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Indirect detection of anti-acetylcholinesterase compounds in microcolumn liquid chromatography using packed bed reactor with immobilized human red blood cell acetylcholinesterase and choline oxidase*

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Abstract: The inhibiting compounds were separated by micro-column liquid chromatography in the mobile phase containing the natural substrate acetylcholine. A home-made packed bed microbioreactor system containing immobilized enzyme acetylcholinesterase (ACHE) in human red blood cell membrane and choline oxidase (CHO) from alcaligenes was used for the post-column conversion of acetylcholine to hydrogen peroxide which was detected by an electrochemical detector. The inhibition effect of the solutes caused a decrease in the acetylcholinesterase activity, a decrease in the formation of hydrogen peroxide and also a decrease in the response corresponding to the concentration of the solutes. The rate of the enzyme regeneration was also recorded. The micro-system was compared with a conventional LC system comprising commercially prepared enzyme reactor. The stability of the enzymes is at least 3 weeks at ambient temperature. The limit of detection depends on biological activity of inhibition and for galanthamine was 1 pmol.

Keywords: *Micro-column liquid chromatography; human red blood cell acetylcholinesterase; enzyme immobilization; post-column reaction; indirect detection; enzyme inhibition.*

Introduction

The toxic action of some insecticides and the therapeutic action of some drugs is associated with their ability to inhibit acetylcholinesterase (ACHE) in the central or peripheral nervous system. The activity of ACHE, as modified by the anti-ACHE compound present, can be assayed by a variety of methods [1].

Acetylcholine (ACH) sensors used for the detection of ACHE inhibiting compounds have been described [2–4]. They are mainly based on immobilized ACHE in a polymer matrix and on a pH sensor. However, such methods are not specific. Specificity may be improved or obtained if these methods are combined with a separation step, e.g. with thin-layer chromatography [5] or LC [6, 7]. The TLC method used radiometry and is rather complicated and time-consuming. The LC method [6] was based on coupling with a cholinesterase inhibition AutoAnalyzer. The substrate and the enzyme were added to the effluent after the column, and the reactions took place in the AutoAnalyzer. The enzyme consumption was rather high and also long reaction times were needed. The second approach is the use of an easily detectable or reactive [7] substrate instead of ACH.

Several LC methods have been published [8–10] on the determination of choline (CH) and acetylcholine (ACH) using packed bed reactors with the immobilized enzymes acetylcholinesterase and cholinoxidase. The released hydrogen peroxide was then detected by an electrochemical detector with a platinum electrode. The enzyme consumption and the cost of the method can be significantly reduced by the miniaturization of the system.

This paper describes a very sensitive and selective method for the detection and determination of ACHE inhibiting compounds in micro LC. The inhibitors are separated on an analytical microcolumn in the mobile phase containing the substrate — ACH. ACHE is immobilized together with choline oxidase

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(CHO) in a packed bed post-column microbioreactor and the produced hydrogen peroxide (reflecting the activity of ACHE) is continuously monitored by an electrochemical detector. As examples, the separation and the monitoring of the inhibiting activity of tetramethylammonium (TMA), galanthamine (Gal) and physostigmine (Phy) are shown.

Experimental

ACH iodide, choline (CH) chloride, Phy and TMA perchlorate were from Sigma (Deisenhofen, Germany), Gal hydrobromide was from Waldheim Pharmazeutika (Vienna, Austria) and ethylenediaminetetra-acetic (EDTA) sodium salt was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Chromatographic separations were performed at ambient temperature on a Gynkotek Model 600/200 liquid chromatograph (Gynkotek, Munich, Germany) equipped with a 2 or 20 μl six-port injection valve and with a Model EP 30 electrochemical detector with platinum electrode (Biometra, Gottingen, Germany) (Fig. 1). The oxidation potential was set at +0.45 V vs an Ag/AgCl electrode. The (150 × 1 mm) micro-LC column and the (20 × 4.6 mm) column were packed with 12-μm Spheron Micro-DEAE 300 (exclusion limit 300 kD) from Lachema (Brno, Czechoslovakia) by upward displacement as a 10% (w/v) suspension in concentrated ammonium sulphate at 60 bar. The 20 × 1 mm i.d. home-made microreactor (Fig. 2) was used for the monitoring of ACHE activity. The human red blood cell membranes containing ACHE were prepared by the procedure similar to [11]. A 2 ml volume of blood was mixed with 20 ml of

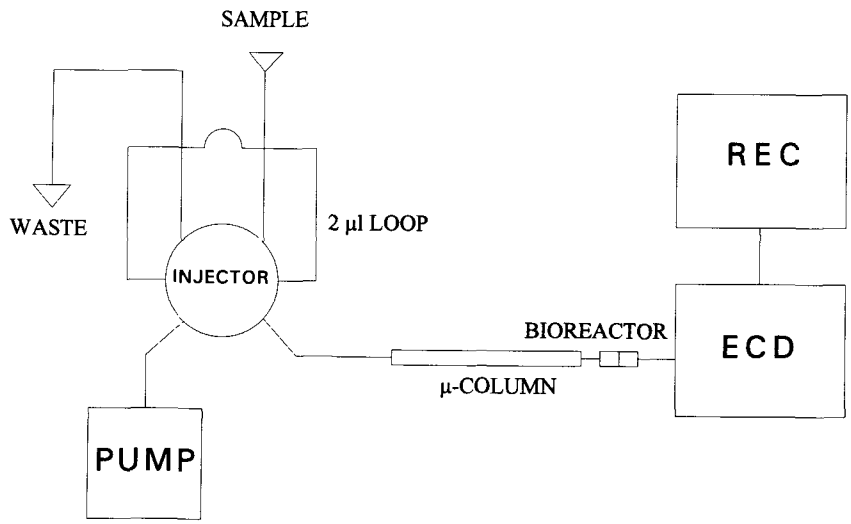

Figure 1
Scheme of the chromatographic apparatus.

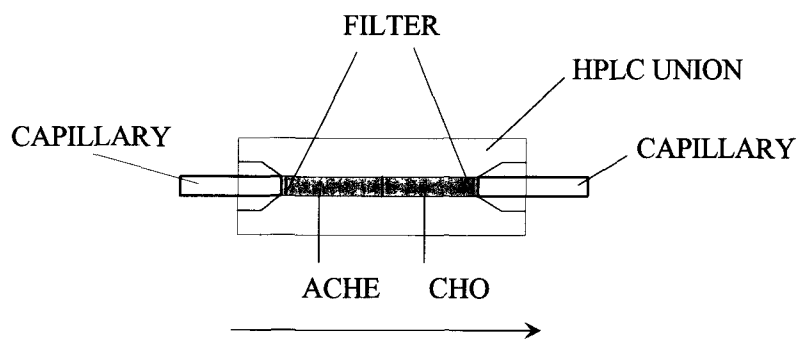

Figure 2
Scheme of the microbioreactor.

sodium phosphate buffer (0.005 M, pH 8.5) and centrifuged at 16,000*g* for 20 min. The upper liquid containing haemoglobin was discarded and the remaining membranes were mixed with 20 ml of the buffer and again centrifuged. The procedure was repeated (*ca* four times) until the colourless membranes were obtained. The prepared membranes were concentrated by the centrifugation in 200 μl of the buffer. A 50 μl volume of the mixture was added to 20 mg of the glass microfibre filter GF/B (Watman, Maidstone, UK) packed in a 30 × 2.1 mm i.d. column. The column was washed with 2 ml of water and 10 ml of sodium phosphate buffer (0.1 M, pH 7.4). The fibres with adsorbed membranes were packed into one half of the microreactor plastic Silon body. Beads (d_p = 40 μm) with immobilized CHO from alcaligenes species (Biometra, Gottingen, Germany) were packed into the second half of the microreactor. The mobile phase for the analytical column consisted of sodium phosphate buffer (0.1 M, pH 7.4) containing ACH (1×10^{-4} M) and EDTA (0.1 mM). The flow rate was 60 μl min^{-1} (flow splitting). The chromatography was made at ambient temperature.

For comparison an LC system was used comprising a Supelguard column (20 × 4.6 mm i.d.) packed with hydroxyethylmethacrylate gel modified with diethylaminoethyl groups (Spheron Micro-DEAE 300, d_p = 12 μm, as noted above). A commercially prepared cartridge (30 × 2.1 mm i.d.) with immobilized ACHE from electric eel, and CHO from alcaligenes (Biometra) was employed. The mobile phase described above was used at a flow rate of 0.8 ml min^{-1}. The variable UV detector SP-4 (Gynkotek) was set at 250 nm.

The percentage inhibition of ACHE (%*I*) was calculated from the relationship:

$$\%I = (I_O - I_I)/I_O \times 100,$$

where I_O is the baseline current in equilibrium before sample injection and I_I is the current in the minimum of an inhibiting peak.

Results and Discussion

The microbioreactor behaviour

The optimum concentration of the ACH in the mobile phase was 0.1 mM (Fig. 3). Lower substrate concentrations resulted in a decrease in signal and at concentrations of ACH above 1 mM the substrate inhibition effect decreased the baseline signal and the dynamic range of detection. Figure 4 shows the dependence of the rate of choline oxidation by CHO. The range below 100 μM is linear, i.e. the hydrogen peroxide produced by the reaction on CHO reflects linearly the changes of the CH concentration and also the inhibition of ACHE.

Not only ACHE but also CHO can be inhibited by some solutes. The CHO behaviour was monitored in the mobile phase containing CH instead of ACH. The studied compounds did not inhibit CHO.

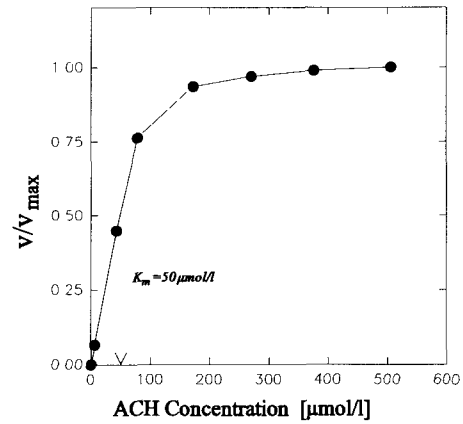

Figure 3
Dependence of the rate of acetylcholine hydrolysis by human red blood cell ACHE. Conditions: mobile phase: sodium phosphate buffer (0.1 M, pH 7.4) containing variable concentration of ACH; flow rate: 0.8 ml min^{-1}; reactor dimensions: 30 × 2.1 mm i.d. For other conditions see Experimental.

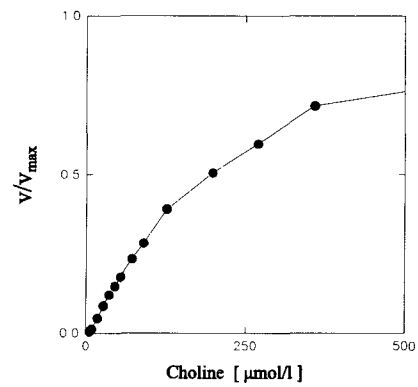

Figure 4
Dependence of the rate of CH oxidation by CHO. Conditions: mobile phase: sodium phosphate buffer (0.1 M, pH 7.4) containing variable concentration of CH; other conditions as in Fig. 3.

The lifetime of the microbioreactor is mostly dependent on the lifetime of the immobilized ACHE and mainly on the nature of the injected samples. For example, the injection of more than 20 μl of absolute ethanol caused significant changes to the enzymes and irreversible signal decrease. The stability of the immobilized enzymes is at least 3 weeks at ambient temperature.

The stability of the mobile phase

ACH can spontaneously hydrolyse in aqueous solution at physiological pH and therefore it would be expected that with just CHO in the enzyme reactor a signal would be detected from the CH (originating from spontaneous hydrolysis of ACH). It can cause a baseline drift and mistakes in the calculation of ACHE inhibition. The mobile phase stability was checked in the enzyme system with bioreactor containing only CHO. This current can be subtracted from the total current obtained with both enzyme reactors. However, this value did not change during a day and was very low (about 1 nA) in comparison with total current (usually more than 100 nA).

Influence of oxygen

The CH oxidation is dependent on the oxygen concentration in the mobile phase. This concentration is high enough even in the degassed mobile phase at nanomolar concentrations of ACH or CH. However, the oxygen level in the mobile phase is critical at ACH concentrations above 0.1 mM. The degassing of the mobile phase caused a decrease in response and drifting baselines. When the mobile phase was saturated by air for 10 min at 10 ml min^{-1} before chromatography, this step eliminated the drift and did not cause any gassing problems in our HPLC–ECD system.

Linearity

The dependence of the response of the bioreactor on TMA concentration is shown in Fig. 5. It is slightly nonlinear in the studied range, in accord with theory [12]. The relative standard deviation of TMA peak heights was 1%, as calculated from four injections of 20 μl of 5×10^{-2} mol l^{-1} solution.

Influence of pH

The pH value of the mobile phase influences not only ACH hydrolysis reaction but also the reaction in the detector. The dependence of the response on the mobile phase pH value is shown in Fig. 6. The shoulder at about pH 8.5 corresponds to the maximum activity of the ACHE. However, the pH value of 7.4 was used in order to study the human ACHE at its physiological pH.

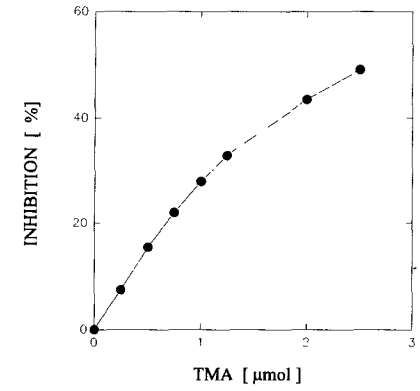

Figure 5
The calibration curve of the TMA. Bioreactor dimensions: 30 × 2.1 mm. For other conditions see Experimental.

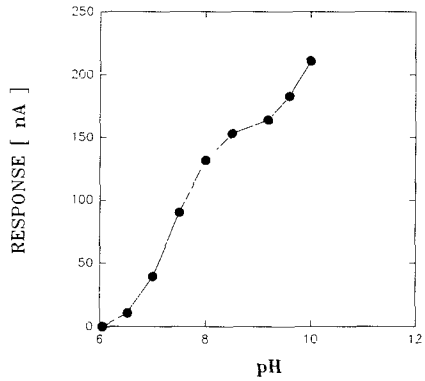

Figure 6
Dependence of the response on the pH of the mobile phase. Conditions: mobile phase: sodium phosphate buffer (0.1 M, pH 6–10) containing ACH (0.1 mM). Other conditions as in Fig. 3.

Separation

The activity of the human ACHE and the response, dynamic range and lifetime is sensitive to the chemical nature of the mobile phase. The maximum response is obtained with the neat aqueous mobile phase containing only sodium phosphate buffer. The addition of acetonitrile, sodium dodecylsulphate or tetramethylammonium bromide significantly decreased the activity of the ACHE and also the sensitivity of the detection. The composition of the mobile phase is rather limited and the choice of the column material plays a

significant role in this chromatographic system. We used hydroxyethylmethacrylate gel modified with diethylaminoethyl groups. The column is not very hydrophobic, allowing the Gal or Phy to elute from the column. The retention decreased as the concentration of the sodium phosphate buffer in the mobile phase increased. The retention mechanism is probably based on ion-exchange interactions of the solutes with ionic groups on the surface of the polymer. The chromatogram of TMA and Gal is shown in Fig. 7. The limit of detection (LOD) (three times the background) was 1 nmol for TMA and 1 pmol for Gal. For comparison the separation of TMA and Phy on 4.6 mm i.d. column both with UV detector and the enzyme technique is shown (Fig. 8). The LOD for physostigmine was 120 pmol and for TMA 100 nmol. The difference of LODs reflects the difference of column diameters and also higher reaction time in the case of microcolumn technique. The chromatogram with UV detector shows only a peak of Phy, TMA is not detectable at 250 nm [Fig. 8(a)].

The enzyme detection technique enables detection not only of Phy but also of TMA. It shows the peaks typical for the technique.

Peak shapes

The shape of the inhibitor peaks detected with the described immobilized enzyme reactor detection method is quite different from the peaks obtained with UV detector. From the difference of the signal level before injection of an inhibitor and the signal level in the minimum, the percentage of ACHE inhibition was calculated. The intensity of the ACHE inhibition is characteristic for the inhibitor (compare TMA, Gal and Phy, Figs 7 and 8). TMA is a reversible, fully competitive ACHE inhibitor reacting with negatively charged sites [13] or with π electrons in the aromatic residues on ACHE [14]. On the other hand, Phy is a reversible covalently bound inhibitor forming carbamyl enzymes [15].

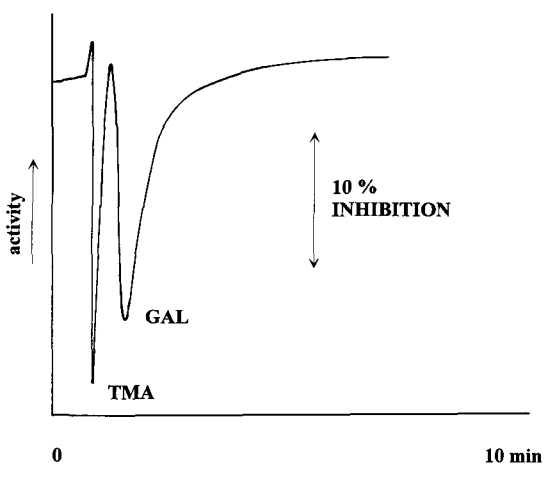

Figure 7
Micro LC chromatogram of the mixture of TMA and Gal. Conditions: column 150 × 1 mm i.d. packed with Spheron Micro DEAE 300, d_p = 12 μm; flow rate: 60 μl min^{-1}; bioreactor: 20 × 1 mm i.d.; sample: 2 μl, TMA (64 nmol), Gal (54 pmol), dissolved in the mobile phase. For other conditions see Experimental.

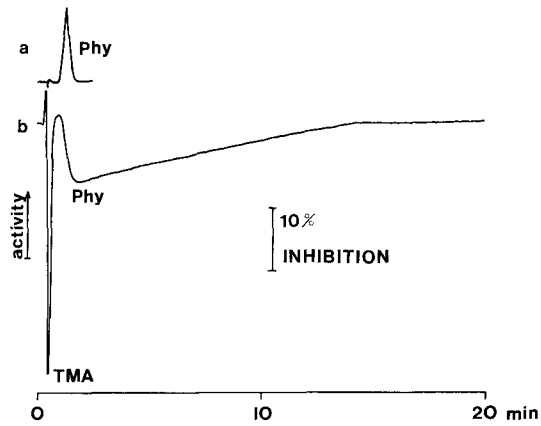

Figure 8
Chromatogram of the mixture of TMA and Phy with 4.6 mm i.d. column with UV 250 nm (a) and enzyme (b) detector. Conditions: 20 × 4.6 mm i.d. packed with Spheron Micro DEAE 300, d_p = 12 μm; flow rate: 0.8 ml min^{-1}; bioreactor: 30 × 2.1 mm i.d.; sample: 20 μl, TMA (1.8 μmol), Phy (500 pmol), dissolved in the mobile phase. For other conditions see Experimental.

Regeneration profiles

The microbioreactor responds not only to the amount of the inhibitor (negative peak height) but also to the rate of the ACHE regeneration (peak broadening). This rate is different for different inhibitors and this factor enhances also the selectivity of the method (Figs 7 and 8). The regeneration profile of the ACHE after the Phy injection is not so steep as in the case of Gal or TMA. The rate of the regeneration for Phy is dependent on the decomposition of the substance–enzyme complex.

Conclusions

Only ACHE inhibiting compounds are detected in the present chromatographic system. The only possible drawback is the potential of the eluted compounds to selectively inhibit CHO, which could be

detected in the mobile phase containing CH instead of ACH. The method described has the advantage over the existing methods [6, 7] for the determination of ACHE inhibitors in that additional post column mixing and long reaction times are not needed. Original natural substrate ACH and human red blood cell ACHE are used to detect ACHE inhibition. The method also provides information concerning the nature of inhibition (reversible vs irreversible). The principle of the described detection method can be also used in flow injection analysis. The miniaturization of the system brings advantages in the enzyme consumption and the costs of the bioreactor.

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