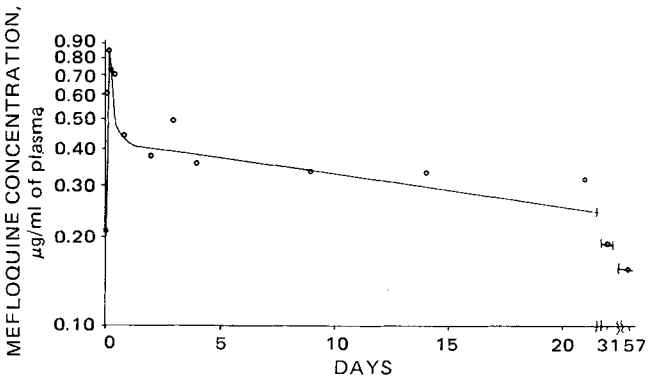

Figure 4—*Plasma I concentrations in a male subject following a single oral dose of 500 mg of I.*

plasma proteins probably caused the apparent disparities in the blood and plasma level data. Further data interpretation will be performed in conjunction with ongoing pharmacokinetic studies. The ability of the assay to follow blood and plasma levels of mefloquine for at least 57 days after a single oral administration of the drug established its utility for studying mefloquine's pharmacokinetic parameters in humans.

Table IV—Mefloquine Excretion in the Urine of an Adult Male after a Single Oral Dose of 500 mg of I [a]

	I Excretion		
Day[b]	μg/ml	μg/Sample	% Dose
1	3.03	527.92	0.12
2	0.76	127.01	0.03

[a] Chromatographic conditions are described under *Experimental.* [b] A random urine sample was obtained on each day.

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Cytolysis of Neuroblastoma Cells *In Vitro* and Treatment of Neuronal Tumors *In Vivo* with Bromoacetylcholine

C. Y. CHIOU

Abstract □ The effect of bromoacetylcholine on mouse neuroblastoma C-1300 was investigated in cell culture as well as in A/J mice. *In vitro,* bromoacetylcholine (1×10^{-5} *M*) was a potent cytolytic agent and produced an additive effect in combination with vincristine (3×10^{-9} *M*). Since the choline acetyltransferase inhibitor, dimethylaminoethyl chloroacetate, does not inhibit neuroblastoma efficiently *in vitro,* the potent cytolytic action of bromoacetylcholine is probably not due to its choline acetyltransferase inhibitory action. Furthermore, the neuroblastoma inhibitory effect of bromoacetylcholine was not affected by atropine. Therefore, the inhibitory action is not related to the interaction of bromoacetylcholine with muscarinic receptors either. In *in vivo* experiments, 1, 10, or 30 mg/kg of bromoacetylcholine was injected directly into the tumors three times daily for 6 weeks. Bromoacetylcholine at 10 and 30 mg/kg gave significant protection of A/J mice from the death induced by neuroblastoma inoculation, and the lifespan was prolonged significantly with these bromoacetylcholine treatments.

Keyphrases □ Bromoacetylcholine—*in vitro* cytotoxic and *in vivo* antitumor activity, mice □ Cytotoxic activity—bromoacetylcholine evaluated, mouse neuroblastoma cell culture □ Antitumor activity—bromoacetylcholine evaluated, mouse neuroblastoma □ Cholinergic agents—bromoacetylcholine, *in vitro* cytotoxic and *in vivo* antitumor activity, mice

Many attempts have been made to treat the highly malignant neuroblastoma in children (1). However, no drug tested, including cyclophosphamide and vincristine, is satisfactory, mainly because the remission rate in neuroblastoma patients is quite low and the duration of drug effectiveness is fairly short (2–4).

The nature of neuroblastoma cells can be cholinergic, adrenergic, or inactive (5–8). Acetylcholinesterase is always present, and these cells possess membranes that respond to acetylcholine and are excitable electrically. These data indicate the existence of cholinergic receptors at the membrane site (7, 9). Attempts were made to destroy these

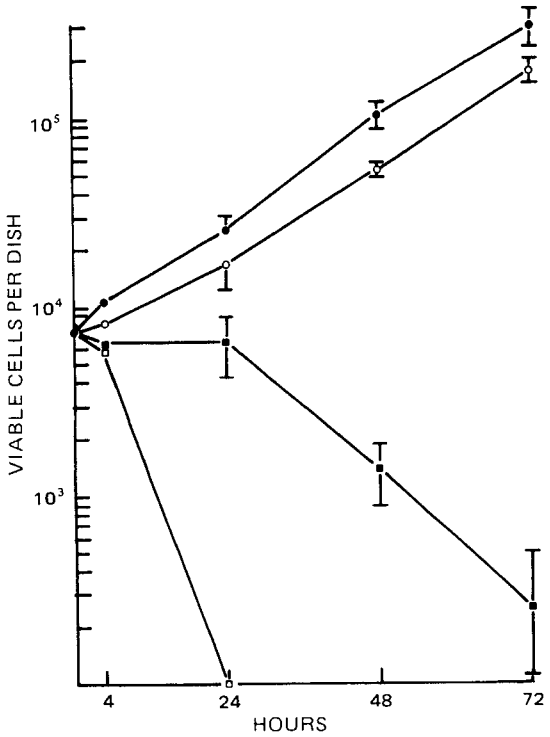

Figure 1—*Inhibition of neuroblastoma by various concentrations of bromoacetylcholine* in vitro. *Bromoacetylcholine was added to the culture medium at time zero and left in the medium throughout the experiment. Key:* ●, *control;* ○, *3 × 10^{-6}* M *bromoacetylcholine;* ■, *1 × 10^{-5}* M *bromoacetylcholine; and* □, *3 × 10^{-5}* M *bromoacetylcholine. Each point is a mean of four values, and bars represent* SEM.

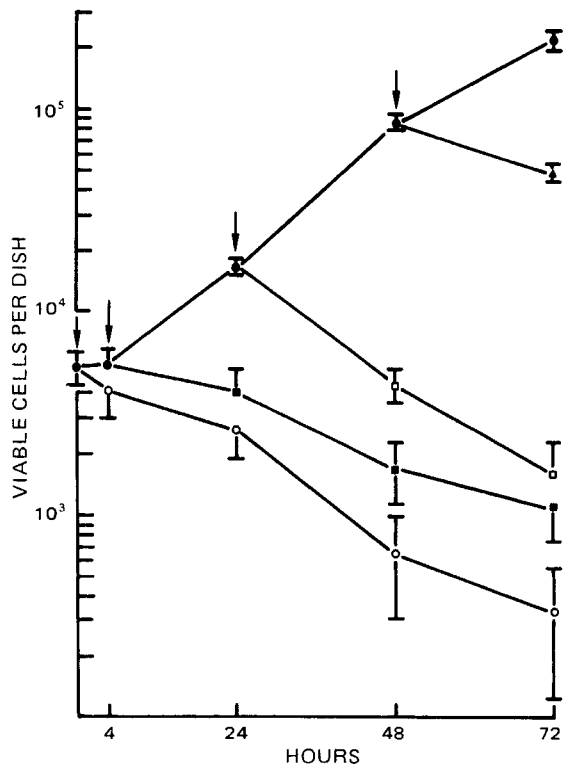

Figure 2—*Inhibition of neuroblastoma by bromoacetylcholine (3 × 10^{-5}* M*)* in vitro *after various stages of incubation. Bromoacetylcholine was added to the medium after 0 (○), 4 (■), 24 (□), or 48 (▲) hr of incubation time (indicated with arrows). Each point is a mean of four values, and bars represent* SEM.

neuronal tumors with an irreversible sympatholytic agent, 6-hydroxydopamine (10), resulting in a brief tumor regression only in the 1st week of treatment. The effectiveness of this treatment then decreased rapidly.

The ineffectiveness of 6-hydroxydopamine to maintain cytolysis of neuroblastoma was confirmed further by Finklestein *et al.* (11). Therefore, instead of an adrenolytic agent, an irreversible cholinolytic agent, bromoacetylcholine (12), was evaluated and is the subject of this report.

EXPERIMENTAL

Materials—Vincristine sulfate, 6-hydroxydopamine hydrochloride, and atropine sulfate were obtained commercially. Bromoacetylcholine perchlorate and dimethylaminoethyl chloroacetate were synthesized using previously published methods (13, 14).

Culture Medium—Mouse neuroblastoma C-1300 was cultivated in Dulbecco modified Eagle medium[1] supplemented with 10% fetal calf serum[2], 200 m*M* L-glutamine, 30 m*M* $NaHCO_3$, 50 units of penicillin/ml, and 50 μg of streptomycin/ml (7). The cells were grown at 37° under 95% air–5% CO_2 with 100% humidity.

For the culture experiments, 1×10^6 cells were placed in a tissue culture flask[3] (75 cm^2) for 3–5 days until the cells were in the logarithmic growth phase. They were then removed from the surface by a 15-min exposure to 0.25% trypsin in modified D_1 solution[4] (7, 15). The cells were then tapped off in fresh medium containing 10% fetal calf serum. Aliquots of the cell suspension were incubated for 5 min with 0.4% trypan blue, and the number of viable and nonviable cells was counted with a hemocytometer.

Appropriate dilution was made, and the cell suspension was plated at

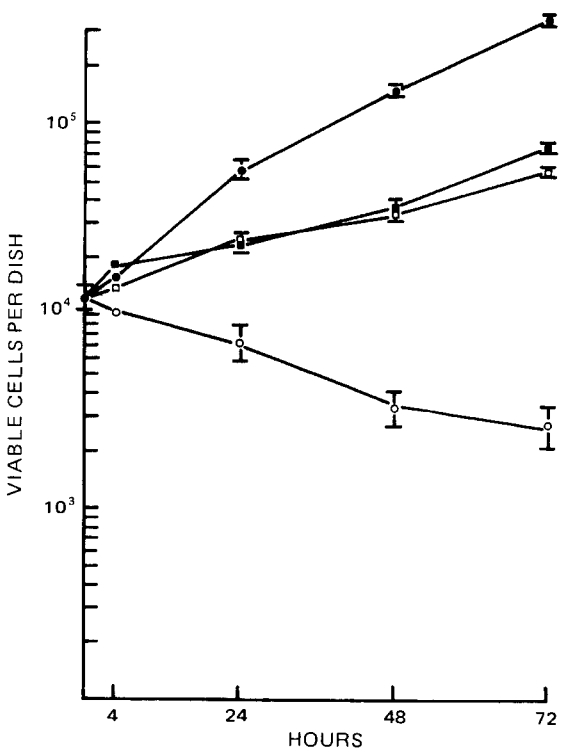

Figure 3—*Additive action of bromoacetylcholine and vincristine to inhibit neuroblastoma. Key:* ●, *control;* ■, *1 × 10^{-5}* M *bromoacetylcholine;* □, *3.2 × 10^{-9}* M *vincristine; and* ○, *1 × 10^{-5}* M *bromoacetylcholine plus 3.2 × 10^{-9}* M *vincristine. Each point is a mean of four values, and bars represent* SEM.

[1] MEM, Earle's Powder, Catalog No. F-11, Grand Island Biological Co., Grand Island, N.Y.

[2] Catalog No. 614, Grand Island Biological Co., Grand Island, N.Y.

[3] Falcon.

[4] Modified D_1 solution contained 1.2 mg of phenolsulfonphthalein, 8 g of sodium chloride, 0.4 g of potassium chloride, 45 mg of dibasic sodium phosphate heptahydrate, 30 mg of monobasic potassium phosphate, 2 g of glucose, 20.2 g of sucrose, 5×10^4 units of penicillin, and 50 mg of streptomycin in 1 liter of double-distilled water.

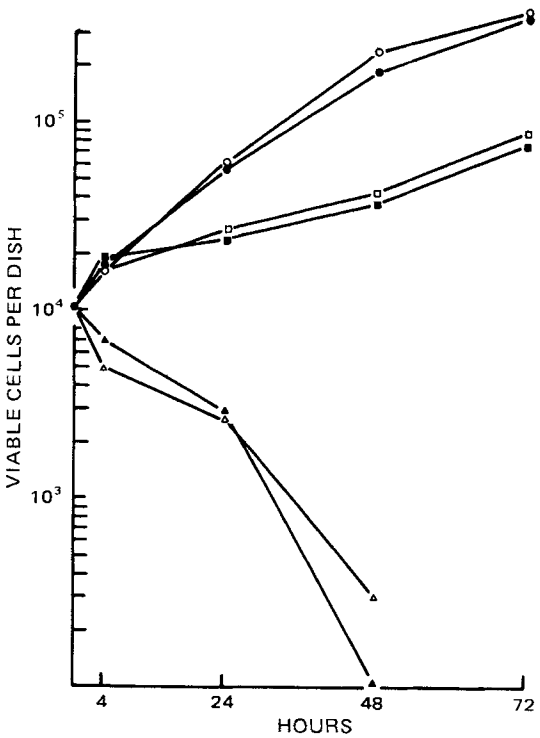

Figure 4—*Effect of atropine (1×10^{-7} M) on the control growth of neuroblastoma and inhibitory action of bromoacetylcholine* in vitro. *Key: ●, control; ○, 1×10^{-7} M atropine; ■, 1×10^{-5} M bromoacetylcholine; □, 1×10^{-5} M bromoacetylcholine plus 1×10^{-7} M atropine; ▲, 3×10^{-5} M bromoacetylcholine; and △, 3×10^{-5} M bromoacetylcholine plus 1×10^{-7} M atropine. Each point is a mean of four experiments.*

5×10^4 cells/dish (tissue culture dishes[3] of 35-mm diameter) and grown for 24 hr. Various concentrations of drugs were added to the medium, and these cultures were incubated further for 72 hr. The cultures were removed at intervals (4, 24, 48, and 72 hr) for cell count.

Inhibition of Neuroblastoma *In Vitro*—Various concentrations of bromoacetylcholine, dimethylaminoethyl chloroacetate, 6-hydroxydopamine, and vincristine were added to the cell culture to determine the potency of these drugs and drug combinations to inhibit neuroblastoma. Drug concentrations required to reduce the initial control viable cells (at zero time) by 50% after 72 hr of incubation were termed CD_{50} (50% cytolytic dose). Bromoacetylcholine (3×10^{-5} *M*) also was added to the culture medium after various incubation times (0, 4, 24, and 48 hr) to study its inhibitory ability at various stages of neuroblastoma culture.

To determine whether the inhibitory action of bromoacetylcholine is mediated through its interaction with the muscarinic site, atropine was added to the medium.

Treatment of Neuroblastoma *In Vivo*—The cell suspension of mouse neuroblastoma C-1300 was injected (1×10^6 cells/mouse) subcutaneously to the interscapular area of A/J mice[5], 16–18 g (11, 16). Ten days later, the animals were divided into tumor-control and tumor-treated groups. The control group was injected three times daily, except Saturdays and Sundays, with saline; the treated groups were injected similarly with bromoacetylcholine at 1, 10, or 30 mg/kg. Injections were made directly into the tumors. The size and body weight were recorded every other day.

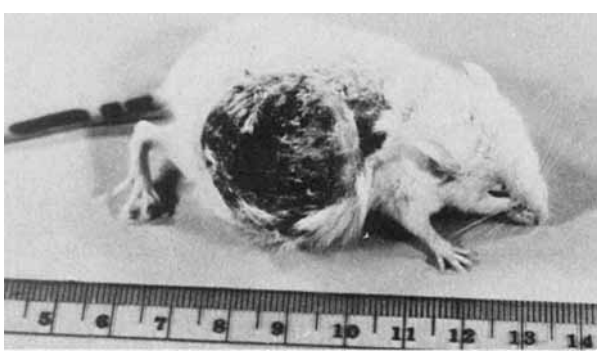

Figure 5—*Control A/J mouse inoculated with 10^6 cells of neuroblastoma for 5 weeks.*

[5] Jackson Lab., Bar Harbor, Me.

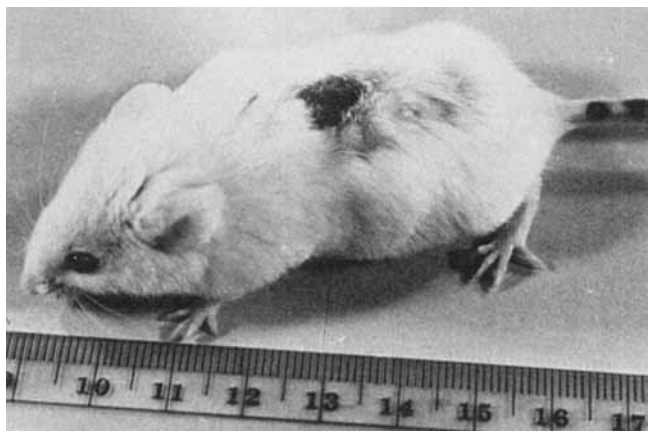

Figure 6—*Treatment of neuroblastoma-inoculated A/J mouse with 30 mg of bromoacetylcholine/kg three times daily for 6 weeks.*

RESULTS

Figure 1 shows a potent cytolytic effect of bromoacetylcholine on neuroblastoma cells at a concentration of 1×10^{-5} *M*. The neuroblastoma cells could be inhibited by bromoacetylcholine (3×10^{-5} *M*) at any stage of the incubation period (0, 4, 24, and 48 hr) (Fig. 2). The CD_{50} of bromoacetylcholine was 5.8×10^{-6} *M*.

Bromoacetylcholine is a potent inhibitor of choline acetyltransferase in cell-free choline acetyltransferase preparations (17). Although bromoacetylcholine does not cross the cell membrane efficiently because of its positive charge in the molecule, it is possible to accumulate enough bromoacetylcholine in the intracellular site to inhibit choline acetyltransferase after prolonged incubation. To determine whether the cytolytic action of bromoacetylcholine is related to its inhibition of choline acetyltransferase in the intracellular site, dimethylaminoethyl chloroacetate was used because it can get into the intracellular site efficiently. Although dimethylaminoethyl chloroacetate inhibited choline acetyltransferase in the intact cellular preparation with an I_{50} of 1.29×10^{-4} *M* (14), it took 1.3×10^{-3} *M* to inhibit the growth of neuroblastoma, indicating that the cytolytic action of dimethylaminoethyl chloroacetate

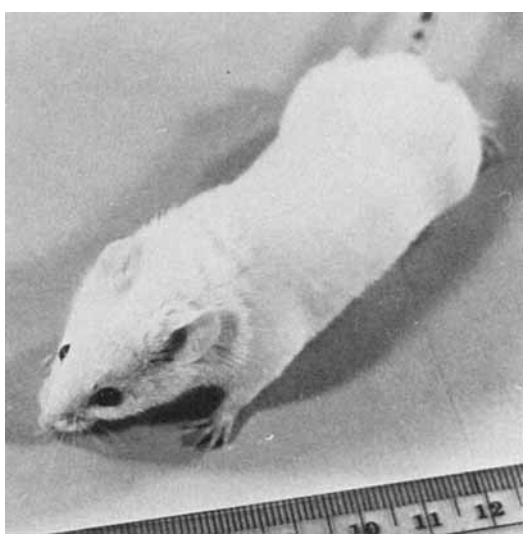

Figure 7—*Complete recovery of neuroblastoma-inoculated A/J mouse. The mouse was treated with 30 mg of bromoacetylcholine/kg three times daily for 6 weeks. Drug treatment was stopped thereafter, and the picture was taken 7 weeks after the drug treatment was stopped.*

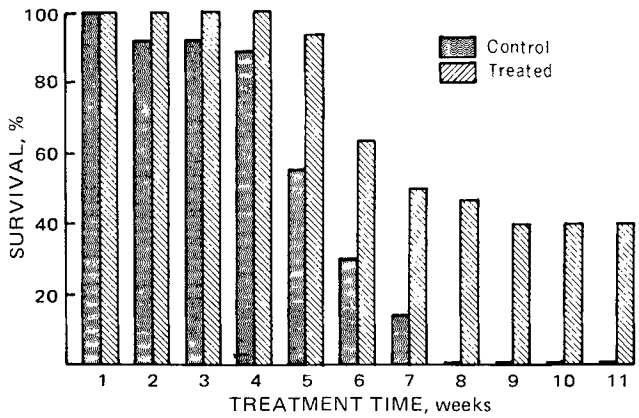

Figure 8—*Comparison of survival rate between treated (bromoacetylcholine, 30 mg/kg) and untreated (control) neuroblastoma-inoculated A/J mice.*

on neuroblastoma is either unrelated to the inhibition of choline acetyltransferase or due to a rise in concentration needed in cancer cells to inhibit choline acetyltransferase.

The sympatholytic, 6-hydroxydopamine, had a CD_{50} of 2.0×10^{-5} *M*. Vincristine, a cellular mitotic arresting agent used clinically to treat neuroblastoma, was very potent, inhibiting neuroblastoma *in vitro* with a CD_{50} of 8.5×10^{-9} *M*. Combined use of bromoacetylcholine (1×10^{-5} *M*) and vincristine (3×10^{-9} *M*) produced an additive effect, which was equivalent to the higher concentration of bromoacetylcholine or vincristine given individually (Fig. 3).

Atropine (1×10^{-7}–1×10^{-5} *M*) did not affect the cytolytic action of bromoacetylcholine on neuroblastoma (Fig. 4), indicating that bromoacetylcholine does not act *via* muscarinic sites to inhibit neuroblastoma. The CD_{50} of atropine was greater than 1.0×10^{-4} *M*.

In *in vivo* experiments, a dramatic difference between control and treated neuroblastoma mice was noted (Figs. 5–8). Marked tumor regression was noted 3 weeks after bromoacetylcholine treatment at doses of 10 and 30 mg/kg (Figs. 5 and 6). The area of tumor became hard, flattened, and necrotic after 6 weeks of treatment. Finally, the fur grew back and looked healthy 3 months after the treatment was initiated (Fig. 7). Treatments were stopped after 6 weeks.

The survival time increased significantly with mice treated with 10 and 30 mg/kg of bromoacetylcholine (Table I and Fig. 8). Although the body weight of the control group was only slightly lower than that of treated ones (Fig. 9), subtraction of tumor weight from the total body weight would make the net body weight of control mice be even lighter than the treated ones.

DISCUSSION

Bromoacetylcholine is promising as a cancer chemotherapeutic agent for metastatic neuroblastoma, because it inhibits effectively the growth

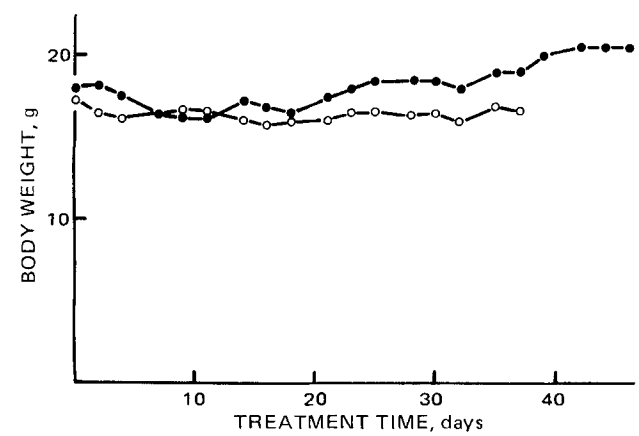

Figure 9—*Comparison of body weights of treated (bromoacetylcholine, 30 mg/kg) and untreated (control) neuroblastoma-inoculated A/J mice. Key: ●, treated; and ○, control.*

Table I—Treatment of Neuroblastoma with Bromoacetylcholine in Adult A/J Mice on Subcutaneously Inoculated Tumors

Treatment	*n*	Survival Time: Range, days	Survival Time: Mean, days	CLS[a], %
Control	12	10–47	34.3 ± 2.9	—
Bromoacetylcholine, 1 mg/kg[b]	7	27–39	33.4 ± 1.7	−2.6
Bromoacetylcholine, 10 mg/kg[b]	7	31–120[c]	56.9 ± 11.7[d]	+65.8
Bromoacetylcholine, 30 mg/kg[b]	10	30–120[c]	64.3 ± 12.6[d]	+87.5

[a] Percent change in lifespan (CLS) of treated over control mice. [b] These doses were administered three times daily, except Saturday and Sunday, for 6 weeks (40 days). [c] The 120 days is not the upper range of survival time, because some mice survived longer; 120 days was chosen arbitrarily as a cutoff date for the purpose of statistical analysis. [d] Statistically different from control ($p < 0.05$).

of neuroblastoma at 5.8×10^{-6} *M* or higher *in vitro* (12).

One major obstacle of using anticancer drugs is toxicity. The main side effect anticipated with the use of bromoacetylcholine is the muscarinic (parasympathomimetic) action, which can be eliminated with atropine and its analogs. Bromoacetylcholine is a cholinergic agonist and stimulates both nicotinic and muscarinic receptors directly (18–20). However, when it is incubated with cells for 15 min or longer, it binds irreversibly to the cholinergic receptor at the nicotinic site but not at the muscarinic site (18, 21).

Atropine (up to 1×10^{-5} *M*) did not affect the cytolytic actions of bromoacetylcholine on neuroblastoma. Therefore, it is possible that bromoacetylcholine can be used along with muscarinic blocking agents as a safe and potent drug to treat neuronal tumors.

Alternatively, reduced doses of more than one cancer chemotherapeutic agent with different action mechanisms can be used in combination. In this way, the chemotherapeutic actions of these drugs can be enhanced while their side effects can be minimized.

Among several agents tested, vincristine was the most potent neuroblastoma inhibitor. When combined with bromoacetylcholine, it showed additive effects, indicating that reduced quantities of bromoacetylcholine (1×10^{-5} *M*) and vincristine (3×10^{-9} *M*) can be used together to get an effect that requires higher concentrations of bromoacetylcholine (3×10^{-5} *M*) or vincristine (1×10^{-8} *M*) when given individually.

Results obtained from *in vivo* experiments are very encouraging, because 50% of the neuronal tumor mice that were treated with bromoacetylcholine at the 30-mg/kg dose injected three times daily for 6 weeks survived after all untreated control mice were dead. Certainly, the dose regimens and the administration route in this study are not ideal, and the combined use of bromoacetylcholine with atropine and/or vincristine should be tried[6].

Since the murine neuroblastoma system used is reported to be a model of human disease (16), the effectiveness of bromoacetylcholine for treating neuroblastoma *in vivo* in this study makes it promising for treating neuroblastoma in children.

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Determination of Triprolidine in Human Plasma by Quantitative TLC

R. L. DeANGELIS, M. F. KEARNEY, and R. M. WELCH ˣ

Abstract □ A chromatographic thin-layer fluorescence procedure, with a sensitivity limit of 0.8 ng/ml, is described for the quantitative analysis of triprolidine in human and rat plasma. Following the intravenous administration of 1 mg/kg of triprolidine to rats, the drug distributed rapidly into tissues and was eliminated from plasma with a half-life of 53 min. The method was used to determine the plasma triprolidine levels in 16 normal human volunteers following oral administration of 3.75 mg of triprolidine hydrochloride in 15 ml of a syrup. The drug obtained a mean peak plasma level of 8.2 ng/ml in 2 hr and was eliminated from the plasma with a half-life of 5 hr. Considerable individual variation was observed in the area under the plasma triprolidine level–time curve; values ranged from 19 to 163 ng hr/ml with a mean value of 75 ng hr/ml.

Keyphrases □ Triprolidine—TLC analysis, human and rat plasma, pharmacokinetic profile □ TLC—analysis, triprolidine, human and rat plasma □ Pharmacokinetics—triprolidine, humans and rats □ Antihistamines—triprolidine, TLC analysis, human and rat plasma, pharmacokinetic profile

Triprolidine hydrochloride [(*E*)-2-[3-(1-pyrrolidinyl)-1-*p*-tolylpropenyl]pyridine hydrochloride] is an unusually potent antihistaminic agent used for the treatment of various allergic conditions. Although animal data (1–3) and several clinical reports (4–8) substantiate the efficacy of triprolidine, its disposition is unknown. One report indicated that triprolidine had a duration of action of approximately 4–6 hr (5), but no confirming plasma level data were given.

In another study on the *in vitro* metabolism of ^{14}C-triprolidine by guinea pig liver, the drug was extensively metabolized (9). Other reports described the TLC (10) and GC (11) properties of triprolidine, but no attempt was made to quantitate the drug in tissues. Considering the extensive clinical use of this antihistamine, alone and in combination with other drugs, a sensitive quantitative analysis in biological fluids is needed.

This report describes a sensitive analytical method for quantitating triprolidine in plasma following oral administration of a therapeutic dose to human subjects. The method was used to determine the pharmacokinetic profile of the drug in animals and humans.

EXPERIMENTAL

Materials—The organic solvents utilized to develop the thin-layer plates were reagent grade and were used as received; the solvents employed for the extraction of triprolidine from plasma were glass distilled[1]. A 0.01% stock solution of triprolidine hydrochloride was prepared by dissolving the drug in 1–2 ml of methanol and then adding an appropriate volume of chloroform. From this solution, a working standard of 1 μg/ml was prepared in chloroform. A spray reagent of 2 *M* ammonium bisulfate, prepared every 2 weeks, was employed to induce the fluorescence of the drug on the TLC plate.

Analytical Procedure—Plasma, 1 ml, and 6 ml of dichloroethane were added to a 15-ml conical glass centrifuge tube, mechanically shaken for 15 min, and centrifuged (1000×*g*). Five milliliters of the organic phase was placed in a disposable glass culture tube, the tube was immersed in a water bath at 45°, and the solvent was evaporated to dryness under a nitrogen stream. The residue was redissolved in 0.1 ml of chloroform, and a suitable aliquot (50–80 μl) was spotted on a prescored silica gel plate[2]. Samples were applied to a thin-layer plate with an automatic spotter[3], while the standards (1–5 ng) were spotted by hand using a 10-μl syringe.

Prior to use, each TLC plate was channeled (scored) into 20 units 1 cm wide. The plate was developed to a height of 15 cm in methanol–ammonium hydroxide–chloroform (10:1:89). The solvent system was prepared fresh each day and allowed to equilibrate for 30 min prior to use. After development, the plate was allowed to air dry for 15 min and then sprayed with a 2 *M* aqueous solution of ammonium bisulfate until shiny. After the plate was air dried for about 1 hr, each channel was scanned, and the fluorescent spot representing triprolidine was quantitated.

Instrumentation—Fluorescence measurements were determined by scanning the TLC plate with a spectrodensitometer[4] in the reflectance mode, utilizing only the sample beam that had been passed through a secondary cutoff filter of 405 nm. The fluorophore was excited at 300 nm, and the total emission above 405 nm was read using a density computer[5].

[1] Burdick and Jackson Laboratories.
[2] Siliplate-22, 20 × 20 cm, 0.25 mm, E. M. Laboratories.
[3] Analytical Instrument Specialties multispotter.
[4] Schoeffel SD3000 with reflectance-mode assembly.
[5] Schoeffel SD 30.