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An HPLC assay procedure of sensitivity and stability for measurement of acetylcholine and choline in neuronal tissue

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A method is presented for the sensitive and specific determination of acetylcholine and choline in neuronal tissue. The method is based on the separation of acetylcholine and choline by reversed-phase HPLC, passing the eluent into a post-column reactor containing choline oxidase and acetylcholinesterase covalently bound to vinyl sulphone bonded onto a hydroxyethyl methacrylate support, and electrochemical detection of the hydrogen peroxide formed. The limit of detection of the procedure is 1 pmol for acetylcholine and 500 fmol for choline. The excellent baseline stability of the method ensures the rapid and reliable processing of a large number of samples.

Levels of acetylcholine and choline can be measured in a number of ways including the traditional approaches of bioassay, radioenzymatic assay or gas chromatography–mass spectrometry (see review by Hanin 1982). Since these methods are in general time consuming and expensive, interest has increased in the development of a suitable technique based on high-performance liquid chromatography (HPLC) for the detection and assay of acetylcholine and choline in the same sample. Such methods are based on the separation of acetylcholine and choline by reversed-phase HPLC, followed by electrochemical detection of the H_2O_2 produced enzymatically by reacting the column eluent with choline : oxygen 1-oxidoreductase; EC 1.1.3.17 (choline oxidase) and acetylcholine acetylhydrolase; EC 3.1.1.7 (acetylcholinesterase). In the method developed by Potter et al (1983), the eluent from the column was mixed with a separate solution containing choline oxidase and acetylcholinesterase. However, the method as reported is expensive, requiring large amounts of enzymes. Subsequent techniques have attempted to reduce the quantity of enzymes required by immobilizing them in a post-column reactor. One of the simplest techniques is to adsorb the enzymes onto an anionic-ion exchanger (Eva et al 1984), but since the enzymes are gradually eluted from the resin, the choice of mobile phases for separation is limited to those of low ionic strength at a pH well-removed from the isoelectric point of the enzymes (Meek & Eva 1984).

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Damsma et al (1985) employed a different approach using cyanobromide-activated Sepharose 4B as an inert carrier on which to immobilize the enzymes. But enzymatic activity was reported to decrease progressively over a two-week period, considerable poisoning of the electrode occurred and slow flow-rates were required to avoid mechanical damage to the Sepharose column. Yao & Sato (1985) obtained a stable reactor by immobilizing the enzymes to alkylamino-bonded silica. However, an additional stream of high-pH buffer between the analytical and post-reactor columns was required and the sensitivity of the electrode to H_2O_2 was reported to decrease over a period of hours.

In the present study we have developed a simple, sensitive, economic and stable HPLC system for the detection and assay of acetylcholine and choline, in which choline oxidase and acetylcholinesterase are covalently bound in a post-column reactor to a vinyl sulphone–hydroxyethyl methacrylate support.

METHODS

Materials

Vinylsulphone–hydroxyethyl methacrylate (HEMA Vinyl Sulphone) was obtained from Anachem Ltd., Luton, UK. Hydrogen peroxide, ammonium sulphate, sodium acetate, sodium chloride and perchloric acid (all Analar grade) and scintillation grade Triton X-100 were purchased from BDH Chemicals Ltd., Poole, UK. Sodium octane sulphonate (HPLC grade) was obtained from Fisons, Loughborough, UK; 1-bromoethane and 3-dimethylamino-1-propanol were obtained from Lancaster Synthesis,

Lancashire, UK; sodium dihydrogen orthophosphate (Analar grade) was obtained from Hopkin and Williams Ltd., Essex, UK; diethyl ether (reagent grade) was obtained from May and Baker, Dagenham, UK; and Bio-Rad protein assay reagent was purchased from Bio-Rad Laboratories, München, West Germany; choline oxidase (EC.1.1.3.17) (*Alcaligenes*) and acetylcholinesterase (EC.3.1.1.7, type VI-S) from electric eel, and all other chemicals were obtained from Sigma Chemical Company, St Louis, MO, USA. All chemicals were used as received and all solutions were prepared using Milli-Q Reagent grade water (Millipore S.A., Molsheim, France).

Assay system

The HPLC system is shown schematically in Fig. 1. The optimized mobile (10 mM Trizma acetate buffer, 140 μM octane sulphonic acid and 1·0 mM tetraethylammonium chloride, pH 7·8) was delivered at a rate of 1·3 mL min^{-1} by a liquid chromatography pump (Kratos Spectroflow 400). An automatic sample injector (Wisp 710B, Waters, or Gilson 231/401, Anachem) was used to introduce samples and standards to the system. A reversed-phase PLRP-S

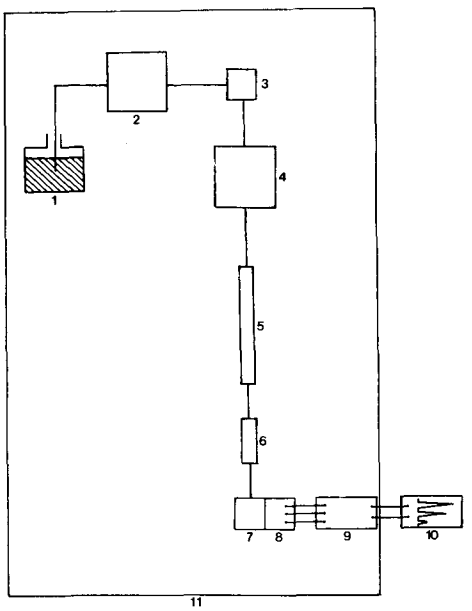

FIG. 1. An HPLC system for the sensitive and specific determination of acetylcholine and choline with the immobilized enzyme reactor: (1) mobile phase; (2) HPLC pump; (3) guard cell; (4) automatic injector; (5) PLRP-S analytical column; (6) enzyme reactor; (7) screening graphite electrode; (8) platinum wall jet electrode; (9) amplifier; (10) recorder; (11) temperature control unit.

(Polymer Laboratories) analytical column (50 × 4·6 mm i.d.) with particle size of 5 μm and porosity 100 Å was used for the separation of choline (Ch), monoethylhomocholine (EHC) and acetylcholine (ACh). The enzyme reactor column (40 × 4·6 mm i.d.) containing covalently bound choline oxidase and acetylcholinesterase was inserted between the analytical column and the detector. The electrochemical detector system employed was the ESA Coulochem 5100A with a model 5012 platinum wall jet electrode. The voltage applied to the platinum electrode was 250 mV (versus the solid state reference electrode incorporated into the analytical cell) and the detector signal was monitored using a Hewlett-Packard 3392A printing integrator. The screening electrode of porous graphite, detector 1, located after the enzyme reactor column was set at 290 mV. The guard cell (ESA Model 5020) was placed between the pump and injector and was set at 700 mV. The assay system (except the integrator) was maintained within a temperature controlled cabinet at 35 °C (Fig. 1).

In preliminary experiments a number of alternative approaches were explored. Thus a Hypersil 5-μm ODS column (100 × 4·9 mm i.d.) (HPLC Tehnology, Macclesfield, Cheshire, UK) was evaluated in addition to the polymer column, but it was necessary for the pH of the mobile phase to be below pH 6·5 to prevent dissolution of the silica; this was below the optimum pH of the enzyme cartridge. Moreover, marked tailing of the acetylcholine peak was observed, possibly reflecting ionic retention on the residual silanol groups of the reversed-phase materials. To some extent this could be overcome by increasing the concentration of the ion-pairing reagent (in this case, 2·0 mM tetraethylammonium chloride) in the mobile phase, but this was at the cost of inhibiting enzymatic activity in the post-column reactor.

In a further modification an ESA model 5011 analytical cell was used to replace the 5012 platinum wall jet electrode. The ESA model 5011 contains two porous graphite electrodes in series: detector 1 was set at a screening potential of 290 mV whilst detector 2 was monitored at 750 mV. However, the background current was found to be considerably increased, leading to a reduction in sensitivity when compared with the platinum wall jet system.

Preparation of the enzyme reactor

The enzyme column (40 × 4·6 mm i.d.) was slurry packed with vinyl sulphone–hydroxyethyl methacrylate, washed with Milli-Q water and then equi-

librated with the enzyme coupling buffer (0·5 M phosphate buffer and 1·0 M ammonium sulphate, pH 9·0). 250 units of choline oxidase (one unit will form 1·0 μmol of H_2O_2 from choline and H_2O per minute at pH 8·0 and 37 °C) and 2000 units of acetylcholinesterase (one unit will hydrolyse 1·0 μmol of acetylcholine to choline and acetate per minute at pH 8·0 and 37 °C) were added to 50 mL of the coupling buffer and this was pumped at a rate of 0·3 mL min^{-1} through the vinylsulphone–hydroxyethyl methacrylate column in a recycling system for 72 h at 4 °C. The column containing the immobilized enzymes was then washed sequentially with 5·0 mL of 0·2 M acetate buffer at pH 5·0, 5·0 mL of 1·0 M sodium chloride and then 5·0 mL of 0·1 M phosphate buffer at pH 9·0. To block any remaining active vinyl sulphone groups on the matrix, 0·1 M ethanolamine with 0·1% v/v Triton X-100 was pumped through the column at 0·3 mL min^{-1} for 2 h. The column was then cleaned with Milli-Q water pumped at 0·3 mL min^{-1} for 1 h. The post-column reactor was then incorporated into the assay system or stored after filling with mobile phase, both ends being capped, at 4 °C.

Preparation of monoethylhomocholine

Monoethylhomocholine was used as an internal standard. The compound was prepared by adding 2·0 mL 1-bromoethane to 2·0 mL of 3-dimethylamino-1-propanol and gently stirring for 30 min at room temperature (20 °C). Diethyl ether (10 mL) was quickly mixed with the solution and the precipitate collected, the crystals being dried under vacuum and stored in a desiccator at −20 °C.

Preparation of standards

Standards of acetylcholine, monoethylhomocholine and choline were freshly prepared in 0·2 M perchloric acid or acidified mobile phase at pH 4·0.

Preparation of tissue samples

Male Sprague-Dawley rats (250 g) (Bradford Strain) were killed by microwave irradiation (output power 5 kW, 2·45 GH_3, 7 s; Elnode Ltd., Luton, UK), the brains removed and the striatum dissected out. The tissues were placed in Eppendorf vials and frozen in liquid nitrogen. Tissues were then homogenized in 200 μL 0·2 M perchloric acid containing 100 μM monoethylhomocholine using an ultrasonic homogenizer (Soniprep 150 MSE) for 3 s and the samples stored in liquid nitrogen.

Extraction of samples and standards

The extraction of acetylcholine, choline and monoethylhomocholine was performed in a similar manner to the method of Eva et al (1984).

Brain samples were removed from storage in liquid nitrogen and centrifuged (15 600 *g*) at 4 °C for 3 min in an Eppendorf centrifuge. 90 μL of the supernatant solution was transferred to a second Eppendorf; if required, the remaining supernatant can be used to assay the catecholamines and indoleamines in the sample. The denatured protein pellet was resuspended in 100 μL 0·1 M phosphate buffer, pH 7·4 and the protein content assayed by the method of Bradford (1976) using bovine serum albumin as a standard.

The perchlorate ions in the 90 μL of supernatant were removed by adding 15 μL 7·5 M potassium acetate followed by the removal of the resultant precipitate by centrifugation (15 600 *g*) at 4 °C for 3 min. The supernatant was transferred to a third Eppendorf for precipitation of the choline, monoethylhomocholine and acetylcholine by the addition of 20 μL 5·0 M tetraethylammonium chloride and 500 μL of cold 2% w/v Reineckate solution. (The Reineckate solution was prepared 1 h before use and left at 4 °C; it was then filtered before use.) The mixtures were left at 4 °C for 1 h and then centrifuged (15 600 *g*) at 4 °C for 3 min. The supernatant was then discarded leaving the precipitated quaternary ammonium compounds which were then gently washed by resuspension in cold (4 °C) 100 mM Trizma acetate buffer, pH 7·4, followed by centrifugation (15 600 *g*) at 4 °C for 3 min. The quaternary ammonium precipitates were dissolved by the addition of 150 μL of a 50% suspension of Dowex-1 acetate in acidified (pH 4·0) mobile phase. The mixture was then vortexed and allowed to stand at 4 °C for 5 min, revortexed and then centrifuged (15 600 *g*) at 4 °C for 3 min. The supernatant was then injected into the HPLC.

Standards made up in 0·2 M perchloric acid containing 100 μM EHC were passed through the extraction procedure to measure the percentage extraction.

RESULTS

The assay procedure using a reversed-phase PLRP-S analytical column connected to the enzyme reactor gave an excellent separation of choline and acetylcholine in striatal brain tissue. Typical chromatograms for the elution profile of A, the injection of a striatal extract, and B, standard solutions of choline and acetylcholine (including monoethylhomocholine as internal standard), are shown in Fig. 2. Each sample was processed within 13 min (Fig. 2) reflecting the flow rate of 1·3 mL min^{-1}. The relatively high flow rate was made possible due to the physical

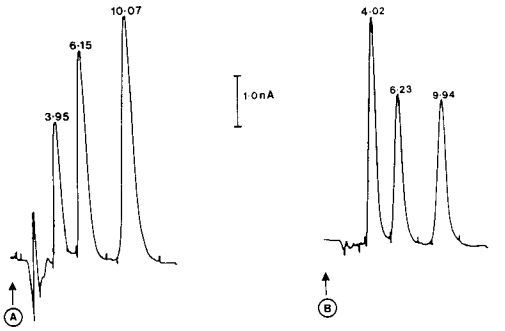

FIG. 2. Typical chromatograms obtained for A, 70 μL of striatal tissue extract. Monoethylhomocholine (20 nmol) was added to the original tissue homogenate and B, 5 μL standard solution of (in elution order) choline (500 pmol), monoethylhomocholine (1250 pmol) and acetylcholine (500 pmol). The figures represent the retention time for each peak (min).

stability of the hydroxymethyl methacrylate support in the enzyme reactor. The chemical stability of the PLRP-S stationary phase in the analytical column allowed the use of eluents within a wide pH range; the mobile phase was adjusted to pH 7·8, this being optimum for enzymatic activity in the post-column reactor.

The conversion of ACh, Ch and EHC to H_2O_2 was assessed by comparing the detector response to injections of standards of H_2O_2, Ch, EHC and ACh. The conversion of ACh and Ch to H_2O_2 was high at 95·4 and 97·8%, respectively, the conversion of EHC at 27·5% being more modest (Table 1).

Table 1. Conversion of acetylcholine (ACh), choline (Ch) and monoethylhomocholine (EHC) to hydrogen peroxide.

Substrate or product	Peak height (mm)	Conversion to H_2O_2 (%)
H_2O_2 (2 nmol)	30·5 ± 0·74	100 ± 2·4
Ch (1 nmol)	29·6 ± 0·73	97·8 ± 2·4
EHC (2·5 nmol)	21·06 ± 0·16	27·5 ± 0·2
ACh (1 nmol)	29·1 ± 1·47	95·4 ± 4·8

Hydrogen peroxide, ACh, Ch and EHC were dissolved in acidified mobile phase (pH 4·0) and injected in a volume of 1·0 μL. Data are presented as the mean ± s.e.m. (n = 5).

To determine the recoveries from the extraction procedure, 90 μL of standard solution containing 150 pmol μL^{-1} ACh and Ch in 0·2 M perchloric acid containing 100 μM EHC was passed through the procedure. The recoveries of ACh, Ch and EHC were 38 ± 4, 47 ± 1 and 46 ± 2% (mean ± s.e.m., n = 5), respectively.

Repeated injection from a pooled sample resulted in an intra-assay relative standard deviation of 1·22 and 1·43% for choline and acetylcholine, respectively (n = 6). Repeated injections of a sample (n = 6) on 6 consecutive days resulted in an interassay relative standard deviation of 1·36 and 1·99% for choline and acetylcholine, respectively.

To investigate the relationship between the amount of ACh and Ch and the detector response, standards were injected containing different amounts of the two compounds along with the internal standard. For ACh and Ch there was a linear relationship between peak height ratio of ACh or Ch to the internal standard and the concentration of ACh and Ch, respectively, over at least 3 orders of magnitude (Fig. 3). The on-column detection limit

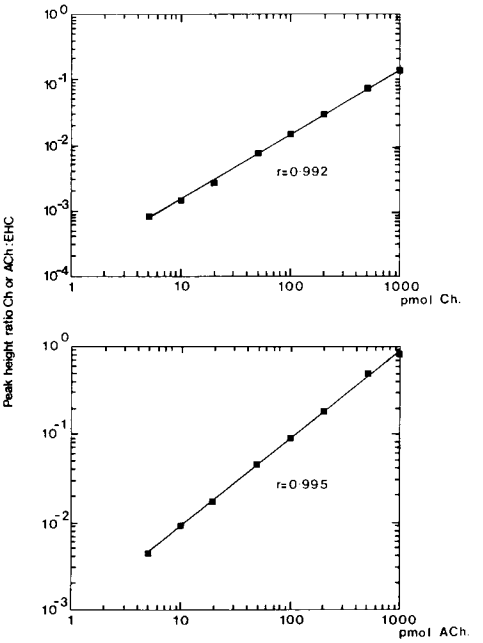

FIG. 3. Calibration graphs for the determination of choline (Ch) and acetylcholine (ACh) by the proposed procedure (r = correlation coefficient). Each value represents the mean of 3 determinations, standard errors were in the range of 0·3–4·7%.

was 500 fmol and 1 pmol for Ch and ACh, respectively (SNR = 2). However, the practical assay limit for automated analysis using brain tissue samples is limited by the baseline noise level, and approximates to 5 pmol and 10 pmol for choline and acetylcholine, respectively.

The actual levels of ACh and Ch determined within striatal tissue were 544 ± 28 and 181 ± 9 pmol mg^{-1} protein, respectively (mean ± s.e.m., n = 9). These are comparable with those reported by Potter et al (1983) when allowance is made for the different methods used for the protein assays.

The preparation and use of the enzyme reactor is straightforward. After the post-column reactor was

connected to the HPLC system the equipment was allowed to equilibrate for 2 days. Within the 8 weeks of experimentation there was no significant diminution in sensitivity, and many hundreds of samples were routinely analysed with the enzyme reactor. Furthermore, and of equal importance, the baseline stability was consistent at a sensitivity of 8·0 nA F.S.D.

DISCUSSION

HPLC assays for the detection of acetylcholine and choline have been based on the electrochemical detection of H_2O_2 enzymatically produced via the action of choline oxidase and acetylcholinesterase (Potter et al 1983). The enzymes were retained on an anionic-ion exchanger or inert support in a post-column reactor. The major disadvantage reported for such techniques is the progressive loss of enzymatic activity (Eva et al 1984; Damsma et al 1985). In the present paper we report an HPLC assay for acetylcholine and choline for which there are several advantages over previous techniques.

The major innovation of the assay is the use of a post-column reactor, where choline oxidase and acetylcholinesterase are immobilized by covalent bonding to vinyl sulphone on a hydroxyethyl methacrylate support. Our use of hydroxyethyl methacrylate was based on the studies of Taylor (1985) who investigated the immobilization and retention of activity of a number of enzymes on a variety of liquid chromatographic supports: hydroxyethyl methacrylate was identified as a particularly useful material. Vinyl sulphone was chosen as having a high degree of reactivity towards the hydroxyl groups. It was reasoned that the pressure stability of hydroxyethyl methacrylate should allow its use in HPLC. The essential finding of the study is that acetylcholinesterase and choline oxidase can be readily immobilized to the vinyl sulphone-hydroxyethyl methacrylate support, when they retain their activity to hydrolyse and oxidize acetylcholine and choline to H_2O_2 over at least 8 weeks, the period of experimentation involved in the present work. This stability of response greatly facilitates the routine assay of acetylcholine and choline. Furthermore, the conversion of acetylcholine, choline and the internal standard monoethylhomocholine to H_2O_2 was found to be quantitative during the 20 s residence time in the post-column reactor under the conditions employed. The intrinsic sensitivity of the assay for acetylcholine and choline was of the order of 500 fmol–1·0 pmol; even when assaying brain tissue samples accompanied by a higher background noise, the sensitivity of the procedure was found to be 10 pmol for acetylcholine and 5 pmol for choline.

The proposed assay has further advantages. (i) The stability of the post-column enzymatic reactor reduces to the minimum requirement for expensive enzymes. (ii) The vinyl sulphone–hydroxyethyl methacrylate system is commercially available and inexpensive. (iii) The pressure stability of the support system is such that normal flow rates can be maintained under elevated pressure conditions to ensure a rapid turnover of results. (iv) The stability of the assay procedure is such that, with the judicious use of periodic standards, automation of the injection process is possible for the routine analysis of large numbers of samples. (v) Absence of poisoning of the platinum electrode was noteworthy; this was probably due to the use of the guard cell and screening electrode, together with the platinum electrode at a potential of 250 mV. (vi) The system proposed is homogeneous, in that the PLRP-S analytical column permitted the use of a mobile phase which was also suitable for the post-column reactor; unlike an earlier report (Yao & Sato 1985) there was no requirement for an additional stream of high pH buffer to be introduced between the analytical and enzyme reactor columns.

In summary we report an assay for the sensitive and specific determination of acetylcholine and choline in neuronal tissue. The reagents used are inexpensive, the procedures can be performed relatively easily, and the assay has excellent long-term baseline stability to ensure the rapid and reliable processing of large numbers of samples. Furthermore, whilst applied to the measurement of acetylcholine and choline, the assay procedure may find application in other assays where the reaction produces an electroactive compound.

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REFERENCES

Bradford, M. (1976) Anal. Biochem. 72: 248–254

Damsma, G., Westerink, B. H. C., Horn, A. S. (1985) J. Neurochem. 45: 1649–1652

Eva, C., Hadjiconstantinou, M., Neff, N. H., Meek, J. L. (1984) Anal. Biochem. 143: 320–324

Hanin, I. (1982) In: Modern Methods in Pharmacology (Spector, S., Back N., eds), pp 29–38. Alan R. Liss, New York

Meek, J. L., Eva, C. (1984) J. Chromatogr. 317: 343–347

Potter, P. E., Meek, J. L., Neff, N. H. (1983) J. Neurochem. 41: 188–194

Taylor, R. F. (1985) Anal. Chim. Acta 172: 241–248

Yao, T., Sato, M. (1985) Ibid. 172: 371–373