buffer, pH 5.6. Two active fractions A and B were eluted at the NaCl concentrations of 0.8 M and 0.95 M, respectively. After diafiltering both the fractions using Amicon UM 05 filter, the active retentates were lyophilized to yield C (188 mg) from A and D (190 mg) from B. Each lyophilizate was purified by HPLC using a column of MCI gel CQP-10 (60 cm × 7.6 mm ID, Mitsubishi Chemical Industries, Ltd) under the condition of 0.15 M ammonium formate as mobile phase and a flow rate of 2 ml/min. The active fractions E ($t_R = 6.4$ min) from C and F ($t_R = 8.6$ min) from D were chromatographed on a column of Sephadex G-25 (0.2 M acetic acid) and lyophilized to afford GTX I (13 mg) and GTX II (12 mg) as colorless powders, respectively.

GTX I, II and their performic acid-oxidized peptides were subjected to cellulose plate electrophoresis in 0.2 M pyridine acetate buffer, pH 6.5 (45 min at 40 V/cm). Only one cathodally moving band (ninhydrin positive) was observed in all cases and a clear separation between them could be achieved under these conditions. The amino acid compositions of the 2 peptide toxins were determined by amino acid analysis of hydrolysates with constant boiling hydrochloric acid for 20 h at 105 °C (table). The compositions of GTX I and II are similar to each other. GTX II possesses methionine residues instead of glutamine (or glutamic acid) residues in GTX I. Furthermore, hydrolysis of dansylated GTX I and II gave only dansylated arginine as the α-labeled amino acid. These results suggest that each toxin is almost homogeneous. It has been reported[9] that all 3 conotoxins isolated from *C. geographus* have glutamic acid in the N-terminal and histidine, serine and glycine as the internal residues. On the other hand, both of GTX I and II have arginine in the N-terminal and threonine and hydroxyproline residues but not histidine, serine and glycine residues.

As shown in the figure the purified toxins GTX I and II at concentrations above 10^{-8} M markedly inhibited twitch responses of the mouse diaphragm to direct stimulation. After washout of both toxins with the fresh medium, the depressed contractile response to electrical stimulation was progressively restored. However, the conotoxins at 2×10^{-7} M have been shown to cause a marked decline of the twitch response of the mouse diaphragm to indirect nerve stimulation without effects on that to direct stimulation[10]. These observations indicate that nature of pharmacological actions of GTX I and II is quite different from that of conotoxins. The detailed chemical and pharmacological studies on GTX I and II are in progress.

1 Acknowledgments. We are grateful to Mr Z. Nagahama of Okinawa island for supplying specimens of *C. geographus* and to Mr H. Kubota of Atto Corp. and Miss K. Nakazawa of this Institute for amino acid analysis.

2 Kohn, A.J., J. Ecol. Monogr. *29* (1959) 47.
3 Kohn, A.J., Hawaii Med. J. *17* (1958) 528.
4 Whyte, J.M., and Endean, R., Toxicon *1* (1962) 25.
5 Endean, R. Parish, G., and Gyr, P., Toxicon *12* (1974) 131.
6 Spence, I., Gillessen, D., Gregson, R.P., and Quinn, R.J., Life Sci. *21* (1977) 1759.
7 Kobayashi, J., Nakamura, H., Hirata, Y., and Ohizumi, Y., Toxicon *20* (1982) 823.
8 Cruz, L.J., Gray, W.R., and Olivera, B.M., Archs Biochem. Biophys. *190* (1978) 539.
9 Gray, W.R., Luque, A., Olivera, B.M., Barrett, J., and Cruz, L.J., J. biol. Chem. *256* (1981) 4734.
10 McManus, O.B., Musick, J.R., and Gonzalez, C., Neurosci. Lett. *24* (1981) 57.
11 Kobayashi, J., Ohizumi, Y., Nakamura, H., and Hirata, Y., Toxicon *19* (1981) 757.

0014-4754/83/060590-02$1.50 + 0.20/0

Stereoselective binding of 4,5-dihydrodiazepam to human serum albumin

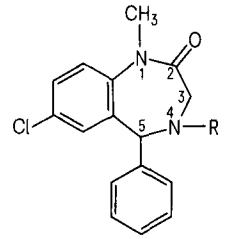
I. Fitos and M. Simonyi[1]

Central Research Institute for Chemistry of The Hungarian Academy of Sciences, P. O. Box 17, H-1525 Budapest (Hungary), September 13, 1982

Summary. S(+)-4,5-dihydrodiazepam was found to have higher binding affinity for human serum albumin than its antipode. The binding of 4-carbamoyl-4,5-dihydrodiazepam is weak and not stereoselective.

Several chiral drugs have been found to exhibit stereoselective binding to serum proteins, indicating the specificity of binding sites[2]. The highest stereoselectivities were obtained for the binding of 3-substituted 1,4-benzodiazepines to human serum albumin (HSA)[2-5]. The phenomenon was interpreted in terms of the inversion of the diazepine ring giving preference to the conformation in which the C_3-substituent is found in the equatorial position. In this work, we studied the stereoselective binding of 4,5-dihydrodiazepam (DHD) as well as 4-carbamoyl-DHD (CDHD) to HSA. These compounds are 1,4-benzodiazepines with a chiral centre at the C_5 position (cf. formula). S(+)-DHD was found[6] to have about 2-3 times stronger anticonvulsant and antiaggressive effects in mice than its antipode.

Materials and methods. Synthesis and resolution of DHD and its 2-^{14}C-labelled form were carried out as previously described[7,8]. Samples of racemic and enantiomeric CDHD were obtained from Chemical Works of Gedeon Richter Ltd, Budapest. ^{3}H-diazepam and ^{14}C-warfarin were purchased from the Institute of Isotopes, Budapest, and The Radiochemical Centre Amersham, respectively. Binding studies with radioactive DHD enantiomers as well as competition experiments were performed by a method[9] applying HSA immobilized on polyacrylamide microparticles. After centrifugation, the concentration of the labelled free drug was measured by liquid scintillation counting of the supernatant. 0.005 M phosphate, 0.1 M KCl buffer



DHD: R = H
CDHD: R = $CONH_2$

(pH = 7.4) was used with 2% ethanol content. Affinity chromatographic studies were performed on a column containing HSA ($\sim 10^{-4}$ M) immobilized on CNBr-activated Sepharose 4B (Pharmacia) using UV-detection. Eluting Ringer buffer (pH = 7.4) contained 0.02% sodium azide.

Results and discussion. The binding of DHD enantiomers in Scatchard representation is shown in figure 1. It can be seen that S(+)-DHD is more strongly bound to HSA than its antipode. The ratio of the vertical intercepts that are characteristic of the overall binding affinities (ΣnK; n: number of binding sites, K: association constant) gives a stereoselectivity factor of 4. The Scatchard plot also shows the considerable contribution of secondary binding sites. In a binding study of DHD enantiomers to mouse and rat sera, the (−)-enantiomer was found[10] to show slightly higher overall affinity. It indicates that the binding stereoselectivity for DHD is species-dependent similarly to tryptophan and warfarin[11].

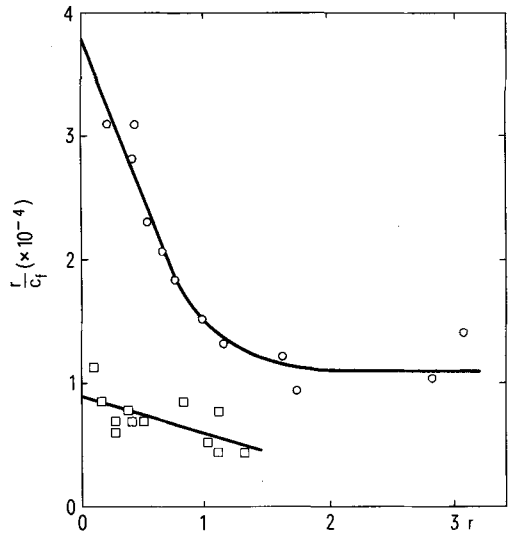


Figure 1. Scatchard plots for the binding of S(+)-DHD (○) and R(−)-DHD (□) to HSA in microparticles (C_{HSA}: 1.45×10^{-5} M, C_{DHD}: 1×10^{-5}–3×10^{-4} M); r, number of moles of the drug bound per mole of HSA; c_f, concentration of the free drug.

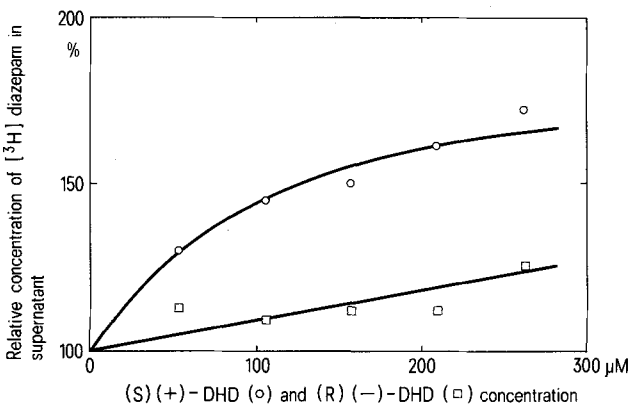


Figure 2. Displacement of ^{3}H-diazepam (11 µM) from HSA in microparticles (13 µM) by S(+)-DHD and R(−)-DHD. 58% of diazepam was bound in the control.

Diazepam is known[12,13] to have 1 specific binding site on HSA and can be used[9] as a marker to study interactions at the 'benzodiazepine' binding site. The other important specific binding site on HSA can be tested by warfarin[9]. Figure 2 shows how the presence of DHD enantiomers influences the binding of radioactive diazepam. It can be seen that both displace diazepam, the effect being larger for the strongly bound S(+)-enantiomer. It suggests that DHD enantiomers do bind at the diazepam binding site. No significant displacement was observed with warfarin marker.

Stereoselective binding can be confirmed by affinity chromatographic resolution of the racemate on immobilized HSA, the elution volumes being characteristic of the binding affinities[11]. On a HSA-sepharose column giving 13 ml for the elution volume of the solvent, R(−)-DHD and S(+)-DHD appeared at 26 ml and 43 ml, respectively. CDHD racemate, however, could not be resolved under the same circumstances. Both enantiomers elute at 17.5 ml, hence binding is not stereoselective and the binding affinity for CDHD is even much lower than that of the less firmly bound DHD enantiomer. The elution volume of CDHD is comparable to that of L-tryptophan. The pronounced decrease in the binding properties brought about by the N_4-carbamoyl substitution on DHD is probably the consequence of different conformations of the derivatives. X-ray analysis[14] proved that the C_5-phenyl group is pseudoequatorial relative to the 7-membered ring in the DHD molecule. In CDHD, however, the C_5-phenyl group was found pseudoaxial due to the inversion of the diazepine ring. It was determined by CD-analysis[15] that (+)-DHD and (−)-CDHD are of (S)-absolute configuration. Results presented suggest that the relative positions of the aromatic rings in the benzodiazepine molecule significantly influence their binding behaviour.

1 Acknowledgment. The authors are indebted to Dr Zs. Tegyey for the preparation of radioactive enantiomers and to Prof. I. Sjöholm for HSA immobilized in microparticles.

2 Alebić-Kolbah, T., Rendić, S., Fuks, Ž., Šunjić, V., and Kajfež, F., Acta pharm. jugosl. *29* (1979) 53.
3 Müller, W., and Wollert, U., Molec. Pharmac. *11* (1975) 52.
4 Simonyi, M., Fitos, I., and Tegyey, Zs., J. chem. Soc. chem. Commun. *1980*, 1105.
5 Simonyi, M., Fitos, I., Tegyey, Zs., and Ötvös, L., Biochem. biophys. Res. Commun. *97* (1980) 1.
6 Pálosi, É., Ürögdi, L., Röhricht, J., Szporny, L., and Kisfaludy, L., Acta pharmac. hung. *43* (1973) 218.
7 Kisfaludy, L., Röhricht, J., Ürögdi, L., Pálosi, É., and Szporny, L., Hung. Path. *160* (1973) 769.
8 Tegyey, Zs., Maksay, G., and Ötvös, L., J. label. Comp. *16* (1979) 377.
9 Sjöholm, I., Ekman, B., Kober, A., Ljungstedt-Påhlman, I., Seiving, B., and Sjödin, T., Molec. Pharmac. *16* (1979) 767.
10 Tegyey, Zs., Maksay, G. Kardos, J., and Ötvös, L., Experientia *36* (1980) 1031.
11 Lagercrantz, C., Larsson, T., and Denfors, I., Comp. Biochem. Physiol. *69C* (1981) 375.
12 Müller, W., and Wollert, U. Naunyn-Schmiedebergs Arch. Pharmak. *280* (1973) 229.
13 Sjödin, T., Roosdorp, N., and Sjöholm, I., Biochem. Pharmac. *25* (1976) 2131.
14 Czugler, M., Kálmán, A., Röhricht, J., Löw, M., Ürögdi, L., and Kisfaludy, L., Tetrahedron Lett. *11* (1977) 917.
15 Ángyán, J., Bánhegyi, Gy., Kajtár, M., and Kiss, Á.I., Magyar Kém. L. *35* (1980) 307.

0014-4754/83/060591-02$1.50 + 0.20/0