Biochemical Pharmacology, Vol. 32, No. 12, pp. 1917–1920, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + .00

STEREOSELECTIVE BINDING OF A 2,3-BENZODIAZEPINE TO HUMAN SERUM ALBUMIN

EFFECT OF CONFORMATION ON TOFIZOPAM BINDING

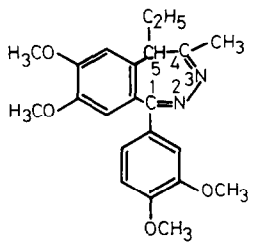
MIKLÓS SIMONYI* and ILONA FITOS
Central Research Institute for Chemistry of the Hungarian Academy of Sciences, PO Box 17, H-1525 Budapest, Hungary

(*Received* 28 *September* 1982; *accepted* 10 *January* 1983)

Abstract—The binding of Tofizopam enantiomers to human serum albumin has been investigated by ultrafiltration and affinity chromatography. In solution, Tofizopam molecules exist in two conformations which slowly interconvert into each other. Both conformers of (*R*)-Tofizopam have the same binding constant of $4.8 \times 10^3\ M^{-1}$. The binding of the (*S*)-enantiomer, however, depends on the conformation. The minor and major (*S*)-conformers were characterized by association constants of 2.3×10^3 and $15.1 \times 10^3\ M^{-1}$, respectively. Thus, the stereoselectivity of binding differs for the two conformations of the enantiomers. Kinetic parameters for the interconversion of conformations have been determined. Tofizopam displaces both bound diazepam and warfarin.

Several chiral drugs have been found to exhibit stereoselective binding to serum proteins [1] indicating the specificity of binding sites. The largest stereoselectivities have been detected [2–7] for the binding of 3-substituted 1,4-benzodiazepines to human serum albumin (HSA). The phenomenon was interpreted in terms of the inversion of the diazepine ring which prefers the conformation in which the C-3-substitutent is found in a pseudo-equatorial position.

In this work, we have studied the binding of 1-(3,4-dimethoxyphenyl)-4-methyl-5-ethyl-7,8-dimethoxy-5H-2,3-benzodiazepine, having a chiral centre at the C-5 position. The racemic compound is used as an anxiolytic agent (Tofizopam WHO, Grandaxin®). Pharmacological tests indicated quantitative differences between the enantiomers and suggested different spectra of their effect [8].



MATERIALS AND METHODS

The synthesis [9] and resolution [10] of racemic Tofizopam have already been described. The absolute configuration has been determined [11] by X-ray analysis, indicating that laevorotatory Tofizopam is of (*S*)-absolute configuration. [^{3}H]Diazepam and [^{14}C]warfarin with radiochemical purities >97% were purchased from the Institute of Isotopes, Budapest, Hungary and The Radiochemical Centre (Amersham, U.K.), respectively. Lyophilized non-defatted HSA was obtained from 'Human' Serum and Vaccine Institute, Budapest, Hungary and used without further purification. Experiments were done in Ringer buffer (pH 7.4) unless otherwise indicated.

Binding studies with Tofizopam enantiomers were performed by ultrafiltration using AMICON YM-10 membranes and UV detection ($A_{310} = 1.62 \times 10^4\ M^{-1}$/cm). A small degree of non-specific membrane binding as well as the leakage of HSA through the membrane was taken into consideration. The buffer contained 1% ethanol.

Competition experiments with diazepam and warfarin were performed by a method [12] applying HSA immobilized in polyacrylamide microparticles. After centrifugation, the concentration of the radioactive-free drug was measured by liquid scintillation counting of the supernatant. In these experiments 0.005 M phosphate, 0.1 M KCl buffer (pH 7.4) was used with 2% ethanol content.

Affinity chromatographic studies were carried out on a column containing HSA ($\sim 10^{-4}$ M) immobilized on CNBr-activated Sepharose 4B (Pharmacia) using UV detection. The eluant contained 0.02% sodium azide. Samples of about 10 μg were applied in ethanol solution.

RESULTS AND DISCUSSION

Ultrafiltration studies with the enantiomers

Binding data of Tofizopam enantiomers obtained by ultrafiltration are shown in Scatchard representation in Fig. 1. It can be seen that (*S*)-Tofizopam is bound about twice as strongly as the (*R*)-enantiomer. The data indicate one primary binding site

* To whom correspondence should be addressed.

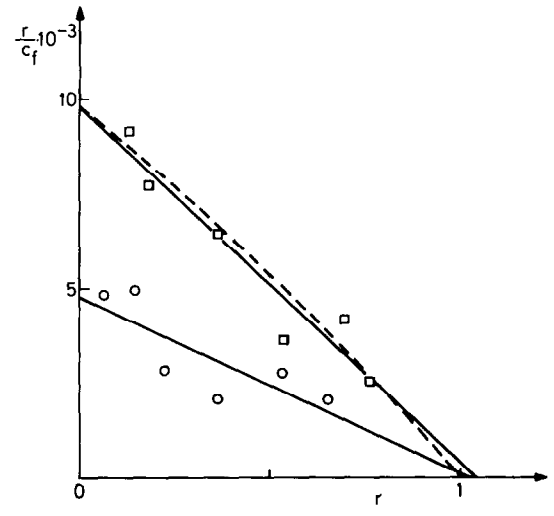


Fig. 1. Binding data of (*S*)-Tofizopam (□) and (*R*)-Tofizopam (○) determined from ultrafiltration experiments ($c_{HSA} = 1.5 \times 10^{-4}$ M, $c_{Tofizopam}$ varied between 2×10^{-5} and 4×10^{-4} M) and plotted according to Scatchard (*r*:number of drug molecules bound per mole HSA; c_f: concentration of the free drug). Least-square fit straight lines indicate one binding site for each enantiomer. The dashed line represents a theoretical curve calculated for equilibrium distribution of (*S*)-Tofizopam conformers; their binding constants were estimated from HSA-Sepharose chromatography and the conformers were assumed to compete for the same binding site.

for both antipodes on HSA and values of 9.8×10^3 and $4.8 \times 10^3\ M^{-1}$ can be given as enantiomeric association constants.

Displacement experiments

Radioactive markers were used in separate experiments to characterize specific binding sites on HSA, and their possible involvement in the binding of Tofizopam enantiomers has been studied. Diazepam was found [13] to have one specific binding site on HSA, and this compound can be used [12] as a marker to study interactions at the 'benzodiazepine' binding site. Another important specific binding site on HSA can be marked by warfarin [12]. The markers have association constants of similar magnitude towards HSA (about $2 \times 10^5\ M^{-1}$ [12]) much larger than those of the Tofizopam enantiomers. The applied concentrations (12 μM for the marker, 15 μM for HSA) brought about a 50% saturation of the specific binding site by the marker. Tofizopam stereoisomers have to be applied in excess (50–250 μM) to compensate for their lower binding affinities. The enantiomers alone would have produced 20–70% saturation. When applied in the presence of markers, 10–20% displacement of both markers occurred, the effect being significantly larger for (*S*)-Tofizopam (data not shown). The degree of displacements is in accordance with the association constants obtained. Though Fig. 1 indicates one primary binding site for both Tofizopam antipodes, displacements of the markers suggest that both specific binding sites of HSA are influenced. It may be of pharmacological importance for patients receiving long-term treatment of anticoagulants.

Affinity chromatography

The chromatographic resolution of racemic Tofizopam on immobilized HSA is shown in Fig. 2. The appearance of three peaks instead of the expected two is highly surprising. The chromatograms of the enantiomers (Fig. 3) reveal that the weakly bound minor component belongs to (*S*)-Tofizopam, the major part of which is more strongly bound. The (*R*)-enantiomer, however, can be eluted as one peak, indicating a somewhat higher affinity than that of the minor component present in the (*S*)-enantiomer.

To explain the appearance of the minor component, which is about 20% of (*S*)-Tofizopam and has a much smaller binding affinity to HSA, we must take into consideration the fact that Tofizopam molecules in solution exist in two conformations. NMR studies [14] have proved that the majority of the molecules have a conformation where the C-5 ethyl group is pseudo-equatorial, while in the remaining molecules this group is pseudo-axial with respect to the diazepine ring. The conformers differed in thermodynamic stability by ~ 4 kJ/mole [14]. The activation enthalpy for the conversion of conformation (~ 100 kJ/mole) has been measured by NMR [10]. The interconversion between the conformers has also been detected [10] by chiroptical investigation. After dissolving the resolved enantiomer, the magnitude of optical rotation decreased and, in a day, reached an equilibrium value. This could be explained by assuming (1) that Tofizopam molecules in solid phase practically exist only in one (the more stable) conformation, and (2) that the energetically less stable conformer of a Tofizopam enantiomer has an optical rotation of opposite sign. Consequently, the labels *R*(−)-, and *S*(+)-Tofizopam refer to definite chemical entities which are minor components present in solution only, in contrast to *R*(+)- and *S*(−)-structures which denote the vast majority of Tofizopam molecules in the solid state and indicate the dominant part of solvated molecules when the conformation equilibrium settles (Fig. 4). In chloroform 15–85% equilibrium distribution was

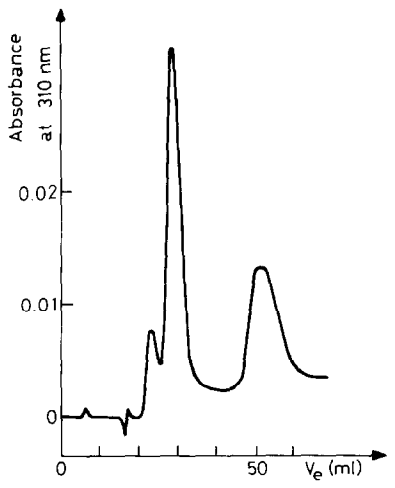


Fig. 2. Chromatographic resolution of racemic Tofizopam on a HSA-Sepharose column (elution volume for solvent: 17.5 ml).

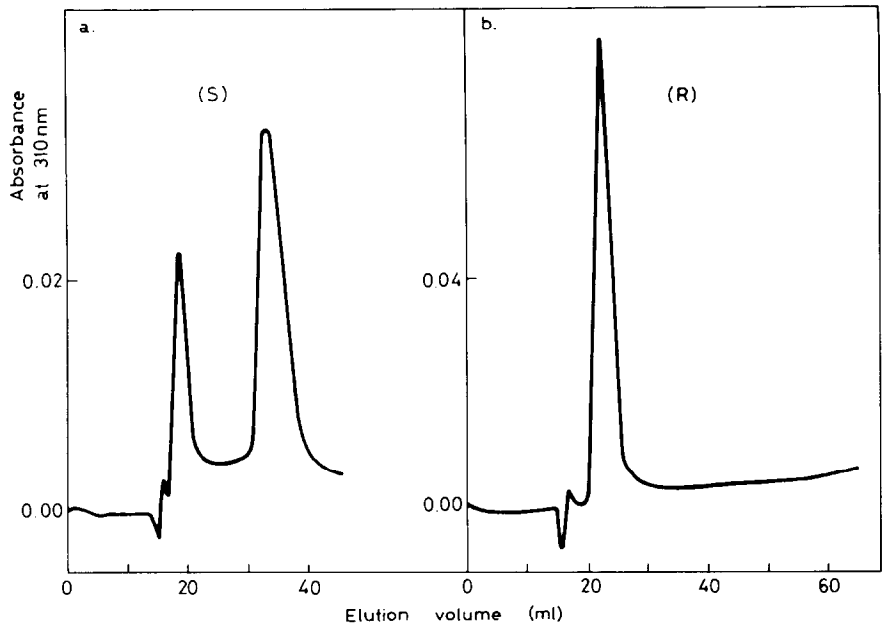


Fig. 3. Chromatogram of Tofizopam enantiomers on HSA-Sepharose column (elution volume for solvent: 15 ml).

detected at room temperature [10]. Tofizopam conformers could also be separated by reversed-phase HPLC technique [15].

The identification of the peaks in Fig. 3a as interconverting conformers of (*S*)-Tofizopam was performed in the following way. Fractions containing the peaks were separately collected, lyophilized, dissolved in ethanol and rechromatographed. The result was similar to Fig. 3a in both cases, i.e. equilibrium occurred between the conformers.

The kinetics of the interconversion were measured too. Having dissolved crystalline (*S*)-Tofizopam in ethanol, chromatograms were recorded at different times. Figure 5a shows the contribution of the first peak in the sample. It was observed that immediately after dissolution, the first peak was practically absent, but its height increased with time and reached an equilibrium value corresponding to 22% of the total material. These data allow us to determine the equilibrium and rate constants of the process according to the following scheme:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \tag{1}$$

$$K = k_1/k_2 = [\bar{B}]/[\bar{A}]. \tag{2}$$

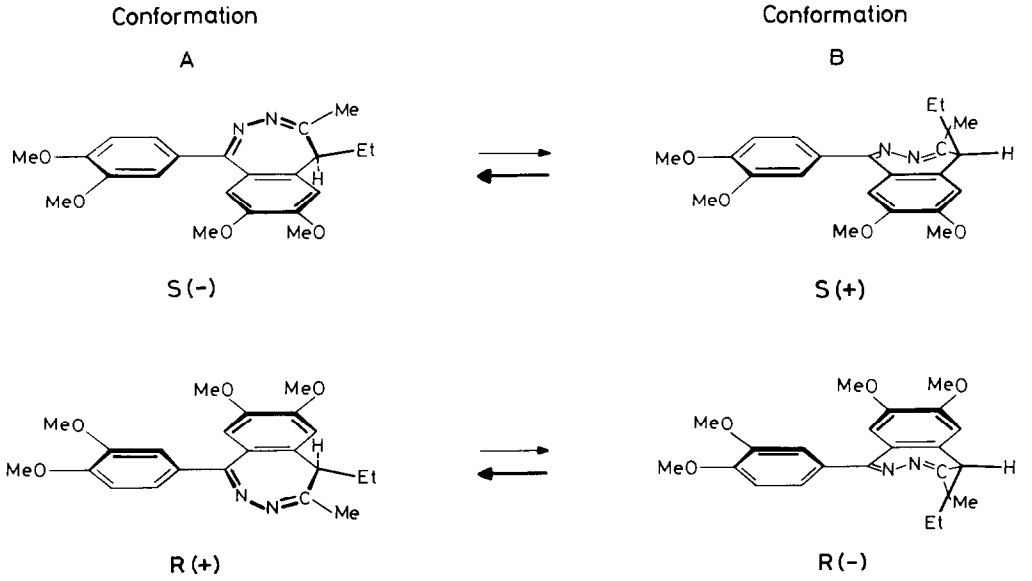


Fig. 4. Scheme for conformational interconversion of Tofizopam enantiomers.

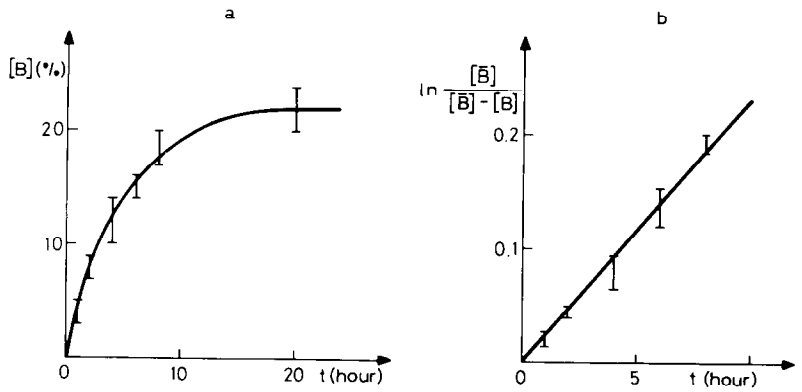


Fig. 5. (a)Time-dependence of the concentration of the minor (*S*)-Tofizopam conformer, [B], determined by affinity chromatography. (b) Kinetic data for the conformational interconversion of the Tofizopam molecule plotted according to equation (3). [B̄] denotes the equilibrium concentration of the minor conformer. The slope equals $(k_1 + k_2)$, the sum of rate constants of interconversion for both directions.

where A and B represent the major and minor conformers, respectively. [A] and [B] represent the actual concentrations at time t, while [Ā] and [B̄] are the equilibrium concentrations of the conformers. The kinetic solution of the above scheme is

$$\ln [\bar{B}]/([\bar{B}] - [B]) = (k_1 + k_2)\, t. \qquad (3)$$

Figure 5b demonstrates that the data are in good accordance with equation (3). The results are shown in Table 1.

On the basis of elution volumes, the binding affinity for both conformers can be estimated [16]. Compared with the (*R*)-enantiomer ($K^{A}_{R} = K^{B}_{R} = 4.8 \times 10^3\,M^{-1}$), the major conformer of the (*S*)-enantiomer can be characterized by a 3.14 times higher binding constant ($K^{A}_{S} = 1.51 \times 10^4\,M^{-1}$), while this ratio is only 0.48 for the minor conformer ($K^{B}_{S} = 2.3 \times 10^3\,M^{-1}$). The dashed line in Fig. 1 shows the theoretical Scatchard curve for (*S*)-Tofizopam calculated on the basis of the evaluated K^{A}_{S} K^{B}_{S} values, and the equilibrium distribution of the conformers which are presumed to compete for one binding site on HSA. There seems to be satisfactory agreement with the experimental data obtained by ultrafiltration and referring to equilibrium conformer composition.

Summarizing the above results, it was found that (*S*)-Tofizopam molecules in conformation 'A' exhibit much higher binding affinity to HSA than in conformation 'B'. The preference of conformation 'A', however, does not appear in the binding of the (*R*)-enantiomer. The stereoselectivity (ratio of binding constants of mirror image chemical entitites) is thus different for the two conformations, giving the average value of 2.2 for the equilibrium distribution (Fig. 1). Hence, Tofizopam is a good model for indicating the importance of the drug conformation in the affinity of its binding to HSA.

Table 1. Equilibrium and kinetic parameters for the interconversion of Tofizopam conformers at 25°

$K = 0.28$
$k_1 = 0.05\ hr^{-1}$
$k_2 = 0.18\ hr^{-1}$
$t_{\frac{1}{2}} = 3$ hr
$\Delta G^{\circ} = 3.2$ kJ/mole

Acknowledgements—Tofizopam (Grandaxin®) samples were obtained from the manufacturer EGYT Pharmaceutical Works, Budapest. We thank Dr. V. Jancsik for the preparation of the HSA-Sepharose gel, Prof. I. Sjöholm (Uppsala) for HSA immobilized in microparticles, and Drs. K. Simon and E. Fogassy for valuable discussion and information prior to publication.

REFERENCES

1. T. Alebić-Kolbah, S. Rendić, Ž. Fuks, V. Šunjić and F. Kajfež, *Acta pharm. jugosl.* **29**, 53 (1979).
2. W. E. Müller and U. Wollert, *Molec. Pharmac.* **11**, 52 (1975).
3. T. Alebić-Kolbah, F. Kajfez, S. Rendić, V. Šunjić, A. Konowal and G. Snatzke, *Biochem. Pharmac.* **28**, 2457 (1979).
4. G. Gratton, S. Rendić, V. Šunjić and F. Kajfež, *Acta pharm. jugosl.* **29**, 119 (1979).
5. A. Konowal, G. Snatzke, T. Alebič-Kolbah, F. Kajfež, S. Rendić and V. Šunjić, *Biochem. Pharmac.* **28**, 3109 (1979).
6. M. Simonyi, I. Fitos and Zs. Tegyey, *J. chem. Soc. Chem. Commun.* 1105 (1980).
7. M. Simonyi, I. Fitos, Zs. Tegyey and L. Ötvös, *Biochem. biophys. Res. Commun.* **97**, 1 (1980).
8. G. Tóth, E. Fogassy, M. Ács, L. Töke, T. Láng, L. Petöcz, I. Kosotzky and K. Grasser, *7th* International Symposium on Medicinal Chemistry, Malaga, p. 147 (1980).
9. J. Körösi and T. Láng, *Chem. Ber.* **107**, 3883 (1974).
10. G. Tóth, E. Fogassy, M. Ács, L. Töke and T. Láng, *J. heterocycl. Chem.* (in press).
11. K. Simon, to be published.
12. I. Sjöholm, B. Ekman, A. Kober, I. Ljungstedt-Pahlman, B. Seiving and T. Sjödin, *Molec. Pharmac.* **16**, 767 (1979).
13. W. Müller and U. Wollert, *Naunyn-Schmiedeberg's Archs Pharmac.* **280**, 229 (1973).
14. A. Neszmélyi, E. Gács-Baitz, Gy. Horváth, T. Láng and J. Körösi, *Chem. Ber.* **107**, 3894 (1974).
15. M. Patthy and J. Salát, *J. Chromat.* **210**, 159 (1981).
16. C. Lagercrantz, T. Larsson and H. Karlsson, *Analyt. Biochem.* **99**, 352 (1979).