Investigation and Determination the Binding Site of Glycyrrhizin of Liquorice to DNA

F. Manouchehri*,1, S. Nafisi1, M. Bonsaii1, K. Abdi2, M. Varavipour3

1Department of Chemistry, Islamic Azad University, Central Tehran Branch (IAUCTB), Tehran, Iran
2Department of Medicinal Chemistry, Tehran University of Medical Sciences, Tehran 14174, Iran
3Department of Irrigation & Drainage Engineering, College of Aboreyhan, University of Tehran, Iran
(Received 19 June 2011; Final version received 18 November 2011)

Abstract
Glycyrrhizin(GL), is a triterpenoid saponin found in glycyrrhiza glabra (liquorice). This compound is a frequently used and very effective drug for the treatment of various malignancies. This study was designed to examine the interactions of glycyrrhizin with calf thymus DNA in aqueous solution at physiological conditions. FTIR spectroscopic method was used to determine the ligand binding modes, using constant DNA concentration (6.25mM) and various drug/DNA (phosphate) ratios of 1/240 to 1/1. UV-Vis spectroscopic method was used to determine the binding constants and the stability of glycyrrhizin-DNA and complexes in aqueous solution, using constant DNA concentration of 5.1×10⁻⁴ M and various glycyrrhizin concentrations of 5×10⁻⁶ - 1×10⁻⁴ M. Spectroscopic evidence showed that the interaction of glycyrrhizin with DNA occurred via G, A-T and PO₄²⁻ group with binding constant of K,GL-DNA = 5.7×10³ M⁻¹ and DNA remains in B-DNA conformation.

Keywords: DNA, Glycyrrhizin, liquoric, FTIR, UV-Visible spectroscopy.

Introduction
The root of liquorice has been used since ancient Egyptian, Greek and Roman times in the West and in ancient China in the East. Liquorice is a common medicinal herb and crude drug in the traditional Chinese medicine used for over 1000 years [1-3]. The main bioactive components of liquorice root is triterpenoid glycoside – glycyrrhizic acid; 3-O-(2-O-β-D-Glucopyranuronosyl-α-D-glucopyranuronosyl)-18β-glycyrrhetinic acid. glycyrrhizic acid has been shown to be active against a variety of viruses including herpes simplex type 1 (HSV-1), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), hepatitis A, B and C (HAV, HBV, and HCV) viruses, influenza virus, and human immunodeficiency virus-1 (HIV-1) [4-12].
addition, GL appears to inhibit helicobacter pylori growth [13] and has been used for many years in Japan, where it is reported to reduce the risk of hepatocellular carcinoma and it is of clinical interest in the treatment of chronic hepatitis C [14].

Even though much reports are available on antivirus activities of glycyrrhizin, little is known about its interactions with individual DNA. We studied the interaction of DNA adducts with glycyrrhizin (glycyrrhizic acid) in aqueous solution at pH 7 with glycyrrhizin/DNA(P) molar ratios of 1/240 to 1/1 by FTIR and UV measurements. The drug binding site, the binding constant, and the effects of drug complexation on the stability and conformation of DNA duplex are discussed.

Preparation of stock solutions
DNA was dissolved to 0.5% w/v (0.0125 M) polynucleotide(phosphate) in 0.1 M NaCl and 1 mM sodium cocodylate/L (pH 7.20) and kept at 5 ºC for 24 h with occasional stirring to ensure the formation of a homogeneous solution. The final concentration of the DNA solution was determined spectrophotometrically at 258 nm using molar extinction coefficient $\varepsilon_{258} = 9250$ cm$^{-1}$ M$^{-1}$ (DNA) (expressed as molarity of phosphate groups) [16]. The appropriate amounts of glycyrrhizin (0.05–12.5 mM) were prepared in distilled water and added drop wise to DNA solution in order to attain the desired ligand/DNA(P) molar ratios (r) of 1/240, 1/120, 1/80, 1/40, 1/20, 1/10, 1/5, 1/2 and 1 with a final DNA(P) concentration of 6.25 mM (path length was 0.03 cm). The pH of the solutions was adjusted at 7.0 ± 0.2 using NaOH solution. The infrared spectra were recorded 2 h after mixing of the glycyrrhizin with DNA solution. For UV measurements, the glycyrrhizin concentrations of $5.1\times10^{-6}$- $1\times10^{-4}$ M at constant DNA concentration of

![Figure 1. Chemical structure of glycyrrhizin](image_url)
5.1×10^{-4} \text{ M} were used.

**FTIR spectroscopic measurements**

Infrared spectra were recorded on a NICOLET-MAGNA-IR-550, FTIR spectrometer equipped with a liquid-nitrogen-cooled HgCdTe(MCT) detector and a KBr beam splitter. The spectra of glycyrrhizin/DNA solutions were taken using a cell assembled with AgBr windows. Spectra were collected and treated using the Spectra Manager software supplied by the manufacturer of the spectrophotometer. Solution spectra were recorded after 2h incubation of the glycyrrhizin with DNA solution, using AgBr windows. The bands were measured in triplicates (three individual samples of the same DNA, and glycyrrhizin concentrations). For each spectrum, 100 scans were collected at a resolution of 4 cm^{-1}. The difference spectra \([\text{polynucleotide solution}+\text{glycyrrhizin solution}] - \text{polynucleotide solution}\) were obtained using a sharp DNA band at 968 cm^{-1} as internal reference \([17-18]\). These bands, which are due to sugar C-C and C-O stretching vibrations, exhibit no spectral change (shifting or intensity variation) upon glycyrrhizin – DNA complexation, and cancelled out upon spectral subtraction \([17-18]\). The intensity ratios of the bands due to several DNA inplane vibrations related to A-T, G-C base pairs and the \(\text{PO}_2\) stretching vibrations were measured with respect to the reference bands at 968 cm^{-1} (DNA) as a function of glycyrrhizin concentrations with an error of ±3%. Similar intensity variations have been used to determine the ligand binding to DNA bases and backbone phosphate groups \([19]\). The plots of the relative intensity \((R)\) of several peaks of DNA in-plane vibrations related to A-T, G-C base pairs and the \(\text{PO}_2\) stretching vibrations versus glycyrrhizin concentrations were obtained after peak normalization using, \(R_i = I_i/I_{968}\), where \(I_i\) is the intensity of the absorption peak for pure DNA in the complex with \(i\) as ligand concentration, and \(I_{968}\) is the intensity of the 968 cm^{-1} peak (DNA internal reference).

**Absorption spectroscopy**

The absorption spectra were recorded on a LKB model 4054 UV–Visible spectrometer, Quartz cuvettes of 1 cm were used and the absorption spectra recorded with drug concentrations of \(5\times10^{-6}-1\times10^{-4} \text{ M}\) and constant polynucleotide concentration of \(5.1\times10^{-4} \text{ M}\). The values of the binding constants \(K\) were obtained according to the methods described by Zhong \([20]\). By assuming that there is only one type of interaction between drug and DNA in aqueous solution, \(I_e\) Equations (1) and (2) can be established:

\[\text{DNA} + \text{ligand} \rightarrow \text{DNA:ligand} \quad (1)\]

\[K = \frac{[\text{DNA:ligand}]}{[\text{DNA}][\text{ligand}]} \quad (2)\]

where \(K\) is the binding constant and ligand =glycyrrhizin. Assuming \([\text{DNA}:\text{ligand}]=C_0\),
K = \frac{C_B}{(C_{DNA} - C_B)}(C_{ligand} - C_B) \quad (3)

where $C_{DNA}$ and $C_{ligand}$ are the analytical concentration of DNA and ligand in solution, respectively. According to the Beer–Lambert law:

\begin{align*}
C_{DNA} &= \frac{A_0}{(\varepsilon_{DNA} - 1)} \quad (4) \\
C_B &= \frac{(A - A_0)}{(\varepsilon_B - 1)} \quad (5)
\end{align*}

where $A_0$ and $A$ are the absorbance of DNA at 258 cm$^{-1}$ in the absence and presence of ligand, respectively. $\varepsilon_{DNA}$ and $\varepsilon_B$ are the molar extinction coefficient of DNA and the bound ligand, respectively. $l$ is the light path length (1 cm). By displacing $C_{DNA}$ and $C_B$ in equation (3) by equations (4) and (5), equation (6) can be deduced:

\begin{equation}
\frac{A_0}{(A - A_0)} = \frac{\varepsilon_{DNA}}{\varepsilon_B} + \frac{\varepsilon_{DNA}}{(\varepsilon_B - 1)K} \times \frac{1}{C_{ligand}} \quad (6)
\end{equation}

Thus, the double reciprocal plot of $1/ (A - A_0)$ versus $1/C_{ligand}$ is linear, and the binding constant ($K$) can be estimated from the ratio of the intercept to the slope.

**Results and Discussion**

**Infrared spectra of glycyrrhizin –DNA complexes**

The IR spectral features of glycyrrhizin–DNA interaction are presented in Figure 2.

![FTIR spectra](image-url)

*Figure 2. FTIR spectra in the region of 1800-600 cm$^{-1}$ for calf thymus DNA, and glycyrrhizin adduct in aqueous solution at pH=7. DNA and three complex spectra obtained at various GL-DNA (phosphate) molar ratios (top four spectra), ligand and two difference spectra (bottom three spectra).*
The spectral changes (intensity and shifting) of several prominent DNA in-plane vibrations at 1712 (G, T, mainly G), 1660 (T, G, A, C, mainly T), 1610 (A, C, mainly A), 1490 (C, G, mainly G) and 1227 (PO$_2^-$ asymmetric stretch) and 1088 cm$^{-1}$ (PO$_2^-$ symmetric stretch) [21-23] were monitored at different glycyrrhizin – DNA molar ratios, and the results are shown in Figures 2 and 3.

![Figure 3](image.jpg)

**Figure 3.** Intensity ratio variations for several DNA in-plane vibrations as a function of GL concentration. Intensity ratios for the DNA bands at 1712 (G, T), 1662 (T, G, A, C), 1610 (A), 1490 (C,G) and 1227 (PO$_2^-$ asymmetric) referenced to the DNA band at 968 cm$^{-1}$.

At low glycyrrhizin concentration ($r = 1/240$), the intensity of guanine, thymine decreased and PO$_2^-$ vibrations were observed as a result of glycyrrhizin interaction with DNA (Figure 3) and helix stabilization. Evidence for this originates from the minor spectral changes of glycyrrhizin –DNA complexes. The intensity of guanine (34%), thymine (19%), cytosine (7%), and asymmetric and symmetric phosphate bands (23% and 21%) decreased, respectively.

Minor interaction was observed between glycyrrhizin and adenine base. Evidence for this comes from the small intensity increase of the adenine vibrations and the intensity of adenine band (6%) increased. At $r=1/120$, the guanine band at 1712 shifted to 1706 cm$^{-1}$, the thymine band at 1660 shifted to 1662 cm$^{-1}$, adenine band at 1612 shifted to 1608 cm$^{-1}$ and phosphate asymmetric band at 1227 shifted to 1234 cm$^{-1}$. The shifting was accompanied by intensity increase for the mainly guanine and thymine bands. The observed spectral changes can be related to glycyrrhizin interaction with bases; guanine, adenine N7 and thymine O2 and backbone phosphate group.

At $r=1/80$, the intensity of the bands decreased that can be related to DNA stabilization upon glycyrrhizin interaction.

At $r=1/40$, the intensity of G, T, A bases and phosphate bands increased that can be related to major glycyrrhizin interaction with guanine and adenine N7, thymine O2, and the backbone PO$_2^-$ group at this concentration (Figure 2 and 3). Evidence for this comes from the spectral changes (intensity and shifting) observed for guanine band at 1712 shifted to 1700cm$^{-1}$ ($r=1/40$), thymine at 1660 shifted to 1658, adenine at 1612 shifted to 1608 cm$^{-1}$, and phosphate asymmetric at 1227 shifted to 1230cm$^{-1}$. The observed shifting was accompanied by major increase in intensity of the bands at guanine, thymine, adenine,
phosphate, upon glycyrrhizin interaction. At \( r = 1/20 \) reduction of intensities were observed for the bases and phosphate vibrations which is attributed to DNA aggregation in the presence of high glycyrrhizin concentrations (Figure 3). In the difference spectra of glycyrrhizin-DNA complexes positive features at \( 1/40, 1/20 \) 1702, 1657, 1236 and 1091 cm\(^{-1}\) are due to an increase in intensity of the DNA vibrations as a result of glycyrrhizin interaction with the G and A-T base pair and phosphate backbone group (Figure 2, 3).

It should be noted that glycyrrhizin-PO\(_2\) binding occurred at all concentrations. Evidence for this comes from the major shifting of the PO\(_2\) asymmetric vibration from 1227 to 1230-34 cm\(^{-1}\) (\( r = 1/240 \) to 1/20) (Figure 2). The observed shifting was accompanied by the variations in the intensity of the phosphate band at lower concentrations (\( r = 1/240 \) to 1/80) and major increase in intensity at \( r = 1/40 \). The major intensity increase at \( r = 1/40 \) is related to the maximum drug-phosphate interaction at this concentration.

In addition to the major spectral shifting of the PO\(_2\) asymmetric band, the relative intensities of the asymmetric (\( \nu_{as} \)) and symmetric (\( \nu_s \)) vibrations were altered upon phosphate interaction [21]. The \( \nu_s \) PO\(_2\) (1088 cm\(^{-1}\)) and \( \nu_{as} \) PO\(_2\) (1227 cm\(^{-1}\)) were changed, with the ratio \( \nu_s/\nu_{as} \) going from 1.7 (free DNA) to 2.1 (glycyrrhizin-DNA complexes) at a high glycyrrhizin concentration. This showed that the maximum binding of glycyrrhizin to backbone phosphate group occurs at \( r = 1/40 \).

No major intensity changes were observed for the cytosine band at 1490 cm\(^{-1}\) that is indicative of no major participation of cytosine in glycyrrhizin-DNA binding.

As the concentration increased, \( r = 1/10 \) (Figure 2), (\( r = 1/5, 1/2, 1/1 \) are not shown here) the DNA bases vibrations in the region of 1750–1680 cm\(^{-1}\), 1666-1550 cm\(^{-1}\) were covered by glycyrrhizin C=O stretching band at 1727 cm\(^{-1}\) and C=C stretching band at 1591 cm\(^{-1}\).

Thus in the spectra of glycyrrhizin-DNA (\( r = 1/10, 1/5, 1/2, 1/1 \)), the absorption bands at 1724-1722 , 1591-1597, 1416-1421, 1142, 1113, 1063, 1041, 875 and 758 cm\(^{-1}\) and in difference spectra, the bands at 1728, 1589, 1410-1421, 1142, 1113, 1063, 1039, 875 and 758 cm\(^{-1}\), are related to glycyrrhizin vibrations and we could not draw a certain conclusion on the glycyrrhizin binding at high glycyrrhizin.

It is worth to mention that the absorption bands with medium intensity at 1653 cm\(^{-1}\) in the IR spectrum of free DNA and 1651–1654 cm\(^{-1}\) in spectra of the glycyrrhizin–DNA adducts and in difference spectra are due to water deformation mode, and they are not coming from DNA vibrations [24].

**DNA conformation**

No alterations of B-DNA structure were observed upon glycyrrhizin-DNA complexation as a result of the absence of
major spectral changes for B-DNA marker bands at 1227 cm$^{-1}$ (PO2 stretch), 1707 cm$^{-1}$ (mainly guanine) and 836 cm$^{-1}$ (phosphodiester mode) upon glycyrrhizin complexation (Figure 2).

In a B to A transition, the marker band at 836 cm$^{-1}$ shifts towards a lower frequency at about 825-800 cm$^{-1}$ and the guanine band at 1707 cm$^{-1}$ appears at 1700-1695 cm$^{-1}$, while the phosphate band at 1227 cm$^{-1}$ shifts towards a higher frequency at 1240-1235 cm$^{-1}$. In the glycyrrhizin-DNA complexes, shifting of B-DNA marker bands at 1227 to 1230–1238 cm$^{-1}$ is indicative of drug interaction with phosphate and not arising from B to A-DNA conformational change (Figure 2).

**Stability of glycyrrhizin-DNA**

Absorption spectroscopy revealed that addition of aqueous glycyrrhizin to DNA solution resulted a red shift of the DNA band at 258 to 260-263 nm in complexes, which is the additional evidence for glycyrrhizin-DNA interaction (Figure 4).

The drug binding constants were determined as described in experimental (UV-visible spectroscopy). The calculations of the overall binding constants were carried out using UV spectroscopy as reported. Concentrations of the complexed drug were determined by subtracting absorbance of the free DNA at 258 nm from those of the complexed. Concentration of the free drug was determined by subtraction of the complex drug from total drug used in the experiment. Our data of $1/\text{[drug complex]}$ almost proportionally increased as a function of $1/\text{[free drug]}$ (Figure 4). The double reciprocal plot of $1/(A-A_0)$ versus $1/(\text{drug concentration})$ is linear, and the binding
constant (K) can be estimated from the ratio of the intercept to the slope (Figure 4), where $A_0$ is the initial absorbance of the free DNA at 258 nm, and $A$ is the recorded absorbance of DNA in the presence of different glycyrhizin concentrations. The overall binding constant is estimated to be $K_{GL-DNA} = 5.7 \times 10^3 \text{ M}^{-1}$.

**Conclusions**

The results showed that the interaction of glycyrhizin with DNA occurred via external binding with overall binding constant of $K_{GL-DNA} = 5.7 \times 10^3 \text{ M}^{-1}$. Glycyrhizin interacts with G, A, T and PO$_2$ group. No biopolymer secondary structural changes were observed upon glycyrhizin interaction and DNA remains in the B-family structure in these complexes.

**Acknowledgements**

We thank Islamic Azad University, Central Tehran Branch, for the financial support of this work, and University of Tehran, Pharmaceutical Department and Miss Sh. Hassani (Lab. Manager) for allowing us to use their facilities.

**References**


