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QSAR analysis of chemical and serum-catalyzed hydrolysis of phenyl ester prodrugs of nipecotic acid

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Summary

Chemical and serum-catalyzed hydrolysis of an appropriately designed series of substituted X-phenyl esters of nipecotic acid were studied. A pseudo-first-order kinetics has been observed in the rates of both kinds of hydrolysis and for the serum-catalyzed hydrolysis the following quantitative structure-activity relationship was derived: $\log t_{1/2} = -0.99\sigma^- - 0.21\pi + 2.25$. In this equation $t_{1/2}$ is the half-life, σ^- is the "trough resonance" Hammett constant and π is the Hansch hydrophobic constant. The correlation equation pointed out the great influence of the electronic properties of X-substituents on the rate of hydrolysis which is only slightly influenced by the hydrophobicity of X. A group of suitably selected esters **4** was tested for antagonism of convulsions and death induced by bicuculline and a correlation between in vitro rates of enzymatic hydrolysis and anticonvulsant activity could be found. The results have been discussed in relation to the design of ester prodrugs of polar GABA-mimetics and other CNS-active agents.

Introduction

In the design of CNS-active drugs a particular attention has to be devoted to their lipophilic character, which has to be comprised in an optimal range to allow the crossing of the blood–brain barrier (BBB). This is an indispensable condition to elicit any central pharmacological activity unless an active transport is involved. Recently a review has been written on this subject and a new and reliable hypothesis about the principle of "minimum hydrophobicity" for drugs has been proposed (Hansch et al., 1987).

However, at present the possibility to evaluate correctly the CNS in vivo activity of several substances active in vitro is in many cases hampered by the lack of the above fundamental prerequisite and thus, several approaches have been developed to overcome this problem.

A relatively new and promising strategy, based on the use of a site-specific delivery system, has been successfully applied to some CNS-active molecule (Bodor and Brewster, 1983) but classical approaches like the synthesis of suitable analogs and prodrugs are still very active and open research fields (Stella and Himmelstein, 1980). The prodrug approach has been utilized for very polar

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molecules, in which several restrictions about their possible clinical value exist because their poor availability limits the in vivo activity. However, a complete QSAR study aimed at detecting the main physicochemical factors responsible for the observed activities has been rarely undertaken. Among the different polar CNS-active substances, GABA and GABA-related aminoacids like isoguvacine and nipecotic acid have been especially investigated (Wermuth et al., 1982). Unfortunately most derivatives, resulting from alkylation or acylation of the amino function, esterification or amidification of carboxylic group and both variations coupled, did not elicit an anticonvulsant potency comparable with other GABA-agonists such as, for instance, 4,5,6,7-tetrahydroisoxazolo-[5,4-c]-pyridin-3-ol (THIP) (Krogsgaard-Larsen et al., 1984). Recently, however, a good correlation between the in vitro enzymatic hydrolysis and pharmacological effect of isoguvacine ester prodrugs has been found (Falch et al., 1981) and these promising results indicate that the prodrug

TABLE 1

Analytical and physical properties of the newly synthesized X-phenylnipecotates **4**

Compounds	Analysis(%) Calcd. C	H	N	Found C	H	N	M.p. (°C)	Cryst./ solvent [a]	IR [b] (cm^{-1})	^{1}H-MNR [c], δ (ppm)
4b	61.05	7.09	5.48	60.68	7.45	5.58	121–123	B	1765(C = 0)	1.60–2.20(m, 4H, $C\underline{H}_2C\underline{H}_2CH$), 2.30(s, 3H, CH_3), 2.85–3.70(m, 5H, $C\underline{H}_2NH_2^+C\underline{H}_2C\underline{H}$), 7.00(d, 2H, J = 8, ArH), 7.20(d, 2H, J = 8, ArH).
4c	64.56	8.07	4.71	64.21	8.31	4.89	125–127	B	1760(C = 0)	0.80(t, 3H, J = 7, $C\underline{H}_3CH_2$), 1.15(d, 3H, J = 7, $C\underline{H}_3CH$), 1.50(qt, 2H, J = 7, $C\underline{H}_2CH_3$), 1.75–2.20(m, 4H, $C\underline{H}_2C\underline{H}_2CH$), 2.50(qt, 1H, J = 7, $C\underline{H}C_2H_5$), 2.70–3.90(m, 5H, $C\underline{H}_2NH_2^+C\underline{H}_2C\underline{H}$), 6.90(d, 2H, ArH, J = 8), 7.10(d, 2H, ArH, J = 8).
4i	65.61	6.37	4.03	65.25	6.33	3.98	213–215	A	1750(C = 0)	1.60–2.15(m, 4H, $C\underline{H}_2C\underline{H}_2CH$), 2.80–3.60(m, 5H, $C\underline{H}_2NH_2^+C\underline{H}_2C\underline{H}$), 5.05(s, 2H, CH_2–Ø), 7.00(s, 4H, ArH), 7.20–7.45(m, 5H, ArH).
4k	60.50	6.77	4.70	60.15	6.84	4.89	168–170	A	1760(C = 0, ester) 1700(C = 0, ketone)	1.20(t, 3H, J = 6, CH_3), 1.50–2.50(m, 4H, $C\underline{H}_2C\underline{H}_2CH$), 2.70–3.90(m, 5H, $C\underline{H}_2NH_2^+C\underline{H}_2C\underline{H}$), 2.90(q, 2H, J = 6, CH_2CO), 7.10(d, 2H, J = 9, ArH), 7.80(d, 2H, J = 9, ArH).
4m	46.64	5.72	8.37	46.51	5.95	8.19	183–185	C	1750(C = 0) 1340, 1160 (SO_2)	1.70-2.40(m, 4H, $C\underline{H}_2C\underline{H}_2CH$), 3.00–3.80(m, 5H, $C\underline{H}_2NH_2^+C\underline{H}_2C\underline{H}$), 3.15(s, 3H, CH_3SO_2, partially overlapped with the latter m), 7.20(d, 2H, J = 8, ArH), 7.40(d, 2H, J = 8, ArH).
4p	57.46	6.68	5.15	57.09	6.59	5.38	163–165	B	1765(C = 0)	1.65–2.30(m, 4H, $C\underline{H}_2C\underline{H}_2CH$), 2.85–3.70(m, 5H, $C\underline{H}_2NH_2^+C\underline{H}_2C\underline{H}$), 3.75(s, 3H, OCH_3), 6.70–7.00(m, 3H, ArH), 7.25–7.35(m, 1H, ArH).
4r (X = 4-OC_6H_{13})	63.24	8.25	4.10	62.90	8.28	4.19	167–169	B	1760(C = 0)	0.80(t, 3H, J = 7, CH_3), 1.10–2.50(m, 14H, $-(CH_2)_5-$ + $C\underline{H}_2C\underline{H}_2CH$), 2.60–3.70(m, 5H, $C\underline{H}_2NH_2^+C\underline{H}_2C\underline{H}$), 6.75(d, 2H, J = 9, ArH), 6.90(d, 2H, J = 9, ArH).

[a] A, ethanol; B, ethanol-diethylether; C, acetone.

[b] Only the most characteristic absorption bands are reported.

[c] Spectra were recorded in the following solvents: $CDCl_3$ for **4c**, **4k** and **4r**; DMSO-d_6 for **4b** and **4i**; D_2O for **4m** and **4p**. The NH_2^+ protons give rise to a broad signal (exch. with D_2O) in the range 9.35–9.70 δ.

approach might be useful in the field of GABA-related compounds.

Also for nipecotic acid the same approach yielded to some interesting results. (±)-Nipecotic acid is a potent in vitro inhibitor of neuronal and glial GABA uptake; the R-(−) enantiomer possesses an affinity for the GABA uptake carrier approximately 5 times greater than that of the S-(+)-enantiomer (Krogsgaard-Larsen and Johnston, 1975; Johnston et al. 1976). Unfortunately nipecotic acid is devoid of any in vivo activity since it does not easily penetrate the BBB, due to its polar nature (Lodge et al., 1977; Frey et al., 1979). Many prodrugs showing varying degrees of anticonvulsant activity have been reported (Wermuth et al., 1982) and among them some X-phenyl esters **4** (particularly *m*- and *p*-nitro) have been claimed to possess a good anticonvulsant activity (Crider et al., 1982, 1984; Hinko et al., 1984). Since apparently no kinetic data have been published about their enzymatic cleavage, we planned the synthesis of a suitably designed series of substituted X-phenyl esters **4** (see Table 2) and the study of their uncatalyzed and catalyzed (by non-specific esterases from human serum) hydrolysis.

The principal aim of these studies was to determine the main factors governing the serum-catalysed hydrolysis and the pharmacological activity and possibly to establish whether such activity could be due to the intact molecules **4** or to the nipecotic acid deriving from their in vivo hydrolysis. A group of esters representing different rates of hydrolysis was then selected and subjected to pharmacological testing in order to study the correlation between the hydrolysis rate in approximately physiological conditions and the in vivo activity since the ability to predict the in vivo conversion rate of a prodrug to its active form is highly desirable in various pharmacokinetic models.

Materials and Methods

Apparatus

Melting points were determined by the capillary method on a Electrothermal apparatus (Gallenkamp MFB 595) and are uncorrected. IR-spectra were recorded as KBr pellets on a Perkin-Elmer 283 spectrophotometer; only the most significant absorption bands have been reported in Table 1.

^{1}H-NMR spectra were taken on a Varian EM 390 spectrometer using tetramethylsilane as an internal reference. Chemical shifts were expressed in δ values downfield from Me_4Si; the coupling constants are expressed in Hz; exchange with D_2O was used, when necessary, to identify NH and COOH protons. The following abbreviations have been used: s, singlet; d, doublet; t, triplet; q, quartet; qt, quintet; m, multiplet(s). Elemental analyses were performed by the Analytical Laboratory Service of the Dipartimento Farmacochimico of the University of Bari on a Hewlett-Packard 185 C, H, N analyzer and agreed with theoretical values to within ±0.40%. HPLC analyses have been carried out on a Waters Associates Model 510 constant-flow pump equipped with a Waters 481 variable wavelength detector and a Rheodyne Model 7125 injection valve (with a 10 µl loop), by using a µ-bondapack C_{18} (10 µm) column (150 × 3.9 mm i.d.) as stationary phase.

Synthesis of X-phenyl 3-piperidine-carboxylate hydrochlorides ***4***

The substituted X-phenyl esters of nipecotic acid (**4a–r**) were synthesized as shown in Scheme 1, according to the method previously described by Crider et al. (1982, 1984). 1-(Tert-butyloxycarbonyl)piperidine-3-carboxylic acid (**2**) was obtained in 85% yield, m.p. 147–149° C (lit. m.p. 149–151° C) by classical method.

Esterification of BOC-protected nipecotic acids (3): General procedure

To a stirred solution of **2** (1.50 g, 0.011 mol) and appropriate X-substituted phenol (0.011 mol) in acetonitrile (120 ml), dicyclohexylcarbodiimide (2.30 g, 0.011 mol) was added in one portion. A precipitate of dicyclohexylurea (DCU) was formed immediately. The stirring was continued at room temperature (20–25° C) for 48–72 h and DCU was filtered off. The filtrate was concentrated to

TABLE 2

Parameters and kinetic data used for derivation of Eqns. 2–6 for uncatalyzed and serum-catalyzed hydrolysis of X-phenyl nipecotates **4** *at 37 °C, pH 7.40 (0.05 M phosphate buffer)*

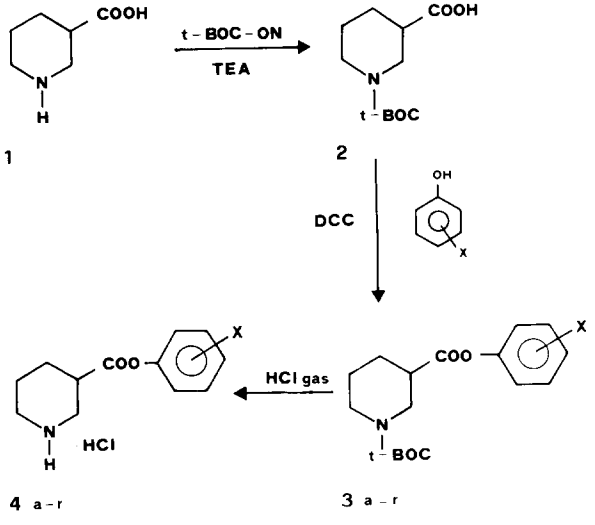
Compounds	X	log k_{OH}		log $t_{1/2}$		σ^{-} [c]	MR[c]	π[c]
		obsd.	calcd. [a]	obsd.	calcd. [b]			
4-a	H	3.53	3.51	2.21	2.25	0.00	0.10	0.00
b	4-CH_3	3.31	3.38	2.34	2.28	−0.15	0.56	0.56
c	4-$CH(CH_3)C_2H_5$	3.37	3.39	1.91	1.95	−0.13	1.96	2.04
d	4-$C(CH_3)_3$	3.40	3.39	2.06	1.96	−0.13	1.96	1.98
e	4-Cl	3.71	3.74	1.88	1.83	0.27	0.60	0.71
f	4-Br	3.90	3.75	1.89	1.79	0.28	0.89	0.86
g	4-I	3.79	3.77	1.82	1.72	0.30	1.39	1.12
h	4-OCH_3	3.41	3.37	2.33	2.41	−0.10	0.79	−0.02
i	4-$OCH_2C_6H_5$	3.53	3.37	1.75	2.06	−0.16	3.22	1.66
j	4-$COCH_3$	4.11	4.26	1.54	1.50	0.87	1.12	−0.55
k	4-COC_2H_5	4.10	4.26	1.54	1.37	0.87	1.58	0.06
l	4-NH_2	3.09	3.38	2.76	2.65	−0.24	0.54	−1.23
m	4-$NHSO_2CH_3$	3.64	3.53	2.29	2.47	0.03	1.82	−1.18
n	4-NO_2	4.56	4.58	0.90	1.08	1.24	0.74	−0.28
o	4-CN	4.42	4.37	1.39	1.38	1.00	0.63	−0.57
p	3-OCH_3	3.51	3.58	2.38	2.16	0.09	0.79	−0.02
q	3-NO_2	4.38	4.12	1.48	1.60	0.71	0.74	−0.28

[a] Calculated using Eqn. 2.

[b] Calculated using Eqn. 6.

[c] Taken from standard compilations (Hansch and Leo, 1979); as usual MR has been scaled by 0.1 to make it more nearly equiscalar with π.

about 30 ml and after the addition of few drops of glacial acetic acid the mixture was stirred for another hour. The further precipitate was filtered off and the solvent evaporated under reduced pressure gave an oil which generally solidified after trituration with diethyl ether. This product was used in the subsequent deprotection reaction without further purification. An additional purification by recrystallization from abs. ethanol was instead necessary for *p*-nitrophenyl-1-(tert-butyloxycarbonyl)-3-piperidine carboxylate (**3n**), m.p. 77–79 °C (lit. m.p. 73–75 °C), since it had to be submitted to a different and more sensitive reaction (catalytic reduction with Pd/C).



Scheme 1

*p-Aminophenyl-1-(tert-butyloxycarbonyl)-3-piperidine carboxylate (**3l**)*

A suspension of **3n** (3.0 g, 0.009 mol) and 0.10 g of 10% palladium on carbon in 120 ml of ethanol was hydrogenated at room temperature under atmospheric pressure with stirring until a TLC analysis revealed the complete disappearance of the starting material. The mixture was gently warmed to dissolve the partially precipitated product, filtered on Celite to remove the catalyst and

evaporated under reduced pressure to give 2.2 g of 3l (76%), m.p. 154–156 °C from abs. ethanol; IR: 3480 and 3380 (NH_2), 1750 (CO, ester), 1670 (CO, carbamate) cm^{-1}; NMR ($CDCl_3$): δ 1.45(s, 9H, $C(CH_3)_3$), 1.60–2.00(m, 4H, $C\underline{H}_2C\underline{H}_2CH$), 2.60–3.35(m, 3H, CHNCH and $C\underline{H}$-COOAr), 3.70–4.40(m, 4H, CHNCH and NH_2, exch.D_2O), 6.65(d, 2H, J = 9, ArH), 6.85(d, 2H, J = 9, ArH). Anal. Calcd. for $C_{17}H_{24}N_2O_4$: C, 63.73; H, 7.55; N, 8.74. Found: C, 63.58; H, 7.81; N, 8.61.

*p-N-methanesulfonylaminophenyl-1-(tert-butyloxycarbonyl)-3-piperidine carboxylate (**3m**)*

Methanesulfonylchloride (0.40 ml, 0.005 mol) was added dropwise to a stirred solution of **3l** (1.60 g, 0.005 mol) in 15 ml of dry dioxane and 3 ml of dry pyridine, at 0–5 °C. After the addition stirring was continued for 5 h. The reaction mixture was then poured into ice-cold 2 N HCl and the resulting solid was collected and crystallized from aqueous ethanol to yield 1.55 g (78%) of **3m**, m.p. 138–140 °C; IR: 1750(CO, ester), 1670(CO, carbamate), 1340 and 1150(SO_2); NMR($CDCl_3$): δ 1.45(s, 9H, $C(CH_3)_3$), 1.60-2.00(m, 4H, $C\underline{H}_2C\underline{H}_2CH$), 2.50-3.40(m, 3H, CHNCH and $C\underline{H}$-COOAr), 2.95(s, 3H, CH_3SO_2), 3.65-4.30(m, 3H, CHNCH and NH, exch.D_2O), 7.00(d, 2H, J = 9, ArH), 7.25(d, 2H, J = 9, ArH). Anal. Calcd. for $C_{18}H_{26}N_2O_6S$: C, 54,26; H, 6.58; N, 7.03. Found: C, 54.64; H, 6.95; N, 7.21.

Removal of the tert-butyloxycarbonyl group

A solution of BOC-protected nipecotic acid X-phenyl ester (**3a–r**) (0.001 mol) in 100 ml of chloroform was saturated with hydrogen chloride at 0–5 °C. The solvent was evaporated and the resulting solid was crystallized to afford the corresponding X-phenyl 3-piperidine carboxylate hydrochlorides **4**.

The physical and spectroscopic data of the already reported compounds agreed with literature data (Crider et al., 1982 and 1984); the analytical properties of the newly synthesized compounds are listed in Table 1. The total yields, calculated with respect to the starting material **2**, were in the range 25–40%.

Kinetic measurements

Chemical hydrolysis

The study of the chemical hydrolysis of esters **4** was performed in 0.05 M phosphate buffer solution (pH = 7.40) at 37.0 ± 0.2 °C. The pH-rate profile of ester **4a** was determined using 0.05 M phosphate (pH 7.00–7.70) and 0.05 M borate (pH 8.50–10.75) buffers. A constant ionic strength (μ = 0.5) was maintained for each buffer solution by adding a calculated amount of potassium chloride. The reactions were initiated by adding a methanolic solution of the ester **4** to the buffer solution pre-equilibrated at 37 °C to give an initial concentration of about 4×10^{-4} M. The concentration of organic solvent in the reaction solution did not exceed 1%. The solution was kept at constant temperature in a water-bath and at appropriate intervals aliquots were withdrawn and mixed with a predetermined amount of 40% w/v phosphoric acid to give an appropriate pH to quench the hydrolysis (pH 4). 10 μl portions of the mixture were analyzed by HPLC.

Serum-catalysed hydrolysis

The hydrolysis of esters **4** was studied in 0.05 M phosphate buffer (pH 7.40) containing 10% of human serum at 37 °C. The hydrolysis kinetics of esters **4f, h, k** were also studied in presence of different percentages of serum (5–80%) and the results obtained are shown in Fig. 5 and Table 4.

The serum protein content was determined by the biuret–tartrate method (Henry, 1968) using bovine serum albumin as a standard. Two different pools of serum were used in our analysis: one, with a total protein content of 70.6 ± 0.6 mg/ml for collecting the data used in the derivation of the multiparametric equations 2–6 and the second (total protein 78.8 ± 0.9 mg/ml) for evaluating the influence of the serum concentration on the hydrolysis of some representative X-phenyl esters **4f, h, k**.

The initial ester concentration and the other experimental conditions in the kinetic runs with serum were as described for the chemical hydrolysis. At various times 1 ml of sample was withdrawn and added to 0.2 ml of 10% w/v trichloroacetic acid in order to deproteinize the serum. After

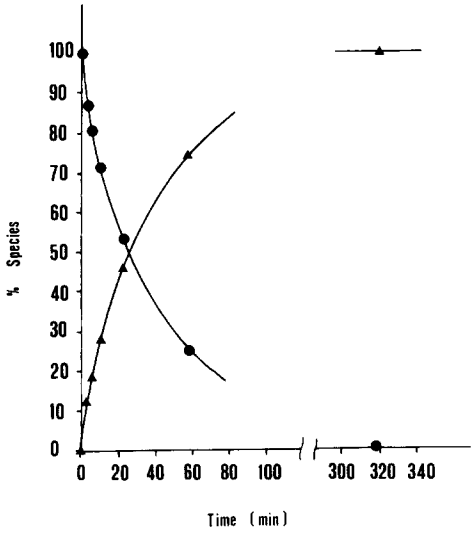


Fig. 1. Time-courses of disappearance of *p*-nitrophenyl nipecotate **4n** (●) and appearance of p-nitrophenol (▲) in 0.05 M phosphate buffer solution (pH 7.40) at 37 °C.

mixing and centrifugation (5 min at 4.000 rpm), 10 μl of the clear supernatant was injected on the column for HPLC analysis. By this procedure the esters **4** did not significantly hydrolyse during the sample preparation. The progress of chemical and serum-catalyzed hydrolysis was followed by simultaneously determining the disappearance of nipecotic acid aryl ester and the appearance of its hydrolysis product (phenol) as a function of time (Fig. 1). The reversed-phase column (μ-bondapack C_{18}, 10 μm, 150 × 3.9 mm i.d.), equipped with a pre-column, was eluted with two different solvent systems depending on the compound under study: (1) acetonitrile–H_2O (80% H_3PO_4, pH 3.00), and (2) methanol–H_2O (80% H_3PO_4, pH 3.00).

In the chromatographic analysis of *p*-aminophenyl nipecotate it was necessary to utilize the pentane sulfonic acid as mobile phase modifier.

The concentration of organic solvent and the flow rate (1.0–2.0 ml × min^{-1}) were adjusted for each compound to give appropriate retention times (2–8 min). The column effluent was monitored at 254 nm. The concentrations of each monitored compound were established from measurement of peak areas by reference to the appropriate calibration curves, which were linear ($r > 0.999$) over the range of concentrations used.

Pseudo-first-order rate constants for the hydrolysis reactions were determined from the slopes of linear plots of the logarithm of the concentrations of residual nipecotic acid phenyl ester against time.

Pharmacological studies

Male CDl mice (Charles River Lab., Calco, Italy) weighing 33–38 g were used. Animals were allowed free access to food and water, were housed at constant room temperature (20–22 °C) and exposed to a light cycle of 12 h/day for a week before the experiment. Bicuculline (3 mg/kg) was injected s.c. in a volume of 0.01 ml/g. The bicuculline solution was freshly prepared on the day of experiments in citrate buffer (pH 4.00) and kept at 4 °C to minimize its degradation to less active products (Olsen et al., 1975).

The animals were placed singly in Plexiglas cages and observed continually for 1 h after the injection of bicuculline. The time to onset of clonic and tonic convulsions as well as the time of death were measured.

A clonic convulsion was defined as a single episode of clonic spasms of at least 5 s duration. A tonic seizure was defined as a brief period of hindlimb flexion followed by a prolonged period of hindlimb extension. Compounds **4a, f, h, k, n** in a dose of 150 mg/kg were dissolved in citrate buffer (pH 4.00) and administered s.c. 1 h before bicuculline, in a volume of 0.01 ml/g. An equal volume of pH-adjusted saline was injected by the same route in controls. Mice treated with bicuculline were considered "protected" when clonic or tonic seizures and death did not occur.

The statistical analysis of data was performed by using Student's *t*-test.

Results and Discussion

The X-substituents in compounds **4** have been selected on the basis of the classical quantitative structure–activity relationships (QSAR) principles of "maximum spread" and minimum collinearity

TABLE 3

Squared correlation matrix of electronic, hydrophobic and bulk substituent parameters of compounds 4

	σ	σ^-	π	*MR*	*L*	B_1	B_5
σ	1.000	0.857	0.053	0.045	0.004	0.003	0.010
σ^-		1.000	0.143	0.059	0.008	0.000	0.010
π			1.000	0.291	0.173	0.444	0.034
MR				1.000	0.832	0.133	0.671
L					1.000	0.031	0.596
B_1						1.000	0.001
B_5							1.000

σ, σ^-, π and MR were taken from standard compilation (Hansch and Leo, 1979); *L*, B_1, B_5 values have been reported by Verloop (1982).

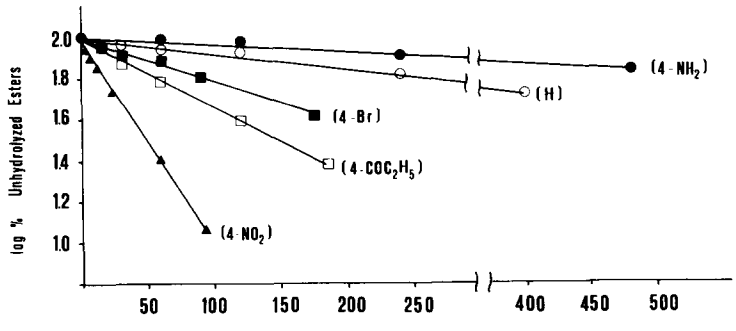
among the physicochemical parameters which describe the electronic, hydrophobic and steric properties of a substituents set. The squared correlation matrix reported in Table 3 shows that the different parameters are almost orthogonal, with the expected and unavoidable exception of a small intercorrelation among "bulk" parameters L, B_1, B_5 and MR.

X-phenyl esters of nipecotic acid (**4a–r**) were prepared according to the classical pathway shown in Scheme 1 (Crider et al., 1982 and 1984). For the preparation of compound **4m** (X = 4-$NHSO_2CH_3$) the intermediate *N-t*-BOC-*p*-nitrophenyl nipecotate (**3n**) was reduced to *p*-aminophenyl ester (**3l**) with H_2 and Pd/C, reacted with methanesulfonylchloride and finally *t*-BOC-deprotected with HCl. Compound **4l** (X = 4-NH_2) was obtained by reduction with H_2 and Pd/C of *p*-nitrophenyl nipecotate hydrochloride **4n**.

Hydrolysis of X-phenyl esters 4 in aqueous buffers

The kinetics of hydrolysis of X-phenyl nipecotates **4a–q** were studied at physiological pH (7.40) and temperature (37 °C). At constant pH and temperature strict first-order kinetics was observed for the hydrolysis of esters **4**.

Some typical first-order plots are shown in Fig. 2. The hydrolysis of phenyl nipecotate **4a** was also studied in 5 different aqueous buffer solutions in the pH range 7.00–10.75 at 37 °C and the results are shown in Fig. 3, where the logarithm of the k_{obs} values are plotted against pH. The pH–rate profile for compound **4a** is straight line of unity slope in the pH range 7.00–8.65. Log k_{obs} measured at the highest pH explored (pH = 10.75, the nearest value to pK_a of the secondary amino group of the piperidine ring) is deviating from linearity. This deviation could be most likely explained by a lesser degree of stabilisation of the



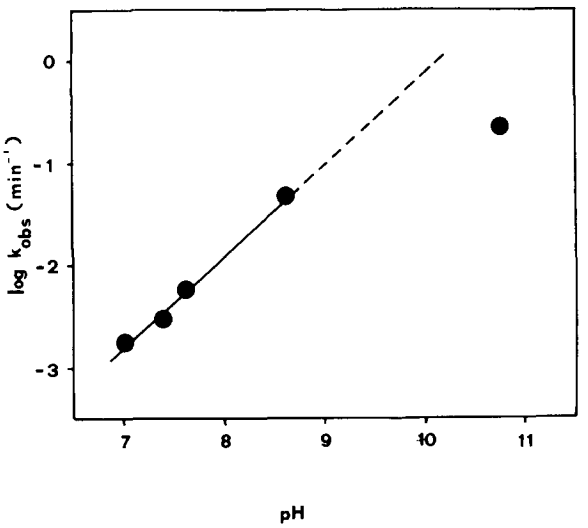


Fig. 3. pH–rate profile for hydrolysis of phenyl nipecotate **4a** at 37 °C and $\mu = 0.5$.

negatively charged tetrahedral intermediate in ester hydrolysis due to a lower concentration of positively charged amino group. This catalytic effect is in fact dependent on the acid–base equilibrium of the ionizable group, as previously demonstrated in many instances (Ascenzi et al., 1982). However, since in our experimental conditions (pH = 7.40) only positively charged molecules are present, the value of the second-order rate constant for hydroxide ion-catalyzed hydrolysis (k_{OH}) may be evaluated according to the following expression:

$$k_{obs} = k_{OH} \times a_{OH} \quad (1)$$

In this expression k_{obs} is the observed pseudo-first-order rate constant and a_{OH} is the hydroxide ion activity, which was calculated from the hydroxide ion concentration according to Harned and Hamer (1933).

The log k_{OH} values at pH 7.40 have been correlated with the Hammett substituent σ^- constant through the following regression equations:

$$\log k_{OH} = 0.87(\pm 0.15)\sigma^- + 3.51(\pm 0.08) \quad (2)$$

$$n = 17 \quad s = 0.135 \quad r = 0.953$$

In the above equations k_{OH} is the second-order rate constant, $t_{1/2}$ is the half-life (in min), n represents the number of data points, s is the standard deviation and r is the correlation coefficient; in parentheses are indicated the 95% confidence intervals. Equations with quite close statistics have been obtained with σ and this could be simply due to the high collinearity ($r^2 = 0.857$) between the two electronic parameters. The coefficient with σ^- ($\rho = 0.87$) is acceptably close to that calculated by us ($\rho = 1.09$), using a more recent compilation of σ^-, on the basis of kinetic data reported for the hydrolysis of a small set of substituted phenyl acetates and determined in slightly different experimental conditions (pH = 8.00; T = 30 °C) (Tommila and Hinshelwood, 1938).

Serum-catalyzed hydrolysis of X-phenyl esters **4**

The hydrolysis of esters **4** was also studied at 37 °C in 0.05M phosphate buffer solution (pH 7.40) containing 10% of human serum. A first-order kinetics over a period of two half-lives was observed using an initial ester concentration of about 4×10^{-4} M. A comparison between the hydrolytic rate data for degradation in human serum and in buffer solution alone reveals that the esters **4** are subjected to enzyme catalyzed hydrolysis by non-specific plasma esterases. The highest acceleration in the hydrolysis rate has been observed for **4i** (about 7-fold increase).

From the kinetic data obtained in the serum-catalyzed hydrolysis the following correlation equations have been formulated:

$$\log t_{1/2} = -0.82(\pm 0.28)\sigma^- + 2.14(\pm 0.15) \quad (4)$$

$$n = 17 \quad s = 0.246 \quad r = 0.853 \quad F_{1,15} = 40.2$$

$$\log t_{1/2} = -0.90(\pm 0.23)\sigma^- - 0.21(\pm 0.14)MR + 2.40(\pm 0.21) \quad (5)$$

$$n = 17 \quad s = 0.195 \quad r = 0.916 \quad F_{1,14} = 9.8$$

$$\log t_{1/2} = -0.99(\pm 0.18)\sigma^- - 0.21(\pm 0.09)\pi$$

In these equations π is the Hansch hydrophobic substituent constant, MR is the substituent molar refractivity and F is the statistic for the significance of each term in the equations. The other terms and symbols have been already defined for Eqns. 2–3.

The influence of the electronic effect on the degradation rates is quite similar in the catalyzed and uncatalyzed hydrolysis of esters **4**, as can be deduced from the comparison of the ρ values with σ^- in Eqns. 3 and 6 (-0.87 and -0.99, respectively). The equation with σ^- is the most significant one-variable equation but accounts for only 73% of the variance in the data. The introduction of the STERIMOL parameters (Verloop, 1982) did not significantly improve the correlation while a slight and more consistent improvement has been obtained by introducing the MR (Eqn. 5) and the π (Eqn. 6) terms. Since the MR and π vectors are not collinear ($r^2 = 0.291$), the higher statistical reliability of Eqn. 6 indicates that the interaction between X-substituents in **4** and serum esterases must be hydrophobic in nature. It has been in fact well documented in several recent studies of enzyme–ligand interactions that MR, which is a measure of polarizability and molar volume of the substituent, mainly accounts for interaction in non-polar spaces, whereas π accounts for interaction in hydrophobic regions (Hansch and Klein, 1986; Carotti et al., 1984, 1988). The hydrophobicity of X-substituents thus plays an important role in determining an higher degradation rate of **4** in the serum-catalyzed hydrolysis, even if the magnitude of this effect is not very large, as can be seen from the low coefficient with π in Eqn. 6.

In previous similar studies on alkyl ester prodrugs of several classes of drug and particularly on CNS-active drugs, the influence of the hydrophobicity of the alkyl group on the enzymatic hydrolysis rates was not always so clear and well-defined as in Eqn. 6, most likely because other factors, like chain branching, higher chain conformational mobility and steric hindrance, could be all operative at the same time giving rise to complex interaction very difficult to be correctly parameterized and evaluated in quantitative terms. However, useful qualitative (or semiquantitative) indications have been derived in the study of isoguvacine (Falch et al., 1981) and L-DOPA prodrugs (Marrel et al., 1985a and b), for the design of new molecules sufficiently resistant to the hydrolysis in the blood stream and able to be hydrolyzed at a suitable rate by brain esterases, after crossing the blood–brain barrier. The set of X-substituents used in the present study are potentially more suitable to undertake a reliable QSAR analysis and, in general, aryl esters like **4** can constitute a more consistent prodrug reference model. Unfortunately, some phenol has its own biological activity but it is worth to point out that in the doses generally required for most of our in vivo test no evident neurotoxic or other observable effects have been detected.

Finally, to verify the predictive capacity of Eqn. 6 and with the aim of detecting a critical upper hydrophobic limit of X-substituents, we synthesized a further congener **4r** (X = 4-OC_6H_{13}) which presents the highest π value, 2.55. It was very gratifying to find that log $t_{1/2}$ for this compound was absolutely well predicted from Eqn. 6 (obs. 1.85 vs calcd. 1.87) and that this equation holds up to very lipophilic compounds like **4r** (see Fig. 4).

The hydrolysis of three X-phenyl nipecotates **4f,k,n**, chosen among the pharmacologically tested

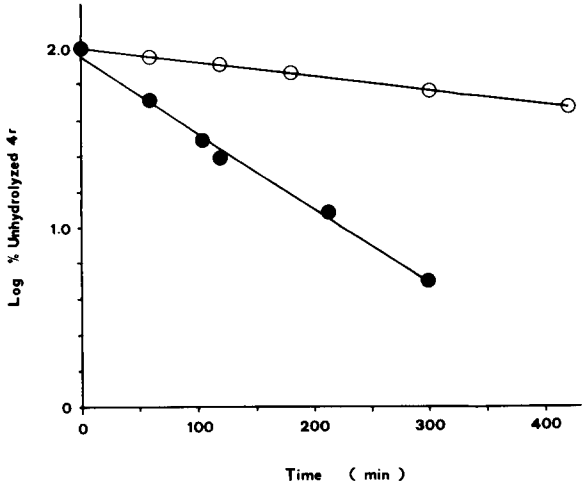


Fig. 4. First-order plots for hydrolysis of *p*-hexyloxyphenyl nipecotate **4r** in 0.05 M phosphate buffer solution, pH 7.40 (○) and in 10% human serum solution (●) at 37 °C.

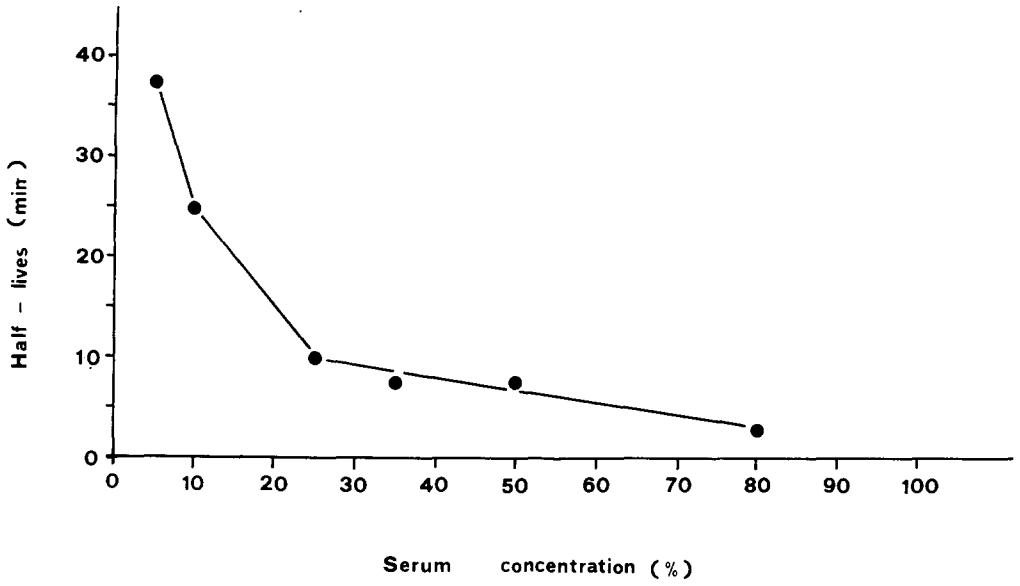


Fig. 5. Influence of the serum concentration on the hydrolysis rate of compound **4k**, pH 7.40 and 37 °C.

TABLE 4

Effect of human serum concentration on the rate of hydrolysis of some X-phenyl nipecotates 4 (4×10^{-4} M) in 0.05 M phosphate buffer solution (pH 7.40) at 37 °C

Serum concentration (%)	Half-life (min)		
	4f (X = 4-Br)	**4h** (X = 4-OCH_3)	**4k** (X = 4-COC_2H_5)
5	89.7	187.0	37.3
10	60.3	114.7	24.8
25	42.3	54.7	9.9
35	23.5	50.0	7.6
50	15.3	32.2	7.8
80	9.7	8.9	2.7

compounds (see after), was studied in presence of diverse amounts of human serum with the aim to observe the influence of the serum concentration on the rate of hydrolysis. A 10–20 fold increase in rate of hydrolysis, by changing the serum concentration from 5 to 80%, could be calculated (see Table 4). At low protein concentrations (< 20 mg/ml), the rate of ester hydrolysis increases more markedly in relation to protein concentration, as illustrated in Fig. 5.

Pharmacological results

Five esters (**4a**, X = H; **4f**, X = 4-Br; **4h**, X = 4-OCH_3; **4k**, X = 4-COC_2H_5; **4n**, X = 4-NO_2) rep-

TABLE 5

Antagonism of bicuculline-induced convulsions and death in CD1 mice by some X-phenyl esters of nipecotic acid 4

Pretreatment	Onset of convulsions (mean ± S.E.M., min)				Time of death (mean ± S.E.M., min)	
	Clonic		Tonic			
Saline	3.36 ± 0.28	(0/20)	5.24 ± 0.26	(0/20)	6.01 ± 0.30	(0/20)
4a	4.46 ± 0.67	(0/8)	6.61 ± 1.11	(0/8)	7.31 ± 1.11	(0/8)
4f	10.22 ± 1.43 ***	(1/9)	14.43 ± 1.41 ***	(2/9)	14.84 ± 1.42 ***	(2/9)
4h	4.84 ± 0.68 *	(0/8)	7.41 ± 1.04 *	(0/8)	8.91 ± 1.30 **	(0/8)
4k	8.16 ± 1.92 ***	(0/8)	11.11 ± 2.43 ***	(0/8)	12.20 ± 2.35 ***	(0/8)
4n	5.68 ± 0.73 **	(1/8)	8.75 ± 1.33 ***	(1/8)	10.04 ± 1.51 ***	(1/8)

Compounds **4** (150 mg/kg s.c.) were administered 1 h before s.c. injection of bicuculline (3 mg/kg). In parentheses the ratio (no. of mice protected)/(no. of mice tested) is reported. Statistical significance: $*P < 0.025$; $**P < 0.005$; $***P < 0.001$ vs saline (Student's *t*-test).

resenting different rates of hydrolysis in vitro were selected in order to evaluate their effects on convulsions and death induced by GABA-antagonist bicuculline.

Our results (Table 5), in contrast to some previous findings (Crider et al., 1982 and 1984), show that the anticonvulsant activity of compounds **4** is not very high at 150 mg/kg s.c. A low activity is detectable in terms of an increase in the onset time of tonic-clonic seizures as well as in the time of death for compounds **4f, h, k, n.**

This increase was highly statistically significant for **4h, k, n.** Moreover, a correlation between in vitro rates of enzymatic hydrolysis and the pharmacological activity (onset time of bicuculline-induced convulsions and time of death) was found. In particular, a good activity was observed with esters having half-lives in serum in the range of about 10–80 min, being the most active compound (**4f**), the most resistant to serum-catalyzed hydrolysis and the most lipophilic molecule tested so far.

It has been reported that nipecotic acid aryl esters and nipecotic acid itself are potent in vitro inhibitors of GABA uptake and most likely the observed anticonvulsant activity of esters **4** could be due to the combined action of the intact and hydrolyzed molecules (Crider et al., 1984).

Our results seem to indicate that the in vivo pharmacological activity is mainly connected to the resistance of ester **4** in the blood stream and to the ability (largely dependent on the lipophilicity) of the intact molecule to cross the BBB, beyond which it could be more or less easily hydrolyzed.

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