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Flow injection determination of neostigmine and galanthamine by immobilised acetylcholinesterase inhibition

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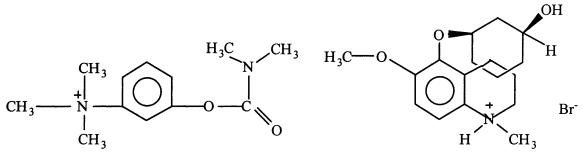
Abstract

Neostigmine (1×10^{-7}–1×10^{-6} M) and galanthamine (5×10^{-7}–6×10^{-6} M) are determined by measuring their inhibition of acetylcholinesterase immobilised on controlled pore glass. The determination is carried out by a flow injection procedure with spectrophotometric detection. The 3σ limits of detection were 0.5×10^{-7} M neostigmine and 5×10^{-7} M galanthamine. The relative standard deviations were 1.3% for five determinations of 5×10^{-7} M neostigmine and 2.0% for six determinations of 2×10^{-6} M galanthamine. Several reagents for achieving on-line reactivation of inhibited enzyme were studied. As a result, 2×10^{-3} M NaF was recommended for reactivation after neostigmine inhibition, and 2×10^{-4} M substrate solution for reactivation after galanthamine inhibition. The efficiency of the reactivation process was interpreted in terms of the inhibitory mechanisms relating to the above drugs.

Keywords: Flow injection; Spectrophotometry; Enzyme inhibition; Galanthamine; Neostigmine; Immobilised AchE

1. Introduction

Neostigmine (I), a carbamate derivative, is a potent acetylcholinesterase inhibitor and is extensively used for treatment of *myasthenia gravis* [1]. It reacts with acetylcholinesterase (AChE) in much the same way as do the substrate acetylcholine and inhibiting organophosphorus compounds. The substrate and inhibitor bind at both the anionic and esteric sites on AChE, following which hydrolysis occurs in a similar manner for substrate and inhibitor. In the case of neostigmine quaternary ammonium phenol is split off by hydrolysis leaving the carbamylated enzyme. The latter is hydrolysed to release a substituted carbamic acid and regenerates the free enzyme [2]. The rate of hydrolysis of the carbamylated enzyme is slower than that of the acylated enzyme formed with the substrate, but the regeneration of the enzyme activity occurs far more readily than with an organophosphorus inhibitor.



(I) (II)

Galanthamine (II) is a reversible inhibitor which binds only to the anionic site of AChE [3]. The compound has safely been used in humans for ca.

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30 years as an anticurare agent in the treatment of various neurological disorders [4]. It is the only member of the large group of *amaryllidaecea* alkaloids that has gained widespread commercial application. Clinically this alkaloid is important as a natural, potent cholinergic substance, showing strong anticholinesterase activity [5]. The alkaloid is extracted from plants. Commercial synthesis of the compound is too expensive and very low yielding. Therefore the supply of the drug depends on natural plant resources [6].

In the last few years this compound has also gained popularity for the treatment of Alzheimer's disease. As progressive memory dysfunction in Alzheimer's disease is associated with a decreased rate of formation of acetylcholine in the brain, it is useful to inhibit acetylcholine hydrolysis to restore synaptic function [7]. A reasonable therapeutic approach, therefore, is to treat this disease with cholinesterase inhibitors. Different inhibitors have been tried. Few of them can pass into the brain and remain effective without any severe side effects. Physiostigmine is one of the most commonly used drugs for the treatment of Alzheimer's disease. While this can produce memory improvement, its clinical application is not without problems. Another drug, tacrine (tetrahydroaminoaccaridine), is less potent than physiostigmine, and is reported to be more useful for the treatment of this disease, but it also produces adverse effects [8,9]. The reason could be that both physiostigmine and tacrine are not selective for AChE and also inhibit butyl(Bu)ChE, which causes adverse effects.

Galanthamine is highly selective for AChE and thus is well tolerated, which might be related to lack of BuChE inhibition. Owing to the high selectivity of this drug for AChE it has achieved considerable superiority to physiostigmine and tacrine for the treatment of Alzheimer's disease. Although neostigmine is also selective for AChE it is inappropriate for Alzheimer's disease because its quaternary structure makes it difficult to cross the blood–brain barrier [10].

Liquid chromatographic methods [11–14] and an enzyme immunoassay and radioimmunoassay [5,6] have been developed for galanthamine. Radioimmunoassay is very sensitive, but has well-known disadvantages.

Neostigmine is most frequently determined by gas chromatography (GC) [15–17] and a few spectrophotometric methods [18,19] and less frequently iodimetrically [20] and potentiometrically [21]. An amperometric enzyme immunoassay has also been developed [22] for the determination of anticholinesterase drugs. The system is sensitive but the lifetime of the enzyme biosensor is only 15 days.

Batch enzymatic inhibition (EI) methods have been described for the determination of neostygmine [23,24], but these methods have been proved to underestimate carbamates [25]. The sensitivity and reliability of these methods could be improved by converting them into flow injection (FI) methods. Such methods have been successfully adopted in this laboratory for the determination of carbamate pesticides [26,27]. No FI-enzyme inhibition (EI) method has yet been described for the determination of routinely and effectively used medicinal carbamates. In a previous paper [28] FI analysis for metrifonate, an organophosphorus compound, based on the inhibition of immobilised AchE, was successfully achieved. In the present paper the same principle is adopted for the determination of neostigmine and galanthamine, which might prove useful for routine analysis for the drugs and analogous compounds of medicinal importance. It is particularly interesting to test such a FI-immobilised AChE procedure on inhibitors that bind to the anionic site, unlike metrifonate which binds to the esteric site of AChE. Emphasis has also been placed on the rapid regeneration of the enzyme after its inhibition to enable the enzyme to be reused efficiently.

2. Experimental

2.1. Reagents

All chemicals and reagents were the same as those described previously [29], except for galanthamine–HBr, which was generously provided by Shire Pharmaceuticals, Foss House, Andover, Hants, and neostigmine, which was purchased from Sigma (Poole, UK). A galanthamine stock solution (3×10^{-4} M) was prepared by dissolving 0.01 g of drug in 100 ml of water. Dilutions were made in sodium phosphate buffer (pH 8.5, 0.1 M). A neostigmine stock solution was prepared by dissolving exactly 0.06 g of drug in 100 ml of water. The solution was stored in a

refrigerator, and freshly diluted before use in the sodium phosphate buffer. A new stock solution was prepared each week. The immobilised acetylcholinesterase was prepared as described previously [28] by binding to 3-aminopropyl-triethoxysilanised controlled pore glass (CPG) via glutaraldehyde linking. The CPG-240 (Sigma) was 80–120 mesh, mean pore diameter 22.6 nm.

2.2. Apparatus and procedure

The basic FI manifold equipped with flow cell and spectrophotometer was the same as that used for the determination of metrifonate [28]. The inhibitor and substrate (acetylthiocholine) zones were injected to coincide in the buffer stream, which then passed through the immobilised AChE column, 2.5 mm long×2.5 mm i.d. The effluent was mixed with chromogen (5,5-dithiobis)(2-nitrobenzoic acid) solution, and the absorbance measured at 405 nm. The enzyme column was thermostated by a water jacked connected to a thermostat bath. The spectrophotometric procedure followed for inhibition studies of AChE with neostigmine or galanthamine was the same as that adopted for metrifonate determination [28]. The percentage enzyme inhibition (*%I*) was calculated by the formula: $\%I=100\ (A_0-A_i)/A_0$, where A_0 is the peak height absorbance in the absence of inhibitor and A_i is that in the presence of inhibitor.

3. Results and discussion

3.1. Effect of reaction parameters

When working with enzymes, pH is an important factor to be optimised. pH optimisation studies were carried out for neostygmine by preparing sodium phosphate buffers of a wide range of pH values (6.0–10.0, 0.1 M). Each buffer was used as a carrier solution and *%I* was calculated. As shown in Fig. 1, inhibition increases with increase in pH up to ca. pH 8.5. It can probably be concluded that binding of neostigmine is greatest at this pH as the mechanism is similar to that of ACh binding and AChI hydrolysis is also maximal at this pH [28]. Neostigmine and galanthamine inhibition were subsequently studied at pH 8.6, in 0.1 M sodium phosphate buffer.

Temperature was another factor to be optimised for inhibition studies. When the temperature was increased from 15°C to 50°C during studies of inhibition by neostigmine, *%I* continuously increased with temperature (Fig. 2). At temperatures >45°C [28] the

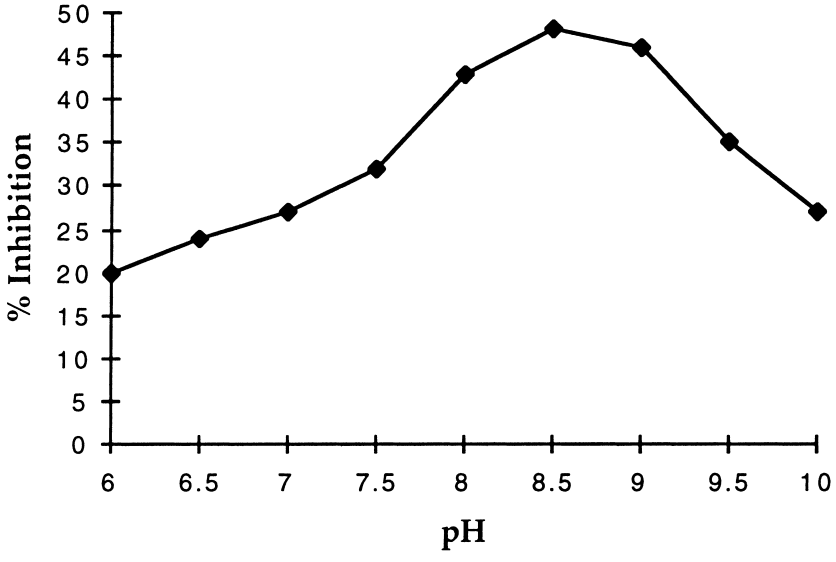

Fig. 1. Effect of pH on % inhibition; 6×10^{-4} M substrate (70 μl) and 2×10^{-7} M neostygmine (160 μl). Drug incubated enzyme for 3 min at 30°C.

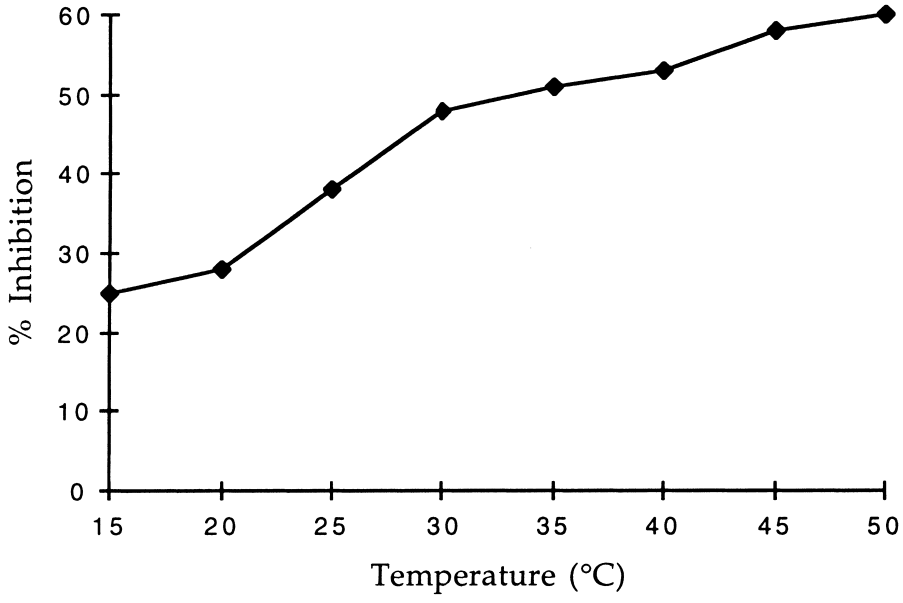

Fig. 2. Effect of temperature on % inhibition; pH 8.6 conditions otherwise as in Fig. 1.

enzyme eventually became denatured, so further work was carried out at 30°C in order to ensure a long active lifetime for the enzyme column, and also to avoid the problem of air bubbles in the flow system which increased with increase in temperature.

The effect of substrate (acetylthiocholine) concentration on *%I* by neostigmine or galanthamine was investigated by injecting different concentrations of substrate with a fixed concentration of inhibitor (1×10^{-7} or 2×10^{-7} M neostigmine or 3×10^{-6} M galanthamine; 3 min incubation). The results showed that over all concentrations of substrate studied (2×10^{-4}–8×10^{-4} M) there was no change in percentage inhibition by either inhibitor with increasing substrate concentration, i.e. the inhibition is non-competitive. Unlike metrifonate, neostigmine and galanthamine inhibit AChE at its anionic site. Thus the inhibitors can bind with free enzyme and also with the acyl–enzyme complex, as in this complex the anionic site is free because choline has dissociated from the anionic site and only the esteric site is blocked. Hence the inhibition is non-competitive, whereas for metrinfonate it is uncompetitive [29]. As a result a substrate concentration of 2×10^{-4} M was used for galanthamine and 4×10^{-4} M for neostigmine determination.

A small range of substrate solution volumes (30–100 μl) was injected to investigate the effect of substrate volume on *%I*. The results showed only a small (ca. 30%) increase with increasing volume over this range. In fact, 30 and 50 μl of substrate were injected for further studies of neostigmine and galanthamine, respectively, although somewhat greater sensitivity would have been achieved with larger substrate volumes.

The extent of inhibition increases with increasing incubation time of enzyme with inhibitor, thus making detection more sensitive. The incubation time for neostigmine was optimised by injecting a definite fixed volume of sample (inhibitor) solution into the carrier stream of phosphate buffer and the flow was stopped after 15 s so that the inhibitor remained in the enzyme column for a fixed period of time (30 s–5 min). After each experiment enzyme activity was completely regenerated and the column reused (see below). The results obtained are shown in Fig. 3, which indicates that inhibition increases significantly with increase in incubation time up to ca. 3 min after which only a modest increase is observed. Galanthamine, likewise, was incubated for 10–180 s in the enzyme column. It was found that the inhibition was very rapid and was not time dependent, so all

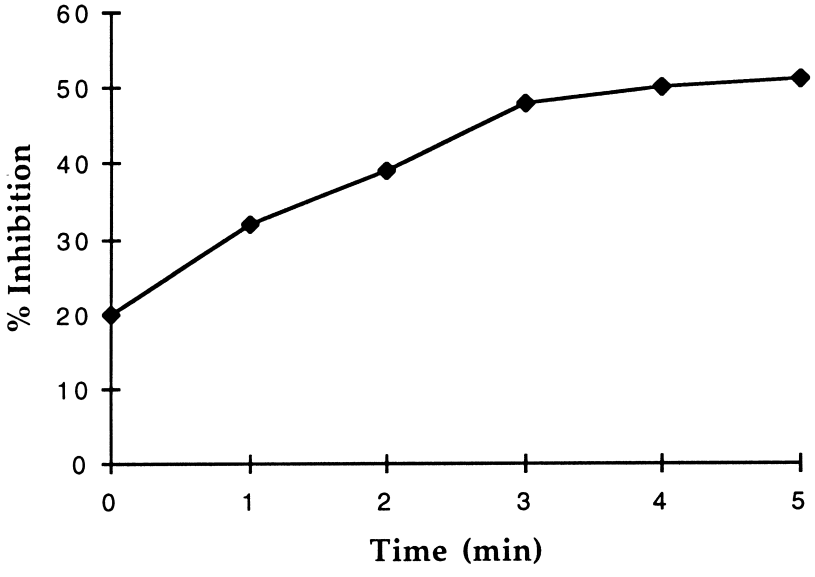

Fig. 3. Effect of incubation time on % inhibition; 4×10^{-4} M substrate (30 μl) and 2×10^{-7} M neostygmine (160 μl), 30°C, pH 8.6.

inhibition measurements were made without incubation, i.e. under continuous flow conditions.

The effect of sample (inhibitor) volume was investigated by increasing the volume from 50 to 200 μl for both inhibitors (Fig. 4). The *%I* increased almost linearly with such an increase in sample volume. For calibration a 180 μl sample volume was used in order to achieve high sensitivity, as there was little increase in sensitivity above this volume.

3.2. Enzyme reactivation

In order to reuse the inhibited enzyme reactor, it is necessary to reactivate the enzyme, preferably without removing it from the flow system. For metrifonate inhibition, the inhibitor could be displaced from the enzyme by passing pH 8.5 sodium phosphate buffer, but the process took 15 min [28]. Displacement of inhibitor by passing substrate gave faster displacement (ca. 6 min), but the use of pyridine-2-aldoxime

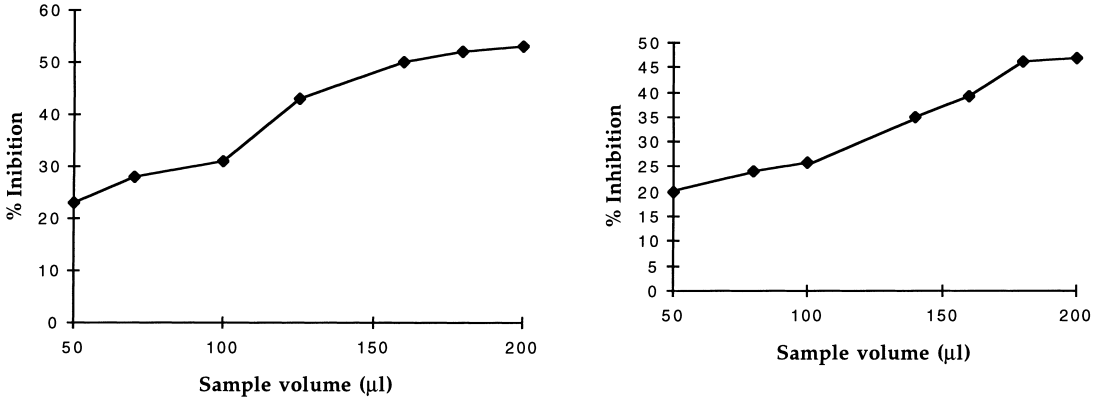

Fig. 4. Effect of sample (drug) volume for (a) neostygmine; (b) galanthamine on % inhibition. Conditions otherwise as in Table 2.

methiodide (2-PAM), which binds preferentially with the inhibitor, gave complete restoration of activity of 90% inhibited enzyme in 3 min. This was needed to regenerate the enzyme so as to allow 30 determinations of metrifonate per hour.

Reactivation of AChE inhibited by galanthamine was found to be almost instantaneous at room temperature (ca. 25°C) in a stream of pH 8.6 buffer, but when the experiment was repeated at 10–15°C complete reactivation took longer (5 min). Potential reactivators such as NH_2OH, (1×10^{-3} M), NaF and $MgCl_2$ (both 2×10^{-3} M) were injected, but they had no effect on the rate of reactivation of the enzyme at 10–15°C. So reactivation by buffer at 25°C was used in further studies.

In contrast to galanthamine, removal of neostigmine inhibition was quite slow, as mentioned by Goldstein and Hamlisch [29], probably because of the additional esteric site binding. For neostygmine, as with galanthamine, the first target factor in order to enhance the regeneration of the enzyme after inhibition was temperature. Regeneration at 20°C was slow, taking 10–15 min for 95–100% regeneration while continuously passing buffer and finally injecting substrate, or by continuously passing the substrate. However, when the inhibited enzyme column was washed with buffer at 30°C or 35°C the enzyme activity completely reappeared in 5–7 min. This shows that temperature is again an important factor in the reversal of inhibition. As there was no difference in the percentage reactivation at 30°C or 35°C, 30°C was used in all further work. No additional reactivating agent was required.

The effect of higher concentrations of substrate than are used in the analytical procedure may also have an effect on the rate of regeneration of the enzyme. When 30 μl injections of 1×10^{-3} M substrate were made into the enzyme column after inhibition by neostygmine, it was observed that there was a little or no change in reversal of inhibition when the substrate was incubated in the column for 30 s–4 min. However, incubation of 2×10^{-3} M substrate with the enzyme for 90 s restored the enzyme to ca. 90% activity (Table 1). None of the other compounds tested for reactivation, as described below, gave significantly improved deactivation.

The regeneration effects of different reactivators on neostygmine inhibition were studied at pH 8.6 and 30°C. The results are summarised in Table 1. In this set of experiments 30 μl of reactivator solution was injected and passed through the enzyme column or stopped in it for some time (30 s–3 min). Finally buffer was passed as carrier and after 5 s substrate was injected to investigate the change in enzyme activity. $MgCl_2$ (2×10^{-3} M), molybdate or hydroxylamine (both 1×10^{-3} M) had no effect on regeneration without incubation, but ca. 80% enzyme activity was recovered after 2 min incubation.

Table 1
Reactivators used for the regeneration of the 70% neostigmine inhibited enzyme

Reactivators	Concentration (M)	Stopped time (min)	Activity recovered (%)
$MgCl_2$	2×10^{-3}	3	80
Molybdate	1×10^{-3}	3	82
Substrate	2×10^{-3}	1.5	90
2-PAM	1×10^{-4}	2	72
NH_2OH	1×10^{-3}	2	83
Atropine+2-PAM	$4\times10^{-5}+2\times10^{-5}$	2	74
Atropine	4×10^{-5}	2	85
NaF	2×10^{-3}	1	92

The oxime (2-PAM) used for the reactivation of a phosphorylated enzyme (e.g. with metrifonate) was tested for reactivation of the carbamylated enzyme (neostigmine inhibition). The reactivating action of 2-PAM in the range 2×10^{-5}–2×10^{-4} M was studied. It was found that a concentration as high as 1×10^{-4} M did not reactivate the enzyme inhibited by neostigmine significantly better than the buffer alone. It has been reported by Naloff [30] that oximes differ in their effectiveness against different carbamates. In addition, it would not be expected that 2-PAM would reverse the carbamylation of the enzyme.

It was also observed in this work that frequent injections of 2-PAM even in the absence of inhibitor decreased the enzyme activity, presumably via interaction with the anionic site. If the enzyme was then washed with buffer, however, its normal activity was restored. In addition, reactivation of the enzyme with the oxime decreased the degree of subsequent inhibition by neostigmine even after complete reactivation of the inhibited enzyme.

Atropine is well known as a compound that restricts AChE inhibition, and has been reported to be a reactivator of the enzyme inhibited by neostigmine.

Table 2
Optimised conditions for the determination of neostigmine and galanthamine

	Neostigmine	Galanthamine
Substrate concentration (M)	4×10^{-4}	2×10^{-4}
Temperature (°C)	30–35	25
Phosphate buffer (M)	0.1	0.1
pH	8.6	8.5
Flow rate (ml min^{-1})	0.6	0.65
Column length (mm)	2.5	2.5
Column i.d. (mm)	2.5	2.5
Substrate loop size (μl)	30	50
Sample loop size (μl)	160	180
Incubation time (min)	1.0	0

As shown in Table 1, when 4×10^{-5} M atropine or a mixture of atropine (4×10^{-5} M) and 2-PAM (2×10^{-5} M) was injected it was found that atropine alone was a better reactivator than 2-PAM alone or a mixture of 2-PAM and atropine.

As can be seen from Table 1 NaF was found to be the most effective reactivator for neostigmine-inhibited AChE. Thus 2×10^{-3} M NaF was selected for use, but its performance was only marginally better than the use of substrate. Indeed, NaF is unnecessary when the enzyme is inhibited by <50%.

3.3. Analytical performance

On the basis of the optimisation experiments described above, the conditions recommended for the determination of neostigmine and galanthamine are those given in Table 2. The use of stopped flow increased the sensitivity for neostigmine; however, a 1 min incubation time was selected for calibration as a compromise between sample throughput rate and sensitivity. The conditions recommended for reactivation after neostigmine inhibition are given in Table 3.

Under the recommended conditions, the relative standard deviation was 1.3% for five determinations of 5×10^{-7} M neostigmine. The linear response range was 1×10^{-7}–1×10^{-6} M. Below 1×10^{-7} M the slope changed markedly. Thus, the limit of detection, defined as the concentration of analyte giving a signal equal to the blank signal plus three standard deviations of the blank, was 0.5×10^{-7} M, which gave 8% inhibition. The least squares calibration equation was $\%I=13+7.4\times10^{7}$ [neostigmine] (M) and the linear correlation coefficient was 0.999 (n=9). Each analysis took 2–3 min, the sample throughput rate was 20 samples per hour, and the immobilised enzyme remained useable for several months, if stored in a refrigerator at 4°C in contact with the buffer solution, when not in use. Under the optimised conditions the calibration graph for galanthamine was linear over the range 5×10^{-7}–6×10^{-6} M. The least squares linear equation was $\%I=6.9+11.2\times10^{6}$ [galanthamine] (M) and the correlation coefficient was 0.998 (n=7). The sampling rate was >40 samples per hour and the limit of detection 5×10^{-7} M (inhibition ca. 10%). The relative standard deviation for measurements of six replicate injections of 2×10^{-6} M drug was 2.0%.

Table 3
Recovery conditions for neostigmine (if inhibition is more than 50%)

Temperature (°C)	30
NaF concentration (M)	2×10^{-3}
Incubation time (min)	1.0
Recovery time (min)	2.0

4. Conclusions

Drugs which inhibit actylcholinesterase at the anionic site can be determined spectrophotometrically by measuring the inhibition in a flow system. The procedure is straightforward, with a sample throughput of 20 per hour. The reactivation of the enzyme is much simpler than with metrifonate, which binds at the esteric site. Passage of substrate solution generally completely removes the inhibitor in 1 min for both drugs studied, although 2×10^{-3} M NaF was slightly more effective for neostigmine.

References

[1] R.D. O'Brien, Insecticides: Action and Metabolism, Academic Press, New York, 1972.
[2] L.S. Goodman, A. Gillman, The Pharmacological Basis of Therapeutics, 4th ed., Macmillan, London, 1970.
[3] T. Thomsen, H. Kewitz, J. Life Sci. 46 (1990) 1553.
[4] T. Thomsen, U. Bickel, J.P. Fisher, H. Kewitz, Eur. J. Clin. Pharmacol. 39 (1990) 603.
[5] T. Tanahashi, A. Poulev, M.H. Zenk, Planta Medica 56 (1990) 77.

[6] A. Poulev, B. Deus-Neumann, M.H. Zenk, Planta Medica 59 (1993) 442.
[7] S.Y. Han, J.E. Sweeney, E.S. Bachman, E.J. Schweiger, G. Forloni, J.T. Coyle, B.M. Davis, M.M. Joullie, Eur. J. Med. Chem. 27 (1992) 673.
[8] G. Chatellier, L. Lacomblez, Br. Med. J. 300 (1990) 495.
[9] J.L. Mark, Science 238 (1987) 1041.
[10] T. Thomsen, B. Zendh, J.P. Fischer, Biochem. Pharmacol. 41 (1991) 139.
[11] H.A. Claessens, M. Van Thiel, P. Westra, A.M. Soeterboek, J. Chromatogr. 275 (1983) 345.
[12] J. Tencheva, I. Yamboliev, Z. Zhivkova, J. Chromatogr. 421 (1987) 396.
[13] P. Westra, M.J.S. van Thiel, G.A. Vermeer, A.N. Soeterboek, Br. J. Anaesth. 58 (1986) 1303.
[14] U. Bickel, T. Thomsen, W. Weber, J.P. Fisher, R. Bachue, M. Nitz, H. Kewitz, Clin. Pharmacol. Ther. 50 (1991) 420.
[15] T. Lukazewski, J. Anal. Toxicol. 9 (1985) 101.
[16] M. Kokko, J. Chromatogr. 648 (1993) 501.
[17] K. Chan, A. Dehghan, J. Pharmacol. Methods 1 (1978) 311.
[18] S.F. Belal, M. Elsayes, Sci. Pharm. 49 (1981) 397.
[19] S.I. Kulikov, T.N. Bokovikova, L.K. Karopa, V.E. Chichiro, Farmatysiya (Moscow) 36 (1987) 43.
[20] F.A. Michenko, L.O. Kirichenko, Farm. Z. (Kiev) 4 (1983) 60.
[21] E.P. Diamandis, T.K. Christopoulos, Anal. Chim. Acta 152 (1983) 281.
[22] L. Campanella, R. Cocco, M. Tomassetti, J. Pharm. Biomed. Anal. 10 (1992) 741.
[23] E. Reiner, W.N. Aldridge, Biochem. J. 105 (1967) 171.
[24] J.R. Atack, Q.S. Vu, T.T. Soncrat, J. Pharmacol. Exp. Ther. 249 (1989) 194.
[25] F.P.W. Winsteringham, K.S. Fowler, Biochem. J. 101 (1966) 127.
[26] I.A. Takruni, A.M. Almuaibed, A. Townshend, Anal. Chim. Acta 282 (1993) 307.
[27] M.E. Leon-Gonzales, A. Townshend, Anal. Chim. Acta 236 (1990) 267.
[28] T. Ghous, A. Townshend, Anal. Chim. Acta 332 (1996) 179.
[29] A. Goldstein, R.E. Hamlisch, Arch. Biochem. Biophys. 35 (1952) 12.
[30] I.L. Naloff, in Medical Protection against Chemical Warfare Agents, Stockholm, 1976, pp. 53–60.