Chapter 7

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

7.1. Metabolism of isoflavones

Isoflavones are polyphenolic compounds and undergo conjugation reactions readily. Glucuronidation is the major conjugation pathway of phase II metabolism of isoflavones [1-3]. The conjugated metabolites of isoflavones get excreted in urine, while some part gets reabsorbed after entero hepatic circulation [4-8]. The phase I metabolism of isoflavones has been reported to produce active metabolites by reduction, hydroxylation and demethylation pathways. Equol is an active metabolite obtained by reduction of daidzein in its phase I metabolism [9]. Similarly, methoxylated isoflavones formononetin and biochanin have been reported to be metabolized to daidzein and genistein respectively by demethylation reactions of phase I metabolism [10]. Thus, the metabolic study of isoflavones in microsomes is important to understand the role of phase I metabolism in determining the stability and the activity of isoflavones. Metabolic stability study involves incubation of the drug candidate in an *in vitro* hepatic model followed by quantification of the parent compounds remaining after metabolism. The results are generally expressed as the percentage of the parent compound disappeared. The most stable compound is the one which is least metabolized.

7.2. Experimental methods

7.2.1. Chemicals and reagents

Tris base, Tris hydrochloride, and β-Nicotinamide adeninine dinucleotide phosphate hydrogen sodium salt (β-NADPH) were obtained from Sigma chemicals (St. Louis, MO, USA). All other reagents, chemicals, and reference standards are same as those described in section 4.1.1 of Chapter 4.

7.2.2. Buffer for incubation and homogenization of rat liver

0.472g of Tris base, 2.54g Tris hydrochloride, 2.3g of potassium hydrochloride and 0.0744gm of EDTA were dissolved in 100mL of MilliQ water (0.1 M) and the pH was adjusted to 7.4 with concentrated hydrochloric acid. The solution was filtered through 0.22 μ membrane filter before use.

7.2.3. Preparation of rat liver S9 fraction

Young healthy female *S.D.* rats were procured from the Laboratory Animal Services Division of the institute. Animals were fasted for 12 h, but water was provided *ad libitum*. The rats were sacrificed by decapitation and the liver was isolated and placed in ice cold homogenizing solution. Adhering non liver tissues and blood were carefully removed and the liver pieces were then homogenized in ice cold homogenizing buffer in 1: 3 w/v ratio. The pooled homogenate was then centrifuged at 9000 g for 30 min at 4°C. The supernatant (S9 fraction) was used for the preparation of microsomes by ultracentrifugation method. The S9 fraction was stored at -80°C till use.

7.2.4. Preparation of rat liver microsomal fraction

The aliquots of S9 fraction were transferred to ultracentrifuge tubes and centrifuged at 100000 g for 1 hour. After centrifugation, the supernatant is decanted and the microsomal pellet suspended gently in 0.1M tris buffer by using homogeniser to get the final mocrosomal suspension. Aliquots of microsomes were stored at -80°C till use [11].

7.2.5. Protein Estimation

The protein content of microsomes utilized in the experiment was determined by Bradford method [12]. The concentration of the proteins in microsomes was determined by the standard curve constructed using bovine serum albumin.

7.2.6. Chromatographic conditions

The analyses were carried out by liquid chromatography with photo diode detector (LC-PDA) using Shimadzu LC 6AD pumps, a CBM-20A controller, a SIL-20A autosampler and SPD-M20A prominence PDA detector. The chromatographic data obtained were recorded and analyzed by Class-VP software. The chromatographic conditions applied for the separate analysis of K095, K054 and S006-1709 is summarized in Table-7.1. The injection volume was 50µl.

Table-7.1: HPLC-PDA conditions for K095, K054 and S006-1709

Markers	Column	Mobile Phase (%)		λ_{max}[c]	Flow rate (mL/min)
		A[a]	B[b]		
Medicarpin (K095)	Discovery (100 x 4.6mm, 5µm)	40	60	285	1.0
Cladrin (K054)	Lichrocart (250 x 4.6mm, 5µm)	50	50	250	0.4
S006-1709	Discovery (100 x 4.6mm, 5µm)	40	60	285	1.0
IS= 2µg/mL of biochanin for K095 and S006-1709; 2µg/mL of 7-hydroxy isoflavone for K054					

Note: a- acetonitrile, b- 0.1% acetic acid, c- wavelength of maximum absorption

7.2.7. Incubation, Sampling and Analysis

Five microliters of a working solution (200µg/mL) of K095/K064/ S006-1709 was added to 495 µL of 0.1 mM Tris buffer (pH 7.4) containing 2mM NADPH. The mixture was shaken for 5 min for equilibration in a water bath at 37°C. The incubation was then initiated by adding a 500-µL solution containing 1mg of rat microsomes in 0.1 mM Tris buffer into the above mixture. The final concentrations of the test compound, NADPH and the microsomal protein were 1µg/mL, 1mM and 1 mg/mL, respectively.

Aliquots of 100 µL of the incubation mixture were collected at 0, 15, 30, 45, 60, 90, 120, 150 and 180 min and the reaction was quenched by adding each aliquot into 400 µL of ice cold acetonitrile containing internal standard (IS). The samples were vortex-mixed and centrifuged at 12000 g for 10 min. The supernatant (400 µL) was transferred to test tube and evaporated to dryness. The dry residue was reconstituted in mobile phase and the samples were then analyzed by a HPLC-PDA method.

7.2.8. Data Treatment

The analyte to IS peak area ratio was used to determine the percent drug remaining in sample at time 'T' by comparison with the peak area ratio at time T=0 as 100%. To determine the *in vitro* half life (t ½), the percentage drug remaining versus time plot was fitted to one phase exponential decay using GraphPad Prism software (Ver.4) [13, 14].

The following equation (Eq 9.1) was used for nonlinear regression analysis of the data in one phase exponential decay.

$$Y = Plateau + Span.e^{-k.X} \quad \text{(Eq-7.1)}$$

Where, Y is % concentration remaining,

X is time in min,

K is the elimination rate constant,

Plateau is the value of Y at terminal sampling time,

Span is the difference in value of Y at T=0 and the plateau.

The half life was computed from the value of elimination rate constant (K) using the following equation (Eq-9.2)

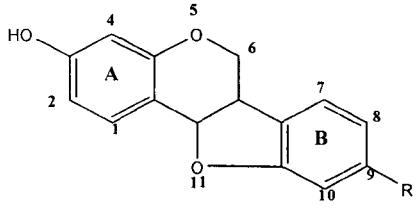
$$t_{1/2} = 0.693/K \quad \text{(Eq-7.2)}$$

7.3. Results and Discussion

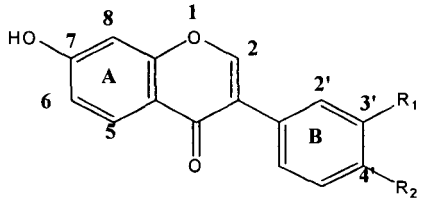
The results obtained in the metabolic stability studies of K095, K054 and S006-1709 in rat liver microsomes are summarized in Table-7.2. The study shows that the order of stability in accordance with their half life is K054> S006-1709> K095 (Table-7.2, Fig-7.2.1 to 7.2.3).

Among the three compounds, K095 was metabolized faster, while K054 was slowly metabolized. As discussed in Chapter-6, K095 and S006-1709 differ from each other by a single methoxy group. Methylated flavonoids have been determined to be more stable than unmethylated isoflavones (18). However, in the present study, K095 is less stable in comparison to unmethylated analogue S006-1709. K095 has methoxyl group substituted in 9^{th} position, which corresponds to that present in 4' position of formononetin (Fig-7.1). The methoxy group at 4' position has been found to be more susceptible for demethylation reaction. Formononetin has been reported to undergo demethylation of 4'-methoxyl substituent in phase I metabolism to give daidzein (Fig-7.1) [10]. Similarly, the decreased metabolic stability of K095 in rat liver microsomes might be due to increased rate of demethylation.

The marker K054 was metabolized at a very slow rate and this is also structurally related to formononetin but it has an additional methoxyl group at 3' position. As discussed in Chapter-6, the presence of ortho substituted methoxyl groups might have caused steric hindrance, thereby reducing or inhibiting the demethylation reaction in K054. Thus compounds S006-1709 and K054 undergo metabolism at a slower rate than K095 and they might get metabolized by reduction or other pathways of phase I metabolism.



Medicarpin: R= OCH_3
S006-1709: R=H



K054: R_1= OCH_3, R_2=OCH_3
Formononetin: R_1=H, R_2=OCH_3,
Daidzein: R_1=H, R_2=OH

Fig-7.1: Structures of pterocarpans and isoflavones

Table-7.2: Metabolic stability of K095, K054 and S006-1709

Parameters	K095	K054	S006-1709
Span (%)	77.44±1.92	15.96±2.52	53.26±0.35
K	0.03±0.004	0.01±0.001	0.019±0.003
Plateau (%)	22.16±1.72	84.26±1.79	45.72±0.97
Half-life (min)	23.42±3.19	69.72±3.54	35.71±4.70

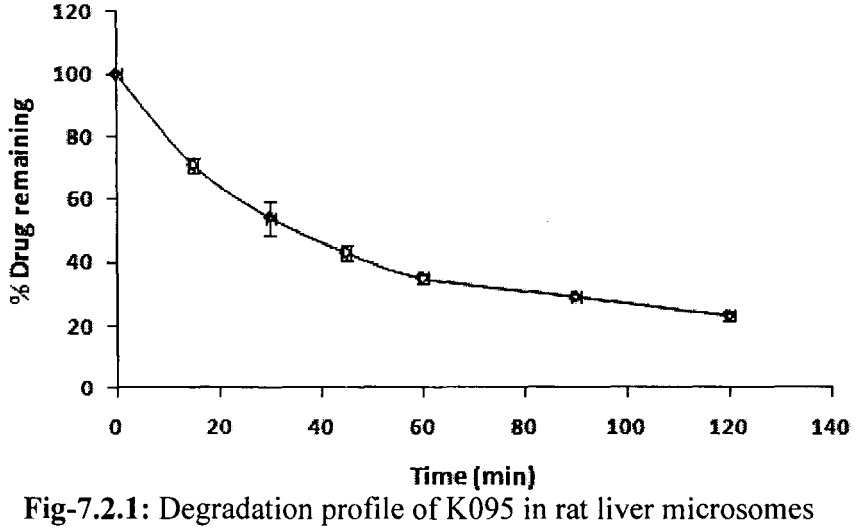


Fig-7.2.1: Degradation profile of K095 in rat liver microsomes

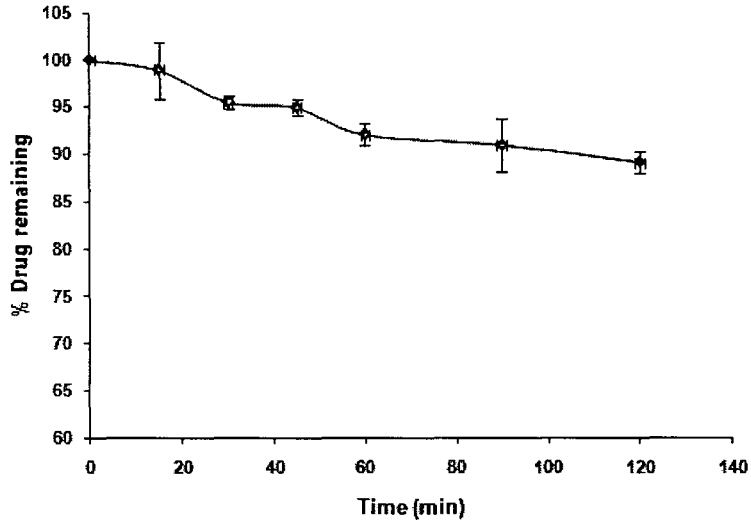


Fig-7.2.2: Degradation profile of K054 in rat liver microsomes

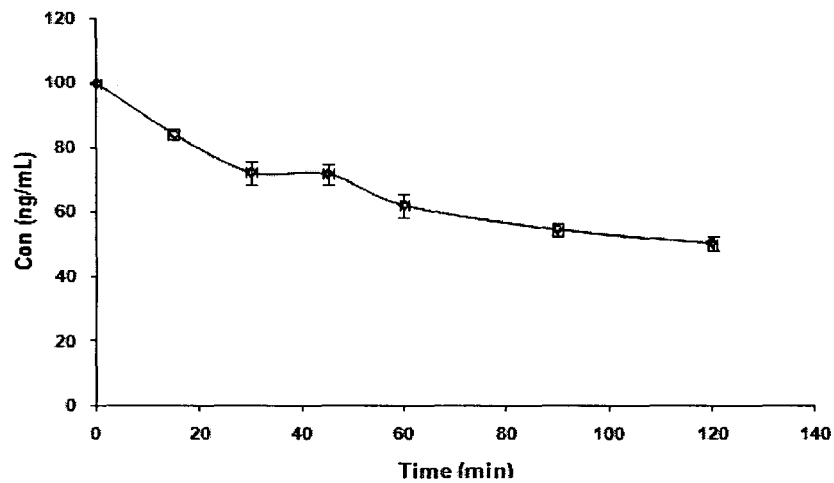


Fig-7.2.3: Degradation profile of S006-1709 in rat liver microsomes

7.4. Conclusion

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 be more stable than its natural analogue K095 and may be a potential candidate for development as an osteogenic agent.

References

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