Vol. 97, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 17, 1980 Pages 1-7

STATIONARY ULTRAFILTRATION: A RADIO-TRACER TECHNIQUE FOR STUDYING STEREOSELECTIVE BINDING OF RACEMIC LIGANDS

M. Simonyi, I. Fitos, Zs. Tegyey and L. Ötvös

Central Research Institute for Chemistry of the
Hungarian Academy of Sciences, Budapest, Pf. 17, H-1525.

Received September 8,1980

SUMMARY

The continuous exchange of the radioactive free ligand by its non-labelled isotopomer of identical concentration does not affect the position of the overall-equilibrium. The replacement of the enantiomer of higher binding affinity lags behind the replacement of the other one that leads to stereoselective labelling of racemates. Radioactivity, as a function of filtrate volume can be fitted to theoretical expressions. The method allows to study stereoselectivity of binding equilibria set by racemic ligands.

INTRODUCTION

Stereoselective binding of drugs to serum proteins is a problem of increasing pharmacological interest. The ability of chiral 1,4-benzodiazepines to bind enantioselectively has been well recognized in studies applying resolved enantiomers (1-3). Stationary ultrafiltration (SUF) is applicable to establish the degree of stereoselectivity in binding of racemates to serum proteins. The principle of the method is the selective radio-isotopic exchange of enantiomers without disturbing the racemic composition of the ligand (4).

MATERIALS AND METHODS

Lyophilized human serum albumin (HSA) was obtained from "Human" Serum and Vaccine Institute, Budapest and was used without further purification. The HSA concentration was calculated according to the molecular weight, 66248 (5). Salicylic acid (SA) was of analytical grade. 5-[^{3}H]-salicylic acid (1.18 mCi/mmol = 43.6 GBq/mmol) and *d,l*-3-acetoxy-7-chloro-5-(2-chloro-phenyl)-1,3-dihydro-3H-1,4-benzodiazepin-2-one-2-^{14}C = 2-[^{14}C]-*d,l*-lorazepam acetate (19 mCi/mmol = 703 MBq/mmol) were purchased from the Institute of Isotopes, Budapest. *d,l*-3-acetoxy-7-chloro-5-phenyl-1,3-dihydro-3H-1,4-benzodiazepin-2-one = *d,l* oxazepam acetate (OAc) and its 2-[^{14}C]-labelled form (2.6 mCi/mmol = 96.2 MBq/mmol) were synthetized according to (6) and (7), respectively. *d,l*-lorazepam acetate (LAc) was a gift from Mrs. E. Simon-Trompler. Radiochemical purities of 2-[^{14}C]-LAc and 2-[^{14}C]-OAc were 97% and 99%, respectively.

0006-291X/80/210001-07$01.00/0

Solutions were prepared in Ringer buffer pH 7.4. OAc solutions contained 1%, while LAc solutions 2% ethanol. Equal concentrations for the radioactive and inactive solution-pairs were adjusted by UV absorbance (A^{230}_{OAc}: $3.4 \cdot 10^4$ $M^{-1}cm^{-1}$; A^{232}_{LAc}: $2.6 \cdot 10^4$ $M^{-1}cm^{-1}$). In case of SA the inactive solution was labelled. Initial specific radioactivity was chosen in the range of 0.1-1.0 μCi/ml (3,7-37 kBq/ml). SUF was carried out in Amicon Model 8MC cell. Feed solution was prepared in Amicon 202 cell in advance. PM-10 membranes were used for SA, while YM-10 for OAc and LAc. Filtrate was collected in 0.1-1 ml fractions.

Radioactivity was measured by liquid scintillation counting in a Packard model 2650 instrument. Actual radioactivity content of the cell as a function of the filtrate volume was calculated as a difference of the initial radioactivity and that found in the cumulated filtrate. Values of α^* are exact within $\pm$ 0.02, while errors in α_l and α_d are believed to be within $\pm$ 0.01.

LAc samples for CD analysis were prepared by a simple UF of 400 ml racemate - HSA solution (c_o: $1.4 \cdot 10^{-4}$ M, c_p^o: $1.5 \cdot 10^{-4}$ M); LAc was extracted by chloroform from both the filtrate and retentate and purified by thin-layer-chromatography. The quantity of pure LAc was determined from the UV spectra. CD spectra were taken on a Jouan Quetin Dichrographe III. Curve fittings were done by a HP-97 calculator using the method of least-squares.

RESULTS

In continuous operation (8,9), the protein-free feed solution entering the UF cell and the protein-free filtrate leaving the cell have identical flow rates. As a result, the volume of the cell content (V) as well as the protein concentration in the cell (c_p^o) remain constant. The total concentration of the ligand in the cell, c_o, is the sum of bound and free ($c_f = \alpha c_o$) components; the latter being ultrafiltrable equals the concentration of the ligand in the filtrate. Let the feed solution and the filtrate have identical compositions: you end up with constant ligand concentration in the cell throughout the experiment. We suggest the name stationary ultrafiltation (SUF) for this operation involving stationary equilibrium; it is particularly useful for studying the isotopic replacement of ligands.

Having radioactive ligand in the cell, the replacement by non-labelled ligand during SUF is reflected by the concentration of radioactivity in the filtrate, viz.,

$$c_f^* = \alpha c_o \exp\left(-\frac{\alpha x}{V}\right) \qquad (1)$$

where x is the volume of the filtrate. Eq.(1) is widely applicable because its validity does not depend on such details as the quality of binding sites or number of equi-

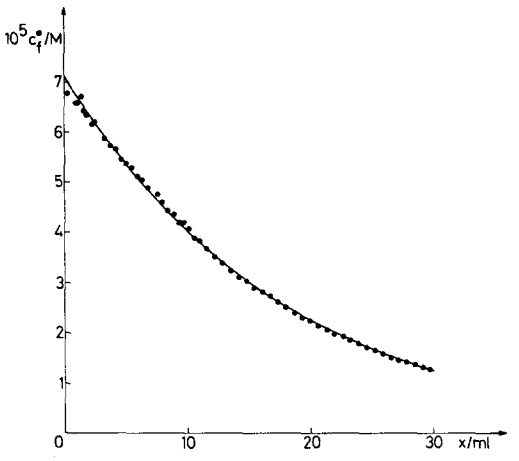


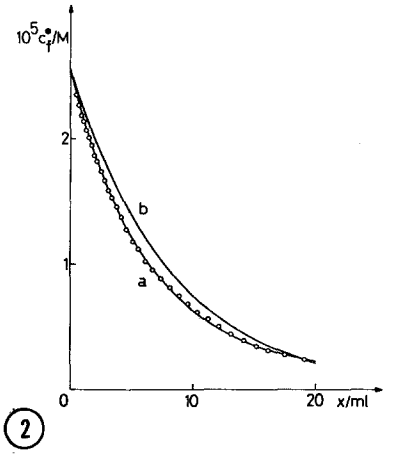
Figure 1. Replacement of 5-[^{3}H]-SA in SUF. Experimental data (closed circles) fit eq. (1) (curve drawn). $c_o = 2.1 \cdot 10^{-4}$ M; $c_p^o = 1.51 \cdot 10^{-4}$ M; V = 5.9 ml; α= 0.34.

libria as far as only one ligand is involved. An example is given in Fig.1 for the replacement of 5-[^{3}H]-SA binding to HSA.

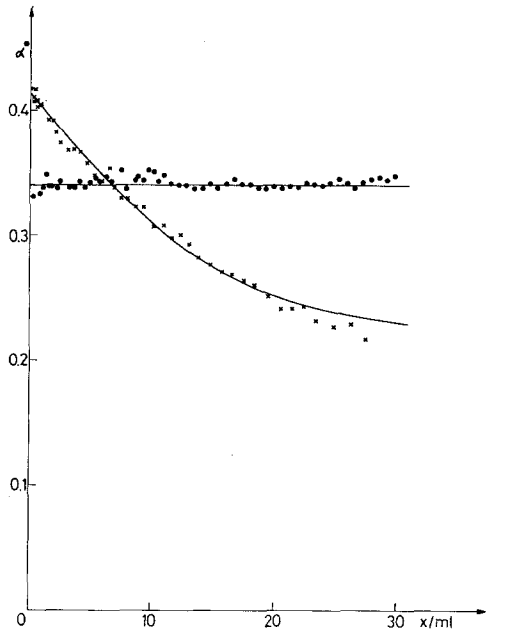
If a chiral ligand is applied in racemic form and the binding is stereoselective, eq. (1) should be modified. Simultaneous enantiomeric equilibria result in a sum of exponentials for the radioactivity of the filtrate as a function of filtrate volume:

$$c_f^* = \frac{c_o}{2} \left[\alpha_l \exp\left(-\frac{\alpha_l x}{V}\right) + \alpha_d \exp\left(-\frac{\alpha_d x}{V}\right)\right] \qquad (2)$$

where α_l and α_d are constants in the given conditions and characteristic of levo- and dextrorotatory enantiomers, respectively. Eq.(2) allows for the possibility of stereoselective labelling. Having exchanged a critical volume, the exponential involving the higher α value can be neglected indicating that the corresponding enantiomer of lower binding affinity has practically been replaced while the other one remained labelled in the cell. Consequently, the radioactive degree of dissociation, α^* depends on the filtrate volume [eq.(3)] approaching the smaller enantiomeric α value, i.e.



(2)



(3)

Figure 2. Replacement of 2-[^{14}C]-LAc in SUF. Experimental data (open circles) fit eq.(2) (curve a) and are incompatible with eq.(1) (curve b). c_o = 6.9·10^{-5} M; c_p^o = 1.51·10^{-4} M; V = 3.0 ml; α = 0.37; α_l = 0.57; α_d = 0.17.

Figure 3. Dependence of the radioactive degree of dissociation (α^*) on the filtrate volume in SUF. Data of OAc (x) fit eq. (3) (curve drawn). c_o = 4.9·10^{-5} M; c_p^o = 8.9·10^{-5} M; V = 3.2 ml; α = 0.40; α_l = 0.61; α_d = 0.19. Data of SA (closed circles) taken from the experiment shown in Fig.1. scatter around α = 0.34.

$$\alpha^*(x) = \frac{Vc_f^*(x)}{Vc_o - \int_0^x c_f^*(x)dx} = \frac{\alpha_l \exp(-\frac{\alpha_l x}{V}) + \alpha_d \exp(-\frac{\alpha_d x}{V})}{\exp(-\frac{\alpha_l x}{V}) + \exp(-\frac{\alpha_d x}{V})} \qquad (3)$$

that of the enantiomer possessing higher binding affinity.

The fit of experimental values to eq.(2) (Fig.2), or to eq.(3) (Fig.3) provides the values of α_l and α_d. Assignment can be made by CD spectra of the ligand partially resolved (10) in simple ultrafiltration (Fig.4). The enantiomers of higher binding affinity of both LAc and OAc were found to be dextrorotatory and of S absolute configuration (11), similarly to other 3-substituted 1,4-benzodiazepines (1-3). Provided that

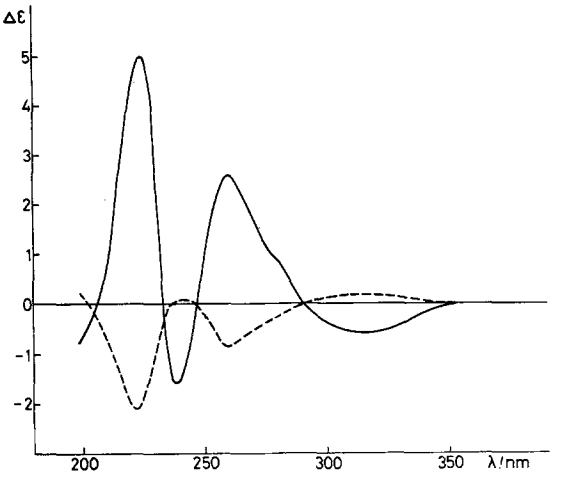


Figure 4. CD spectra of LAc partially resolved in simple UF. Heavy line represents S(+) enantiomer (11) extracted from retentate (cf. Materials and Methods). Broken line represents R(-) enantiomer extracted from filtrate. Spectra were taken in MeOH solvent.

the enantiomers bind to the same site on HSA, the degree of stereoselectivity, π, is given by

$$\pi = \frac{K^d}{K^l} = \frac{1-\alpha_d}{\alpha_d} \frac{\alpha_l}{1-\alpha_l} \qquad (4)$$

DISCUSSION

The success of SUF is subject to several conditions. The radiochemical purity of the labelled ligand has to be high. Simple UF of the system should provide filtrate the concentration of which is independent of filtrate volume; then feed solution can easily be prepared. At last, the attainment of the dynamic equilibrium has to be much faster than the rate of filtration. These conditions are met also for SA binding to HSA (cf. Fig.1) though a time period of 4-12 hours was indicated (12) to be necessary to reach equilibrium.

The application of the method to LAc binding to HSA resulted in experimental data which can be fitted to eq.(2) but are clearly incompatible with eq.(1) (cf. Fig.2), hence the binding process is stereoselective represented by largely differing α_l and α_d values. The transformation of experimental data into α^* values was made for OAc as well as for SA binding

Table 1. Stereoselectivity from SUF of racemic 3-substituted--1,4-benzodiazepines binding to HSA

Ligand	$10^5 c_p^o$/M	$10^5 c_o$/M	α_l	α_d	π §
LAc	15.1	6.9	0.57±0.01	0.17±0.01	6.5±0.7
OAc	8.9	4.9	0.61±0.01	0.19±0.01	6.7±0.7

§ According to eq.(4)

to HSA. The satisfactory fit to the theoretical expression for OAc proves that α^* decreases with filtrate volume as a consequence of stereoselectivity, in the lack of which α^* is constant (cf. Fig.3).

Table 1 gives the degrees of stereoselectivity for the two 3-acetoxy-1,4-benzodiazepines; the values are close to 7 reported for a 3-isopropyl-derivative (3) but are much smaller than 49 [oxazepam hemisuccinate (1)] or 40 [3-methyl-derivative (2)]. Care should be taken in comparing the stereoselectivities, since our values refer to simultaneous binding of racemic ligands and thus, could be influenced by competition if the binding sites are common for the two enantiomers, an assumption suggested earlier (1,13) but questioned recently (2,14). The application of SUF for systems in which the stereoselectivity is known from experiments with resolved enantiomers may contribute to the elucidation of the nature of binding sites.

ACKNOWLEDGEMENTS

The authors thank Mrs. E. Simon-Trompler for the synthesis of *d,l*-lorazepam acetate, and Dr. M. Kajtár for taking CD spectra. This work was supported by a project of the Hungarian Academy of Sciences.

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