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Protein Binding of Piroxicam Studied by Means of Affinity Chromatography and Circular Dichroism

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Abstract

The protein binding of piroxicam, a widely used non-steroidal anti-inflammatory drug has been investigated by high-performance liquid affinity chromatography, with phenylbutazone and diazepam used as markers for binding-site characterization, and by circular dichroism titration.

It was found that piroxicam binds to high-affinity phenylbutazone-binding sites and to high-affinity diazepam-binding sites. No binding to the low-affinity sites of either marker was established. High values of the primary (high-affinity) binding constants corresponding to both types of binding site were obtained by means of a mathematical method cited in the literature. The circular dichroic spectra of piroxicam were studied at a given albumin concentration and various drug concentrations. A new Cotton effect was observed and was ascribed to the binding of piroxicam to the protein molecule. The values of differential molar ellipticity ($\Delta\theta$) were treated by a new mathematical procedure for analysis of the data obtained. A high affinity constant was calculated for one class of binding site. Its value is in good agreement with the values obtained by affinity chromatography.

These results reveal that circular dichroism is an acceptable method for investigation of protein binding.

Oxicams are long-lasting non-steroidal anti-inflammatory drugs (NSAIDs) which act by inhibiting enzymes involved in the biosynthesis of prostaglandins. An enolic oxicam derivative piroxicam—4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide (Figure 1) is currently among the ten most popular NSAIDs on the market. It is lipophilic and achiral and is almost completely absorbed under fed or fasting conditions (Ishizaki et al 1979). Piroxicam is extensively (> 99%) bound to albumin (Lin et al 1987) and after single doses the volume of distribution, Vd, is low (Vd/F $\approx$ 10 L/70 kg). It is eliminated predominantly by hepatic metabolism (Woolf & Radulovic 1989) with a long elimination half-life, $t_{1/2}$, of 55 h (approx.).

The protein binding of piroxicam has been the subject of several in-vitro and in-vivo studies. Binding to serum albumin in man has been studied by ultrafiltration (Kanavi & Seibler 1988) and the affinity constants and the percentage of unbound drug were determined. After a competition study performed with a human serum albumin (HSA)-based HPLC column, using markers as displacing agents, Rahim & Aubry (1995) suggested that piroxicam binds equally to sites I and II and to other sites on HSA. Binding at other sites includes non-specific attachment to the protein, that is binding not associated with a well defined three-dimensional site. Piroxicam is included in the list of commonly used drugs that bind to site I on albumin (Srinivasan et al 1995); the extent of binding is 99·3%. Karim et al (1997) investigated the effect of this binding behaviour on the disposition kinetics of piroxicam at steady state and it was shown that HSA binding was not dependent on plasma concentration.

In the current study data were obtained by two methods: circular dichroism titration and high-performance liquid affinity chromatography. The usefulness of the circular dichroism technique as a

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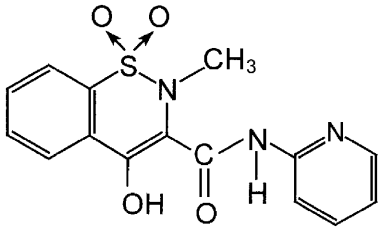

Figure 1. The chemical structure of piroxicam.

very sensitive method for study of HSA binding has been widely discussed (Sjoholm & Sjodin 1972; Ascoli et al 1995). The basis of the method is that binding of a small symmetric ligand to an asymmetric macromolecule (protein) can induce optical activity in the ligand–macromolecule complex leading to extrinsic Cotton effects which can be used to estimate binding constants and number of binding sites. Small changes in the drug–protein complex can have large influences on the circular dichroism spectra (Chignell 1969a, b; Muller & Wollert 1973). We recently used this method to study the binding characteristics of sulindak, ketoprofen, indomethacin and diclophenac sodium (Russeva et al 1994a, b; Russeva & Michailova 1996).

Many biological events involving interactions between small ligands and biopolymers are often influenced by the presence of other small molecules. Affinity chromatography (Noctor et al 1992) seems to be an attractive alternative approach in which the protein is immobilized on a suitable support, the ligand is injected on to the column, and competing agent is added to the mobile phase. Small changes in the binding affinity affect chromatographic retention and can be precisely monitored. We recently reported the use of phenylbutazone and diazepam as markers in an affinity chromatography study of albumin binding sites (Russeva & Zhivkova 1998).

After measuring the affinity constants of piroxicam binding to albumin by the two methods an attempt was made to determine the number of binding sites and to characterize the sites using phenylbutazone and diazepam as markers.

Materials and Methods

Drugs and chemicals

Piroxicam, phenylbutazone and diazepam were obtained from the National Drugs Institute (Sofia, Bulgaria). Propan-1-ol for affinity chromatography and NaH_2PO_4 and Na_2HPO_4 of purest grade for phosphate buffers were obtained from Merck (Darmstadt, Germany).

HSA used in circular dichroism measurements was obtained from Koch Light Laboratories (England). To remove any remaining adsorbed impurities HSA was treated with activated charcoal at pH 3. The albumin monomer was then isolated by repeated gel filtration on Sephadex G-200 and G-100 in 0·1 M KCl. The purity of the monomer fraction was monitored by electrophoresis on 7% polyacrylamide gel at pH 8·3. The albumin monomer was stored at $-20°C$ until required. Albumin solutions were prepared in 0·05 M, pH 7·4, phosphate buffer immediately before use, assuming a molecular weight of 69 000 Da and 98% purity. The concentration of HSA was checked by measuring the optical density of solutions at 280 nm using $E^{1\%}_{1\ cm}$ (the extinction of a 1% solution in 1 cm cuvette) = 5·8.

Circular dichroism measurements

Circular dichroism experiments were performed with a Dichrographe III spectropolarimeter (Jobin Ivon, Long Jumeau, France) at room temperature. Spectra were recorded in the range 400–250 nm using cylindrical cells with 10-nm path length. The scanning velocity was $2\ mm\ n^{-1}$.

Affinity chromatography

Affinity chromatography was performed with a Shimadzu (Japan) modular HPLC system comprising an LC-10A pump, a DGU-3A solvent degasser, a Rheodyne injector with 20-μL loop, a CTO-10A column oven, a SPD-M10A diode-array detector and a CBM-10A communication bus module. The immobilized HSA-based column (150 mm × 4·6 mm) was from Shandon Scientific (Runcorn, UK). The analysis was controlled and the data acquired by Class LC-10 software. Chromatography was performed isocratically at $34 \pm 0·1°C$ and a flow rate of $1·2\ mL\ min^{-1}$. The mobile phase was based on NaH_2PO_4 and Na_2HPO_4 (67 mM, pH 7·4) modified with 8% (v/v) propan-1-ol.

Mathematical analysis

Binding results obtained by means of circular dichroism were treated according to a new mathematical procedure (Vandev et al 1998). Affinity chromatography data were treated by a mathematical approach developed by Noctor et al (1992).

Results and Discussion

Affinity chromatographic studies of competitive binding with phenylbutazone as mobile-phase additive (marker)

The influence of increasing phenylbutazone concentration (range 0–30 μM) on the chromatographic retention of piroxicam was studied. The results are

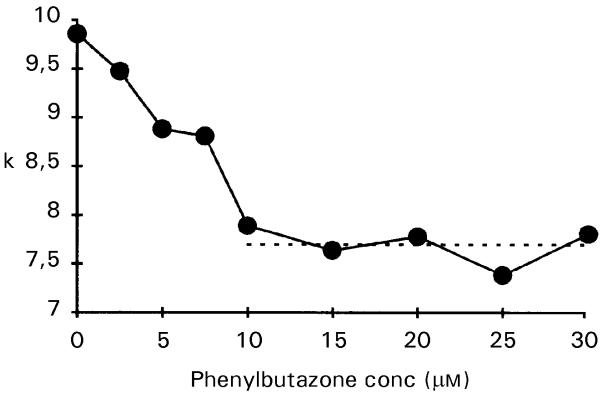

Figure 2. Influence on the capacity factor of piroxicam of the concentration of the marker phenylbutazone in the mobile phase.

shown in Figure 2. Initially the capacity factor, k, decreases almost linearly with increasing of marker (phenylbutazone) concentration then, above 7·5 μM, remains constant—the binding sites probably become saturated. During the experiments the quantity of the displaced marker changes in the same manner and reaches a considerable value (10–15 μM) at saturation. An affinity constant of $4{\cdot}42 \pm 0{\cdot}57 \times 10^5\ \text{M}^{-1}$ was calculated for the binding of piroxicam to high-affinity phenylbutazone-binding sites; this corresponds to (approx.) 23% primary binding. There was no evidence that piroxicam binds to low-affinity phenylbutazone-binding sites.

Affinity chromatography studies of competitive binding with diazepam as mobile-phase additive (marker)

Similar behaviour (Figure 3) was established when the binding of piroxicam to sites marked by diazepam (range 0–40 μM) was studied. Initially k decreases, then after the concentration of diazepam reaches 10 μM it remains constant (as saturation is reached). It was found that piroxicam binds only to the high-affinity diazepam-binding sites. Nearly 13% of the total amount of the drug is bound to this type of site. Although the affinity constant for binding to these sites is rather high, $1{\cdot}05 \pm 0{\cdot}33 \times 10^6\ \text{M}^{-1}$, very small amounts of marker were observed to be displaced during the experiments because of the small concentration of diazepam-binding sites previously established (Russeva & Zhivkova 1998). Again there was no evidence of binding to the low-affinity diazepam-binding sites.

Circular dichroism measurements

The molecule of piroxicam (Figure 1) is not optically active. The circular dichroic spectra of piroxicam were studied for a fixed concentration of albumin and different concentrations of the drug. When a drug binds to the albumin molecule new extrinsic Cotton effects are obtained at wavelengths where the drug has absorption bands. In the circular dichroic spectrum given in Figure 4 it is apparent that a positive extrinsic Cotton effect arises at 322 nm. Figure 5 shows the differential circular dichroic spectrum obtained when HSA is titrated against piroxicam at 322 nm. As piroxicam has no ellipticity of its own the new effect is ascribed to its binding to the protein molecule. The molar ellipticity change $\Delta\theta$ is proportional to the concentration of drug–protein complex. The values of differential molar ellipticity ($\Delta\theta$), obtained by subtracting the circular dichroic spectrum of HSA from that of the drug–HSA complex, were treated according to the method of Vandev et al (1998). The estimated affinity constant was $9{\cdot}37 \pm 0{\cdot}26 \times 10^5\ \text{M}^{-1}$, which is indicative of one class of binding site.

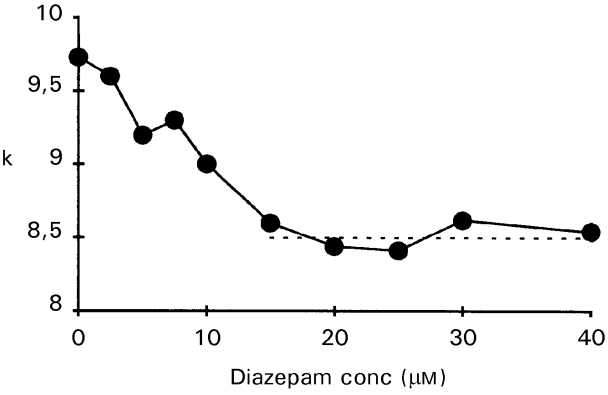

Figure 3. Influence on the capacity factor of piroxicam of the concentration of the marker diazepam in the mobile phase.

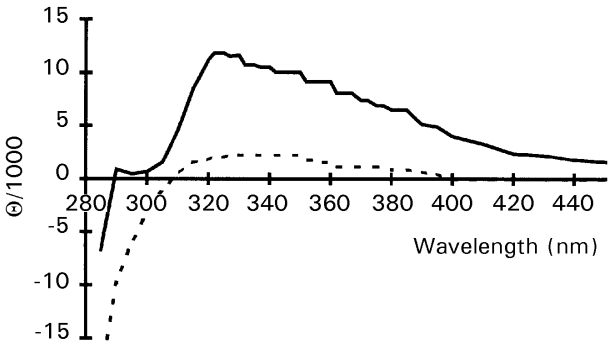

Figure 4. Circular dichroic spectra of serum albumin from man, $1{\cdot}5 \times 10^{-5}$ M (- - -) and of piroxicam, $1{\cdot}1 \times 10^{-5}$ M (—) when bound to albumin. All solutions are in 0·05 M phosphate buffer, pH 7·4.

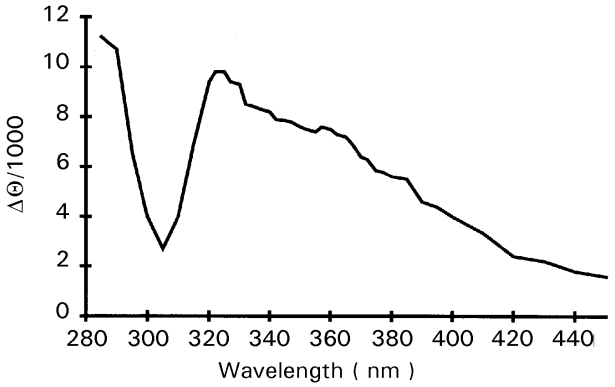

Figure 5. Differential circular dichroic spectrum of piroxicam bound to serum albumin from man (molar ratio 1 : 1). The HSA concentration is $0{\cdot}5 \times 10^{-5}$ M; all solutions are in 0·05 M phosphate buffer, pH 7·4.

Conclusions

Quantitative characterization of the protein binding of piroxicam has been performed by two different methods. The affinity constant for binding is rather high, which explains the strong binding affinity of the drug to serum albumin in man. The albumin binding sites were characterized by use of phenylbutazone and diazepam as markers. Although both types of site can have high and low affinity our results reveal binding to high-affinity phenylbutazone- and diazepam-binding sites only. In previous work (Russeva & Zhivkova 1998) we stated that the binding sites for phenylbutazone and diazepam on HSA cannot be entirely differentiated and that they probably overlap to some extent.

Because circular dichroism can be used to study systems in which binding of the drug alters the conformation, and hence the ellipticity, of the protein, or systems in which binding occurs within a chiral environment, the method reveals only those binding sites causing a change in ellipticity. Taking into account these considerations the single affinity constant for binding obtained by circular dichroic measurements can be regarded to be as valid as the affinity constants found by affinity chromatography, and as they do not differ significantly they can be regarded as characterizing one class of binding site.

The data obtained show that circular dichroism is an appropriate method for studying the protein binding of drugs.

References

Ascoli, G., Bertucci, C., Salvadori, P. (1995) Stereospecific and competitive binding of drugs to human serum albumin: a difference circular dichroism approach. J. Pharm. Sci. 84: 737–741

Chignell, C. F. (1969a) Optical studies of drug-protein complexes. II. Interaction of phenylbutazone and its analogues with human serum albumin. Mol. Pharmacol. 5: 224–252

Chignell, C. F. (1969b) Optical studies of drug-protein complexes. III. Interaction of flufenamic acid and other *N*-arylanthranilates with serum albumin. Mol. Pharmacol. 5: 455–462

Ishizaki, T., Nomura, T., Abe, T. (1979) Pharmacokinetics of piroxicam, a new non-steroidal anti-inflammatory agent, under fasting and postprandial states in man. J. Pharmacokinet. Biopharm. 7: 369–381

Kanavi, A., Seibler, D. (1988) Zur bindung von oxicam-derivaten an HSA. Arzneim. Forsch. 38: 1089–1092

Karim, A., Noveck, R., McMahon, F. G., Smith, M., Crosby, S., Adams, M., Wilton, J. (1997) Oxaprozin and piroxicam, non-steroidal antiinflammatory drugs with long half-lives: effect of protein-binding differences on steady-state pharmacokinetics. J. Clin. Pharmacol. 37: 267–278

Lin, J. H., Cocchetto, D. M., Duggan, D. E. (1987) Protein binding as a primary determinant of the clinical pharmacokinetic properties of non-steroidal anti-inflammatory drugs. Clin. Pharmacokinet. 12: 402–432

Muller, W. E., Wollert, U. (1973) Interactions of benzodiazepines with human serum albumin. Circular dichroism studies. Naunyn Schmiedebergs Arch. Pharmacol. 283: 301–312

Noctor, T. A. G., Wainer, I. W., Hage, D. S. (1992) Allosteric and competitive displacement of drugs from human serum albumin by octanoic acid, as revealed by high-performance liquid affinity chromatography, on a human serum albumin-based stationary phase. J. Chromatogr. B 577: 305–315

Rahim, S., Aubry, A.-F. (1995) Location of binding sites on immobilized human serum albumin for some non-steroidal anti-inflammatory drugs. J. Pharm. Sci. 84: 949–951

Russeva, V., Michailova, D. (1996) Modelling of indomethacin and diclophenac sodium binding to the molecule of human serum albumin. Arzneim. Forsch. 46: 288–292

Russeva, V., Zhivkova, Z. (1998) Albumin binding sites studied by high-performance liquid affinity chromatography. Int. J. Pharm. 168: 23–29

Russeva, V., Stavreva, N., Rakovska, R., Michailova, D. (1994a) Binding of sulindak to human serum albumin studied by circular dichroism. Arzneim. Forsch. 44: 159–162

Russeva, V., Rakovska, R., Stavreva, N., Michailova, D., Berova, N. (1994b) Binding of ketoprofen to human serum albumin studied by circular dichroism. Pharmazie 49: 519–522

Sjoholm, I., Sjodin, T. (1972) Binding of drugs to human serum albumin—I. Circular dichroism studies on the binding of some analgesics, sedatives and antidepressive agents. Biochem. Pharmacol. 12: 3041–3052

Srinivasan, S. K., Tewary, H. K., Iversen, P. L. (1995) Characterization of binding sites, extent of binding, and drug interactions of oligonucleotides with albumin. Antisense Res. Dev. 5: 131–139

Vandev, D., Prodanova, K., Russeva, V. (1998) New mathematical procedure for analysis of data obtained by means of circular dichroism titration method. Arzneim. Forsch. 48: In press

Woolf, T. F., Radulovic, L. (1989) Oxicams: metabolic disposition in man and animals. Drug Metab. Rev. 21: 255–276