Proc. Indian Acad. Sci. (Chem. Sci.), Vol. 99, No. 3, September 1987, pp. 187–193.

Binding of ketoprofen with bovine serum albumin

MEENAKSHI MARUTHAMUTHU* and SANKARAN KISHORE
Department of Physical Chemistry, University of Madras, Guindy Campus, Madras 600 025, India

MS received 23 February 1987; revised 15 June 1987

Abstract. The binding of Ketoprofen, i.e. 2-(3-benzoylphenyl) propionic acid, with bovine serum albumin, BSA, was investigated by equilibrium dialysis. Limiting the studies to low drug (10 to 100 μM) concentrations, the binding data correspond to a single set of binding sites, namely, the high-affinity sites. Scatchard and Klotz methods of analysis have been employed to determine the binding parameters for the high-affinity sites. The binding constant does not vary significantly with [BSA] in the concentration range 7·25 to 21·5 μM. The molar ratio, [drug]/[protein] is found to be a critical factor in determining the nature and number of binding sites. The quenching of intrinsic fluorescence of the protein at the emission wavelength of 346 nm indicates the presence of tryptophan in the binding site.

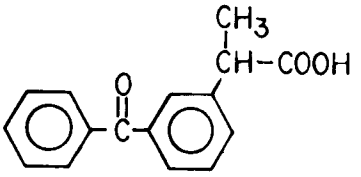
Keywords. Ketoprofen; bovine serum albumin; binding constant; high-affinity sites.

1. Introduction

Serum albumin is a very interesting receptor macromolecule in the study of drug-receptor interactions. It has a number of relatively specific binding sites for drugs and endogeneous compounds and is characterized by the presence of several binding regions (Krag-Hansen 1983). Two specific sites for acidic drugs have been characterized in serum albumin (Sudlow *et al* 1975, 1976; Birkett *et al* 1980); they are known as the azopropazone/warfarin site and the diazepam/benzodiazepine site in terms of the drugs or groups having specific binding affinity for the sites. Drugs which bind to the diazepam site are aromatic carboxylic acids largely ionized at physiological pH. The configuration of these molecules is extended and the negative charge is located at one end of the molecule away from the nonpolar region.

In the present work, we have investigated the interaction of 2-(3-benzoylphenyl)-propionic acid with bovine serum albumin, BSA. The substrate is a new non-steroidal anti-inflammatory drug having the pharmaceutical name, Ketoprofen. The interaction of Ketoprofen with BSA as the receptor would be of interest to gain insight into the pharmacodynamics and toxicology of the drug which is useful for arthritis and related conditions (Fowler *et al* 1980). 2-(3-benzoylphenyl)-propionic acid or Ketoprofen belongs to the class of aryl propionic acid drugs which selectively bind to the diazepam site. The drug-macromolecule interaction has been examined employing equilibrium dialysis and fluorescence quenching.

* To whom all correspondence should be addressed



2-(3-benzoylphenyl)propionic acid or Ketoprofen

2. Experimental

Bovine serum albumin fraction V (Loba Chemie Co., India), a defatted sample and Ketoprofen (The Pharmaceutical Company of India, Ltd.) were used without further purification. Dialysis membranes (Sigma Chemical Co., USA) were subjected to washing in hot water for about two hours before use. The experiments were carried out at pH = 7·1 using an Na_2HPO_4-KH_2PO_4 mixture of 0·1 M concentration. The concentration of BSA is expressed on the basis of its molecular weight (69,000). [BSA] ranging from 7·25 μM to 21·5 μM have been used in the present study. The initial concentration of the drug (C_0) is varied from 10 μM to 300 μM.

Equilibrium dialysis experiments were carried out as described in the literature (Rosenberg and Klotz 1960). Dialysis experiments were performed at 30°C for 48 hours. Experiments were carried out in the absence of BSA and the binding of the drug onto the dialysis membrane was found to be negligible. Equilibrium concentrations of the drug, C, were found out from the measurements of absorbance in the uv region at 261 nm using a Carl-Zeiss Specord UV-VIS spectrophotometer. Blanks were obtained from control experiments carried out under the same conditions in the absence of the drug. The concentration of the bound drug (C_B), and hence, the molar ratio of bound drug to BSA ($r = C_B$/[BSA]) have been obtained from these to calculate the binding parameters. To evaluate the binding constant for the high-affinity sites, observations were restricted to [drug] = 10 μM to 100 μM.

Fluorescence measurements have been made with an Aminco Bowman spectrophotofluorometer. Fluorescence-quenching titrations were carried out by addition of Ketoprofen to a known concentration of BSA. [Ketoprofen] = 6 to 150 μM, and [BSA] = 7·25 μM and 21·5 μM, have been employed for fluorescence studies. The protein fluorescence spectrum due to tryptophan was obtained using an excitation wavelength of 280 nm. The maximum fluorescence values at the emission wavelength of 346 nm were measured as a function of [Ketoprofen]. Correction for quenching due to bulk-absorption or self-absorption (Velick *et al* 1960) has been applied and the actual quenching curve corresponds to corrected fluorescence values.

3. Results and discussion

The binding of a substrate to several classes of sites on a protein molecule is represented by the Scatchard equation (Scatchard 1949) in terms of the molar ratio of the bound substrate to protein r, as

$$r = \sum_i \frac{n_i K_i C}{1 + K_i C}, \tag{1}$$

where $n_i K_i$ is the binding constant corresponding to the ith class of sites with intrinsic binding constant K_i and number of binding sites n_i and C is the equilibrium or free concentration of the substrate. Inspection of (1) reveals that a plot of r/C versus r would be a curve if there are more than one class of binding sites, and the analysis of data of BSA-Ketoprofen system proved to be so in the C_0 range 10 μM to 300 μM. It has been evident that the data corresponding to low values of r, i.e., $r < 4$ may be regarded as belonging to one set of binding sites, namely, the high-affinity sites. Considering only this single set of binding sites, (1) is transformed into the Klotz equation (Klotz *et al* 1946) or the Scatchard equation.

$$\frac{1}{r} = \frac{1}{n_1} + \frac{1}{n_1 K_1 C}, \quad \text{Klotz equation,} \tag{2}$$

$$\frac{r}{C} = n_1 K_1 - r K_1, \quad \text{Scatchard equation.} \tag{3}$$

We have evaluated the binding constant $n_1 K_1$, the number of binding sites n_1 and the intrinsic binding constant K_1 for the high-affinity sites making use of (2) and (3). The other set of binding sites, termed the low-affinity sites, is not subjected to any detailed analysis to determine $n_2 K_2$ since the error involved in these values would be greater.

The multiplicity of binding sites, evident when the [drug] is varied widely from 10 to 300 μM, is no longer observed when [drug] is limited to < 100 μM corresponding to $r < 4$. Table 1 shows the data obtained for [BSA] = 21·5 μM. Figure 1 shows the above data analyzed in terms of the Klotz equation and figure 2 in terms of the Scatchard equation. Analysis of the data by the method of least squares yields $n_1 K_1$ and n_1 and the binding parameters (table 2) by both Klotz and Scatchard methods are in good agreement. The binding constants do not change significantly with [BSA]. The mean value of the intrinsic binding constant is found to be $(1{\cdot}24 \pm 0{\cdot}33) \times 10^5$ M^{-1} (table 2). The number of binding sites is found to be $n_1 \simeq 5$ at [BSA] = 7·25 μM and $n_1 \simeq 4$ at [BSA] = 21·5 μM. Considering the uncertainty associated with n_1 (table 2), the two values of n_1 do not differ significantly. However, a probable explanation for the difference in the value of n_1

Table 1. Binding of Ketoprofen to BSA in 0·1 M phosphate buffer. [BSA] = 21·5 μM; temperature = 30°C; pH = 7·1

C_0 (μM)	C (μM)	r	$(1/r)$	$(r/C) \times 10^{-4}$ (M^{-1})	$(1/C) \times 10^{-4}$ (M^{-1})
40·00	12·30	2·57	0·389	20·89	8·10
50·00	19·00	2·88	0·347	15·15	5·26
60·00	25·80	3·18	0·314	12·32	3·87
70·00	34·40	3·31	0·302	9·62	2·90
80·00	44·10	3·33	0·300	7·55	2·26
90·00	52·00	3·53	0·283	6·78	1·92

C_0 = Initial concentration of the drug; C = equilibrium concentration of the drug; r = molar ratio of bound drug to albumin.

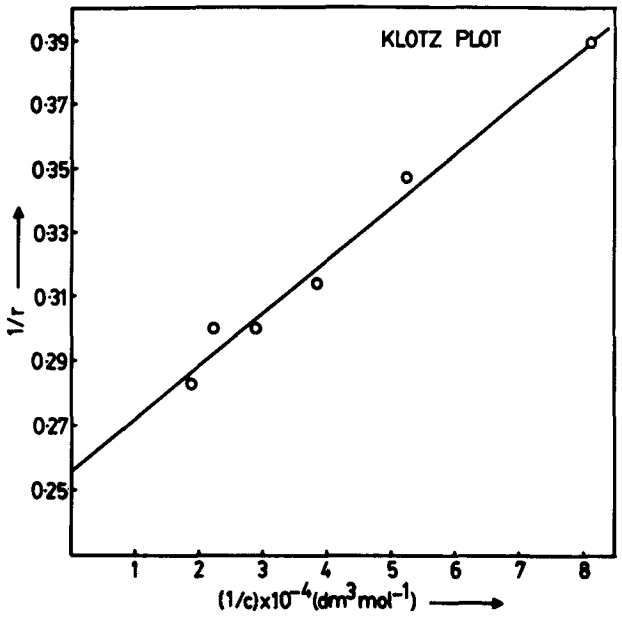


Figure 1. Klotz plot for the BSA-Ketoprofen system corresponding to the high-affinity binding sites. [BSA] = 21·5 μM, [Ketoprofen] = 10 to 100 μM, pH = 7·1 and 30°C.

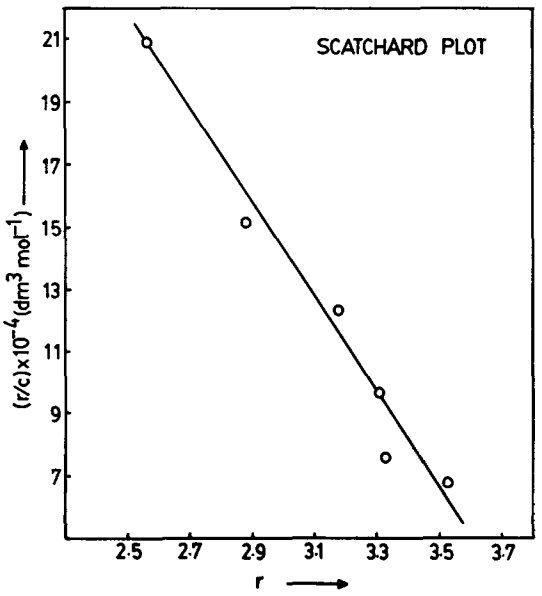


Figure 2. Scatchard plot for the evaluation of binding parameters relating to the high-affinity binding sites. [BSA] = 21·5 μM, [Ketoprofen] = 10 to 100 μM, pH = 7·1 and 30°C.

Table 2. Binding parameters for the BSA-Ketoprofen system. Temperature = 30°C; pH = 7·1; [Buffer] = 0·1 M; [Drug] = 10 to 100 μM.

Method of analysis	[BSA] (μM)	$n_1K_1 \times 10^{-5}$ (M^{-1})	n_1	$K_1 \times 10^{-5}$ (M^{-1})	Mean value $K_1 \times 10^{-5}$ (M^{-1})
Klotz method	7·25	6·24(± 0·87)	4·80(± 0·14)	1·30	1·24 ± 0·33
Scatchard method	7·25	4·83(± 0·36)	5·26(± 0·63)	0·92	
Klotz method	21·50	6·09(± 0·30)	3·89(± 0·02)	1·57	
Scatchard method	21·50	6·13(± 0·03)	3·90(± 0·31)	1·57	

The values given in parenthesis are standard deviations.

would be the following. Since the initial concentration of the drug, C_0, is the same at both 7·25 μM and 21·5 μM of BSA, the relative concentrations of the drug to BSA, C_0/[BSA] would be three times more at low [BSA] than at high [BSA]. This would have led to the utilization of the fifth binding site in the protein molecule which is otherwise inactive at low C_0/[BSA] ratio. In other words, the molar ratio of substrate to protein also appears to determine the number of active binding sites in the macromolecule.

The low-affinity sites are involved in binding only at high values of r, namely, $4 < r < 8$ and at [drug] = 100 to 300μM. It is evident from this that the relative ratio C_0/[BSA] and hence C_B/[BSA], i.e., r are important in determining the nature of binding sites. The second set of binding sites would have been left unnoticed if the study has not been performed at high C_0/[BSA]. The binding constant, n_2K_2, for the low-affinity sites is found to be of the order of 10^4.

Ketoprofen is an aryl propionic acid drug. Since the carboxylic acid group would exist in the form of the anion at the experimental pH, the anion is expected to interact electrostatically with cationic amino acids of the type lysine, arginine and histidine. In addition to this, the non-polar part of the drug would interact with the hydrophobic aryl residues of albumin. Recent studies (Wanwimolruk *et al* 1983) show that aryl propionic acid non-steroidal anti-inflammatory drugs and medium chain fatty acids (C_8 to C_{10}) bind strongly to the diazepam site, which is a hydrophobic cleft about 16 Å deep and about 8 Å wide in the albumin molecule with a cationic group located near the surface. The molecular size as well as the presence of both anionic and hydrophobic groups in Ketoprofen suggest the diazepam site as the probable binding site for Ketoprofen.

The intrinsic binding constant for BSA-Ketoprofen system is about five times smaller than that for the related system involving human serum albumin, HSA-F 1594 (Urien *et al* 1984) as evident from table 3. In the case of Ketoprofen, the carbonyl group is in between the two aryl groups and this reduces the interaction of the aryl residues with the hydrophobic residues of albumin. The position of carbonyl group is not so unfavourable in the case of F 1594. For the interaction of Flurbiprofen, Ibuprofen and Naproxen with HSA immobilized in microparticles (Kober and Sjoeholm 1980), the binding constants are of the order of 10^6 (table 3). The absence of the hydrophilic carbonyl group in the above drugs explains the higher binding constants relative to Ketoprofen since hydrophilic groups such as hydroxyl and carbonyl have been shown to reduce the binding affinity of such aryl propionic acid drugs (Urien *et al* 1984).

Table 3. Comparison of the binding constants for BSA-Ketoprofen and related systems.

Substrate	Pharmaceutical name	$K_1 \times 10^6$ (M^{-1})	Reference
2-(3-benzoylphenyl)propionic acid	Ketoprofen	0·12	This work
2-methyl-3-(4-phenylcarboxyphenyl)propionic acid	F 1594	0·54	Urien *et al* (1984)
2-(6-methoxy-2-naphthyl)-propionic acid	Naproxan	1·8	Kober and Sjoeholm (1980)
2-(4-isobutylphenyl)propionic acid	Ibuprofen	1·3	Kober and Sjoeholm (1980)
2-(2-fluoro-4-biphenyl)-propionic acid	Flurbiprofen	5·0	Kober and Sjoeholm (1980)

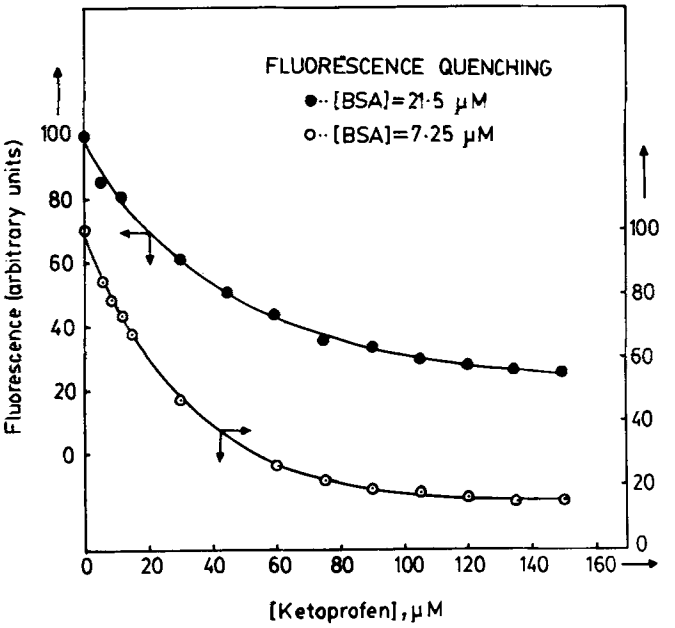


Figure 3. The quenching of fluorescence of BSA as a function of [Ketoprofen]. [Ketoprofen] = 6 to 150 μM, pH = 7·1 and 30°C. BSA concentration: (●) 21·5 μM; (○) 7·25 μM.

For BSA-Ketoprofen system, fluorescence quenching studies have also been made to gain insight into the nature of the binding site. BSA contains two tryptophan residues that emit fluorescence with maximum at 346 nm by excitation at 280 nm. This intrinsic tryptophanyl fluorescence of the protein can be utilized to monitor drug-protein interactions (Teale and Weber 1957). The absence of quenching of fluorescence does not imply the absence of drug-protein interaction whereas the quenching of protein fluorescence by the drug can be taken to indicate drug-protein interaction, in the absence of any conformational change in the protein favouring quenching.

A progressive decrease in the intensity of the intrinsic protein fluorescence has been observed with increase in the concentration of Ketoprofen (figure 3). The maximum quenching at saturation reaches 80–90%. As the drug exhibits no absorbance at $\lambda > 300$ nm, there is no appreciable overlap of the emission spectrum of the protein and the absorption band of Ketoprofen. Hence it is not likely that the quenching phenomenon is due to nonradiative energy transfer. Quenching has its origin at the source of fluorescence emission, namely, in the direct perturbation of the fluorophore. In other words, the drug comes in direct proximity with the tryptophan residue. This implies that tryptophan is a part of the binding site in the interaction of Ketoprofen with BSA and also leads to the conclusion that hydrophobic forces are involved in binding.

Acknowledgement

The authors acknowledge the CSIR, New Delhi, for financial support and the Pharmaceutical Company of India Ltd., for the gift sample of Ketoprofen.

References

Birkett D J, Ray S, Sudlow G and Hagedorn J 1980 *Acta Pharm. Suec.* **17** 78
Fowler R J, Moncada S and Vans J R 1980 in *The pharmacological basis of therapeutics* (eds) A G Gilman, L S Goodman and S Gilman (New York: MacMillan) p. 710
Klotz I M, Walker F M and Pivan R B 1946 *J. Am. Chem. Soc.* **68** 1486
Kober A and Sjoeholm I 1980 *Mol. Pharmacol.* **18** 421
Krahg-Hansen U.1983 *Biochem. J.* **209** 135
Rosenberg R M and Klotz I M 1960 in *Analytical methods in protein chemistry* (eds) P Alexander and R J Block (London: Pergamon) p. 133
Scatchard G 1949 *Ann. N.Y. Acad. Sci.* **51** 660
Sudlow G, Birkett, D J and Wade D N 1975 *Mol. Pharmacol.* **11** 824
Sudlow G, Birkett D J and Wade D N 1976 *Mol. Pharmacol.* **12** 1052
Teale F and Weber G 1957 *Biochem. J.* **65** 476
Urien S, D'Athis P and Tillement J P 1984 *Biochem. Pharmacol.* **33** 2283
Velick S F, Parker L W and Eisen H N 1960 *Proc. Natl. Acad. Sci. USA* **46** 1470
Wanwimolruk S, Birkett D J and Brooks P M 1983 *Mol. Pharmacol.* **24** 458