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Note

Resolution by affinity chromatography: stereoselective binding of racemic oxazepam esters to human serum albumin

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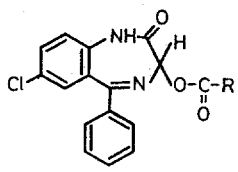
The detection of stereoselective binding of racemic drugs to serum proteins is of great importance for the elucidation of chiral drug action. Applying resolved enantiomers of oxazepam hemisuccinate, Müller and Wollert[1] found that the binding of the *S*(+)-enantiomer to human serum albumin (HSA) is about 30 times stronger than that of the *R*(−)-antipode. Ultrafiltration techniques developed specifically for the study of racemic compounds[2–4] have been successfully applied for the quantitative determination of stereoselective binding of oxazepam acetate. Increasing the hydrophobicity of the acyl moiety in oxazepam esters:



however, causes systematic errors due to non-specific binding of the ligand to ultrafiltration membranes.

Proteins immobilized in hydrophylic gels usually maintain a specific binding affinity towards small molecules[5]. Hence, affinity chromatography can be used to detect the stereoselectivity of binding to serum albumin[6–8]. This method even provides quantitative data on binding affinity if certain column parameters are known[9]. As non-specific binding of the drugs can be avoided and very small amounts of racemic samples are applicable, the chromatographic technique seems superior to ultrafiltration in most instances. A micro-preparative resolution of racemic oxazepam acetate on an HSA–Sepharose column has been performed, which allowed the chiroptical parameters for the enantiomers to be determined[4].

In this paper we compare different methods for studying the stereoselective binding of a number of oxazepam esters to HSA.

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EXPERIMENTAL

Racemic oxazepam esters were prepared from oxazepam with acyl chlorides using pyridine-catalysed acylation[10]. Lyophilized HSA was obtained from the "Human" Serum and Vaccine Institute, Budapest, Hungary. Ringer buffer (pH 7.4) with 0–2% of ethanol was used.

Affinity chromatographic resolution of racemic samples (5 μg in 20 μl of ethanol) was performed on a column containing HSA immobilized on cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) using UV detection. The HSA concentration was about 10^{-4} *M*. The eluent contained 0.02% of sodium azide. The elution of ethanol was indicated by a small negative peak.

Preparative ultrafiltration was carried out in an Amicon 202 cell using PM-10 membranes. Partially resolved esters were extracted with chloroform from both the filtrate and the retentate and purified by thin-layer chromatography. Concentrations were determined from UV spectra. Circular dichroism spectra were taken in ethanol solution on a Roussell-Jouan (Jobin-Yvon, Longjumeau, France) Dichrograph No. III instrument.

RESULTS

All racemic oxazepam esters studied could be eluted as two equivalent peaks. Fig. 1 shows the chromatographic resolution of racemic oxazepam propionate, and also that of the samples partially resolved by ultrafiltration. The filtrate contains more of the weakly bound enantiomer, while the ligand obtained from the retentate is enriched with the strongly bound stereoisomer. Table I shows the elution volumes of the enantiomers obtained by the resolution of different racemic oxazepam esters. Elution volumes over 50 ml could not be determined accurately owing to the broad and flat character of the peaks (see Fig. 1). The elution volume for diazepam is also given which corresponds to a binding constant (K)[11] of about $1.8 \cdot 10^5$ M^{-1}. The ratio given in Table I, which includes the elution volumes of corresponding enantiomers corrected for the elution volume of a ligand with no specific interaction (*e.g.*, solvent), is characteristic of the stereoselectivity[9]. Considering separately the variation in the elution volumes V_R (first peak) and V_S (second peak) due to the increasing hydrophobicity of the acyl moiety from acetate to phenylacetate, it can be observed that the binding affinity increases for both enantiomers, leaving the stereoselectivity almost unchanged. Methylsuccinate ester shows a low binding affinity, in accordance with its low hydrophobicity. The relative decrease is, however, more pronounced for the (*S*)-enantiomer (*cf.*, acetate), resulting in a smaller stereoselectivity. The hemisuccinate with an anionic nature exhibits a strikingly higher stereoselectivity than the other esters, because the binding affinity is small for the (*R*)-enantiomer, in accordance with its polar character, whereas it is relatively high for the (*S*)-enantiomer.

These results can be compared with those obtained by a combined ultrafiltration–circular dichroism method[4] (Table II). Because of non-specific binding to the membrane, which is increasingly disturbing with increasing hydrophobicity and the decreasing water solubility of the ligand, the binding constants of the enantiomers could not be calculated separately and only their ratio, *i.e.*, their stereoselectivity, is indicated. The stereoselectivity is the lowest for methylsuccinate ester

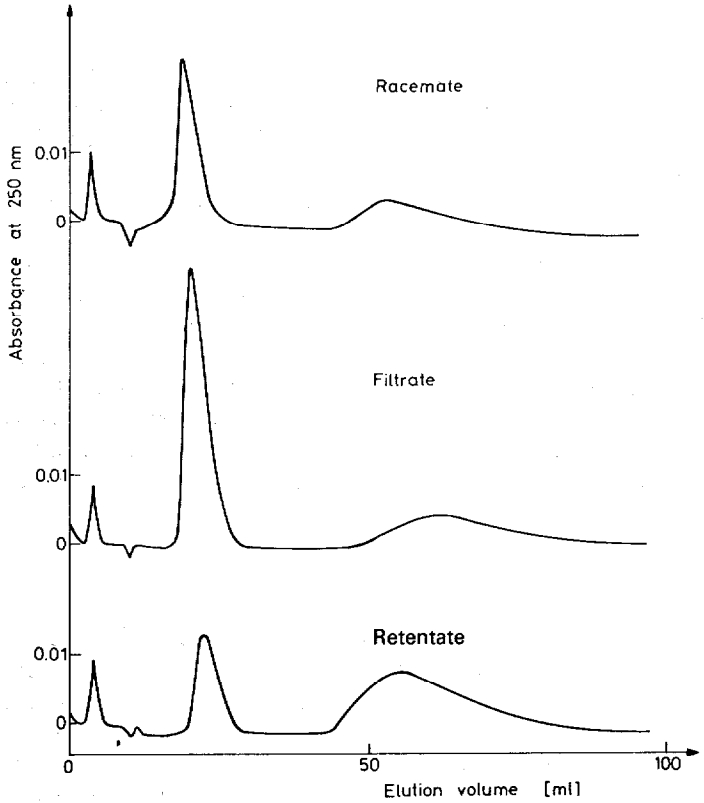

Fig. 1. Chromatographic resolution of oxazepam propionate on an HSA–Sepharose column. Elution profiles for racemate and for samples partially resolved by ultrafiltration.

TABLE I

RESOLUTION OF OXAZEPAM ESTERS ON AN HSA–SEPHAROSE COLUMN

Elution volumes for $R(-)$-enantiomers (V_R) and $S(+)$-enantiomers (V_S) and their application for characterizing stereoselectivity.

Oxazepam ester	V_R *(ml)*	V_S *(ml)*	$\frac{V_S - 11}{V_R - 11}$
(Solvent)	11		
Acetate	19	46	4.4
Propionate	23	53	3.5
n-Butyrate	28	85	4.4
Isobutyrate	28	85	4.4
Pivaloate	37	96	3.3
α-Ethylbutyrate	65	> 150	
Phenylacetate	73	> 150	
Methylsuccinate	20	34	2.6
Hemisuccinate	16	70	11.8
(Diazepam)	65		

TABLE II

STEREOSELECTIVITY FOR THE BINDING OF OXAZEPAM ESTERS TO HSA

Optical purities for the ligand in the filtrate (ξ_f) and retentate (ξ_r) were measured by circular dichroism after ultrafiltrating solutions of racemic oxazepam esters and HSA.

Oxazepam ester	C_{ester} ($M \times 10^{-5}$)	C_{HSA} ($M \times 10^{-5}$)	ξ_f	ξ_r	$\frac{K_S}{K_R}$
Acetate	8.1	15.0	−0.565 ± 0.01	0.265 ± 0.01	6.5 ± 1
Propionate	3.0	4.5	−0.41 ± 0.02	0.58 ± 0.03	10 ± 3
n-Butyrate	3.2	3.0	−0.31 ± 0.01	0.67 ± 0.03	12 ± 3
Isobutyrate	2.9	3.8	−0.35 ± 0.01	0.56 ± 0.03	8 ± 3
Pivaloate	1.1	1.5	−0.39 ± 0.02	0.44 ± 0.01	6 ± 2
Methylsuccinate	5.1	4.5	−0.26 ± 0.02	0.37 ± 0.02	4 ± 2
Hemisuccinate*					30**

* Hydrolysed during purification.
** From ref. 1.

and the largest for hemisuccinate, in agreement with the chromatographic results. The variation of the stereoselectivity in going from acetate to pivaloate does not seem to be significant owing to the large uncertainty of the K_S/K_R values.

Oxazepam, the parent 3-hydroxy compound, was also investigated. It cannot be resolved into enantiomers because of its very rapid racemization in solution[12]. Its peculiar elution profile, shown in Fig. 2, is presumably a manifestation of its stereoselective binding and tends to indicate the lack of racemization for the bound enantiomer.

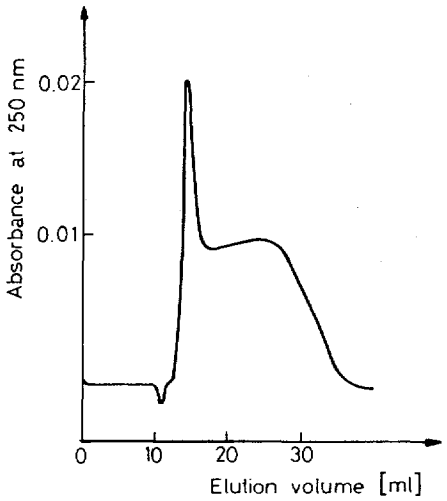

Fig. 2. Elution profile of oxazepam on an HSA–Sepharose column. Elution volume of the solvent, 11 ml.

DISCUSSION

Summarizing the changes obtained by varying the acyl moiety, the following conclusions can be drawn.

(1) Increasing hydrophobicity of the acyl group increases the binding of both enantiomers without a significant change in the stereoselectivity. This is in accordance with the observation[10] that the binding of racemic oxazepam esters to HSA gives a good correlation with the hydrophobicity. On the other hand, our findings seem to be in contradiction with the results found for 3-alkyl-substituted compounds. The stereoselectivity factor of 7 for the 3-isopropyl derivative[13] compared with the factor of 40 for the 3-methyl compound[14] was explained[13] by steric hindrance that occurs only with the (*S*)-enantiomer.

(2) The presence of polar atoms in the acyl moiety (*cf.*, methylsuccinate ester) decreases both the binding affinities of the enantiomers and the stereoselectivity.

(3) A negative charge on the acyl moiety (*cf.*, hemisuccinate) decreases the binding of the (*R*)- and enhances the binding of the (*S*)-enantiomer, resulting in a high stereoselectivity.

Elution volumes obtained by affinity chromatography are characteristic of the overall binding affinity of the strongest binding site at the protein. Hence, it cannot substitute techniques for detailed studies on binding. Nevertheless, it is a rapid, useful and economic method when relative values are to be investigated. The results with oxazepam showed that phenomena that cannot be observed in solution might be detected for the bound molecule by chromatography.

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