Chapter 6

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

The protein binding of drugs in plasma are generally determined by equilibrium dialysis, ultrafiltration, ultracentrifugation methods [1-3]. All these methods involve the separation of free drugs from protein bound under equilibrium condition and have been applied for the protein binding study of various drugs. However, these methods cannot be used for the drug that shows non-specific adsorption to the membrane or lipid partitioning into low density lipoproteins after ultracentrifugation [4]. For such compounds, charcoal adsorption method has been reported to determine the percentage of protein binding. In charcoal adsorption method, the time course of decline of bound drug concentration is determined while the free drug is being removed by charcoal adsorption [5].

6.1. Experimental methods

6.1.1. Chemicals and Reagents

Dextran coated charcoal was obtained from Sigma Chemicals, USA. Dulbecco's Phosphate Buffered Saline (DPBS) (Ca^{2+} and Mg^{2+} free) was purchased from Hi Media Laboratories Pvt. Ltd., Mumbai. Drug-free heparinised rat plasma was obtained from healthy rats as described in section 4.1.1. of Chapter 4. Details of other reagents and chemicals are same as that mentioned in section 4.1.1. of Chapter 4.

3.1.2. Preparation of dextran coated charcoal suspension

The dextran coated charcoal suspension was prepared by adding 0.66 g of dextran-coated charcoal to 100 ml of DPBS (9.5 g/L). The mixture was stirred with a magnetic stirrer at room temperature until the charcoal was suspended. This suspension was prepared at least 18 hours before use and stored at 5-10°C for not more than 30 days. The stored charcoal mixture was re-suspended before use.

3.1.3. Preparation of stock solutions

A stock solution (1mg/mL) of test compounds (K054/K095/S006-1709) was prepared separately in methanol: DMSO (9:1) and diluted to get the concentration of 200µg/mL in methanol. This working solution was used for spiking in normal rat plasma so as to get a final concentration of 1µg /mL of test compound.

3.1.4. Charcoal adsorption assay

The protein binding was estimated using the modified charcoal adsorption method reported by Khurana *et al.* [6]. The drug was spiked to normal plasma (6mL) and the spiked plasma was allowed to equilibrate for 15 minutes before the start of study. Charcoal suspension (6 mL) was transferred into a 30 ml glass tube, centrifuged at 3000 g for 15 minutes at 25°C, and the supernatant DPBS was carefully decanted off. Spiked plasma (6 mL) was then added on to the charcoal pellet under continuous stirring at 37 ± 1°C. Serial samples (300 µl) were withdrawn at 0, 5, 10, 20, 30, 40, 50min, 1 h, 1.5, 2, 2.5, and 3 h in 0.5 ml polypropylene micro-centrifuge tubes and centrifuged immediately at 11,000 g for 5min at 37°C. The supernatant was separated and immediately transferred into 5 ml glass centrifuge tubes and stored at -50°C until analyzed. The study was performed separately for each candidate drug (K054/K095/S006-1709), in three replicates.

3.1.5. Sample analysis

The samples were analyzed by validated LC-MS/MS assays for rat plasma as described in Chapter 4.

8.1.6. Data treatment

The percent drug remaining in plasma was determined by comparison with the initial concentrations (prior to charcoal treatment). Percent drug remaining in the supernatant plasma versus time data was fitted to a two-compartment open model with intravenous bolus input by nonlinear regression program using WinNonlin software (Version 5.1) which mimics the events pertaining to charcoal adsorption assay as depicted in Fig-6.1 [5].

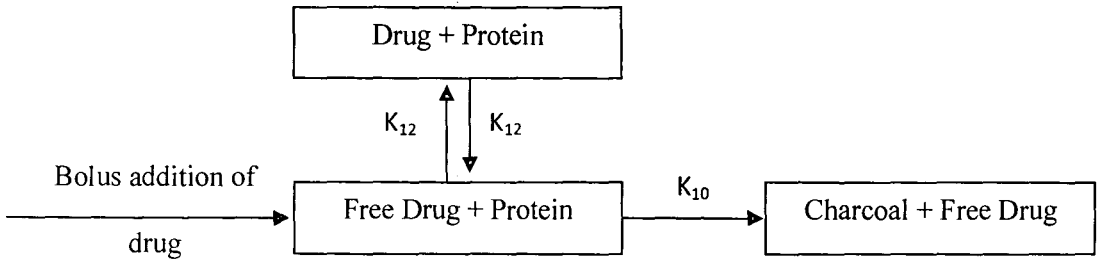


Fig-6.1: The hypothetical two-compartment model describing the basis of charcoal adsorption assay for protein binding estimation.

The model is described by the following bi-exponential equation:

$$B_t = A_1 e^{-\alpha t} + A_2 e^{-\beta t}$$

Where B_t is % bound at time t, A_1 and A_2 are Y intercepts, α and β are distribution and disposition rate constants for the two phases respectively. The extent of protein binding was given by the C_{max} value (at time t = 0). Statistical comparison was performed at 95 % confidence interval wherever necessary.

6.2. Results and Discussion

Ultra filtration method was not applied for the protein binding study of three test compounds because of non-specific adsorption of these test compounds to the membrane of the ultra filtration units. So modified charcoal adsorption method was used for their protein binding studies. The percent drug remaining in the supernatant plasma versus time data for each compound is given in Fig-6.2.1, 6.2.2 and 6.2.3. The protein binding of K095, K054 and S006-1709 was determined to be 12.17±1.71%, 3.37± 0.15 % and 16.28±3.73% respectively.

K054 (7-hydroxy, 3', 4'-dimethoxy isoflavone) has the least degree of protein binding (Fig-6.2.1). K054 is structurally similar to another isoflavone, calycosin (Fig-6.3) and the binding of calycosin to human serum albumin (HSA) had been reported to be 39.8% [7]. In another study, it has been reported that the binding affinity of calycosin to bovine serum albumin (BSA) was lesser than that of flavonol quercetin [8]. These three compounds (K054, calycosin and quercetin) differ in their substituent in ring B (Fig-6.3). There are various factors that affect the binding of a drug to plasma proteins. The two important factors responsible for plasma protein binding are hydrophobic interactions and hydrogen bonding [9-11]. However, some other types of interactions including electrostatic interactions, van der Waals interactions and steric constants may also be involved in protein binding of drugs [12]. The binding affinity of flavonoids to albumin has been reported to decrease with increase in molecular size, polarity, steric hindrance and nonplanarity of strucuture [8, 13]. Similarly, in this study, the presence of adjacent

methoxyl groups in K054 might have increased molecular size, polarity, steric hindrance, thereby reduced its binding affinity to plasma protein leading to very low degree of protein binding.

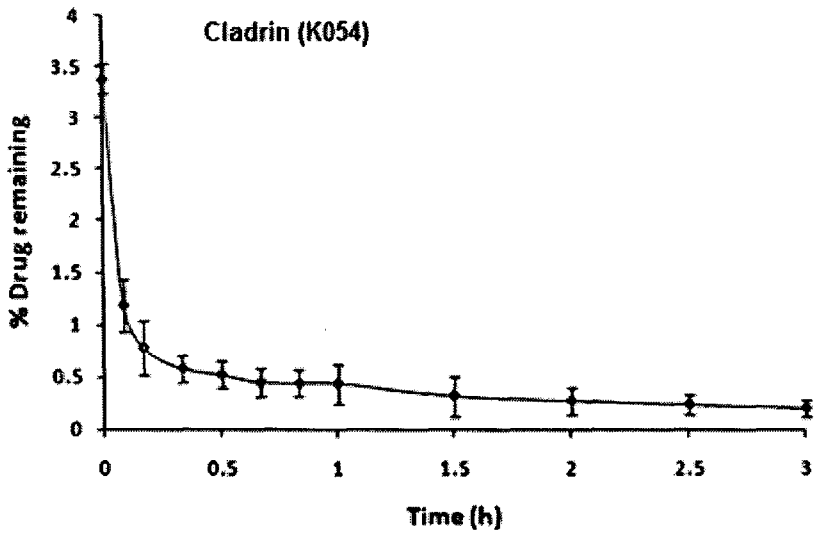


Fig-6.2.1: Percentage drug remaining versus time profile of K054

The percentage of protein binding for K095 and S006-1709 were not significantly different (Fig-6.2.2 and Fig-6.2.3). These two compounds are pterocarpans and are structurally similar (Fig-6.3). K095 has an additional methoxy group in its ring B, but this difference in the structure has not caused significant difference in the protein binding of these two compounds.

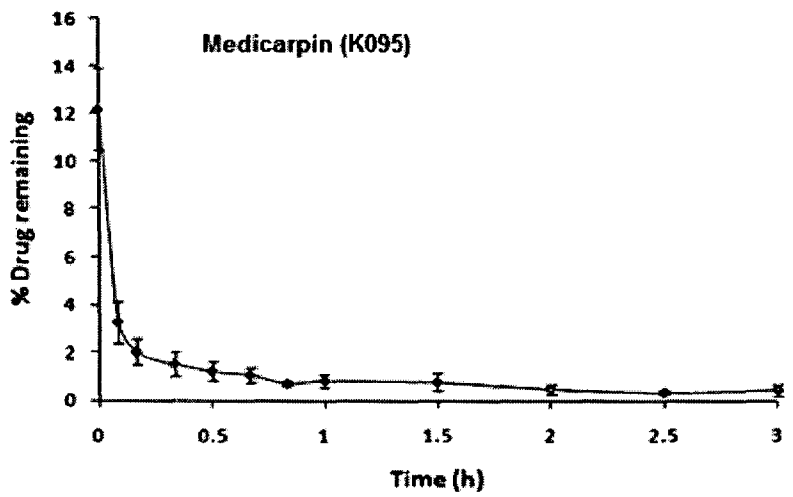


Fig-6.2.2: Percentage drug remaining versus time profile of K095

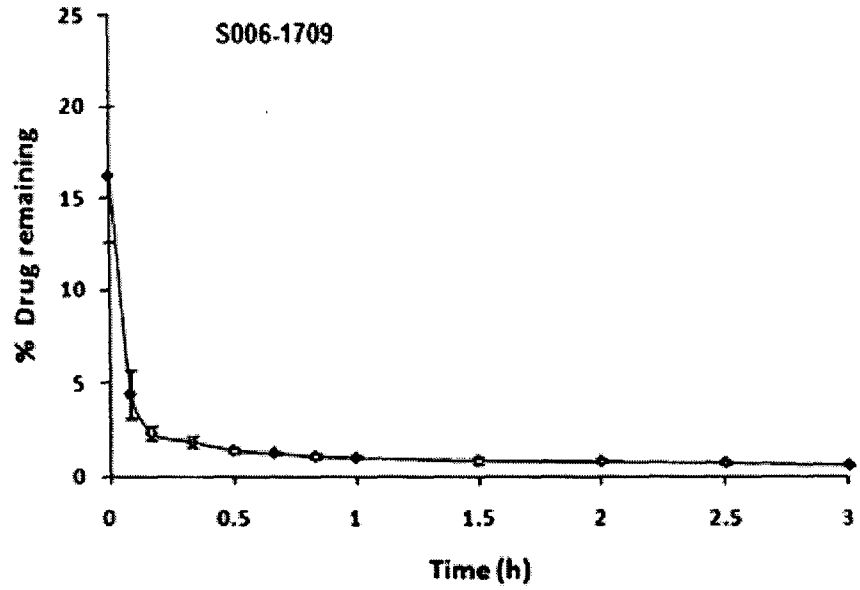
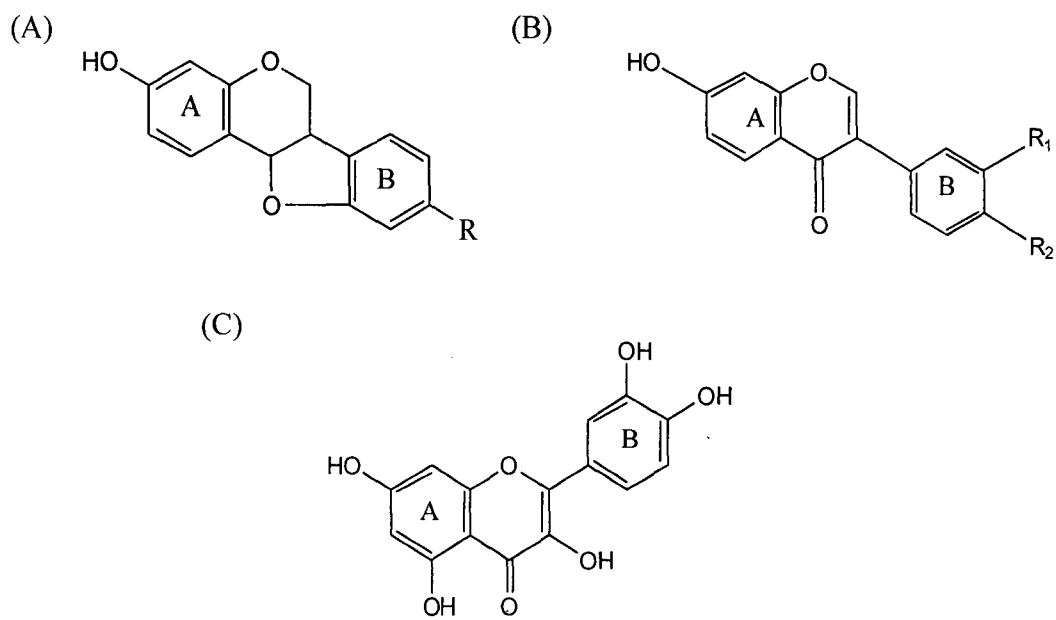


Fig-6.2.3: Percentage drug remaining versus time profile of S006-1709



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

K054: R_1= OCH_3, R_2= OCH_3
Calycosin: R_1= OH, R_2= OCH_3

Fig-6.3: Structures of (A) K095 and S006-1709 (B) K054 and Calycosin (C) Quercetin

Overall, the percentage of protein binding for K095, K054 and S006-1709 were low. When the percentage of protein binding is low, the free drug concentration in plasma would be higher and could cross the cell membranes to get distributed in tissues. This is evident in their pharmacokinetics studies (Chapter-5). The parameter volume of distribution at steady state (V_{ss} > 0.7L/kg, Table-7.1-Table-7.3) shows that all these compounds have extensive distribution in tissues. As a result, more drugs would be available to interact with receptors or active site in tissues causing intense pharmacological effect [14]. All these compounds, with low degree of protein binding and high tissue distribution exhibit more potent oesteogenic activity.

6.3. Conclusion

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.

References

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