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Note

Propranolol binding to human serum proteins studied by high-performance liquid chromatography

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Several studies have reported the usefulness of chromatographic methods for the determination of drug binding parameters to biological macromolecules [1—3]. However, most results have been obtained with other methods, mainly equilibrium dialysis Thus, we thought it would be interesting to know if these two methods gave identical or different values The present paper deals with a comparison of the Hummel and Dreyer method [4] with the equilibrium dialysis technique for the determination of propranolol binding parameters to human plasma macromolecules As the chromatographic method used here involves a dilution of the injected protein sample, the change in the concentration of the protein during elution could modify the association characteristics [5, 6] This was another reason to verify that the results obtained by chromatography agree with those of the dialysis method, in which protein concentration remains constant

EXPERIMENTAL

Materials

Human serum albumin (HSA) HSA [Sigma, St. Louis, MO, U.S.A.; A 1887,

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purity 99%; free fatty acid (FFA) molar ratio = 0.04] dissolved in 0.067 *M* (μ = 0.284) phosphate buffer (pH 7.4) was used. When pooled human serum was used, HSA concentration was estimated by the bromocresol-green method (according to the instructions for bromocresol-green, Sigma Technical Bulletin No. 630, 1780)

α_1-Acid glycoprotein (AAG) AAG (Behring Marburg, F.R.G., purity 99%) was used in 0 067 *M* phosphate buffer (pH 7.4). The AAG concentration in serum was measured by radial immunodiffusion on M-Partigen plates (Behring).

Lipoproteins. Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) concentrations in serum, corresponding to apolipoproteins B (LDL) and A (HDL), were measured by radial immunodiffusion on M-Partigen plates (Behring) Each isolated lipoprotein was obtained by ultracentrifugation from pooled normolipidaemic human serum No chylomicrons were present. Plasma lipoproteins were isolated by sequential ultracentrifugation flotation of plasma at increasing density, as described previously by Havel [7]. The purity of each fraction was tested according to the method of Ouchterlony [8], checked by electrophoresis and estimated to be > 95%. The concentration of each lipoprotein was measured by the method of Lowry et al. [9].

Chemicals (±)-Propranolol hydrochloride was obtained from ICI Pharma

Apparatus and experimental conditions

A 6000A pump, U6K injector and UV Type 450 detector (Waters Assoc., Milford, MA, U.S.A.) were used. Detection was for dilute solutions at 291 nm, i.e. the maximum peak absorption of propranolol, or at 254 nm when propranolol concentrations ($\geqslant 1 \cdot 10^{-4}$ *M*) were high A 15 cm × 4 7 mm I D column filled by a slurry-packing technique with LiChrosorb Diol (10 μm diameter, 100 Å porosity) support (Merck, Darmstadt, F.R.G) was maintained at 37°C in a thermostated bath Binding parameters were calculated using a Tektronix 4051 computer by a previously described method [10]

Methods

The binding of propranolol to the different proteins of interest was measured by using a drug solution saturating a high-performance size exclusion column (LiChrosorb Diol) according to the method of Hummel and Dreyer [4] Injection of a small volume (25 μl) of a protein sample dissolved in the eluent onto the column after UV detection led to a positive peak corresponding to the protein and a negative peak representing the drug removed from the eluent by binding to the protein. In order to quantify the drug binding, we used an internal calibration process successive protein samples of the same volume are injected onto the column, the protein samples containing increasing concentrations of propranolol in phosphate buffer The first positive peak remains unchanged, whereas the second one varies and becomes positive. By plotting the area of the latter peak as a function of the concentration of propranolol in the sample, and interpolating it to zero, we determine the concentration for which there is no "drug peak". Then, the amount of this bound drug represents the exact excess of drug injected relative to the eluent concentration

RESULTS AND DISCUSSION

Fig 1a shows the elution pattern obtained when a few microlitres (25 μl) of an LDL (0 96 μM) buffered solution are injected onto the column and eluted with a propranolol ($2 \cdot 10^{-4}$ M) buffered solution. The area of the

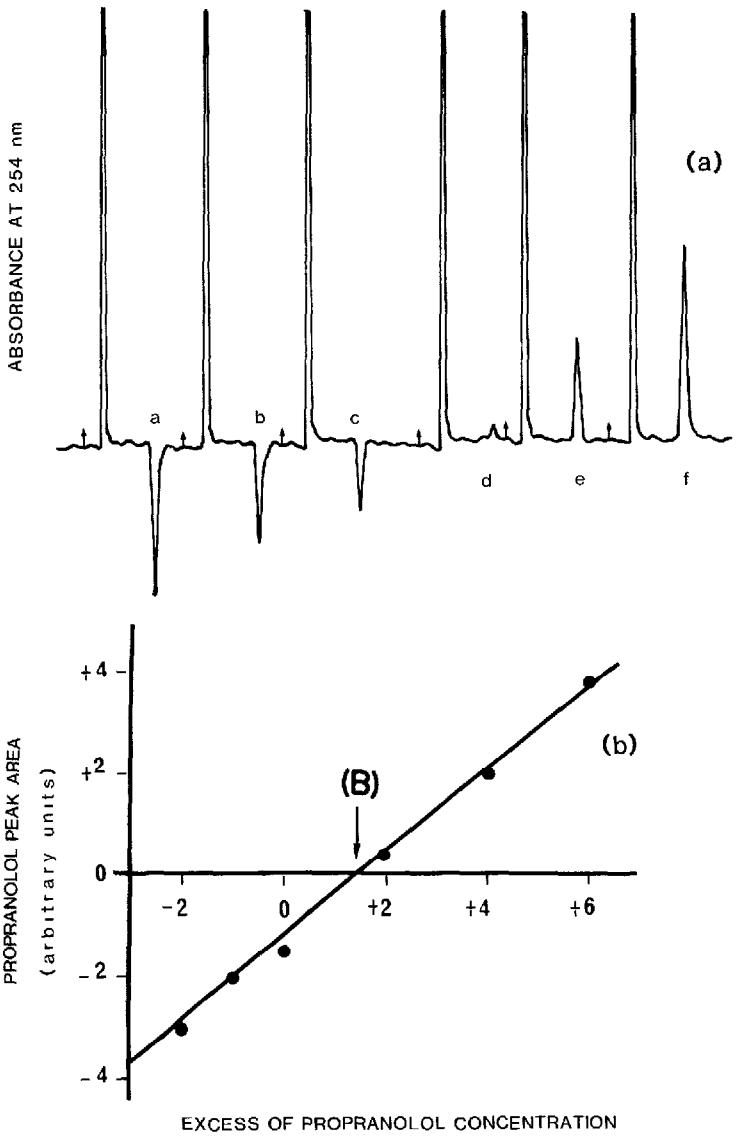

Fig 1 (a) Hummel and Dreyer chromatogram obtained with propranolol LDL interaction Column LiChrosorb Diol (15 cm × 4 7 mm I D), flow-rate 1 ml/min, eluent $2 \cdot 10^{-4}$ M propranolol in phosphate buffer (pH 7 4) Samples 25 μl of LDL (0 96 μM) buffered solution (a), b, c, d, e, f with increasing concentrations of 1, 2, 4, 6 and 8 10^{-4} M propranolol in phosphate buffer Temperature 37° C Time axis 1 5 cm = 10 min (b) Internal calibration for binding of propranolol to LDL Peak area (at 254 nm) as a function of the excess (relative to eluent concentration) of propranolol injected with 0 96 μM LDL into the column Eluent $2 \cdot 10^{-4}$ M propranolol in phosphate buffer (pH 7 4)

ligand peak, negative (a, b, c) or positive (d, e, f) (Fig. 1a), is plotted versus the excess of propranolol relative to the eluent concentration (Fig. 1b). As described above, the plot is then interpolated to zero, and the corresponding excess of propranolol (point B, Fig. 1b) is the exact amount of propranolol bound to LDL when the propranolol concentration in the eluent is $2 \cdot 10^{-4} M$. With each drug concentration in the eluent, we made five or six injections of the same protein sample containing increasing concentrations of propranolol (points a—f, Fig. 1a), in order to determine the interpolated point with the best accuracy. The correlation coefficient of the straight line thus obtained for any protein studied was $0.980 < r < 0.999$ Tables I and II show values of the concentration of propranolol bound to AAG and HDL for each free propranolol concentration in the eluent

TABLE I

DETERMINATION OF THE BINDING OF PROPRANOLOL TO AAG USING THE INTERNAL CALIBRATION METHOD

Binding parameters K_{a_1} = 399 52 · 10^3 ± 76 31 · 10^3 M^{-1} (r = 0 9953), n = 0 81 ± 0 12, nK_{a_2} = 6 544 · 10^3 ± 0 253 · 10^3 M^{-1}

Propranolol concentration (μM)		Coefficient of variation on bound (× 10^6) (%)	r
Free	Bound		
0 50	2 64	33 06	0 9990
0 75	3 27	10 84	0 9999
1 00	3 89	10 15	0 9999
2 50	6 33	0 79	0 9996
5 00	7 99	0 46	0 9999
7 50	9 15	0 40	0 9996
10 00	10 69	0 23	0 9991
25 00	12 92	0 52	0 9997
100 00	20 40	0 01	0 9999
175 00	29 64	4 20	0 9999

TABLE II

DETERMINATION OF THE BINDING OF PROPRANOLOL TO HDL USING THE INTERNAL CALIBRATION METHOD

Binding parameters nK_a = 43 269 · 10^3 ± 4 498 · 10^3 M^{-1} (r = 0 9939)

Propranolol concentration (μM)		Coefficient of variation on bound (× 10^6) (%)	r
Free	Bound		
40 00	4 05	1 11	0 9992
50 00	4 78	1 55	0 9993
80 00	6 41	1 23	0 9989
100 00	6 83	1 09	0 9979
200 00	10 70	1 11	0 9991
300 00	16 51	0 62	0 9850
500 00	26 68	0 22	0 9991
800 00	35 20	0 42	0 9910

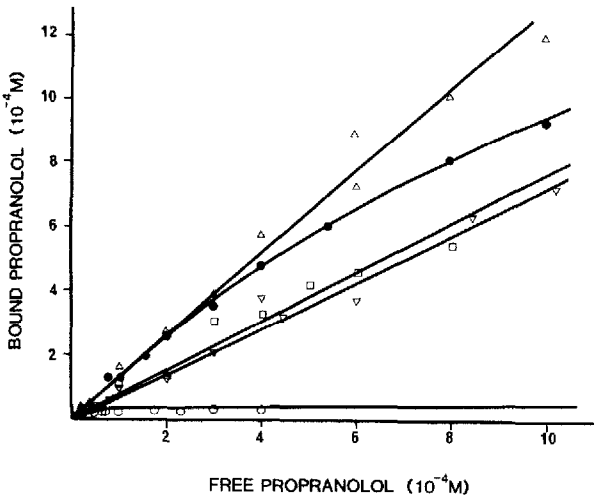

Fig 2 Binding curves of propranolol to different human serum proteins Serum at 56 g/l (•), AAG at 0 6 g/l or 15 μM (○), HDL at 2 4 g/l or 10 4 μM (▽), LDL at 2 4 g/l or 0 96 μM (△) and HSA at 32 g/l or 464 μM (□)

The curves for propranolol binding to isolated human serum proteins and serum are shown in Fig 2 The protein concentrations used are very close to those measured in humans. It appears that AAG is the only protein that shows saturable binding and then non-saturable binding when the propranolol concentration increases These results are in accordance with those previously reported [11, 12] The other proteins bind propranolol only by a non-saturable process Thus, propranolol binding in serum can be considered as the sum of one saturable and non-saturable process

Scatchard analysis was applied to these results The corresponding binding parameters are expressed as the product $N \times K$, where N is the concentration of binding sites in the protein solution and K is the corresponding affinity constant Our data are listed in Table III, together with previously reported data from equilibrium dialysis [11, 13—16] There appears to be some discrepancies with the AAG propranolol interaction. This has been previously reported [11—16] and may be due to the different purities of the protein samples used. Our present results and those previously reported by Soltes et al [12] obtained with another AAG sample are in good accordance with the results of Belpaire et al. [11] and Sager et al [14] For the other proteins, the differences observed in the results are within the precision of the methods

Owing to the relatively large difference between the values of K measured by equilibrium dialysis, comparison with our results is rather difficult. But it is apparent that in general our chromatographic measurements lead to values of the same order of magnitude as the dialysis determinations It should be stated that the bound propranolol concentrations, determined chromatographically using the calibration process described above, have a very small coefficient of variation (Tables I and II). This reproducibility permits characterization of binding curves (Fig. 2) with good correlation coefficients serum 0 997, HSA 0 977, AAG 0.997, LDL 0 977 and HDL 0 993.

All these values demonstrate the good precision of the high-performance

TABLE III

PROPRANOLOL BINDING PARAMETERS OF SERUM AND ISOLATED SERUM PROTEINS

Sample	$N \times K^*$			
	HPLC method	Reference	Equilibrium dialysis method	Reference
Serum	1 31	Our results	1 49	Glasson et al [13]
			9 61	Belpaire et al [11]
HSA	0 66	Our results	1 01	Glasson et al [13]
HDL	0 45	Our results	0 25	Glasson et al [13]
LDL	0 18	Our results	0 21	Glasson et al [13]
AAG	4 84	Our results	16 8	Calculated from Wong and Hsia [16]
			2 6	Calculated from Belpaire et al [11]
			4 39	Calculated from Sager et al [14]
			0 69	Calculated from Glasson et al [13]
			0 62	Calculated from Kornguth et al [15]
	4 45	Soltes et al [12]		

*N is the concentration of binding sites in the protein solution and K is the corresponding affinity constant

liquid chromatographic (HPLC) procedure for binding determinations The major advantage of the application of the HPLC technique for the estimation of drug protein binding is that it is possible to use UV detection for the quantification of bound drug in the same range of concentations which, in dialysis experiments, would require radiolabelled propranolol. This is possible because the molar extinction coefficient of propranolol is sufficiently high to be readily detectable In fact, this is the case with very many drugs, which can also be studied by a similar methodology.

It has been reported previously [14—17] that binding to AAG is stereoselective for (−)-propranolol In the present experiment, however, the separation of the two isomers is not possible on the LiChrosorb Diol column, thus our results relate to the overall binding of the two isomers to the proteins.

CONCLUSION

The method of Hummel and Dreyer with a size exclusion LiChrosorb Diol column and UV detection was used for the determination of propranolol protein binding parameters The results obtained are in the range of those previously reported from equilibrium dialysis experiments and show that protein dilution during chromatographic elution has no dramatic effect on the values of the binding parameters This conclusion has been drawn for other drugs bound to HSA [1, 2] Thus, HPLC can be considered as a valuable method for drug protein binding determination, avoiding the dialysis disadvantages of possible membrane adsorption, protein degradation, instability of the drug during the long period of time (2—24 h) needed to reach equilibrium and, in numerous cases, avoiding the use of radiolabelled drugs.

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