J. Pharm. Pharmacol. 1992, 44: 745–749
Received November 29, 1991

Nicotinate Esters: Their Binding to and Hydrolysis by Human Serum Albumin

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Abstract—Nicotinate esters were studied for their binding to, and hydrolysis by, human serum albumin. Some esters (ethyl, isopropyl, *t*-butyl, cyclohexyl, benzyl) were bound but not hydrolysed, while others (2-chloroethyl, 2-butoxyethyl) displayed the opposite behaviour; 1-carbamoylethyl ester was neither bound nor readily hydrolysed. Only *p*-methoxyphenyl nicotinate was both a ligand and a substrate, and its rate constants of binding and hydrolysis were calculated in a stepwise procedure using a kinetic model.

A number of authors have reported on the esterase activity of human serum albumin (HSA) towards substrates such as phenyl acetates and *p*-nitrophenyl esters (Means & Bender 1975; Kurono et al 1979, 1988, 1991) and aspirin derivatives (Kurono et al 1982). Inhibitors of this activity could be classified into three groups, affording a preliminary understanding of the catalytic sites on HSA (Ozeki et al 1980; Kurono et al 1981, 1982, 1987). In contrast, the binding parameters of the hydrolysed substrates were not determined, nor does the literature give much information on the binding of unstable compounds to HSA in particular and proteins in general. A first step in this direction is the work of Nouravarsani & Cobby (1982), who proposed a model describing the binding of an unstable drug to bovine serum albumin, but assumed absence of hydrolysis when the drug was bound to HSA.

In the present study, some nicotinate esters were selected as model compounds and their binding to and hydrolysis by HSA investigated. Application of the model proposed by Nouravarsani & Cobby (1982) was feasible only in a stepwise approach and for one compound whose binding and rate of hydrolysis were both of sufficient magnitude.

Materials and Methods

Chemicals

Solvents and chemicals were of analytical or HPLC grade. Human serum albumin (quality A 1887, fatty acid-free) was purchased from Sigma Chemical Company (St Louis, MO, USA). Esters of nicotinic acid (Table 1) were synthesized in our laboratory following published methods (Badgett et al 1945; Badgett & Woodward 1947; Charonnat et al 1948; Hassner & Alexanian 1978; Haslam 1980; Reymond et al 1987), except ethyl, butoxyethyl, and benzyl nicotinate, which were purchased from or kindly donated by Aldrich Chemie AG (Steinheim, Germany), Böhringer Ingelheim AG (Basel, Switzerland) and Fluka AG (Buchs, Switzerland), respectively. Phosphate and 3-morpholinopropane sulphonic acid (MPS) buffers and dimethylsulphoxide (DMSO) were purchased from Merck AG (Darmstadt, Germany), nicotinic acid from Fluka and acetonitrile from Romil Chemicals (Loughborough, UK).

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General conditions for all experiments

Solutions of the nicotinates were prepared in the same phosphate buffer (pH 7·4, ionic strength 0·17) as the HSA solution. DMSO (5%) was added to permit dissolution of the cyclohexyl, benzyl and *p*-methoxyphenyl esters. It had been shown that cosolvents in concentrations up to 5% do not alter the binding parameters to HSA (Zini 1984). All dialysis experiments were performed using an equilibrium dialyser (Diachema AG, Rüschlikon, Switzerland) under constant stirring (12 rev min^{-1}) at 37°C. The two chambers (half-cells) were separated by a semi-permeable membrane (Diachema 10·14, 5000 mol. wt cut-off).

The time to reach transport equilibrium between the two half-cells was determined by dialysis of a solution of nicotinate (20 μM) against buffer. Samples were taken at different times from both half-cells and analysed by UV-HPLC (see below). The mass balance indicated that no binding to the dialysis membrane or cell walls had occurred.

The optimal HSA concentration was obtained with a binding percentage equal to about 50%. The free ligand concentrations were chosen in the range 0·1 K_d to 10 K_d for optimal saturation of binding sites. Approximate K_d($K_d = 1/K_a$) values were determined in preliminary experiments.

After dialysis, the concentration of nicotinic acid and its esters was measured in the buffer compartment by reversed-phase HPLC. The equipment was a Data System 450 (Kontron AG, Zürich, Switzerland) equipped with an autosampler 460, a column LiChroCart 125-4 filled with 5 μm Lichrospher 100 (Merck AG, Darmstadt, Germany) and a type 430 UV detector set at 262 nm for all compounds. The very good reproducibility (s.d. < 3%) of the injections by the autosampler made the use of an internal standard superfluous. The mobile phases were mixtures of acetonitrile and MPS buffer (0·01 M; pH 7·4) in proportions depending on the ligand under study (25/75–80/20). The flow-rate was fixed at 1·5 mL min^{-1}.

For nicotinates with negligible hydrolysis, graphs of bound ligand concentrations (B) vs free ligand concentrations (F) were drawn by direct representation and the binding parameters (n_1 and K_{a1}) calculated with MICROPHARM, a nonlinear regression program based on a Gauss-Newton algorithm (MICROPHARM, Créteil, France).

The kinetics of binding and hydrolysis of *p*-methoxyphenyl nicotinate was studied in detail by taking samples from

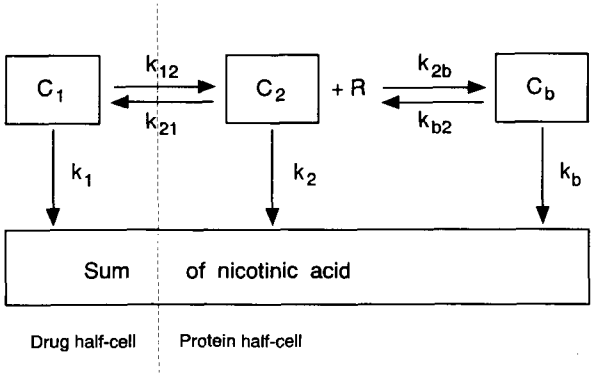
both half-cells at different times. In the protein half-cell, HSA was first precipitated with an equal volume of acetonitrile, and then centrifuged at 10000 rev min^{-1} (14000 *g*) for 10 min. The use of flow and solvent gradients allowed a good separation and quantitation of nicotinic acid and ester. The mobile phase was MPS buffer 0·01 M (pH 7·4) containing 20% acetonitrile for the first 5 min (flow-rate 1·0 mL min^{-1}) and then 40% acetonitrile (between 5 and 20 min, flow-rate 1·5 mL min^{-1}). The detector was set at 262 nm. Chemical hydrolysis of *p*-methoxyphenyl nicotinate was determined in buffer (Wernly-Chung et al 1990) and subtracted from hydrolysis in HSA solutions to obtain the esterase activity of HSA.

Models for the binding and hydrolysis of p-*methoxyphenyl nicotinate*

Using the program SIPHAR version 3·3 (SIMED, Créteil, France; weighting factor of 1 for the weighted least-squares algorithm), the concentrations of *p*-methoxyphenyl nicotinate and its metabolite nicotinic acid at different times in both half-cells were fitted by the various mathematical models described below.

General model

Model A. Scheme 1 shows the general model proposed by Nouravarsani & Cobby (1982) and represents the dynamics of drug-macromolecule binding as well as hydrolysis in an equilibrium dialysis system. Both half-cells are described and the dialysis membrane is represented by the dashed line. The symbols are as follows: C_1, free ester concentration in buffer half-cell; C_2, free ester concentration in protein half-cell; C_b, bound ester concentration in protein half-cell ($C_T = C_2 + C_b$); C_{bmax}, maximum bound ester concentration representing all available binding sites on HSA; R, concentration of unoccupied binding sites on the protein ($R = C_{bmax} - C_b$); ΣA, sum of nicotinic acid formed from C_1, C_2 and C_b; k_{12} and k_{21}, rate constants of diffusion of free ester through the dialysis membrane; k_{2b}, rate constant of formation of the complex; k_{b2}, rate constant of dissociation of the complex; k_1, rate constant of chemical hydrolysis in buffer half-cell; k_2, rate constant of chemical hydrolysis in protein half-cell; and k_b, rate constant of enzymatic hydrolysis.



SCHEME 1. General model (model A) representing the dynamics of drug-macromolecule binding as a multiple, rapid equilibrium dialysis system. For details and symbols, see text.

The variations in concentration of free and bound esters and acid are described by the following rate equations:

$$\frac{dC_1}{dt} = k_{21}C_2 - (k_1 + k_{12})C_1 \quad (1)$$

$$\frac{d(C_2 + C_b)}{dt} = k_{12}C_1 - (k_{21} + k_1)C_2 - k_bC_b \quad (2)$$

$$\frac{d\Sigma A}{dt} = k_1C_1 - k_2C_2 - k_bC_b \quad (3)$$

$$\frac{dC_2}{dt} = k_{12}C_1 - (k_{21} + k_2)C_2 - k_{b2}C_b - k_{2b}C_2(C_{bmax} - C_b) \quad (4)$$

$$\frac{dC_b}{dt} = k_{2b}C_2(C_{bmax} - C_b) - (k_{b2} + k_b)C_b \quad (5)$$

As the diffusion rates through the dialysis membrane should not be different when entering or leaving the buffer half-cell, we can assume that $k_{12} = k_{21}$. In addition, the rate constant of chemical hydrolysis should be equal in both half-cells i.e. $k_1 = k_2$. For substrates with low binding, R can be considered as a constant since in this case C_{bmax} is much higher than C_b.

Model without HSA

Model B. The measurement of the distribution of the ester and acid in absence of HSA in both dialysis half-cells allows the determination of k_{12}, k_{21} and k_1 ($k_1 = k_2$). These parameters should not be affected by the presence of HSA. The rate equations describing this situation are:

$$\frac{dC_1}{dt} = k_{21}C_2 - (k_1 + k_{12})C_1 \quad (6)$$

$$\frac{dC_2}{dt} = k_{12}C_1 - (k_{21} + k_1)C_2 \quad (7)$$

$$\frac{d\Sigma A}{dt} = k_1C_1 - k_2C_2 \quad (8)$$

General model with HSA, R being constant, k_1, k_2, k_{12} and k_{21} being fixed

Model C. This model is analogous to model A, except that k_1, k_2, k_{12} and k_{21} are given fixed values previously determined using the model without HSA. Additionally, R is assumed to be constant ($C_{bmax} \gg C_b$) and therefore can be included in k_{2b}. Thus equations 4 and 5 become 9 and 10:

$$\frac{dC_2}{dt} = k_{12}C_1 - (k_{21} + k_1 + k_{2b})C_2 + k_{b2}C_b \quad (9)$$

$$\frac{dC_b}{dt} = k_{2b}C_2 - (k_{b2} + k_b)C_b \quad (10)$$

Results and Discussion

The first column in Table 1 indicates the optimal HSA concentrations for which the binding percentage was about 50%. Preliminary measurements showed that a dialysis time of 90 min was necessary for aliphatic nicotinates, while cyclohexyl or aryl nicotinates necessitated 120 min (Table 1). The difference of dialysis times between aliphatic and

Table 1. Nicotinate esters: binding to and hydrolysis by human serum albumin (HSA).

Nicotinate ester	Human serum HSA (μM)[a]	Dialysis time (min)	Percentage hydrolysed		Percentage bound[d]	K_{a1} (M^{-1})	Binding parameters (n_1)	n_2K_{a2} (M^{-1})
			Buffer[b]	HSA[c]				
Ethyl	600	90	0·1	4·1	35·6	3560 ± 1070	0·34 ± 0·07	619 ± 25
Isopropyl	300	90	0	0	34·1	868 ± 435	1·1 ± 0·6	863 ± 101
t-Butyl	300	90	0·8	2·5	41·4	150 ± 16	17·7 ± 1·5	—
Cyclohexyl	50	120	0	0	35·9	13000 ± 1500	1·2 ± 0·1	9020 ± 280
Benzyl	30	120	0·6	6·4	25·2	75000 ± 17000	0·95 ± 0·16	13000 ± 967
2-Chloroethyl	400	90	0·5	50·3	0		no binding	
2-Butoxyethyl	30	90	0·4	28·6	0		no binding	
1-Carbamoylethyl	600	90	0·8	< 5	< 5		no binding	
p-Methoxyphenyl	30	120	7·1	21·6	14·0		see Table 2	

[a] Optimal concentration for 50% binding.
[b] Calculated from data of Wernly-Chung et al (1990) (MPS buffer 0·05; pH 7·4; $\mu = 0{\cdot}1$; 37°C).
[c] Measured in dialysis cells at dialysis time.
[d] Binding percentage taking hydrolysis into account.

cyclohexyl or aryl nicotinates probably depends on the higher molecular weight of the latter.

The percentages of nicotinate hydrolysed during equilibrium dialysis in buffer (chemical hydrolysis) and in a solution of HSA (chemical plus enzymatic hydrolysis) under the same conditions are listed in Table 1. Three categories of nicotinates can be distinguished. Esters of the first category (ethyl, isopropyl, *t*-butyl, cyclohexyl, benzyl and 1-carbamoylethyl nicotinate) undergo negligible chemical and enzymatic hydrolysis (usually < 5%). The ester moiety of these compounds is stabilized by functional groups having a positive inductive effect and no (or weak, benzyl nicotinate) mesomeric effect. All these compounds except the 1-carbamoylethyl ester show some affinity for HSA.

Esters of the second category, namely 2-chloroethyl and 2-butoxyethyl nicotinate, are rapidly hydrolysed by HSA. Both nicotinates have polar groups with negative inductive effects presumably favouring enzymatic hydrolysis. When the hydrolysis of these nicotinates is taken into account, no binding to HSA is found to occur.

p-Methoxyphenyl nicotinate displays intermediate behaviour. This compound undergoes relatively marked chemical and enzymatic hydrolysis (Fig. 1), and it is calculated to be moderately bound to HSA once total hydrolysis is taken into account.

Table 1 shows the binding parameters obtained for the nicotinates which exhibit affinity to HSA, K_{a1} and n_1 being the association constant and the number of binding sites of the high affinity binding site, respectively. The binding curve of cyclohexyl nicotinate drawn with the MICROPHARM computer program is presented as an example (Fig. 2). Except for the *t*-butyl ester, two families of binding sites are seen. The second family represents low-affinity, non-saturable sites, whose total affinity is expressed by the parameter n_2k_{a2}.

The affinity of nicotinates for HSA is highly variable, with the cyclohexyl and benzyl esters having a higher affinity than the alkyl esters perhaps for reasons of lipophilicity or steric effects. Strangely, the n_1 value of ethyl nicotinate is a fractional number. This could be due to the heterogeneity of HSA, with only a fraction of the macromolecules binding the ligand. In contrast, the n_1 value of *t*-butyl nicotinate is very large due to a very low affinity ($K_a < 200\ M^{-1}$) and perhaps to a non-specific binding.

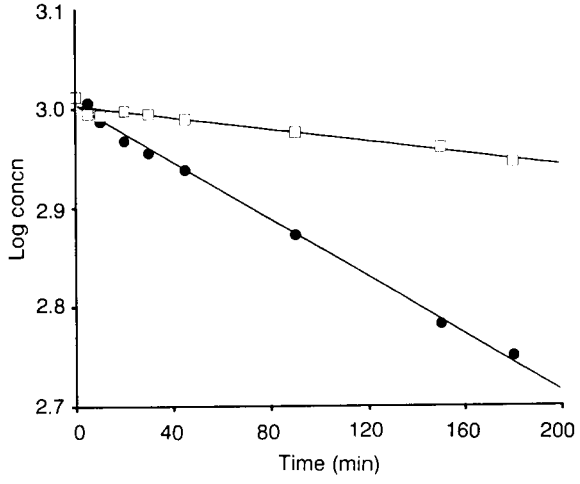


FIG. 1. Hydrolysis of *p*-methoxyphenyl nicotinate in buffer (□) and in HSA 300 μM (●). pH = 7·4; 37°C.

Among the nine nicotinic acid derivatives investigated, only six bind to HSA. The affinity of a drug for HSA is

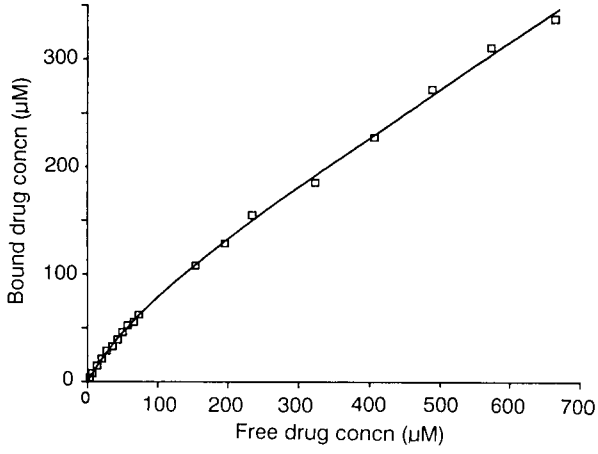


FIG. 2. Direct representation of the binding curve of cyclohexyl nicotinate.

considered as weak if $K_a \leqslant 20\,000\ M^{-1}$, intermediate if $20\,000\ M^{-1} \leq K_a \geqslant 100\,000\ M^{-1}$, and strong if $K_a > 100\,000\ M^{-1}$. Thus only benzyl nicotinate has an intermediate affinity for HSA, five nicotinates have a low affinity, while the others do not bind to HSA.

Models for the binding and hydrolysis of p*-methoxyphenyl nicotinate during dialysis*

p-Methoxyphenyl nicotinate was chosen for a full investigation because this compound is not only hydrolysed but also bound to HSA. The fitting of the kinetic data to the general model A gave no satisfactory results, the main problem being that there are six unknown variables and an insufficient number of known parameters. For this reason, an experimental design corresponding to model B (the absence of HSA, dialysis against buffer) was first used. This allows the determination of parameters k_1, k_{12} and k_{21} which should remain constant in the presence or absence of HSA.

The results of the regression (Table 2) indeed show that k_{12} and k_{21} had almost the same values, and that k_1 was very small indicating a slow rate of chemical hydrolysis. Since these rate constants should not be affected by the presence of HSA, they were introduced as fixed values in model C in order to reduce the number of unknown variables. The fact that *p*-methoxyphenyl nicotinate is only moderately bound to HSA allows further simplification of the general model. Indeed, in this case C_{bmax} is much higher than C_b, implying that $(C_{bmax} - C_b)$ has a constant value and can be included in k_{2b}. With these assumptions, a relatively good fit was obtained between experimental points and calculated curves (Fig. 3), despite some standard deviations being relatively large (Table 2).

It thus appears that the first-order rate of enzymatic hydrolysis is about 60-fold the rate of chemical hydrolysis, a modest increase when compared with carboxylesterase and human plasma (Durrer et al 1991, 1992). Despite the large standard deviation associated with k_{b2}, Table 2 indicates that the drug-HSA complex is dissociated faster than it is formed.

Interactions between xenobiotics and biological systems are always manifold and complex, obliging workers to investigate underlying phenomena separately and under artificial experimental conditions. In this study, we show that HSA can bind or hydrolyse nicotinate esters, and an attempt is made to investigate the two phenomena simultaneously. This approach proved feasible for one such ester, *p*-methoxyphenyl nicotinate, whose affinity to and rate of hydrolysis by HSA are both of sufficient magnitude to permit quantitative assessment. For this purpose, simplified forms of a general kinetic model had to be used at the expense of complete mathematical rigour. However, we believe that the approach and first results reported here afford an important warning and should stimulate further research.

Table 2. *p*-Methoxyphenyl nicotinate: first-order rate constants of binding to and hydrolysis by HSA.

Rate constants (min^{-1}) as calculated with the kinetic models B or C.

Model B (no HSA)		Model C (with HSA)	
k_{12}	0·0414 ± 0·0039		
k_{21}	0·0432 ± 0·0047		
k_1	0·0003 ± 0·0001		
		k_b	0·0195 ± 0·0036
		k_{2b}	0·0042 ± 0·0040
		k_{b2}	0·0084 ± 0·0217

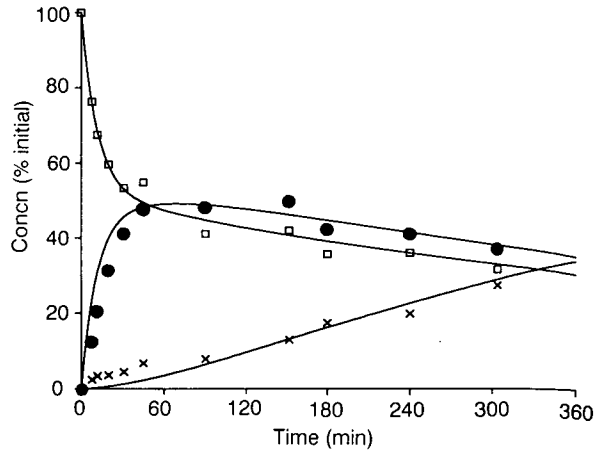


FIG. 3. Kinetics of *p*-methoxyphenyl nicotinate obtained from model C. Ester in drug half-cell, □; ester in protein half-cell, ●; total acid, ×.

The warning should be clear, namely that premature conclusions can be drawn from binding studies that neglect other essential aspects of the ligand-protein interaction. The esterase activity of HSA is precisely such a critical phenomenon whose study may prove as rewarding as its neglect can be misleading.

Pharmacologically, the main side-effects of nicotinates are vascular. These effects could perhaps be decreased if the drugs were bound to plasma proteins. However, the nicotinates investigated here have a low affinity for HSA. Binding to α_1-acid glycoprotein and lipoproteins was not investigated but could, if high enough, result in a different pharmacokinetic behaviour. Another solution could be the synthesis of nicotinates whose chemical structure would favour binding to HSA but prevent or decrease hydrolysis mediated by the protein, allowing selective hydrolysis in the liver, which is the site of the lipid-regulating action of nicotinic acid.

Acknowledgements

The authors are indebted to Professor Jean-Paul Tillement, Créteil, for his interest and fruitful discussions. B. Testa acknowledges support by the Swiss National Science Foundation.

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