*Pharmacology Biochemistry & Behavior*, Vol. 31, pp. 627–631. © Pergamon Press plc, 1989. Printed in the U.S.A. 0091-3057/88 $3.00 + .00

# Toxicity Study of Continuous Administration of Physostigmine Salicylate

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LIM, D. K., Y. ITO, T. STEWART, B. HOSKINS AND I. K. HO. *Toxicity study of continuous administration of physostigmine salicylate.* PHARMACOL BIOCHEM BEHAV **31**(3) 627–631, 1988.—The present study demonstrates that continuous administration with physostigmine salicylate (0.12 or 0.24 mg/kg/hr via mini-osmotic pumps) induces toxicities (e.g., body weight loss, decreased water consumption, tremors, decreased body temperatures, mortality) in guinea pigs. Both blood and brain cholinesterase activity is inhibited dose-dependently by physostigmine salicylate. The signs of toxicity in the guinea pigs which received the low dose appeared within 2 or 3 days and then the animals recovered, while toxic signs in the guinea pigs treated with the high dose of the drug persisted throughout the experiments. The study further shows that continuous administration of physostigmine salicylate also caused down-regulation of muscarinic receptors in the striata of the guinea pigs.

Physostigmine Mini-osmotic pumps Toxicities ChE activity Muscarinic receptors

PHYSOSTIGMINE is an alkaloid from the seeds of the calabar bean which inhibits acetylcholinesterase. The inhibition of plasma cholinesterase by physostigmine was studied as early as 1946 by Koster (11). The clinical uses and mechanism of action of physostigmine have been reported (4,17). Physostigmine appears to improve memory function in patients with Alzheimer's disease (1, 19, 23). Physostigmine can also reverse the toxic effects associated with overdosage of other drugs, such as tricyclic antidepressants (18), morphine (24) and benzodiazepines (12). It also has potential use as a prophylactic agent against organophosphate intoxication (8–10). However, the duration of action of physostigmine is very short (22); and thus its use in Alzheimer's disease has involved multiple injections (23).

It is, therefore, of potential significance to develop a model for studies of continuous administration of physostigmine as a prophylactic regimen against drug overdoses. Little is known about the toxicity of repeated administration of physostigmine although it has been reported to produce myoclonus in Alzheimer's patients (16). The present study was designed to determine the general toxicity of continuous administration of physostigmine via mini-osmotic pump implantation. The data obtained should provide valuable information on the potential use of continuous administration of physostigmine as a prophylactic measure against organophosphate intoxication.

## METHOD

### *Animals*

Male Hartley guinea pigs weighing 200–250 grams were obtained from Charles River Breeding Laboratories, Wilmington, MA. The guinea pigs were chosen because they are more sensitive than other rodents (13) and provide a better reference for human application. Upon arrival, they were housed two to a cage (9×20×7 inches) and maintained in a room with controlled humidity of 55%, temperature regulated at 74±2°F, 100% fresh air continually circluating and 12 hour light/dark cycles. The guinea pigs were given constant access to food and water.

### *Animal Treatment*

Three groups, each consisting of 10 guinea pigs, were implanted with mini-osmotic pumps (Model 2001, Alza Corp., Palo Alto, CA). The presealed mini-osmotic pumps containing physostigmine salicylate (in solvent consisting of 40% propylene glycol, 10% alcohol and water) were preincubated in saline overnight at room temperature before implantation. The back of each guinea pig was shaved and lidocaine hydrochloride was applied topically. The mini-osmotic pumps were implanted after the animals had been anesthetized. The doses of physostigmine salicylate used were:

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0.12 and 0.24 mg/kg/hr. The control group was implanted with mini-osmotic pumps containing vehicle only.

*Monitoring of Body Weight and Water Consumption*

Four hundred ml of tap water were available for each cage every day. The body weight of each guinea pig and water consumption per cage were recorded daily. The daily percent change in body weight of each animal was calculated. The mean body weight change (±S.E.M.) was obtained for all guinea pigs in a given group. Daily water consumption per 100 g of body weight was determined.

*Hypothermia*

As an index of body temperature, rectal temperature was measured by a thermistor mounted in a rectal probe connected to a Tele-thermometer (Bailey Instrument Co., Saddle Brook, NJ). The flexible thermistor probe was inserted 15 mm into the rectum. The body temperature of each guinea pig was recorded immediately prior to implantation of the mini-osmotic pumps and 1, 3 and 7 days after the implantation.

*Determination of the Number of Red Blood Cells and Red Blood Cell Acetylcholinesterase Activity*

A blood sample (10 $\mu$l) from each guinea pig was collected from the front leg using heparinized microliter pipettes (Accupette, Dade Diagnostics Inc., Aguada, Puerto Rico) before the mini-osmotic pump implantation and 1,3 and 7 days after the implantation. Four guinea pigs from each group were used at each time point. Red blood cell numbers were estimated using a Coulter counter (Coulter Electronics Limited). Red blood cell acetylcholinesterase activities were measured according to the method of Ellman *et al.* (5). Red blood cell acetylcholinesterase activities were expressed on the basis of red blood cell numbers.

*Determination of Acetylcholinesterase Activity in Brain*

On the seventh day after the mini-osmotic pump implantations, guinea pigs were sacrificed by decapitation and the brains were rapidly removed. Striatum and frontal cortex were dissected out according to the method of Glowinski and Iversen (7). Brain acetylcholinesterase activities were measured according to the method of Ellman *et al.* (5). Brain acetylcholinesterase activities were expressed as nmoles acetylthiocholine hydrolyzed/mg protein/min.

*Membrane Preparation for Binding Assays*

Membranes were prepared according to the method of Zukin *et al.* (27) with slight modification. The animals were decapitated, the brains were rapidly removed, and the striata were dissected out. The striata were pooled and homogenized in 15 volumes of ice-cold 0.32 M sucrose using a Brinkman Polytron PT-10 at low speed (setting 3). The homogenate was centrifuged at 1,000×g for 10 min; the pellet was discarded and the supernatant fluid was centrifuged at 20,000×g for 20 min to obtain a crude mitochondrial pellet. The crude mitochondrial pellet was resuspended in double-distilled deionized water dispersed with a Brinkman Polytron PT-10 (setting 6) for 30 sec. The suspension was centrifuged at 8,000×g for 20 min. The supernatant including the buffy layer was collected and centrifuged at 48,000×g for 20 min to obtain a pellet. The pellet was resuspended in water and centrifuged at 48,000×g for 20 min. The final pellet (membrane preparation) was suspended in Tris-HCl buffer (pH 7.4) and stored at −20°C for 1–4 days.

*[$^3$H]QNB Binding*

The frozen membranes were thawed and centrifuged at 25,000×g for 15 min and the pellet was suspended in 50 mM sodium phosphate buffer (pH 7.4). The binding of [$^3$H]QNB was carried out according to the method of Yamamura and Snyder (26) with minor modifications. In brief, the binding assay was performed in 50 mM sodium phosphate buffer (pH 7.4), with different concentrations (0.01–2 nM) of [$^3$H]QNB to generate saturation curves, in a final volume of 1 ml. Specific binding was calculated as the total binding minus that occurring in the presence of 1 $\mu$M atropine. The binding was initiated by addition of 0.2 ml of membrane preparation (0.2–0.4 mg/ml), and incubations were allowed to proceed for 1 hr at 25°C in a shaking water bath. The reaction was terminated by rapidly filtering through Whatman GF/B glass fiber filters using a cell harvester (Model M-24, Brandel, MD). Each filter was washed twice with 5 ml buffer, and the dried filter was transferred to scintillation vials containing 10 ml of Safety Solve (Research Products International Corp., Mount Prospect, IL). The radioactivity retained in the filters was determined by liquid scintillation spectrophotometry.

*Determination of Protein Concentration*

The protein content of the tissue homogenates and the membrane preparations was determined by the method of Lowry *et al.* (15) using bovine serum albumin as a standard.

*Statistics*

Linear regression analyses were used to obtain all values of the dissociation constant ($K_d$) and maximum number of binding sites ($B_{max}$). When applicable, data were analyzed for significance by Student's *t*-test: a $p$ value $<0.05$ between two means was considered significant.

## RESULTS

*In Vitro Determination of the Stability of Physostigmine Salicylate*

There were no significant changes in potency of physostigmine salicylate in terms of inhibition of AChE activity even after 7 days of incubation at 37°C in the vehicle (data not shown). These results indicate that physostigmine salicylate is fairly stable in the vehicle used.

*Effects of Physostigmine Salicylate on Tremor and Mortality in Guinea Pigs*

All guinea pigs implanted with mini-osmotic pumps containing the high dose of physostigmine salicylate (0.24 mg/kg/hr) exhibited tremors within 24 hr. The incidence of tremors disappeared after 2 days of physostigmine infusion. No tremor was detected in controls or in the low dose of physostigmine salicylate-implanted groups. The effects of continuous administration of physostigmine salicylate on cumulative mortality show that 40% of the guinea pigs treated with the high dose of physostigmine salicylate died within 24 hr, and an additional guinea pig died on the 4th day. None of the animals treated with the vehicle alone or with the low dose of physostigmine salicylate (0.12 mg/kg/hr) died.

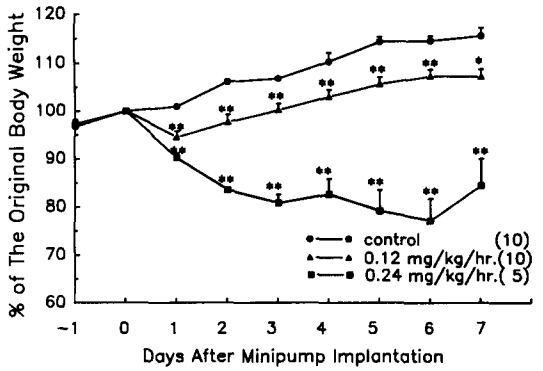


FIG. 1. Percent of original body weight during continuous infusion of physostigmine salicylate via mini-osmotic pumps. Numbers in parentheses indicate number of guinea pigs in each treatment group. Asterisks denote significant ($*p<0.05$ and $**p<0.01$) differences from the vehicle-infused group.

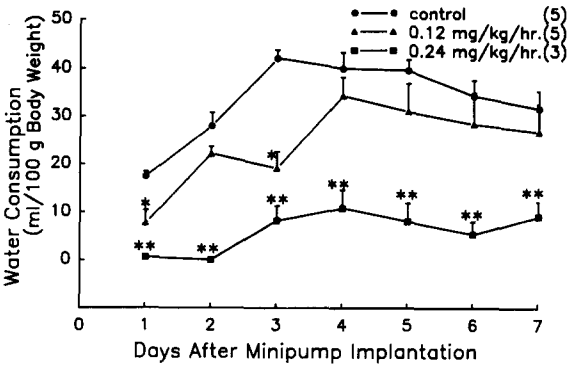


FIG. 2. Water consumption by each treatment group during continuous infusion of physostigmine salicylate via mini-osmotic pumps. Numbers in parentheses indicate the number of cages (each containing animals) in each treatment group. Asterisks denote significant ($*p<0.05$ and $**p<0.01$) differences from the vehicle-infused group.

*Effects of Continuous Administration of Physostigmine Salicylate on Body Weight and Water Consumption in Guinea Pigs*

The effects of physostigmine salicylate on guinea pig body weights are shown in Fig. 1. In guinea pigs treated with different doses of physostigmine salicylate, the body weights were significantly lower that those of the control group. The loss of body weight was dose-dependent. In the group treated with the low dose of physostigmine salicylate, the animals started to gain body weight after the second day of treatment. The rate of body weight gain in this group was comparable to the control group. However, the body weights of animals which were treated with the high dose of physostigmine salicylate stayed around 15% lower than their original body weights from the 2nd through the 7th day.

Figure 2 shows that the effect of physostigmine salicylate on water consumption in guinea pigs was also dose-dependent. In guinea pigs which received the high dose of physostigmine salicylate, water consumption was significantly lower throughout the period of physostigmine infusion. In guinea pigs which were treated with the low dose of physostigmine salicylate, water consumption returned to the control level 3 days after the implantation of mini-osmotic pumps.

*The Effect of Physostigmine Salicylate on Guinea Pig Body Temperatures*

Body temperatures of the animals treated with the low dose of physostigmine salicylate were not significantly different from those of the control animals (Fig. 3). However, the body temperatures of those treated with the high dose of physostigmine salicylate were significantly lower that those of the control animals throughout the period of infusion.

*Effect of Physostigmine Salicylate on Red Blood Cell and Brain Acetylcholinesterase Activity*

Figure 4 shows dose-dependent inhibition of red blood cell acetylcholinesterase activity after continuous administration of physostigmine salicylate via mini-osmotic pumps. In the group of guinea pigs treated with the low dose of physostigmine salicylate, red blood cell acetylcholinesterase

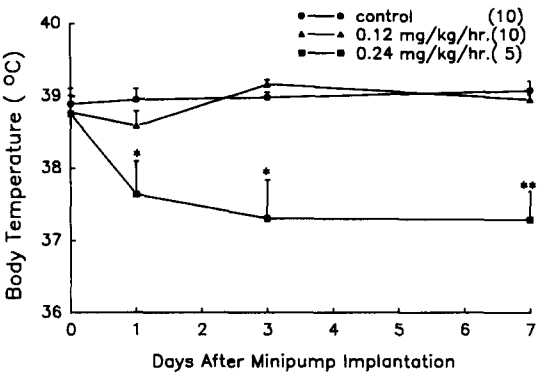


FIG. 3. Changes in body temperature during continuous infusion of physostigmine salicylate via mini-osmotic pumps. Numbers in parentheses indicate the numbers of guinea pigs in each treatment group. Asterisks denote significant ($*p<0.05$ and $**p<0.01$) differences from the vehicle-infused group.

activity was 20–40% lower than that of the control group. Around 45% of red blood cell acetylcholinesterase activity was inhibited in the group treated with the high dose of physostigmine salicylate starting from the first day and continuing throughout the entire experimental period.

Figure 5 shows the effects of physostigmine salicylate on striatal and frontal cortex acetylcholinesterase activity. The acetylcholinesterase activity in both brain regions was significantly inhibited 7 days after the mini-osmotic pump implantation. The degree of inhibition was also dose-dependent. The results show that 20 and 40% of acetylcholinesterase activity was inhibited after low and high doses of physostigmine salicylate, respectively. Both regions were inhibited equally by physostigmine salicylate treatment.

*Effect of Physostigmine Salicylate on Muscarinic Receptors of Guinea Pig Striatal Membranes*

Table 1 shows the effects of physostigmine salicylate on striatal muscarinic receptors. The density of muscarinic receptors in striata of guinea pigs was significantly reduced 10

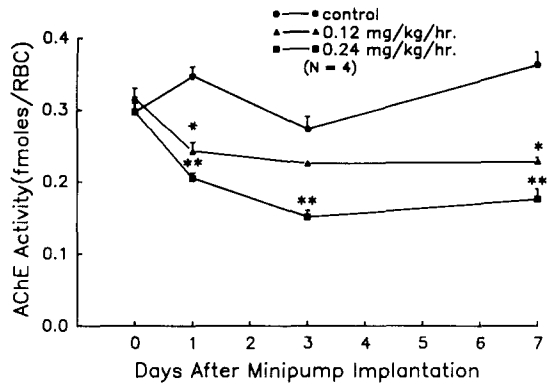


FIG. 4. AChE activities in red blood cell during continuous infusion of physostigmine salicylate via mini-osmotic pumps. Blood was collected from the front leg of the guinea pigs in each group before and after implantation of the mini-osmotic pumps. Red blood cell AChE activities were measured as described in the Method section. The values are means±S.E. of four determinations. Asterisks denote significant (*$p<0.05$ and **$p<0.01$) differences from the vehicle-infused group.

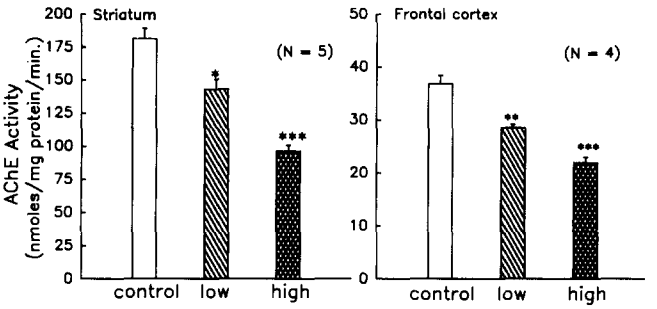


FIG. 5. Effects of the continuous administration of physostigmine salicylate on AChE activities in striata and frontal cortex of guinea pigs. Guinea pigs were sacrificed 7 days after the implantations. AChE activities were measured as described in the Method section. The values are means±S.E. of N determinations. Asterisks denote significant (*$p<0.05$, **$p<0.01$ and ***$p<0.001$) differences from the vehicle-infused group.

and 15% after 7 days of continuous treatment with the low and high doses of physostigmine salicylate, respectively. There were no changes in receptor affinity ($K_d$).

## DISCUSSION

The present results demonstrate that continuous treatment with physostigmine salicylate produces certain adverse effects ranging from decreased water consumption to increased mortality. Physostigmine salicylate also inhibits blood and brain acetylcholinesterase activity in a dose-dependent manner. However, the density of striatal muscarinic receptors was reduced by the physostigmine salicylate infusion for 7 days. The signs of toxicity in guinea pigs which received a low dose appeared within 2 or 3 days and then the animals recovered, while toxicity signs in the guinea pigs treated with a high dose of the drug persisted at the same levels throughout the seven-day period.

It has been reported that during continuous exposure to organophosphates, animals develop tolerance in terms of drinking behavior (14, 20, 21), thermoregulation (3), learning (2,20) and motor function (6). In those reports, body weights, water consumption, body temperatures and motor function of tolerant animals were not different from those of controls. The present results indicate that tolerance does not develop during continuous administration of a high dose of physostigmine for up to 7 days.

Physostigmine has a very short half-life in plasma (17 min) and brain (16 min) and it easily penetrates the blood-brain barrier (22). Somani and Khalique (22) also reported that maximum ChE inhibition in plasma occurred in 7 min, with recovery to 81% in 2 hr. Since we found that physostigmine was stable in vitro, our results indicate that acetylcholinesterase activity during the continuous exposure of physostigmine was dose-dependently inhibited to the same extent. Although acetylcholinesterase activity at early time points remains to be investigated, the same inhibition rate of acetylcholinesterase activity during continuous administration of physostigmine might be due to rapid clearance of this drug.

TABLE 1

EFFECT OF CONTINUOUS INFUSION OF PHYSOSTIGMINE SALICYLATE ON [$^3$H]QNB BINDING IN STRIATA OF GUINEA PIGS

| | $K_d$ (nM) | $B_{max}$ (pmol/mg protein) |
|---|---|---|
| Control | 0.124 ± 0.021 | 4.073 ± 0.094 |
| 0.12 mg/kg/hr | 0.116 ± 0.010 | 3.679 ± 0.119* |
| 0.24 mg/kg/hr | 0.109 ± 0.009 | 3.449 ± 0.055† |

Animals received physostigmine salicylate via implantation of mini-osmotic pumps for 7 days.

The values are mean ± S.E. of 4 determinations performed in duplicate.

*$p<0.05$ compared to the control value.

†$p<0.01$ compared to the control value.

Yamada *et al.* (25) had reported that the repeated administration of physostigmine to guinea pigs has no effect on muscarinic receptors. However, the present study shows that continuous administration of physostigmine salicylate to guinea pigs resulted in decreased muscarinic receptor density. The discrepancy might be due to the different treatment protocols used by Yamada *et al.* (25), i.e., that intermittent treatment allowed recovery of muscarinic receptors to take place whereas our continuous treatment did not.

The results of the present study suggest that even though physostigmine salicylate does induce some toxicity, continuous infusion of the proper dose of this drug using mini-osmotic pumps or other devices should be attempted when repeated treatment with physostigmine is warranted.

## ACKNOWLEDGEMENT

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## REFERENCES


1. Bartus, R. T.; Dean, R. L.; Beer, B. An evaluation of drug for improving memory in aged monkeys: Implications for clinical trials in humans. Psychopharmacol. Bull. 19:168–184; 1983.
2. Costa, L. G.; Murphy, S. D. Passive avoidance retention in mice tolerant to the organophosphorus insecticide disulfoton. Toxicol. Appl. Pharmacol. 65:451–458; 1982.
3. Costa, L. G.; Schwab, B. W.; Murphy, S. D. Differential alterations of cholinergic muscarinic receptors during chronic and acute tolerance to organophosphorus insecticides. Biochem. Pharmacol. 31:3407–3413; 1982.
4. Daunderer, M. Physostigmine salicylate as an antidote. Int. J. Clin. Pharmacol. Ther. Toxicol. 18:523–535; 1980.
5. Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Featherstone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88–95; 1961.
6. Fernando, J. C. R.; Hoskins, B.; Ho, I. K. Effect on striatal dopamine metabolism and differential motor behavioral tolerance following chronic cholinesterase inhibition with diisopropylfluorophosphate. Pharmacol. Biochem. Behav. 20:951–957; 1984.
7. Glowinski, J.; Iversen, L. L. Regional studies of catecholamines in the rat brain. I. The disposition of $^{3}$H-norepinephrine, $^{3}$H-dopamine and $^{3}$H-DOPA in various regions of the brain. J. Neurochem. 13:655–669; 1966.
8. Gordon, J. J.; Leadbeater, L.; Maidment, M. P. The protection of animals against organophosphate poisoning by pretreatment with a carbamate. Toxicol. Appl. Pharmacol. 43:207–216; 1978.
9. Harris, L. W.; Heyl, W. C.; Stitcher, D. L.; Moore, R. D. Effect of atropine and/or physostigmine on cerebral acetylcholine in rats poisoned with soman. Life Sci. 22:97–100; 1978.
10. Harris, L. W.; Lennox, W. J.; Talbot, B. G. Toxicity of anticholinesterase: Interactions of pyridostigmine and physostigmine with soman. Drug Chem. Toxicol. 7:507–526; 1984.
11. Koster, R. Synergisms and antagonisms between physostigmine and diisopropylfluorophosphate in cats. J. Pharmacol. Exp. Ther. 88:39–46; 1946.
12. Larson, G. F.; Rutebert, B. J.; Wingard, D. W. Physostigmine reversal of diazepam-induced depression. Anesth. Analg. 56:348–351; 1977.
13. Lennox, W. J.; Harris, L. W.; Talbot, B. G.; Anderson, D. R. Relationship between reversible acetylcholinesterase inhibition and efficacy against soman lethality. Life Sci. 37:793–798; 1985.
14. Lim, D. K.; Fernando, J. C. R.; Hoskins, B.; Ho, I. K. Quantitative assessment of tolerance development to diisoprophylfluorophosphate. Pharmacol. Biochem. Behav. 26:281–286; 1987.
15. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
16. Mayleux, R.; Albert, M.; Jenike, M. Physostigmine-induced myoclonus in Alzheimer's disease. Neurology 37:345–346; 1987.
17. Muramoto, O.; Sugishita, M.; Ando, K. Cholinergic system and constructional praxis: A further study of physostigmine in Alzheimer's disease. J. Neurol. Neurosurg. Psychiatry 47:485–491; 1984.
18. Nattel, S.; Bayne, L.; Ruedy, J. Physostigmine in coma due to drug overdose. Clin. Pharmacol. Ther. 25:96–102; 1979.
19. Parsons, D. S.; Peagler, A.; Barlow, T. S.; Harrell, L. E. Failure of chronic physostigmine to ameliorate working memory deficits after medial septal lesions. Exp. Neurol. 96:456–461; 1987.
20. Russell, R. W.; Vasquez, B. J.; Overstreet, D. H.; Dalglish, F. W. Consummatory behavior during tolerance to and withdrawal from chronic depression of cholinesterase activity. Physiol. Behav. 7:523–528; 1971.
21. Russell, R. W.; Overstreet, D. H.; Cotman, C. W.; Carson, V. G.; Churchill, L.; Dalglish, F. W.; Vasquez, B. J. Experimental tests of hypotheses about neurochemical mechanisms underlying behavioral tolerance to the anticholinesterase diisoprophylfluorophosphate. J. Pharmacol. Exp. Ther. 192:73–85; 1975.
22. Somani, S. M.; Khalique, A. Distribution and pharmacokinetics of physostigmine in rat after intramuscular administration. Fundam. Appl. Toxicol. 6:327–334; 1986.
23. Thal, L. J.; Fuld, P. A.; Masur, D. M.; Sharpless, N. S. Oral physostigmine and lecithin improve memory in Alzheimer's disease. Ann. Neurol. 13:491–496; 1983.
24. Weinstock, M.; Erez, E.; Roll, D. Antagonism of the cardiovascular and respiratory depressant effects of morphine in the conscious rabbit by physostigmine. J. Pharmacol. Exp. Ther. 218:504–508; 1981.
25. Yamada, S.; Isogai, M.; Okudaira, H.; Hayashi, E. Correlation between cholinesterase inhibition and reduction in muscarinic receptors and choline uptake by repeated diisopropylfluorophosphate administration: antagonism by physostigmine and atropine. J. Pharmacol. Exp. Ther. 226:519–525; 1983.
26. Yamamura, H. I.; Snyder, S. H. Muscarinic cholinergic binding in rat brain. Proc. Natl. Acad. Sci. USA 71:1725–1729; 1974.
27. Zukin, S. R.; Young, A. E.; Snyder, S. H. Gamma-aminobutyric acid binding to receptor sites in rat central nervous system. Proc. Natl. Acad. Sci. USA 71:4802–4807; 1974.