Protein Binding Studies of Gossypin by Equilibrium Dialysis

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Abstract
Gossypin is a glucosyl flavone obtained from the flowers of Hibiscus pitifolius (Malvaceace). It has potent analgesic and anti-inflammatory activity. It has shown cytotoxic activity when tested against human lung adrenocarcinoma cell lines (A549). It has been shown to suppress angiogenesis, inflammation, and carcinogenesis. The mechanisms of these activities, however, are unknown. Because of these effects, to improve the efficacy of gossypin we made an attempt in the protein binding of gossypin in this study. In-vitro protein binding of gossypin in purified bovine serum albumin was investigated by equilibrium dialysis. The drug was highly protein bound; approximately 99.2% and the extent of protein binding remained constant at gossypin concentration in the range of 1-5µg/ml. The extent of binding tends to decrease at lower albumin and higher drug concentrations. Scatchard plot indicates the presence of two binding sites.

Key words
Protein binding, gossypin, equilibrium dialysis.

Introduction
Bioflavonoids exhibit varied biological activities such as analgesic, antipyretic, anti-inflammatory, local anesthetic, and anti-hypertensive etc /1,2,3/. A wide range of effects have been studied including inhibition of neoplastic transformation, 4-nitro quinolone-1 oxide induced rat oral carcinogenesis, proliferation of human breast and colon carcinoma /4,5/. Some were shown to provide protection against coronary heart disease /6,7,8/. They have been reported to inhibit important cytochrome P450 enzymes such as CYP1A1 and 1A2 there by prevent transformation of procarcinogens into carcinogens. They were implicated in impaired absorption and metabolism of some drugs by inhibiting P-glycoprotein and CYP 3A4 respectively /9/. Most of these reported activities were observed under in vitro conditions or in animal systems where disposition of the compounds did not matter /10,11,12/. Most or many of the bioflavonoids seem to have problem with one or many of the pharmacokinetic processes. Bioflavonoids are often found to be considerably bound to plasma proteins. Gossypin (shown fig 1) is a 3,5,8,3',4'-pentahydroxy-7-O glucosyl flavone obtained from the flowers of Hibiscus pitifolius (Malvaceace) /13/. It has potent analgesic and anti-inflammatory activity /14/. It has shown cytotoxic activity in human lung adrenocarcinoma cell lines (A549). It reduces the tumor burden in solid tumor harboring animals and effectively inhibits the formation of new blood vessels on tumor mass /1/. Gossypin is also known to induce opioid mediated anti-nociceptive response in experimental models /15/ and appears to be a promising candidate for treatment of cancer associated with inflammation and pain. So far no data is available on the protein binding of gossypin and hence systematic in-vitro protein binding studies of gossypin have been undertaken in the present investigation.
Materials and Methods

Materials

Gossypin pure substance was a kind gift from Department of Pharmaceutical Sciences, Andhra University, Vizag, India), Dialysis membrane (12,000-14,000 M.W cut off, Himedia Laboratories Limited, Mumbai), Isopropanol (Qualigens, Mumbai, India), Bovine Serum Albumin (Cohn’s fraction V, Sigma Chemicals, St. Louis, MO, USA) and Rotary Shaker (Remi instruments, Mumbai, India).

Protein binding determinations were carried out by equilibrium dialysis. Dialysis membrane was pretreated by sequential washings with distilled water (twice 2hr each), 45 min in 30% isopropanol and stored in distilled water at 40ºC for 2 days. Dialysis was carried out in Teflon micro dialysis cell consisting of two chambers (1.5 ml each) separated by the membrane. Experiments were conducted by using bovine serum albumin (BSA) in phosphate buffer pH 7.4. The membrane was allowed to equilibrate at room temperature for 1hr prior to use. Human plasma used in this investigation was obtained by pooling plasma of three healthy male volunteers in equal volumes. Drug samples of different concentrations were mixed with albumin and plasma and equilibriated at room temperature for 30min. 1.5 ml of drug samples containing albumin or plasma was dialyzed against the same volume of phosphate buffer (pH 7.4) for 12hr at 37ºC and 15 rpm on rotary shaker. All determinations were done six times /16/.

Gossypin content in albumin and buffer was determined using reversed phase HPLC. Shimadzu high performance liquid chromatography unit equipped with the LC-8A solvent delivery module, SPD-10AVP UV-Visible spectrophotometer detector, Class CR-10 Data Processor, Rheodyne (with 20 µl capacity loop) injection port and Wakosil II C-18 column (stainless steel column of 25 cm length and 4.6 mm internal diameter packed with porous silica spheres of 5µ diameter, 100 Å pore diameter) were used for analysis of samples. Mobile phase consisting of 0.01 M potassium dihydrogen phosphate pH -3 and methanol mixture (40:60 v/v) was used at a flow rate of 1 ml/min. Elute was monitored using a UV/VIS detector set at 376nm /17/.

Determination of extent of protein binding

The extent of protein binding in albumin solution (3.5 g/L) was studied using 6 concentrations (5, 10, 25, 50, 100, 250 µg/ml)

Gossypin and albumin concentration dependent study and binding parameters

The extent of binding of 50 µg/ml concentration of gossypin at different albumin concentrations (1.31, 2.63, 5.27, 10.5, 21 g/L) was evaluated. Initial estimates of the number of binding sites were obtained using Scatchard plot and then fitted to the equation.

\[
r = \frac{\hat{O}_i}{n} = \frac{n \cdot K_i \cdot D_i}{1 + K_i \cdot D_i}
\]

where

- r – ratio between the two molar concentrations of bound drug and albumin,
- n – number of classes of binding sites,
- i and K_i - number of binding sites respectively.
- D_i - concentration of drug (moles/L)

Results

Results obtained from binding to human plasma proteins and BSA at different concentrations of gossypin was presented in table 1& 2. Gossypin was highly bound (99.31%) to human plasma proteins and only a small fraction was present in free form. The binding was reduced to 85% at higher concentration of gossypin (250 µg/ml) and at low concentration of albumin (3.25g/L). The extent of binding of gossypin (50 µg/ml) with different concentrations of BSA is shown in figure 2. The binding
remained constant above 10.5 g/L BSA with 50 µg/ml concentration of gossypin. The Scatchard plot of number of moles of gossypin bound to total moles of BSA (table 3) had shown the existence of two binding sites with high and low affinities respectively.

![Fig 1: Structure of gossypin](image)

**Fig 2:** Extent of binding of gossypin at 50 µg/ml concentration

![Fig 3: Scatchard plot of the binding of gossypin with BSA](image)

**Table 1** Mean percentage of gossypin bound at 3.5g/L BSA (n=6)

<table>
<thead>
<tr>
<th>Conc (µg/ml)</th>
<th>Amount (µg) of gossypin in 0.1ml (donor compartment)</th>
<th>Amount (µg) of gossypin in 1.5ml (donor compartment)</th>
<th>Mean % bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>14.26</td>
<td>213.9</td>
<td>85.56</td>
</tr>
<tr>
<td>125</td>
<td>7.98</td>
<td>119.7</td>
<td>95.76</td>
</tr>
<tr>
<td>100</td>
<td>6.52</td>
<td>97.8</td>
<td>97.8</td>
</tr>
<tr>
<td>50</td>
<td>3.31</td>
<td>49.65</td>
<td>99.3</td>
</tr>
<tr>
<td>10</td>
<td>0.661</td>
<td>9.915</td>
<td>99.15</td>
</tr>
<tr>
<td>5</td>
<td>0.384</td>
<td>5.76</td>
<td>100</td>
</tr>
</tbody>
</table>
So far, little is known about the affinity of bioflavonoids for plasma proteins, but due to the low polarity of many aglycones would probably bind to serum albumin /18/. It is clear from fig.2 that the percentage binding of gossypin increased with increase in concentration of albumin up to 10.5 g/L indicating albumin concentration dependent binding. It remained constant (100%) beyond this concentration.

The Scatchard plot obtained may be regarded as the sum of two or more straight lines each representing a different class of binding site /8/. In the present case one binding site with \( V_1 = 1.028 \) is of high affinity and has an association constant \( K_1 = 2.289 \times 10^{-7} \text{M} \) and a second class of binding site \( V_2 = 1.064 \) of low affinity with an association constant \( K_2 = 0.33 \times 10^{-7} \text{M} \) are observed. Probably the latter class suggests

### Table: 2 Mean percentage of gossypin bound to BSA in-vitro and human plasma protein (n=6)

<table>
<thead>
<tr>
<th>Conc of gossypin</th>
<th>Mean percent bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BSA n=6 (3.25 g/L)</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>99.3</td>
</tr>
<tr>
<td>50</td>
<td>99.1</td>
</tr>
<tr>
<td>100</td>
<td>97.8</td>
</tr>
<tr>
<td>125</td>
<td>95.76</td>
</tr>
<tr>
<td>250</td>
<td>85.56</td>
</tr>
</tbody>
</table>

### Table: 1 No. of moles of gossypin bound

<table>
<thead>
<tr>
<th>No. of moles of gossypin</th>
<th>No. of moles gossypin bound</th>
<th>Free gossypin (D)</th>
<th>BSA - Gossypin complex (moles) PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000102</td>
<td>0.000102</td>
<td>0</td>
<td>0.053</td>
</tr>
<tr>
<td>0.000203</td>
<td>0.000201</td>
<td>1.812927E-06</td>
<td>0.953201423</td>
</tr>
<tr>
<td>0.001016</td>
<td>0.001009</td>
<td>7.11382E-06</td>
<td>0.054009146</td>
</tr>
<tr>
<td>0.002033</td>
<td>0.001988</td>
<td>4.47154E-05</td>
<td>0.054987805</td>
</tr>
<tr>
<td>0.002541</td>
<td>0.002433</td>
<td>0.000107724</td>
<td>0.055432927</td>
</tr>
<tr>
<td>0.005081</td>
<td>0.004329</td>
<td>0.000752033</td>
<td>0.057329268</td>
</tr>
</tbody>
</table>

### Discussion

So far, little is known about the affinity of bioflavonoids for plasma proteins, but due to the low polarity of many aglycones would probably bind to serum albumin /18/. It is clear from fig.2 that the percentage binding of gossypin increased with increase in concentration of albumin up to 10.5 g/L indicating albumin concentration dependent binding. It remained constant (100%) beyond this concentration.
electrostatic interaction between gossypin and protein due to hydrogen bonds or Vander Waals forces. In conclusion Scratched plot explains that the in-vitro protein binding improves the efficacy and reduces the dose of Gossypin.

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References


