Biochemical Pharmacology, Vol. 35, No. 16, pp. 2834–2836, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

Binding of 7-hydroxy-methotrexate to human serum albumin

(*Received* 9 *December* 1985; *accepted* 6 *February* 1986)

In the last decade, the folic antagonist Methotrexate (MTX) has been widely used in very high doses with citrovorum rescue for the treatment of a variety of neoplasma [1–2]. MTX metabolism rate, lower at conventional doses increases at higher doses [3]. Plasma pharmacokinetic studies have identified substantial amounts of 7-hydroxy-methotrexate (7OH-MTX) (Fig. 1A), an oxidative metabolite principally formed by hepatic aldehyde oxidase [4]. The plasma concentration of 7OH-MTX may actually exceed the MTX concentration a few hours after high-dose MTX infusion [5]. At drug doses of 300 mg/kg of body weight, the 7OH-MTX peak plasma level has been found to reach $5.6\,10^{-4}$ M [6].

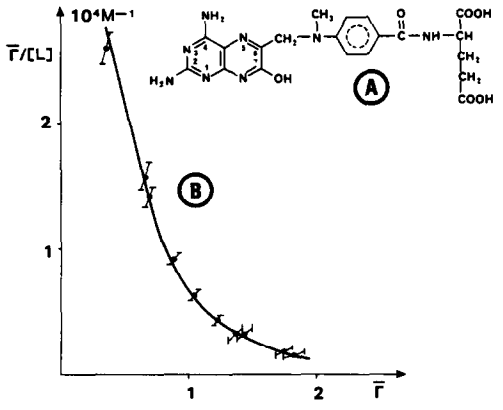

Fig. 1. (A) 7OH-MTX formula; (B) Scatchard plots of dialysis results on 7OH-MTX binding to HSA: — theoretical curve; ● experimental plots. [HSA] = $5.6\,10^{-4}$ M, pH = 7.4, $\mu = 0.154$, $\theta = 37°$.

Initially 7OH-MTX had been considered to be an inactive compound because of its low affinity with dihydrofolate reductase, the molecular target [7]. However, this metabolite has a lower aqueous solubility than the parent compound and may precipitate in the renal tubules, inducing nephrotoxicity [4]. In addition, recent *in vitro* studies have made obvious that 7OH-MTX was able to reduce MTX cellular uptake, to increase MTX efflux and to influence MTX polyglutamate formation [8–11], all of which might be related to the drug resistance encountered following high-dose therapy. So, monitoring 7OH-MTX plasma levels during MTX treatment will certainly improve our understanding of MTX time-dependent pharmacokinetics and drug reactions; moreover, it will certainly help to identify high-risk patients.

It is well established that the affinity of a drug for plasma proteins and the extent to which it is bound at therapeutic concentrations can greatly influence its distribution, excretion and interaction with other drugs [12]. To our knowledge, no results have yet been published dealing with 7OH-MTX plasma protein binding, except for the ultrafiltration data from Breithaupt and Küenzlen [6] claiming that 7OH-MTX plasma protein binding rate was 93% ± 2% [6]. This result is very high compared with MTX plasma binding rate [13–15]. In addition, MTX has been shown to bind in plasma primarily to serum albumin (HSA) [13–14]. Hence, firstly, our aim was to study *in vitro* 7OH-MTX binding to HSA by dialysis experiments in order to determine its binding parameters by Scatchard's analysis. Secondly, our purpose was to make obvious a possible competition between MTX and 7OH-MTX binding to HSA, so as to quantify, in therapeutic conditions, the free plasma concentrations of both compounds, since these free plasma species are commonly thought to be the only ones able to participate in pharmacological activity and in toxicity.

Materials and methods

Reagents. HSA, a Koch-Light product, was obtained from Sochibo S.A.. The absence of fatty acids, which can interfere with protein binding of many drugs, was verified as previously described [16]. The concentration of HSA was determined by absorbance measurements at 280 nm ($E_{cm}\% = 5.3$).

Purified MTX was kindly supplied by Lederle Labora-.ories; the [^{3}H]-MTX (As = 238 Ci/M) was from Amersham. 7OH-MTX was synthesized from MTX using partially purified aldehyde oxidase according to a modified Johns and Ti Li Loo method [17]. The modifications were the following: this enzymatic preparation was obtained from beef liver (400 g) instead of rabbit liver; 40 ml of a 10^{-2} M MTX solution in phosphate buffer (pH = 8.4) containing 5 mg of EDTA was added dropwise to the protein extract, with stirring, at a rate of 5 ml/hr and at room temperature; the solution was then allowed to stand at room temperature for 24 hr; then at 40° for the same time period; the yellowish floculent precipitate was collected by centrifuging, dissolved in 50 ml of 0.1 M ammonium hydrogenocarbonate solution (pH = 8.4) and applied to a 1.6 × 20 cm column of DEAE cellulose (DEAE Sephacel Pharmacia) equilibrated with the same solution; after loading at a rate of 40 ml/hr, the column was eluted with a linear gradient (800 ml total volume) of NH_4HCO_3 from 0.1 to 0.4 M, pH = 8.4; absorption spectra of fractions of 8 ml were obtained and those with spectra identical to 7OH-MTX were pooled and mixed with 4 ml of AGX4 resin (Biorad) previously equilibrated with distilled water; after centrifuging, the supernatant was discarded and the AGX4 pellet was washed by distilled water; this step was repeated twice; 10 ml of glacial acetic acid were added and the AGX4 pellet was resuspended; after centrifuging, the lemon-yellow coloured supernatant was retained; this step was repeated until no more colour was detectable in glacial acetic acid eluent; all supernatant fractions were collected with 1.2 volume of distilled water and this solution was then allowed to stand at 4° for 24 hr at least; 7OH-MTX precipitate thus obtained was separated by a final centrifugation, washed with cold water and lyophilized until dryness to a fluffy yellow solid. The molar yield in a typical preparation was 70%. The absorption spectra of the final product at pH 1.7 and 13 were identical to those published in the literature for 7OH-MTX [18]. This purification scheme easily separated 7OH-MTX from trace residual MTX, a finding then verified by reversed phase HPLC analysis of the final product [19].

Methodology

Equilibrium dialysis was carried out in a 1 ml macrocell using a rotative Dianorm apparatus, the two compartments of each cell being separated by a Diachema membrane (5000 D). At 37°, equilibrium was reached within 3 hr. The concentrations of free and bound 7OH-MTX were

determined in each compartment by absorbance measurements of solution dilutions in the range 1/3–1/200 at $\lambda = 306$ nm ($\varepsilon = 24{,}574\ M^{-1}\ cm^{-1}$) on a Uvikon 820 spectrophotometer (Roche Kontron). When tritium radiolabelled MTX solutions were used in competitive binding experiments, the concentrations of free and bound fractions of this drug in each compartment were determined by liquid scintillation counting in 10 ml Aquasol (New England Nuclear) on a Beckman LS 100 C apparatus. It was then possible to calculate at 306 nm the absorbance due to MTX in each compartment ($\varepsilon = 24{,}254\ M^{-1}\ cm^{-1}$) and to deduce the concentration of free and bound 7OH-MTX by spectrophotometric measurements.

The ionic strength of the different solutions was maintained at 0.154 with phosphate buffer pH 7.4. HSA concentrations were always about $5\ 10^{-4}$ M. 7OH-MTX solutions were in the 10^{-4}–$1.5\ 10^{-3}$ M concentration range. The results of dialysis experiments were plotted according to Scatchard's method [20], each plot represented the mean result obtained from five experiments operated in the same experimental conditions. The theoretical curve was then fitted to experimental plots by a non-linear least squares computation. Thus the 7OH-MTX binding parameters were determined with and without MTX.

Results and discussion

The computed results of dialysis experiments using a $5.6\ 10^{-4}$ M HSA solution showed two classes of HSA binding sites for 7OH-MTX: the first involving (0.8 ± 0.1) site with an apparent binding constant value of (5.0 ± 0.3) $10^4\ M^{-1}$, the second (1.6 ± 0.2) sites with a K_a value equal to (1.3 ± 0.2) $10^3\ M^{-1}$ (Fig. 1B). This result was quite different from those obtained for MTX; as a matter of fact, only class of HSA binding sites was made obvious for this drug ($N = 1.6 \pm 0.2$ and $K_a = (1.0 \pm 0.1)10^3\ M^{-1}$) which is in agreement with data previously published [15]. Thus at a $5.6\ 10^{-4}$ M 7OH-MTX concentration, corresponding to the peak plasma level after high dose MTX infusion [6], 65% of the 7OH-MTX bound to a $5\ 10^{-4}$ M HSA solution; this percentage was calculated using the kinetic parameters determined above. At a concentration of 10^{-5} M, corresponding to the plasma level at 60 hr after a continuous 6 hr infusion [6], this ratio was 95%. This latter result did not differ from the one published by Breithaupt and Küenzlen [6] relating a plasma binding ratio of 7OH-MTX equal to (93 ± 2)%. This was a proof that 7OH-MTX in plasma is mainly bound to HSA, as has equally been shown for MTX [15]. This result could be interpreted as suggesting that this binding is not modified by other plasma components; this was not the case for MTX for which plasma binding has been found to be decreased by chloride ions [15]. On the other hand, *in vivo*, the percentage of 7OH-MTX bound to plasma proteins would always be higher than that of MTX.

In other experiments, binding of 7OH-MTX to HSA ($4.77\ 10^{-4}$ M) was studied in presence of MTX ($5\ 10^{-4}$ M in dialysis cells), to determine any competition between both compounds. The experimental results were plotted according to Scatchard's method (Fig. 2). Due to the methodology described herein spectrophotometric measurements were not reliable for 7OH-MTX concentrations lower than $2\ 10^{-4}$ M. Nevertheless, the experimental plots were fitted as previously described for 7OH-MTX alone: two classes of HSA binding sites were observed once again, the first involving (0.6 ± 0.1) site with an apparent binding constant value of (8.7 ± 0.6) $10^4\ M^{-1}$, the second (1.8 ± 0.1) sites $K_a = (3.2 \pm 0.3)\ 10^3\ M^{-1}$. So, no decrease of 7OH-MTX binding to HSA was observed at an MTX concentration as high as $5\ 10^{-4}$ M; furthermore, on account of these binding parameters, the binding ratio of 7OH-MTX would be slightly higher in presence of MTX.

Concerning MTX, its binding to HSA was decreased when 7OH-MTX was present as is shown in Fig. 3.

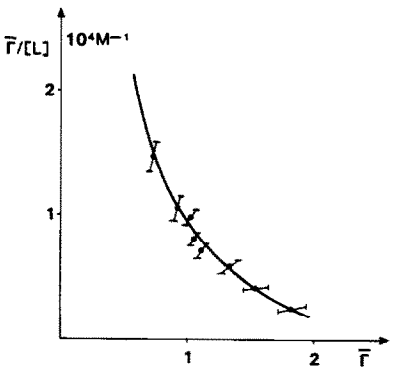

Fig. 2. Scatchard plots of dialysis results on 7OH-MTX binding to HSA in presence of a $5\ 10^{-4}$ M MTX concentration: — theoretical curve; ● experimental plots. [HSA] = $4.77\ 10^{-4}$ M; pH = 7.4; $\mu = 0.154$; $\theta = 37°$.

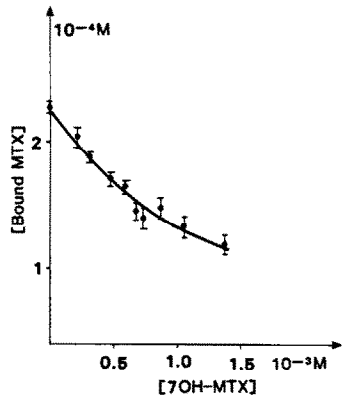

Fig. 3. HSA bound MTX concentrations versus 7OH-MTX concentrations. [HSA] = $4.77\ 10^{-4}$ M, [MTX] = $5\ 10^{-4}$ M; pH = 7.4; $\mu = 0.154$; $\theta = 37°$.

Our results suggested that MTX and 7OH-MTX binding to HSA would be somewhat mutually dependent, which is not surprising because of the structural similarity of both compounds. However, *in vivo*, the percentage of their free forms would not be expected to change considerably when 7OH-MTX metabolite is present in plasma.

In addition, 7OH-MTX did in fact bind to an additional site ($N = 0.8$, $K_a = 5.0\ 10^4\ M^{-1}$) when compared with MTX. This must be related to the presence of a hydroxyl group at position 7 (Fig. 1A), one not present for MTX. The corresponding proton must be somewhat acidic but no pK_a value was found by titrimetry in the pH range 5.5–9, as was the case for MTX. So no additional charge was thought to be present for 7OH-MTX at pH 7.4, the pH at which the experiments were performed. Nevertheless, this 7-hydroxyl group may contribute to hydrogen bond formation. Also, an enol-amide tautomeric equilibrium might be thought to take place for 7OH-MTX molecule, though the amide form would *a priori* seem rather unlikely to occur because of a loss of stability consequent to a decreased conjugation. If this form did exist, the pteridine ring would not conserve its rigidity and would no longer be plane. This flexibility would permit the metabolite to more easily enter inside the HSA macromolecule where binding would then occur. At the moment, no definitive explanation of the additional 7OH-MTX-HSA binding site can be provided; work is going on in this field.

Albeit, the binding rate of this metabolite to HSA is high, so the rather new concept that 7OH-MTX can modify

the chemotherapeutic activity of MTX, in particular because both compounds compete for cellular uptake [10] must be reconsidered in view of the low *in vivo* plasma concentrations of 7OH-MTX not bound to HSA.

Acknowledgements—The author thank Dr Maldonado for helpful discussions in the field of organic chemistry, Dr Sarrazin who provided programs for computation and H. Bouteille for his graphical work.

This study was supported by a grant from "Fédération des Centres de Lutte contre le Cancer".

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Biochemical Pharmacology, Vol. 35, No. 16, pp. 2836–2839, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

De novo analysis of receptor binding affinity data of β-carbolines

(*Received* 28 *October* 1985; *accepted* 10 *February* 1986)

β-Carbolines are a class of compounds, chemically unrelated to benzodiazepines (BDZs), able to interact at various affinity degrees with BDZ receptors in mammal Central Nervous System. Although several researches have been devoted to the study of quantitative structure–activity relationships (QSAR) for BDZs [1, 2], such an attempt has never been clearly carried out for the β-carbolines class of drugs. In this paper we report a QSAR study, making use of the Free and Wilson approach [3], on receptor binding affinity data for a series of 33 β-carbolines. The results of the analysis are briefly discussed in terms of the interactions of these compounds with the BDZ receptor.

Methods

(i) De novo *Model.* For QSAR studies the LFER (Linear Free Energy Relationships) approach [4] and the *de novo* model [3, 5, 6] are widely used. However, many of the independent substituents for the present series of compounds cannot be described by LFER continuous parameters. Therefore the *de novo* model, in its Free and Wilson version was preferred to the Hansch's approach. According to this model, the biological activities of molecules belonging to a homologous series can be expressed as the sum of a constant contribution, μ, and of as many individual contributions, α_{ij}, as there are substituents i in the different positions j. The group contributions were calculated by means of a modified version [7] of the Fortran program written by Purcell *et al.* [8].

(ii) *Data set.* β-Carbolines' receptor binding affinities (RBAs) are expressed by their ability to displace ^{3}H-flunitrazepam from synaptosomal rat brain membranes as the concentrations of the test compounds required to displace 50% of specific ^{3}H-flunitrazepam binding (IC_{50}). IC_{50} (μM) values used here were taken from Braestrup and Nielsen [9] for compounds 1–6, 9, 11–18 of Table 1, from Cain *et al.* [10] for compounds 21–23, 27–30, 33, from Loew *et al.* [11] for compound 27, from Lippke *et al.* [12] for compounds 25 and 26, or measured in our laboratory for compounds 7, 8, 10–13, 24, 31 and 32 by the method given by Karobath and Supavilai [13]. Data are not completely homogeneous in the sense that they have been obtained by several researchers. However, our redeterminations for some reference compounds show that possible systematic differences due to small changes of experimental procedure are very small, in agreement with what is generally observed for binding experiments.

Results and discussion

Table 1 shows the 33 β-carbolines employed in the Free and Wilson analysis together with the observed and calculated values of -log IC_{50}. The atoms or groups selected as independent variables are shown in Table 2 together with the values of the individual group contributions, α_{ij}, obtained from the analysis.

The two independent variables, AR and NAR, take into account, respectively, the aromaticity or not of the C ring. Compounds 12 and 13 are 3,4-H while compounds 14–20, 25 and 29 are 1,2,3,4-H β-carbolines. The AR/NAR variable is conceived as an indicator both of the planarity of the three rings system and of the coplanarity of the esteric function with ring C. X-Ray diffraction studies show that the three rings system is almost perfectly planar when