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DETERMINATION OF THE BINDING CHARACTERISTICS OF DICOUMAROL TO BOVINE SERUM ALBUMIN BY UV /VISIBLE SPECTROSCOPIC METHOD

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ABSTRACT

8-Anilinonaphthalene-1-Sulfonic acid (ANS) was used as a probe to study the binding characteristics of Dicoumarol to Bovine Serum Albumin (BSA). The absorbance of ANS-BSA complex was decreased by the binding of Dicoumarol. This suggests that there is competition between Dicoumarol and ANS for the binding sites in the BSA. It means that ANS and Dicoumarol shared some of the binding sites on BSA. From the scatchard plot for the binding of Dicoumarol to BSA it is seen that Dicoumarol has three high affinity binding sites with association constants K_l=14.5245 x 10^6, 10.6281x10^6,11.6811x10^6 respectively, and three secondary binding sites with association constants K_l =14.0084 x 10^6 15.8816 x 10^6 and 19.2058x10^6 respectively and numerous low affinity binding sites.

KEYWORDS: Binding characteristics, 8-Anilinonaphthalene-1-Sulfonic acid, Dicoumarol, Bovine Serum Albumin, Scatchard plots.

INTRODUCTION

Serum is plasma from which the clothing proteins have been removed. Antiserum is blood serum with specific antibodies for passive immunity. Serum albumin, is a globular protein, produced by the liver, and found dissolved in blood plasma. It is the most abundant blood

protein in mammals. About half of the serum protein is albumin (Wikipedia,2015). It is regarded as the main drug binding protein in serum hence the binding of drugs to pure albumin and whole serum or plasma are often regarded as synonymous (Ejele, 2004). Albumin has a low pH of 4.7. It buffers the pH of the blood. Albumin, when ionized in water at pH 7.4 (as found in the body) is negatively charged (Wikipedia, 2015).It can exist in two main forms within the neutral and slightly alkaline region. The neutral form occurs almost exclusively near pH 6 while the basic form exists predominantly at pH 9 (Ejele & Anusiem, 2001).

Serum albumin contains eleven boarding domains for hydrophobic compounds. One hemin and six long chain fatty acids can bind to serum albumin at the same time (Wikipedia, 2015).

TYPES OF ALBUMIN

Some common types of albumin are:

- Human serum Albumin (HSA) found in human blood
- Bovine serum Albumin (BSA) found in Cow's blood
- Chicken Serum Albumin (CSA) found in chicken's blood.

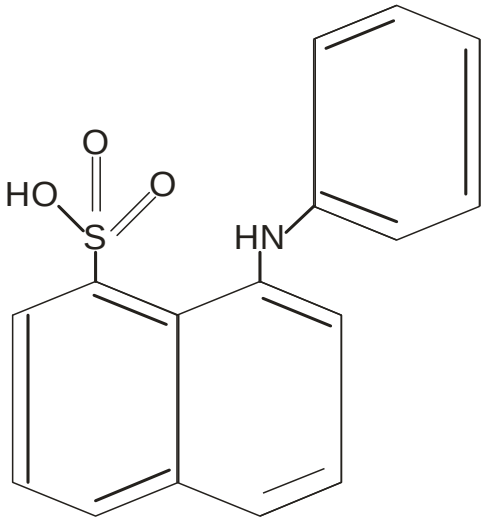
Other animals such as goats, sheep, rat, rabbit, reptiles (e.g. lizard) and amphibians (e.g. frog and toad) have serum albumins.

Albumin is soluble and monomeric. The reference range for albumin concentrations in serum is approximately 35-50g/l. It has a serum half-life of 20days (Wikipedia, 2015).

Bovine Serum Albumin comprises three homologous domains. Based on probe displacement method, there are, at least, three relatively high specific binding sites on the BSA molecule. These sites are generally called the warfarin-binding site, the benzodiazepine-binding site and digitoxin-binding site – denoted as site- I, II and III respectively (Uddin *et al.,2004).*

BSA has several biochemical applications which include its usage as a nutrient in cell and microbial culture. It is commonly used to determine the quantity of other proteins by comparing an unknown quantity of protein to known amounts of BSA (Wikipedia, 2015).

8 - Anilinonaphthalene - 1 -Sulfonic Acid (ANS)



8 - Anilinonaphthalene -1-sulfonic acid (or 1-Anilino-8-naphthalenesulfonate) IUPAC name:

8 - (Phenylamino) -1 -naphthalenesulfonic acid

This is an organic compound containing both a sulfonic acid and an amine group. It has the chemical formula ($C_{16}H_{13}N0_3S$ and mass of 299.34gmol^{-1}. It is a fluorescent dye that binds with high affinity to hydrophobic surfaces of proteins. This compound is used as a fluorescent molecular probe to study conformational changes induced by ligand binding in proteins (Mainasi-Csizmadia, Hegyi, Tolgyesi, Szent-Gyorgyi, & Nyitray, 1999).

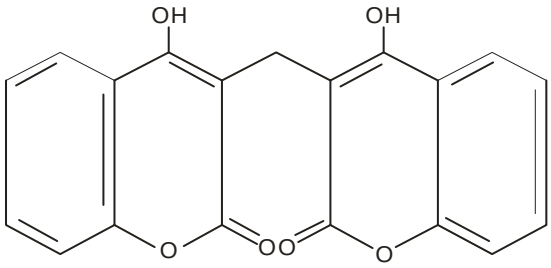
Also using ANS as a model ligand, the micro environment of the binding sites can also be ascertained because the binding of ANS is considered a suitable probe of protein surface hydrophobicity.

Uses of 8 – Anilinonaphthalene – 1 – Sulfonic Acid

The most common uses of ANS (and bis ANS) are:

- To monitor conformational changes in proteins
- To follow the kinetics of protein folding and unfolding
- To detect and characterize partially folded intermediates of proteins (both transcient and equilibrium).
- To measure changes in membrane properties
- To detect the presence of exposed hydrophobic accessible surfaces and,
- To study ligand binding through displacement assays.

Dicoumarol



IUPAC (Systematic) Name: 3,3 – Methylenebis (4 – hydroxy – 2H – Chromen – 2 – one)

Dicoumarol, according to Wikipedia, is a naturally occurring anti – coagulant that functions as a functional Vitamin K depleter (similar to warfarin). It is a natural substance of combined plant and fungal origin, a derivative of coumarin. It is a bitter tasting but sweet smelling substance made by plants that do not affect coagulation but are transformed in mouldly feeds or silages by many species of fungi into dicoumarol (Wikipedia, 2015). Its chemical formula is $C_{19}H_{12}O_6$ and its molecular mass is 336.295gmol$^{-1.}$

Dicoumarol is soluble in aqueous alkaline solutions, organic bases, pyridine (50mg/ml), 0.1N Sodium hydroxide (15mg/ml), very slightly soluble in acetone, ethanol and di-ethyl ether, and slightly soluble in benzene and chloroform. Its solubility in water at 25^{o}C is 0.0018/100g of water (Chemister.ru/Database/Properties-en).

Dicoumarol is used therapeutically as an anti – coagulant as well as, a rodenticide. It is also used to prevent blood clots in the brain or from going to the brain etc. Dicoumarol is prescribed for people with artificial heart valves and people suffering from heart attack.

MATERIALS AND METHODS

Materials

BOVINE Serum Albumin (BSA, fraction V) Lot No. 05813, CAS No (9048 – 48 – 8) was obtained from Kem Light Laboratories PVT. LTD., India while 8-Anilino-1-naphathalenesulfonic acid (ANS), Lot#SLBGO546V, CAS:82-76-8 and Dicoumarol No. D2628, Lot: 76c-0184 were obtained from sigma chemical company, St. Louis, USA. Sodium di-hydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate used were obtained from Mallinckrodt Inc. Paris. Distilled water was used in the preparation of the 0.05 M phosphate buffer of pH 7.4.

METHODS

Two methods were employed for this work as reported by Odoemelam, Anununso & Ejike (2004) and Togashi & Ryder (2008) as amended and described below: Bovine Serum Albumin (BSA), 8-Anilinonaphthalene-1-sulfonic acid (ANS) and dicoumarol's concentrations for stock solutions were calculated using their molecular mass of 66000 for BSA and 289.34 and 336.3 for ANS and dicoumarol respectively.

Absorbance measurements were performed on Spectronic 20D spectrophotometer, M-Tech instrument, USA, using 4 cm^3 quartz cuvet. The buffer solution's pH was checked with chem. Cadet JrColeparmer pH meter. Titrations were done in 4cm^3 quartz cuvet using 1ml pipette. The absorbance measurements were made at the wavelengths of 275 nm and 280 nm for ANS and dicoumarol respectively. All solutions (except that of dicoumarol) were made up in 0.05 M phosphate buffer at pH 7.4. Dicoumarol could not dissolve in the phosphate buffer, and as such, it was dissolved in 0.1N sodium hydroxide and made up to mark with the 0.1N sodium hydroxide.

TITRATION OF DICOUMAROL WITH BSA

To determine when all the dicoumarol was bound to BSA dicoumarol was titrated with BSA.A 2.0 cm^3 solution of 1.43 x 10^{-4} M BSA was successively titrated with aliquots (0.002-0.040 cm^3) of dicoumarol solution ($1x10^{-8}$ M).Like in titration of ANS with BSA the concentration of BSA was made constant while that of dicoumarol was increased. After each addition the dicoumarol absorbance was measured at 280nm. A blank titration of 0.1 M sodium hydroxide was run to correct the values of dicoumarol titrated against BSA since it was dissolved and made up with 0.1M sodium hydroxide.

BINDING OF DICOUMAROL TO BSA

The absorbance of dicoumarol in presence of 2 cm^3 BSA and 1 cm^3 ANS was used to investigate dicoumarol's binding to BSA. Initially cuvet cell contained 1cm^3 ANS ($1x10^{-4}$ M) together with 2.0 cm^3 of $1.43x10^{-6}$ M BSA solution. Absorbance was taken with successive additions of 2 μl of $1x10^{-2}$ M solution of Dicoumarol. After each addition the absorbance was measured at 280 nm.

RESULTS AND DISCUSSION

Absorption spectrum of Dicoumarol (3,3-Methylenebis (4-hydroxy-2H-Chromen-2-one) for its wavelength of maximum absorption determination is shown in figure 1 below.

Table 1: Determination of the wavelength of maximum absorption (λmax) of Dicoumarol (at 2 x 10^{-8} M) concentration.

Wavelength (nm)	Absorbance (A)
225	0.053
230	0.056
235	0.057
240	0.054
245	0.050
250	0.054
255	0.056
260	0.056
265	0.057
270	0.057
275	0.0585
280	0.059
285	0.0575
290	0.053
295	0.053
300	0.053
305	0.052

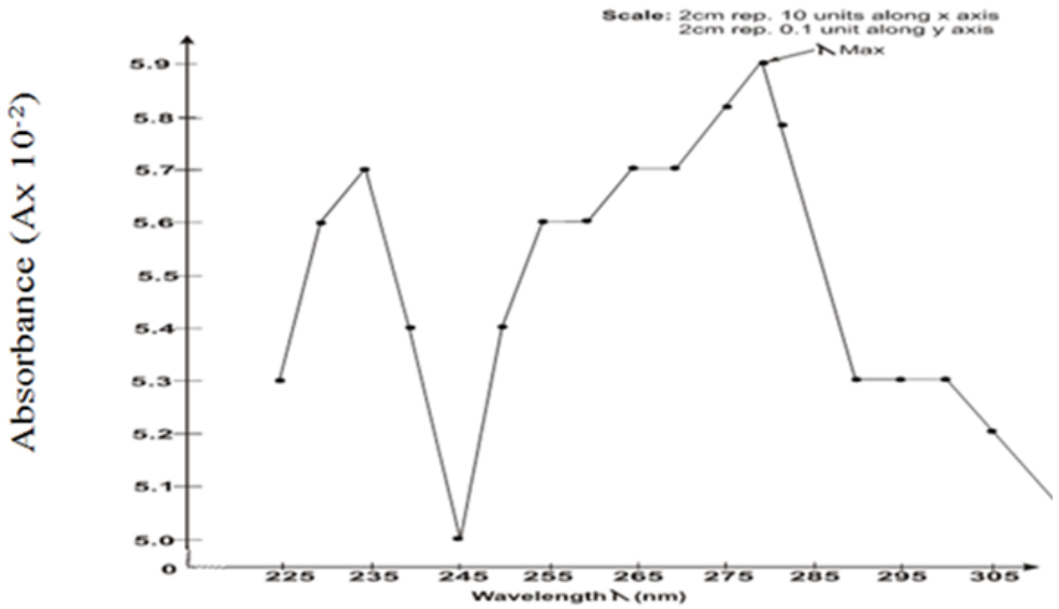


Figure 1: A graph for the determination of wavelenghth of maximum absorption of Dicoumarol.

Concentration of Dicoumarol used = 2x10^{-8} M

From the graph it was seen that the wavelength of maximum absorption (λmax) of Dicoumarol is at 280 nm (scanned from 225 – 300 nm). It was this wavelength that was used

in determining its binding characteristics to Bovine Serum Albumin through ultra-violet visible spectroscopy.

Table 2: Table of value for the graph of titration of BSA with dicoumarol

Molar concentration of BSA used: 1.43 x 10^{-4} M

Molar concentration of Dicoumarol used: 1 x 10^{-8} M.

Volume of BSA in the cuvet: 2 cm^3

Millilitres of Dicoumarol titrated at 280 nm: 0.01 cm^3

MOLAR CONCENTRATION (M X 10^{-7} M)	ABSORBANCE (A)
0	0.01
0.2	0.044
0.4	0.078
0.6	0.114
0.8	0.121
1.0	0.126
1.2	0.129
1.4	0.134
1.6	0.138
1.8	0.147
2.0	0.149
2.2	0.149
2.4	0.152
2.6	0.156
2.8	0.158
3.0	0.159
3.2	0.160
3.4	0.162
3.6	0.162
3.8	0.162
4.0	0.162

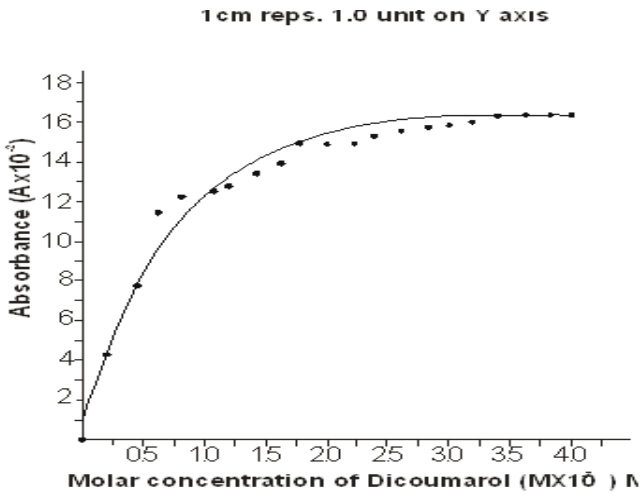


Figure 2: Titration of BSA against Dicoumarol.

It can be seen from table 2 and Figure 2 that as concentration of dicoumarol increased its absorbance increased progressively until it reached the absorbance of 0.162 at the concentration of 3.4 $x10^{-7}$ M. At this, further increment of the concentration of dicoumarol did not lead to any increment in the absorbance. It can be said that all the binding sites of dicoumarol on the BSA must have been occupied at this concentration hence the constancy of the absorbance from the concentration of 3.4 $x10^{-7}$ M to $4.0x10^{-7}$ M.

Table 3: Table Of Value For The Binding Of Dicoumarol Bsa

Volumes of BSA and ANS in the cuvet = 2 cm^3 and 1 cm^3 respectively. Molar concentration of stock ANS =1×10^{-4} M; Molar concentration of stock BSA=1.43×10^{-6} M; Molar concentration of dicoumarol = 2 x10-^{8} M; Volumes of dicoumarol successively titrated = 2 µl.

Molar concentration ($M\times10^{-7}$) M	Absorbance (A)
0	0.018
0.2	0.024
0.4	0.031
0.6	0.037
0.8	0.057
1.0	0.066
1.2	0.074
1.4	0.082
1.6	0.090
1.8	0.099
2.0	0.105
2.2	0.113
2.4	0.119
2.6	0.127
2.8	0.137
3.0	0.142
3.2	0.142
3.4	0.142

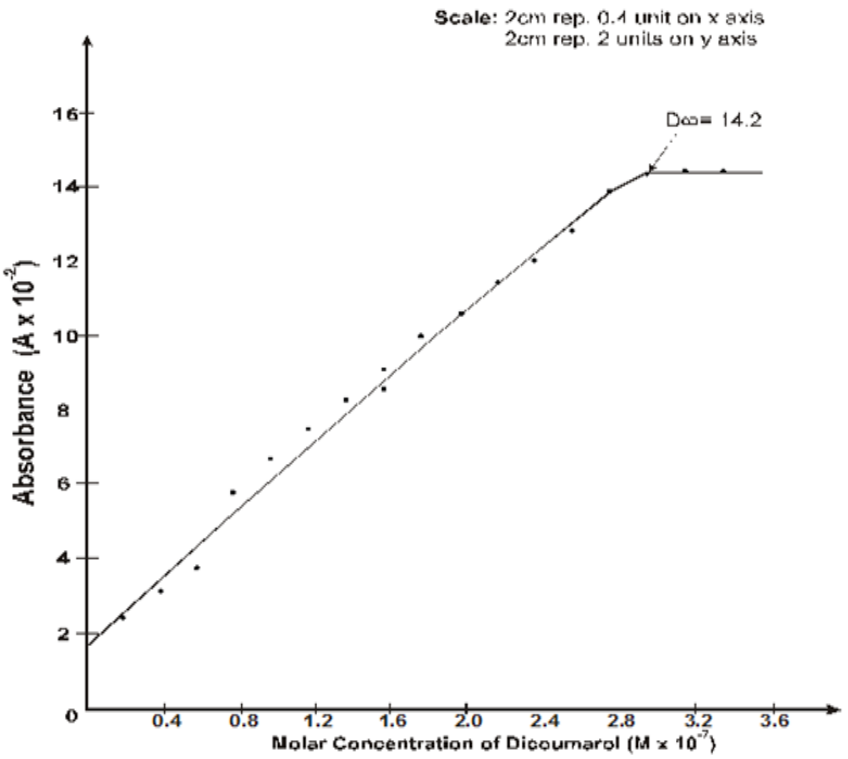


Figure 3: Binding of Dicoumarol to Bovine Serum Albumin
(Titration of ANS and BSA against Dicoumarol)

It can be seen that as the concentration of Dicoumarol increased in the presence of ANS and BSA the absorbance increased. This also implies exposure of more binding sites on BSA until the curve reached a plateau at the absorbance of 0.142 and concentrations of 3.0-3.4x10^{-7} M which means that all the binding sites on BSA may have been occupied.

The shapes are in agreement with the shapes of graphs drawn in research work done by fluorescence technique (Chignell, 1970; Odoemelam*et al.*, 2004).

Since fluorescence intensity is proportional to the part of the light absorbed by the solution (Kuznetsova, Sulatskaya, Pavaro &Turoverov, 2012), one can say that the titration curves are comparable to works done on drugs binding to serum albumins using florescence techniques. However, from the concentration and absorbance all the binding sites of dicoumarol seemed to have been occupied in table 2 figure 2 ,it appears there is competitition for the same binding site between ANS and Dicoumarol in table 3, figure 3.This is because absorbance became constant at lesser concentration (3.0 – 3.4 x 10^{-7} M).This implies that ANS and dicoumarol have some common binding sites on the BSA which got occupied at lower

absorbance than when Dicoumarol was titrated with BSA without the presence of ANS (0.142 and 0.162).

Figure 4 and Table 4 showed the Scatchard plot for the binding of dicoumarol to Bovine Serum Albumin (BSA).

Table 4: Table For The Scatchard Plot For The Bidning Of Dicoumarol To Bsa.

$Lb/Lf \times 10^{-2}$	$Lb \times 10^{-8}$ (bound Dicoumarol)
12.7	0.9
12.5	1.1
12.1	1.4
11.8	1.5
11.6	1.7
11.6	1.9
11.2	2.0
11.1	2.2
10.7	2.3
10.7	2.5
10.9	2.7
10.5	2.9

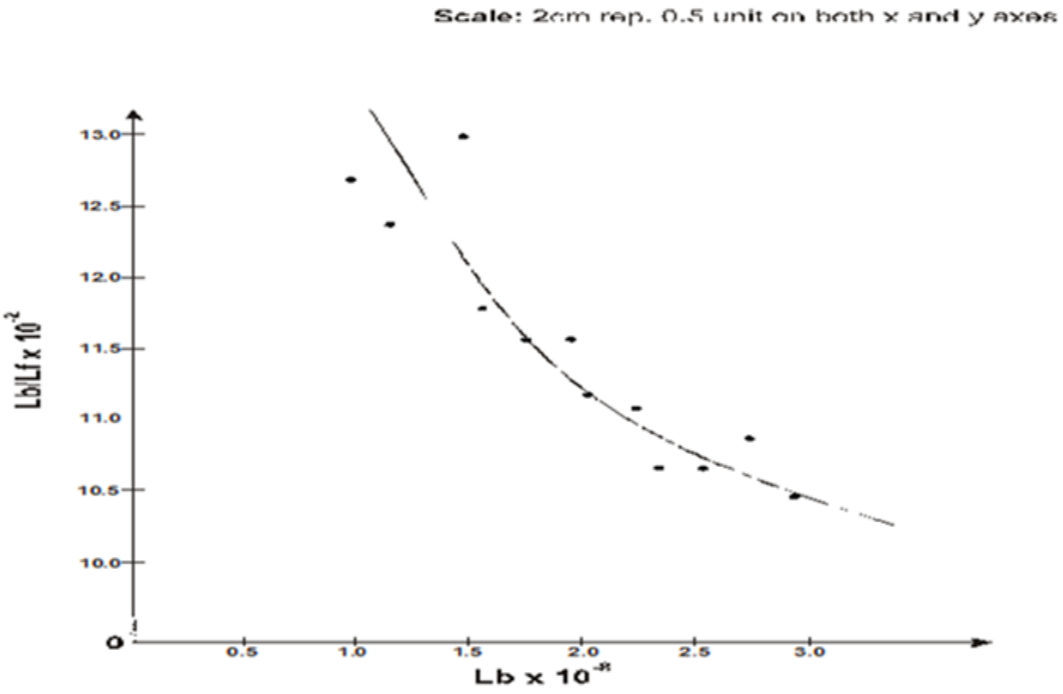


Figure 4: A scatchard plot for the binding of Dicoumarol to Bovine Serum Albumin.

Efficacy, as well as toxic profile of a drug depends largely on its ability to bind with serum protein (Alam *et al.*, 2011). From this plot it is seen that dicoumarol has three high affinity binding sites with association constants $K=14.5245 \times 10^6$, 10.6281×10^6 and 11.6811×10^6

respectively and three intermediate (secondary) binding sites with association constants $K=14.0084x10^6$, $15.8816x10^6$ and $19.2058x10^6$ respectively and six low affinity binding sites. Though no published work was found using 8-Anilinonaphalene-1-Suphonic acid as a probe to study the binding of dicoumarol to Bovine Serum Albumin by Ultra violet spectroscopy, the scatchard plot of the binding of dicoumarol to HSA in the work done by Chignell (1970) titled "The Interaction of Warfarin and Dicoumarol with Human Serum Albumin," by circular dichroism measurements showed that dicoumarol was bound to three binding sites on HSA. Since from literature Bovine Serum Albumin is found to be structurally homologous with human serum albumin (Agudelo, *et al.*, 2012); Rahman & Sharker, 2009; Uddin, *et al.*, 2004). I can say that this result is in agreement with a published work (Chignell, 1970). The low affinity sites could not be saturated by the addition of a large excess of dicomarol.

As discussed earlier in this work for ANS the non-linearity of the scatchard plot showed that BSA has many binding sites for dicoumarol. The non-linear shape of the scacthard plot describes both high and low affinity binding sites of the drug on the BSA molecule. This is in agreement with other works which reported that there are two main types of protein binding: strong affinity binding to a small number of sites and weak affinity binding to a large number of sites (Uddin *et al.*, 2004; Alam *et al.*, 2011). It suggests that the binding of dicoumarol to BSA caused conformational changes in BSA thereby exposing its secondary binding sites.

Also, from the values of the binding constants of dicoumarol when titrated with BSA in the absence of ANS and when titrated in the presence of ANS it can be said that there is a competition existing between dicoumarol and ANS in the binding site on the BSA which reduced the value of the binding constants of dicoumarol.

When dicoumarol was titrated in the absence ANS it has much higher values of associated constants than when done in presence of ANS.

Table 5: Table Of Value For The Scatchard Plot Of The Ratio Of The Bound Dicoumarol To That Of The Free Dicoumarol In The Absence Of Ans.

$Lb/Lf \times 10^{-1}$	$Lb \times 10^{-8}$
4.7	0.6
4.7	1.3
4.8	2.0
3.5	2.1

2.8	**2.2**
2.3	**2.2**
2.0	**2.2**
1.8	**2.4**
1.7	**2.6**
1.5	**2.6**
1.4	**2.6**
1.3	**2.7**
1.2	**2.8**
1.1	**1.8**
1.0	**2.8**
1.0	**2.8**
0.9	**2.9**
0.9	**2.9**
0.8	**2.9**
0.8	**2.9**

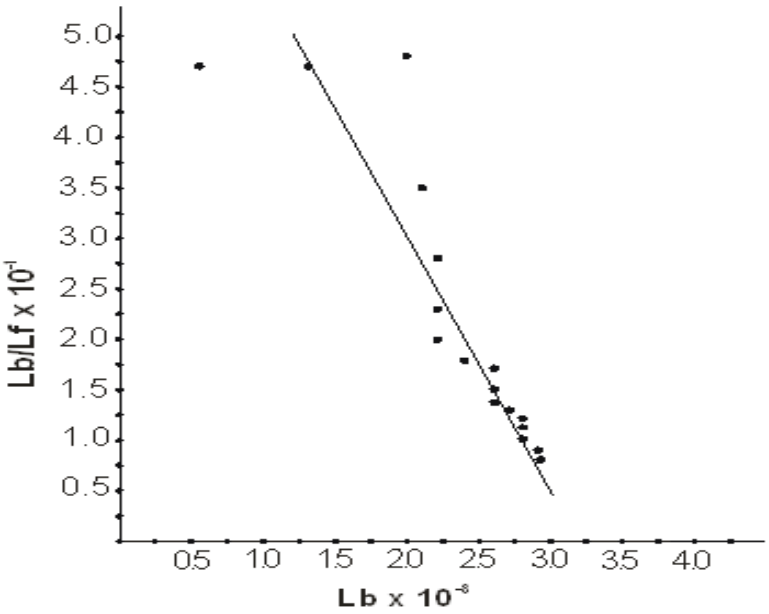


Figure 5: A scatchard plot for the binding of Dicoumarol to BSA in the absence of ANS.

CONCLUSION AND RECOMMENDATION

In conclusion, the binding characteristics of Dicoumarol to bovine serum albumin was successfully determined by ultraviolet-visible spectroscopy. It was found that Dicoumarol has three (3) high affinity binding sites with association constant $k=14.5245x10^6$. $10.6281x10^6$ and $11.6811x10^6$ respectively and three secondary binding sites with association Constants $K=14.0084x10^{6,}$ $15.8816x10^6$ and $19.2058x10^6$ respectively. It was also found that Dicoumarol and ANS competitively bind to some binding sites on the Bovine Serum Albumin.

AN UNDERTAKING

I affirm that this manuscript has not been published elsewhere except in the form of an abstact.

REFERENCES

1. Agudelo, D., Bourassa, P., Bruneau, J., Berube, G., Asselin, E. & Tajmir-Riahi, H.A. Probing the Binding sites of Antibiotic Drugs: Doxorubicin and N-(trifluoroacetyl) Doxorubicin with Human and Bovine Serum Albumins Plos ONE, 2012; 7(8): e 43814.doi:10, 1371/*Journal*. Pone. 0043814.
2. Alam,K.D.,Hossain,M.K.,Kabir,S.,Chowdhury,R.M.A.A.,Mahjabeen,S.,Mondal,M.S.,Ab uzar,S.M & Rahman,M.F. (2011).In vitro Binding Chemistry of Amlodipine Besylate (Calcium Channel Blocker) and Atorvastatin Calcium (HMG – CoA Reductase Inhibitor) to Serum Albumin and their Mutual Effect to Displace Each Other from the binding site. *American Journal of Drug Recovery and Development*, 2011; 1(4): 220-230.
3. Chemister.ru/Database/Properties-en…
4. Chignell, C. F. (1970). Fluorescence Studies of drug interactions with Biological systems - A review. Fluorescence News 5(2): 1.
5. Ejele, A.E. &Anusiem, A.C.I. Thermodynamics of the Neutral-to-base transition in Human Serum Albumin *Alvana Journal of Science*, 2001; 1(4): 43 – 48.
6. Ejele,A.E. (2004).Effect of pH on the binding characteristics of drugs to human serum albumin. *J. Chem .Soc. Nigeria*, 2004; 29: 2.
7. Ejele, A.E. Drug – Protein Binding in Human Serum: The Effect of pH.*Journal of Chemical Society of Nigeria*, 2004; 29: 1.
8. Hossain, Md. K., Khatun, A., Rahman, M., Akter, Md.N.,Chowdhury, S.A. &Alam, SM.M (2016). Characterization of the Effect of Drug – Drug Interaction on Protein Binding in concurrent administration of sulfamethoxazol and diclofenac sodium using Bovine Serum Albumin.*Adv. Pharm Bull, 2016;* 6(4): 589 – 595.
9. Kuznetsova, IM.,Sulatskaya Al.,PovarovaOl. &Turoverov KK. Reevaluation of ANS Binding to Human and Bovine Serum Albumins: Key Role of Equilibrium Microdialysis in Ligand – Receptor Binding Characterization. Plos ONE, 2012; 7(7): e40845. doi:10.1371/ *Journal*.pone.0040845.
10. Mainas – Csizmadia, A.,Hegyi, G.; Tolgyesi, F., Szent-Gyorgyi, A.G. &Nyitray, L. Fluorescence Measurement detect changes in scallop Myosin regulatory domain. *European Journal of Biochemistry*, 1999; *2*61(2): 452 – 458.

11. Odoemelam, S. A., Anununso, C. I. &Ejike, E. N. (2004). Fluorescence studies of Binding of Dansylglycine and Dicoumarol to Bovine Serum Albumin. Bio-Research., 2004; 2(1): 124-131.
12. Rahman, A.A. &Sharker, S. Md. (2009) Determination of the Binding sites of propranolol HCl on Bovine Serum Albumin by direct and reverse procedure S. *Saudi Pharmaceutical Journal, 2009; 17: 249 – 253.*
13. Togashi, D. M. & Ryder, A. G. (2008). A Fluorescence Analysis of ANS Bound to Bovine Serum Albumin: Binding Properties Revisited by using Energy Transfer. *J. Fluoresc.*, 2008; 18: 519-526.
14. Uddin, S.J., Shilpi, J.A., Murshid, G.M.M., Rahman, A.A., Sarder, M.M. &Alam, M.A. Determination of the Binding sites of Arsenic on Bovine Serum Albumin using warfarin (site – 1 specific probe) and diazepam (site – 11 specific probe). *Journal of Biological sciences*, 2004; 4(5): 609 – 612.
15. Wikipedia, (2015).