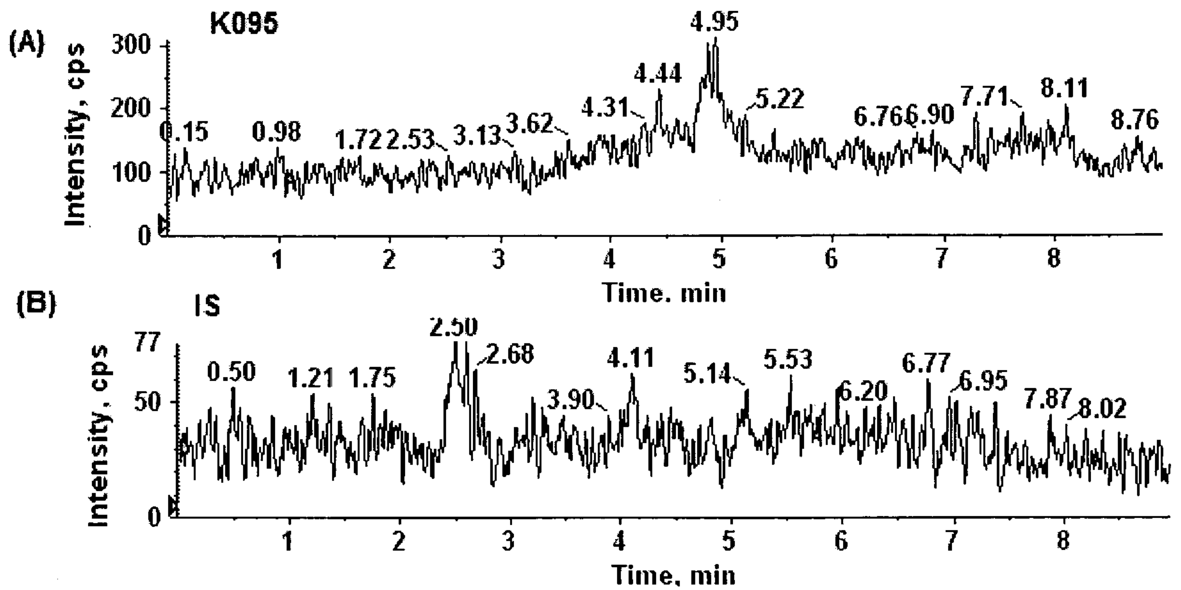


Fig-4.3.1: LC-MS/MS chromatogram of blank rat plasma for MRM of (A) K095 and (B) IS

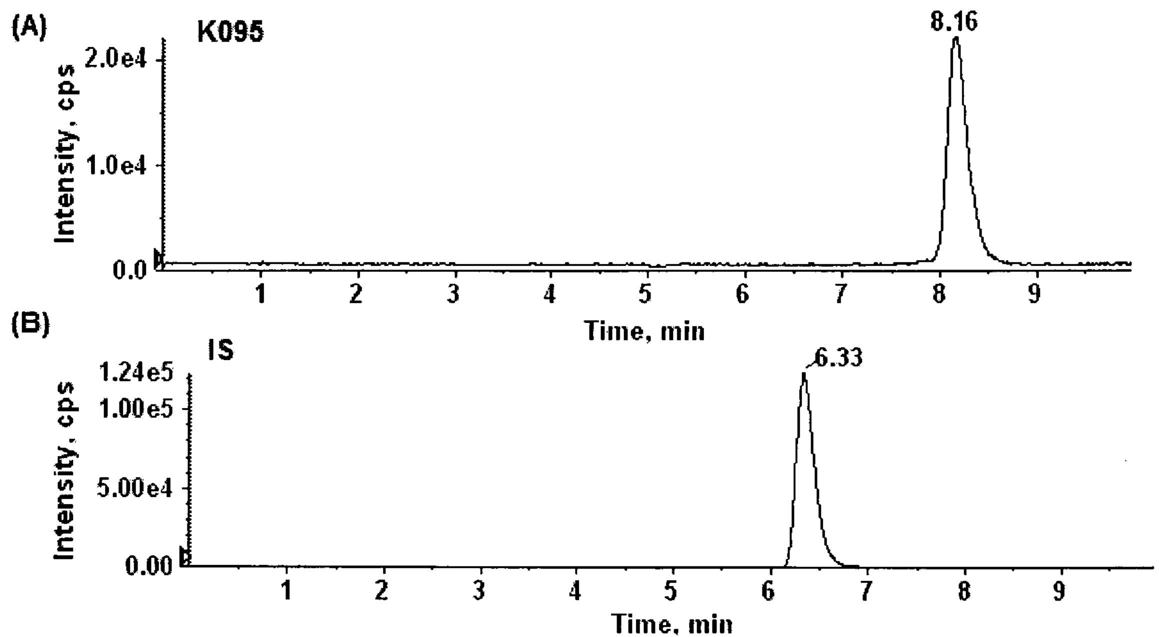


Fig-4.3.2: LC-MS/MS chromatogram of rat plasma spiked with (A) K095 and (B) IS

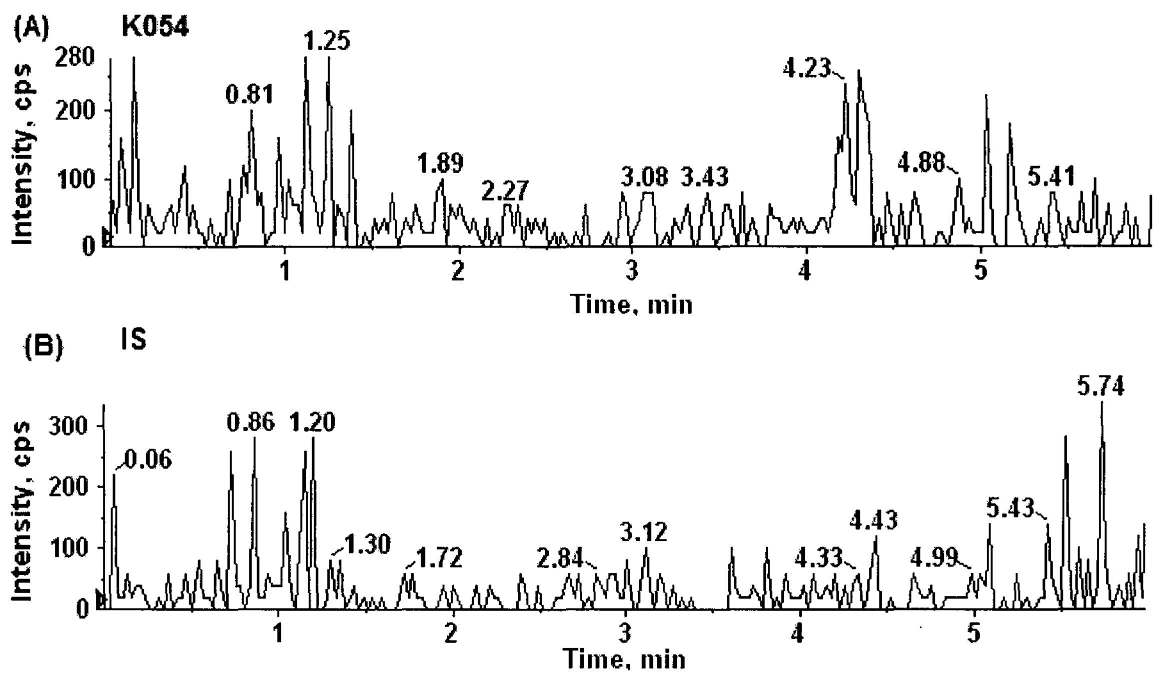


Fig-4.4.1: LC-MS/MS chromatogram of blank rat plasma for MRM of (A) K054 and (B) IS

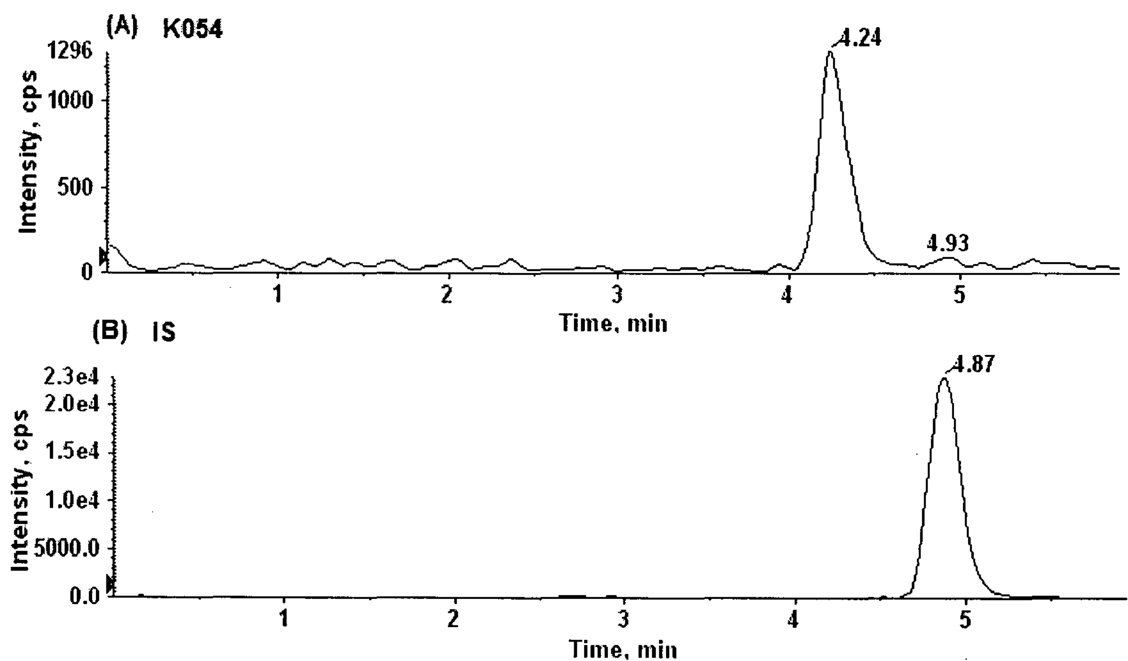


Fig-4.4.2: LC-MS/MS chromatogram of rat plasma spiked with (A) K054 and (B) IS

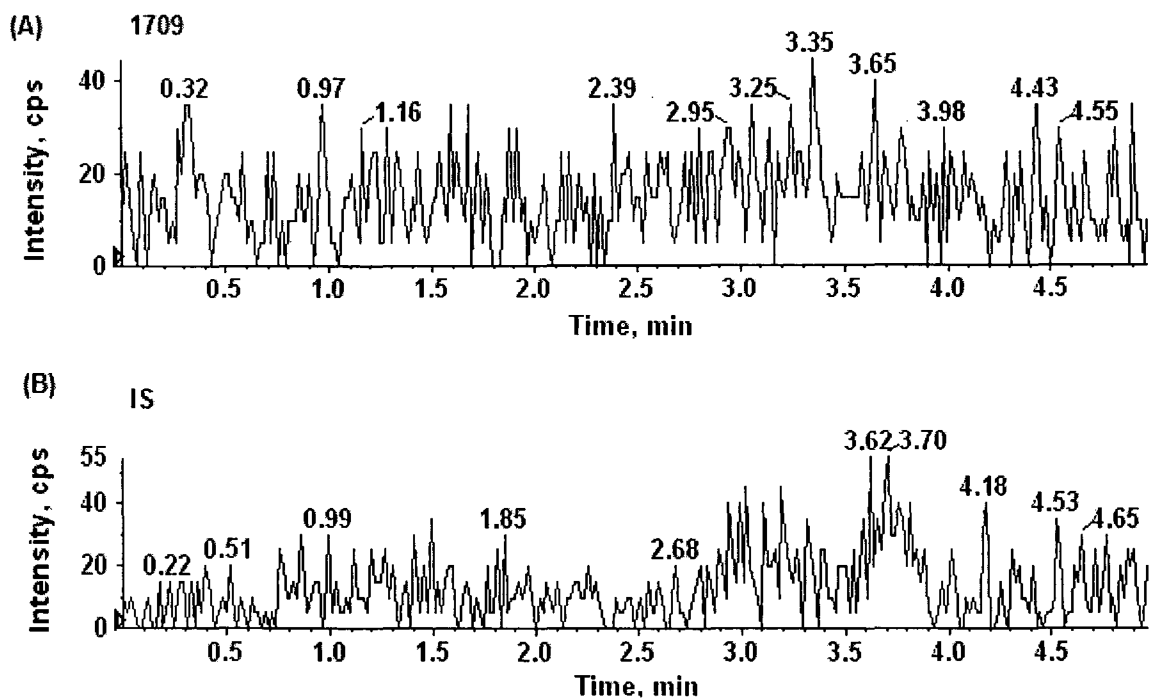


Fig-4.5.1: LC-MS/MS chromatogram of blank rat plasma for MRM of (A) S006-1709 and (B) IS

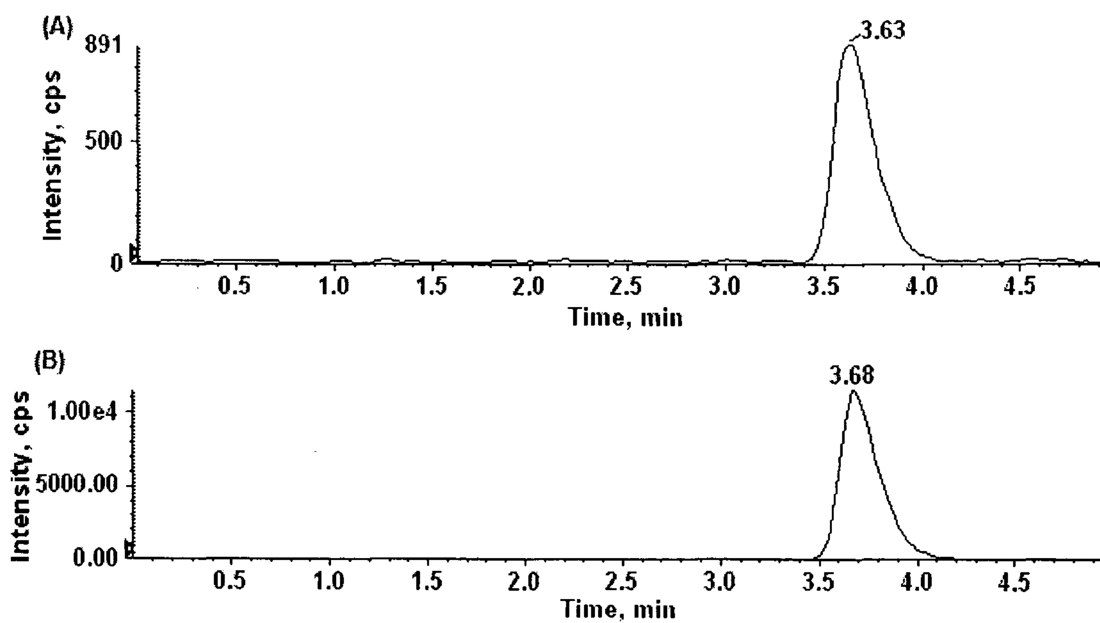


Fig-4.5.2: LC-MS/MS chromatogram of rat plasma spiked with (A) S006-1709 and (B) IS

Recovery and Matrix effect

The extraction of each compound and IS by LLE method using diethyl ether was effective and consistent. The recovery of medicarpin was 89% to 108.4% and 77.3% for IS. (Table-4.7). There was no significant ionic suppression or enhancement observed for K095. The matrix effect calculated for post extracted samples were in the range from 2.39% to 7.30%.

The recovery of K054 and IS was also greater than 80% with less matrix effects (Table-4.7). The values for ionic suppression or enhancement were lying between -1.88 to 12.4%.

The recovery of S006-1709 from rat plasma for quality control samples were in the range from 73% to 79%. The matrix effect at these concentration levels was found to be -29.9% to 34.09%. Though the matrix effect was moderately high, it did not affect the consistency of the absolute recovery as their R.S.D values were less than 15%. The recovery of IS calculated at 50ng/mL was 85.7±3.5 (Table-4.7). These results show that sample clean up procedure involving double extraction with diethyl ether was efficient to provide good recovery for all the three compounds and IS as well.

Table-4.6: Validation data of K095, K054 and S006-1709

Markers	**t_R^a (min)**	**LOD[b] (ng/mL)**	**LLOQ[c] (ng/mL)**	**Linearity (ng/mL)**	**Regression Coefficient (r)**
Medicarpin (K095)	6.05	1.95	3.9	3.9-250	0.9981±0.001
Cladrin (K054)	7.5	3.9	7.8	7.8-1000	0.9991±0.001
S006-1709	6.6	1.95	3.9	3.9-1000	0.9995±0.0003

Note: a-Retention time, b- Limit of Detection, c- Lower Limit of Quantification

Table-4.7: Recovery of K095, K054, S006-1709, 7-hydroxy isoflavone and biochanin

Quality controls	Medicarpin	Cladrin	S006-1709	7-Hydroxy isoflavone	Biochanin
LQC	89.8±11.5	81.9±0.33	74.26±8.10	77.3±9.68[a]	85.7±3.5[c]
MQC	92.5±6.77	81.2±7.82	76.42±2.38	85.3±8.7[b]	
HQC	108.4±10.29	87.5±6.81	71.42±1.65		

Note: a - % recovery calculated at the concentration of 125ng/mL, b- % recovery calculated at the concentration of 50ng/mL, c-% recovery calculated at the concentration of 50ng/mL

Accuracy and Precision

The intra-day and inter-day accuracy (% bias) and precision (% C.V.) of the LC-MS/MS method for each test compound were determined for quality control samples at low, medium and high concentrations. These values are presented in Table-4.8. The results showed that the method was accurate and precise with values of % bias and % C.V. not exceeding 15% for the quality control samples of the three compounds.

Table-4.8: Intra-day and Inter-day accuracy and precision of K095, K054 and S006-1709

Markers	Quality controls	Accuracy (% Bias)		Precision (% C.V)	
		Intra-day	Inter-day	Intra-day	Inter-day
Medicarpin (K095)	LQC	-5.2	-5.5	3.5	3.7
	MQC	3.5	4.0	6.7	5.5
	HQC	6.7	4.9	4.4	6.3
Cladrin (K054)	LQC	-1.0	-1.7	14.5	11.5
	MQC	8.7	2.1	12.0	8.9
	HQC	12.4	10.6	4.1	0.7
S006-1709	LQC	0.2	2.9	13.2	6.2
	MQC	1.4	1.5	6.2	6.2
	HQC	4.2	3.3	6.0	10.3

Stability in plasma

The stability of K095, K054 and S006-1709 in plasma samples was evaluated under various storage conditions. The stability in rat plasma was determined for quality control samples in triplicate. The calculated concentrations of 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 freeze thaw cycles of K095, K054 and S006-1709 were in the range of -0.12 to 7.68, 0.56 to 11.77 and 0.61 to 11.70 respectively. These values indicate their stability in plasma after three freeze-thaw cycles (Table-4.9, Fig-4.6.1, 4.6.2). The % bias values of bench top stability and long term stability data of all the three compounds were also less than ±15 (Table-4.10). The results summarized in Table-4.10 for autosampler and dry residue stability studies did not exceed the value of 9% for any of these compounds, which shows that they were also stable in processed samples.

Table-4.9: Freeze thaw stability of K095, K054 and S006-1709 in rat plasma

Markers	**Quality controls**	**Cycle-1 (%Bias)**	**Cycle-2 (%Bias)**	**Cycle-3 (%Bias)**
Medicarpin (K095)	LQC	5.56	-0.12	7.68
	MQC	-1.23	3.72	-1.54
	HQC	-0.64	1.90	5.11
Cladrin (K054)	LQC	0.56	1.58	6.37
	MQC	1.49	7.52	1.44
	HQC	-7.33	7.27	11.77
S006-1709	LQC	0.61	7.46	5.79
	MQC	2.21	6.02	7.10
	HQC	4.64	10.91	11.70

Table-4.10: Stability of K095, K054 and S006-1709 in rat plasma at different conditions

Markers	Quality controls	Benchtop stability (% Bias)	Long term stability (% Bias)	Auto sampler stability (% Bias)	Dry residue stability (% Bias)
Medicarpin (K095)	LQC	14.12	1.07	-8.12	6.01
	MQC	7.94	1.07	1.79	2.92
	HQC	5.7	3.85	0.25	5.08
Cladrin (K054)	LQC	2.83	-6.07	2.51	-6.22
	MQC	6.43	13.68	3.17	-0.96
	HQC	10.44	7.81	8.83	5.70
S006-1709	LQC	7.33	8.65	-6.98	5.84
	MQC	9.53	2.37	3.31	0.58
	HQC	4.29	6.63	8.14	0.75

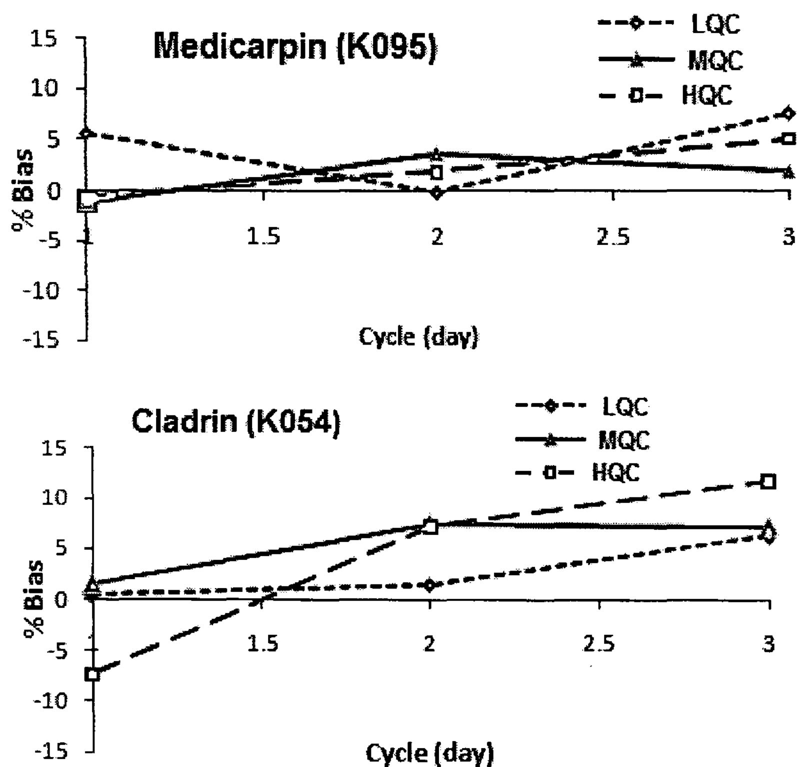


Fig-4.6.1: Graph of freeze thaw stability of K095 and K054 in rat plasma

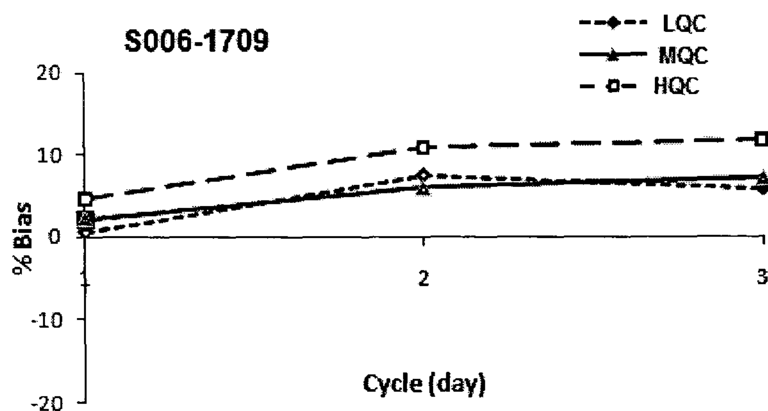


Fig-4.6.2: Graph of freeze thaw stability of S006-1709 in rat plasma

4.3. Conclusion

A selective, sensitive and rapid LC-MS/MS method has been developed and validated separately for the quantitative analysis of three osteogenic compounds; medicarpin (K095), cladrin (K054) and S006-1709 in female rat plasma. The LOD of the LC-MS/MS methods for K095, K054 and S006-1709 was 1.95, 3.9 and 1.95ng/mL respectively. The LLOQ of the LC-MS/MS methods for K095, K054 and S006-1709 was 3.9, 7.8 and 3.9ng/mL respectively. The LC-MS/MS methods was linear for the concentration range from 3.9ng/mL to 250ng/mL for K095, 7.8ng/mL to 1000ng/mL for K054 and 3.9ng/mL to 1000ng/mL for S006-1709 with value of $r > 0.99$. All three methods were accurate and precise with intra-day and inter-day accuracy (% bias) and precision (% C.V.) within the acceptable limit of ±15% for quality control samples.

The extraction of K095, K054 and S006-1709 by liquid-liquid extraction method with diethyl ether was also efficient and consistent. The recovery of K095 and K054 was greater than 80%, while that of S006-1709 was greater than 70%. No stability problems were observed for any of these compounds when their stability was evaluated by freeze thaw, benchtop, long term and dry residue stability studies. The % bias calculated for K095, K054 and S006-1709 in these stability studies did not exceed the permissible limit of ±15%. Thus the developed LC-MS/MS method for the three osteogenic compounds in female rat plasma has been validated for accuracy, precision, recovery and stability. The validated LC-MS/MS methods for K095, K054 and S006-1709 were suitable to apply for the pre-clinical pharmacokinetics studies in female S.D. rats.