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Improvements in the separation and detection of acetylcholine and choline using liquid chromatography and electrochemical detection

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SUMMARY

The liquid chromatoghraphic separation and enzymatic conversion of acetylcholine and choline have been improved by using monosized polymer particles, Dynospheres, for both the separation column and the enzyme reactor. Theoretical expressions for the behaviour and optimization of post-column enzyme reactors have been derived. Some plausible supports and immobilization methods have been tested. Several antimicrobial agents have also been briefly evaluated for use as additives to the mobile phase.

INTRODUCTION

The investigation of the cholinergic transmitter system has been hampered over the years by the lack of simple and reliable methods for the determination of acetylcholine (ACh) and choline (Ch) *in vivo*[1]. Several methods have been developed and tested, mainly bioassays[2] and a variety of chemical methods[3]. They all have more or less severe drawbacks, however, which have limited their use to specialized and dedicated laboratories. New hope arose when Potter *et al.*[4] presented a concept using liquid chromatographic (LC) separation, post-column enzyme reaction and electrochemical detection. Several workers have applied and improved the method, as reported by Tyrefors and Gillberg[5] and Damsma[6]. However, several problems remain to be solved before widespread routine use can be expected.

Microdialysis is a recently introduced and rapidly accepted sampling technique that is useful in a wide variety of *in vivo* sampling situations. The inherent properties of the method place high demands on the subsequent analysis, however[7]. Work is in progress in this laboratory aimed towards developing a useful and reliable method for the determination of ACh and Ch in microdialysis samples. This work necessitates the careful examination of the different components of the analytical system involved. We

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have recently developed a separation system based on Dynospheres, a polymeric packing material with unique properties[8]. Further, several enzyme immobilization techniques have been briefly evaluated. This resulted in two which were adjudged worthy of further investigation. These were also both based on Dynospheres. Microbiological growth has been a major, but rarely recognized, problem in the systems designed earlier. We have tested several plausible poisons and found Kathon CG to be a suitable choice.

THEORY

Several more or less elaborate theories have been developed in order to describe the behaviour of enzyme reactors in flow systems[9–12], but the special considerations needed for a post-column reactor in LC have rarely been addressed[13].

The total dispersion in a chromatographic system is considered to be equal to the sum of dispersions from the different system components[14]. The dispersion in the present system can be described as

$$\sigma^2 = \sigma^2_{\text{inj}} + \sigma^2_{\text{column}} + \sigma^2_{\text{enz}} + \sigma^2_{\text{det}} \tag{1}$$

where σ^2 is the total dispersion in the chromatographic system (μl^2) and σ^2_{inj}, σ^2_{column}, σ^2_{enz} and σ^2_{det} are contributions from the injector, separation column, enzyme reactor and detector, respectively. Within the Gaussian approximation, the peak height obtained when injecting a sample plug varies with dispersion (variance) according to

$$h = ck_1 \cdot \frac{1}{\sigma\sqrt{2\pi}} \cdot \exp[-(x-m)^2/2\sigma^2] \tag{2}$$

where h = peak height, c = sample concentration, k_1 = detector sensitivity and $x - m$ = distance from peak maximum. Since only the maximum peak height is considered, $x - m = 0$, the expression reduces to

$$h = ck_2(1/\sigma) \tag{3}$$

where $k_2 = k_1/\sqrt{2\pi}$.

If the reactor length (l) is used as a single variable, the expression for the total system dispersion can be rearranged to

$$\sigma^2 = \sigma^2_{\text{sys}} + \sigma^2_{\text{enz}} l \tag{4}$$

where σ^2_{sys} is the dispersion from all components except the enzyme reactor and σ^2_{enz} the specific dispersion from the enzyme reactor (μl^2 mm^{-1}). Combining eqns. 3 and 4 yields

$$h = ck_2 \cdot \frac{1}{\sqrt{\sigma^2_{\text{sys}} + \sigma^2_{\text{enz}} l}} \tag{5}$$

The degree of conversion of an enzyme reactor under pseudo-first-order conditions can be described as[10]

$$X = 1 - \exp(-K_{ps}^{app}\tau) \tag{6}$$

where $X = (S_0 - S)/S_0$, S_0 is the starting substrate concentration, S the instantaneous substrate concentration, K_{ps}^{app} the apparent pseudo-first-order rate constant (s^{-1}) and τ the residence time in the enzyme reactor (s). The reason for using an apparent pseudo-first-order rate constant, K_{ps}^{app}, is to account for mass-transfer limitations. The relationship between the pseudo-first-order, K_{ps}, and the apparent pseudo-first-order rate constant[12] is

$$\frac{1}{K_{ps}^{app}} = \frac{d_p^{3/2}}{B\sqrt{F}} + \frac{1}{K_{ps}} \tag{7}$$

where $B = 9.75(1 - \varepsilon_E)D_m^{3/2} - \nu^{-1/6}$, ε_E = external void fraction, D_m = molecular diffusion coefficient ($m^2\ s^{-1}$), ν = kinematic viscosity ($m^2\ s^{-1}$), F = linear flow velocity ($m\ s^{-1}$), d_p = particle diameter (m) and K_{ps} = pseudo-first-order rate constant (no external mass-transfer limitations).

When using a constant flow-rate and varying the reactor length, K_{ps}^{app} can be expressed in the unit mm^{-1}, and we obtain

$$X = 1 - \exp(-\kappa_{ps}^{app}l) \tag{8}$$

where κ_{ps}^{app} is the apparent pseudo-first-order rate constant (mm^{-1}) and l the reactor length (mm). Using $c = n(S_0 - S)$, where n is a stoichiometric coefficient, and inserting eqn. 8 in eqn. 5 results in

$$h = \frac{S_0 n k_2[1 - \exp(-\kappa_{ps}^{app}l)]}{\sqrt{\sigma_{sys}^2 + \sigma_{enz}^2 l}} \tag{9}$$

Eqn. 9 describes how the peak height varies with reactor length and enzyme kinetics. Fig. 1 shows a theoretical plot of peak height (h) *vs.* the degree of conversion (X), and Fig. 2 peak height as a function of reactor length.

The conditions assumed for the separation column are $N = 3000$, $l = 100$ mm, column I.D. = 2 mm and void fraction = 70%. For the enzyme reactor, K_{ps}^{app} is varied according to the figures and $\sigma_{enz}^2 = 6.6\ \mu l^2\ mm^{-1}$. The specific dispersion, σ_{enz}^2, is determined from Fig. 3.

Several interesting observations can be made in Figs. 1 and 2. From Fig. 1 and eqn. 9, we can conclude that 100% conversion efficiency imposes infinite reactor length and dispersion, resulting in zero height. Further, the degree of conversion required for maximum peak height is dependent on k', K_{ps}^{app} and N, and it cannot be determined on an intuitive basis or by rules of thumb.

In Fig. 2, the arrows represent the maximum allowable reactor length if $N > 2600$, *i.e.*, the dispersion caused by the enzyme reactor should not result in a substantial

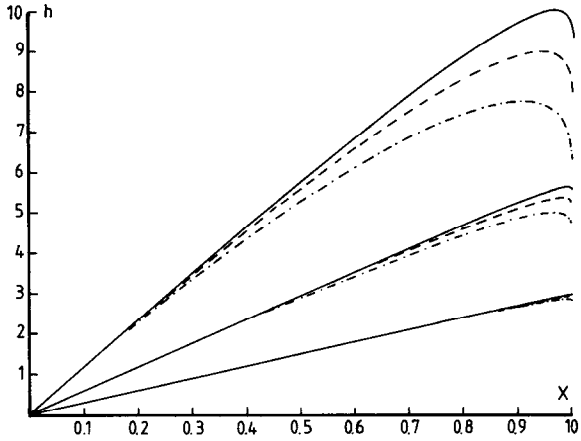

Fig. 1. Peak height as a function of degree of conversion under various conditions. The assumed k' values are 1, 3 and 7 from top to bottom. K_{ps}^{app}: ———, 1 mm^{-1}; – – – –, 0.5 mm^{-1}; – · – · –, 0.25 mm^{-1}. For other conditions, see text.

decrease in total system performance. We can conclude that a maximum peak height is not desirable under all conditions. When using slow enzyme preparations to convert early-eluting compounds, the reactor length required to obtain maximum peak height causes a severe decrease in the resolving power of the system.

In the opposite case, with late-eluting compounds passed through an efficient reactor, maximum peak height can be desirable. When neither sensitivity nor resolution is critical, the maximum degree of conversion can be desirable in order to achieve a long lifetime of the enzyme reactor and immunity to deactivation[12].

Thus, a post-column reactor of any kind needs to be carefully tailored to its application in order to deliver optimum performance.

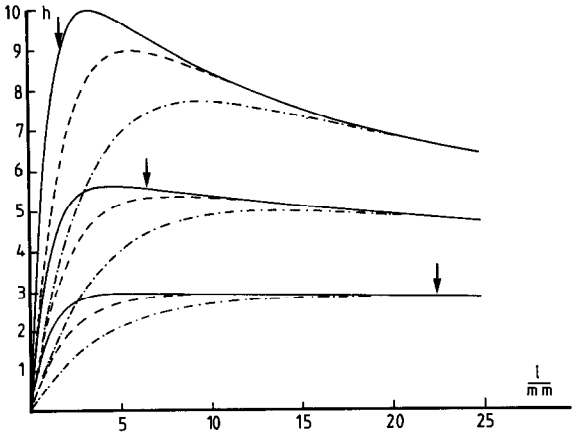

Fig. 2. Peak height as a function of reactor length under various conditions. K_{ps}^{app}: ———, 1 mm^{-1}; – – – –, 0.5 mm^{-1}; – · – · –, 0.25 mm^{-1}. The arrows indicate the maximum tolerable reactor length in order to keep the decrease in separation efficiency under 400 plates/m. For other conditions, see text.

Several factors not discussed here contribute to dispersion, making these assumptions approximate. Tailing peaks, for example, cannot be fully described in terms of σ^2. Further, if the substrate and products show different distributions between the stationary and mobile phases in the enzyme reactor, the dispersion will also be dependent on the degree of conversion.

EXPERIMENTAL

Chemicals

Acetylcholine chloride (AChCl), choline chloride (ChCl), acetylcholinesterase (AChE, EC 3.1.1.7) and cholineoxidase (ChO, EC 1.1.3.17) were obtained from Sigma (St. Louis, MO, U.S.A.). All activity units given refer to Sigma units. Superose 12 and Sephadex G-10 S were provided by Pharmacia (Uppsala, Sweden). Sodium dodecyl sulphate (SDS) with a guaranteed content of 98% C_{12} was obtained from Pierce (Rockford, IL, U.S.A.), 1,1,1-trifluoroethanesulphonyl chloride (tresyl chloride) from Fluka (Buchs, Switzerland) and Kathon CG from Rohm & Haas (Philadelphia, PA, U.S.A.). All other chemicals were from E. Merck (Darmstadt, F.R.G.) of analytical-reagent grade and used as received, unless stated otherwise. The water used for all solutions was distilled in glass and passed through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Chromatographic equipment

The chromatographic system consisted of a Knauer 364.00 high-performance liquid chromatographic (HPLC) pump fitted with a microbore pump head, a CMA/200 microsampler (Carnegie Medicin, Stockholm, Sweden) and an SP-4290 integrator (Spectra-Physics, San Jose, CA, U.S.A.). The column and enzyme reactor were thermostated using a block heater–cooler described elsewhere[15]. Briefly, the device is based on a Peltier element and operates between -5 and $+60$°C. This arrangement makes it possible to run the system at the optimum of 30–35°C and to store it at $+4$°C without removing the enzyme reactor.

For some flow-injection analysis (FIA) experiments and purity control of the enzymes, a Pharmacia FPLC system was used, consisting of two P-500 pumps an LCC-500 controller, two MV-7 injection valves and two MV-8 selector valves.

Mobile phase

The mobile phase used for the chromatographic and FIA experiments consisted of 6% (v/v) HPLC-grade acetone and 94% aqueous buffer containing 0.1 *M* NaH_2PO_4, 0.15 m*M* SDS, 0.2 m*M* EDTA and 0.1% Kathon CG. The pH of the aqueous buffer was adjusted to 7.5 with sodium hydroxide solution.

Detector system

The electronics of the electrochemical detector were constructed in-house according to commonly accepted principles[16] and the thin-layer flow cell closely resembled, with some exceptions, a commercially available model (BAS TL-5A).

The working electrode had an area of 0.79 mm^2 (1 mm diameter) and the thin-layer region was defined by a 23-μm thick Mylar gasket. An Ag/AgCl quasi-reference electrode was used, consisting of an Ag wire fitted downstream of the

Pt working electrode in the same Kel-F block, so the potential of the reference electrode was determined by the activity of Cl^- in the mobile phase.

Kathon CG contains 23% $MgCl_2$, which results in 4.8 m*M* Cl^- in the mobile phase; this concentration gives the reference electrode a potential of *ca.* 160 mV *vs.* Ag/AgCl (3 *M* KCl). All reported potentials were recalculated in order to refer to Ag/AgCl (3 *M* KCl).

Separation column

The separation column consisted of Dynospheres PD-051-R (Dyno Industrier, Lillestrøm, Norway) packed in a 60 × 2 mm I.D. or a 30 × 2 mm I.D. Knauer Vertex column. The particles are spherical with a diameter of 5 ± 0.2 μm and porous with a surface area of *ca.* 700 $m^2 g^{-1}$. The nominal pore size is 500 Å, but it can vary slightly from batch to batch. The particles can be made with a coefficient of variation of < 1% in particle size as measured with a Coulter Counter. This is equal to or better than the particles made by NASA under weightless conditions.

The columns were packed in accordance with the recommendations from Dyno; a 10% (v/v) slurry in methanol was packed at a constant pressure of 100 bar for 45 min. The resulting columns were only tested in the ACh system.

For comparison purposes, a 100 × 4.6 mm I.D. PLRP-S column (Polymer Labs., Shropshire, U.K.) was operated together with a silica-based enzyme reactor prepared according to Tyrefors and Gillberg[5].

Enzyme reactor

Both ChO and AChE were immobilized on 10-μm porous, hydrophilized Dynospheres (HD) using the aliphatic OH groups which are incorporated during the process of hydrophilization. The particles are an intermediate in the production of Pharmacia Mono-Q and Mono-S ion exchangers. The HD were suspended in a 40–50% (v/v) slurry in 4% acetic acid, from which aliquots were taken for activation. ChO was immobilized using tresyl coupling[17] and AChE by the carbonyldiimidazole (CDI) method[18]. The buffer used for all couplings was 0.1 *M* NaH_2PO_4–NaOH (pH 7.0) with 0.1% Kathon CG added as a preservative.

Solvents and reaction media were changed by trapping and rinsing the particles on 1/8-in. membrane filters, removing the filter with the thin cake of particles, suspending them in the new solvent and then picking up the filter. Three to four such transfers were easily performed without significant losses. The particles were thoroughly washed between each reaction step.

As both the ChO and AChE preparations contain Tris buffer salts, they were passed through a PD-10 desalting column (Pharmacia) in order to change to phosphate buffer before coupling.

Coupling of ChO was done by taking 80 μl of HD slurry and changing the solvent to 500 μl of acetone–pyridine (90:10), adding 40 μl of tresyl chloride and allowing reaction to proceed for 30 min at 4°C. The reaction medium was then changed to 500 μl of buffer with 3.5–4 mg of ChO added and allowed to couple for 1 h at +4°C.

The capacity of the enzyme reactor to bind protein was assessed by using a breakthrough technique similar to that described by Damsma *et al.*[19]. A 37 × 2 mm I.D. reactor packed with either tresyl chloride-activated HD or glutaraldehyde-activated silica was connected between the injector and a UV–VIS detector. The

detector was operated at 450 nm, and portions of ChO dissolved in buffer were injected until breakthrough was detected. The amount of ChO eluted was measured by integrating the resulting peaks.

AChE was coupled by taking 40 μl of HD, changing the solvent to 100 μl of dioxan, adding 6–7 mg of CDI and allowing reaction to proceed for 30 min at 22°C. The particles were then washed and suspended in 400 μl of buffer with 160–200 U of AChE and allowed to react for 1 h at 22°C.

The coupling of enzymes to silica was done for comparison purposes and has been described elsewhere[5].

RESULTS AND DISCUSSION

Comparison of immobilization supports

There are several reasons to doubt the choice of silica or porous glass as a support for the immobilization of AChE and ChO. Both hydrolyse at the optimum pH for enzyme activity (pH 7.0–8.5), and residual silanol activity causes tailing and band broadening in the absence of competing salts (*e.g.*, tetramethylammonium chloride).

We have tried some of the more plausible candidates as supports for immobilized enzymes. The requirements are mainly the same as those raised for use in high-performance liquid affinity chromatography applications[20], *i.e.*, small spherical particles with a narrow size distribution, large surface area and a narrow pore size distribution in a range where the protein has access to the pores. When this specification is applied to commercially available materials, we are left just a few possible candidates.

In addition to silica and Dynospheres, we also tried Pharmacia Superose 12 and Sephadex G-10 Superfine, but none of them was rigid enough to withstand the pressure at the applied flow-rates.

Fig. 3 shows peak profiles for a 14 × 2 mm I.D. silica-based ChO reactor and

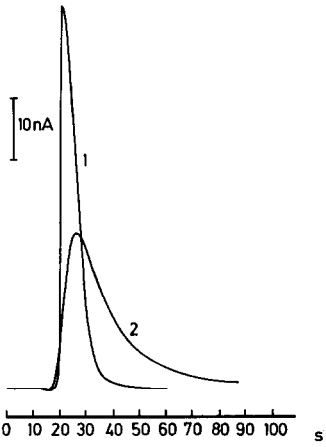

Fig. 3. Comparison of elution profiles for (1) polymer-based and (2) silica-based ChO reactors. The silica-based reactor shows severe tailing but gives approximately the same area as the polymer-based reactor. For details, see text.

a 15 × 2 mm I.D. polymer-based reactor operated at a flow-rate of 200 μl min^{-1} and a temperature of 35°C. The working electrode potential was 450 mV and the sample was 10 μl of 8 μM ChCl. It is observed that the dispersion (σ) in the silica-based reactor is 2.5 times that in the polymer-based reactor. However, the difference in conversion efficiency is negligible. The difference can be related to mass transport limitations, adsorption, or probably both, but the result remains that the polymer-based reactor is superior with respect to dispersion.

The tresyl chloride-activated HD showed a capacity of 140 mg protein per ml bed volume, and the glutaraldehyde-activated silica 100 mg/ml. This can be compared with the 10.4 mg/ml that is achieved using 5-μm particles with 120 Å pore size[19]. We did not observe any leakage of protein before the respective breakthrough, indicating fast coupling reactions.

Methods for immobilization

We tried several common methods for enzyme immobilization in order to find some suitable for our application.

Every attempt to use diazo linkage[11] of ChO to any support resulted in high protein binding, but a complete loss of activity. The CDI method refused to bind ChO at all, leading to complete elution of the enzyme from the reactor. Glutaraldehyde[21], cyanogen bromide[22] and tresyl coupling retained both ChO and its activity, but both cyanogen bromide and glutaraldehyde raise serious doubts according to the structure and stability of the binding[23–25]. Further, the use of glutaraldehyde necessitates the incorporation of a primary amine on the carrier particle surface. This obviously makes the process of coupling the enzymes more laborious and complex.

AChE, on the other hand, did not retain much of its activity either when tresyl or diazo coupling was used. Glutaraldehyde, cyanogen bromide and CDI couplings retained both enzyme and activity, but the same objections against glutaraldehyde and cyanogen bromide as raised for ChO are valid for AChE.

This screening leaves tresyl coupling for ChO and CDI coupling for AChE.

Characteristics of the enzyme reactor

Enzyme reactors can be characterized by their degree of conversion, but that is a limited measure, valid only under strictly defined conditions. If pseudo-first-order kinetics can be assumed (linear calibration graphs), the pseudo-first-order rate constant is a valuable measure of the reactor efficiency[12]. The rate constant is reasonably independent of flow-rate and can be corrected for temperature variations and different reactor lengths. In our case, the AChE have a much higher specific activity (1000–2000 U/mg) than ChO (10–15 U/mg), and therefore we only considered the kinetics of ChO.

The degree of conversion is more difficult to determine than it appears at first sight. The obvious way would be to inject a Ch standard and compare the area of the resulting peak with that obtained on injecting a hydrogen peroxide standard. We have not been able to achieve reproducible results from such experiments using stainless-steel components in an HPLC system.

When using the FPLC system where wetted parts are made of Kel-F, Tefzel or glass (and titanium in some pump parts), it was possible to inject hydrogen peroxide and obtain reproducible results. However, the pumps and tubing do not cover the range of pressures needed for studies of kinetics in these enzyme reactors.

One way to circumvent the problem is to measure the amount of Ch consumed by the reactor instead of the hydrogen peroxide produced. This was done by injecting a known amount of Ch and then collecting the mobile phase with the eluted peak in a weighed microvial and then weighing the vial again. Then a portion of the collected mobile phase was reinjected and the resulting peak area was determined. Finally, we calculated how much Ch was left after passage through the reactor. This is possible because injection of Ch gave reproducible results and straight calibration graphs, whereas injection of hydrogen peroxide did not.

Another fundamental complication is the reaction mechanism. The oxidation of Ch to hydrogen peroxide and betaine is a two-step reaction proceeding via betaine aldehyde[26]:

$$(CH_3)_3N^+CH_2CH_2OH + O_2 \rightarrow (CH_3)_3N^+CH_2COH + H_2O_2$$

$$(CH_3)_3N^+CH_2COH + O_2 + H_2O \rightarrow (CH_3)_3N^+CH_2COOH + H_2O_2$$

Despite this complication, the simplification involved when assuming first-order conditions has been shown to be valid to a useful extent.

Fig. 4 shows a chromatogram obtained with a 30 × 2 mm I.D. separation column and an 11.5 × 2 mm I.D. ChO reactor obtained when reinjecting a portion of a previously collected peak. As can be seen, betaine aldehyde is separated from Ch and is clearly visible. The conditions for the first passage were: flow-rate 400 μl min^{-1}, temperature 22°C and 10 μl of 60 μM ChCl injected.

Fig. 5 shows a plot of $-\ln(1 - X)$ *vs.* τ for an 11.5 × 2 mm I.D. ChO reactor operated at 22°C. The slope of the regression line gives $K_{ps}^{app} = 0.58\ s^{-1}$, indicating that a residence time of about 8 s will result in 99% conversion of Ch to hydrogen peroxide and betaine. When using a flow-rate of 200 μl min^{-1} and a 30 × 2 mm I.D. reactor, a residence time of about 20 s will result in 99.999% conversion.

The residence time was determined by comparing the time from injection to peak

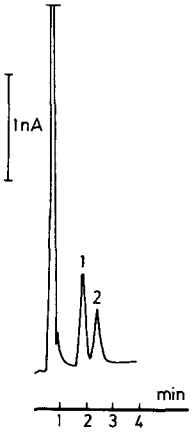

Fig. 4. Chromatogram obtained on reinjecting a portion of a previously injected ChCl sample. (1) Betaine aldehyde; (2) Ch. For details, see text.

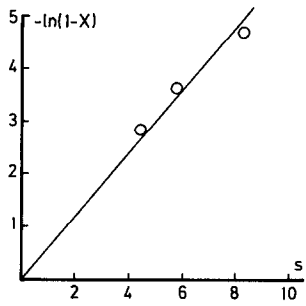

Fig. 5. Plot of $-\ln(1 - X)$ *vs.* residence time for an 11.5 × 2 mm I.D. polymer-based ChO reactor. The transformation is valid only under pseudo-fist-order conditions; under higher order conditions the plot would deviate from a straight line.

maximum with and without the reactor when injecting hydrogen peroxide. Changing the flow-rate instead of the reactor length is fully justified if eqn. 7 is evaluated in the range of flow-rates that we used.

Bacterial growth

It has been reported elsewhere[27] that bacterial growth in the mobile phase can cause severe problems with high background currents, noise and a rapid decline in reactor performance. The acetone that we use as an organic modifier in the mobile phase has some bacteriostatic properties, but not enough to eliminate completely the microbiological activity in the mobile phase.

We searched for a good poison to be used as additive to the mobile phase without disturbing any part of the chromatographic system. Sodium azide, ascorbic acid and the different Parabens are all readily oxidized by the Pt electrode at the potentials used and give rise to high and unstable background currents. Thiomersal rapidly reduces the ChO activity to zero, and chlorhexidine is strongly retained by the separation column, causing the retention times to shift. Formaldehyde reacts readily with primary amine residues of the immobilized enzymes, forming Schiff bases, and denatures the enzymes.

After some experimenting with these common bacteriostats, we found that Kathon CG was a suitable candidate that does not interfere with the separation or detection. The active component of Kathon CG is a mixture of two isothiazolines, stabilized with 23% magnesium chloride in aqueous solution. For further information, Rohm & Haas provide extensive documentation on request. So far we have not encountered any problem that can be associated either with the presence of 0.1% Kathon CG or with bacterial growth.

Chromatographic separation

Fig. 6 shows a chromatogram obtained on injecting 20 μl of 2 μM ChCl and AChCl onto a 100 × 4.6 mm I.D. PLRPS column followed by a 30 × 4 mm I.D. enzyme reactor as described previously[5]. The flow-rate was 1.0 ml min^{-1}, the temperature 35°C and the working electrode was 3 mm diameter and operated at 450 mV. The chromatogram is representative of what is achieved with this system; the main features are 3000–7000 plates/m and severe tailing.

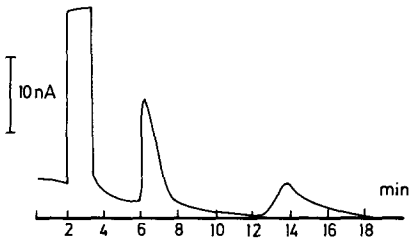

Fig. 6. Chromatogram obtained with a 100 × 4.6 mm I.D. PLRPS column and a 30 × 4 mm I.D. silica-based enzyme reactor.

Fig. 7 shows a chromatogram obtained with the polymer-based system using a 60 × 2 mm I.D. separation column and a 30 × 2 mm I.D. enzyme reactor operated at 35°C, using a flow-rate of 200 μl min^{-1}. The working electrode was a 1 mm diameter Pt electrode operated at 450 mV and 10 μl of an 8 μM ChCl and AChCl solution were injected.

A calculation of the number of theoretical plates[28] based on Fig. 7 results in 30 000–40 000 plates/m. The result can be corrected for the contribution from the enzyme reactor, assuming the variance (σ^2) to be additive, and using Fig. 1 to estimate the variance from the enzyme reactor the result is 40 000–50 000 plates/m for the separation column alone. The slight fronting observed is probably due to channels in an imperfectly packed column, the fronting becoming more pronounced at large k'.

Fig. 8 shows log k' *vs.* SDS concentration at 2% acetone and log k' *vs.* acetone concentration at 0.2 mM SDS for ACh and Ch. The reason for the non-linear behaviour of log k' *vs.* SDS concentration is probably micelle formation and the adsorption isotherm for adsorption of SDS to the stationary phase. The critical micellar concentration for SDS in water is found in this concentration region[29].

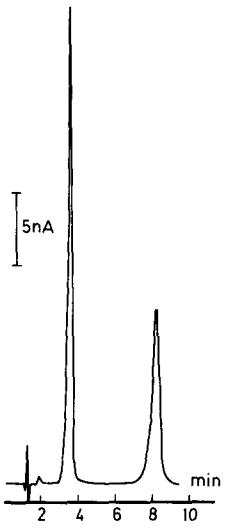

Fig. 7. Chromatogram obtained with a 60 × 2 mm I.D. separation column and a 30 × 2 mm I.D. enzyme reactor, both based on Dynospheres. For details, see text.

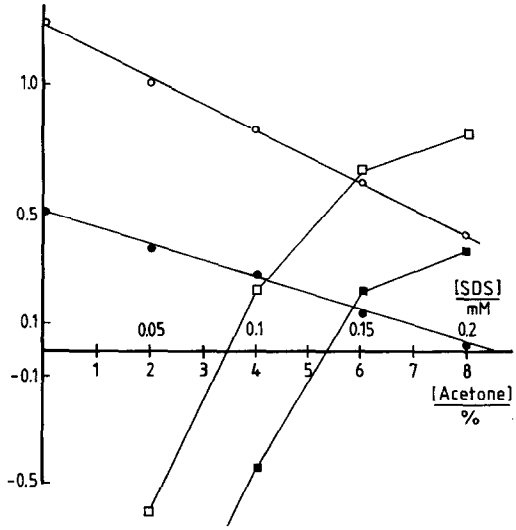

Fig. 8. Log k' *vs.* mobile phase composition. (●) ACh and (○) Ch *vs.* acetone concentration; (■) ACh and (□) Ch *vs.* SDS concentration.

CONCLUSIONS

The need for careful design of post-column enzyme reactors has been demonstrated. Useful relationships for performing such designs have been derived. The described application of monosized particles seems to be a valuable solution to some of the problems associated with efficient separation and enzymatic derivatization of Ch and ACh. Kathon CG is a suitable poison for use in conjunction with immobilized enzymes and electrochemical detectors.

ACKNOWLEDGEMENTS

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