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CALCULATION OF COMPETITIVE INHIBITION OF SUBSTRATE BINDING TO CYTOCHROME P-450 ILLUSTRATED BY THE INTERACTION OF *d,l*-PROPRANOLOL WITH *d,l*-HEXOBARBITAL

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Abstract—Various forms of cytochrome P-450 exist. A substrate may bind to these forms completely or partially. In addition, during the interaction of two substrates, several cytochrome P-450 forms may also be involved, either partially or completely. Now a method is described which allows the estimation of the spectral dissociation constants for the binding of two compounds to cytochrome P-450 subforms common to both. The method is illustrated by the competitive interaction of *d,l*-hexobarbital and *d,l*-propranolol with cytochrome P-450 which show only a partial overlap for type I binding sites using rat hepatic microsomes. Different subforms of cytochrome P-450 may be characterized by means of cross-over studies with several type I compounds using this method.

It is generally agreed that the type I difference spectrum usually represents the highly specific interaction of a hydrophobic substrate with the enzyme-active site of one or more forms of cytochrome P-450 [1, 2]. Inhibition of type I binding of substrates will inhibit the metabolism of those compounds [3, 4].

In the present paper, we describe a method for determining the spectral inhibition constant (K_I), when there is a competitive interaction between two compounds for cytochrome P-450. This is an expansion of the method of Van den Berg *et al.* [5], in which it was shown that, if the inhibition is competitive, the classical equations for competitive inhibition, as determined by means of difference spectroscopy [6], do not apply. In their calculations, moreover, it had to be assumed that both compounds bind to the same binding sites. This might not always be true, since Grasdalen *et al.* [7] showed that there is an overlap in the binding-specificities of different subforms of cytochrome P-450. Consequently, substrates may not only bind to common subforms, but to others as well.

Our method deals with this partial competition between substrates and is illustrated by the interaction of *d,l*-hexobarbital and *d,l*-propranolol, both relatively pure type I compounds [8]. Although little is known about this interaction [e.g. 9–11], it could be due to competition for cytochrome P-450 binding sites.

With our method, the affinity constants for binding to cytochrome P-450 subforms, common to both hexobarbital and propranolol, can be estimated. Thus it has been possible to correlate inhibition of drug metabolism with interaction at cytochrome P-450 binding sites.

MATERIALS AND METHODS

Chemicals. *d,l*-Propranolol–HCl was a gift from ICI, *d,l*-sodium-hexobarbital (Na-Evipan) was obtained from Bayer. All other chemicals and solvents used were of analytical grade purity.

Preparation of microsomes. Male Wistar rats, weighing approximately 250 g (TNO, Zeist) were used. The animals were killed by decapitation, livers removed and the latter homogenized in three volumes of ice-cold phosphate buffer (50 mM and pH 7.4) containing 0.1 mM EDTA, using glass Potter tubes with a Teflon pestle. The homogenate was centrifuged at 9000 *g* for 20 min at 4°. The microsomal fraction was sedimented from the supernatant by 90 min centrifugation at 75,000 *g* and 4°. The pellet was resuspended in the same ice-cold buffer so that the microsomal preparations contained approximately 3 nmoles P-450/ml.

Spectral measurements. Difference spectra were recorded at 37° [12] using an Aminco DW-2 UV-Vis spectrophotometer in the split beam mode. For each determination the livers of at least four animals were pooled. Microsomal suspensions were equally divided between sample and reference cuvettes and a baseline of zero absorbance was established. Substrates, dissolved in water, were added as indicated in the text.

The concentration of cytochrome P-450 was estimated according to Estabrook *et al.* [6]. Both cuvettes were bubbled with CO and sodium dithionite was added to the sample cuvette, thereby preventing interference from any hemoglobin which might have contaminated the samples. Mean changes in absorbance were expressed per nmole cytochrome P-450 per ml from three experiments.

RESULTS AND DISCUSSION

Difference spectroscopy as a method for studying competitive inhibition of binding to cytochrome P-

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450. Although it is of importance to quantitate the interaction of compounds to cytochrome P-450, several difficulties may arise.

Thus, there is the problem described by Van den Berg *et al.* [5]. They showed that if two substrates bind to the same binding sites, thereby eliciting the same type of spectral change, the contribution to the spectral change observed (ΔA_{obs}) of each substrate, if added simultaneously to a microsomal suspension, cannot be separated.

To obtain, nevertheless, the spectral inhibition constant (K_I), they derived the equation

$$\Delta A_{obs} = \Delta A_{SI} - \Delta A_I = \frac{\Delta A_{max}[S]}{[S]\left(1 + \frac{[I]}{K_I}\right) + K_S\left(1 + \frac{[I]}{K_I}\right)^2} \quad (1)$$

in which ΔA_{SI} is the total spectral change in the sample cuvette, elicited by both the substrate *S* and the inhibitor I. ΔA_I represents the spectral change in the reference cuvette produced by I. ΔA_{max} is the maximum possible spectral change caused by S. K_S and K_I are the dissociation constants of S and I, respectively, for type I binding to cytochrome P-450 [5]. The limitation of this method [5] is the requirement that both compounds bind to the same binding sites.

However, because of the heterogeneity of the cytochrome P-450 system, S and I will probably have, in most cases, only a fraction of the several cytochrome P-450 species in common (indicated by cyt SI). Other cytochrome P-450 species will bind only S or only I and may be represented by cyt S and cyt I, respectively.

The observed difference spectrum (ΔA_{obs}) caused by S in the sample cuvette and by I in both the sample and the reference cuvettes is composed of:

$$\Delta A_{SI}\text{ (cyt SI)} - \Delta A_I\text{ (cyt SI)} + \Delta A_S\text{ (cyt S)} = \Delta A_{obs}. \quad (2)$$

It is clear that ΔA_I (cyt I) does not contribute to ΔA_{obs}, because this spectral change is similar in both the sample and reference cuvette.

Insertion of equation (1) in equation (2) is allowed because the same total number of binding sites are involved in binding of both S and I to the cyt SI pool and thus the following equation is obtained:

$$\frac{\Delta A_{max}[S]}{[S]\left(1 + \frac{[I]}{K_I}\right) + K_S\left(1 + \frac{[I]}{K_I}\right)^2} + \Delta A_S(\text{cyt S}) = \Delta A_{obs}, \quad (3)$$

in which all parameters of the first term apply to cyt SI pool only. It can be seen that, at an infinitely high (saturating) inhibitor concentration, ΔA_{obs} in equation (3) cannot be driven to zero but reduces to:

$$\Delta A_{obs} = \Delta A_S(\text{cyt S}) = \frac{\Delta A_{max}(\text{cyt S})[S]}{[S] + K_S(\text{cyt S})}. \quad (4)$$

Using equation (4), the cyt S pool can be characterized by plotting $1/\Delta A_{obs}$ (equation 4) vs 1/S in the presence of a saturating concentration of I. Thus, both ΔA_{max} (cyt S) and K_S (cyt S) are obtained.

By subtracting the appropriate ΔA_S (cyt S) from ΔA_{obs}, equation (2) can be rearranged to give:

$$\Delta A_{SI}\text{ (cyt SI)} - \Delta A_I\text{ (cyt SI)} = \Delta A_{obs} - \Delta A_S\text{ (cyt S)}, \quad (5)$$

which is essentially analogous to equation (1). Consequently, the method of Van den Berg *et al.* [5] can now be applied.

Using this method the dissociation constants and maximum spectral changes for binding of S and I to pool cyt SI and for binding of S to the cyt S pool can be determined.

It should be realized that, using this method, only apparent K_S values are obtained. As discussed by Parry *et al.* [13], lipid solubility of the substrates plays an important role in substrate binding, and in the case of very lipophilic compounds, e.g. propranolol, the apparent K_S may be a considerable underestimation of the real K_S.

Interaction of d,l-*propranolol and* d,l-*hexobarbital to cytochrome P*-450. There are some indications that propranolol can interact with other drugs, via a central [14] or a hemodynamic [15] mechanism, but also through an influence on the activity of the hepatic microsomal cytochrome P-450 system [10, 11]. The latter effect may be due to a competitive interaction. In investigating the influence of propranolol (I) on hexobarbital (S) type I binding, we only used the so-called high affinity binding [16, 17] of propranolol (Fig. 1). A K_S of 0.09 μM and a ΔA_{max}/nmole P-450/ml of 0.0053 were obtained for type I binding of propranolol. At high substrate concentrations the spectral shift is reversed, probably by a reverse type I binding of propranolol, which is definitely not present at low propranolol concentrations. The values for K_S and ΔA_{max} are in agreement with those of Topham [8], but not with those of Schneck *et al.* [17]. The spectra obtained, using high concentrations of propranolol, as determined by Schneck *et al.*, were confirmed. However, some doubts should be expressed over the meaning of these spectra, because changes in membrane properties may occur when high concentrations of lipophilic compounds are used [18].

Using equation (4), the characteristics of the pool of cytochrome P-450 that bind hexobarbital (cyt S), but not propranolol, can be determined.

The contents of the sample cuvette were titrated with propranolol. The results are presented in Fig. 2. The curves are similar to the ones presented by Segel [19], who showed a situation in which two enzymes catalyse a reaction and only one is inhibited competitively, so an apparent partial inhibition is seen.

By plotting the reciprocal of the estimated values of ΔA_S (cyt S), which is found by extrapolation to infinite propranolol concentration (Fig. 2), against the reciprocal of the appropriate hexobarbital concentration, we obtained a ΔA_{max} (cyt S)/nmole P-450/ml of 0.0126 and a K_S (cyt S) of 87 μM (Fig. 2, inset), using equation (4).

In order to examine the mutual effects of hexobarbital and propranolol on their binding to cytochrome P-450, the contents of the sample cuvette were titrated with hexobarbital in the presence of different fixed concentrations of propranolol in the sample and reference cuvette. The results are shown

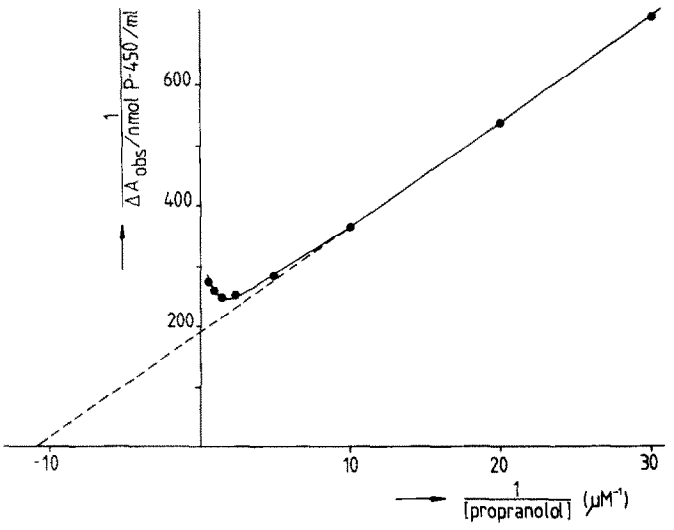

Fig. 1. Double reciprocal plot of $\Delta A_{385-420}$/nmole P-450/ml against the propranolol concentration. Extrapolation yields a ΔA_{max}/nmole P-450/ml of 0.0053 ± 0.0007 and a K_S of 0.093 ± 0.01 μM.

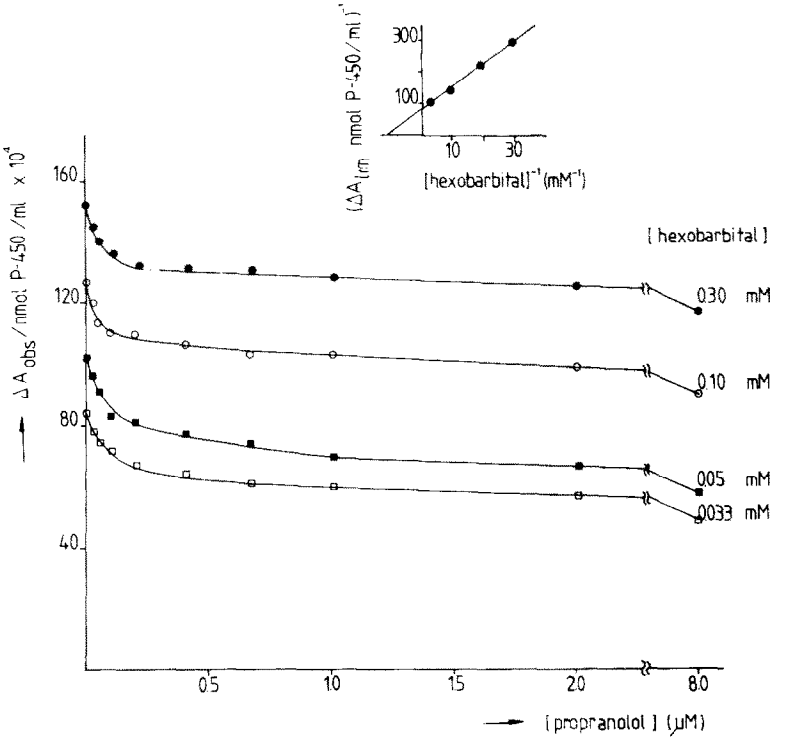

Fig. 2. Influence of propranolol on hexobarbital type I binding. The sample cuvette contained a fixed concentration of hexobarbital and the contents of both sample and reference cuvette were titrated with propranolol. The magnitude of the difference spectrum ($\Delta A_{385-420}$/nmole P-450/ml) is plotted against the propranolol concentration. Inset: Reciprocal plot of ΔA_S (cyt S)/nmole P-450/ml (equation 4) vs the appropriate hexobarbital concentration, yielding a ΔA_{max} (cyt S)/nmole P-450/ml of 0.0126 and a K_S of 87 μM.

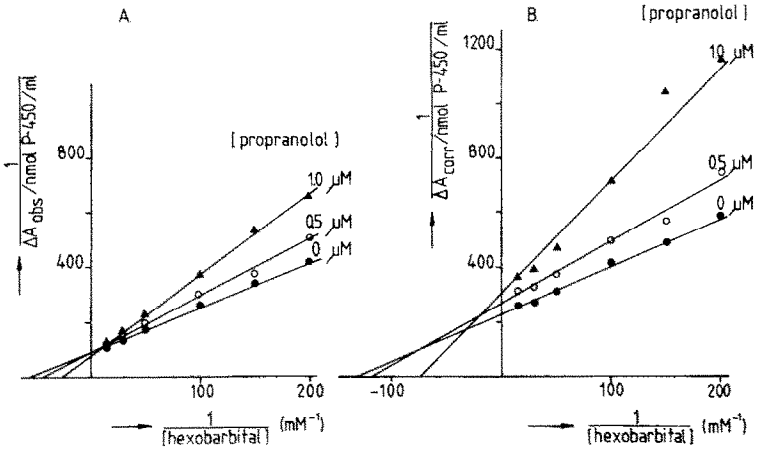

Fig. 3. (A) Effect of propranolol upon the difference spectrum ($\Delta A_{385-420}$/nmole P-450/ml) produced by hexobarbital. Propranolol was present in a fixed concentration in both sample and reference cuvettes. The contents of the sample cuvette were titrated with hexobarbital. Plot as in Fig. 1. (B) Data as in A, except that $\Delta A_{385-420}$/nmole P-450/ml was corrected for binding of hexobarbital to cytochromes P-450 which do not bind propranolol (cyt S), as shown in inset Fig. 2 and according to equation (5).

in Fig. 3A. By subtracting ΔA_S (cyt S) for each hexobarbital concentration, calculated from the previous experiment (Fig. 2, inset), from the ΔA_{obs} (Fig. 3A), the data shown in Fig. 3B were obtained. This situation is described by equation (5) and is analogous to the one described by Van den Berg *et al.* [5].

A replot of the apparent $1/\Delta A_{maxSI-I}$ (cyt SI) against the propranolol concentration [5] yielded a K_I of 3.0 μM (not shown). This value is only applicable to the pool of cytochromes P-450 binding both hexobarbital and propranolol (cyt SI).

From Fig. 3B, a K_S (cyt SI) of 8 μM and a ΔA_{max} (cyt SI)/nmole P-450/ml of 0.0043 can be calculated for hexobarbital. Consequently, it can be tentatively concluded that almost all high affinity propranolol binding with a ΔA_{max}/nmole P-450/ml of 0.0053 can interact with hexobarbital binding. This suggests that propranolol should be displaced to a very large extent from its high affinity type I binding places by hexobarbital. This is indeed the case, as shown in Fig. 4.

In conclusion, it can be said that propranolol can occupy, in a competitive manner, only a fraction of the cytochromes P-450 that bind hexobarbital. If binding to the cyt S pool as well as to the cyt SI pool are both involved in the metabolism of hexobarbital and these binding sites can be occupied independently, only partial competitive inhibition by propranolol is to be expected.

When using an inhibitor which binds to both type I and type II sites (cf. Van den Berg *et al.* [5]), the method described by Van den Berg *et al.* [5] may still be applicable. This of course is provided that type I binding of S and I is independent of type II binding of I.

The method described here can also be used to quantitate and to characterize the different subforms

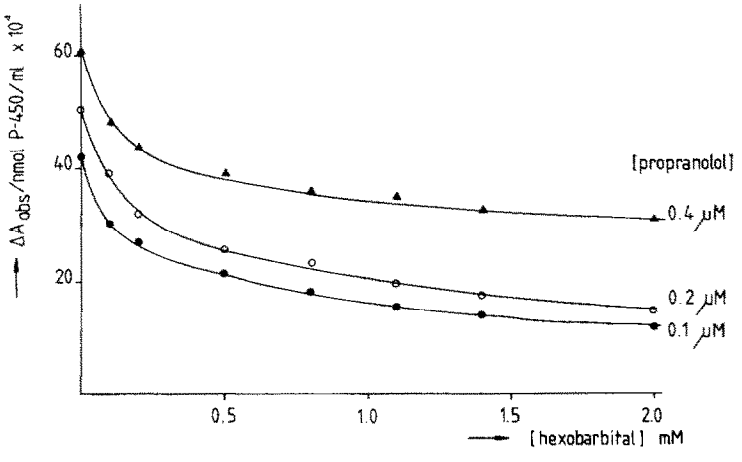

Fig. 4. Influence of hexobarbital on high affinity propranolol type I binding. The sample cuvette contained a fixed concentration of propranolol and the contents of both sample and reference cuvettes were titrated with hexobarbital. The magnitude of the difference spectrum ($\Delta A_{385-420}$/nmole P-450/ml) is plotted against the hexobarbital concentration.

of cytochrome P-450 by means of cross-over studies with several type I compounds. It is also applicable, for example, in studying both qualitative and quantitive effects of various enzyme inducers on cytochrome P-450 or for checking the results of a purification method of P-450 [20].

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REFERENCES

1. S. Orrenius, B. J. Wilson, C. von Bahr and J. B. Schenkman, in *Biological Hydroxylation Mechanisms* (Eds. G. S. Boyd and R. M. S. Smellie), p. 55. Academic Press, New York (1972).
2. Y. C. Martin and C. Hansch, *J. med. Chem.* **14**, 777 (1971).
3. J. B. Schenkman, D. L. Cinti, P. W. Moldeus and S. Orrenius, *Drug Metab. Dispos.* **1**, 111 (1973).
4. I. Roots and A. G. Hildebrandt, *Naunyn-Schmiedeberg's Arch. Pharmak.* **277**, 27 (1973).
5. A. P. van den Berg, J. Noordhoek and E. Koopman-Kool, *Biochem. Pharmac.* **28**, 37 (1978).
6. R. W. Estabrook, J. A. Peterson, J. Baron and A. Hildebrandt, in *Methods in Pharmacology* (Ed. C. F. Chignell), Vol. 2, p. 303. Appleton-Century-Crofts, New York.
7. H. Grasdalan, D. Bäckström, L. E. G. Eriksson, A. Eehrenberg, P. Moldeus, C. von Bahr and S. Orrenius, *Fedn. Eur. biochem. Soc. Lett.* **60**, 294 (1975).
8. J. C. Topham, *Biochem. Pharmac.* **19**, 1695 (1970).
9. M. A. Peters, *J. Pharmac. exp. Ther.* **181**, 417 (1972).
10. D. J. Greenblatt, K. Franke and D. H. Huffman, *Circulation* **57**, 1161 (1978).
11. W. Klinger, D. Müller and H. Ankermann, *Acta biol. med. germ.* **34**, 661 (1978).
12. R. E. Ebel, D. H. O'Keeffe and J. A. Peterson, *J. biol. Chem.* **253**, 3888 (1978).
13. G. Parry, D. N. Palmer and D. J. Williams, *Fedn. Eur. biochem. Soc. Lett.* **67**, 123 (1976).
14. K. Hermansen, *Acta pharmac. tox.* **27**, 453 (1969).
15. R. A. Branch, D. G. Shand, G. R. Wilkinson and A. S. Nies, *J. Pharmac. exp. Ther.* **184**, 515 (1973).
16. C. von Bahr, H. Vadi, A. Norlin and P. Moldeus, *Acta pharmac. tox.* **38**, 513 (1976).
17. D. W. Schneck, J. F. Pritchard and A. H. Hayes Jr., *J. Pharmac. exp. Ther.* **203**, 621 (1977).
18. I. Schuster, C. Fleschurz and I. Helm, *Eur. J. Biochem.* **51**, 511 (1975).
19. I. H. Segel, in *Enzyme Kinetics*, p.197. John Wiley, New York (1975).
20. F. P. Guengerich, *J. biol. Chem.* **253**, 7931 (1978).