Hydrolysis of Nipecotic Acid Phenyl Esters

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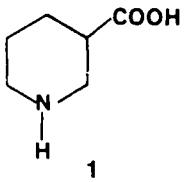
Abstract □ The synthesis and anticonvulsant activity of nipecotic acid esters (**1a–1f**) have been previously reported. It was thought that these prodrug esters underwent hydrolytic conversion to **1** which inhibited GABA uptake, and that both **1** and an intact ester may have caused inhibition of GABA uptake which resulted in the anticonvulsant activity. There is, however, no stability data available to help evaluate these effects. We have determined degradation half-lives ($t_{1/2}$) of these phenyl esters dissolved in 10% serum solution or in pH 7.4 buffer (ionic strength = 0.25 adjusted with KCl) at 37 °C by monitoring the appearance of a phenolic compound for a period of 12 h with an HPLC method. Utilizing a published method, in vitro [^{14}C]GABA uptake was measured. Results show that the hydrolysis rate in 10% serum solution was faster than that in buffer solution and that half-lives varied between 0.20 and 3.84 h. The uptake inhibition varied between 8.2 and 41.7% at 0.02 mM concentration, and percent GABA uptake inhibition correlated with log $t_{1/2}$ ($r = -0.9827$, $p = 0.00045$, based on a t test). Our data suggest that at concentrations ranging from 0.02 to 1 mM, inhibition of GABA uptake is mainly due to **1** formed after hydrolysis of **1a–1f**.

Nipecotic acid (3-piperidinecarboxylic acid; **1**) is a potent gamma-aminobutyric acid inhibitor in rat cerebral cortex[1] and mouse whole brain minislices.[2] Disorders such as Huntington's disease, Parkinsonism, and epilepsy may result from an impairment in GABA-mediated neurotransmission.[3] As a result, enhancement in GABA activity in the central nervous system may have therapeutic potential in these disorders. Nipecotic acid does not cross the blood–brain barrier; but, apparently, its aliphatic and phenyl esters do cross the blood–brain barrier and show anticonvulsant activity.[4–6] It was reported[5] that phenyl esters of **1** inhibit GABA uptake in vitro and protect mice against bicuculine-induced convulsions, and that *m*-nitrophenyl and *p*-nitrophenyl esters were most potent. It was proposed that GABA uptake inhibition would be due to hydrolytic conversion of phenyl esters to nipecotic acid. Although variations in uptake kinetics due to differences in hydrolysis rates were expected, at a concentration of 1 mM and during an incubation period of 15 min[5] all compounds with the exception of the *m*-CN ester, inhibited >80% GABA uptake in vitro.[2,5] Although there was the possibility that sufficient hydrolysis of the ester(s) produced the effect, there was also a likelihood that the inhibition of uptake was due to the presence of intact ester. Stability data, however, was not available to help evaluate these observations. Some data on stability and in vivo activity of the phenyl esters have been recently published,[6] but it is in contrast to previously published results.[4,5] Moreover, in vitro GABA uptake inhibition was not evaluated and the correlation shown between the in vivo activity and hydrolysis rates was borderline. Thus, the purpose of this work was to study the hydrolysis of phenyl esters in 10% serum or in a buffer solution, and to test the in vitro GABA uptake inhibition at a concentration of 0.02 mM.



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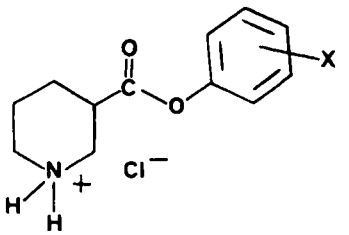
Since it was not necessary to study all nipecotic acid esters, **1a–1f** were selected as representative, based on differences in anticonvulsant activity, chemical structure–reactivity, and GABA uptake inhibition.[5] Prodrugs **1a**, **1b**, and **1e** were more potent, and protected 100, 87.0, and 75%, respectively, of mice against bicuculine-induced death. Compounds **1c**, **1d**, and **1f** were less potent and protected 50, 28.5, and 0.0%, respectively, of mice against bicuculine-induced death. Each phenyl ester (**1a–1d**) had an electron-withdrawing group attached to the benzene ring; on the other hand, **1e** and **1f** had an electron-donating group attached to it. **1a–1e** inhibited >94% of the GABA uptake, **1f** inhibited only 81% of the uptake.

Experimental Section

Materials—Compounds **1a** and **1f** were synthesized, isolated, and purified according to previously published methods.[5] Sodium phosphate dibasic heptahydrate, sodium chloride, acetonitrile, methanol, and water were purchased from Fischer Scientific, Fairlawn, NJ. *p*-Nitrophenol, *m*-nitrophenol, *p*-iodophenol, *p*-chlorophenol, *p*-tertiary-butylphenol, and *p*-methoxyphenol were purchased from Aldrich Chemical, Milwaukee, WI. Sodium phosphate monobasic and acetic acid were obtained commercially from Mallinckrodt, Paris, KY. All solvents and chemicals were ACS analytical grade or HPLC grade.

Standard Solutions—Six standard solutions of each phenol (0.1 to 1×10^{-3} M) were prepared in a phosphate buffer solution (0.067 M) or in a 10% serum solution that contained *p*-chlorophenol (3.30×10^{-4} M) as an internal standard for **1a**, **1c**, and **1e**, or *p*-nitrophenol (1.30×10^{-4} M) as an internal standard for **1b**, **1d**, and **1f**.

Hydrolysis Studies—Three concentrations of each prodrug ester (0.2 to 1×10^{-3} M) were prepared and pre-equilibrated (37 °C) in phosphate buffer solution (0.067 M, pH 7.4, ionic strength = 0.25 adjusted with KCl) or in 10% serum solution containing the appro-



1a: X = p-NO_2
1b: X = m-NO_2
1c: X = p-Cl
1d: X = p-I
1e: X = p-*t*-Bu
1f: X = p-OCH_3

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priate internal standard. Solutions were kept at 37 °C, and aliquots (0.5 mL) were withdrawn at predetermined intervals for 12 h and at infinite time (after 24 h). In order to preclude interference with serum proteins, samples were denatured with acetonitrile (2.5 mL), centrifuged, and filtered.

Instrumentation and Procedure—The HPLC system consisted of a Waters model 6000A solvent delivery system (Millipore, Bedford, MA), equipped with a Waters model U6K variable loop injector, a Waters model 440 absorbance detector (280 nm), a Waters Bondapak C18 column (10-μm particles, 30 cm × 2 mm internal diameter), and a Houston model Omniscribe recorder (Houston Instruments, Austin, TX). The mobile phase consisted of a mixture of methanol:acetonitrile:water:acetic acid (30:10:60:1) pumped at a flow rate of 1.5 mL/min. The injection volume varied between 10 and 20 μL.

In Vitro GABA Uptake Inhibition Studies—These experiments were conducted using mouse whole brain minislices according to a published method.[2]

Data Analysis—Unknown concentrations of a phenol were calculated by determining the peak height ratio of product phenol-to-internal standard and utilizing least square regression parameters of a standard curve obtained by the Lincal program on an Apple IIe computer system.[6] Using a mass balance between concentrations of phenol at various times during hydrolysis and an initial concentration of a prodrug ester, concentrations of an intact ester as a function of time were calculated. Subjecting these data to a first-order kinetics program on an Apple IIe computer system,[7] a degradation half-life of an ester was obtained. To evaluate a correlation between percent GABA uptake inhibition and half-life of an ester, the Microstat program[8] on a Samsung computer system was used.

Results and Discussion

Because nipecotic acid (**1**) and prodrug did not absorb enough UV light at 280 nm, the HPLC detector could not detect them. Nevertheless, because a phenolic degradation product had a good minimum detectability range (7×10^{-7}–1.65×10^{-5} M), good selectivity, and a quantitative range (0.02–10^{-3} M, $r^2 = 0.999$), and because its concentration at infinite degradation (24 h) mass balanced with the initial concentration of an intact ester, hydrolysis could be monitored following a phenolic compound.

The results (Table I) show that $t_{1/2}$ values in 10% serum were shorter than in buffer, yet the trends were similar; $t_{1/2}$ varied between 0.20 and 3.84 h; percent GABA uptake inhibition varied between 8.2 and 41.7% for prodrug esters compared with 58.4% for **1**, and percent GABA uptake inhibition correlated with log $t_{1/2}$ ($r = -0.9827$, $p = 0.00045$, based on a t test). While rapid hydrolysis of a prodrug would increase both the availability of the drug to receptors in brain and its activity, because of systemic turnover, delayed biotransformation of prodrug to drug would decrease it. Compounds **1a** and **1b**, with $t_{1/2}$ values of <1 h (Table I), could offer substantial protection in mice against bicuculine-induced death after subcutaneous injection.[5] Compounds **1c**, **1d**, and **1f**, with $t_{1/2}$ values of >1 h (Table I), were not as effective.[5] Despite having a relatively long $t_{1/2}$ (3.54 h) **1e** protected 75% of the mice in the same experiment (Table I). Several possible explanations can be offered (i.e., prodrug lipophilicity, detergent-like action, and effect on the cell membrane) but in the absence of proline and β-alanine uptake inhibition data, these mechanisms cannot be supported.

Nipecotic acid (**1**), a hydrolytic product of a prodrug, inhibited GABA uptake. While the degree of inhibition depended on the rate of hydrolysis, the chemical structure determined the reactivity of the prodrug to hydrolysis. Indeed, **1a**–**1d**, having electron-withdrawing substituents, degraded faster than **1e**–**1f**, having electron-donating substituents (Table I). When the data were subjected to regression analysis, the regression equation $I = -25.33 \log t_{1/2} + 21.80$ (SE = 2.70) was obtained.

However, the GABA uptake inhibition at a prodrug concentration of 1 mM[5] did not correlate with log $t_{1/2}$ ($r = -0.54$, $p = 0.27$, based on a t test). This apparent discrepancy is from the different concentrations of the prodrugs used in the two studies. Figure 1 shows dose–response curves (percent GABA uptake inhibition versus concentration) of **1** and **1a**. The curve is linear at lower concentrations, but plateaus after 90% inhibition as the concentrations of **1** exceed 0.2 mM. This means that with the 1 mM initial concentration of a prodrug, 20% hydrolysis in 15 min (duration of the GABA uptake experiment) would give 90% uptake inhibition. Using hydrolysis data in buffer (Table I), the concentration of **1** generated due to hydrolysis of a relatively stable prodrug ester, **1f**, in 15 min was calculated (1 mM initial concentration). From that concentration (4.5×10^{-5} M), the predicted uptake inhibition (Figure 1) was 73% which correlates reasonably with the observed value of 81%.[5] Poor correlation between hydrolysis rate and GABA uptake inhibition at the 1 mM concentration of other prodrugs was from the lack of spread in uptake inhibition data resulting from >90% inhibition.

Some prodrug esters were thought to disrupt cell membrane functions, interfere with proline uptake, and inhibit GABA uptake in a nonspecific manner.[4,5] Compounds **1a**–**1f** might also have a nonspecific effect, but it is minimal, as a specific effect of **1** could account for GABA uptake inhibition at low (2×10^{-5} M), as well as at high concentrations (1×10^{-3} M) of prodrugs.

In conclusion, the information gathered provides GABA uptake inhibition and hydrolysis rates of phenyl esters of **1**, presents a relationship between these two parameters, and illustrates that at low and high concentrations of a prodrug GABA uptake inhibition is mostly due to **1** that is formed after hydrolysis.

Table I—Half-life and GABA Uptake Inhibition Data[a]

Compound	Half-life, h		Percent GABA Uptake Inhibition[b]
	10% Serum	Buffer Solution	
1	—	—	58.4 (0.9)
1a	—	0.20 (0.05)[c]	41.7 (1.8)
1b	0.21 (0.05)	0.53 (0.09)	27.1 (2.5)
1c	0.67 (0.20)	1.66 (0.50)	13.8 (1.2)
1d	—	2.13 (0.30)	11.2 (1.2)
1e	—	3.54 (0.19)	10.9 (2.4)
1f	2.3 (0.30)	3.84 (0.07)	8.2 (1.8)

[a] n = 3. [b] At 0.02 mM concentration of a prodrug (n = 6). [c] Number in parentheses indicates standard deviation.

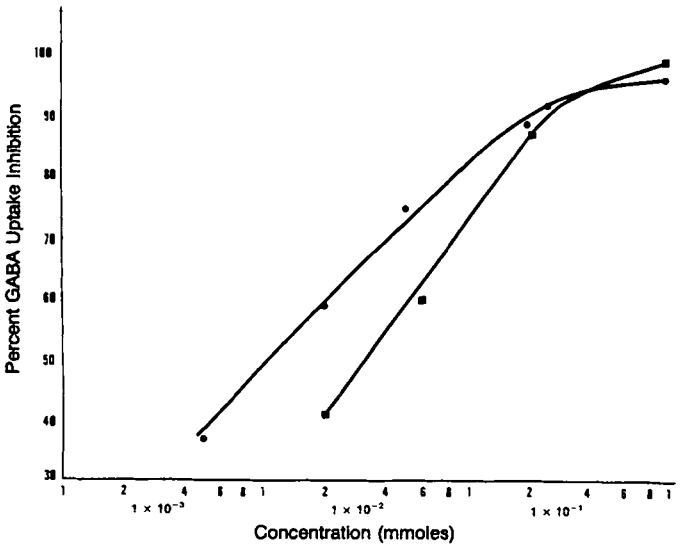

Figure 1—A plot of percent GABA uptake inhibition versus log concentration of **1** (●) or **1a** (■).

References and Notes

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Acknowledgments

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