Journal of Chromatography, 487 (1989) 257–264
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4523

α_1-ACID GLYCOPROTEIN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COLUMN (ENANTIOPAC) AS A SCREENING TOOL FOR PROTEIN BINDING

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(First received June 16th, 1988; revised manuscript received September 30th, 1988)

SUMMARY

An attempt was made to correlate retention behavior on a high-performance liquid chromatographic (HPLC) system employing an immobilized α_1-acid glycoprotein (AAG) column with AAG binding behavior for various compounds Protein binding was assessed by propranolol displacement studies in an equilibrium dialysis system using isolated AAG. HPLC retention behavior poorly correlated with propranolol displacement from AAG. This system is not suitable for use as a screening tool for AAG affinity.

INTRODUCTION

A new high-performance liquid chromatography (HPLC) stationary phase consisting of immobilized α_1-acid glycoprotein (AAG) bound to a silica support was developed by Hermansson [1]; this column is commercially available as EnantioPAC from LKB Produkter. This stationary phase was developed in response to the need to separate stereoisomers of various drugs; it takes advantage of the stereoselectivity in AAG-binding behavior reported for propranolol and other enantiomeric compounds [2,3].

In the present investigation, the EnantioPAC column is used in a different manner. The purpose of the work is to evaluate the ability of the EnantioPAC HPLC column to function as a means of estimating the relative binding affinities of various drugs for AAG. In order to do this, the relationship between the ability of a compound to displace propranolol from AAG and the retention time of the compound on the EnantioPAC column is explored. If retention characteristics

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on the column could be ascribed to AAG binding, then the capacity factor could be used as a rapidly and easily determined indication of the relative affinity of a drug or endogenous substance for interaction with AAG. The compounds selected for this work were either achiral or optically pure in order to avoid the stereoselective AAG binding behavior mentioned above.

EXPERIMENTAL

Materials

The radiolabelled *l*-propranolol (21.3 Ci/mmol) was purchased from Dupont NEN Research Products (Boston, MA, U.S.A.); the purity of this material was verified by thin-layer chromatography to be greater than 95%. Chlorpromazine·HCl was obtained from Smith, Kline and French Labs. (Philadelphia, PA, U.S.A.) and diazepam was obtained from Hoffmann-La Roche (Nutley, NJ, U.S.A.) The remaining compounds as well as the human AAG (Lot No. 104F-9355) were purchased from Sigma (St. Louis, MO, U.S.A.). The isopropanol and phosphate buffer salts were certified ACS grade and purchased from Fischer Scientific (Fair Lawn, NJ, U.S.A.). All chemicals were used as received without further purification. Spectra/Por 2 cellulose dialysis membrane (12 000–14 000 molecular mass cut-off) was manufactured by Spectrum Medical Industries (Los Angeles, CA, U.S.A.).

Equilibrium dialysis

Tritiated *l*-propranolol (1.56 n*M*) in a 0.13 *M* phosphate buffer solution of either pH 6.0 or pH 7.4 was dialyzed at 37°C across a Spectra/Por 2 membrane against a 20 μM human AAG in 0.13 *M* phosphate buffer solution of the same pH. The protein solution without addition served as the control; quantities of the displacing compounds sufficient to make a 40 μM solution were added to aliquots of the protein solution for the displacement determinations. At equilibrium (8 h), each side of each dialysis cell was sampled for liquid scintillation counting (Model 3255 Tri-Carb, Packard Instruments, Downers Grove, IL, U.S.A.). After correction for quenching by the external standard ratio method, the free fraction (f_p) for *l*-propranolol was determined for each cell as the ratio of corrected counts per minute in the buffer side to corrected counts per minute in the protein side. Each f_p was the mean of three or four determinations.

Chromatographic system

Chromatography was performed using a Milton-Roy miniPump (Milton-Roy, Riviera Beach, FL, U.S.A.), a Rheodyne Model 7120 injector with a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.), the EnantioPAC column (10 μm, 100 mm × 4 mm; LKB Produkter, Bromma, Sweden) and a Varian Varichrome variable-wavelength detector at 215 nm (Varian, Palo Alto, CA, U.S.A.). All chromatographic studies were performed at room temperature. The retention times of the compounds were noted after injection of 20 μl of an 80 μM solution of the compound in 0.02 *M* phosphate buffer adjusted to the pH of the mobile phase buffer; t_0 was determined by the appearance of the solvent front after each injection.

Mobile phase composition and flow-rates varied depending upon the pH of analysis. These experiments were performed at two pH levels, 6.0 and 7.4. The conditions at pH 6.0 were isopropanol–0.02 *M* pH 6.0 phosphate buffer (6:94, v/v) at a flow-rate of 0.30 ml/min; at pH 7.4, the conditions were isopropanol–0.02 *M* pH 7.4 phosphate buffer (25: 75, v/v) at a flow-rate of 0.13 ml/min.

Data analysis

Retention times and capacity factors were the expressions of retention on the EnantioPAC column which were related to the protein binding expressions. Propranolol free fraction (the ratio of free to total propranolol concentrations) was the measurement of AAG affinity. In addition, propranolol binding was expressed in transformed fashion, as either K_iI (the product of the affinity constant of the displacer and displacer concentration) or $\%\Delta f_p$ (percentage change in propranolol free fraction). K_iI was calculated using eqn. 1 and making the assumption that free propranolol (< 1.5 n*M*) was much less than the dissociation constant K_d for propranolol binding (2.5 μM [4]) both in the presence and in the absence of the displacing compounds.

$$K_iI = \frac{B/F}{B'/F'} - 1 = \frac{(1-f_p)/f_p}{(1-f_p')/f_p'} - 1 \quad (1)$$

where B is the bound propranolol concentration, F is the free propranolol concentration and f_p is the propranolol free fraction; B', F' and f_p' represent the same quantities in the presence of a potential displacer.

Data analysis involved the use of correlation techniques between an expression of retention behavior (t_R, k') and an expression of propranolol displacement (f_p, $\%\Delta f_p$ or K_iI) at each of the two pH values examined. The correlation coefficients were tested for statistical significance in order to estimate the strength of the relationship.

RESULTS

Table I presents the results of the equilibrium dialysis experiments at both pH 7.4 and pH 6.0. The propranolol displacement potential for each compound can be evaluated by the propranolol free fraction (f_p), by the percentage increase in free fraction ($\%\Delta f_p$) or by the product of the affinity constant of the displacer for AAG and the displacer concentration (K_iI). The decreased range of propranolol free fractions with the various displacers as well as the increased control free fraction at pH 6.0 lead to the conclusion that the binding site on AAG is significantly altered by pH; the pH 7.4 data can be expected to reflect the physiological situation more accurately.

Fig. 1 shows the typical chromatogram resulting from the injection of a buffered displacer solution; there were no qualitative differences between the appearance of the chromatograms at pH 6.0 and pH 7.4. Table II presents the results of the HPLC studies as capacity factors at both pH 7.4 and pH 6.0.

Fig. 2 and 3 are plots of capacity factors against propranolol free fractions at pH 7.4 and pH 6.0, respectively. Plots of the other expressions of the data are not

TABLE I

FREE FRACTION (f_p) FOR THE BINDING OF *l*-PROPRANOLOL (1.56 n*M*) TO AAG (20 μM) IN THE PRESENCE OF POTENTIAL DISPLACERS AT A CONCENTRATION OF 40 μM

Equilibrium dialysis for 8 h in 0.13 *M* phosphate buffer at pH 7.4 or pH 6.0. $n=3$ or 4.

Substance	f_p (mean ± S.D.)[a]	
	pH 7.4	pH 6.0
Control	0.306 ± 0.015	0.867 ± 0.021
Dipyridamole	0.866 ± 0.009	0.916 ± 0.033
Perhexilene	0.799 ± 0.017	0.929 ± 0.012
Perphenazine	0.756 ± 0.007	0.857 ± 0.009
Chlorpromazine	0.750 ± 0.011	0.817 ± 0.027
Triflupromazine	0 740 ± 0.028	0.814 ± 0.103
Nifedipine	0.686 ± 0.011	0.783 ± 0.014
Trifluoperazine	0.676 ± 0.024	0.819 ± 0.056
Dibucaine	0.647 ± 0.020	0.906 ± 0.051
Quinidine	0.564 ± 0.018	0.899 ± 0.024
Lidocaine	0.554 ± 0.030	N.D.[b]
Haloperidol	0 547 ± 0.025	0.888 ± 0.014
Procaine	0.527 ± 0.035	0.791 ± 0.031
Butacaine	0.526 ± 0.039	0.890 ± 0.035
Diazepam	N.D.[b]	0.881 ± 0 020
d,l-Propranolol	N.D.[b]	0.891 ± 0.014

[a] f_p = unbound propranolol concentration/total propranolol concentration.
[b] N.D. = not determined.

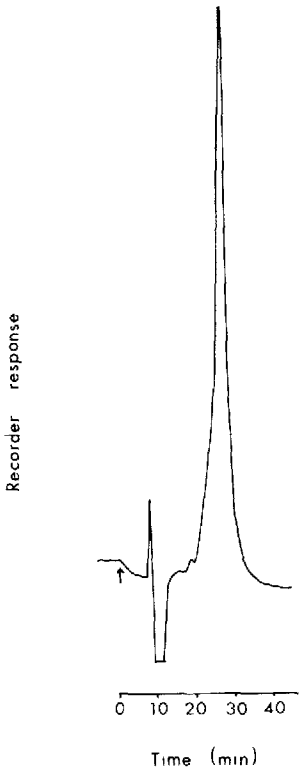

Fig. 1. Chromatogram resulting from injection of 20 μl of an 80 μM solution of quinidine in 0.02 *M* pH 7.4 phosphate buffer. Arrow denotes injection. Mobile phase: isopropanol–0.02 *M* pH 7.4 phosphate buffer (25:75).

TABLE II

CAPACITY FACTORS (k') RESULTING FROM HPLC ANALYSIS USING THE ENANTIOPAC COLUMN AT pH 7.4 AND pH 6.0.

Mobile phase at pH 7.4: isopropanol–0.02 *M* phosphate buffer (25:75); mobile phase at pH 6.0: isopropanol–0.02 *M* phosphate buffer (6:94).

Substance	k' at pH 7.4	k' at pH 6.0
Dipyridamole	0.458	N.D.[a]
Perhexilene	1.25	6.61
Perphenazine	3.89	N.D.[a]
Chlorpromazine	8.64	30.6
Triflupromazine	6.35	28.1
Nifedipine	0.301	N.D.[a]
Trifluoperazine	6.42	6.89
Dibucaine	2.01	6.33
Quinidine	2.13	3.86
Lidocaine	0.771	0.278
Haloperidol	3.59	6.83
Procaine	1 10	0.417
Butacaine	1.37	2.36
Diazepam	0.867	6.00
d,l-Propranolol[b]	3.00	6.78

[a]N.D. = not determined.
[b]Propranolol isomers were not resolved in either case.

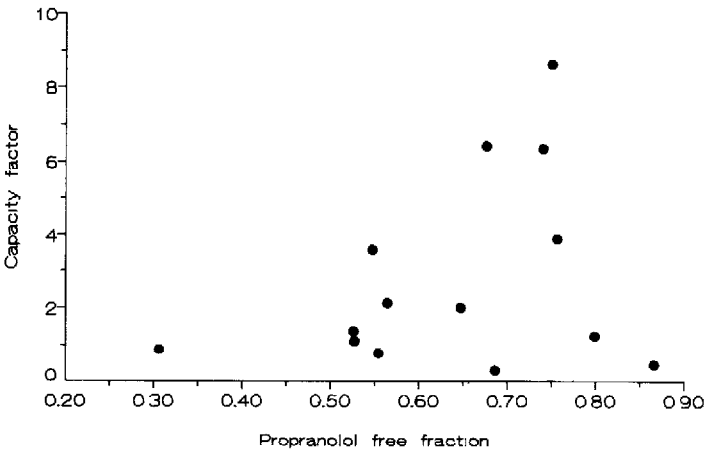

Fig. 2. Plot of capacity factors against propranolol free fractions determined at pH 7.4. HPLC mobile phase: isopropanol–0.02 *M* pH 7.4 phosphate buffer (25:75) at 0.13 ml/min. Protein binding: 0 13 *M* pH 7.4 phosphate buffer against 20 μM AAG in buffer for 8 h at 37°C.

included here but are very similar in appearance. Table III summarizes the correlation coefficients calculated for each pair of expressions at pH 7.4 and pH 6.0, respectively. The p values in the table result from testing the hypotheses that each $r=0$.

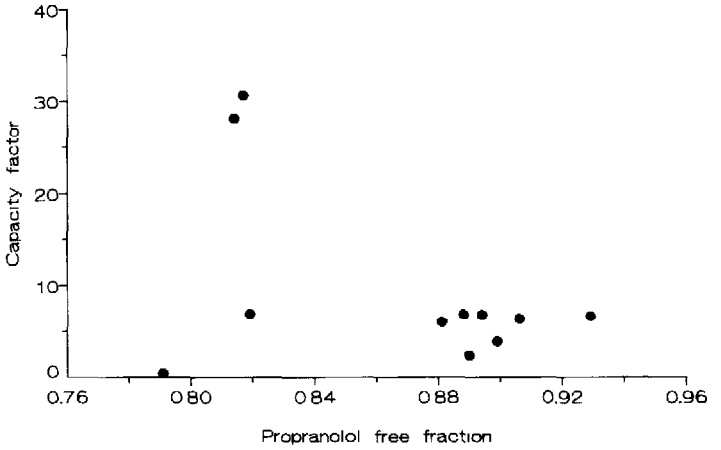

Fig. 3. Plot of capacity factors against propranolol free fractions determined at pH 6.0. HPLC mobile phase: isopropanol–0.02 *M* pH 6.0 phosphate buffer (6:94) at 0.30 ml/min. Protein binding: 0.13 *M* pH 6.0 phosphate buffer against 20 μM AAG in buffer for 8 h at 37°C.

TABLE III

CORRELATION COEFFICIENTS AND *p* VALUES FOR RELATIONSHIPS BETWEEN EXPRESSIONS OF RETENTION AND EXPRESSIONS OF BINDING AFFINITY

The data were collected at pH 7.4 and pH 6.0. All tests failed to show statistical significance at both pH 7.4 and pH 6.0.

	f_p		$\%\Delta f_p$		$K_1 I$	
	r	*p*	*r*	*p*	*r*	*p*
pH 7.4						
t_R	0.33673	0.2197	0.33791	0.2180	0.11373	0.6865
k'	0.33658	0.2200	0.33775	0.2183	0.11367	0.6867
pH 6.0						
t_R	−0.43877	0.1770	−0.43611	0.1799	−0.41774	0.2011
k'	−0.43879	0.1770	−0.43613	0.1799	−0.41776	0.2011

DISCUSSION

The choice of mobile phase for this study was based principally on work by Hermansson [1,5] in selection of buffer ionic strength, organic modifier and flow-rates. The pH values were based on both the column performance variation with pH previously reported [1] and consideration of physiological pH for serum protein binding of drugs. The use of ionic mobile phase modifiers was avoided in order to make the HPLC system as similar as possible to the usual protein binding system. The particular proportions of organic modifier and flow-rates were selected after some experimentation in order to achieve reasonable retention times (< 1.5 h) for all the compounds while allowing for some separation between the compounds. These conditions are representative of mobile phases commonly em-

ployed in using the EnantioPAC column. It must be noted, however, that the use of pH 6.0 or the relatively high proportion of isopropanol in the mobile phase at pH 7.4 give non-physiological conditions.

Examination of Figs. 2 and 3 show that no clear relationship exists between retention behavior for a given compound in the HPLC system used and the ability of the same compound to displace *l*-propranolol from its binding site on AAG. The correlation coefficient tests summarized in Table III are consistent with this observation.

The lack of a strong correlation between the retention of compounds known to bind to AAG in serum and their propranolol displacement potential is puzzling at first. Hermansson [5] suggested that solute retention is caused solely by an interaction between the protein and the solutes; capacity factors increased with increasing protein load on AAG columns, with essentially no retention of compounds on a silica column. The EnantioPAC column is used to separate stereoisomers of compounds presumably based on the differential binding affinity of the immobilized AAG for the enantiomers. One possible explanation of the lack of correlation is an alteration in the AAG, thus altering the binding site, during stationary phase manufacture.

The procedure for the manufacture of the EnantioPAC column is as follows [6]: AAG is ionically linked to activated diethylaminoethyl silica in the amount of 180 mg AAG per g silica. The protein is then oxidized with periodate to form aldehyde functional groups which are cross-linked through Schiff base formation. The resulting enamines are reduced to secondary amines with cyanoborohydride. As Schill et al. [6] pointed out, a number of the carboxylic acid groups of the native protein are tied up in the ionic bonding with the silica and are therefore unavailable for binding to the cationic site of the solute. Thus, although the immobilized protein retains an affinity for cationic compounds, the binding properties of the AAG in the stationary phase could differ from those of the native protein.

The data presented here can be interpreted as indicating that the difference in binding properties about which Schill et al. [6] speculated does indeed occur. It is clear that retention behavior on the immobilized AAG column is not directly related to AAG binding affinity.

CONCLUSION

The retention behavior of several compounds on the EnantioPAC HPLC column was shown not to be a simple function of the AAG binding affinity. This system is not suitable for use as a screening tool for drug–AAG binding.

ACKNOWLEDGEMENT

R.C. Jewell is the 1987 Syntex Pharmaceutics Fellow of the American Foundation for Pharmaceutical Education.

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