action on different receptor sites or states. Although inhibition of adenylate cyclase by opiates is through the delta subtype of opiate receptors [6, 8, 19], multiple enkephalin binding sites exist [21]. Thus, alcohol may inhibit binding of enkephalins to a delta binding site that is not coupled to adenylate cyclase. Similarly, acetylcholine inhibits striatal adenylate cyclase through a muscarinic receptor [9], but the muscarinic receptor is coupled to both adenylate cyclase and phosphoinositide metabolism [22]. Alcohol may, therefore, inhibit agonist binding to the form of the muscarinic receptor that is linked to phosphoinositide metabolism rather than to adenylate cyclase.

Both the increase in adenylate cyclase activity by stimulatory drugs and the reduction in enzyme activity by inhibitory drugs require the activation of regulatory subunits [23, 24]. Although N_S and N_I mediate opposite actions on adenylate cyclase activity, the regulatory subunits share a number of similar properties. Both N_S and N_I require GTP and magnesium for activation, and activation involves a dissociation of the subunit into constituent components [23, 25]. Furthermore, N_S and N_I both contain an identical 35,000-dalton (i.e. β) component [26]. However, whereas dissociation of N_S results in activation of the catalytic subunit of adenylate cyclase [23], dissociation of N_I attenuates adenylate cyclase activity by forcing the dissociative activation reaction of N_S toward formation of the inactive holoenzyme [25]. Although ethanol appears to enhance the activation of N_S [5, 27], the present study indicates that ethanol does not alter either the activation of N_I or the reassociation (i.e. inactivation) of N_S. Ethanol thus appears to specifically enhance the dissociative activation of N_S.

In summary, ethanol did not alter the inhibition of striatal adenylate cyclase activity by morphine, Leu-enkephalin and acetylcholine. Thus, while ethanol has been shown to alter the activation of N_S, ethanol does not affect either the activation or activity of N_I. The discrepancy between the previously reported alcohol-induced inhibition of agonist binding and the lack of an effect of ethanol on the inhibition of adenylate cyclase activity may be due to an action of ethanol on different forms of the delta opiate and muscarinic receptors.

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REFERENCES

1. M. Rodbell, *Nature, Lond.* **284**, 17 (1980).
2. L. E. Limbird, *Biochem. J.* **195**, 1 (1981).
3. R. E. Gorman and M. W. Bitensky, *Endocrinology* **87**, 1075 (1970).
4. R. A. Rabin and P. B. Molinoff, *J. Pharmac. exp. Ther.* **216**, 129 (1981).
5. R. A. Rabin and P. B. Molinoff, *J. Pharmac. exp. Ther.* **227**, 551 (1983).
6. D. M. F. Cooper, C. Londos, D. L. Gill and M. Rodbell, *J. Neurochem.* **38**, 1164 (1982).
7. K. Kamikuba, M. Nozaki and H. Fujimura, *Jap. J. Pharmac.* **31**, 175 (1981).
8. P. Y. Law, J. Wu, J. E. Koehler and H. H. Loh, *J. Neurochem.* **36**, 1834 (1981).
9. M. C. Olianas, P. Onali, N. H. Neff and E. Costa, *Molec. Pharmac.* **23**, 393 (1983).
10. M. C. Olianas, P. Onali, N. H. Neff and E. Costa, *J. Neurochem.* **41**, 1364 (1983).
11. M. Waelbroeck, P. Robberecht, P. DeNeff and J. Christophe, *Biochem. J.* **219**, 563 (1984)
12. B. Tabakoff and P. L. Hoffman, *Life Sci.* **32**, 197 (1983).
13. J. M. Hiller, L. M. Angel and E. J. Simon, *Science* **214**, 468 (1981).
14. P. L. Hoffman, C. T. Chung and B. Tabakoff, *J. Neurochem.* **43**, 1003 (1984).
15. J. M. Hiller, L. M. Angel and E. J. Simon, *Molec. Pharmac.* **25**, 249 (1984).
16. Y. Salomon, C. Londos and M. Rodbell, *Analyt. Biochem.* **58**, 541 (1974).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. C. W. Dunnett, *J. Am. statist. Ass.* **50**, 1096 (1955).
19. P. L. Hoffman, G. R. Luthin, D. Theodoropoulos, P. Cordopatis and B. Tabakoff, *Pharmac. Biochem. Behav.* **18**, (Suppl. 1) 355 (1983).
20. R. B. Rothman and T. C. Westfall, *Molec. Pharmac.* **21**, 538((1982).
21. R. B. Rothman, W. D. Bowen, M. Herkenham, A. E. Jacobson, K. C. Rice and C. B. Pert, *Molec. Pharmac.* **27**, 399 (1985).
22. J. H. Brown and S. L. Brown, *J. biol. Chem.* **259**, 3777 (1984).
23. J. K. Northup, M. D. Smigel, P. C. Sternweis and A. G. Gilman, *J. biol. Chem.* **258**, 11369 (1983).
24. T. Katada, G. M. Bokoch, J. K. Northup, M. Ui and A. G. Gilman, *J. biol. Chem.* **259**, 3568 (1984).
25. T. Katada, J. K. Northup, G. M. Bokoch, M. Ui and A. G. Gilman, *J. biol. Chem.* **259**, 3578 (1984).
26. D. R. Manning and A. G. Gilman, *J. biol. Chem.* **258**, 7059 (1983).
27. G. R. Luthin and B. Tabakoff, *J. Pharmac. exp. Ther.* **228**, 579 (1984).

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Study of propranolol binding to human α_1-acid glycoprotein by high-performance liquid chromatography

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Propranolol binding to human plasma proteins is known to be high (>90% [1]) with α_1-acid glycoprotein (α_1-AGP) playing a major role in this binding. To determine binding parameters for propranolol bound to human α_1-AGP mostly the equilibrium dialysis (ED) has been used [2–4].

Several high-performance liquid chromatographic (HPLC) methods have been introduced for the study of biomacromolecule–ligand interactions [5–8]. The present paper reports on the application of HPLC as a comparable approach for studying the binding of propranolol to human α_1-acid glycoprotein.

Materials and methods

Materials and chemicals. Human α_1-acid glycoprotein (Op.-Nr. 8376, mol. wt. 44100*) was purchased from Behring Institute. Racemic propranolol hydrochloride was donated by ICI Pharmaceuticals (Macclesfield, U.K.).

KH_2PO_4 and $Na_2HPO_4 \times 12\,H_2O$ were of analytical grade. Water used for buffer solutions was of Millipore Q quality.

Apparatus and chromatographic conditions. A Touzart and Matignon pump (Model Chromatem 380), and a Waters Assoc. universal injector (Model U6K) and a variable-wavelength photometric detector (Model 450) were used for all the experiments. Separations were carried out at 37° in a stainless-steel column (15 cm × 4.7 mm i.d.) filled with LiChrosorb Diol (Merck, Darmstadt, F.R.G.), mean particle size 10 µm. Solutions of propranolol (5×10^{-7}–1×10^{-3} M) in a phosphate buffer (0.067 M, pH 7.4) used as eluents were pumped at a flow-rate of 1 ml/min. Detection was effected at 291 nm, the absorption maximum of the eluent, or at 254 nm, since higher propranolol concentrations ($\geq 1.75 \times 10^{-4}$ M) saturated the detector. The samples injected were solutions of human α_1-AGP (0.6 g/l) in phosphate buffer containing various amounts of propranolol. Volumes of the injected samples were 25 µl for higher concentrations of propranolol in eluent ($\geq 1 \times 10^{-5}$ M), 50 µl, or 100 µl for lower drug concentrations ($\leq 1 \times 10^{-6}$ M).

Evaluation of binding data. At the thermodynamic equilibrium the concentration of propranolol bound by a mole of the human α_1-AGP (B) depends on the number of binding sites (n) available on the latter, their affinity (k) for propranolol, and the concentration of the free propranolol (F). To express our data with a binding isotherm we considered the occurrence of specific (saturable) and nonspecific (unsaturable) binding of propranolol to human α_1-AGP. This isotherm can be described by the following equation

$$B = \frac{nkF}{1 + kF} + n'k'F. \qquad (1)$$

The iterative program NONLIN [9] was used for the weighted nonlinear least-squares curve fitting.

Results

A typical chromatogram obtained by injecting a solution of human α_1-AGP in the eluent onto the column is shown in Fig. 1c. The first, positive peak, emerging at the void volume of the column, belongs to a protein–drug complex formed under the used experimental conditions. A trough appearing at the retention time of propranolol, is a manifestation of the ligand deficiency in the analysed sample caused by propranolol binding to the human α_1-AGP. The concentration of the bound species of propranolol equaling the necessary amount of ligand which compensates this deficiency was determined by the internal calibration technique proposed by Hummel and Dreyer [10]. This amount of ligand was assayed using a set of trough or peak heights obtained after the injections of the human α_1-AGP solution containing various amounts of propranolol (Figs. 1a–e). Since the dependence of peak heights on amounts of ligand added to the injected samples was linear (each $r > 0.999$) the concentration of bound propranolol can be easily calculated from that amount of ligand which abolishes the detector response.

The plot B/F against B shown in Fig. 2 indicates that propranolol does not bind only to a single specific class of binding sites of human α_1-acid glycoprotein. Therefore the binding data obtained were evaluated according to the equation (1) by using the program NONLIN. Computer assisted guesses and optimalised values of binding parameters for propranolol bound to human α_1-acid glycoprotein are given in Table 1.

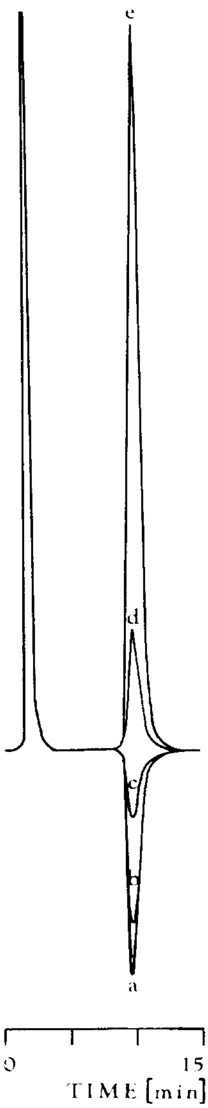

Fig. 1. HPLC elution profiles obtained after application of 25 µl of the human α_1-AGP solution (0.6 g/l) in phosphate buffer (0.067 M, pH 7.4) containing propranolol a, b, c, d, and e, of 1×10^{-5}, 2.5×10^{-5}, 5×10^{-5}, 1×10^{-4}, and 2.5×10^{-4} M, respectively, injected onto the column equilibrated with solution of propranolol (5×10^{-5} M) in phosphate buffer.

Discussion

Recently, several reports on *in vitro* binding of propranolol to the human α_1-acid glycoprotein by means of equilibrium dialysis have appeared [2–4]. These data and those based on HPLC are given in Table 1. Some discrepancy among the reported [2–4] binding parameters is obvious. This may be due to different experimental conditions used, and/or inadequacy of the model utilized for evaluation of binding data. Considering the latter possibility Müller and Stillbauer [2] as well as Wong and Hsia [3] assumed only a single specific class of binding sites, while Belpaire *et al.* [4] suggested the existence of two specific classes. In the same paper the authors maintained that within the feasible experimental conditions no saturation occurs [4]. Our results confirmed this finding (cf.

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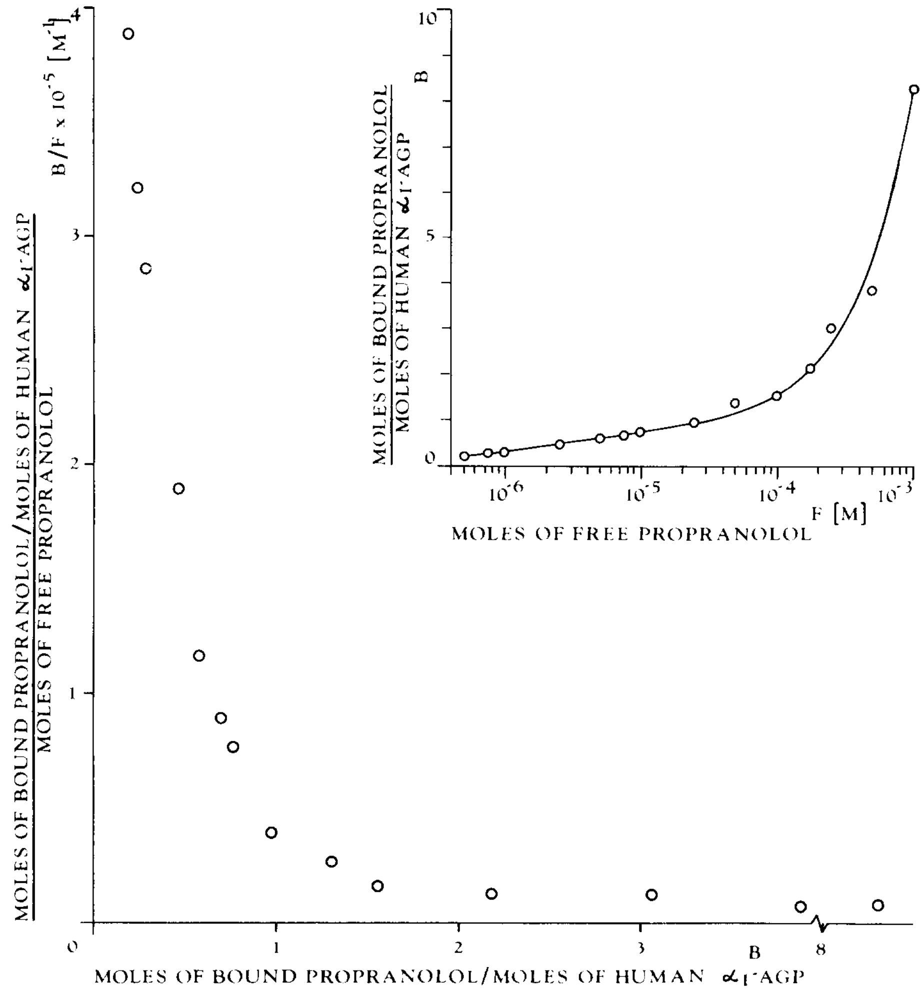

Fig. 2. Binding of propranolol by human α_1-AGP at 37° in phosphate buffer (0.067 M, pH 7.4).

Table 1. Estimated binding parameters for propranolol bound to human α_1-acid glycoprotein

Investigators	n	$k \times 10^{-5}$ (M^{-1})	$n'k' \times 10^{-3}$ (M^{-1})	Method and experimental conditions
Müller and Stillbauer [2]	1.05	11.3		ED, 0.07 M phosphate buffer pH 7.4, 22° [11], 16 hr
Wong and Hsia [3]	≈1	8.4		ED, phosphate-buffered saline pH 7.5, 37°, overnight
Belpaire *et al.* [4]	0.67*	2.44	†	ED, 0.15 M phosphate buffer pH 7.4, 25°, 4 hr
Present work				HPLC, 0.067 M phosphate buffer
Initial guesses‡	1.1	2.7	7.4	
Optimalized values§	0.81 ± 0.05	5.4 ± 0.7	7.4 ± 0.4	pH 7.4, 37°, minutes

* The value of n was calculated from the published data [4] on assuming the mol. wt. of human α_1-AGP to be 44100.

† Evaluation attempted, for details see "Discussion" of this paper.

‡ The initial guesses of binding parameters were calculated from the co-ordinates of the cross sections of a plane $B/F = f(F, B)$ with the axis F, B, and B/F.

§ The values of binding parameters and their approximate SDs were optimalized by means of the iterative program NONLIN [9] using statistical weight equal to $1/B_i^2$.

Fig. 2) yet for the evaluation of our binding data a model considering a single specific and a nonspecific class of binding sites on the human α_1-AGP for propranolol was utilized. This model can be validated only indirectly, because no information on the binding sites of human α_1-AGP for propranolol at molecular level is available as yet. According to Klotz [12], the semilogarithmic plot $B = f(F)$, illustrating the binding of a ligand to specific sites, is an S-shaped curve with the inflection point appearing at half-maximum binding. Considering our results shown in Fig. 2 in terms of this mode of presentation, the total number of sites originating exclusively in specific drug–protein interactions is to be rejected.

The present paper demonstrates that HPLC is an efficient method for studying the binding of propranolol to human α_1-acid glycoprotein. Considering a single specific and a nonspecific class of binding sites on human α_1-AGP for propranolol the estimated mean number of specific binding sites was $n = 0.81$, with an affinity of $k = 5.4 \times 10^5\ \mathrm{M}^{-1}$, and for the nonspecific binding their product $n'k'$ was $7.4 \times 10^3\ \mathrm{M}^{-1}$.

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REFERENCES

1. L. S. Goodman and A. Gilman (Eds.), *The Pharmacological Basis of Therapeutics*, 5th Edn, p. 551. Macmillan, New York (1975).
2. W. E. Müller and A. E. Stillbauer, *Naunyn-Schmiedeberg's Arch. Pharmac.* **322**, 170 (1983).
3. A. K. L. Wong and J. C. Hsia, *Can. J. Biochem. Cell Biol.* **61**, 1114 (1983).
4. F. M. Belpaire, R. A. Braeckman and M. G. Bogaert, *Biochem. Pharmac.* **33**, 2065 (1984).
5. B. Sebille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.* **167**, 159 (1978).
6. B. Sebille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.* **180**, 103 (1979).
7. B. Sebille, N. Thuaud and J.-P. Tillement, *J. Chromatogr.* **204**, 285 (1981).
8. S. F. Sun, S. W. Kuo and R. A. Nash, *J. Chromatogr.* **288**, 377 (1984).
9. C. M. Metzler, G. K. Elfring and A. J. McEwen, *Biometrics* **30**, 562 (1974).
10. J. P. Hummel and W. J. Dreyer, *Biochim. biophys. Acta* **63**, 530 (1962).
11. W. E. Müller, A. E. Stillbauer and S. El-Gamal, *J. Pharm. Pharmac.* **35**, 684 (1983).
12. I. M. Klotz, *Science* **217**, 1247 (1982).

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Characterisation of a new transposon-mediated trimethoprim-resistant dihydrofolate reductase

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Plasmid-mediated resistance to trimethoprim (Tp) is due to the plasmid directed synthesis of additional Tp-insensitive dihydrofolate reductases (DHFR) (5,6,7,8-tetrahydrofolate: NADP+ oxidoreductase (EC 1.5.1.3)) [1, 2]. The resistant enzymes are much larger than the sensitive chromosomal DHFR with molecular weights (MW) of about 35,000 as compared to the *Escherichia coli* chromosomal enzyme which has a MW of only 21,000 [3, 4]. The plasmid encoded enzymes have since been classified into two major groups — type I and type II [3]. Although both enzyme types have a molecular weight of about 35,000 the subunit composition of the enzymes differs — type I enzymes consisting of two identical subunits molecular weight 18,000 and type II enzymes comprising four subunits molecular weight 9000 [5]. The enzymes also differ in several other properties. A third plasmid-mediated Tp-resistant DHFR (type III) has recently been reported by Fling *et al* [6]. This enzyme has monomeric structure, low molecular weight (16,900) and mediates only a moderate level of Tp resistance [7].

The type I DHFR is often encoded by a transposon (Tn7) which also encodes resistance to streptomycin [8]. The new 2 megadalton resistance transposon, Tn4132, conferring high level resistance to Tp alone, was extracted from a clinical plasmid isolated from a urinary pathogen in Edinburgh [9]. In this paper, the DHFR encoded by this transposon has been characterised and compared with the type I enzyme encoded by a Tn7-like transposon, Tn4130.

Materials and methods

Materials. The auxotrophic organism *Escherichia coli* K-12 strain J62 [10] harbouring plasmid RP4 [11] containing either Tn4130 or Tn4132 were used from our previous study [9]. NADPH and dihydrofolate were purchased from the Sigma Chemical Company (London) and Sephadex G-75 superfine from Pharmacia Fine Chemicals AB, Sweden. Trimethorpim lactate was kindly donated by the Wellcome Research Laboratories (Beckenham, U.K.).

Enzyme assay. Dihydrofolate reductase activity was assayed by the method of Osborn and Huennekens [12] as described previously [1]. Protein concentrations were estimated by the method of Waddell [13].

Enzyme preparation. The enzyme was prepared from ten litre batches of exponential phase bacteria by the method of Amyes and Smith [1]. Removal of NADPH oxidase activity and subsequent precipitation of DHFR activity was