Chapter 2

Bioanalytical method development and validation for markers of F147

The osteogenic herbal fraction F147 from the stem bark of *Butea monosperma* contains isoflavones and pterocarpan as active constitutents (markers). The compounds daidzein (K040), cajanin (K051), genistein (K053), cladrin (K054), formononetin (K080), isoformononetin (K082), medicarpin (K095) and prunetin (K098) have been identified as the active constituents of F147. The percentage content of these markers in F147 has been determined by applying validated LC-MS/MS method (Chapter-1, Part-I). However, the validated LC-MS/MS method for percentage content analysis of markers in F147 was a long method of 60 min run time. To perform preclinical studies, LC-MS/MS method of shorter run time would be preferable for determination of markers of F147 in female rat plasma. As the percentage of markers was low ranging from 0.3% to 0.002%, a pilot study was performed in female rats to find out the absorption of markers in plasma samples. The samples of pilot study were analyzed by LC-MS/MS method of 60min run time as a preliminary step and the results showed that the marker K052 was not at all detected and K054 was below limit of quantification (BLOQ). Therefore, it was planned to develop and validate another gradient of shorter run time for the simultaneous analysis of only six markers (K040, K051, K053, K080, K095 and K098) and excluding K052 and K054 of F147, in female rat plasma to apply it for pre-clinical pharmacokinetic studies.

2.1. Experimental methods

2.1.1. Chemicals and Reagents

The internal standard (IS) 7-hydroxy isoflavone was obtained from Indofine Chemicals, New Jersey, U.S.A. HPLC grade acetonitrile was obtained from SRL, Mumbai, India; glacial acetic acid and Dimethyl sulphoxide (DMSO) were obtained from S.D. Finechem limited, Mumbai, India. Purified water was prepared in our division from Millipore Milli-Q system. Female S.D. rats were procured from Laboratory Animal 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.

2.1.2. Herbal preparation and marker compounds:

The herbal preparation F147 was extracted from the stem bark of *Butea monosperma* at MPC division of CDRI, Lucknow, India. The marker compounds of F147 were isolated as pure compounds from the extract and also synthesized at MPC division. The marker compounds synthesized at MPC division includes cajanin and medicarpin. The reference standards of other markers daidzein, genistein, formononetein and prunetin were obtained from Indofine Chemicals, New Jersey, USA.

2.1.3. Preparation of stock and working solutions

The stock solutions of all the standards were prepared in methanol and dimethyl sulfoxide (DMSO) in the ratio of 9:1 at the concentration of 1mg/mL. The composite working solution consisting of six standards; daidzein, cajanin, genistein, formononetin, medicarpin, and prunetin were prepared by spiking 10µl of each stock solution in methanol to get the concentration of 10µg/mL in methanol. This working solution was serially diluted in methanol to get the concentration of 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, 0.0195 and 0.00975µg/mL. These working solutions (WS) of isoflavones were used to prepare calibration standards (CS). The composite working solution of six standards of concentration 8 µg/mL was also prepared in methanol by spiking 8 µl of each stock solution (1mg/mL). This solution was diluted in methanol to get the working solutions at the concentration of 4, 1 and 0.05 µg/mL. These working solutions were used to prepare quality control samples in plasma. The stock solution of 7-hydroxy isoflavone (IS) of 1mg/mL was also prepared in methanol. The working solution of IS (20µg/mL) was prepared from this stock solution in methanol.

2.1.4. Preparation of calibration standards and quality control samples

10µl of these working solutions of concentration range from 5µg/mL to 0.00975µg/mL were spiked in 190µl of female rat plasma to get the calibration standards of 250ng/mL to 0.487ng/mL. 10µl of the composite working solutions of concentration 4, 1 and 0.05 µg/mL were spiked in 190µl of plasma for the preparation of quality control samples to get the concentration of 200 (quality control high-QCH), 50 (quality control medium-QCM) and 2.5ng/mL (quality control low-QCL) respectively (Table-2.1). 10µl of IS

working solution was spiked in each calibration standard and quality control samples of markers of F147.

2.1.5. Sample preparation

Liquid-liquid extraction (LLE) method was used for processing of calibration standards, quality control and test samples of markers of F147 using ethyl acetate. The processing volume of plasma sample was 200µL. To each standard and sample, 10µL of IS working solution (20µg/mL) was added and vortexed for 15 s. After addition of IS, 1.5mL of ethyl acetate 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 100µL of mobile phase, vortexed and 20µl of it was injected for analysis.

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 (ng/mL)**
0.00975	10µL + 190µL	CS 0.487
0.0195	10µL + 190µL	CS 0.975
0.039	10µL + 190µL	CS 1.95
0.078	10µL + 190µL	CS 3.9
0.156	10µL + 190µL	CS 7.8
0.3125	10µL + 190µL	CS 15.6
0.625	10µL + 190µL	CS 31.25
1.25	10µL + 190µL	CS 62.5
2.5	10µL + 190µL	CS 125
5	10µL + 190µL	CS 250
0.05	10µL + 190µL	QC 2.5
1	10µL + 190µL	QC 50
4	10µL + 190µL	QC 200
To each CS and QC, 10µL of IS (20 µg/mL) was added		

Note: a - Working Solution of mix of K040, K051, K053, K080, K095 and K098

2.1.6. Chromatographic and mass spectrometric conditions:

Shimadzu UFLC pump (LC-20AD) with degasser (DGU-20A3) and autosampler (SIL-HTc) coupled to API 4000 Q trap (Applied Biosystems, MDS Sciex, Toronto, Canada) mass spectrometry was used for liquid chromatography tandem mass spectrometric analysis (LC-MS/MS). The chromatographic separation was carried out on Phenomenex C18 column (150x4.6mm, 5µm). The mobile phase composition was acetonitrile as mobile phase A and 0.1% acetic acid in water (v/v) as mobile phase B in gradient condition (Table-2.2) at the flow rate of 0.5mL/min. The injection volume was 20µL.

Table-2.2: Gradient condition for LC-MS/MS method of F147 markers

Time (min)	A[a] (%)	B[b] (%)
0.1	40	90
2.0	45	40
7.0	45	40
8.0	50	90
13.0	50	50

Note: a – acetonitrile, b - 0.1% acetic acid

Mass spectrometric analysis was performed in electro spray ionisation (ESI) source. The source temperature was set at 500°C and the ionisation voltage was adjusted at -4500 V. MRM in both negative and positive mode of ionisation was applied for simultaneous determination of six marker compounds in a single LC-MS/MS run by polarity switching method. The MRM transition selected for the markers of F147 and internal standard (IS) are given in Table-2.3. The mass spectrometer was operated in unit resolution at dwell time of 40ms for each MRM transition. The other parameters optimized for tandem mass analysis includes the curtain gas and nebuliser gas at 25 units each. Analyst software (version 1.4.2) was used for data acquisition and analysis.

Table-2.3: Mass spectrometric conditions for LC-MS/MS method of F147 markers

Markers	Molecular weight	MRM transitions (Ionization mode)	D.P.[a]	C.E.[b]
Daidzein (K040)	254	253/132 (-)	-95	-55
Cajanin (K051)	300	299/164.7 (-)	-90	-30
Genistein (K053)	270	269/133(-)	-90	-44
Formononetin (K080)	268	269/254(+)	-80	-25
Medicarpin (K095)	270	269/254(-)	-80	-25
Prunetin (K098)	284	283/239(-)	-90	-40
IS[c]	238	239/137 (+)	81	44
IS[c]	238	237/117 (-)	-90	-46

Note: a - Declustering Potential, b - Collision Energy, c - Internal Standard

2.1.7. Validation of LC-MS/MS method

Selectivity, Sensitivity and Linearity

The method was evaluated for the selectivity and specificity by processing the blank female plasma obtained from different female rats similar to the processing of calibration standard and comparing the response obtained for blank plasma with those of markers in LLOQ of calibration standard. The sensitivity of the method was determined from the signal to noise ratio (S/N) of the response of each analyte in calibration standard. The S/N ratio should be greater than three for limit of detection (LOD) and greater than ten for lower limit of quantitation (LLOQ).

The ratio of peak area of each compound to IS of calibration standards (0.487ng/mL to 250ng/mL) was used for the construction of calibration curve to check linearity. Calibration standards in female rat plasma were prepared and analyzed on three different days. The method was considered to be linear for the concentration range of calibration standards when the regression coefficient (r) value was greater than 0.99

Extraction recovery and Matrix effect

The recovery of six markers and IS from female rat plasma by liquid-liquid extraction (LLE) method using ethyl acetate was calculated by comparing the peak area of the analyte in quality control (QC) samples in rat plasma (A) with the peak area of analyte in dilution solvent (B). The recovery of IS was also determined similarly at the concentration of 1000ng/mL. The effect of matrix components on ionic suppression or enhancement was also evaluated by comparing the area of standards obtained after spiking in blank plasma post extraction (C) with the area obtained for standards prepared in dilution solvent (B).

$$Recovery = \frac{A}{B} x100 \qquad (2.1)$$

$$Matrix\ effect = \left(1 - \frac{C}{B}\right) x\ 100 \qquad (2.2)$$

Accuracy and Precision

The intra-day and inter-day accuracy and precision of the method for all six markers of F147 were determined for QC samples in plasma at three concentration levels. 2.5, 50 and 200 ng/mL were used as QC low (QCL), QC medium (QCM) and QC high (QCH) respectively (N=5 for each QC]. This procedure was repeated for three days. To determine accuracy, % bias was calculated and to determine precision, % C.V. was calculated by ANOVA.

Stability in plasma

The stability of all six markers in female rat plasma was determined for the QC samples (QCL, QCM and QCH) in plasma in triplicate. These QC samples were subjected to three 24-hour freeze-thaw cycle, on storing at -80^{0}C. Short term stability was carried out by keeping samples spiked with composite working solutions of six markers at room temperature for 4 hrs. Long term stability test was also performed in triplicate after storing the spiked plasma samples for two months at -80^{0}C. The stability of marker compounds in dry residue and in processed samples kept in autosampler for 24 hrs was also evaluated for QC samples.

2.2. Results and discussions

2.2.1. Optimization of chromatographic and mass spectrometric conditions

K040, K051, K053, K080, K095 and K098 are the active constituents of F147 herbal preparation. The other two active compounds K052 and K054 were present in lower quantity in F147 and based on the pilot pharmacokinetics study, these markers were not included in development of bio-analytical LC-MS/MS method. As K052, the isomer of K080 was not included; the 60 min gradient developed for the resolution of K052 and K080 was not needed. Since F147 contains compounds C1-C14, in addition to markers, it was necessary to develop a gradient method for chromatographic separation of the six markers to avoid interferences from C1-C14. Therefore, a gradient of shorter run time has been developed for the simultaneous determination of six markers of F147. Acetonitrile and acetic acid were found to be suitable mobile phase for the present LC-MS/MS method that ensured proper separation and good peak shape of markers. These isoflavones have high sensitivity in negative mode of electrospray ionisation and $[M-H]^-$ was the most abundant ion observed in the MS/MS spectra of these markers. However, for K080, the MRM selected in negative mode was not selective because of interferences from biomatrix. So, the positive mode of ionisation was selected for K080. Thus, the LC-MS/MS developed was performed in both negative and positive mode of ionisation in a single run to analyse six bioactive markers simultaneously. The internal standard, 7-hyrdoxy isoflavone was used for both negative and positive ionisation because of its good sensitivity and selectivity. The run time was 13 min and shorter than that applied for pattern profiling of F147.

Liquid-liquid (LLE) extraction method was suitable for the clean-up of plasma samples of isoflavones. Diethyl ether and ethyl acetate were used to optimise the extraction process. The extraction recovery was good and similar with both diethyl ether and ethyl acetate for all isoflavones and IS, except for K051. In the case of K051, extraction with diethyl ether resulted in the ionisation enhancement and variable percentage of recovery. So, ethyl acetate was selected for the extraction process.

2.2.2. Validation of LC-MS/MS method

Selectivity, sensitivity and linearity

Comparison of the chromatogram of blank plasma and chromatogram of plasma spiked with each isoflavones and IS showed that the MRMs selected for quantitative analysis were selective with no interferences from female rat plasma (Fig-2.1.1 to 2.1.3 and Fig-2.2.1, 2.2.2). The LOD and LLOQ of the markers K040, K051, K053 and K095 were 0.487ng/mL and 0.975ng/mL respectively. 0.975ng/mL was the LOD and 1.95ng/mL was LLOQ for the markers K080 and K098. The method was linear for the concentration range from 0.975ng/mL to 250ng/mL for K040, K051, K053 and K095 while the linearity of K080 and K098 range from 1.95ng/mL to 250ng/mL. The LOD, LLOQ and linearity of LC-MS/MS method for the simultaneous analysis of markers of F147 are summarized in Table-2.4.

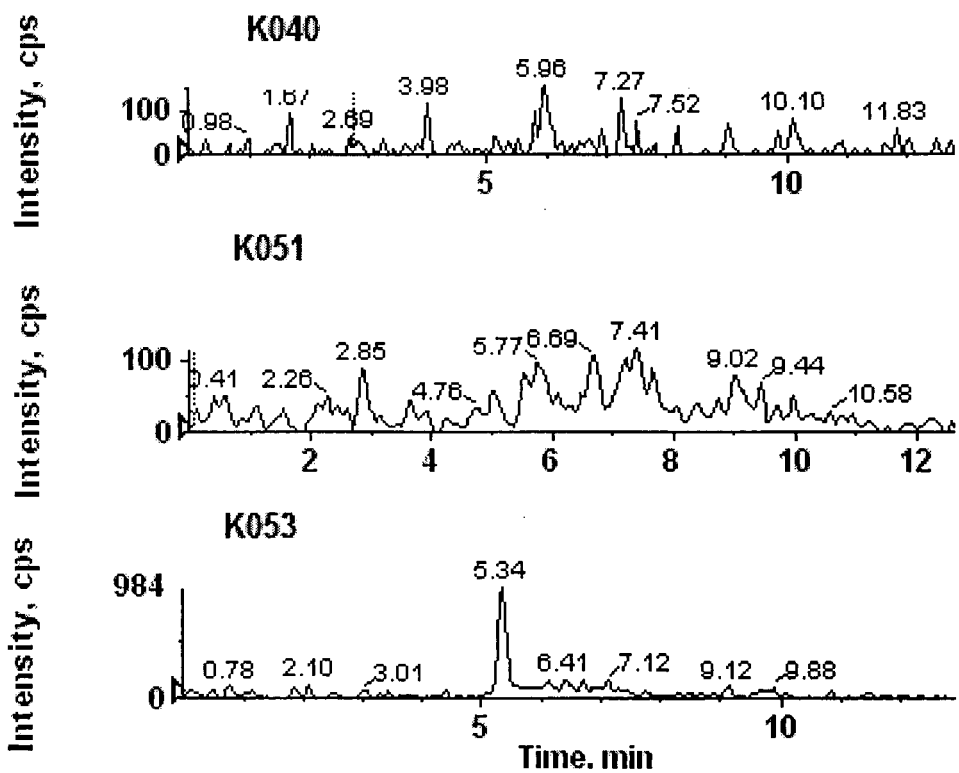

Fig-2.1.1: LC-MS/MS chromatogram of blank plasma for MRM of markers K040, K051 and K053

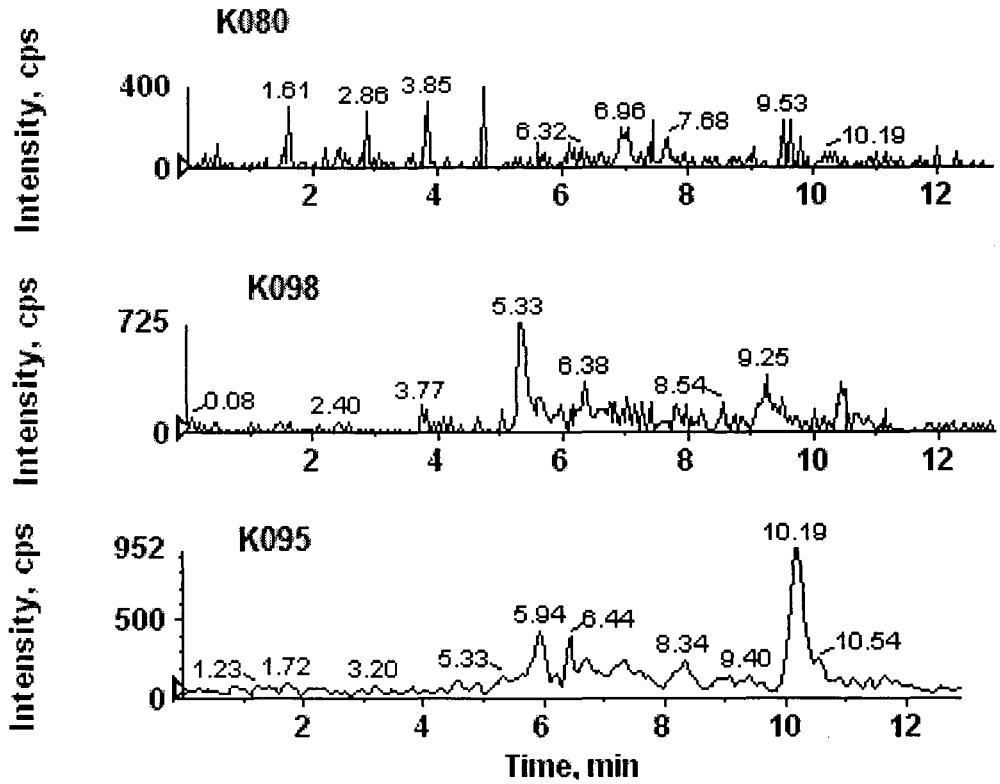

Fig-2.1.2: LC-MS/MS chromatogram of blank plasma for MRM of markers K080, K098 and K095

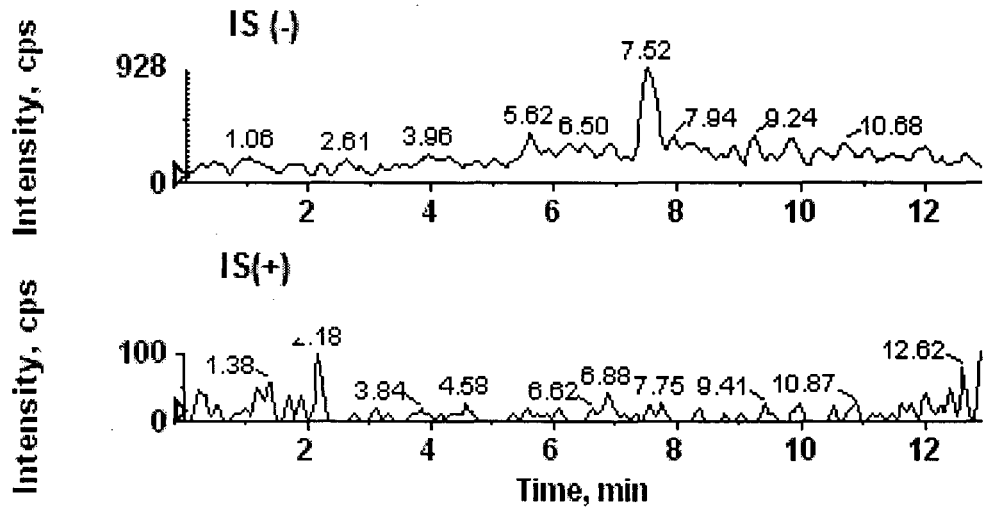

Fig-2.1.3: LC-MS/MS chromatogram of blank plasma for MRM of IS in negative and positive mode

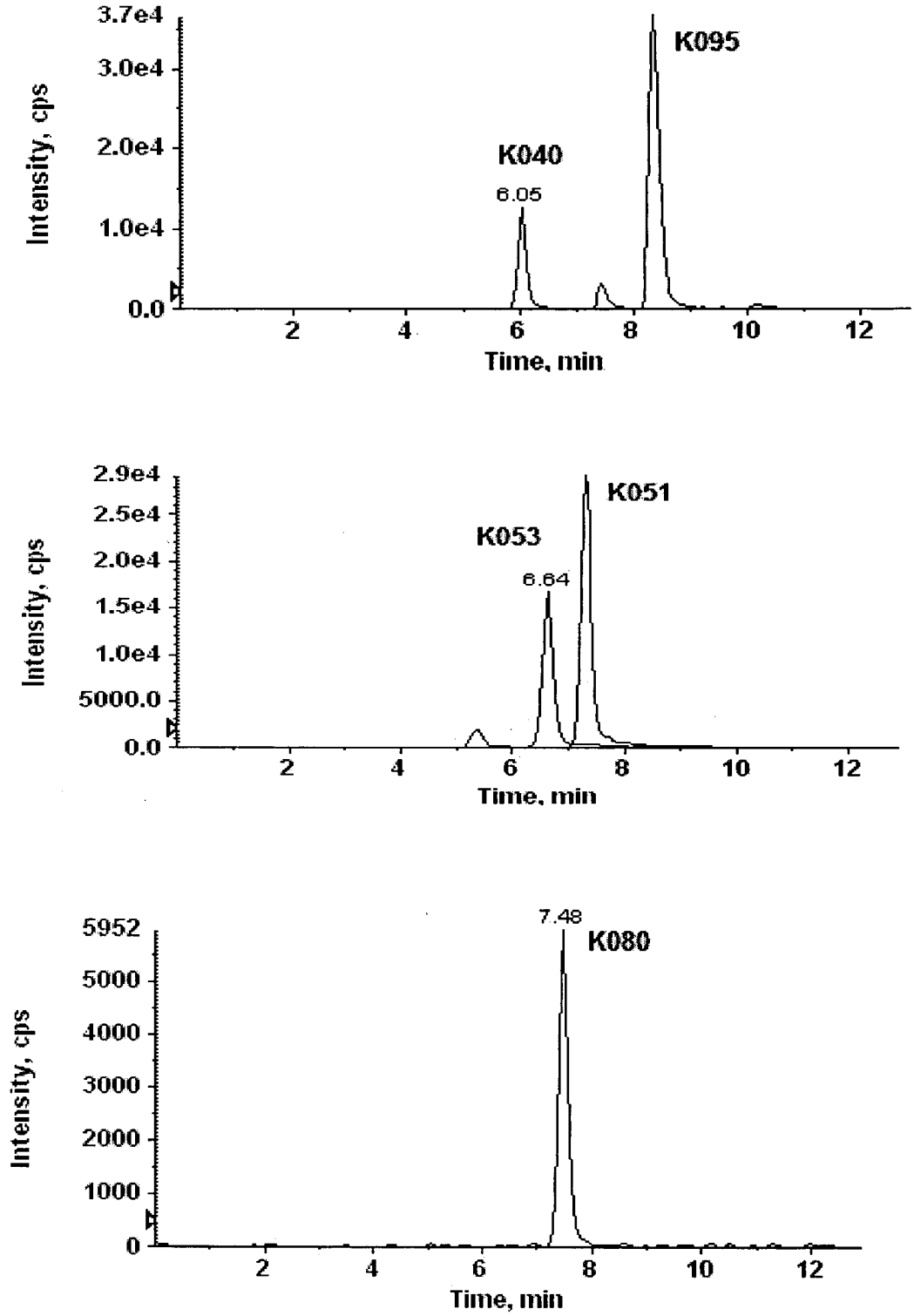

Fig-2.2.1: LC-MS/MS chromatogram of markers K040, K095, K051, K053 and K080 spiked in rat plasma

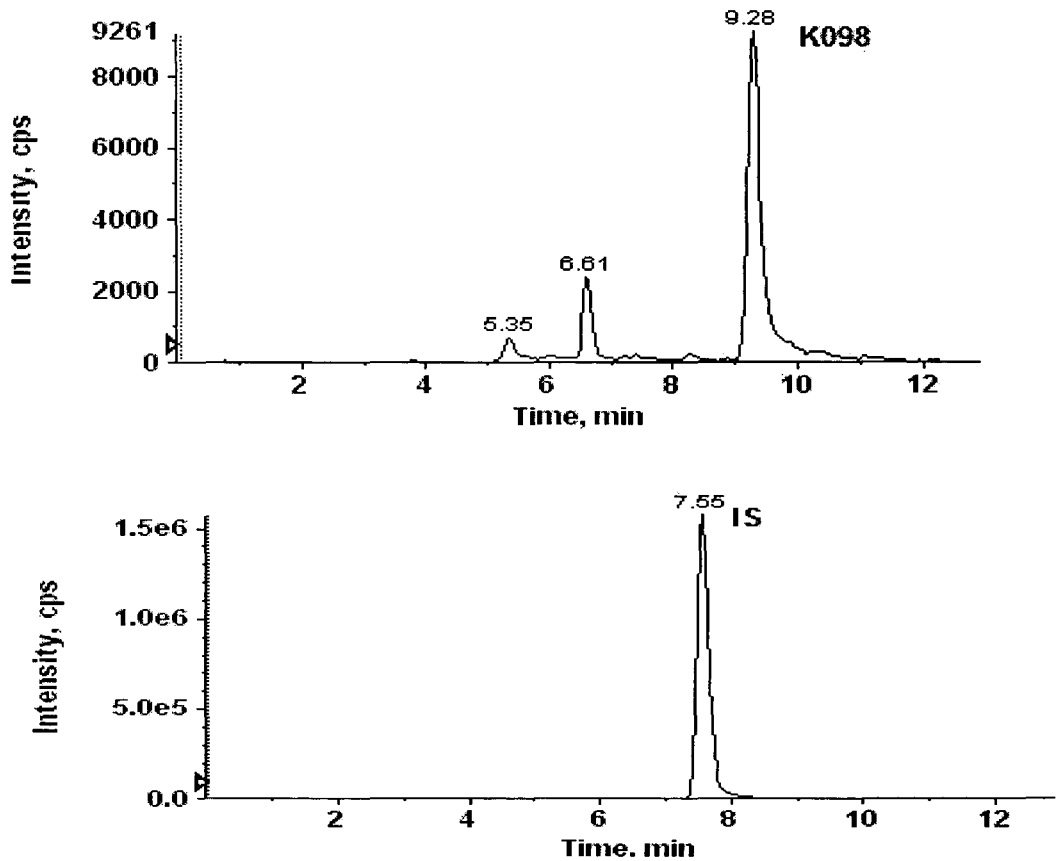

Fig-2.2.2: LC-MS/MS chromatogram of markers K098 and IS spiked in rat plasma

Table-2.4: Validation parameters of F147 markers

Markers	t_R[a] (min)	LOD[b] (ng/mL)	LLOQ[c] (ng/mL)	Linearity (ng/mL)	r[d]
Daidzein (K040)	6.05	0.487	0.975	0.975-250	0.9987±0.0004
Cajanin (K051)	7.5	0.487	0.975	0.975-250	0.9965±0.002
Genistein (K053)	6.6	0.487	0.975	0.975-250	0.9961±0.004
Formononetin (K080)	7.48	0.975	1.95	1.95-250	0.9988±0.0004
Medicarpin (K095)	8.5	0.487	0.975	0.975-250	0.9973±0.003
Prunetin (K098)	9.3	0.975	1.95	1.95-250	0.9963±0.003

Note: a - Retention time, b - Limit of Detection, c –Lower limit of quantitation,
d - Regression coefficient

Extraction recovery and matrix effect

The recovery of all the constituents of F147 was greater than 75% and consistent for the quality control samples analyzed at three levels of concentration (Table-2.5). The matrix effect was also within ± 15%, indicating absence of significant matrix effect for all analytes and IS.

Table-2.5: Recovery and matrix effect of markers of F147

Markers	Concentration (ng/mL)	Recovery % (Mean±S.D.)	Matrix effect %
K040	2.5	102 ± 11.4	1.12
	50	94.42 ± 6.43	-9.16
	200	78.93 ± 2.36	-10.12
K051	2.5	113.45 ± 14	1.54
	50	107.45 ± 8.38	2.03
	200	91.62 ± 7.57	-5.58
K053	2.5	113.07 ± 8.94	11.82
	50	97.13 ± 2.07	6.53
	200	86.54 ± 10.97	-10.93
K080	2.5	92.99 ± 6.57	-3.89
	50	82.47 ± 10.20	-11.64
	200	78.34 ± 6.12	-13.78
K095	2.5	110.27 ± 18.77	13.93
	50	107.73 ± 2.89	0.74
	200	88.81 ± 6.53	-12.59
K098	2.5	94.25 ± 2.13	-9.89
	50	111 ± 9.26	1.65
	200	89.55 ± 7.36	-9.12

Accuracy and precision

The values of % bias for intra-day and inter-day accuracy and the % C.V. values for inter-day and intra-day were within the acceptable limits of ±15% for all the six bioactive markers (Table-2.6).

Table-2.6: Intra-day and Inter-day accuracy and precision of F147 markers

Markers	Concentration (ng/mL)	Accuracy (% Bias)		Precision (% C.V.)	
		Intra-day	Inter-day	Intra-day	Inter-day
K040	2.5	-2.43	2.17	10.41	9.45
	50	1.46	2.21	4.74	4.87
	200	0.14	3.61	3.87	12.74
K051	2.5	-0.32	0.65	7.85	10.77
	50	3.10	10.17	5.72	13.38
	200	-0.62	0.35	4.84	12.52
K053	2.5	-1.63	-1.77	2.94	1.53
	50	0.66	-0.93	4.65	6.82
	200	1.35	2.06	6.08	3.05
K080	2.5	2.56	4.51	7.14	7.06
	50	-1.25	-2.05	5.31	9.32
	200	-1.14	-0.52	3.63	4.51
K095	2.5	1.88	3.30	8.97	7.90
	50	0.38	1.86	6.20	6.51
	200	-6.85	-7.76	5.05	11.93
K098	2.5	-0.80	-5.34	12.14	7.65
	50	0.78	1.71	5.44	11.02
	200	-1.78	-3.07	5.04	4.43

Stability

The stability of all six marker components in female 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 the values of % bias for three freeze thaw cycles were less than 15% (Table-2.7, Fig-2.3.1 and Fig- 2.3.2). Long term stability study was also performed after storage at -80°C for 60 days and the % bias obtained from this study indicated no significant degradation of any constituents. The %bias values obtained for autosampler stability and dry residue stability were lying in the range from -9.27 to 8.46%, which indicates that the markers were stable in processed quality control samples and in dry residue (Table-2.8).

Table-2.7: Freeze thaw stability of markers of F147

Markers	Concentration (ng/mL)	Cycle-1 (%Bias)	Cycle-2 (%Bias)	Cycle-3 (%Bias)
K040	2.5	8.41	-2.21	-5.41
	50	-4.61	6.27	5.73
	200	3.31	-0.41	-0.50
K051	2.5	-3.74	-2.21	0.67
	50	-10.73	6.28	5.08
	200	-1.89	-0.87	6.01
K053	2.5	-6.88	-3.53	0.60
	50	-2.70	6.51	-3.74
	200	-0.60	3.16	4.78
K080	2.5	9.14	7.20	-3.55
	50	-4.65	3.11	1.15
	200	-3.98	2.10	-7.23
K095	2.5	6.28	3.11	9.72
	50	2.93	0.15	-1.23
	200	-11.31	-11.86	8.79
K098	2.5	5.76	-8.8	0.88
	50	4.83	10.21	-9.97
	200	-2.89	-6.27	5.61

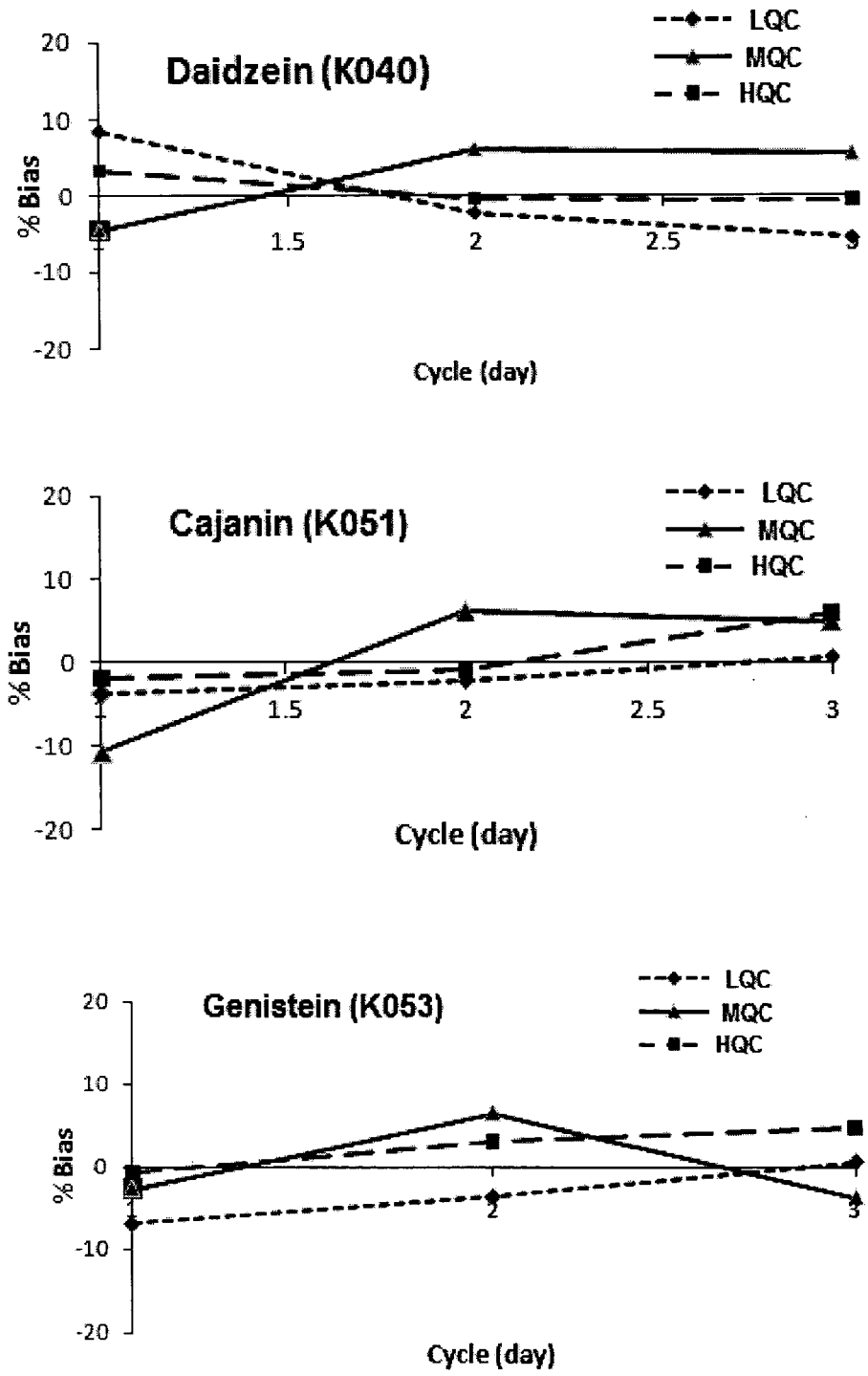

Fig-2.3.1: Graph of freeze thaw stability of marker K040, K051 and K053

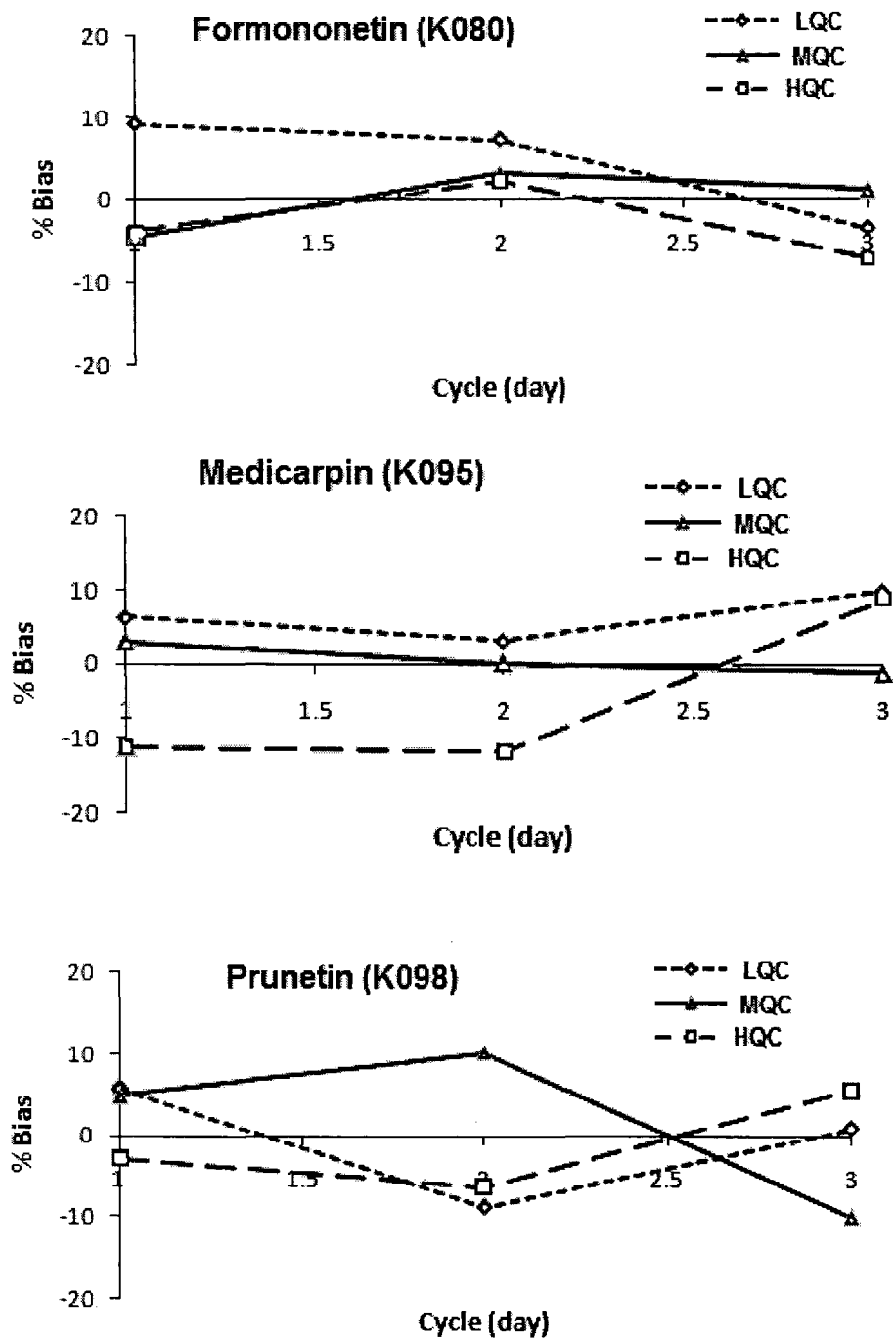

Fig-2.3.2: Graph of freeze thaw stability of marker K080, K095 and K098

Table-2.8: Stability of markers of F147 in plasma and processed samples

Markers	Concentration (ng/mL)	Benchtop stability (% Bias)	Long term stability (% Bias)	Auto sampler stability (% Bias)	Dry residue Stability (% Bias)
K040	2.5	5.08	-9.50	1.37	-4.97
	50	1.24	7.04	-6.29	3.89
	200	6.36	8.18	7.51	-4.53
K051	2.5	4.54	2.53	4.37	0.67
	50	-10.46	-5.65	3.18	-5.72
	200	1.20	1.84	8.46	7.95
K053	2.5	-10.71	4.76	-2.95	1.87
	50	1.31	0.21	-9.27	-2.60
	200	1.22	0.72	-6.67	7.66
K080	2.5	0.42	-9.81	6.37	-4.61
	50	-9.40	4.74	-4.65	-1.95
	200	2.10	8.40	6.67	-0.08
K095	2.5	10.99	8.67	8.10	4.32
	50	3.39	3.27	4.62	3.52
	200	6.83	8.96	1.16	-1.31
K098	2.5	-7.33	6.13	4.24	-6.40
	50	-6.03	-3.79	8.01	-1.19
	200	1.02	-4.70	6.81	-2.35

2.3. Conclusion

A selective and sensitive LC-MS/MS method has been developed and validated for the simultaneous analysis of daidzein (K040), cajanin (K051), genistein (K053), formononetin (K080), medicarpin (K095) and prunetin (K098) in female rat plasma. The LOD and LLOQ of the LC-MS/MS method for markers K040, K051, K053 and K095 were 0.487ng/mL and 0.975ng/mL respectively. 0.975ng/mL was the LOD and 1.95ng/mL was LLOQ for the markers K080 and K098. The method was linear for the concentration range from 0.975ng/mL to 250ng/mL for K040, K051, K053 and K095 while the linearity of K080 and K098 range from 1.95ng/mL to 250ng/mL.

The method was accurate and precise with % bias and % C.V. values for intra-day and inter-day evaluation lying within the acceptable limit of ±15%. The extraction of the bioactive markers from rat plasma by LLE using ethyl acetate was greater than 75%. The stability of these compounds, as evaluated by various stability studies, show that they were stable in plasma after short and long term storage at -80°C. The bioactive markers of F147 were stable in processed samples also as the values of % bias for auto sampler and dry residue stability were lying in the range from -9.27 to 8.46%. The validated LC-MS/MS method for the simultaneous analysis of bioactive markers of F147 was sensitive, selective, accurate and precise so that it can be applied to perform the pharmacokinetic studies of F147 herbal fraction.