Pergamon

Biochemical Pharmacology, Vol. 47, No. 2, pp. 415–417, 1994.

0006-2952/94 $6.00 + 0.00

Analysis of free fatty acid effect on methotrexate binding to albumin

(*Received* 22 *July* 1993; *accepted* 1 *October* 1993)

Abstract—The binding of methotrexate to human serum albumin and the inhibitory effect of serum free fatty acids (FFA) have been studied by equilibrium dialysis with radiolabeled methotrexate. Methotrexate was bound to albumin via a single site (1.03 ± 0.02) with a low affinity (1350 ± 60 M^{-1}). The effect of FFA on binding by albumin of methotrexate was analysed according to the classical inhibition models with computation of the free inhibitor concentration and was ascribed to an uncompetitive type of inhibition. These results were in agreement with the observed serum binding of methotrexate (45–50%) and allowed the simulation of the effect of various concentrations of FFA on methotrexate albumin binding in human serum.

Long chain free fatty acids (FFA*) and many drugs bind to human serum albumin (HSA). *In vivo*, drugs bind to HSA in the presence of 1–3 mol of FFA/mol of HSA. FFA have been reported to modify the binding of many drugs although they do not share drug binding sites on HSA, and both enhancement and inhibition of drug binding to albumin have been reported [1]. For instance, at low concentrations FFA enhanced warfarin or glafenic acid binding to HSA [2, 3], whereas they inhibited the binding of salicylic and salicyluric acids [4].

In the present study, the effects of FFA on methotrexate binding to human serum albumin have been analysed *in vitro*. The results of such binding interactions are usually presented and interpreted in terms of linearization procedures, with the inhibitor free concentration assumed to be the same as the total inhibitor concentration. On this note, an alternative approach is suggested for analysis of the interactions, equations have been derived to calculate the free inhibitor concentration (FFA) at each actual concentration of methotrexate, according to the three classical inhibition models, competitive, non-competitive and uncompetitive inhibition, and the untransformed binding data have been fitted to the model equations.

Materials and Methods

Radiolabeled [3′5′,7-^{3}H]methotrexate was purchased from Amersham (U.K.). The radiochemical purity was >95% by HPLC. The dried compound was dissolved in 20% dimethyl sulfoxide–80% methanol solution and stored at −20°. An isotopic dilution was made with unlabeled methotrexate to obtain a specific activity of 40 mCi/mmol. HSA without FFA (Sigma A1887) and HSA with FFA (Sigma A6909) were used.

Methotrexate binding to HSA with different amounts of FFA was studied by equilibrium dialysis (Dianorm apparatus, 0.25 mL teflon cells, Spectrapor membranes). HSA was dissolved in dialysis buffer (120 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM Tris) at pH 7.4 at physiological concentration (620 μM), and different FFA/HSA solutions were prepared (0/620, 389/620, 868/620) by mixing the two batches of HSA. Dialysis was performed against various concentrations of methotrexate in buffer (60–1750 μM), at 37°, under constant rotation (20 rpm). The dialysis time was 2.5 hr since preliminary studies showed equilibrium was achieved within 2 hr. Each sample was dialysed in duplicate. There was no significant volume shift nor methotrexate binding to the dialysis apparatus.

* Abbreviations: FFA, free fatty acids; HSA, human serum albumin.

The binding parameters (see below) were estimated by non-linear regression (least-squares criterion, Gauss–Newton algorithm) of data on model equations using MicroPharm©, a commercially available software [5].

Inhibition Models

For a better understanding of further reading, the following notations are used.

Concentration terms. B and F are the bound and free ligand concentrations, P_t the total protein concentration, I_t, I_b and I_f the total, bound and free inhibitor concentrations.

Parameters. n is the number of binding sites, K_a is the ligand association constant, K_i is the inhibition constant, and β is the proportionality constant for the non-specific binding.

In each model, it is assumed that the ligand and the inhibitor have similar effects on the binding of each other. The equations describing the binding of drug and inhibitor are the following.

Competitive inhibition.

$$B = \frac{n \cdot K_a \cdot F}{1 + K_i \cdot I_f + K_a \cdot F} P_t + \beta \cdot F \quad (1)$$

$$i_b = \frac{n \cdot K_i \cdot I_f}{1 + K_i \cdot I_f + K_a \cdot F} \cdot P_t + \beta \cdot I_f. \quad (2)$$

I_f was calculated at each step of the analysis as follows:

$$I_f = (I_t - I_b)A \quad (3)$$

where A is set to 1 (FFA did not diffuse in the buffer side of the membrane).

Then

$$I_f = (-q + \sqrt{\Delta})/(A + \beta)/2 \quad (4)$$

with

$$q = n \cdot P_t - I_t + (A + \beta)(1 + K_a \cdot F)/K_i \quad (5)$$

and

$$\Delta = q^2 + 4 \cdot I_t \cdot (A + \beta)(1 + K_a \cdot F)/K_i. \quad (6)$$

Non-competitive inhibition.

$$B = \frac{n \cdot K_a \cdot F}{(1 + K_i \cdot I_f)(1 + K_a \cdot F)} \cdot P_t + \beta \cdot F \quad (7)$$

$$I_b = \frac{n \cdot K_i \cdot I_f}{(1 + K_i \cdot I_f)(1 + K_a \cdot F)} \cdot P_t + \beta \cdot I_f. \quad (8)$$

Table 1. Model adequation and parameter estimates for methotrexate binding to HSA and inhibition by FFA

Model	Competitive	Uncompetitive	Non-competitive
n	0.84 ± 0.06	1.03 ± 0.02	0.83 ± 0.05
K_a (M^{-1})	1990 ± 300	1350 ± 60	2030 ± 240
K_i (M^{-1})	1980 ± 290	3640 ± 205	1690 ± 290
RSS	9731	882	8159
r	0.985	0.998	0.986

Values are given as means ± SD.
n, number of binding sites; RSS, residual sum of squares.

Then

$$I_f = (q + \sqrt{\Delta})/\{2(1 + K_a \cdot F)(A + \beta)\} \quad (9)$$

with

$$q = (1 + K_a \cdot F)\{I_t - (A + \beta)/K_i\} - n \cdot P_t \quad (10)$$

and

$$\Delta = q^2 + 4 \cdot I_t \cdot (A - \beta)(1 + K_a \cdot F)^2 / K_i. \quad (11)$$

Uncompetitive inhibition.

$$B = \frac{n \cdot K_a \cdot F}{1 + (1 + K_i \cdot I_f) \cdot K_a \cdot F} \cdot P_t + \beta \cdot F \quad (12)$$

$$I_b = \frac{n \cdot K_i \cdot I_f}{1 + (1 + K_a \cdot F) \cdot K_i \cdot I_f} \cdot P_t + \beta \cdot I_f. \quad (13)$$

Similarly, we expressed I_f from Eqns 3 and 13:

$$I_f = (-q + \sqrt{\Delta})/\{2(1 + K_a \cdot F)(A + \beta)\} \quad (14)$$

with

$$q = (A + \beta)/K_i - I_t \cdot (1 + K_a \cdot F) + n \cdot P_t \quad (15)$$

and

$$\Delta = q^2 + 4 \cdot I_t \cdot (A + \beta)(1 + K_a \cdot F)/K_i. \quad (16)$$

Eqns 1, 7 and 12 were deduced from classical model equations of enzyme inhibition [6].

Results and Discussion

A one-site Scatchard model proved adequate for the data analysis, with no non-specific binding ($\beta = -0.0009 \pm 0.065$, not significant). Our estimation of methotrexate–HSA interaction indicates that methotrexate was HSA-bound to a single site ($n = 1.03$) with a low association constant (1350 M^{-1}). Two binding sites ($n = 2$, one class of sites) have been reported previously with a lower affinity, resulting in a similar extent of binding to HSA [7], or with a higher affinity, resulting in a higher extent of binding [8]. In the last study, the authors used a spectroscopic method, and this could explain the difference. The difference we found in the number of binding sites, $n = 1$, could result from the use of a different buffer, whose ionic composition is closer to plasma ionic composition than the more commonly used Sörensen's buffer (phosphate buffer).

Methotrexate (1–2 μM) binding to a serum pool (HSA 620 μM, FFA 372 μM) was 46.2 ± 2.0%. Methotrexate binding to serum was reported to be more than 90% by one group [9] or in the range 40–55% by most authors [8, 10, 11].

Analyses of the data with the different models of inhibition indicated that the inhibition of methotrexate binding by FFA was uncompetitive (Table 1). Accordingly,

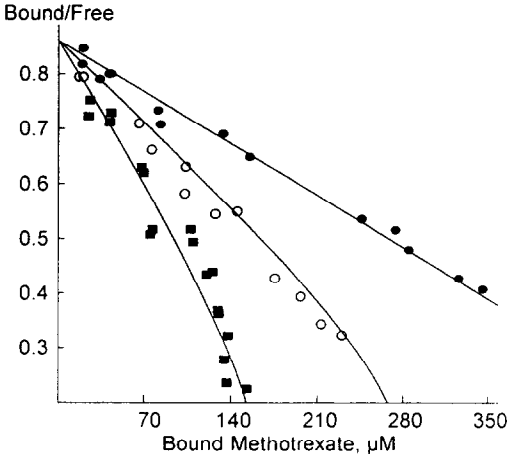

Fig. 1. Scatchard plots for the FFA-induced uncompetitive inhibition of methotrexate binding to albumin. Ordinate, bound/free methotrexate concentration ratio; abscissa, albumin-bound concentration of methotrexate, in the presence of 0 (●), 389 (○) or 868 (■) μM FFA.

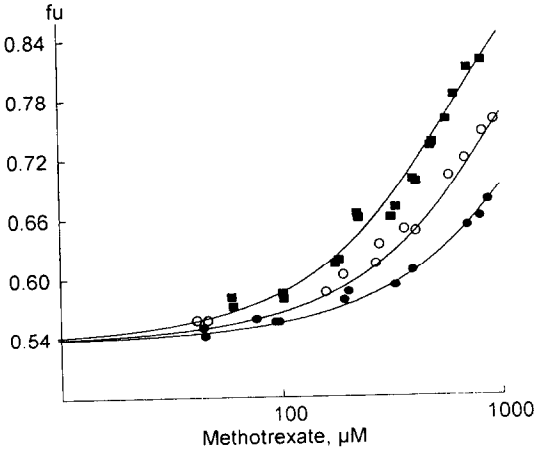

Fig. 2. Effect of FFA on methotrexate unbound fraction in human serum as a function of total methotrexate concentration. The albumin concentration is 620 μM and FFA are 0 (●), 389 (○) or 868 (■) μM.

the K_i value estimated cannot be interpreted as being equal to the equilibrium constant for the FFA–HSA interaction (actually K_i reflects the equilibrium constant between the ternary HSA–ligand–inhibitor complex on one hand and the binary HSA–ligand complex and the inhibitor on the other hand). The uncompetitive inhibition of methotrexate binding to HSA by FFA indicated that methotrexate and FFA do not share common sites on the HSA molecule. When the data were plotted via the Scatchard linearization procedure (Fig. 1), the non-linear aspect of inhibition curves (especially for the highest FFA concentration) showed that HSA-bound FFA were displaced when the methotrexate concentration was increased, and this validated the use of equations that take into account variation of inhibitor free concentration. According to these results and assuming methotrexate was exclusively bound to HSA, the HSA-bound fraction of methotrexate in the serum pool could be simulated, i.e. 46.3%, which is very close to the observed value, 46.2 ± 2.0%.

Given the parameters of methotrexate binding to HSA together with the FFA inhibitory model and parameters, the methotrexate unbound fraction in serum (f_u) was plotted as a function of methotrexate total serum concentration at three levels of FFA. As depicted in Fig. 2, the effect of FFA is negligible at low methotrexate concentration, i.e. below 10 μM; however, f_u is markedly increased above 40–50 μM total methotrexate.

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