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Investigation of Chloride Anion Binding Properties of Glipizide Drug

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Keywords

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Abstract

This study addresses the anion binding property of Glipizide (GLP), an oral antidiabetic a second-generation drug member of the sulphonylurea (SU) family. GLP effectively interacts with Cl^- anion according to ^{1}H-NMR spectroscopic titrations of successive tetrabutylammonium chloride (TBACl) in deuterated chloroform ($CDCl_3$) and dimethyl sulfoxide (d_6-DMSO). Upon the addition of TBACl, the change in chemical shift was observed for both N-H protons of SU in $CDCl_3$, whereas it causes a difference in the shift of only one of N-H proton in SU in d_6-DMSO. In addition, the data obtained from ^{1}H-NMR spectroscopic titrations was analyzed by DynaFit program to calculate the binding constant (K_a) value between GLP and Cl^- anion. It was found that GLP binds Cl^- anion in $CDCl_3$ with higher affinity (K_a=77.37 M^{-1}, Fitplot for N-H_h proton at δ=6.47 ppm) than in d_6-DMSO (K_a=38.53 M^{-1}, Fitplot for N-H_h proton at δ=6.32 ppm).

Cite

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1. INTRODUCTION

A variety of amides (Bondy & Loeb, 2003; Kang et al., 2006), sulfonamides (Huggins et al., 2009), (thio)ureas (Amendola et al., 2010; Li et al., 2010), squaramides (Cai et al., 2018; Marchetti et al., 2019) and sulfonylurea (SU)s (Barišić et al., 2021) can be used as neutral receptors because they displayed remarkable interaction with different anions such as Cl^- via H-bonding. Especially, aromatic synthetic SU derivatives displayed the characteristics of good anion binders with weakly basic anions (NO_3^-, HSO_4^-, Cl^-, Br^-, I^-) while N-Hs of SU compounds were deprotonated with strongly basic anions (dihydrogen phosphate and acetate), leading to hinder the anion binding possibilities of SUs in DMSO. However, anion binding and ligand deprotonation were noticed in deuterated acetonitrile (ACN) by adding dihydrogen phosphate or acetate salts. The different behaviors in deuterated ACN and DMSO and the detection of large differences between the binding constants have been attributed to the nature of the solvents due to the polarity difference affecting the ability of their H-bond acceptor (Barišić et al., 2021). Several distinctive signals of Glibenclamide (GLIB) were monitored in course of the titration by ^{1}H-NMR analyses to detect the interaction type and its strength between GLIB (Host) and various monomers such as tetrabutylammonium methacrylate (TBAM), acrylamide (AAM), and methacrylic acid (MAA) (Guest), and it was found that a very strong interaction between GLIB and TBAM was established due to the deprotonation of SU by the formation of GLIB dimers when compared to GLIB-AAM and GLIB-MAA. The molecularly imprinted polymer, prepared from TBAM, MAA, and ethylene glycol dimethacrylate (EDMA), displayed high selectivity for GLIB over Gliclazide (GLIC) and Glipizide (GLP), and very high-recoveries for GLIB from blood serum up to 92.4% over GLP and GLIC (Hasanah et al., 2015). Very strong interactions of GLIB with 4-vinylbenzyltrimethylammonium methacrylate (VBTMA) were also acquired with a 1:1 complex formation having stability constant $K_a > 10^5$ M^{-1} by ^{1}H-NMR titration. Similarly,

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the imprinted polymers prepared from VBTMA exhibited high affinity and binding for GLIB, and selective extraction of GLIB with recoveries of up to 98% over GLIC and GLP from blood serum samples (Pessagno et al., 2018).

Glipizide, the second-generation SU family of the drug, is an oral hypoglycemic agent (Shuman, 1983; Pahwa et al., 2010) and highly effective in treating non-insulin-dependent diabetes mellitus (Lebovitz & Feinglos, 1983; Shuman, 1983). GLP, which helps the production of insulin from the pancreas and thus decreases glucose levels in blood, is rapidly absorbed and completely metabolized after its action (Brogden et al., 1979). GLP displays hypoglycemic (Pahwa et al., 2010), anticancer (Qi et al., 2014), and radical scavenging activities (by theoretical approach) (Hussan et al., 2022), and controlled release behaviors (Emami et al., 2014). Besides, a wide range of spectroscopic analyses of GLP has been performed in the literature (Ming et al., 2008; Adhikari et al., 2012; Prasad et al., 2013; Prakash & Iqbal, 2015; Ganesh et al., 2016; Jena et al., 2017; Ambadekar & Keni, 2018; Anwer et al., 2021).

The insulinotropic role of GLP is related to a high-affinity to SU receptor (SUR1), inhibiting the K_{ATP} channel on β cell plasma membrane, causing depolarization of the membrane potential, triggering [Ca^{2+}] influx, and thus, insulin release is ensured. This lowers glucose levels in plasma and maintains this effect even with a short half-life (Norris, 1979; Shuman, 1983; Aguilar-Bryan et al., 1995; Best & Benington, 1998; Gribble & Reimann, 2002; Renström et al., 2002; Pahwa et al., 2010). Drug binding to granule membrane may also cause a change in the transport of Cl^- known to modulate islet electrical activation and insulin release (Best et al., 2004). Consequently, SUs are likely to have a further ionic effect on β cell. Thus, Cl^- is suggested to be important for islet function during the release of insulin provided by SUs drugs (Sehlin, 1981; Kinard & Satin, 1995; Best et al., 2004).

Cl^- anion and its transportation has crucial importance for biological systems such as the regulation of membrane potential, homeostasis, pH regulation, cellular proliferation, and cellular differentiation (Davis et al., 2007; Valdivieso & Santa-Coloma, 2019). Moreover, the abnormal chloride transportation was observed in various diseases including cystic fibrosis (Rowe et al., 2005). Considering the importance of Cl^- anion in biological systems (Rowe et al., 2005; Davis et al., 2007; Hosogi et al., 2014; Martin et al., 2018; Valdivieso & Santa-Coloma, 2019) and a relationship between Cl^- anion transport and insulin release (Sehlin, 1981; Kinard & Satin, 1995), Cl^- was chosen as the anionic guest to investigate the anion binding property of the antidiabetic drug GLP in the current work. Although there have been several attempts about anion binding studies related to SUs (Hasanah et al., 2015; Pessagno et al., 2018; Barišić et al., 2021) as mentioned above, as far as known, no Cl^- binding study on GLP drug, which may form supramolecular complexes through non-covalent interactions with Cl^-, has been performed before. The solution of GLP drug (as host) in $CDCl_3$ and d_6-DMSO was titrated with 0.2 to 26 equivalents of TBACl salt (guest), and the chemical shift change (Δδ) of N-H protons in GLP was continuously monitored by ^{1}H-NMR analysis after each addition of TBACl salt. Finally, the association or binding constants (K_a's) were calculated for N-H protons in both deuterated solvents using the DynaFit software. This study has demonstrated that GLP, a drug with known antidiabetic properties, which can interact non-covalently with Cl^-, can be used as a potential agent in the transport and regulation of the concentration of Cl^- anion, which is very important for biological systems.

2. MATERIAL AND METHOD

Materials and Instruments: Glipizide (Santa Cruz Biotechnology) and tetrabutylammonium chloride (TBACl, 98% purity, Sigma-Aldrich) were used for ^{1}H-NMR spectroscopic titrations. Moisture sensitive TBACl was weighed in a glove bag filled with nitrogen atmosphere, and then, Bruker Avance III 400 MHz NMR spectrometer was used to perform spectroscopic titrations in $CDCl_3$ and d_6-DMSO.

NMR Analyses

^{1}H-NMR analyses of GLP in $CDCl_3$ and d_6-DMSO were performed, and signals of the protons of GLP in d_6-DMSO were found to be in agreement with the literature (Ming et al., 2008). The protons of GLP taken ^{1}H-NMR in $CDCl_3$ were accordingly assigned based on their chemical environments. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.25 (s, 1H), 8.66 (s, 1H), 8.35 (t, 1H), 7.89 (t, J = 8 Hz., 1H), 7.82 (d, J = 8 Hz., 2H), 7.39 (d, J = 8 Hz.,

2H), 6.47 (d, *J* = 8 Hz., 1H), 3.75 (q, *J* = 8 Hz., 2H), 3.60 (m, 1H), 3.04 (t, *J* = 8 Hz., 2H), 2.64 (s, 3H), 1.56 – 1.84 (m, 5H), 1.36 – 1.13 (m, 5H).

^{1}H-NMR (400 MHz, d_6-DMSO) δ 10.31 (s, 1H), 9.09 (s, 1H), 8.82 (t, 1H), 8.60 (s, 1H), 7.79 (d, *J* = 8.1 Hz., 2H), 7.45 (d, *J* = 8.1 Hz., 2H), 6.33 (d, *J* = 8.1 Hz., 1H), 3.59 (m, 2H), 3.52 (s, 1H), 2.96 (t, *J* = 7.2 Hz., 2H), 2.58 (s, 3H), 1.72 – 1.41 (m, 5H), 1.37 – 0.95 (m, 5H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 163.05, 156.87, 150.59, 145.11, 142.86, 142.43, 142.07, 138.24, 129.20, 127.32, 48.09, 34.79, 32.30, 25.01, 24.21, 21.35.

Both H_h and H_n protons were observed in 0.0533 M solution of GLP in $CDCl_3$ before and after titration. However, H_h proton of GLP was visible at 6.33 ppm but H_n proton of SU somehow did not appear at 10.31 ppm at 0.0533 M solution of GLP drug in the ^{1}H-NMR spectrum before starting the titration in d_6-DMSO. The H_n proton was visible at 10.31 ppm in d_6-DMSO when 0.165 M GLP was used. However, the concentration at 0.165 M is too high to continue titration of TBACl up to 26 equiv. Therefore, H_h proton of GLP was monitored when ^{1}H-NMR of GLP was recorded in d_6-DMSO.

The solution of 1 M TBACl salt in $CDCl_3$ was added as an anionic guest in specific aliquots starting from 0.2 to 26 equiv. to a solution of 0.0533 M GLP receptor to evaluate the anion binding property of GLP. The changes in selected peaks of GLP were monitored by ^{1}H-NMR after each addition of Cl^- anion. ^{1}H-NMR titrations experiments were also performed in d_6-DMSO to see the effect of the solvent nature on the binding ability of receptor to anion. Then, the data from these ^{1}H-NMR spectroscopic titrations was analyzed, and stoichiometric K_a of Cl^- anion to GLP were calculated by open-access DynaFit software by fitting 1:1 model (Kuzmič, 2009; Thordarson, 2011; Ulatowski et al., 2016) as described in literature (Kuzmič, 2009; Mercurio et al., 2015; Zapata et al., 2017).

The concentration of GLP varied gradually from 0.0533 to 0.0203 M with subsequent addition of TBACl solution. By adding TBACl, the concentration of Cl^- increased from 0 to 0.528 M at the end of titration. The shifts of N-H protons of SU throughout titration were recorded as ppm values. These three parameters (conc. of GLP and TBACl and ppm values of related protons) were used for the calculation of K_a, and plotting of fitplots, residual and relative sum of squares (SSQ/SSQ min.).

3. RESULTS AND DISCUSSION

The ^{1}H-NMR analysis of GLP drug (host) was performed in $CDCl_3$ and d_6-DMSO as a reference point before titration with TBACl salt (guest) in order to investigate Cl^- anion recognition ability of GLP drug. The protons in the structure of GLP were labelled as shown in the spectrum in Figure 1A. The area under the peak of each proton signal was observed to be proportional to the number of hydrogen atoms in the structure of GLP. The difference in chemical shifts for both N-H protons of SU (H_h and H_n), amide N-H proton (H_l), aromatic protons (H_i and H_j) of the sulfonyl attached *para*-substituted ring, and the protons (H_k and H_m) in the pyrazine ring in $CDCl_3$ or d_6-DMSO (Figure 1 and Table 1) were analyzed after each TBACl addition from 0.2 to 26 equiv. to GLP drug.

The downfield shifts from 8.66 ppm to 10.98 ppm for H_n in Figure 2 and from 6.47 ppm to 7.41 ppm for H_h in Figure 3 were observed by adding TBACl up to 6 equiv. in $CDCl_3$ in ^{1}H-NMR spectra, respectively, indicating that the binding sites via the formation of a H-bonds (Ramalingam et al., 2008; Amendola et al., 2010; 2011; Bao et al., 2018; Picci et al., 2020; Barišić et al., 2021; Kumawat et al., 2021) between H_n and H_h protons of GLP and Cl^- anion. Interestingly, when TBACl was continuously added from 8 to 26 equiv., slightly highfield shifts were observed from 10.98 ppm to 10.89 ppm for H_n and from 7.41 ppm to 7.33 ppm for H_h as shown in Figures 2 and 3 respectively. This might be interpreted as saturation of the H-bond interactions after the addition of more than six equiv. of TBACl and a very slight weakening of the H-bond interaction due to a partial increase in the electron density in N-H bond.

When the aromatic protons (H_i and H_j) of sulfonyl attached *para*-substituted ring were examined in $CDCl_3$, H_i was shifted to highfield slightly from 7.39 ppm to 7.13 ppm with successive addition of TBACl (from 0 to 26 equiv.) as shown in Figure 3, however, aromatic proton H_j was firstly shifted to downfield from 7.82 ppm to 7.95 ppm upon to 2 equiv. of TBACl, then, to highfield from 7.95 ppm to 7.77 ppm from 3 to 26 equiv. of TBACl in $CDCl_3$ as indicated in Figure 4. H-bonding between N-H_n proton adjacent to SU and Cl^- anion affects

electron density of the aromatic ring directly attached to SU, and as a natural consequence of this interaction, slight differences in the chemical shifts of the H_i and H_j protons occur with the successive addition of TBACl in ^{1}H-NMR.

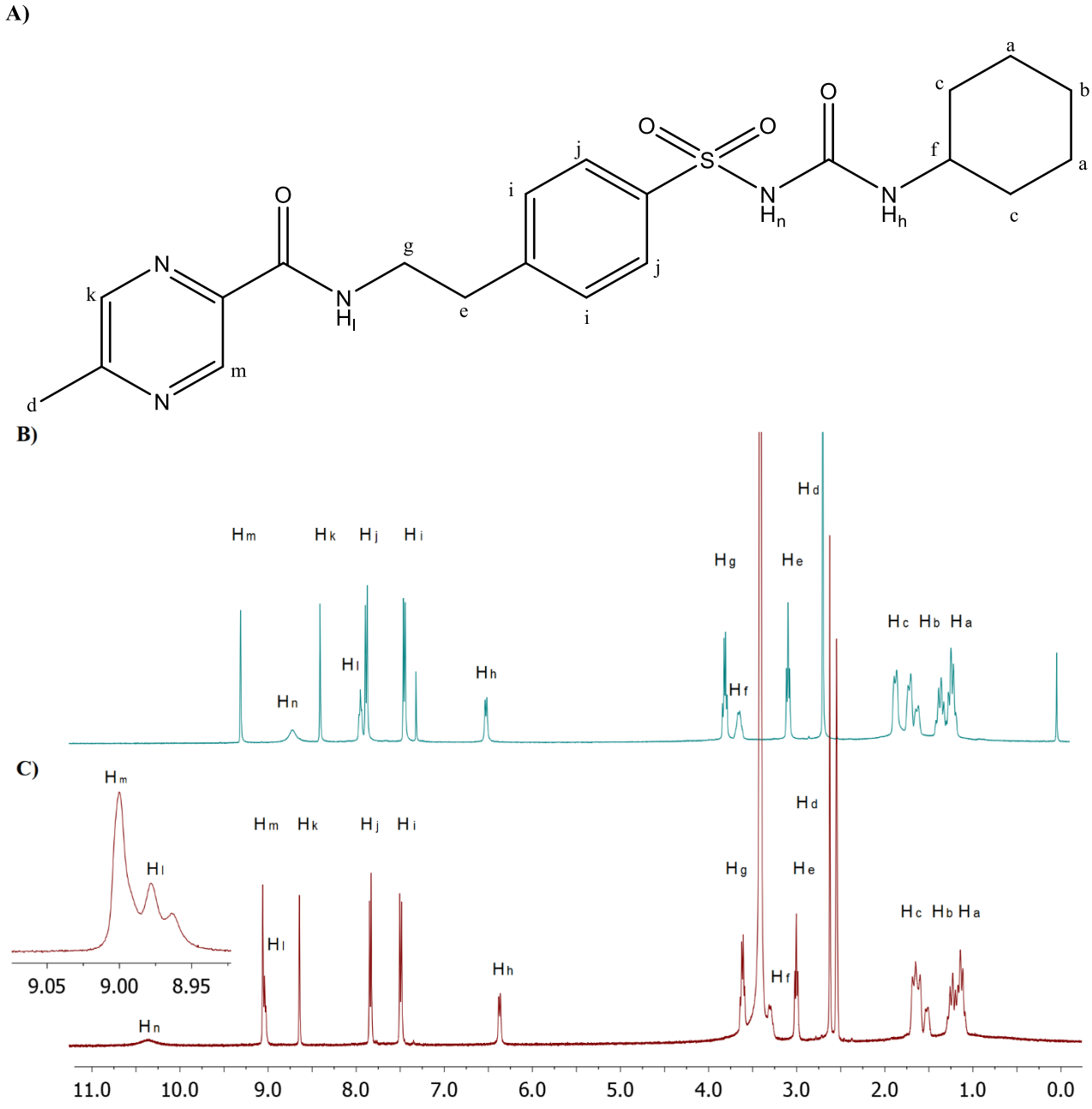

Figure 1. A) *Structure of Glipizide,* ***B)*** *^{1}H-NMR Spectra of Glipizide in $CDCl_3$ and* ***C)*** *in d_6-DMSO*

Table 1. *Selected signals of protons from ^{1}H-NMR to monitor chemical shift changes in GLP in $CDCl_3$ and d_6-DMSO*

Solvent	h	i	j	k	l	m	n
$CDCl_3$	6.47 ppm	7.39 ppm	7.82 ppm	8.35 ppm	7.89 ppm	9.25 ppm	8.66 ppm
d_6-DMSO	6.33 ppm	7.45 ppm	7.79 ppm	8.60 ppm	8.82 ppm	9.09 ppm	10.31 ppm

The slight unexpected highfield change in chemical shifts of amide proton H_l from 7.89 to 7.75 ppm with the addition of 26 equiv. of TBACl in $CDCl_3$ indicated no H-bonding interaction between amide proton and Cl^- as shown in Figure 4. In a similar situation, where the complex stability of a single amide is low, but with the addition of urea functional group in addition to the amide in a compound, the ^{1}H-NMR titration results emphasize that Cl^- anion makes the H-bond with urea instead of amide (Barišić et al., 2022), which is compatible with the preference of Cl^- for N-H of SU over amide in our work.

When the shifts of H_m proton in the pyrazine ring, close to the amide group, were examined, very weak highfield shifts for H_m from 9.25 ppm to 9.02 ppm were noticed with the successive addition of 26 equiv. of TBACl in $CDCl_3$ as shown in Figure 2. However, when the shift of H_k proton on pyrazine ring, which is farther from the amide proton, was examined, H_k was unaffected up to the addition of 1 equivalent of TBACl in $CDCl_3$. Further addition of TBACl (1.2 to 26 equiv.) resulted in slight highfield shifts of the H_k signal from 8.34 ppm to 8.21 ppm as shown in Figure 4. The weak interaction between H_l proton of amide and Cl^- anion slightly affects resonance structure of the pyrazine ring, and thus, e^- density of the ring, leading to a slight change in chemical shifts of H_k and H_m protons in the pyrazine ring in ^{1}H-NMR with successive addition of TBACl.

When the titration of GLP drug with Cl^- was examined in d_6-DMSO as indicated in Figure 5, a significant downfield shift of only H_h from 6.32 ppm to 7.21 ppm by adding TBACl up to 8 equiv., indicating the existence of a H-bond (Ramalingam et al., 2008; Amendola et al., 2010; 2011; Bao et al., 2018; Picci et al., 2020; Kumawat et al., 2021) between GLP and Cl^- anion, as expected. H_h proton signal at 7.21 ppm almost remained unchanged when the 8 to 14 equiv. of TBACl was added. Then, a very slight highfield shift from 7.18 to 7.04 ppm was observed in the signal of N-H_h proton when TBACl is added from 16 to 26 equiv. (Figure 5).

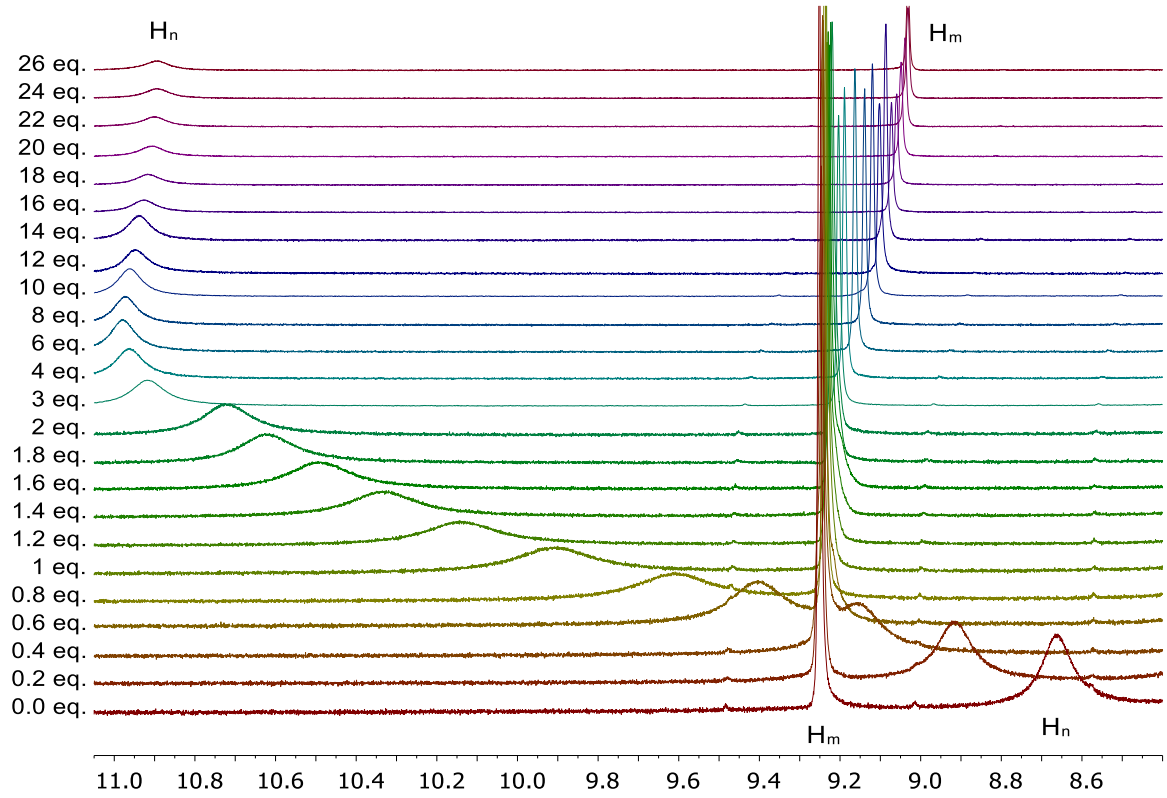

Figure 2. *Chemical shift of H_n, H_m in stack plots of ^{1}H-NMR spectra with the addition of TBACl (0–26 equiv) to GLP in $CDCl_3$ at 25°C*

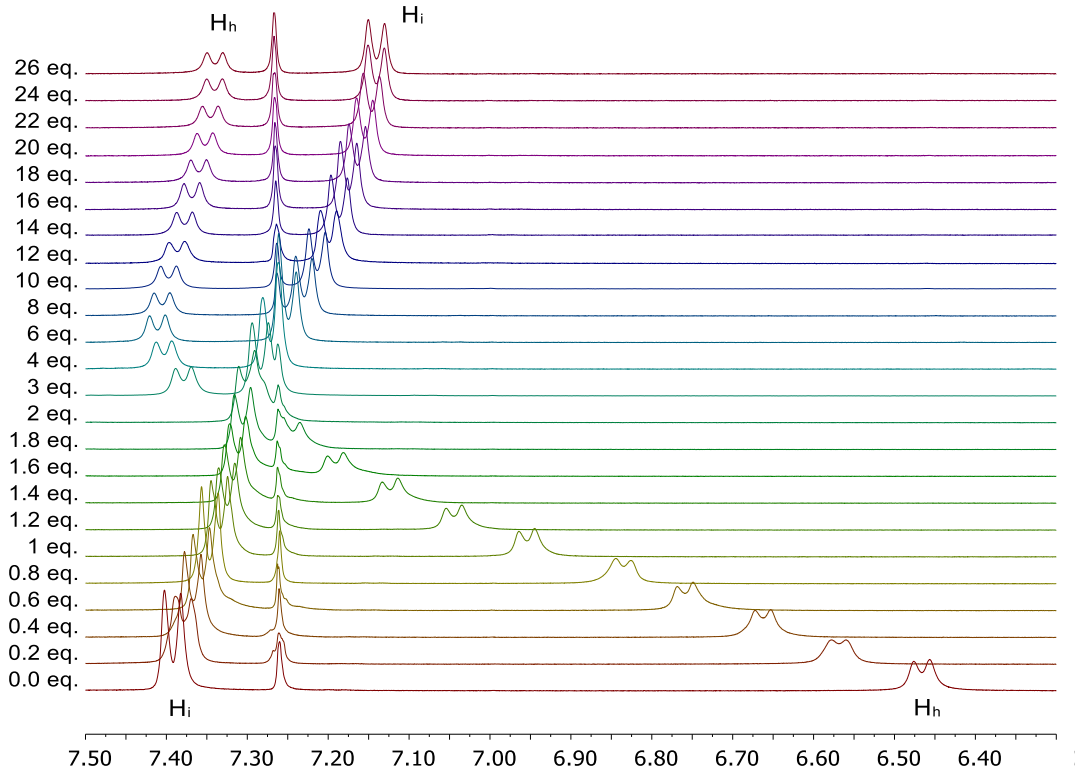

Figure 3. *Chemical shift of H_h, H_i in stack plots of ^{1}H-NMR spectra with the addition of TBACl (0–26 equiv) to GLP in $CDCl_3$ at 25°C*

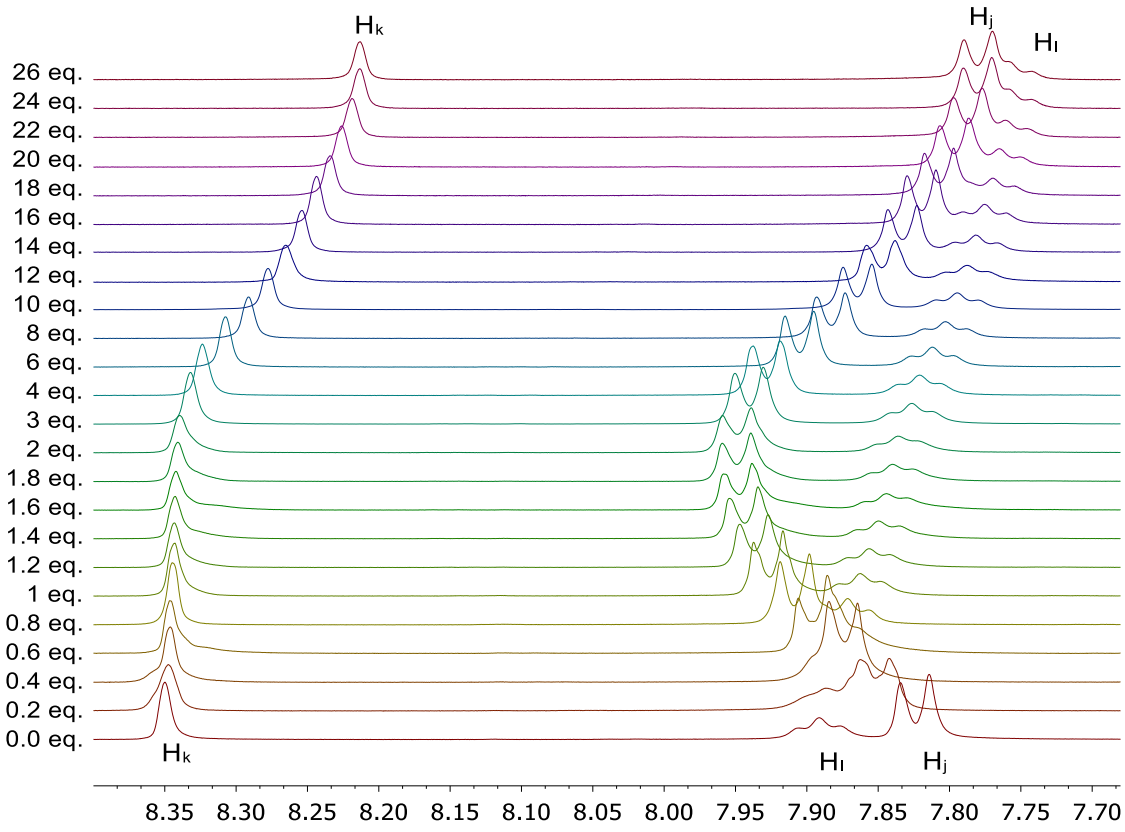

Figure 4. *Chemical shift of H_j, H_l, H_k in stack plots of ^{1}H-NMR spectra with the addition of TBACl (0–26 equiv) to GLP in $CDCl_3$ at 25°C*

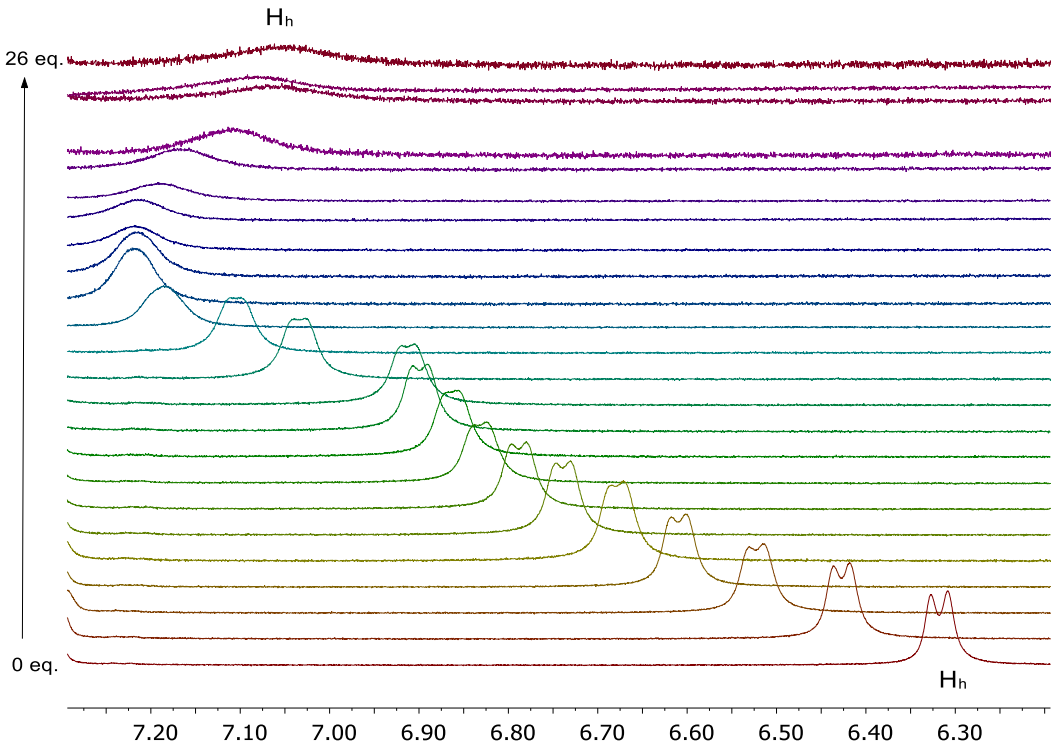

Figure 5. *Chemical shift of H_h in stack plots of ^{1}H-NMR spectra of GLP after the addition of TBACl (0 to 26 eqv.) in d_6-DMSO having <0.02% H_2O at 25°C*

The difference in H_n and H_h chemical shifts (Δδ) of GLP in response to increasing equiv. of Cl^- anions in $CDCl_3$ and d_6-DMSO in ^{1}H-NMR was shown in Figure 6. Significant downfield changes of H_n and H_h in $CDCl_3$ and H_h in d_6-DMSO in ^{1}H-NMR confirm the binding site via the formation of H-bond between GLP and Cl^-. When only 3 equiv. of TBACl anion were added to the GLP solution, a very large chemical shift difference of 2.23 ppm for H_n in $CDCl_3$ reflects very strong H-bonding between H_n and Cl^- anion (Figure 6). By adding 3 equiv. of TBACl to GLP solution, chemical shift differences of 0.91 ppm in $CDCl_3$ and of 0.71 ppm in d_6-DMSO were observed for H_h. No significant Δδ difference was observed for H_h when 8-18 equiv. of TBACl were added. The slightly different Δδ values at the initial stage of the titration (1-4 eq.) indicate the different strength of the H-bond interactions between GLP and Cl^- anion due to the nature of the solvent. The reason why Δδ in H_n is larger than in H_h in $CDCl_3$ may be due to the direct bonding of N-H_n proton to the sulfonyl group.

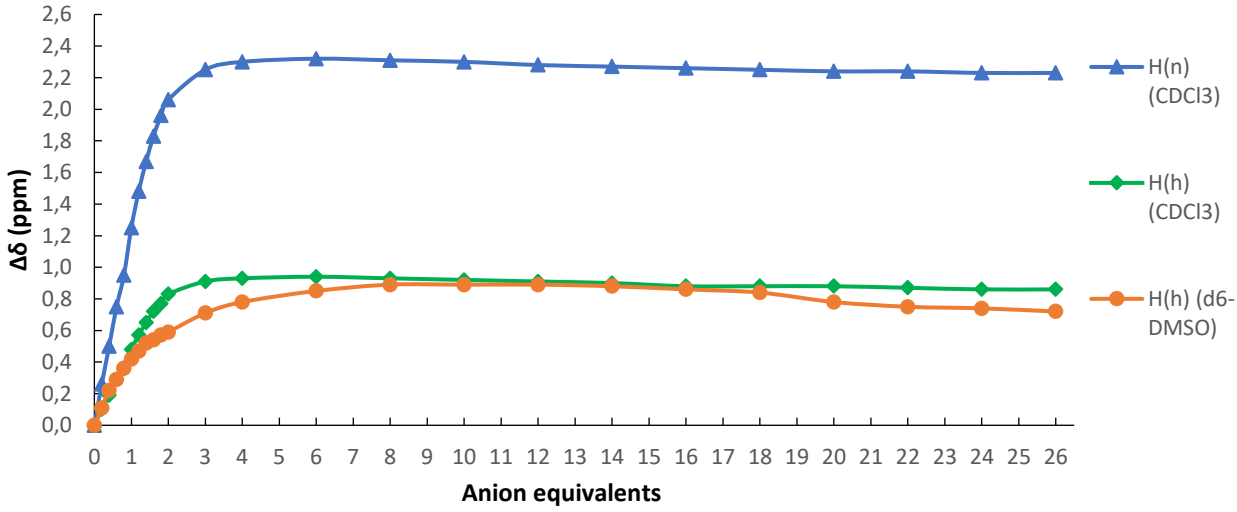

Figure 6. *Chemical shift differences of H_n and H_h of GLP with the successive addition of TBACl (0–26 equiv) in $CDCl_3$ and d_6-DMSO at 25°C*

Deprotonation of N-H of SU was not observed in our study while performing the titration experiments either in d_6-DMSO or in $CDCl_3$. When the titration experiment of GLIB, another derivative of GLP, with TBAM was carried out, the deprotonation of N-H bond in SU with the addition of TBAM was observed by disappearance of N-H peak in $CDCl_3$ or d_6-DMSO (Hasanah et al., 2015). The difference between deprotonation in the work of Hasanah et al. (2015) and anion binding in the current work is most probably related to the basicity of anions used during the titration because strong basic anions like acetate and dihydrogen phosphate could deprotonate N-H of SUs while weakly basic anions like NO_3^-, HSO_4^-, Cl^-, Br^-, I^- showed high binding ability with SUs (Barišić et al., 2021).

The stoichiometric K_a value of GLP and Cl^- anion according to the 1:1 model (Kuzmič, 2009; Thordarson, 2011; Ulatowski et al., 2016) were calculated as 77.59 M^{-1} for N-H_n proton at δ= 8.66 ppm in $CDCl_3$ (Table 2) by analyzing of the titration data with DynaFit program (Kuzmič, 2009; Mercurio et al., 2015; Zapata et al., 2017). For the calculation of K_a values, three variants namely concentrations of GLP and TBACl, and chemical shift values of N-H protons of SU as ppm for each addition of TBACl were all used in the DynaFit program. Therefore fitplots, residual and relative sum of squares (SSQ/SSQ min.) obtained from DynaFit software using these variants were shown in Figures 7 and 8.

Although SU protons were unexpectedly shifted downfield at different rates (Figure 6) when titrated with TBACl, the K_a values were found very close to each other (77.59 M^{-1} for H_n and 77.37 M^{-1} for H_h) in $CDCl_3$ by the DynaFit program. Confidence intervals for K_a values were 95% from DynaFit software. Moreover, ^{1}H-NMR spectroscopic titration showed that GLP can bind Cl^- anion in $CDCl_3$ with 77.37 M^{-1} higher K_a value (for N-H_h proton at δ = 6.47 ppm) than in d_6-DMSO, 38.53 M^{-1} (for N-H_h proton at δ = 6.32 ppm) for the same N-H_h proton. The reason why the K_a value calculated by the DynaFit program for H_h in d_6-DMSO was considerably lower than in $CDCl_3$ was due to the nature of d_6-DMSO, competing with Cl^- anion for H-bond with GLP receptor, and this situation partially inhibits the shifts of H_h in ^{1}H-NMR spectrum. Even more H_n proton of SU does not appear in ^{1}H-NMR spectrum before and during the titration, so K_a value of this proton could not be calculated in d_6-DMSO. The amide proton H_l of GLP at 7.89 ppm was not analyzed with DynaFit program because it was not suitable for DynaFit analysis due to its shift to the upfield with the addition of TBACl in $CDCl_3$.

Table 2. *K_a values in different deuterated solvents*

	$CDCl_3$	**Δδ[a]**	**K_a[a,c]**	**d_6-DMSO**	**Δδ[b]**	**K_a[b,c]**
H_h	6.47 ppm	0,86	77.37	6.32 ppm	0,72	38.53
H_n	8.66 ppm	2.23	77.59	-		-

[a:] in $CDCl_3$, [b:] d_6-DMSO, [c:] binding constant generated using DynaFit software.

Fitplots, residual, and relative sum of squares (SSQ/SSQ min.) were obtained from DynaFit software by using a 1:1 model for the binding of Cl^- with GLP in $CDCl_3$ as indicated in Figure 7. The randomness of the residual plot obtained from DynaFit program demonstrated the compatibility of the data with the program (Ulatowski et al., 2016). The program determines the corresponding number of independent least squares for all possible combinations (Kuzmič, 2009). The results were sorted by residuals (Figure 7B, 7E) and sum of squares (Figure 7C, 7F) to obtain a high confidence interval for the calculated K_a value. Confidence intervals for K_a values were given as 95% from DynaFit software. Furthermore, Cl^- binding by GLP in d_6-DMSO was also studied using DynaFit program in a similar manner to that in $CDCl_3$ mentioned above (Figure 8). DynaFit scripts for each analysis were given in APPENDIX.

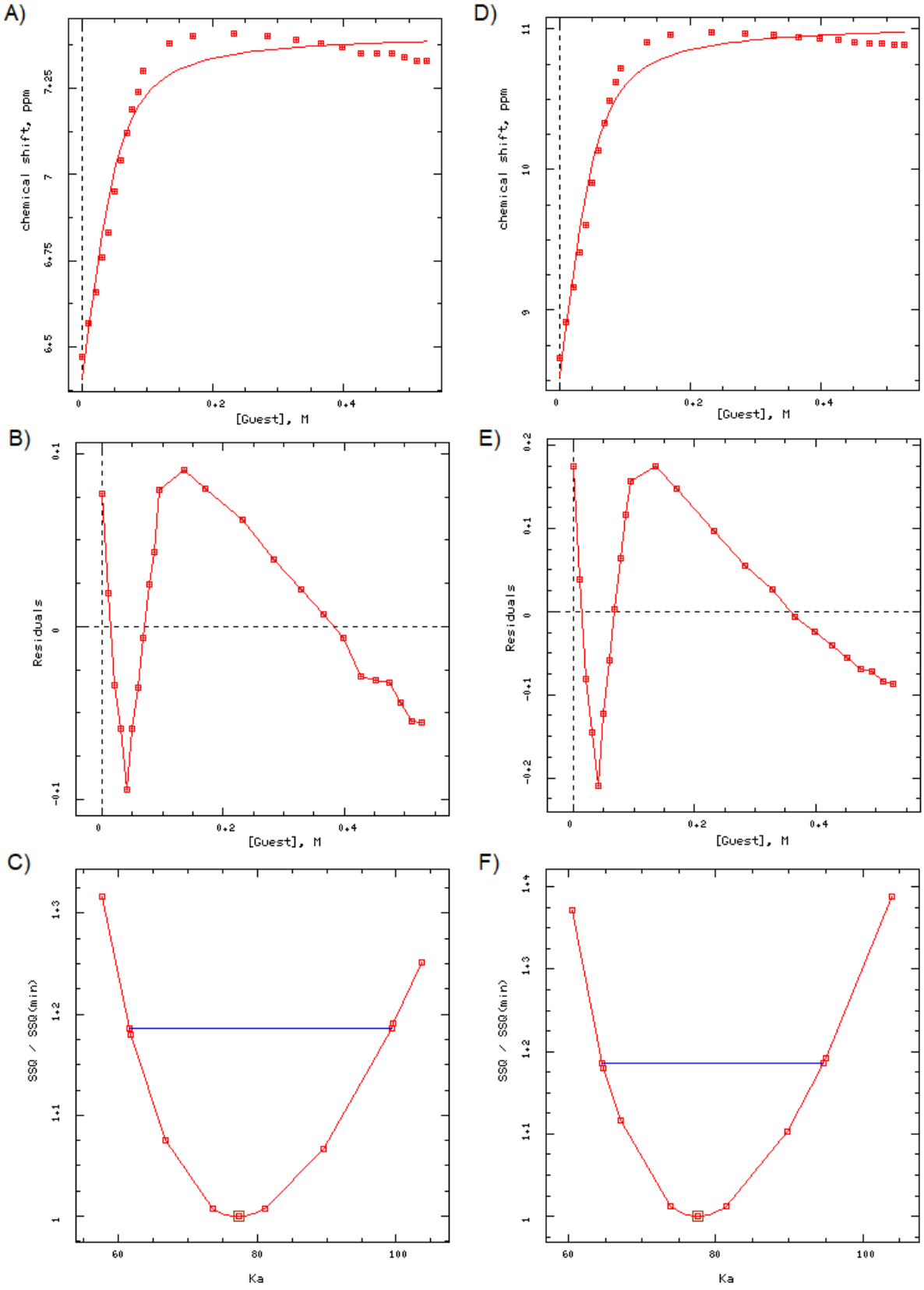

Figure 7. *^{1}H-NMR spectroscopic titration in $CDCl_3$* ***A)*** *Plot of downfield shifts of N-H_h proton at δ = 6.47 ppm in GLP versus TBACl concentration* ***B)*** *Residual* ***C)*** *Relative sum of squares (SSQ/SSQ min.) K_a = 77.37 M^{-1} (Fitplot for N-H_h proton at δ = 6.47 ppm)* ***D)*** *Plot of downfield shifts of N-H_n proton at δ = 8.66 ppm in GLP versus TBACl concentration* ***E)*** *Residual* ***F)*** *Relative sum of squares (SSQ/SSQ min.) K_a = 77.59 M^{-1} (Fitplot for N-H_n proton at δ = 8.66 ppm).*

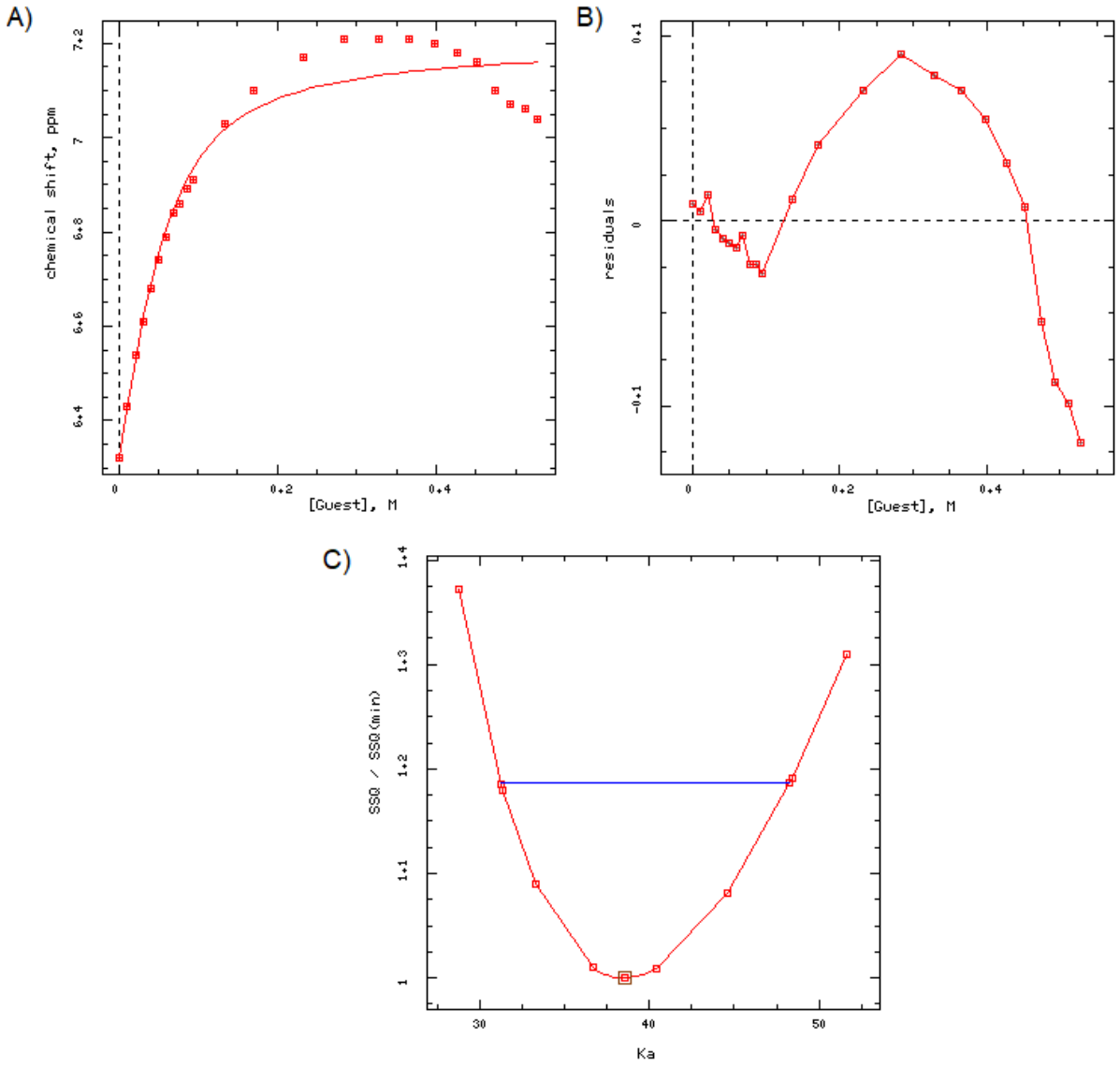

Figure 8. *^{1}H-NMR spectroscopic titration in d_6-DMSO* ***A)*** *Plot of downfield shifts of N-H_h proton at δ = 6.32 ppm in GLP versus TBACl concentration* ***B)*** *Residual* ***C)*** *Relative sum of squares (SSQ/SSQ min.) K_a = 38.53 M^{-1} (Fitplot for N-H_h proton at δ = 6.32 ppm).*

4. CONCLUSION

In this study, the anion binding property of GLP was studied by ^{1}H-NMR spectroscopic titration with TBACl salt in $CDCl_3$ and d_6-DMSO. It was found that GLP could bind to Cl^- anion in both deuterated solvents. Namely, the binding site via formation of H-bond between GLP and Cl^- anion was confirmed by significant downfield changes of H_n and H_h in $CDCl_3$ and H_h in d_6-DMSO in ^{1}H-NMR when N-H protons of SU (H_h and H_n), amide N-H proton (H_l), aromatic protons (H_i and H_j) of the sulfonyl attached *para*-substituted ring, and the protons (H_k and H_m) in the pyrazine ring were examined with successive TBACl addition from 0.2 to 26 equiv. to GLP drug. An incredibly significant Δδ value of 2.25 ppm for H_n in $CDCl_3$ was observed even only 3 equiv. of TBACl were added. On the other hand, a smaller Δδ values of 0.91 ppm in $CDCl_3$ and 0.71 ppm in d_6-DMSO were observed for H_h when 3 equiv. of TBACl was added to related GLP solution. According to binding constant (K_a) value obtained by using DynaFit program for GLP and Cl^- anion, it can be concluded that GLP can bind Cl^- anion in $CDCl_3$ with higher affinity (K_a=77.37 M^{-1}, Fitplot for N-H_h proton at δ = 6.47 ppm) than in d_6-DMSO (K_a=38.53 M^{-1}, Fitplot for N-H_h proton at δ = 6.32 ppm) for N-H_h proton. The difference between the binding constants between GLP and Cl^- anion in $CDCl_3$ and d_6-DMSO might be associated with the nature of solvent.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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APPENDIX

DynaFit Script (Kuzmič, 2009)

1. GLP in $CDCl_3$, H_h at δ = 6.47 ppm

```
[task]
  data = equilibria
  task = fit
[mechanism]

  H + G    <= =>    H.G    :    Ka   assoc
 [constants]
  Ka = 1 ??
[responses]
  intensive ; ... NMR chemical shifts
[data]
  variable G, H
  plot titration ; ... [H] and [G] are varied simultaneously

  graph 1H ; proton chemical shifts
  set aH | response H = 6.47 ?, H.G = 7.33 ?

[output]
  directory ./examples/pt03/NMR/output/aa
[settings]
{Output}
  XAxisLabel = [Guest], M
  YAxisLabel = chemical shift, ppm

[end]
```

2. GLP in $CDCl_3$, H_n at δ = 8.66 ppm

```
[task]
  data = equilibria
  task = fit
[mechanism]

    H + G    <= =>    H.G    :    Ka   assoc

[constants]
  Ka = 1 ??
[responses]
  intensive ; ... NMR chemical shifts
[data]
  variable G, H
  plot titration ; ... [H] and [G] are varied simultaneously

  graph 1H ; proton chemical shifts
  set aH | response H = 8.66 ?, H.G = 10.89 ?

[output]
  directory ./examples/pt03/NMR/output/aa
[settings]
{Output}
  XAxisLabel = [Guest], M
  YAxisLabel = Chemical shifts, ppm

[end]
```

3. GLP in d_6-DMSO H_h at δ = 6.32 ppm

```
[task]
  data = equilibria
  task = fit
[mechanism]

 H + G    <= =>    H.G    :     Ka   assoc

[constants]
  Ka = 1 ??
[responses]
  intensive ; ... NMR chemical shifts
[data]
  variable G, H
  plot titration ; ... [H] and [G] are varied simultaneously

  graph 1H ; proton chemical shifts
  set aH | response H = 6.32 ?, H.G = 7.04 ?

[output]
  directory ./examples/pt03/NMR/output/aa
[settings]
{Output}
  XAxisLabel = [Guest], M
  YAxisLabel = chemical shift, ppm

[end]
```