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Cation-exchange liquid chromatography of choline and acetylcholine on free shielded silanols of silica-based reversed-phase stationary phases

Jaroslav Šalamoun*,☆

Institute of Analytical Chemistry, Veveri 97, 611 42 Brno (Czechoslovakia)

Phuc Trung Nguyen and Jörg Remien

Walther-Straub Institute of Pharmacology and Toxicology, Nussbaumstrasse 26, W-8000 Munich 2 (Germany)

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ABSTRACT

Free anionic functions present on the surface of reversed-phase packing materials were used for the selective cation-exchange preconcentration and separation of the neurotransmitters choline and acetylcholine from a biological matrix. The cation-exchange behaviour of different reversed-phase packing materials in the neat aqueous mobile phase, the properties of an end-capped column, the dependence of capacity factors and peak shape on the concentration of counter ions, ionic strength, pH and the addition of acetonitrile and optimum conditions for enzymatic conversion of solutes to hydrogen peroxide were studied. The studied reversed-phase columns exhibit better pH stability and longer lifetimes than normal silica-based cation exchangers. Acetylcholine is an effective and sensitive test sample for the measurement of adsorption on silica support. A large sample volume was injected onto a precolumn inserted instead of an injection valve and after injection the solutes were focused and separated on an analytical column with a mobile phase containing tetramethylammonium perchlorate as the counter ion.

INTRODUCTION

Acetylcholine [$CH_3COOCH_2CH_2N(CH_3)_3^+$, ACH] and choline [$HOCH_2CH_2N(CH_3)_3^+$, CH] are endogenous substances with a tetramethylammonium group present in very low concentrations in biological materials. A number of high-performance liquid chromatographic (HPLC) methods have been published [1–7] on the determination of these substances, using mainly packed-bed reactors with the immobilized enzymes acetylcholinesterase and cholinoxidase. The released hydrogen peroxide is then detected by an electrochemical detector with a platinum electrode. Cation-exchange chromatography [3,5] or the ion-pair technique on reversed-phase columns [1,2,4,6] was the most often used technique for the separation of both compounds. Reversed-phase liquid chromatographic (RPLC) separation on an octadecyl column without the addition of an ion-pairing reagent to the mobile phase was reported, suggesting a reversed-phase mechanism of the separation [7].

Many RPLC methods are performed by using porous silica chemically modified with alkyl groups bonded to the surface hydroxyl groups. About half or about 4 μmol/m^2 of the available silanols can be substituted even after end-capping because of steric hindrance [8–10]. The high concentration of the remaining surface silanols significantly influences the behaviour of many solutes and can cause

☆ Present address: Walther-Straub Institute of Pharmacology and Toxicology, Nussbaumstrasse 26, W-8000 Munich 2, Germany.

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irregular retention and peak broadening [11,12]. This undesirable effect of silanols is usually partly eliminated by adding a silanol blocking agent such as amines or tetraalkylammonium salts to the mobile phase or by varying the pH, ionic strength, etc. [13,14].

The pK_a of silica ($\equiv SiO^- \rightleftharpoons \equiv SiOH$) has been determined [15] to be 7.1. Iler [16] noted that the pK_a varies from 6.5 at 0% neutralization to about 9.5 at 50%. The silica surface is more strongly acidic than monosilicic acid, which has a pK_a of 9.8. As a weak acid, silica exhibits properties typical of weakly acidic cation exchangers.

The behaviour of basic amino compounds on silica and silica reversed phases has been reported and the contribution of the cation-exchange properties of silica matrix has been demonstrated [17,18]. Weber and Tramposch [19] studied Spherisorb ODS and reported that it has properties similar to a strong cation exchanger of low capacity and that the pK_a of silanols vary with the degree of neutralization, salt concentration and the microenvironment.

Scott and Simpson [20] showed that the "brush-type" reversed phases are slow to come to equilibrium with pure water, and in contact with water it would appear that the hydrocarbon chains dispersively interact with themselves and are lying almost flat on the surface.

We used the remaining shielded silanol functions of the RPLC materials for the cation-exchange chromatographic separation of CH and ACH. The samples were pretreated on a larger volume precolumn inserted instead of an injection loop and packed with coarser RPLC material. The mobile phase composition was evaluated with regard to the function of the precolumn, column and reactor.

EXPERIMENTAL

ACH iodide, CH chloride and tetramethylammonium (TMA) perchlorate were obtained from Sigma (Deisenhofen, Germany) and ethylenediaminotetraacetic (EDTA) sodium salt from Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade.

Chromatographic separations were performed at ambient temperature on a Gynkothek (Munich, Germany) Model 600/200 liquid chromatograph equipped with a Gynkothek 20- or 50-μl six-port injection valve and with a Model EP 30 electrochemical detector set at 0.45 V (Biometra, Göttingen, Germany). A 30 × 2.1 mm I.D. cartridge with immobilized acetylcholinesterase and cholinoxidase from Biometra was used for the detection of CH and ACH. A CGC column (150 × 3 mm I.D.) packed with LiChrosorb RP-18, particle diameter, d_p = 5 μm (Merck) was used for the study of its cation-exchange behaviour. The other analytical columns used were Hamilton PRP-X 200 (250 × 4.1 mm I.D.) packed with polymer-based strong cation exchanger, d_p = 10 μm, 250 × 4 mm I.D. packed with Supelcosil LC-8, d_p = 5 μm (Supelco, Bellefonte, PA, USA) and LiChroCART (125 × 2 mm I.D.) packed with Superspher 60 RP-Select B, d_p = 5 μm (Merck). A Supelguard LC-8, d_p = 5 μm, precolumn (20 × 4.6 mm I.D.) was used in conjunction with the Supelcosil LC-8 column. A Pelliquard LC-8, d_p = 40 μm column (20 × 4.6 mm I.D.) was used in conjunction with LiChrosorb RP-18 columns and large volumes of the sample were injected on to the precolumn inserted instead of an injection valve. The void volume of the precolumn was 250 μl and that of the LiChrosorb RP-18 column was 1 ml. The precolumn was equilibated with 0.005 *M* sodium phosphate buffer (pH 7.4) and volumes of sample up to 250 μl were injected. The precolumn was then washed with 250 μl of 0.005 *M* sodium phosphate buffer and all the contents of the precolumn were injected into the analytical column. The mobile phase for the analytical column was of 0.1 *M* sodium phosphate buffer (pH 7.4.) containing 1 m*M* TMA and 0.1 m*M* EDTA.

Brain and heart tissues were mixed with 2 ml of 6% trichloroacetic acid homogenized for 10 min at 0°C and kept for 10 min in an ice-cold bath. The homogenate was mixed with 2 ml of 0.1 *M* sodium phosphate buffer (pH 7.4) and after 10 min in an ice-cold bath was centrifuged for 25 min at 30 000 *g*. The supernatant was diluted tenfold with 0.1 *M* sodium phosphate buffer (pH 7.4) and 50 μl were injected on to the Supelcosil LC-8 column.

A 10-μl volume of erythrocytes was mixed with 10 μl of 3 *M* trichloroacetic acid and 180 μl of 0.1 *M* sodium phosphate buffer (pH 7.4) were added. The mixture was centrifuged for 10 min at 10 000 *g* and the supernatant, after further dilution, was injected on to the precolumn.

RESULTS AND DISCUSSION

The number of silanol groups accessible and their contribution to the retention in RPLC depend on the size and chemical character of the compounds to be analysed. CH and ACH are polar compounds containing a tetramethylammonium moiety. These small ionic molecules can easily penetrate silanol or other ionic groups hidden under the bonded layer of a reversed-phase stationary phase and interact with them on the cation-exchange principle. Not only the chromatographic behaviour but also the enzyme reactions are functions of many variables and experimental conditions. All these variables should eventually be considered whenever one wishes to utilize immobilized enzyme reactors in HPLC.

Influence of TMA on retention

We studied whether the mechanism of retention is based on hydrophobic or cation-exchange interactions. Optimum conditions for the separation of CH and ACH were established by varying the concentration of TMA. An increasing salt concentration in the mobile phase results in increased retention of hydrophobic solutes in RPLC. On the other hand, the capacity factor (k') in ion-exchange chromatography is a function of the selectivity coefficient or equilibration constant (K_A^B), total ion-exchange capacity (Q) and counter ion concentration in the mobile phase (c) [21]:

$$k' = V_s/V_m(K_A^B)^{1/a}Q^{b/a}c^{-b/a} = (V_s/V_m)K_D \qquad (1)$$

where V_s and V_m are the volumes of stationary and mobile phase in the column, respectively, a and b are charges of the counter ion in the mobile phase and solute, respectively, and K_D is the distribution constant. The logarithmic dependence of the capacity factor on the concentration of an eluted ion is linear, the slope of which is determined mainly by the ratio of the charges of the chromatographed and counter ions. The dependence for CH and ACH on LiChrosorb RP-18 is shown in Fig. 1. The higher the TMA concentration, the lower is the retention. The dependence is linear in accord with the suggested cation-exchange mechanism. A pure ion-exchange mechanism should give a slope of unity. The slopes of these plots are in fact 0.191 for CH and 0.226 for ACH. This shows that a mixed ion-exchange and reversed-phase mechanism is responsible for the chromatographic behaviour of CH and ACH in this system.

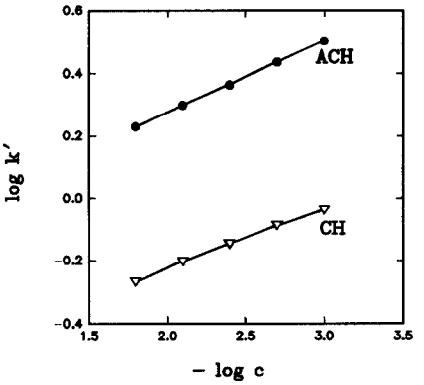

Fig. 1. Dependence of the capacity factor (k') of CH (▽) and (●) ACH on molar concentrations of TMA by cation-exchange chromatography on LiChrosorb RP-18. Mobile phase, 0.1 *M* phosphate buffer (pH 7.4) containing 0.1 m*M* EDTA. For other conditions, see Experimental.

A decrease in TMA concentration in the mobile phase to <2 m*M* results in significant peak tailing (the asymmetry factor at 10% of the ACH peak height is 3.3 for 1 m*M* TMA; see Fig. 2). This indicates that TMA inhibits the activity of strong cation-exchange sites. The activity of these groups is suppressed at higher concentrations of TMA when these high-activity sites are saturated with TMA, whereas the less reactive anionic sites remain for the cation-exchange chromatography of ACH and CH.

Linear range of ACH response

The dependence of the ACH response on the amount of ACH injected is linear up to about 1 nmol for the LiChrosorb RP-18 column. This value is significantly lower than that usually found in RPLC. Higher amounts decrease the retention time and result in peak asymmetry (see Fig. 2). The peak asymmetry factor is in the range 1.3–1.6, depending on the batch of the column used up to 1 nmol of ACH injected. For the injection of 10 and 200 nmol ACH, the peak asymmetry factor was 2.0 and 2.75 and the capacity factor decreased from the normal value of 2.35 to 2.30 and 1.8, respectively. This means that we work in the linear part of a convex absorption isotherm only at low concentrations of ACH. However, it is sufficient in the trace analysis of CH and ACH to have a linear range of three orders of magnitude.

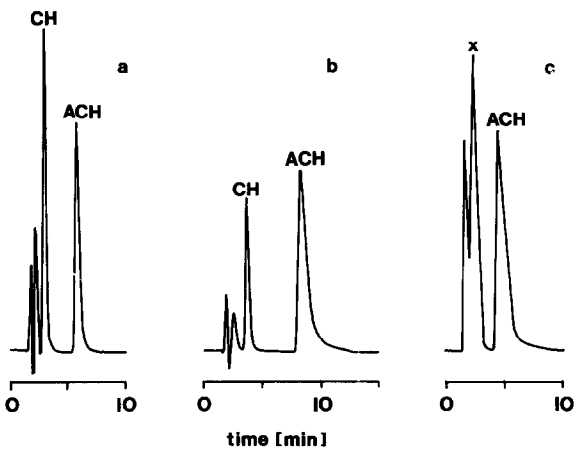

Fig. 2. Peak broadening in cation-exchange chromatography of ACH on LiChrosorb RP-18. (a) Mobile phase, 0.1 *M* sodium phosphate buffer (pH 7.4) containing 4 m*M* TMA and 0.1 m*M* EDTA; amount of ACH injected, Q = 400 pmol; detector range = 80 nA full-scale. (b) Mobile phase as in (a), containing 1 m*M* TMA; Q = 400 pmol; detector range = 160 nA full-scale. (c) Mobile phase as in (a); Q = 200 nmol; detector range = 8 μA full-scale. × = Unknown.

Influence of TMA on detection sensitivity

The dependence of ACH response on TMA concentration in the mobile phase is shown in Fig. 3. The higher the TMA concentration, the lower is the response. TMA is not only a competitor of ACH for the active sites on silica surface but also inhibits enzyme activity in the enzyme bioreactor. The advantage of reversed-phase low-capacity cation exchangers is that a lower concentration of TMA in the mobile phase (4 m*M*) can be used in comparison with normal cation exchangers (7 m*M*), resulting in higher response.

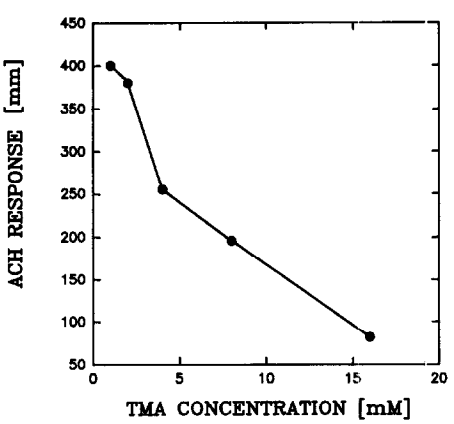

Fig. 3. Dependence of the ACH response on the concentration of TMA. Conditions as in Fig. 1.

Influence of sodium phosphate buffer concentration

The concentration of sodium phosphate buffer in the mobile phase was 0.1 mol l^{-1}. A tenfold decrease in Na^+ concentration had only a slight influence on the increase in ACH retention and did not affect its peak shape. The diameter of the solvated sodium ion is comparable to that of TMA. Anyway, the methyl groups present in the TMA molecule make TMA more hydrophobic than the solvated sodium ion [22], and it can easily penetrate through the hydrophobic layer and interact effectively with remaining silanols.

Influence of EDTA

The silica surface can be enriched by a number of metal oxide impurities. Twenty metals in the ppm range and fifteen in ppb range have been reported [12]. These metal impurities can cause both increases and decreases in enzyme activity. In our experience, a new column, even if preconditioned consecutively with methanol, water and buffer, brings about degradation of the enzyme reactor activity after the immediate application of the mobile phase. The EDTA present in the mobile phase complexes the metal impurities, making the silanol surface free, and protects not only both enzymes from inactivation but also the hydrogen peroxide from metal-catalyzed decomposition.

Influence of pH on retention

At lower pH of the mobile phase the silanol groups lose their ionic character and, as a result, the capacity of the column and also the capacity factor decrease. The influence of pH on the capacity factor of ACH is illustrated in Fig. 4. The retention of CH and ACH decreased only gradually, with no clear break in the pH range studied, which is in accord with the theory that the silanol groups behave like a polyelectrolyte [16].

Influence of pH on column lifetime

A major drawback of silica-based cation exchangers is a very short lifetime under the assay conditions (pH 7.4–8). Silica is dissolved at alkaline pH. Silica in a reversed-phase stationary phase is

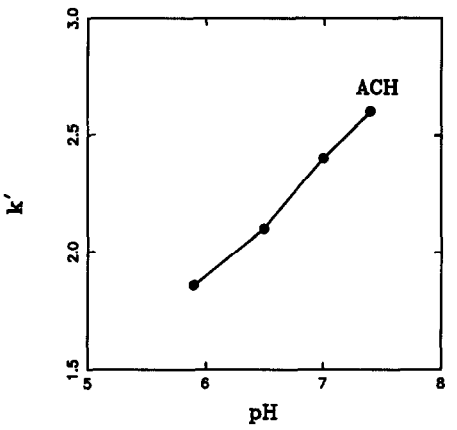

Fig. 4. Dependence of the capacity factor (k') of ACH on the pH of the mobile phase. Conditions as in Fig. 1; the mobile phase contained 4 mM TMA.

protected from intensive contact with the mobile phase by hydrophobic chains because the penetration of water molecules into the bonded layer is unfavourable [10,20]. This significantly prolongs the lifetime of the column from several days for normal silica-based cation exchangers [5] to several months for reversed-phase materials.

Effect of shielded anionic groups

The hydrophobic phase influences not only the pH stability of the column but also its deterioration by the sample matrix. The Supelcosil LC-8 column has been used in our laboratory for the determination of CH and ACH in heart and brain tissues and during 6 months hundreds of unextracted homogenate samples have been injected with the efficiency of the column gradually decreasing from 3000 to 2000 theoretical plates. In comparison, the Hamilton cation-exchange column was not able to separate ACH completely from biological components in the same samples because active sites soon deteriorate owing to the compounds present in the sample matrix. The active groups on the surface of RPLC packings have a net negative charge, causing ion exclusion, and moreover are hardly accessible to large negatively charged macromolecules because of size exclusion.

Performance of cation-exchange columns

The performance of the analytical columns used for the separation of CH and ACH was relatively low for all cation exchangers [5] and also for ion-pair RPLC [4]. The efficiency of our RPLC column was 3000 theoretical plates for ACH and the 25 × 0.4 cm I.D. Supelcosil LC-8 column and 1300–2000 for the 15 × 0.3 cm I.D. LiChrosorb RP-18 column. This is approximately the same efficiency as for silica-based cation exchangers [5]. The solutes diffuse to the surface silanols through the bonded layer with all the silica-based columns and the effect of stationary phase mass transfer contributes to the decrease in efficiency.

Influence of acetonitrile

Acetonitrile molecules at a low concentration in the eluent penetrate into the bonded layer and can cause rearrangement of the bonded layer structure [10]. The addition of 3% of acetonitrile to the mobile phase caused a decrease in the ACH capacity factor from 2.6 to 1.6. This behaviour can be explained by the competing adsorption of acetonitrile on accessible silanol groups by the polar part. Anyway, the low concentration of acetonitrile present in the mobile phase causes gradual damage to the immobilized enzyme reactor.

Sample focusing

Large-volume injections of very dilute samples in a non-eluting solvent have been successfully applied in conventional RPLC [23] and in microbore HPLC [24–26]. We applied this technique to the preconcentration of solutes on the precolumn and the top of the analytical column. The solutes were at first adsorbed on the precolumn silanols. With the injection of the precolumn eluent (*i.e.*, diluted buffer containing no TMA which, if used as the mobile phase on the chromatographic column, would lead to very long retention times), the top of the analytical column was conditioned. Simultaneously, the mobile phase enters the precolumn and CH and ACH are eluted in a sharp gradient on to the analytical column. Consequently, an increase in retention volume equal to the precolumn volume was observed. This technique allows one to vary the sample injection volume and preseparate the solutes from the non-retained sample matrix without any extra-column band broadening. The recovery of CH and ACH for erythrocytic samples was more than 95%.

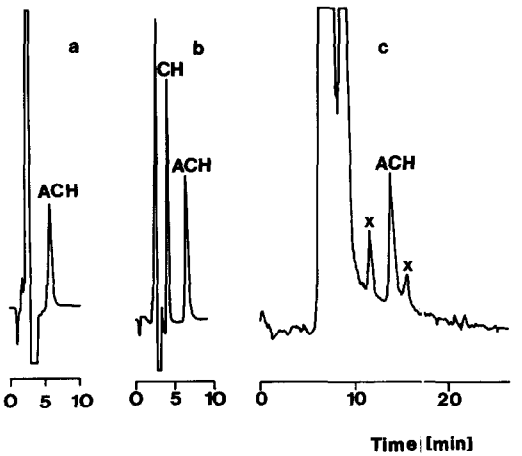

Fig. 5. Cation-exchange chromatography of CH and ACH on different RPLC columns. (a) Chromatogram of ACH standard solution on Superspher 60 RP-select B. Mobile phase, 0.1 *M* phosphate buffer (pH 7.4) containing 1 m*M* TMA and 0.1 m*M* EDTA; flow-rate, 0.1 ml/min; amount of ACH injected, Q = 84 pmol. (b) 200 μl of erythrocytic homogenate separated on LiChrosorb RP-18 column. Q = 423 pmol of ACH; mobile phase, 0.1 *M* phosphate buffer (pH 7.4) containing 4 m*M* TMA and 0.1 m*M* EDTA; flow-rate, 0.5 ml/min. For other conditions, see Experimental. (c) 50 μl of heart homogenate separated on a Supelcosil LC-8 column. Q = 17 pmol of ACH; conditions as in (b). × = Unknown.

Behaviour of other RPLC columns

Superspher 60 RP-select B, the column suggested for the separation of basic compounds, showed a considerably lower ability to retain CH and ACH. The concentration of TMA in the mobile phase had to be decreased to 1 m*M* to reach a capacity factor of 1.7 for ACH. The Supelcosil LC-8 column gave the same capacity factors as the LiChrosorb RP-18 column.

Chromatograms of some samples on these three columns are shown in Fig. 5. The detection limit of acetylcholine was 60 fmol on the LiChrosorb RP-18 column. The reproducibility of the retention time was better than 1% within day and better than 2.5% day to day.

CONCLUSIONS

Although it is widely recognized that interactions of basic or ionic substances with anionic sites of reversed-phase column occur, the magnitude of such effects is not always fully appreciated. This study is an example of the usefulness of these interactions. The present mode of cation-exchange chromatography of CH and ACH has several advantages over the conventional silica-based cation-exchange chromatography: better pH stability, shielded active sites, lower capacity of anionic groups allowing better optimization of the composition of the mobile phase for enzymatic reactions. The present results also show how incomplete the elimination of these interactions with silanols by addition of TMA to the mobile phase can be. The method involves very low concentrations of small and charged solute molecules and their detection in a packed-bed reactor system with immobilized enzymes and neat aqueous mobile phase. It also permits a better study of the behaviour of RPLC materials. ACH is an effective and sensitive test sample for the measurement of adsorption on silica supports. Apparently ACH is an even more sensitive probe than other basic compounds, because it is smaller and sterically more able to interact with surface silanol groups that are shielded by alkyl ligands.

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