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DETERMINATION OF ACETYLCHOLINE AND CHOLINE IN MICRODIALYSATES FROM SPINAL CORD OF RAT USING LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method for the determination of acetylcholine and choline in microdialysates from rat spinal cord has been developed using liquid chromatography, enzyme-catalysed post-column derivatization and electrochemical detection. The sensitivity required was achieved by a certain degree of miniaturization, careful temperature control and a slight modification of the amperometric detector. Determinations were performed on 5-μl samples.

INTRODUCTION

Liquid chromatography (LC) with electrochemical detection (ED) is a method with an increasing number of applications for the determination of organic substances at trace levels. The selectivity of the detector is both an advantage and a limitation: an advantage because of the reduction of interferences and a limitation owing to the relatively few substances that can be detected. Several pre- and post-column derivatization techniques have been developed to make possible the detection of otherwise undetectable substances [1].

Acetylcholine (ACh) is the earliest discovered neurotransmitter, and choline (Ch) is both its precursor and metabolite. Several assay methods have been developed for their determination. A handbook of available methods for the determination of ACh and Ch, edited in 1974 by Hanin [2], is still very useful.

Since 1983, when Potter et al. [3] presented the concept of LC with post-

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column enzyme reaction and ED, several investigators have focused their interest on this method [4–8]. In this novel approach [3] the reactions of solubilized acetylcholinesterase (AChE) and cholineoxidase (ChO) with ACh and Ch are used to generate hydrogen peroxide in the eluate of a high-performance liquid chromatography (HPLC) column. The hydrogen peroxide is detected in a thin-layer amperometric detector equipped with a platinum working electrode. Although Potter et al. [3] used solubilized enzymes, they suggested the use of immobilized enzymes instead, and in subsequent studies several investigators have tried various immobilization techniques and support materials, combined with different chromatographic separations [4–8].

Recently, microdialysis has been introduced as a very powerful in vivo sampling technique [9]. Most of its applications are still to be explored, but it generally requires that analyses be performed on very small samples containing analytes in concentrations lower than endogenous.

We have developed an LC separation and designed a detection system for ACh and Ch which makes it possible to analyse these substances in microdialysates from the spinal cord of rat. This has been achieved by using a 2 mm I.D. analytical column instead of the conventional 4.6 mm I.D., thus reducing system volume and sample dilution by a factor of five [10]. Use of an amperometric thin-layer cell with reduced electrode area and spacer thickness leads to further enhancement of sensitivity [11]. Finally, the use of a saturator column makes the separation possible at pH 7.7 [12], thus eliminating the need for a make-up flow and further sample dilution.

EXPERIMENTAL

Reagents and chemicals

ACh chloride, Ch chloride, AChE (EC 3.1.1.7, type VI-S) and ChO (EC 1.1.3.17) were obtained from Sigma (St. Louis, MO, U.S.A.). All activity units given refer to Sigma units. Tetramethylammonium chloride (TMACl) was obtained from Janssen Chimica (Beerse, Belgium). Sodium dodecyl sulphate (SDS), glutaraldehyde (25% in water) and 3-aminopropyltriethoxysilane (APTS) were obtained from Fluka (Buchs, Switzerland). All other chemicals were of p.a. grade (E. Merck, Darmstadt, F.R.G.) and used as received unless otherwise stated. LiChrospher Si-500 (10-μm spherical silica with 500 Å pore size) was obtained from E. Merck. The water used for all solutions was distilled in glass and further purified in a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Chromatography

The chromatographic system consisted of an LKB 2150 solvent delivery module (LKB, Bromma, Sweden) followed by a 50×4.6 mm I.D. saturator column packed with uncoated 47–63 μm silica. The sample was introduced with a Valco C6W injector fitted with a 13-μl sample loop. Chromatographic separations were carried out on a 100×2 mm I.D. Sperisorb S5 C_6 column (Phase Separations, Queensferry, U.K.) at 200 μl/min. The mobile phase was 7.1% (v/v) HPLC-

grade acetone and 92.9% aqueous buffer containing 50 m*M* sodium dihydrogenphosphate, 15 m*M* TMACl and 0.30 m*M* SDS, and adjusted to pH 7.7 with sodium hydroxide.

The aqueous solution was filtered through a 0.45-μm Millipore membrane filter prior to mixing with acetone. The separation was followed by enzymatic hydrolysis of ACh to Ch and acetic acid, and subsequent oxidation of Ch to hydrogen peroxide and betaine. Hydrogen peroxide was detected at 480 mV using a laboratory-made thin-layer amperometric flow-cell fitted with a platinum working electrode. All reported potentials refer to an Ag/AgCl (3 *M* potassium chloride) reference electrode. The detector cell was in all aspects compatible with a commercially availble model (BAS TL-5 A) except that the spacer was made from 23-μm Mylar film and the working electrode had a diameter of 1 mm. The cell was controlled by a laboratory-made potentiostat and I/E-converter constructed according to commonly accepted principles [13]. The current signal was recorded using a Chromatopac C-R3A computing integrator (Shimadzu, Kyoto, Japan). The complete flow-system, from the saturator column to the waste bottle, was housed in a forced air circulation thermostat, able to control the temperature between 25 and 50°C to $\pm$0.1°C. The system was operated at 32°C, and samples were injected by partial filling of the loop with 5 μl using a 25-μl SGE syringe.

Preparation of the enzyme reactor

The considerations concerning the reactor are based on the general conclusions of Johansson et al. [14]. LiChrospher Si-500 was chosen as the support for the immobilized enzymes. The support was selected both with respect to the molecular mass of ChO [15], assuming a fairly globular shape of the protein, and the mass transport in a packed bed [16].

Since AChE exhibits a very high specific activity, the same consideration concerning its size [17] was found unnecessary. The means and methods for immobilization were adopted from Robinson et al. [18] and Johansson et al. [14]. Except for the fact that these methods are based on porous glass as support, the chemistry is similar: silanol groups are used for coupling the reagents to the carrier.

The silica was refluxed in 5 *M* nitric acid for 1 h, dried at 95°C, refluxed for 10 h in 5% APTS (5 ml/g of silica) and stirred in a 5% solution of glutaraldehyde in 0.1 *M* sodium carbonate buffer (pH 8.0) for 1 h (10 ml/g of silica at 22°C). The silica was carefully washed after each step. Then 25 U of ChO and 167 U of AChE in 350 μl of 0.1 *M* sodium phosphate buffer (pH 8.0) were added to 100 mg of derivatized silica, and the mixture was stirred for 2 h at 22°C. The preparation was then slurry-packed in a 35$\times$1.8 mm I.D. glass-lined stainless-steel column and stored in 0.1 *M* phosphate buffer with 3 m*M* sodium azide at 6°C when not in use.

Several attempts were made to pack the enzymes in series, with the AChE first since there is not an equilibrium problem with the hydrolysis of ACh [14]. The AChE preparation showed poor flow characteristics with extreme back-pressures, and the idea was abandoned.

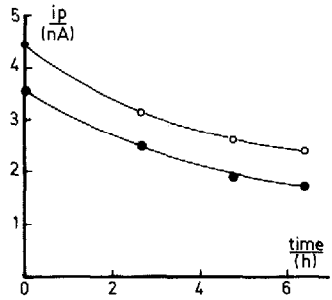

Fig. 1. Severe decrease in sensitivity of the platinum electrode during a day's work. The curves connecting the points have been constructed using the model $ip = A + Be^{-tC}$; 5 pmol each of Ch (o) and ACh (•) were injected.

Preparation and maintenance of the working electrode

The platinum working electrode used to detect hydrogen peroxide needed special care to work properly. The oxidation of hydrogen peroxide is suggested to be mediated by platinum(II) oxide on the electrode surface [19]. Thus a reproducible oxide layer, rather than a reproducible platinum surface is required for reproducible determinations of hydrogen peroxide.

The electrode was polished with 1-μm alumina before the cell was assembled. The potential was set to +200 mV, the cell was switched on, and the potential was then turned to +460 mV. It was found that even a short potential pulse reaching +900 mV would cause rapid loss of sensitivity. There still remained a loss of sensitivity during operation due to electrode deactivation, but in a rather predictable way. Since the integrator used had a built-in clock, it could be programmed to print the time of day for every injection. Thus, for every peak, the hour, minute and second of its elution could be determined. Since every fourth or fifth sample was a reference, a graph describing the decrease of sensitivity during the day could be constructed (Fig. 1). This was done using a standard non-linear regression model and assuming an exponential decay of sensitivity ($ip = A + Be^{-tC}$, where *ip* is peak current). A correction factor for each time of the day could thus be calculated. The constants calculated for the equation were different from day to day, and thus had to be determined for every correction graph.

Microdialysis

Male Sprague–Dawley rats weighing 250–350 g were anaesthetized with chloral hydrate (300 mg/kg and then 120 mg/kg per hour). The laminae of vertebrae Th_{13}-L_1 were removed and a microdialysis probe (CMA-10, Caregie Medicin, Solna, Sweden) with a 1-mm long dialysis membrane was inserted into the spinal cord at the L_3 segment (2 mm deep, 0.5 mm medial of dorsolateral sulcus).

The probe was perfused (1 μl/min) with Ringer solution, with the addition of 0.2 m*M* physiostigmin, and the perfusate was collected in 20-μl fractions and stored at −20 °C until analysed.

RESULTS AND DISCUSSION

Separation system

The pH optimum of the immobilized enzymes lies between 7.8 and 8.3 [7], which makes necessary either that the separation be carried out at this pH or the use of a make-up flow from a second pump to raise the pH after the separation. We decided to use the first alternative to decrease system complexity and sample dilution. The problem with dissolution of the silica in mobile phases of high pH was overcome by the use of a "saturator column" [12]. This column was packed with uncoated silica, which slowly dissolved and thus protected the analytical column, although the mobile phase was probably not actually "saturated" with silica. Without the saturator column, the analytical column failed to work properly after less than a week of continuous use, but when the former was installed the lifetime of the latter exceeded three months. Furthermore, the separation of quaternary ammonium compounds at this high pH required that the column had minimal residual silanol activity, which was usually not the case with the columns tested. The addition of 20 m*M* TMACl to the mobile phase was found to suppress the silanol activity and peak tailing. SDS was used to get any retention at all for Ch, and acetone was used to speed up the elution of ACh.

Acetone was found to be a useful organic modifier. Common primary alcohols are oxidized at the platinum electrodes at the potential used to detect hydrogen peroxide, and acetonitrile denatures the enzymes. The final concentrations of SDS and acetone was selected on a trial-and-error basis.

Characteristics of the enzyme reactor

The reactor showed no degradation for the first week of continuous use, but during the following two months it lost ca. 40% of its activity. Some reactors showed a very rapid degradation, the main cause probably being bacterial growth. When 3 m*M* sodium azide was added to the storage buffer, the problem disappeared. The operating temperture of 32°C was a compromise; a higher temperature gave a slightly higher signal, but also more problems due to gas bubbles, increased background current and baseline noise.

Sensitivity

The calibration graphs had to be corrected for the decrease in electrode sensitivity, as described in Experimental. The corrected graph was linear in the range 0.2–20 μM with a slope of 0.67 ± 0.01 and 0.58 ± 0.01 and an intercept of 0.004 ± 0.03 and 0.08 ± 0.1 for Ch and ACh, respectively (concentration, μM; response, nA; $n = 10$, 95% confidence limit). With a noise of 3 pA, this corresponds to a limit of detection of ca. 70 fmol for Ch and 90 fmol for ACh at three times the noise level. This theoretical value compares well with the practical limit, about twice as high achieved during routine operation of the system. However, the correction applied makes it difficult to draw further conclusions from statistical treatment of the observations.

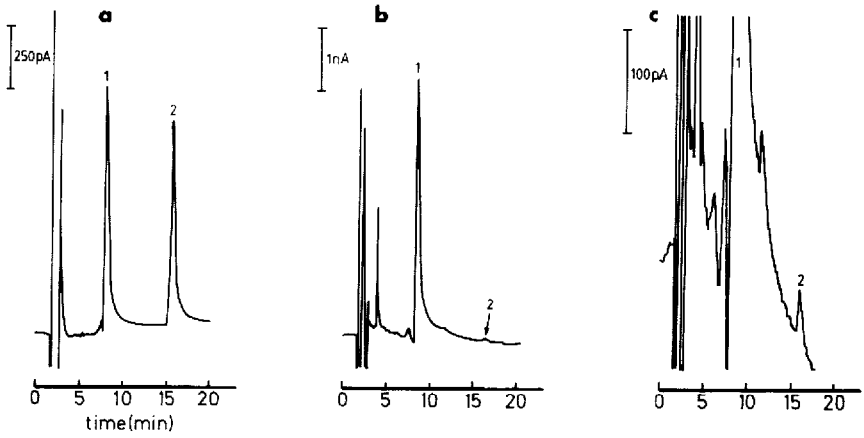

Fig. 2. (a) Separation of a standard mixture containing 5 pmol each of Ch and ACh in 5 μl of water. (b) Separation of 5 μl of microdialysate from rat spinal cord. (c) Same as Fig. 2b, but reintegrated with 16 times less attenuation. Peaks: 1 = Ch; 2 = ACh. Note that the ACh peak is barely visible at the sensitivity used to determine Ch.

In vitro recovery from dialysis probe

The probe was immersed in a 0.1 m*M* solution of Ch chloride and ACh chloride and perfused at 1 μl/min with Ringer solution. The perfusate was collected in 20-μl fractions and analysed for Ch and ACh. The recovery in vitro was found to be 15.8% for Ch and 14.9% for ACh.

Validation

Verification of purity and identity of the peaks can be done simply by removing the enzyme reactor and injecting standards and samples. When this was done, no peaks were detected at the retention times of ACh and Ch. The possibility of the coelution of an unknown substrate for the enzymes still exists, but it seems unlikely.

Chromatographic determination of acetylcholine and choline

Chromatograms were recorded for references and samples (Fig. 2). The total analysis time was 16–17 min, but it could easily be adjusted by changing the acetone or SDS concentrations. The use of an integrator with reintegration capability or a two-channel recorder is necessary to determine ACh and Ch in microdialysates. The concentration of Ch is ca. 50 times the concentration of ACh, and it is quite difficult to change the detector sensitivity manually during an analysis.

In Fig. 2c, the chromatogram from Fig. 2b is reintegrated with a decreased attenuation to visualize the ACh peak. The integration and peak detection parameters also have to be changed, depending on which of the two analytes is to be determined. Typical levels are in the ranges 2–3 μM for Ch and 40–60 nM for ACh.

Work is in progress to analyse larger series of samples, and different kinds of manipulation of the transmitter system are performed.

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