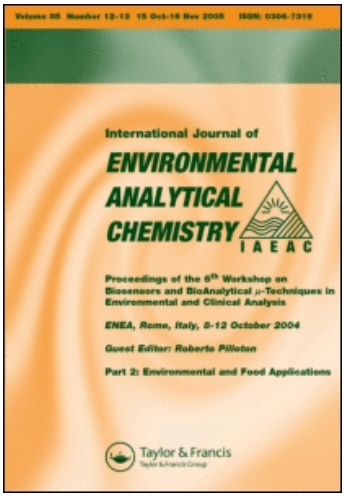

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Detection of Anticholinesterase Agents in Drinking Water by use of Tandem Packed Bed Reactors with Human Red Blood Cell Acetylcholinesterase and Choline Oxidase in a Switching Flow System

J. Šalamoun[ab]; J. Remien[a]
[a] Walther-Straub Institute of Pharmacology and Toxicology, Munich, Germany [b] Chemie Brno, s.r.o., Brno, Czech Republic

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DETECTION OF ANTICHOLINESTERASE AGENTS IN DRINKING WATER BY USE OF TANDEM PACKED BED REACTORS WITH HUMAN RED BLOOD CELL ACETYLCHOLINESTERASE AND CHOLINE OXIDASE IN A SWITCHING FLOW SYSTEM

J. ŠALAMOUN* and J. REMIEN

Walther-Straub Institute of Pharmacology and Toxicology, Nussbaumstrasse 26, 80336 Munich, Germany

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A flow system for the sensitive monitoring of acetylcholinesterase (ACHE) inhibitors is reported. The mobile phase contained acetylcholine (ACH) and choline (CH) as substrates. Red blood cell membrane ACHE was adsorbed on glass fibres. A tandem packed bed bioreactor system with reactors containing ACHE and choline oxidase (CHO), respectively, converted acetylcholine and choline selectively to hydrogen peroxide which was continuously detected by an electrochemical detector. The presence of ACHE inhibitors caused decreases in ACHE activity and hydrogen peroxide production, the decrease of the response being proportional with the concentration, reaction time and inhibiting strength of the solutes. Switching of the reactors enabled the detection of substances which inhibit CHO (e.g. the oxime HLö 7, which is used for the regeneration of ACHE), stopped-flow injection of inhibitors and their long time reaction with ACHE. Flow kinetic studies of both CHO and ACHE were performed to determine optimum conditions for the measurement. The limits of detection for paraoxon and physostigmine in drinking water were found to be 1 nM, and for malathion and parathion-methyl, 2 μM and 8 μM, respectively.

KEY WORDS: Acetylcholinesterase, choline oxidase, enzyme immobilization, organophosphates, flow injection analysis, inhibitors.

INTRODUCTION

Organophosphates and carbamates inhibit ACHE in central and peripheral nervous tissues[1], resulting in acute toxic effects mediated by excessive accumulation of ACH at nicotinic and muscarinic receptors. ACH accumulation causes a spectrum of acute toxic effects mediated by the nicotinic and muscarinic receptors. Unfortunately, a certain percentage of pesticides from agriculture reaches the aquatic environment in spite of their rapid degradation and low bioaccumulation potential[2].

*Author for correspondence. Present address: Chemie Brno, s.r.o., Nopova 5, 615 00 Brno, Czech Republic.

There have been numerous reports on the development of enzymatic methods for the monitoring of antiacetylcholinesterase substances in recent years. They are based on the determination of ACHE activity in the presence of an inhibitor. The activity of ACHE can be assayed by a variety of methods[3]. In general, the most commonly used manual method is the electrometric method of Michel[4]. The colorimetric method of Ellman *et al.*[5] is the basis for a number of automated methods. ACH sensors used for the detection of ACHE inhibiting compounds have also been described[6–8]. They are mainly based on ACHE immobilized in a polymer matrix and on a pH sensor. Kiffer and Minard[9] described a continuous method for the monitoring of ACHE activity by means of immobilized electric eel ACHE and an assay adapted from Ellman's method.

Several methods have been published[10–19] on the determination of choline (CH) and acetylcholine (ACH) by HPLC, using bed reactors packed with the immobilized enzymes ACHE and choline oxidase (CHO). The released hydrogen peroxide was detected by an electrochemical detector with a platinum electrode:

$$\text{Acetylcholine} + H_2O \xrightarrow{\text{ACHE}} \text{Choline} + CH_3COO^- \quad (1)$$

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\text{CHO}} \text{Betaine} + 2H_2O_2 \quad (2)$$

$$H_2O_2 \xrightarrow{\text{Pt/450 mV}} O_2 + 2H^+ + 2e^- \quad (3)$$

Formerly, CHO present in the solution was used for the determination of choline. Okabe *et al.*[20] measured the hydrogen peroxide concentration photometrically after an oxidative reaction with 4-aminoantipyrine and phenol. Mizutani and Tsuda[21] measured the decrease of the oxygen concentration in the system with an oxygen electrode containing CHO solution [see reaction (2)].

Schmid and Kindervater[22, 23] published a method for the detection of antiACHE substances using a magnet reactor as exchangeable immobilized enzyme reactor in combination with the Ellman method or choline oxidase [reactions (1)–(3)] in a flow injection analysis (FIA) system. They stressed the advantages and reliability of biological FIA tests involving ACHE inhibition over other enzymatic or expensive physical methods such as gas chromatography or HPLC. They measured the peak heights observed after the injection of ACH. This was done before and after application of the sample. They did not show kinetics of ACHE and CHO and did not check the CHO activity during measurement. Kumaran and Tran-Minh[24] determined organophosphorous and carbamate insecticides by FIA with immobilized ACHE. The detector was a simple pH electrode with a wall-jet entry.

The principle of the detection of ACHE inhibitors by HPLC is based on an on-line bioreactor with immobilized ACHE and CHO and the mobile phase with ACH.[25]. The possible incompatibility of the mobile phase with the enzymes, however, may limit the choice of chromatographic techniques.

This paper describes a very sensitive, selective and relatively less time consuming method for the detection of anti-ACHE compounds in a flow system that simulates *in vivo* inhibition.

Red blood cell membranes with their ACHE are adsorbed on glass fibres in a short microbore column reactor. A second reactor contains the second enzyme CHO. The mobile phase contains substrates—ACH and CH. The hydrogen peroxide produced (reflecting the activity of ACHE) is continuously monitored by an electrochemical detector. As examples, the detection of paraoxon (Par) and physostigmine (Phy) in drinking water in a FIA system is shown.

EXPERIMENTAL

ACH iodide, CH chloride, Phy, Par, Malathion (Mal) and Parathionmethyl (ParMe) were from Sigma (Deisenhofen, FRG). HLö-7 dimethanesulphonate (1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2,4-bis[(hydroxyimino)methyl]pyridiniumdi-methanesuphonate)[26] was obtained from Prof. I. Hagedorn (Freiburg, Germany). All other chemicals were of analytical grade.

The experiments were performed at ambient temperature, using a Gynkotek (Munich, Germany) Model 600/200 HPLC pump equipped with a six-port injection valve and a Model EP 30 electrochemical detector (ECD) with a platinum electrode (Biometra, Göttingen, Germany) (Figure 1). The oxidation potential was set at +0.45 V vs an Ag/AgCl electrode.

The kinetic studies were performed with a Shimadzu Model LC-9A ternary gradient pump (Duisburg, Germany) with programmed step gradients. The mobile phase was sodium phosphate buffer (0.1 M, pH 7.4), (reservoir A) and 1 mM CH or ACH dissolved in the same buffer (reservoir B). CHO or ACHE activity was measured at twelve gradient steps from 0 to 100% each lasting 5 min. The mobile phases with ACH were cooled in an ice bath during the measurement.

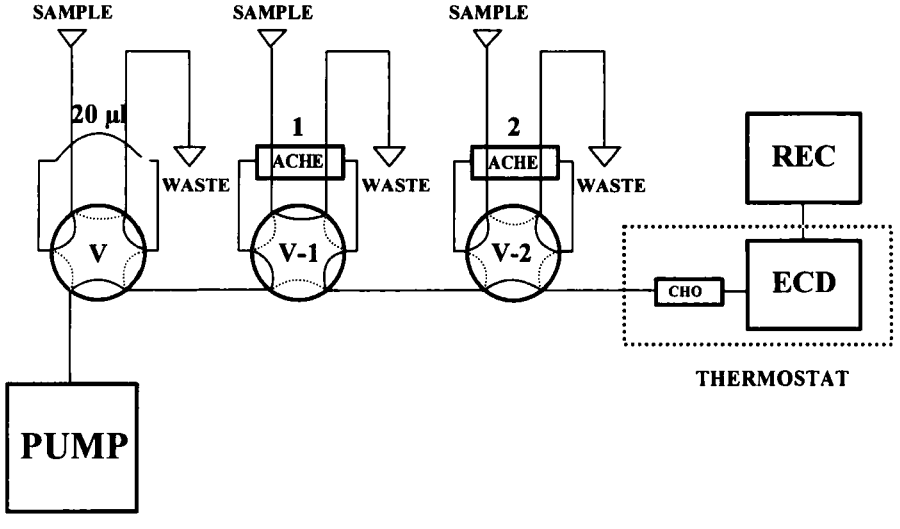

Figure 1 Scheme of the apparatus. Configuration (a): both ACHE 1 and CHO in the flow line, ACHE 2 off line.

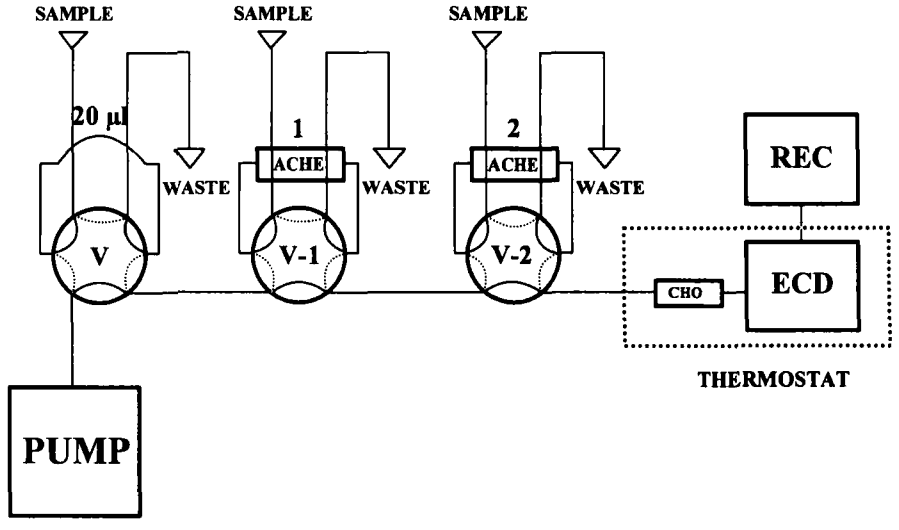

Figure 1 Scheme of the apparatus. Configuration (b): only CHO on-line, i.e. ACHE 1 (or ACHE 2) is in a stopped flow state = inject position for the sample or regeneration agent onto e.g. ACHE 1. Abbreviations: V, six-port injection valve for a direct sample injection, V-1 and V-2 six-port injection valves 1 and 2; ECD, electrochemical detector; REC, recorder.

Human red blood cell membranes were prepared as described in ref. 25. 2 ml of blood were mixed with 20 ml of sodium phosphate buffer (0.005 M, pH 8.5) and centrifuged at 16,000 g for 20 min. The upper layer was discarded and the remaining membranes were mixed with 20 ml of the buffer and centrifuged again. The procedure was repeated five times until colourless membranes were obtained. The prepared membranes were concentrated by centrifugation in 200 µl of buffer. 50 µl of the mixture were added to 20 mg of a glass microfibre filter GF/B (Whatman, Maidstone, England) packed by hand in a 30 ×2.1 mm i.d. stainless-steel column reactor (Biometra). The bioreactor was washed with 20 ml of the mobile phase consisting of a sodium phosphate buffer (0.1 M, pH 7.4) with ACH (0.1 mM), CH (0.02 mM) and EDTA (0.1 mM). For optimum detection the mobile phase was prepared at least 5 h before analysis to achieve constant concentration of dissolved oxygen. The flow rate was 0.8 ml/min. The second bioreactor containing immobilized CHO from a species of Alcaligenes (Biometra) was then connected. CHO reactor and ECD cell were inserted in a column thermostat STH 585 (Gynkotek) set at 10°C.

The organophosphates (Par, Mal and ParMe) were dissolved in ethanol (1 mg/ml) and then diluted with drinking water from the water supply of Munich. 50 µl of sodium citrate buffer (2 M, pH 7.4) were added to 4.95 ml of the above solution. The carbamate Phy was dissolved in water (0.1 mg/ml) and then diluted in the same way as the solutions of the organophosphates.

The samples (800µl) were injected into ACHE reactor 1 in the load position (Figure 1, configuration b) of the six-port injection valve V-1 (i.e. stopped flow in ACHE reactor 1); they remained in the reactor for up to 10 min. Then, ACHE reactor 1 was flushed with

distilled water and switched to inject position (Figure 1, configuration a). Samples with higher inhibitor concentrations were injected via another six-port injection valve V (20 µl loop) inserted between pump and injection valve V-1.

In order to detect the inhibition of the second enzyme, CHO, the ACHE inhibitors were injected directly via the injection valve V into the CHO reactor.

The inhibited ACHE reactor 1 was regenerated in the stopped-flow mode (configuration b in Figure 1) with 1 mM HLö-7 for 15 min. The ACHE reactor was then washed with 2 ml of the mobile phase before being switched in line with the CHO reactor. Reactor 2 was used while reactor 1 was being regenerated in order to increase sample throughput.

The graphs of the kinetic studies of ACHE and CHO were constructed by the use of EnzFitter program version 1.03 (Elsevier-Biosoft). The activity of ACHE was calculated from:

$$\text{Activity} = c(CH)*F_M/1000 \tag{4}$$

where c(CH) is the concentration of the produced CH [µM] and F_M is the flow rate of the mobile phase in ml/min. One unit hydrolyses 1.0 µmol of ACH to CH per minute.

The inhibition (%) of ACHE activity was calculated by using the relationship:

$$\%\ \text{Inhibition} = 100*(I_o-I_i)/I_o \tag{5}$$

where I_o is the baseline current corresponding to ACHE response in equilibrium before inhibitor injection and I_i is the current after inhibition.

The regeneration (%) was calculated from: $\%\ \text{Regeneration} = 100*(I_r-I_i)/(I_o-I_i)$ (6)

where I_r is the baseline current after reactivation.

The rate of ACH hydrolysis (v) and also the amount of CH produced in reactor 1 are dependent on the residence time of the substrate in the reactor, on the total enzyme concentration (E_t), i.e. on the volume of immobilized ACHE (membranes) in the reactor, and also on the substrate concentration [S]. Michaelis and Menten formulated a simple rate equation:

$$v = v_{max}*[S]/(K_m+[S]) = k_3*[E_t]*[S]/(K_m+[S]); \tag{7}$$

where K_m = Michaelis constant; v_{max} = reaction rate at [S] much greater than K_m; k_3 = rate constant for the final product formation. The amount of product (CH) formed during the passage through the ACHE reactor is time dependent and is given by the integrated form of the Michaelis-Menten equation:

$$v_{max}*t=([S_o]-[S])-K_m*\,ln\,[S]/[S_o] \tag{8}$$

where t is the residence time in the reactor and $[S_o]$ and [S] are the concentrations of substrate (ACH) at the entrance and exit of the reactor, respectively.

RESULTS AND DISCUSSION

Behaviour of the enzyme reactors

Hydrogen peroxide formation was a linear function of the CH concentration at ≤ 100 μM CH (Figure 2). This means that the ACHE load in reactor 1 must be kept such that the CH concentration does not exceed 100 μM. The Michaelis-Menten dependence and its Lineweaver-Burk double reciprocal plot were more convenient for the calculation of our results than the integrated form of equation (8) which showed significant divergence. The K_m value for CHO was 509 μM (relative standard deviation, 0.25%, 11 measurements). The Lineweaver-Burk plots are approximately linear in the studied range. With a residence time of 6 sec as chosen in our experimental device, 30 μM CH were produced from 100 μM ACH (Figure 3). Figure 4 shows that linearity of hydrogen peroxide formation was also formal

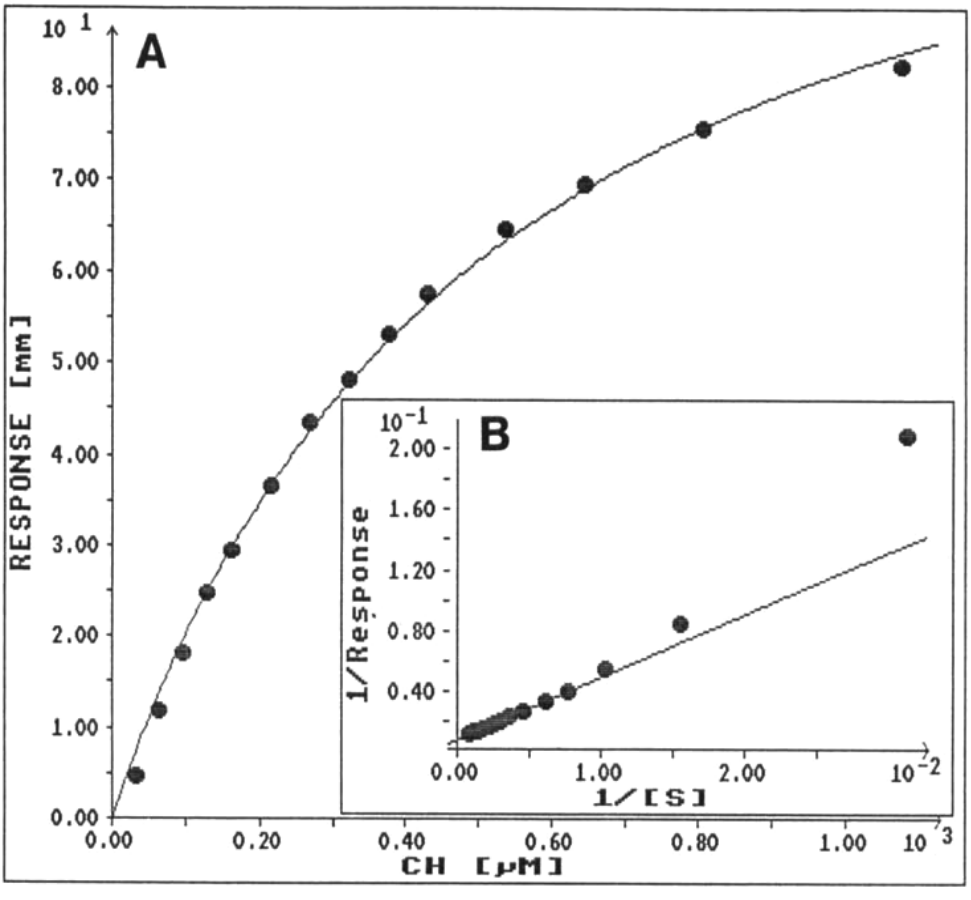

Figure 2 Dependence of the rate of CH oxidation by CHO. A, Michaelis-Menten dependence; B, Lineweaver-Burk plot. For conditions, see Experimental.

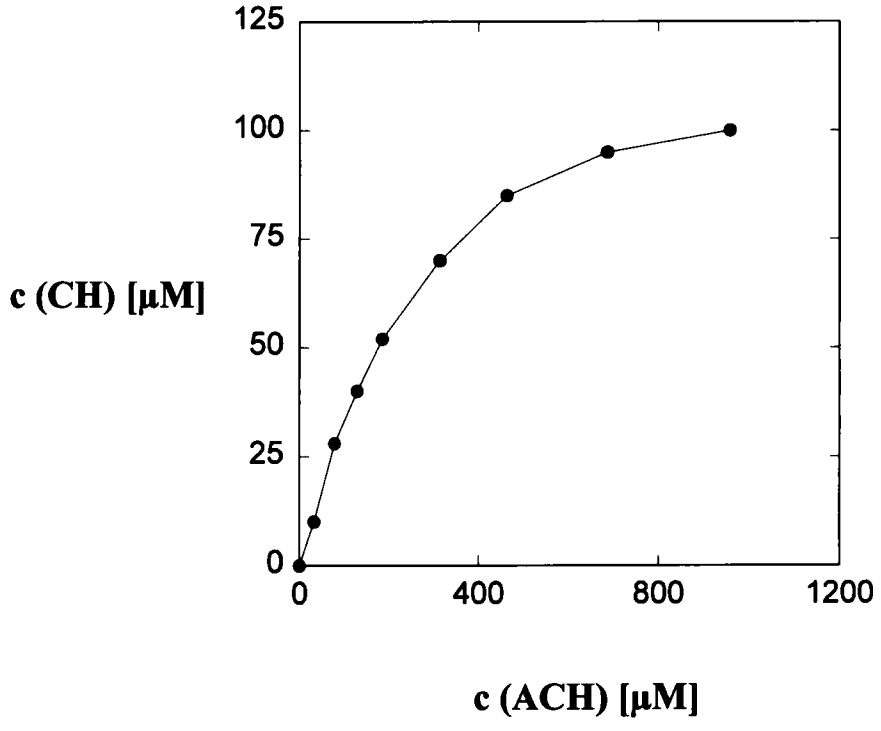

Figure 3 Dependence of the production of CH in the ACHE reactor on ACH concentration in the mobile phase.

with ACH when its concentration was ≤ 100 μM. The K_m value for ACHE was dependent on the origin of the cells and in the case shown was 194 μM (relative standard deviation, 1.0%, 6 measurements).

The calibration of the second reactor allows to determine the concentration of the produced CH (Figure 3) and also the activity of the ACHE in the first reactor containing red blood cell membranes. It was only ca. 0.08 units, calculated from equation (4) for c(CH)=100μM as a maximum and F_M=0.8 ml/min. However, this apparently low activity actually produced relatively high concentrations of CH in the reaction time of 6 sec thanks to the low void volume (80 μl) of the bioreactor. An ACH concentration of over 1 mM causes substrate inhibition and decrease of the CH production.

The activity of the immobilized ACHE did not change during 6 month storage at −30°C or during 2 months at ambient temperature. The activity of CHO in our system decreased very slowly and was higher than 90% of the original activity with storage at 10°C during analysis and at 4°C at night in a cool room in an original solution (containing 50% 1M phosphate buffer and 50% glycerine supplied by Biometra) after 3 month use.

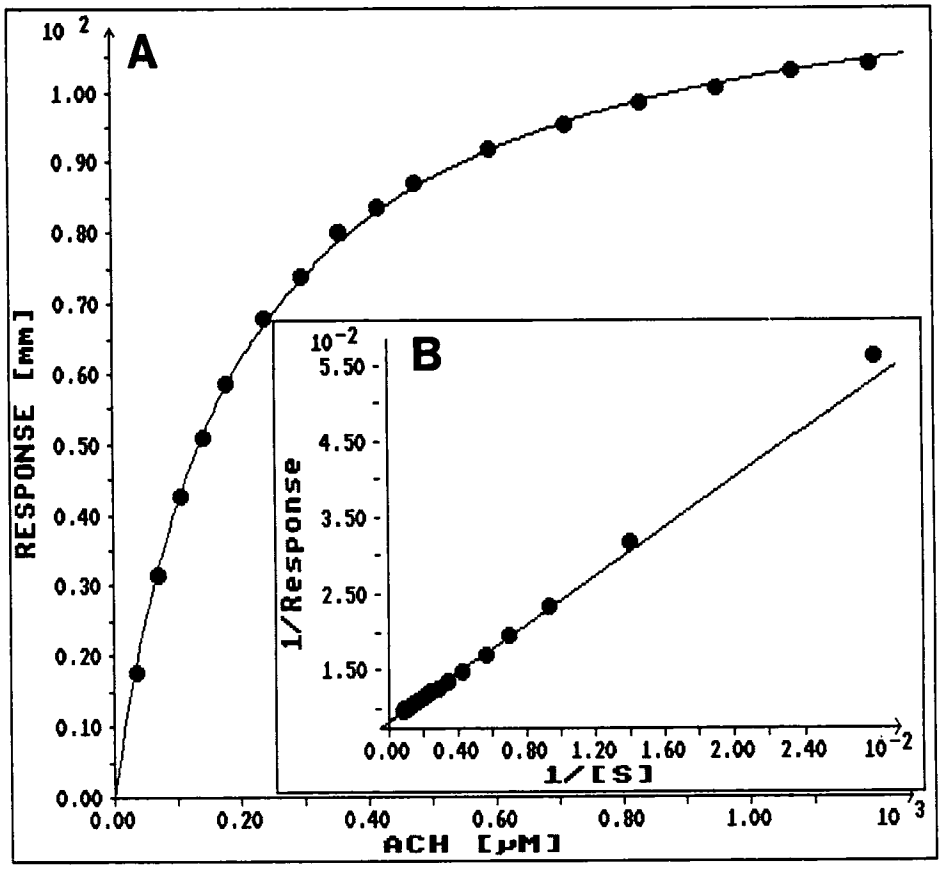

Figure 4 Dependence of the rate of ACH hydrolysis by human red blood cell ACHE. A, Michaelis-Menten dependence; B, Lineweaver-Burk plot. For conditions, see Experimental.

Switching of ACHE reactor

Not only ACHE but also CHO can be inhibited by some solutes. For example, the reactivator HLö-7 irreversibly inhibited CHO and therefore contact was prevented by switching of the reactors. The other compounds used did not inhibit CHO in the concentration range (nM) studied and did not respond to the ECD at the low potential of +0.45 V. The switching technique also enabled stopped flow injection of the inhibitors directly onto the ACHE reactor and long time reaction with ACHE.

The activity of CHO was monitored and checked during the stopped flow injection of inhibitors with ACHE out of flow line. Long time drifts sometimes occurred in the part of the system containing CHO. The decrease of the CHO reactor temperature to 10°C minimised the drifting to less than 0.33%/10 min.

The mobile phase contains three compounds which are important for the enzyme

reactions: ACH and CH as substrates for ACHE and CHO, respectively, and oxygen as a reagent for oxidation on CHO. The stable concentration of both these compounds is of great importance. ACH spontaneously hydrolyses in aqueous solution at physiological pH.

The ACH stability was checked in the enzyme system configuration containing only the CHO reactor on line and only ACH in the mobile phase or during repeated kinetic studies of ACHE. The current increase (about 1 nA/h) was detected with CHO and compared with the total current (usually more than 100 nA at 100 μM ACH) obtained with both enzyme reactors. However, the repeated kinetic studies revealed a gradual increase of the K_m value for ACHE (2.5%/h). Cooling the mobile phase with ice resulted in constant K_m values and in better baseline stability.

The oxidative enzymatic reaction on CHO is dependent on the oxygen concentration in the mobile phase. Degassing of the mobile phase caused a long time drift as oxygen from the air continuously dissolved in the liquid phase and the baseline signal increased. Equilibration of the mobile phase with air before analysis prevented this problem.

Some cations can be precipitated from the mobile phase in contact with phosphate buffer. As a result, prolonged stabilisation of the baseline is required when measuring the ACHE activity. Citrates do not have this disadvantage. Therefore, the citrate buffer was preferred to adjust the pH value of drinking water samples, despite, its lower buffering capacity at pH 7.4.

Applications

A common feature of many organophosphates and carbamates is their ability to inhibit ACHE. The detection of total anti-ACHE activity does offer the possibility of screening water samples to see if there are toxic substances present that will inhibit ACHE. The detection of Phy is shown in Figure 5. The ACHE baseline (activity) decrease responds to the concentration and reaction time of the inhibitor. At constant reaction time the decrease was dependent on the type and concentration of the inhibitor. The inhibition curves of

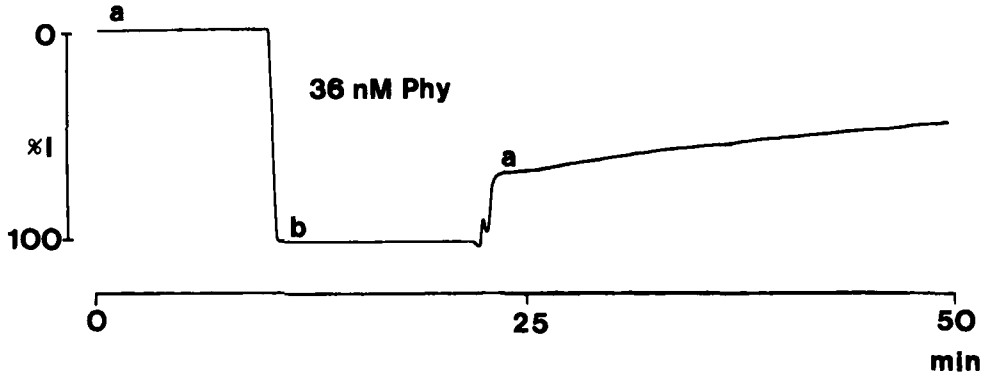

Figure 5 Stopped-flow injection for the measurement of ACHE inhibition by the injection of drinking water containing 36 nM physostigmine (Phy). Abbreviations: a, system configuration as in Figure 1a; b, configuration as in Figure 1b. For conditions, see Experimental.

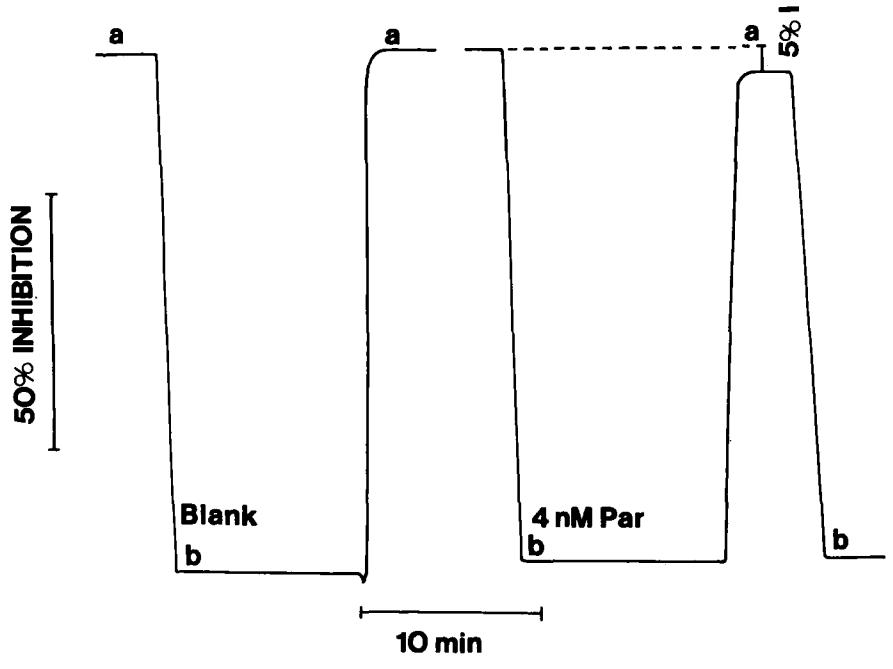

Figure 6 Stopped-flow injection of drinking water (blank) and the same drinking water spiked with Par (4 nM). For conditions, see Experimental. Abbreviations as in Figure 5.

Figures 5 and 6 demonstrate the reversibility of carbamylated ACHE and relative 'irreversibility' of paraoxon-inhibited ACHE. The calibration curve for Par inhibition in the ACHE reactor at 10 min reaction time was linear up to 100 nM Par (Figure 7). The inhibition rate decreased with time. We chose 10 min as maximum reaction time; however, longer inhibition time brings in higher sensitivity (Figure 8).

Malathion and parathion methyl were also determined. Their graphs are similar to those of Par. The limits of detection (3 times background) for 10 min incubation of Par and Phy in drinking water were 1 nM and, for Mal and ParMe, 2 μM and 8 μM, respectively, or 50 pmol of Par after a direct injection of 20 μl. The relative standard deviation for five direct injections was 2.6% for 20μl of 10 μM Par, 2.5% for 50 nM Par and 5 min incubation or 3.2% for 20 nM Par and 10 min incubation. Up to 20 samples per hour can be analysed by the direct injection technique or 4 samples per hour with the 10 min stopped flow incubation technique.

Regeneration of ACHE

The rate of ACHE bioreactor regeneration can be recorded too. The regeneration profile of ACHE after Phy injection is not steep (1%/min), but the enzyme is regenerated after a certain

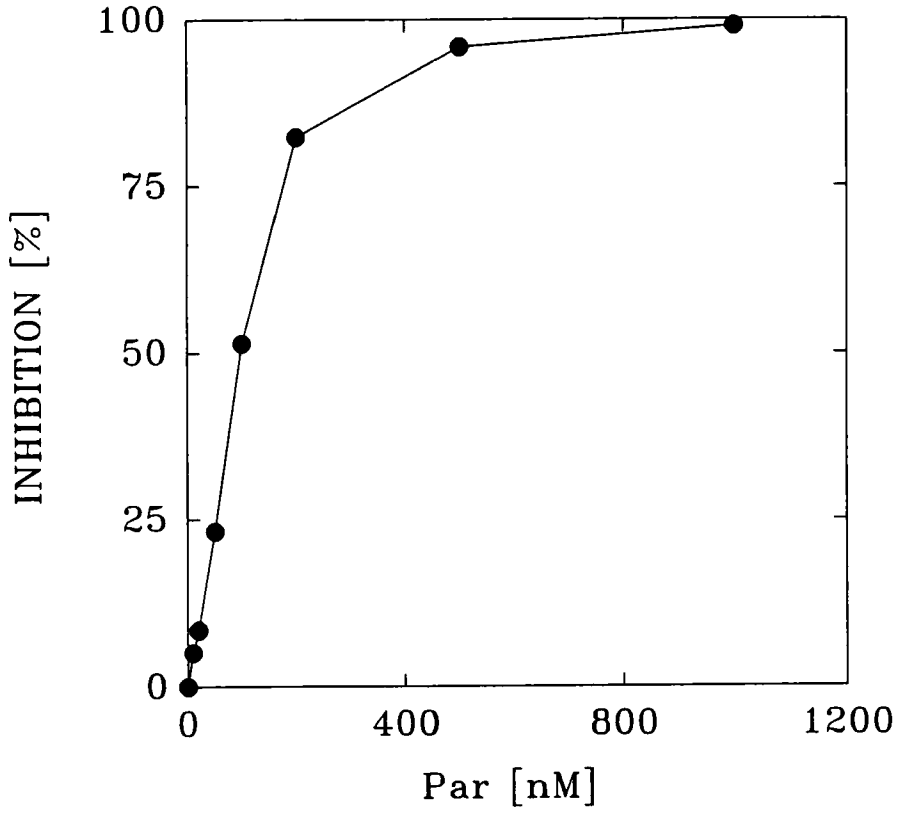

Figure 7 Calibration curve of Paraoxon (Par) in drinking water. For conditions, see Experimental.

time. In the case of Par, HLö-7 was used for regeneration. Its efficiency depended on the time interval between inhibition and start of regeneration. More than 90% of the inhibited ACHE could be regenerated by HLö-7 within 2 h. The regeneration of the carbamylated enzyme will reduce the sample frequency. Switching the inhibited ACHE reactor off line and the second reactor in flow line can increase the sample throughput. In the meantime, the inhibited reactor can be regenerated or exchanged.

CONCLUSIONS

The method described has the advantage over the existing methods for the monitoring of anti-ACHE substances that no long reaction times are needed. The preparation of a new ACHE reactor is very simple and rapid. Moreover, the immobilized human red blood cell ACHE can be used repeatedly. The regeneration step may bring additional information about the aging of the human ACHE-inhibitor compound, i.e. transformation to a form that can

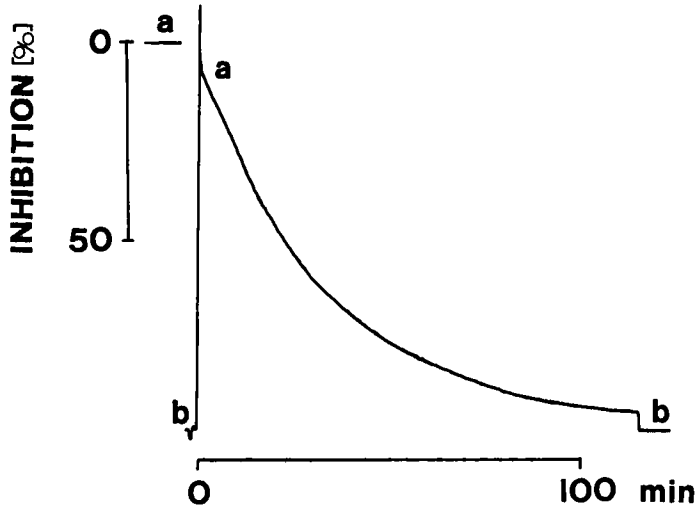

Figure 8 Time dependence of ACHE inhibition with 44 nM Paraoxon (Par) present in the mobile phase. For other conditions, see Experimental. Abbreviations as in Figure 5.

not be reactivated. The rate of the aging is different for different species[27–29]; therefore human ACHE has to be used. The low volume of the microbore reactor enables the analyses of small samples and minimises the consumption of ACHE. ACH can be used as a natural substrate to detect ACHE activity. The switching technique with stopped-flow injection allows one to prolong the reaction time in the ACHE reactor. It also enables monitoring of the CHO activity and injection of substances that may inhibit CHO. An ACHE reactor can be exchanged during the measurement when its function has become lost. The method can be easily automated. The principle of the method was also used for the study of ACHEs of different origin[30] (e.g. brain, ganglion, etc.) and has turned out to be suitable for kinetic studies with ACHE.

Acknowledgements

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