BIOCHEMICAL PHARMACOLOGY 72 (2006) 358–365

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Development of a dynamic model for real-time determination of membrane-bound acetylcholinesterase activity upon perfusion with inhibitors and reactivators

Saskia Eckert [a], *Peter Eyer* [a,*], *Harald Mückter* [a], *Franz Worek* [b]

[a] *Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University of Munich, Goethestr. 33, D-80336 Munich, Germany*

[b] *Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstr. 11, D-80937 Munich, Germany*

ARTICLE INFO

Article history:
Received 16 February 2006
Accepted 7 April 2006

Keywords:
Acetylcholinesterase
Bioreactor
Perfusion
Paraoxon
Obidoxime
Real-time determination

Abbreviations:
AChE, acetylcholinesterase (EC 3.1.1.7)
AU, absorbance units
DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)
HPLC, high performance liquid chromatography

ABSTRACT

Quantitative predictions of the course of acetylcholinesterase (AChE) activity, following interference of inhibitors and reactivators, are usually obscured by the time-dependent changes of all reaction partners. To mimic these dynamics we developed an in vitro model. Immobilized human erythrocyte ghosts in a bioreactor were continuously perfused while AChE activity was monitored by a modified Ellman method. The perfusion system consisted of two HPLC pumps with integrated quaternary low-pressure gradient formers that were programmed by a computer using commercial HPLC software. The combined eluates passed a particle filter (Millex®-GS, 0.22 μm) containing a thin layer of erythrocytes that was immersed in a temperature-controlled water bath. The effluent passed a flow cell in a UV–vis detector, the signal of which was digitized, written to disc and calculated with curve fitting programs. AChE activity decreased by 3.4% within 2.5 h. The day-to-day variation of the freshly prepared bioreactor using the same enzyme source was ±3.3%. Residual activity of 0.2% marked the limit of quantification. Following perfusion with paraoxon, pseudo first-order rate constants of inhibition were established that did not differ from results obtained in conventional assays. The same holds true for reactivation with obidoxime. The set-up presented allows freely programmable time-dependent changes of up to eight solvents to mimic pharmacokinetic profiles without accumulation of products. Due to some hysteresis in the system, reaction half-lives should be >3 min and concentration changes in critical compounds should exceed half-lives of 5 min. Otherwise, the system offers much flexibility and operates with high precision.

1. Introduction

The exposure to anticholinesterase agents results in the inhibition of various serine hydrolases, of which acetylcholinesterase (AChE, EC 3.1.1.7) is considered to be the main target of the toxic effects [1,2]. Basic knowledge on the mechanism of action of inhibitors and reactivators stems from in vitro experiments with well-defined experimental conditions and varying enzyme sources [3]. These results have been frequently used to explain the effects observed in vivo. While this approach is helpful for understanding the basic principles, quantitative predictions are usually obscured by the various simultaneous changes of all reaction partners. More recently, physiologically based models have been introduced to predict

DOI of related article: 10.1016/j.bcp.2006.04.015
* *Corresponding author*. Tel.: +49 89 218075 702; fax: +49 89 218075 701.
E-mail address: peter.eyer@lrz.uni-muenchen.de (P. Eyer).
0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.04.016

more precisely the effects of anticholinesterase compounds in vivo [4]. These models require valid kinetic data of all major reaction processes, which were usually generated in particularly designed in vitro experiments [5]. While the results of the theoretical calculations are generally subjected to plausibility criteria, some uncomfortable feeling remains, whether all effects have been considered properly.

In fact, a closer look indicates a high complexity of all the simultaneous and/or sequential reactions [6]. In case of an organophosphorus compound, inhibition of AChE is initiated by formation of a reversible Michaelis-type complex followed by phosphylation of the active site serine alcohol. Spontaneous reactivation and/or 'aging' may ensue. The latter denotes a dealkylation of the bound organophosphorus moiety. Carbamates may be present that are given in a prophylactic regimen to protect part of the enzyme from phosphylation [7]. Here, formation of a Michaelis-type complex, followed by carbamylation and decarbamylation leads to a steady-state equilibrium. Oximes may be given as reactivators that also react in a two-step manner, finally resulting in a highly toxic phosphyloxime [8] that may decompose or re-inhibit AChE [9,10]. In real life, all reaction partners change in their concentrations time-dependently, making calculations even more complicated and experimental confirmation a formidable task when considering the possible combinations.

This aporia brought us to consider the development of a dynamically working in vitro model that allows real-time determination of enzyme activity while the reaction partners were changed in a programmable manner. For this, the concentrations of the reaction partners should be varied with the reaction products not accumulating. These prerequisites suggested the use of a perfusion model with an enzyme reactor allowing activity determination in real time. We adopted the colorimetric Ellman assay and perfused the bioreactor with buffer, the chromogen and substrate at constant flow rate and analyzed the eluate in a common HPLC flow-through detector. The composition of the perfusate could be varied by use of two quaternary low-pressure gradient formers that were computer-programmed. The detector signal was digitized and analyzed by computer. The enzyme reactor was composed of a particle filter containing a thin layer of human erythrocyte ghosts and was kept at constant temperature in a water bath.

The system worked reproducibly, was stable over 3 h and suitable to calculate kinetic constants that were consistent with data obtained in a conventional stationary system.

2. Materials and methods

Usual chemicals were obtained from commercial sources at the purest grade available. Paraoxon-ethyl was from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and freed from disturbing *p*-nitrophenol as described in detail elsewere [11]. Briefly, 75 μmol paraoxon-ethyl suspended in 15 mL 10 mM hydrochloric acid were vortexed with 20 mg activated charcoal (Norit® A, Serva, Heidelberg, Germany), followed by centrifugation at 3000 rpm. The colorless supernatant was filtered (Whatman), adjusted to pH 5 with 1 M Tris, and its concentration determined photometrically ($\varepsilon_{274\ nm}$ $8.9 \times 10^3\ M^{-1}\ cm^{-1}$). The solution was aliquoted and stored at −20 °C. Obidoxime dichloride monohydrate was procured from Duphar (Amsterdam, The Netherlands), Decon 90™ concentrate from Zinsser Analytic GmbH (Frankfurt, Germany), and gelatin from Fluka-Sigma–Aldrich, CatNo. 48719 (Steinheim, Germany).

Particle filters employed were Millex®-GS, 0.22 μm, Millex®-GV, 0.22 μm (Millipore; Eschborn, Germany) and Minisart®, 0.2 μm (Sartorius AG; Göttingen, Germany).

The conventional determination of erythrocyte AChE was performed by a modified [12,13] Ellman method [14] and the hemoglobin content was determined as cyanomethemoglobin [12].

The perfusion system (Fig. 1) was composed of two HPLC pumps (LaChrom L-7100 equipped with a quaternary low-pressure gradient former; E. Merck KGaA; Darmstadt, Germany) that fed a T-type connecting piece attached to a stainless-steel coiled restriction capillary (0.2 mm i.d.; 100 cm). This capillary was immersed in the water bath to equilibrate the temperature and to build up a backpressure of some 5 bar, necessary for constant flow rates of the pumps. The restriction capillary was connected with the particle filter by a male Luer lock, while the effluent passed the photometer (LaChrom L-7420 UV-VIS detector; E. Merck). The data retrieval was computerized using a D-7000 Interface and a D-7000 HPLC-System-Manager software, Vers. 4.1 (E. Merck). Digitized

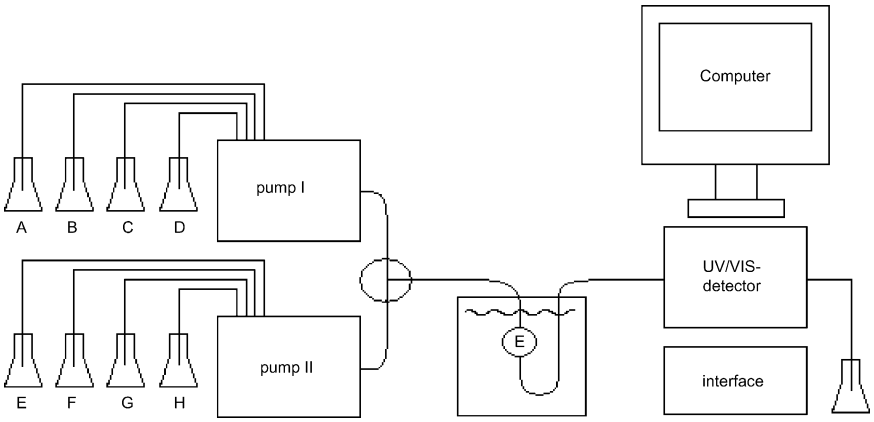

Fig. 1 – Scheme of the assembly of the dynamic model with enzyme reactor "E".

Table 1 – Composition of the perfusion medium for determination of AChE activity

Channel	Pump I (0.25 mL/min)				Pump II (0.25 mL/min)			
	A	B	C	D	A	B	C	D
Solution	G-PB	ASCh	H_2O	DTNB	G-PB	PB	H_2O	PB
Flow rate (mL/min)	0.1125	0.0125	–	0.125	0.25	–	–	–
Final concentration (mM)	100	0.45	–	0.3	100	–	–	–

G-PB: gelatin-containing phosphate buffer; ASCh: acetylthiocholine. For inhibition with paraoxon pump II was switched from 0.25 mL/min (A) to 0.1125 mL/min paraoxon (B) and 0.1375 mL/min (A); for reactivation with obidoxime pump II was switched to 0.1125 mL/min obidoxime (D) with 0.1375 mL/min (A).

absorbance values were collected at 1.6 s intervals, written to an ASCII-file, organized with Microsoft Excel XP and calculated with curve fitting programs using Prism™ Vers. 3.0 (GraphPad Software; San Diego, CA). The pumps and the gradient formers were programmed by the D-7000 HPLC-System-Manager (E. Merck). Means are presented along with standard deviation (S.D.) and the number of experiments.

The enzyme reactor usually consisted of a Millex®-GS particle filter, 0.22 μm, Ø 33 mm containing a thin layer of erythrocytes. Erythrocytes were prepared from freshly drawn heparinized whole blood following centrifugation at 3000 rpm for 15 min. Plasma was discarded by suction and the cells washed five-times with a three-fold volume of 0.1 M phosphate buffer (0.1 M disodium phosphate was mixed with 0.1 M potassium dihydrogen phosphate to give pH 7.4). The final sediment was re-suspended in the phosphate buffer at a hemoglobin concentration of 50 g/L and kept in ice water in the cold room for up to 100 days. Of this preparation, 80 μL were diluted in 5 mL of the phosphate buffer of which 3.2 mL were slowly layered on the Millex filter within 10 min by means of a peristaltic pump (Varioperpex® II; LKB; Bromma, Sweden). The filter had been previously filled retrogradely with buffer to prevent trapping of air bubbles. On pumping the erythrocytes on the filter (orthogradely) the layer usually turned evenly reddish, indicating uniform filling (failure rate < 5%). Usually, the loaded filter was slowly perfused with buffer for additional 5–10 min to prevent formation of air bubbles.

Before the filter was inserted, the whole system was filled with all components and flushed. Care was taken that no buffer contaminated the substrate line to minimize hydrolysis. Finally, the system with a dummy filter was perfused with buffer only, before the erythrocyte-loaded reactor was inserted and immersed into the water bath (37 ± 0.5 °C) with the restriction capillary connected.

The enzyme reactor was perfused with buffer to zero the baseline, before a 5 min pulse of distilled water resulted in immediate hemolysis. This step was included to prevent hemoglobin leaching that would otherwise disturb the baseline. Thereafter, the plain buffer was substituted by a gelatin-containing phosphate buffer (0.2%, w/v) while acetylthiocholine and the chromogen DTNB were added. (Acetylthiocholine, 18 mM, was dissolved in water and kept on ice to minimize hydrolysis; DTNB, 1.2 mM, was dissolved in phosphate buffer, pH 7.4, and kept in an amber bottle to prevent photodegradation [15]; gelatin was added to stabilize AChE activity of the ghosts.) The total flow rate through the enzyme reactor was 0.5 mL/min. Photometric detection was routinely at 470 nm with a mean absorbance of the colored reaction product of about 500 mAU (background without enzyme 4–6 mAU). The usual composition and the flow rates of the single channels are shown in Table 1. The gelatin-buffer was filtered daily using 0.45 μm particle filters (Millipore®).

3. Results

3.1. Specification of the system

When buffer only was perfused through the complete system, including the plain filter, the photometric noise was <0.008 mAU and the drift within 10 h <0.2 mAU. Absorbance was linear up to 1.7 AU and the effective path of the flow-cell 0.899 ± 0.002 cm (colorimetry; specification by the supplier 1.0 cm).

The flow precision of the pumps was individually followed volumetrically over 30 min and was 0.255 ± 0.001 mL/min ($n = 4$), each, at a nominal flow of 0.250 mL/min. The mixing ratio of the gradient formers was controlled (colorimetry) at a flow rate of 0.25 mL/min. At a nominal 10/90 ratio, 10.1/89.9 was found with pump I and 9.1/90.9 with pump II. At a nominal ratio of 50/50, 51.1/48.9 was found with pump I and 50.7/49.3 with pump II.

Due to various dead volumes the hysteresis of the system was considerable. As indicated in Fig. 2 switching from buffer

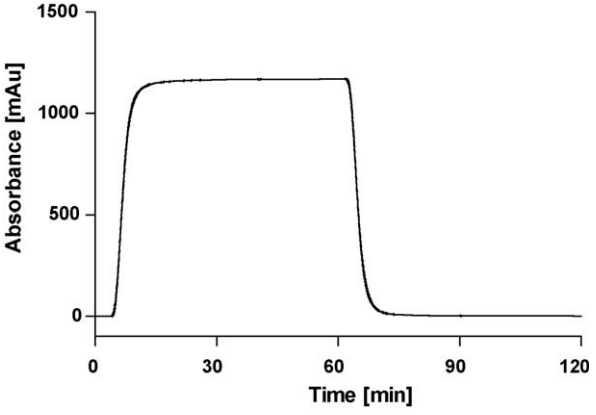

Fig. 2 – Time course of absorbance after addition of a solution of $K_3[Fe(CN)_6]$ at time $t = 2$ min and after discontinuing at $t = 60$ min. (Pumping 100% buffer or 100% $K_3[Fe(CN)_6]$ in buffer with each pump at a flow rate of 0.25 mL/min.)

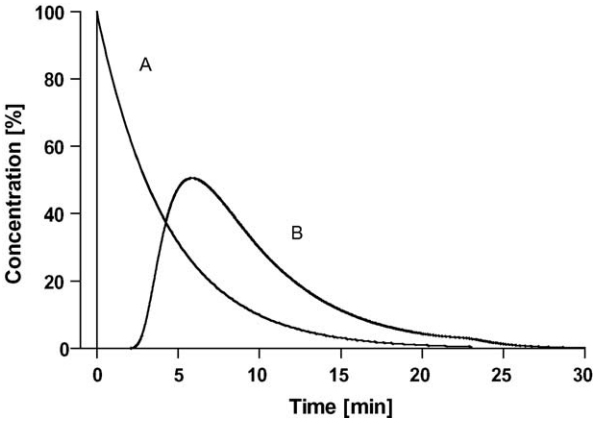

Fig. 3 – Time course of concentration of a colored test solution in the system, switching immediately from buffer to the indicator followed by its monoexponential diminution. (A) Theoretical course with immediate increase of the indicator concentration at $t = 0$, followed by a monoexponential fading of the color with a half-life of 3 min; area under curve set to 100%. (B) Actually determined course of concentration following immediate switching to the colored solution at $t = 0$ and gradual reduction of the indicator using the "dynamic gradient program" with $t_{1/2} = 3$ min; area under curve 97.6%.

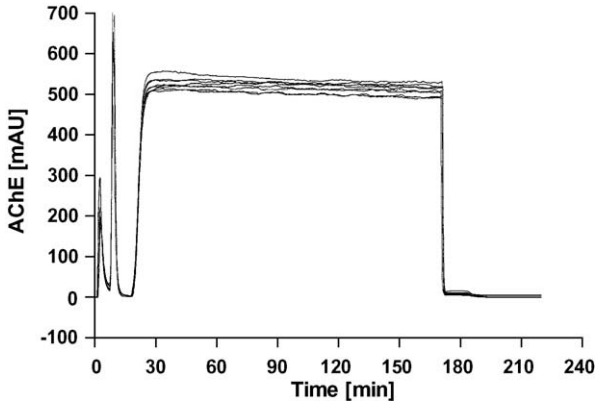

Fig. 4 – Reproducibility of AChE determination in the dynamic model. Change of absorbance on different days (within 3 weeks) using the same batch of erythrocytes and preparing individual enzyme reactors for each experiment (523.9 ± 17.5 mAU, $n = 10$, coefficient of variance 3.3%).

to a solution of $K_3[Fe(CN_6)]$ in buffer showed a delay of some 3 min before the increase in absorbance at 420 nm appeared (two pumps with 0.25 mL/min each). Following this lag period, 2.8 min elapsed until 90% of the final absorbance was reached, but 18 min to end up at 99.1%. Final absorption was reached at $t = 60$ min, when the change in absorption was <0.1 mAU/min. When the colored solution was discontinued it took about 7 min to attain 1.5% and 15 min to end up at 0.7% of the maximal absorbance. It became clear, that rapid changes in the perfusate composition could not be mimicked with sufficient accuracy. The same holds true for kinetic analyses of rapid inhibition or reactivation reactions when the half-life is below 2 min.

Finally, we tested the system by simulating a rapid decrease of the influent concentration. At $t = 0$ the system was switched to a colored test solution and the system programmed to allow monoexponential fading with a half-life of 3 min. Fig. 3 shows the result: instead of the theoretical monophasic decay function, a Bateman-type curve was observed. The maximum absorbance was only half the theoretical value, the comparison of the areas under the curve (planimetry), however, revealed no difference, as expected.

3.2. *Stability of AChE activity in the enzyme reactor*

When the red cell suspension was layered onto the Millex®-GS 0.22 μm filter virtually all AChE-containing membrane particles were retained on the filter because the filtrate showed no enzyme activity. In contrast, when using Minisart® 0.2 μm filters almost half the enzyme activity was found in the eluate. Interestingly, this part was retained by a Millex®-GS filter connected in series. A similar behavior was found with Millex®-GV filters which also did not retain the complete activity. We have not yet analyzed the reason for the different behavior but preferred Millex®-GS filters in the following experiments.

Fig. 4 shows the day-to-day reproducibility of the system. Following introduction of the loaded filter a short-lasting peak was found, indicating the disequilibrium of the system. The second, higher peak between 7 and 10 min was due to hemolysis following the water pulse. After 15 min, when DTNB and substrate were included the absorbance increased to a maximum of 523.9 ± 17.5 mAU ($n = 10$) at $t = 30$ min and very slowly decreased within 140 min to 506.0 ± 14.6 mAU ($n = 10$). When the enzyme reactor was replaced with a plain filter the reagent blank was recorded until 180 min, followed by perfusion with the gelatin-containing buffer without chromogen and substrate. The records obtained on 10 different days using the same enzyme preparation gave almost identical curves. This absorbance corresponds to a concentration of 133.7 μM of the colored product ($\varepsilon_{470\,nm} = 4.36 \times 10^3\ M^{-1}\ cm^{-1}$ [13], $d = 0.889$ cm) and hence to an enzyme activity of 66.8 mU (0.5 mL/min).

The washed red cell suspension kept at 0 °C for up to 100 days exhibited reproducible activity when assayed in the enzyme reactor, indicating that AChE was reproducibly retained by the filter membrane (Fig. 5).

The blank reaction of the system without ghosts is shown in Fig. 6. After perfusion with DTNB and the substrate an initial increase in 3.9 ± 1.0 mAU ($n = 4$) was apparent followed by a gradual increase up to 5.7 ± 0.7 mAU ($n = 4$) within 2.5 h. This increase is most probably due to a slow non-enzymatic substrate hydrolysis, corresponding to 0.007% degradation/h.

An annoying problem arose at the beginning of our study when the photometric signal occasionally did not return to zero at the end of the experiment, but remained varyingly high at some 10 mAU. This phenomenon occurred most often at the end of the week and was due to a fouling phenomenon with some deposits/biofilm on the flow cell of the detector. After carefully cleaning the system this phenomenon disappeared.

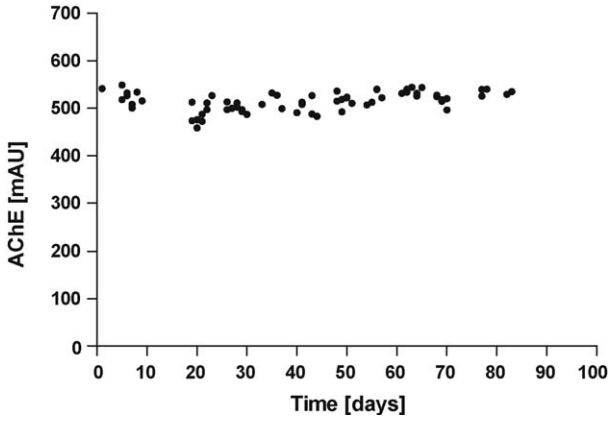

Fig. 5 – Maximum AChE activity in the enzyme reactor depending on the storage time of the erythrocytes (0.45 mM substrate).

We therefore introduced the following routine: at the end of the working day the system was flushed with distilled water and all lines were filled with 70% isopropanol overnight. At the end of the week, the system was cleaned by 3% Decon 90™ for 90 min, rinsed with distilled water and preserved with 70% isopropanol. Using this maintenance procedure, fouling phenomena were not observed any longer.

3.3. *Application of the system to inhibition and reactivation experiments*

When the maximum activity had been reached at $t = 30$ min, paraoxon (1 μM final concentration) was included for 33 min, which resulted in gradual inhibition of the enzyme activity approaching $0.32 \pm 0.12\%$ ($n = 13$) residual activity by 33 min (Table 2). Then paraoxon was discontinued and after 3 min wash-out obidoxime (10 μM final concentration) added for reactivation, resulting in $91.3 \pm 2.5\%$ ($n = 8$) of the maximal activity. Such an experiment is shown in Fig. 7.

It was tempting to analyze the curves to determine the kinetic parameters of inhibition and reactivation. In considering the hysteresis of the system, arbitrarily only those parts of the curves were analyzed when the reaction was already more than 50% complete. Table 3 gives the apparent first-order rate constant of inhibition in the presence of 0.45 mM substrate, the residual absorbance read after 33 min paraoxon perfusion

Table 2 – Inhibition of human erythrocyte AChE by paraoxon

Experiment	$AChE_{max}$ (mAU)	$AChE_{inhib}$ (mAU)	BV (mAU)	Inhibition (%)
1	522.7	9.4	7.2	99.57
2	477.5	12.0	11.0	99.79
3	474.5	8.7	6.8	99.59
4	510.8	13.1	12.0	99.78
5	503.5	12.1	10.8	99.74
6	547.4	9.9	8.4	99.72
7	531.1	13.6	10.7	99.44
8	512.7	5.4	4.0	99.72
9	518.4	5.3	3.9	99.73
10	541.8	5.2	3.8	99.74
11	518.3	7.6	6.5	99.79
12	500.3	6.2	3.6	99.48
13	528.0	6.7	5.5	99.77
Mean ($n = 13$)	514.4	8.9	7.2	99.68
S.D.	21.8	3.1	3.1	0.12

$AChE_{max}$ indicates the maximum enzyme activity; $AChE_{inhib}$ the enzyme activity following inhibition by paraoxon (1 μM) for 33 min; BV the blank value of the complete perfusion medium without enzyme. Substrate concentration was 0.45 mM acetylthiocholine.

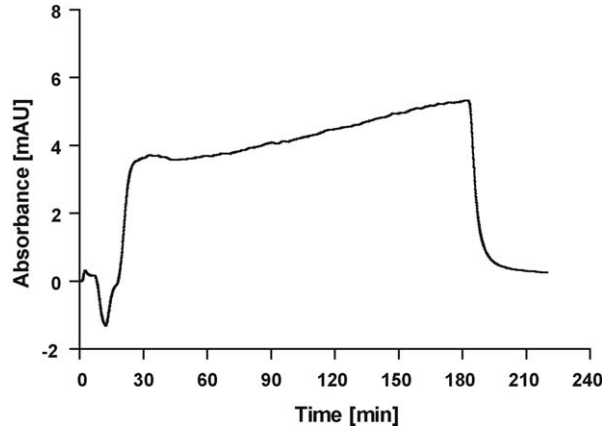

Fig. 6 – Time course of blank reaction in the dynamic model. After inserting the filter, perfusion started with 5 min of gelatin buffer followed by 5 min of water and again 5 min of gelatin buffer. Then the blank reaction was determined by perfusing with substrate (0.45 mM) and chromogen (0.3 mM) until 180 min. To return to baseline, the program was switched to gelatin buffer only after 180 min.

Table 3 – Rate constants of inhibition of human erythrocyte AChE by paraoxon (1 μM), substrate concentration 0.45 mM

Experiment	Interval[a] (min)	$k_{i\ obs}$ (min^{-1})	$t_{1/2}$ (min)	R^2
1	29	0.297	2.34	0.9993
2	29	0.310	2.24	0.9994
3	29	0.294	2.36	0.9994
4	29	0.291	2.38	0.9994
5	28	0.280	2.48	0.9996
6	28	0.270	2.57	0.9997
7	28	0.258	2.69	0.9994
8	28	0.275	2.52	0.9997
9	28	0.270	2.57	0.9998
10	27	0.296	2.34	0.9997
11	27	0.273	2.54	0.9997
12	27	0.245	2.83	0.9999
13	27	0.273	2.54	0.9999
Mean ($n = 13$)		0.279	2.49	
S.D.		0.018	0.16	

For fitting a monoexponential function the individual blank values were fed into the calculation as 'plateau' (cf. Table 2). R^2 indicates the quality of the curve fitting.

[a] Collection period of the data for calculation of $k_{i\ obs}$.

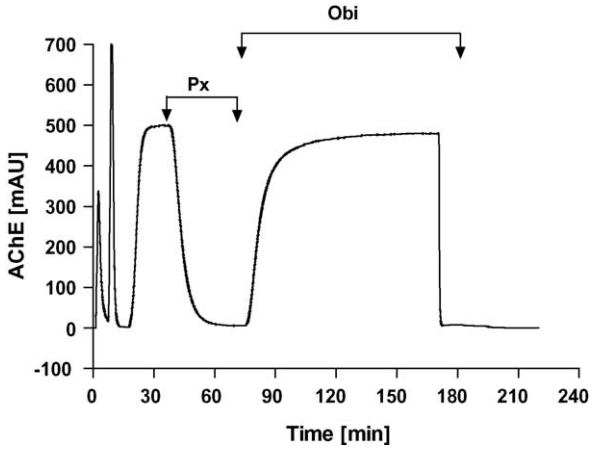

Fig. 7 – Time course of human erythrocyte AChE activity after inhibition with paraoxon (1 μM) and reactivation with obidoxime (10 μM). After inserting the enzyme reactor, perfusion started with 5 min of gelatin buffer followed by 5 min of water to ensure total hemolysis, before maximum activity was determined by perfusing with substrate (0.45 mM) and chromogen (0.3 mM). Following the equilibration phase, the enzyme was inhibited with paraoxon (Px) for 33 min. Then paraoxon was discontinued and reactivation was started upon addition of 10 μM obidoxime (Obi). After 170 min, the enzyme reactor was replaced by a plain filter, and the blank values of the complete perfusion medium, the medium minus obidoxime, and gelatin buffer only were determined.

(the individual blank values were fed into the calculation as 'plateau') and the quality of curve fitting, R^2. The interval indicates the collection period of the data for curve analysis. Table 4 presents the observed first-order rate constant of obidoxime-induced reactivation of the paraoxon-inhibited enzyme.

Table 4 – Rate constants of reactivation of paraoxon-inhibited (1 μM) human erythrocyte AChE by obidoxime (10 μM); substrate concentration 0.45 mM

Experiment	Interval[a] (min)	$k_{r\ obs}$ (min^{-1})	$t_{1/2}$ (min)	R^2
1	25	0.164	4.24	0.9994
2	25	0.159	4.35	0.9995
3	25	0.175	3.97	0.9990
4	25	0.182	3.82	0.9995
5	25	0.175	3.96	0.9996
6	25	0.168	4.13	0.9980
7	25	0.174	3.98	0.9991
8[b]	25	0.182	3.82	0.9978
Mean (n = 8)		0.172	4.03	
S.D.		0.008	0.19	

[a] Collection period of the data for calculation of $k_{r\ obs}$.
[b] Change of experimental procedure: in experiment 8, after discontinuing paraoxon (60 min) the system was perfused with DTNB, substrate and gelatin buffer (0.2%) only for 10 min, before reactivation was started with obidoxime (from 70 min).

4. Discussion

A stable AChE reactor was established with intact human erythrocytes. We opted for human red cells rather than ghosts for layering on particle filters, because the even distribution without clumping and clustering could conveniently be followed by eye. Thereby we succeeded in preparing reactors reproducibly from day-to-day. Washed human red cells in slightly hypertonic buffer (0.1 M phosphate) were surprisingly stable over 3 months when kept on ice water, rather than in the fridge. On average, the loss of enzyme activity was 15 ± 3% within 100 days (n = 5, same donor).

The enzyme activity layered on the Millex®-GS filter was theoretically some 85 mU as determined conventionally. The enzyme activity calculated from the absorbance in the dynamic system was 66.8 mU only. We cannot explain the difference of 20%. Conceivably, some enzyme was detached from the insertion of the glycolipid anchor in the membrane while loading the filter and/or during hemolysis. We were not able to detect enzyme activity in the eluates, which, however, could have escaped because of the marked dilution. Some lability of the glycolipid anchor in human erythrocytes is known from the fact that old erythrocytes in circulation have significantly lower activity compared with young red cells [16].

Another unexpected phenomenon was the different retention of enzyme activity by the three particle filters with virtually identical exclusion limits (0.22 and 0.2 μm). About 50% of the applied activity was not retained by the Minisart® and Millex®-GV filters. Interestingly, this part could be retained by the subsequent Millex®-GS filter connected in series. Since the latter filter gave reproducible results, we were not keen to study the cause of these differences in more detail.

The enzyme activity in the perfused reactor was remarkably stable over 3–4 h, provided that the buffer contained some colloid. We adjusted the cheap gelatin to 0.2%, which did not interfere with Ellman's reagent and maintained the enzyme activity at 96.6% for 3 h. The small losses perhaps stem from some 'bleeding' or inactivation of AChE. In the absence of gelatin the activity dropped to 82% (n = 2).

A prerequisite for recording stable and reproducible activity was a constant flow through the system. Our first experiments with peristaltic pumps were disappointing even under steady-state conditions; the results were detrimental when the perfusate composition was changed by the use of three-way valves. Hence, we adopted an HPLC-system with low-pressure gradient formers. Using two quaternary systems allowed individual component mixing of eight different eluents. The system was freely programmable in 0.1 min steps and was run at a total flow rate of 0.5 mL/min. The flow rate could be increased up to 1 mL/min, which would reduce the photometric signal to 54%. Doubling the erythrocyte count on the filter might compensate this reduction. In such case, however, doubling of cell count and flow rate resulted in a backpressure of some 10 bar, which may reach the breaking limit of the filters. Therefore, we preferred a flow rate of 0.5 mL/min with a backpressure of some 3 bar and included a

restriction capillary to enhance the total pressure at the pumps to about 6 bar. Thereby the constancy of the flow rate was increased to a level of ±0.4%. Moreover, we considered shear stress smaller at lower flow-rate, which should result in increased stability of enzyme on the filter.

Another concern was constancy, reproducibility and validity of the gradient formers. While validity was not too good, particularly at a low percentage of a single channel, which could only be programmed in 1% steps, constancy and reproducibility were excellent, thus, resulting in stable recordings without major undulating lines, cf. Figs. 4 and 7. Separate addition of substrate at sub-optimal concentration (0.45 mM, i.e. $5K_m$) was of particular concern, because the unbuffered, ice-cold solution (to reduce spontaneous hydrolysis) should comprise only a small part of total flow and was thus added at 12.5 μL/min, i.e. 5% of the total flow of a single pump. Nevertheless, the enzyme activity appeared stable. Compounds that determined the rates of inhibition or reactivation concentration-proportionally were usually added at flow rates that made up 45% of a single pump to assure nominal concentrations as close as possible.

Cross-contamination between the four channels of a single pump was not a problem as tested with colored solutions, but the hysteresis of the system when switching from one to another channel was a major problem. Of course, there were many different dead volumes in the entire system and one can expect regions with laminar and/or turbulent flow. In experiments using a rectangular pulse of a colored solution significant increase in absorbance was not observed before some 3 min and it took additional 3 min to reach 90% of the final absorption. Hence, it is obvious that calculations which are based on data earlier than 6 min after switching the solvent are prone to an error of >10%. Reactions with apparent half-lives below 2 min are thus not suitable to precise analysis. This hysteresis of the system has marked impacts when the concentration of a component is continuously changed to mimic certain pharmacokinetic behavior. When the changes are too fast, a completely different profile is obtained as illustrated in Fig. 3. Accordingly, the program has to be tuned to result in reaction rates that are manageable by the system. Odd to say that disconnection of a component also results in a sluggish output beginning some 3 min after stop and being complete by 99% after some additional 10 min.

The system was tested with paraoxon as AChE inhibitor and obidoxime as reactivator.

Following perfusion with 1 μM paraoxon for 33 min, the enzyme was inhibited by 99.7%. Considering the lapse of more than 10 half-lives for inhibition and a spontaneous reactivation half-life of 31 h [17], 99.87% would have been expected. Analysis of the decay curve as shown in Fig. 7 revealed clear first-order kinetics with an apparent half-life of 2.49 ± 0.16 min ($n = 13$). This value is considerably higher than found in experiments in a stationary system performed in the absence of substrate under otherwise identical conditions, yielding 0.315 min [17]. When the influence of substrate acting as a competitor [18] is considered a six-times longer half-life would result theoretically, i.e. 1.89 min. The somewhat longer half-life observed in the dynamic model (+25%) could be the result of the above-mentioned hysteresis. In fact, when the paraoxon concentration was lowered to 0.2 μM the apparent half-life was 9.83 ± 0.14 min ($n = 4$), a rate that is more suitable for correct analysis. This rate is close to the theoretical value of 9.45 min on the assumption that the K_d of the Michaelis complex with paraoxon is in the range of 5–10 μM [19] and the half-life hence inversely concentration proportional.

The reactivation of the paraoxon-inhibited AChE with 10 μM obidoxime was also analyzed. When paraoxon was discontinued and substituted by buffer for 3 min, obidoxime resulted in gradual reactivation that approached 91.3 ± 2.5% ($n = 8$) of the maximal activity. Curve analysis between 10 and 35 min after start with obidoxime gave a clear pseudo-first order reaction with a reactivation half-life of 4.03 ± 0.18 min ($n = 8$). Under similar conditions, a reactivation half-life of 4.3 min was observed in a stationary system under otherwise identical conditions, but in the absence of substrate [10,17]. It is not known whether the presence of substrate at such a low concentration has any impact on the reactivation kinetics. Stimulation of oxime-mediated reactivation of diethylphosphorylated butyrylcholinesterase (EC 3.1.1.8) by quaternary compounds has been attributed to some interaction with the peripheral anionic binding site [20]. At any rate, we consider the observed unambiguous first-order kinetics as indication that the reaction product, diethylphosphoryl obidoxime [10] did not accumulate during the single-pass perfusion.

In conclusion, the presented dynamically working model of an AChE bioreactor that can be perfused simultaneously with eight different solutions in a freely programmable manner is suitable for pharmacokinetic studies, mimicking the actions of anticholinesterase agents and reactivators. The major limitation of the system is an inevitable hysteresis, which cannot cope with apparent half-lives of reactions <3 min and half-lives of changes in critical reactant concentrations <5–10 min, depending on the accuracy desired. Otherwise, the system offers much flexibility and operates with high precision.

REFERENCES

[1] Holmstedt B. Pharmacology of organophosphorus cholinesterase inhibitors. Pharmacol Rev 1959;11:567–688.
[2] Quinn DM. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. Chem Rev 1987;87:955–79.
[3] Taylor P. Anticholinesterase agents. In: Hardman JG, Limbird LE, editors. Goodman and Gilman's the pharmacological basis of therapeutics. New York: McGraw-Hill; 1996. p. 161–76.
[4] Gearhart JM, Jepson GW, Clewell HJ, Andersen ME, Conolly RB. Physiologically based pharmacokinetic model for the inhibition of acetylcholinesterase by organophosphate esters. Environ Health Perspect 1994;102(Suppl 11):51–60.
[5] Worek F, Thiermann H, Szinicz L, Eyer P. Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes. Biochem Pharmacol 2004;68:2237–48.
[6] Eyer P. The role of oximes in the management of organophosphorus pesticide poisoning. Toxicol Rev 2003;22:165–90.
[7] Wetherell J, Hall T, Passingham S. Physostigmine and hyoscine improves protection against the lethal and incapacitating effects of nerve agent poisoning in the guinea-pig. Neurotoxicology 2002;23:341–9.

[8] Hackley Jr BE, Steinberg GM, Lamb JC. Formation of potent inhibitors of AChE by reaction of pyridinaldoximes with isopropyl methylphosphonofluoridate (GB). Arch Biochem Biophys 1959;80:211–4.
[9] De Jong LPA, Ceulen DI. Anticholinesterase activity and rate of decomposition of some phosphylated oximes. Biochem Pharmacol 1978;27:857–63.
[10] Kiderlen D, Eyer P, Worek F. Formation and disposition of diethylphosphoryl-obidoxime, a potent anticholinesterase that is hydrolysed by human paraoxonase (PON1). Biochem Pharmacol 2005;69:1853–67.
[11] Kiderlen D. On the phosphoryl oxime hydrolase of plasma, an enzyme that markedly increases the efficacy of oximes in organophosphate poisoning. Doctoral Thesis. Munich: Ludwig-Maximilians-University; 2004.
[12] Worek F, Mast U, Kiderlen D, Diepold C, Eyer P. Improved determination of acetylcholinesterase activity in human whole blood. Clin Chim Acta 1999;288:73–90.
[13] Eyer P, Worek F, Kiderlen D, Sinko G, Stuglin A, Simeon-Rudolf V, et al. Molar absorption coefficients for the reduced Ellman reagent: reassessment. Anal Biochem 2003;312:224–7.
[14] Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
[15] Walmsley TA, Abernethy MH, Fitzgerald HP. Effect of daylight on the reaction of thiols with Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid). Clin Chem 1987;33:1928–31.
[16] Prall YG, Gambhir KK, Ampy FR. Acetylcholinesterase: an enzymatic marker of human red blood cell aging. Life Sci 1998;63:177–84.
[17] Worek F, Bäcker M, Thiermann H, Szinicz L, Mast U, Klimmek R, et al. Reappraisal of indications and limitations of oxime therapy in organophosphate poisoning. Human Exp Toxicol 1997;16:466–72.
[18] Aldridge WN, Reiner E. Enzyme inhibitors as substrates. In: Interactions of esterases with esters of organophosphorus and carbamic acids. Amsterdam: North-Holland Publ. Co.; 1972.
[19] Eyer F, Eyer P. Enzyme-based assay for quantification of paraoxon in blood of parathion poisoned patients. Human Exp Toxicol 1998;17:645–51.
[20] Masson P, Froment M-T, Bartels CF, Lockridge O. Importance of aspartate-70 in organophosphate inhibition, oxime re-activation and aging in human butyrylcholinesterase. Biochem J 1997;325:53–61.