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MICROCALORIMETRIC STUDY OF A COMPETITIVE EFFECT BETWEEN SOME INHIBITORS WITH A MAMMALIAN DIHYDROFOLATE REDUCTASE.

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ABSTRACT

An original microcalorimetric method is developed to evaluate the association constant ratio of two competitors with a great affinity for the same macromolecule. This method is applied to two antifolic drugs, Methotrexate and Pyrimethamin : these compounds bind to dihydrofolate reductase with association constant in the range of 10^7 - 10^8 M^{-1} and their constant ratio is about 12 in favour of Methotrexate.

INTRODUCTION

Methotrexate (MTX) and Pyrimethamin (PYR) are drugs used as inhibitors of dihydrofolate reductase (DHFR), an enzyme involved in protein biosynthesis. These molecules are both competitors with dihydrofolic acid (DHF) the natural substrate of the enzyme.

MTX, a widely used anticancer agent, is a structural analog of DHF, but PYR, a well known antimalarial drug, is chemically quite different from DHF. So it seems interesting to determine the thermodynamic binding parameters of these compounds, and their ability to compete for DHFR binding.

A microcalorimetric enthalpic titration method (1) has been developed to calculate binding parameters, stoechiometry (n), association constant (K) and ΔH values of biological complexes, as well as to determine competitive binding (2). The association constants of these complexes range from 10^7 to 10^8 M^{-1} (3). The sensitivity of microcalorimetry measurements does not permit today the evaluation of such great constants with sufficient accuracy. So, we have determined K from fluorescence measurements, the number of binding sites and ΔH values from microcalorimetric measurements. On the other hand, an original microcalorimetric method was developed to determine the affinity constant ratio of two substrates which present a competitive effect and great affinity for a binding macromolecule.

MATERIAL AND METHODS

Beef liver DHFR, which presents noticeable analogies with human DHFR was extracted and purified according to KAUFMAN's method (4). PYR and MTX were graciously provided by Specia laboratories.

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All experiments were performed at 10° C and pH 6.80 ; the ionic strength (0.154) was brought by phosphate buffer.

Fluorescence measurements were performed on a SFM 25 spectrofluorimeter KONTRON apparatus (λex = 280 nm, λem = 330 nm). The data were analyzed according to a method described elsewhere (3).

Microcalorimetric measurements were carried out with a flow apparatus LKB Bioactivity Monitor. Whenever it was necessary, the data were corrected from dilution and neutralization heat values. The two pumps were operated at the same flow rate of 20 ml. h^{-1}. The sensitivity used was 10 μ w full scale with a background noise of $\pm$ 0.1. Before the outset of each experiment, the base line was drawn when a drug phosphate solution was pumped into the first circuit while phosphate buffer solution was pumped into the second circuit. A sample of one ml of DHFR was injected between two bubbles to avoid diffusion phenomenon into the phosphate buffer flow, so the reagents volume came up to 2ml.

The heat measurements were obtained with a constant protein concentration and increasing ligand concentrations.

In the case in point let us call :

P_t : *the constant protein concentration.*

n : *the number of "equivalent" binding site per protein molecule (the term equivalent means that K values are identical or too similar to be differentiated by the technique used).*

L_i : *the total ligand concentration which is a variable.*

C_i : *the complex concentration at equilibrium.*

Q_i ($J.l^{-1}$) : *the experimental heat measurements corresponding to* L_i.

ΔH : *the enthalpy variation per mole of ligand bound.*

Under conditions in which temperature, pH, and ionic strength, remain constant for each ligand concentration, the apparent association constant and the heat evolved for each L_i *value can be written :*

$$K = \frac{C_i}{(nP_t - C_i)\ (L_i - C_i)} \quad ; \quad Q_i = C_i\ \Delta H \qquad (1)\ (2)$$

From relationships (1) and (2) one obtains the mathematical expression of the enthalpic titration curve $Q_i = f\ (L_i)$.

$$Q_i = \left[\frac{1}{K} + nP_t + L_i - \sqrt{\left(\frac{1}{K} + nP_t + L_i\right)^2 - 4\ nP_tL_i} \right] \frac{\Delta H}{2}$$

Fig. 1 shows theoritical curves obtained for binary complexes (n = 1) with a constant protein concentration, an arbitrary ΔH *value equal to 100 KJ. Mole^{-1} and different K values.*

The sensitivity of Bioactivity Monitor and the ΔH *values allowed us to use a protein concentration ranging from* 10^{-5} *to* 5.10^{-5} *M. With such concentration, fig. 1 shows that is was impossible to determine with a good accuracy association constants higher than* 10^6 M^{-1} *for binary complexes.*

Nevertheless ΔH *values can be calculated :* $\Delta H = \dfrac{Q_{sat}}{nP_t}$. Q_{sat} (Jl^{-1}) *is the heat measurement at substrate saturation of enzyme .*

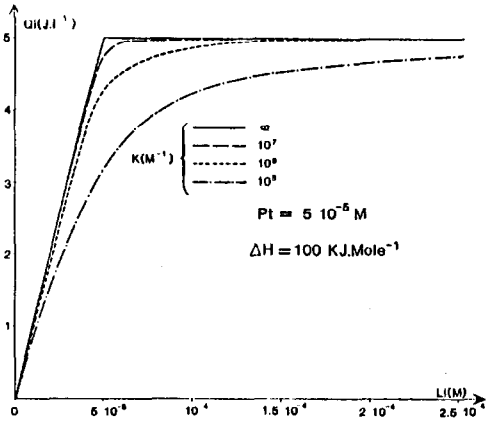

Fig. 1 Enthalpic titration curve

To determine Q_{sat} *the substrate concentrations were 5 to 10 fold higher than those of enzyme.*

To calculate n, additional heat measurements (Q_i) *were made with a substrate/protein ratio (i) lower than stoechiometry. If K value is high enough, the free drug concentration could be considered as negligeable and n could be written :* $n = i.Q_{sat}/Q_i$.

Moreover, microcalorimetric experiments made it possible to evaluate the K_1/K_2 *ratio of two substrate competitors,* S_1 *and* S_2*, with the same number of binding sites.*

Let us call : L_1 *and* L_2 *the total concentrations of competitors,* C_1 *and* C_2 *the concentrations of complexes at equilibrium,* ΔH_1 *and* ΔH_2 *the enthalpy variations,* Q $(J.l^{-1})$ *the experimental heat quantity,* P_t *and* P_1 *the total and free protein concentrations respectively. We can write :*

$$Q = C_1 \Delta H_1 + C_2 \Delta H_2 \qquad P_t = C_1 + C_2 + P_1 \qquad (3)\ (4)$$

$$K_1 = \frac{C_1}{P_1\ (L_1 - C_1)} \qquad K_2 = \frac{C_2}{P_1\ (L_2 - C_2)} \qquad (5)\ (6)$$

When S_1 *and* S_2 *are in large excess with regard to* P_t*,* P_1 *is always negligeable. So from relation ships 3, 4, 5 and 6 one obtains :*

$$\frac{K_1}{K_2} = \frac{(P_t - a)\ L_2 - a\ (P_t - a)}{a\ (L_1 - (P_t - a))} \qquad \text{with} \quad a = \frac{Q - P_t\ \Delta H_1}{\Delta H_2 - \Delta H_1} \qquad (7)$$

RESULTS

In fig. (2) are shown the experimental records obtained from a DHFR preparation of which specific activity was equal to 15.2 UI/mg.

PYR and MTX association constants calculated from fluorescence measurements were respectively equal to 1.6 10^7 M^{-1} and 3 10^8 M^{-1}.

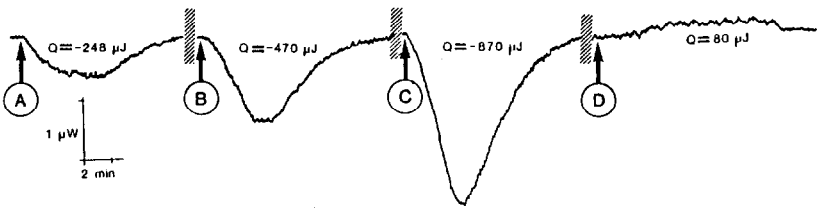

Fig. 2 : DHFR : 2.65 10^{-5} M ; A : DHFR (1ml) + PYR 6.64 10^{-6} M ; B : DHFR (1ml) + PYR 2.46 10^{-4} M ; C : DHFR (1ml) + MTX 9.26 10^{-5} M; D : (DHFR (1ml) + MTX 9.26 10^{-5} M) + PYR 2.46 10^{-4} M.

The number of PYR binding site determined from experiments A,B fig (2) was equal to 0.47. This value was identical for MTX. This fractional binding site can be explained by a partial denaturation of DHFR.

ΔH values calculated from experiments B,C fig (2) were - 37.6 kJ site $^{-1}$ and -70 kJ site^{-1} for PYR and MTX respectively.

Experiment D (fig (2) shows that PYR is a competitor for MTX binding site ; otherwise experiment D would be equal to B.

Using Q = (- 870 10^{-6} J + 80 10^{-6} J) .500 = - 395 10^{-3} J.
$[MTX]$ = 4.63 10^{-5} M, $[PYR]$ = 12.32 10^{-5} M, n $[DHFR]$ = 6.23 10^{-6} M, relation ship (7) lead to K_{MTX}/K_{PYR} = 12. This ratio is in keeping with the value obtained from fluorescence measurements.

CONCLUSION

Microcalorimetry appears to be a complementary technique to fluorescence and dialysis equilibrium in the estimation of the binding parameters of protein-substrate complex formation. At last, because of its simplicity and speed, this method would appear to hold promises for studies involving all pharmacological competitive binding particularly between a drug and its different metabolites.

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