Biochemical Pharmacology, Vol. 38, No. 14, pp. 2259–2262, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INVERSE STEREOSELECTIVITY IN THE BINDING OF ACENOCOUMAROL TO HUMAN SERUM ALBUMIN AND TO α_1-ACID GLYCOPROTEIN

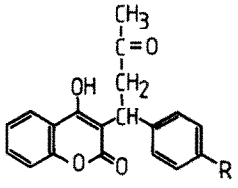
ILONA FITOS, JULIA VISY, ANNA MAGYAR, JUDIT KAJTÁR* and MIKLÓS SIMONYI†

Central Research Institute for Chemistry of The Hungarian Academy of Sciences, P.O. Box 17, H-1525 Budapest, Hungary, and *Institute of Organic Chemistry, Eötvös University, Budapest, Hungary

(*Received* 18 *October* 1988; *accepted* 23 *December* 1988)

Abstract—Stereoselective binding of *rac*-acenocoumarol to human serum albumin (HSA) and α_1-acid glycoprotein (α_1-AGP) was investigated by affinity chromatography and by combined ultrafiltration (UF) and circular dichroism (CD) methods. For HSA, the ratio of the enantiometric constants was $K^R/K^S = 2$, while for α_1-AGP, $K^S/K^R = 3$.

Acenocoumarol is an anticoagulant used as a racemate like other chiral 4-hydroxycoumarin derivatives (e.g. warfarin, phenprocoumon), but unlike them its (*R*)-enantiomer proved to be more potent than the (*S*)-stereoisomer [1]. This discrepancy was explained by the rapid elimination and high clearance [2] of the intrinsically more active (*S*)-acenocoumarol [3]. In rat plasma no significant difference was measured [3] between the binding of the enantiomers. *rac*-Acenocoumarol and *rac*-warfarin were found [4] to have high affinities both to human serum albumin (HSA) and to α_1-acid glycoprotein (α_1-AGP). While the binding of warfarin enantiomers both to HSA [5] and to α_1-AGP [6] has been investigated indicating a slight preference for the (*S*)-enantiomer, we found no information for the binding of acenocoumarol enantiomers. The simple experimental methods used in this work allow unambiguous determination of the binding stereoselectivity.



R=NO2 Acenocoumarol

R=H Warfarin

MATERIALS AND METHODS

rac-Acenocoumarol was obtained from Alkaloida Chemical Factory (Tiszavasvári, Hungary). Its resolution was made through its quinidine salt [7] leading to (−)-(*S*)-acenocoumarol. The (+)-(*R*)-enantiomer was prepared via crystallization of the quinine salt by analogy to (+)-(*R*)-warfarin [8] with the final recrystallization made from ethanol. The optical purities of the enantiomers were checked by CD-spectra shown in Fig. 1. The spectrum obtained in ethanol is in good agreement with those data obtained in methanol [7]. Binding experiments were made in Ringer buffer pH:7.4 containing 0.01% sodium azide, at room temperature. All forms of acenocoumarol were dissolved in NaOH, so they existed as sodium salts. When the drug solution was prepared with buffer containing 1% of ethanol, the CD-spectra and the binding data did not change. *rac*-Warfarin was purchased from Sigma Chemical Co. (St. Louis, MO) and resolved into its enantiomers as described [8]. HSA (fatty acid free) and α_1-AGP were obtained from Miles Labs (Elkhart, IN) and Sigma, respectively.

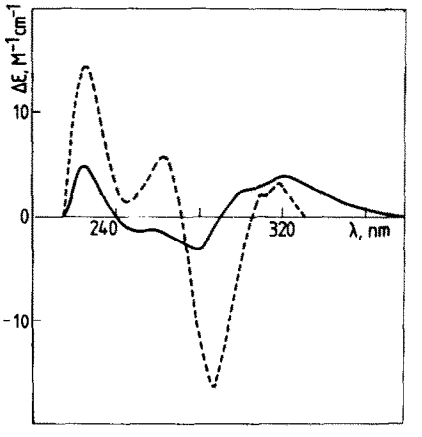


Fig. 1. CD-spectra of (*R*)-acenocoumarol in ethanol (dotted line) and in Ringer buffer (pH: 7.4).

Binding studies by affinity chromatography were performed following the method of Lagercrantz *et al.* [9]. HSA in about 1% was immobilized on CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Elution volumes (V_e) were measured by UV detection.

Ultrafiltrations were carried out in Amicon 10 cells using PM 10 membranes. The concentration of the free drugs in the filtrate was determined by UV spectroscopy ($A_{300} = 2.2 \times 10^4\ M^{-1}\ cm^{-1}$).

Circular dichroism spectra were taken on a Rousell–Jouan (Jobin-Yvon, Longjumeau, France) Dichrograph No. III.

Quantitative determination of the binding stereoselectivity was carried out as described ([10], cf. Eqns (1)–(3)). The experimental parameters were the

† To whom correspondence should be addressed.

degree of dissociation for the racemate ($\alpha_{rac} = c_{free}/c_{total}$) and the optical purity of the ligand in the ultrafiltrate (ξ_f).

$$\alpha_S = \alpha_{rac}(1 - \xi_f) \quad (1)$$

$$\alpha_R = \alpha_{rac}(1 + \xi_f) \quad (2)$$

$$\frac{K^R}{K^S} = \frac{\alpha_S(1 - \alpha_R)}{\alpha_R(1 - \alpha_S)} \quad (3)$$

The ξ_f values were calculated from the molar $\Delta\varepsilon$ values of the pure enantiomers obtained in the same buffer (cf. Fig. 1) at 320 nm.

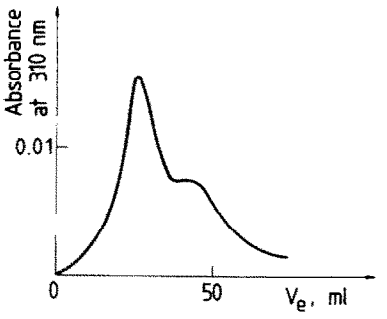


Fig. 2. Elution profile of *rac*-acenocoumarol on a HSA-Sepharose column (V_o: 4.5 ml).

RESULTS

Binding to HSA studied by column affinity chromatography

Figure 2 shows the elution profile of *rac*-acenocoumarol on a short HSA-Sepharose column. The resolution indicates stereoselective binding. The assignment of the peaks were done with resolved enantiomers and their binding affinities (K) were estimated by comparing the elution volumes of standard compounds with known association constants. Data collected in Table 1 indicate that (*R*)-acenocoumarol has the higher binding affinity and the stereoselectivity factor (K^R/K^S) is about 2. It can also be seen that the stereoselectivity is much smaller for the warfarin enantiomers which elute in reversed order ($K^S > K^R$).

Binding to HSA and α_1-AGP studied by combined UF-CD method

In Tables 2 and 3 the free ligand ratio and optical purity values are collected for the ultrafiltrates of *rac*-acenocoumarol with HSA and α_1-AGP, respectively. The stereoselectivities calculated according to Eqns (1)–(3) are also given. Figures 3 and 4 show CD-spectra of ultrafiltrates (i.e. free ligands) obtained from solutions containing *rac*-acenocoumarol as well as HSA or α_1-AGP, respectively. It is striking that they are in mirror image relation. According to the CD-spectra of the pure enantiomers (Fig. 1) the HSA filtrate indicates an enantiomeric excess for the free molecules of (*S*)-configuration. It is in agreement with the results obtained by the chromatographic method that already demonstrated (*R*)-acenocoumarol to have the higher affinity to HSA. In the case of the α_1-AGP filtrate, the molecules of (*R*)-configuration are in excess, i.e. (*S*)-acenocoumarol is of the higher affinity. In Tables 2 and 3 data of the free ligand

Table 1. Elution volumes and corresponding association constants on an HSA-Sepharose column ($V_{gel} \sim 3$ ml)

Compounds	V_e (ml)	K (M^{-1})	Ref.
Solvent	4.5		
(*R*)-Oxazepam acetate	6.5	1.1×10^4	[11]
(*S*)-Oxazepam acetate	23.5	5.5×10^4	[11]
Diazepam	45	1.8×10^5	[12]
(*RS*)-Warfarin	45	2.1×10^5	[12]
(*R*)-Warfarin	40	1.5×10^5	Estimated
(*S*)-Warfarin	52	2.4×10^5	Estimated
(*R*)-Acenocoumarol	41	1.6×10^5	Estimated
(*S*)-Acenocoumarol	25	6.5×10^4	Estimated

Table 2. Binding stereoselectivity of acenocoumarol to HSA determined by combined UF-CD method from the degree of dissociation, and optical purity in the filtrate

c_{rac} (10^{-5} M)	c_{HSA} (10^{-5} M)	α_{rac}	ξ_f (at 320 nm)	K^R/K^S
5.7	3.0	0.39	−0.24	2.2
5.0	5.0	0.21	−0.30	2.1
2.5	2.5	0.32	−0.21	1.9

Table 3. Binding selectivity of acenocoumarol to α_1-AGP determined by combined UF-CD method from the degree of dissociation, and optical purity in the filtrate

c_{rac} (10^{-5} M)	c_{AGP} (10^{-5} M)	α_{rac}	ξ_f at 320 nm	K^S/K^R
5.0	5.0	0.50	+0.26	2.9
5.7	5.8	0.52	+0.33	4.1
10.0	10.0	0.45	+0.28	2.9

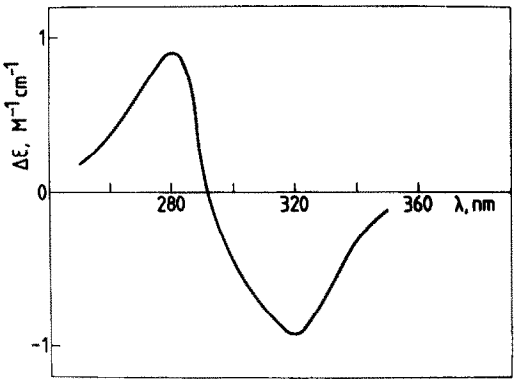


Fig. 3. CD-spectra of the ultrafiltrate ($c_f = 2.2 \times 10^{-5}$ M) of *rac*-acenocoumarol ($c_{rac} = 5.7 \times 10^{-5}$ M) and HSA ($c_{HSA} = 3 \times 10^{-5}$ M).

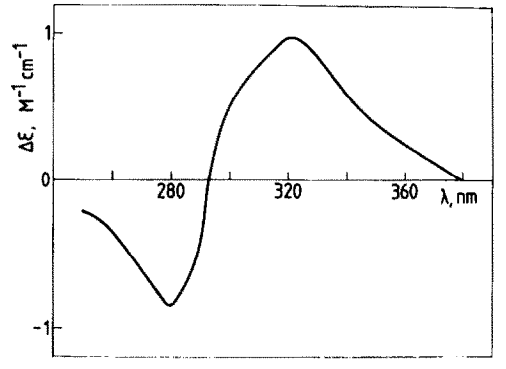


Fig. 4. CD-spectra of the ultrafiltrate ($c_f = 2.5 \times 10^{-5}$ M) of *rac*-acenocoumarol ($c_{rac} = 5 \times 10^{-5}$ M) and α_1-AGP ($c_{AGP} = 5 \times 10^{-5}$ M).

ratios and of the optical purities measured for the ultrafiltrates of *rac*-acenocoumarol as well as HSA and α_1-AGP, respectively, are collected; the stereoselectivities calculated according to Eqns (1)–(3) are also given. It can be seen that for HSA, values of $K^R/K^S \sim 2$ were obtained, while for α_1-AGP, $K^S/K^R \sim 3$–4. Similar experiments were done with *rac*-warfarin but neither HSA, nor α_1-AGP filtrate had significant optical activity, i.e. the binding stereoselectivity is very small to be detected by this method. Separate UF experiments performed with [^{14}C]warfarin enantiomers showed no significant differences in their binding to α_1-AGP either. In displacement studies [6] (*S*)-warfarin was found to have somewhat higher affinity to α_1-AGP. It should be noted that our binding data obtained for the binding of both *rac*-acenocoumarol and *rac*-warfarin to α_1-AGP correspond to lower association constants (3–5×10^4 M^{-1}) than previously reported (2×10^5 M^{-1}) [4, 13]. It may be the consequence of differences in the quality of the protein.

DISCUSSION

Inverse stereoselectivity in serum protein binding has been reported [14, 15] for propranolol, where the enantiomeric ratio of unbound drug in human plasma proved that the effect of α_1-AGP dominated over HSA. In the case of *rac*-acenocoumarol, the binding to HSA is expected to prevail since both its physiological concentration and binding affinity are much higher compared with α_1-AGP. Urien *et al.* [4] who measured similarly high binding constants for the binding of *rac*-acenocoumarol to HSA and α_1-AGP, estimated that in plasma, only about 10% of the bound drug belongs to α_1-AGP. The sensitivity of the CD-method we used is not high enough to detect the optical activity of this highly bound drug in serum filtrate.

Apart from any clinical consequences, the phenomenon that a chiral drug might have reversed binding enantioselectivity on different serum components calls attention to the possibility that an overall lack of enantioselectivity might be the result of selective molecular events of opposite sign. Since the enantioselectivities published for the binding of drugs to α_1-AGP have been generally low [5, 6], the difference between acenocoumarol enantiomers presented here seems to be the greatest found so far.

Acknowledgements—*rac*-Acenocoumarol (Syncumar®) was kindly donated by Dr Vilmos Galamb of Alkaloida Chemical Factory (Tiszavasvári, Hungary). This work was supported by a project No. OTKA 1021.

REFERENCES

1. Meinertz T, Kasper W, Kahl C and Jähnchen E, Anticoagulant activity of the enantiomers of acenocoumarol. *Br J Clin Pharmacol* **5**: 187–188, 1978.
2. Godbillon J, Richard J, Gerardin H, Meinertz T, Kasper W and Jähnchen E, Pharmacokinetics of the enantiomers of acenocoumarol in man. *Br J Clin Pharmacol* **12**: 621–629, 1981.
3. Thijssen HHW, Baars LGM and Drittij-Reijnders MJ, Stereo-selective aspects in the pharmacokinetics and pharmacodynamics of acenocoumarol and its amino and acetamido derivatives in the rat. *Drug Metab Dispos* **13**: 593–597, 1985.
4. Urien S, Albengres E, Zini R and Tillement JP, Evidence for binding of certain acidic drugs to α_1-acid glycoprotein. *Biochem Pharmacol* **31**: 3687–3689, 1982.
5. Müller WE, Stereoselective plasma protein binding of drugs. In: *Drug Stereochemistry Analytical Methods and Pharmacology* (Eds. Wainer IW and Drayer DE), pp. 227–244. Marcel Dekker, New York and Basel, 1988.
6. Brunner F and Müller WE, The stereoselectivity of the "single drug binding site" of human α_1-acid glycoprotein (orosomucoid). *J Pharm Pharmacol* **39**: 986–990, 1987.
7. Wheeler CR and Trager WF, Absolute configuration of acenocoumarin. *J Med Chem* **22**: 1122–1124, 1979.
8. West BD, Preis S, Schroeder CH and Link KP, Studies on the 4-hydroxycoumarins. XVII. The resolution and absolute configuration of warfarin. *J Am Chem Soc* **83**: 2676–2679, 1961.
9. Lagercrantz C, Larsson T and Karlsson H, Binding of some fatty acids and drugs to immobilized bovine serum albumin studied by column affinity chromatography. *Anal Biochem* **99**: 352–364, 1979.
10. Simonyi M, Fitos I, Kajtár J and Kajtár M, Application of ultrafiltration and CD spectroscopy for studying stereoselective binding of racemic ligands. *Biochem Biophys Res Commun* **109**: 851–857, 1982.
11. Fitos I, Tegyey Zs, Simonyi M, Sjöholm I, Larsson

T and Lagercrantz C, Stereoselective binding of 3-acetoxy-, and 3-hydroxy-1,4-benzodiazepine-2-ones to human serum albumin. *Biochem Pharmacol* **35**: 263–269, 1986.
12. Sjöholm I, Ekman B, Kober A, Ljungstedt-Pahlman I, Seiving B and Sjödin T, Binding of drugs to human serum albumin: XI. The specificity of three binding sites as studied with albumin immobilized in microparticles. *Mol Pharmacol* **16**: 767–777, 1979.
13. Schley J, Inhibitory effect of phenothiazines on the binding of [^{3}H]perazine to α_1-acid glycoprotein. *J Pharm Pharmacol* **39**: 132–134, 1987.
14. Walle UK, Walle T, Bai SA and Olanoff LS, Stereoselective binding of propranolol to human plasma, α_1-acid glycoprotein, and albumin. *Clin Pharmacol Ther* **34**: 718–723, 1983.
15. Albani F, Riva R, Contin M and Baruzzi A, Stereoselective binding of propranolol enantiomers to human α_1-acid glycoprotein and human plasma. *Br J Clin Pharmacol* **18**: 244–246, 1984.