Summary

PART-I

Pattern profiling and pharmacokinetic studies of osteogenic herbal fraction F147 and bioactive markers

Pharmacokinetics is the study of the time course of absorption, distribution, metabolism and elimination (ADME) of the drugs in biological system and helps to understand the relationship between pharmacological and toxicological effect and concentration of a drug and its metabolites in the body fluid. In CDRI, bioassay guided acetone soluble herbal fraction F147 from *Butea monosperma* and the markers medicarpin, caldrin of F147, synthetic analogue S006-1709 has been found to exhibit potent osteogenic activity. The quality control (QC), quality assurance (QC) and pharmacokinetic studies of herbal preparations is essential to generate scientific data to support their safety and efficacy. Therefore, studies were planned to perform chromatography based pattern profiling and quantitative analysis of markers for the standardization and quality control of herbal preparation F147 to identify and quantify the active constituents present in them. The pharmacokinetic studies of herbal preparation and their markers in animals were also planned to understand their behaviour within the biological system and to establish PK-PD correlation to support their development as potential osteogenic or osteoprotective agents.

Pattern profiling of osteogenic herbal fraction F147 from *Butea monosperma*

Quantitative methods and/or qualitative pattern profiling or fingerprint analysis are applied for the QC and QA of herbal medicines. Qualitative pattern profiling or fingerprinting analysis is an analytical technique by which the chromatographic pattern of various compounds in complex mixtures of herbal preparations can be obtained. Pattern profiling provides comprehensive and accurate assessment of large number of active constituents in herbal extracts. To perform both quantitative and qualitative analysis of F147, a LC-MS/MS method has been developed and validated for simultaneous determination of eight markers; daidzein (K040),cajanin (K051), isoformononetin (K052), genistein (K053), cladrin (K054), formononetin (K080), medicarpin (K095) and prunetin (K098) and for identification of compounds C1-C14 of herbal preparation F147.

The LC-MS/MS method was accurate and precise with % bias and % C.V. values for intra-day and inter-day analysis lying within the acceptable limit of ±15%. The LOD of the method was 0.487ng/mL and 0.975ng/mL for K080 and K054 respectively. The LOD for markers K051, K052 and K095 was 1.95ng/mL while it was 3.9ng/mL for K040 and K098. The linearity ranged from 0.975 – 500ng/mL for K080; 3.9-1000ng/mL for markers K051, K052 and K095; 7.8-500ng/mL for K051 and 7.8-1000ng/mL for K040 and K098. The method has been applied for the percentage content determination of markers and to determine the relative percentage of peak area of all compounds in F147. The percentage content of marker K080 was highest (0.3%), K095 and K098 were found to be in almost same quantity (0.2%). The percentage content of markers K051, K053 and K040 were in between 0.75 to 0.1%. K054 and K052 were present in very low quantity in the range from 0.002% to 0.004%. The relative percentage of peak area of K080 was highest (100%) and the relative percentage of peak area of other compounds was less and ranged from 16% to as low as 0.02%.

The structural characterization was also done for the compounds C1-C14 using their fragmentation pattern. The type of substitution and position of substitution has been predicted for C1-C14 and their structure has been proposed. It has been found that F147 contains hydroxy, methoxy isoflavone (C1-C2), dimethoxy isoflavone (C3-C7), hydroxyl derivatives of K052 and K080 (C8-C11), dimethoxy, methyl isoflavone (C12/C13) and trihydroxy methoxy isoflavone (C14). One of the compounds of F147, C7 has been confirmed to be 7, 4'-dimethoxy isoflavones. These compounds could also contribute to the osteogenic activity of F147 along with the known bioactive markers and may also form a lead compound for osteogenic activity, if further evaluation is done for the compounds C1-C14. Thus, in this study, a combined approach of qualitative pattern profiling and quantitative analysis has been developed and applied for systematic and efficient assessment of constituents in osteogenic herbal fraction F147.

Bioanalytical method development and validation for markers of F147

A selective, sensitive, accurate and precise bioanalytical method for the compounds in biological matrix is important to perform pre-clinical and clinical pharmacokinetic

studies. A LC-MS/MS method for simultaneous analysis of bioactive markers; daidzein (K040), cajanin (K051), genistein (K053), formononetin (K080), medicarpin (K095) and prunetin (K098) of F147 has been developed and validated in female rat plasma to apply it for the pharmacokinetic studies in female S.D. rats.

The LOD and LLOQ of the validated 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 bioactive markers from rat plasma by liquid-liquid extraction using ethyl acetate was greater than 75%. The stability of these compounds as evaluated by various stability studies show that they are 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 was were lying in the range from -9.27 to 8.46%. Therefore, 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.

Pharmacokinetic studies of osteogenic herbal fraction F147

Generally for herbal medicines, pharmacokinetics is performed for one or two of its major active constituents but this approach based on few marker compounds does not reflect the overall efficacy of herbal extracts. Pharmacokinetics investigation of maximum number of active compounds in herbal medicines provides more scientific data for their safe use in therapy. Therefore, pharmacokinetic studies were planned for F147 based on six bioactive markers daidzein (K040), cajanin (K051), genistein (K053), formononetin (K080), medicarpin (K095) and prunetin (K098) in female S.D. rats.

Single dose intravenous and oral pharmacokinetic studies of F147 were performed in female S.D rats at the dose of 100mg/kg and 1g/kg respectively. The pharmacokinetic parameters of F147 were derived from the six active components; daidzein (K040), cajanin (K051), genistein (K053), formononetin (K080), medicarpin (K095) and prunetin (K098), which were used as markers of F147. The intravenous study revealed that the tissue distribution of all six markers were high. The clearance of four markers (K040, K053, K080 and K098) was low following intravenous dosing, while it was moderately high for K051 and K098. In the oral pharmacokinetics study of F147, absorption of all markers was rapid and their T_{max} were lying between 0.25h to 2.0h. The half-life of all markers was moderately high, ranging from 3h to 7h. Two peaks were observed in the oral plasma concentration time profile of the markers of F147, indicating their entero hepatic circulation, a general phenomenon reported for isoflavones. All markers were detected in plasma till 18h or 24h, after oral dosing. The percentage bioavailability of markers were variable, the highest being 31% for the marker cajanin (K051), while the marker prunetin (K098) had the least bioavailability (2.4%). Overall, the six markers of F147 showed good absorption, high tissue distribution, low to moderate clearance and better systemic exposure. The pharmacokinetics of F147 based on bioactive markers dadizein, cajanin, genistein, formononetin, medicarpin and prunetin correlated well with the single dose pharmacodynamics in female S.D. rats, thus meeting the objective of PK-PD correltation.

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

The bioactive marker compounds medicarpin (K095), cladrin (K054) of F147 and synthetic analogue S006-1709 have also exhibited promising activity for bone formation in animal models without any adverse effect on uterus. To perform pharmacokinetic studies of these osteogenic compounds, LC-MS/MS method has been developed and validated separately for each compound 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 the 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 recovery of K095 and K054 by liquid-liquid extraction 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%. 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 it for the pre-clinical pharmacokinetics studies in female S.D. rats.

Pharmacokinetic studies of osteogenic candidates K095, K054 and S006-1709

Single dose oral pharmacokinetic studies were performed for medicarpin (K095) at 5mg/kg, cladrin (K054) and S006-1709 at 10mg/kg each, in female S.D. rats. The intravenous pharmacokinetic studies were also performed at the single dose of 1mg/kg for each compound. K095 showed rapid absorption, large tissue distribution (12.27 L/kg) and low clearance (2.76 L/h/kg). This pharmacokinetic behaviour of K095, when administered separately, was similar to that observed in the pharmacokinetic study of herbal fractionF147. The absorption of K054 was also rapid with large tissue distribution (6.72 L/kg) and low clearance (2.25 L/h/kg). For, S006-1709, the tissue distribution (17.93 L/kg) and clearance (7.19 L/h/kg) were greater. The half-life of all three compounds was between 4h to 5h and the T_{max} values between 0.5h to 0.75h. The bioavailability of K095 was highest (22.34%) while the bioavailability of other two compounds K054 and S006-1709 were comparable and greater than 10%. The single dose pharmacokinetic studies performed for K095, K054 and S006-1709 indicates that

these compounds have favourable pharmacokinetic parameters and correlate well with their activity *in vivo*. Thus, the three active compounds promise to be potential candidate for treatment and management of osteoporosis, even when they are used as an individual compound.

Protein binding studies of osteogenic candidates K095, K054 and S006-1709

The protein binding study was performed for K095, K054 and S006-1709 by charcoal adsorption method. The percentage of protein binding was low for all three compounds. There was no significant difference in protein binding of K095 (12.17±1.71%) and its synthetic analogue S006-1709 (16.28±3.73%), while the protein binding of K054 (3.37± 0.15 %) was lower than these two compounds.

Metabolic stability studies of osteogenic candidates K095, K054 and S006-1709

The metabolic stability of K095, K054 and S006-1709 was performed in rat liver microsomes. The study showed that the dimethoxy substituted compound K054 was more stable with half-life of 69.72±3.54min. The synthetic compound S006-1709 was found to more stable than its natural analogue K095 and may be a potential candidate for development as an osteogenic agent.

PART-II

Quantitative analysis of osteoprotective markers in 914 and pharmacokinetic studies of bioactive marker K058

The aqueous (914/C008) and butanol (914/F009) extracts obtained from *Ulmus wallichiana* have shown positive anabolic effect on bone and promoted peak bone mass in ovariectomized rats. These extracts were found to contain C-glycosylated flavonoids, which were isolated and assessed for their activity separately. Quercetin-6-C-β-D-glucopyranoside (K012), (2S,3S)-(+)-3'4'5,7-tetrahydroxydihydroflavonol-6-C-β-D-glucopyranoside (K058), naringenin-6-C-β-D-glucopyranoside (K068) and (2S,3S)-(+)-4'5,7-trihydroxydihydroflavonol-6-C-β-D-glucopyranoside (K100) are the C-glycosylated flavonoids isolated from *Ulmus wallichiana* and they have exhibited promising osteoprotective effect *in vitro*. The *in vivo* studies showed that K058 is more

effective in increasing the bone mineral density (BMD). Therefore studies were planned to perform quantitative analysis of osteoprotective markers in active fractions of *Ulmus wallichiana* (914) and evaluation of pharmacokinetics of bioactive marker K058 in female S.D. rats.

Quantitative analysis of osteoprotective markers in 914

HPLC-PDA method has been developed and validated for the simultaneous analysis of osteoprotective markers; quercetin-6-C-β-D-glucopyranoside (K012), (2S,3S)-(+)-3'4'5,7-tetrahydroxydihydroflavonol-6-C-β-D-glucopyranoside (K058), naringenin-6-C-β-D-glucopyranoside (K068) and (2S,3S)-(+)-4'5,7-trihydroxydihydroflavonol-6-C- β -D-glucopyranoside (K100).

The validated HPLC-PDA method was selective, accurate and precise with % bias and % RSD values within acceptable limits of ±15%. The LOD and LLOQ of the method were 0.19µg/mL and 0.39µg/mL respectively for each C-glycoside. The linearity of the HPLC-PDA method ranged from 0.39µg/mL to 25µg/mL for all the four C-glycosides. The validated method was applied to determine the percentage content of these four active constituents in different herbal extracts obtained from *Ulmus wallichiana.* The percentage content of four markers was found to be highest in the butanolic extract of stem bark while their level was very low in the aqueous extract. In herbal fractions obtained from the twig of *Ulmus wallichiani*a, the level of each marker was less than 1%. Thus by applying the validated HPLC-PDA method, the variations in the content of four active markers in different herbal extracts of *Ulmus wallichiana* were evaluated.

Bioanalytical method development and validation for osteoprotective candidate K058

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 was suitable to apply for the pharmacokinetics study of K058 in female rats.

Pharmacokinetic studies of osteoprotective candidate K058

The intravenous and oral pharmacokinetic studies of K058 were performed in female S.D. rats at the dose of 1mg/kg and 5mg/kg respectively. The oral absorption of K058 was moderate with the T_{max} of 1.0h and the C_{max} was 40.66ng/mL. The volume of distribution at steady state was low (0.98 L/kg) indicating lower tissue distribution of K058. The clearance of K058 was found to be very low (0.38 L/h/kg) and could be detected in plasma till 30h in oral pharmacokinetics study. The half life of K058 was 1.65h and 5.07h after i.v. and oral dosing respectively. The bioavailability was determined to be 1.04%, which shows that the systemic exposure of K058 was very low. The low clearance of K058 is a desirable pharmacokinetic behaviour but its tissue distribution and bioavailability were low, which might affect its activity. However, the *in vivo* pharmacodynamic studies at the dose of 5mg/kg have shown that K058 exhibit potent osteoprotective activity despite low tissue distribution and bioavailability. This indicates that K058 may be effective even at very low concentration *in vivo*. Therefore, if suitable dosage form is formulated incorporating K058 to enhance its bioavailability, it may be more, or equally effective at still lower doses.