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Note

Measurement of acetylcholine and choline in cerebrospinal fluid by high-performance liquid chromatography: failure to detect acetylcholine in normal human cerebrospinal fluid

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In recent years several papers have been published on the levels of acetylcholine (ACh) and choline (Ch) in normal and pathological cerebrospinal fluid (CSF). Although the presence of Ch in CSF is well established, results on the occurrence of ACh in CSF are conflicting. Concentrations of ACh ranging from non-detectable [1,2] to values as high as 250 pmol/ml [3,4] have been described in normal human CSF. Under pathological conditions the levels of ACh have been reported to increase in cerebral trauma, epilepsia and tumours [1,4] and to decrease in Alzheimer's disease [5,6] for example.

Several methods have been employed to quantify ACh and Ch in these CSF studies, including bioassays [1,3,7], radiometric assays [4] and gas chromatography–mass spectrometry (GC–MS) [5,6,8]. Recently high-performance liquid chromatographic (HPLC) method for the analysis of ACh and Ch was

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reported [9]. After separation of ACh and Ch on a reversed-phase column the components were mixed with an enzyme solution consisting of a mixture of acetylcholinesterase (AChE) and choline oxidase (ChO). In this process ACh is hydrolysed to acetate and Ch, and the latter is converted into betaine and hydrogen peroxide; the peroxide is detected electrochemically. An improvement in the method involved the immobilization of the enzymes in a post-column reactor [10–12].

This paper describes the measurement of ACh and Ch in CSF by HPLC, an enzyme reactor and electrochemical detection, and the use of this sensitive method to investigate the presence of ACh in human CSF.

EXPERIMENTAL

Reagents and chemicals

Acetylcholine chloride, choline chloride, acetylcholine esterase (EC 3.1.1.7, type VI-S), choline oxidase (EC 1.1.3.17) and neostigmine bromide were obtained from Sigma (St. Louis, MO, U.S.A.). 11-Aminoundecanic acid (AUA), 1-heptanesulphonic acid sodium salt (HSA) and 1-octanesulphonic acid sodium salt (OSA) were obtained from Fisons (Loughborough, U.K.). Tetramethylammonium (TMA) chloride was obtained from Janssen (Beerse, Belgium), and cyanogen bromide (CNBr)-activated Sepharose 4B was from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.). Water purified by a Milli-Q system (super-C cartridge, ion-exchange cartridge, orgamex-Q cartridge, 0.2-μm filter; Millipore, Bedford, MA, U.S.A.) was used for aqueous solutions.

Chromatography

The high-performance liquid chromatograph consisted of the following components: a Beckman 112 solvent-delivery system (Beckman, Anaheim, CA, U.S.A.), a 20-μl Rheodyne 7152 injector (Rheodyne, Cotati, CA, U.S.A.), a Chromsep separation system containing a guard column (reversed-phase, 10 mm $\times$ 2.1 mm I.D.) and a separation column (Nucleosil $5C_{18}$, 100 mm $\times$ 3.1 mm I.D.) both from Chrompack (Middelburg, The Netherlands), an enzyme reactor (75 mm $\times$ 2.1 mm I.D.), Sepharose 4B-immobilized acetylcholinesterase and choline oxidase according to Damsma et al. [11], and a laboratory-made rotating electrochemical detector. The elution buffer contained 0.2 *M* phosphate and 0.1 m*M* EDTA adjusted to pH 7.9 and was filtered at 0.45 μm. Variable amounts of AUA, HSA, OSA and TMA were added to the elution buffer as described in Results.

Patients and samples

CSF samples were obtained from six patients (mean age 45.0 years, range 27–68 years) with disc herniation by lumbar puncture. The lumbar punctures were performed in the lateral decubitus position. A 2-ml volume of the CSF was collected in a tube containing 2 μl of 10 m*M* neostigmine. Each of the three CSF samples was divided into two portions of 1 ml, to one of which were added 10 μl of a standard ACh (1 nmol/ml) solution in 0.01% acetic acid.

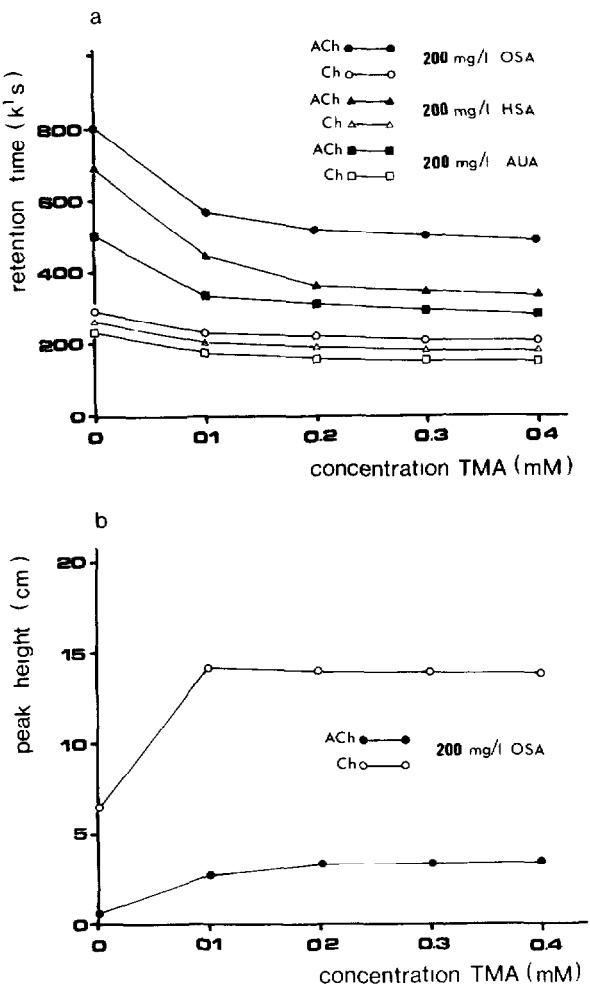

Fig. 1. Effect of different ion-pair formers and different TMA concentrations on retention times (a) and peak heights (b) of ACh and Ch. Each point represents the average of at least three standard injections (Ch, 16 pmol per 20 μl; ACh, 13 pmol per 20 μl).

RESULTS

Chromatography

Optimal conditions for separation of Ch and ACh were established by varying the concentrations of ion-pair formers and TMA. Increasing concentrations of OSA, HSA and AUA up to 200 mg/l improved the resolution of the Ch and ACh. From Fig. 1a it can be seen that good resolution was obtained with OSA and without TMA. However, without TMA, Ch and ACh were absorbed on the column and were eluted as broad peaks. Addition of small amounts TMA (submillimolar) improved dramatically the peak shape as is shown in Fig. 1b. Peak shapes could also be enhanced by the addition of potassium ions, but this effect was obtained only after addition in the submolar range and remained less effective than TMA. In the subsequent analyses, 200 mg/l OSA and 0.4 m*M* TMA were added to the elution buffer.

Fig. 2 shows typical elution patterns for Ch and ACh standards and CSF. The sensitivity of the assay using the conditions as described was 50 fmol per 20-μl injection for both Ch and ACh. The response was linear from 50 fmol to

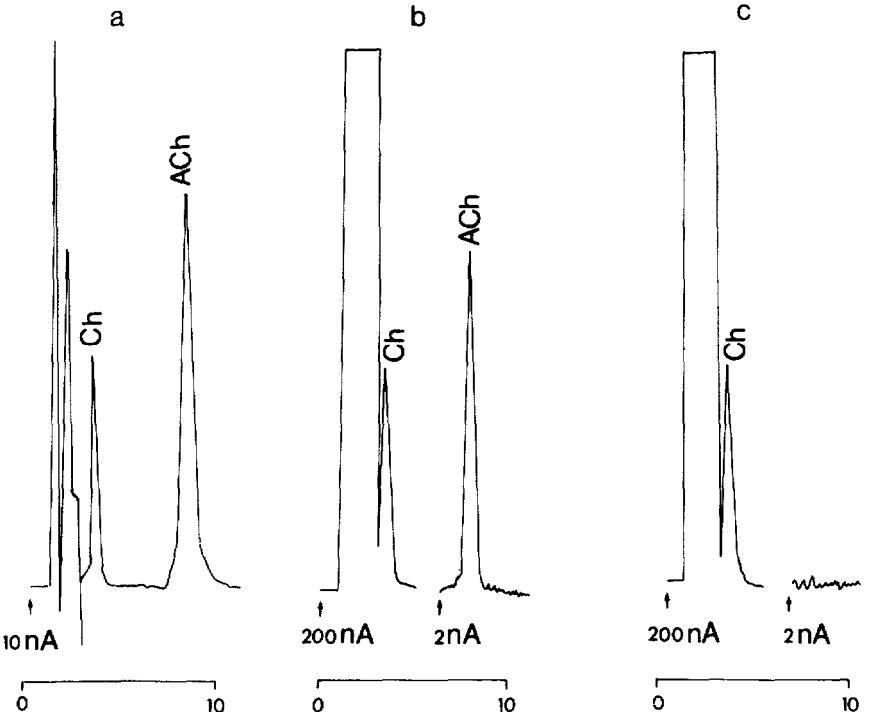

Fig. 2. Chromatograms of (a) a standard mixture of Ch (3 pmol per 20 μl) and ACh (15 pmol per 20 μl), (b) a CSF sample containing 10^{-5} *M* neostigmine and spiked with ACh (0.2 pmol per 20 μl) and (c) the same CSF sample without spiking.

at least 50 pmol per 20-μl injection. The reproducibility of the method was demonstrated by injection of a five-fold standard solution containing 16 pmol Ch and 13 pmol ACh per 20-μl injection. The within-day coefficients of variation for Ch and ACh were 1.3 and 1.8%, respectively.

CSF acetylcholine esterase

AChE is present in abundant amounts in human CSF [4]. We evaluated the presence of this enzyme in normal CSF by investigating the degradation of ACh added at a concentration of 55 nmol/ml. As can be seen from Fig. 3a, ACh

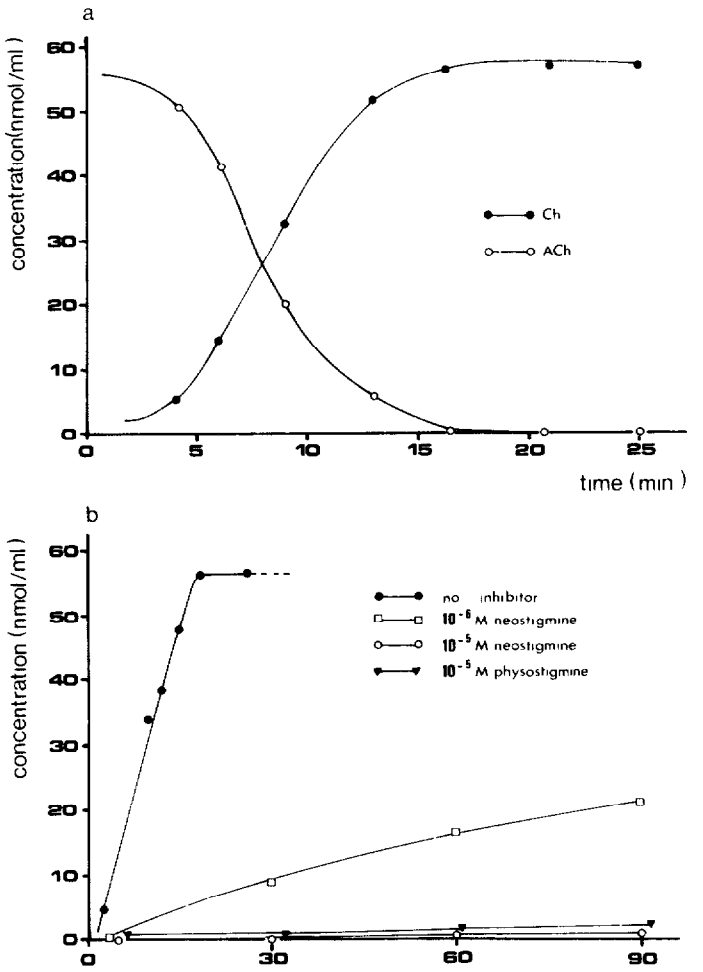

Fig. 3. Effect of AChE activity in CSF on ACh hydrolysis. (a) ACh is added at a concentration of 55 nmol/ml, and the conversion of ACh into Ch is measured. (b) Effect of AChE inhibitors on the concentration of Ch. Each point represents the average of three injections adequately diluted before HPLC analysis. The samples were incubated for different times at 37 °C.

was converted in a comparable nanomolar amount within 20 min. The calculated activity of AChE from these data was ca. 0.22 μmol/ml Ch per h, which is very close to the value reported in normal CSF by Haber and Grossman [4] (0.32 μmol/ml Ch per h).

The activity of AChE is proportionally blocked by the addition of inhibitors such as neostigmine or physostigmine. The degradation of supplemental ACh added to CSF (Fig. 3a) was prevented to the extent of 67, 96 and 98%, after 1 h of incubation, by the addition of 10^{-6} *M* neostigmine, 10^{-5} *M* physostigmine and 10^{-5} *M* neostigmine, respectively. Concentrations of up to 10^{-3} *M* in the samples do not inhibit the enzyme reactor of the HPLC system [13].

CSF acetylcholine and choline

CSF from six patients with disc herniation was analysed for the presence of ACh and Ch. To prevent hydrolysis of ACh a reversible AChE inhibitor, neostigmine, at a final concentration of 10^{-5} *M* was added to CSF-collecting tubes. Despite this we were not able to detect any ACh in the patient samples with the overall detection limit of ACh in CSF of 2.5 pmol/ml. The levels of Ch in CSF (Table I) were in the same range as previously reported [4,14,15]. To confirm that ACh was preserved during the sample preparation, ACh was added to the CSF (10 pmol/ml). The results show that the added ACh was recovered and could be quantified (Table I). The mean recovery of ACh added to CSF was $98 \pm 2\%$ ($n=3$).

TABLE I

CONCENTRATIONS OF Ch AND ACh IN NORMAL HUMAN CSF AND THE EFFECT OF ADDITION OF ACh

ACh was added to a concentration of 10 pmol/ml. CSF samples were collected in the presence of 10^{-5} *M* neostigmine. N.D. means not detectable: detection limit, 2.5 pmol/ml.

Patient	Spiking	Concentration (pmol/ml)	
		Ch	ACh
1, female	–	2120	N.D.
	+	2100	9.6
2, female	–	1610	N.D.
	+	1660	10.1
3, male	–	2530	N.D.
	+	2630	9.7
4, female	–	1150	N.D.
5, female	–	2490	N.D.
6, male	–	2930	N.D.

DISCUSSION

An HPLC method to measure ACh and Ch using a post-column enzyme reactor and an electrochemical detector is described. The method is sensitive, specific and simple. The preparation of the enzyme reactor has been described previously [11]. Instead of absorbing sodium dodecyl sulphate (SDS) on the analytical column [13,16] we accomplished good resolution of Ch and ACh by manipulation with other ion-pair formers, such as OSA. Relatively high concentrations of OSA (200 mg/l) were needed to achieve a good separation of Ch from the front. Small amounts of TMA (>0.2 mM) yielded clear peak shapes.

In other reports [9,10,12] lower concentrations of OSA and higher concentrations of TMA are described for the HPLC analysis of ACh and Ch. These methods were, however, intended for the analysis of purified brain extracts. Moreover, the pH values of the elution buffers were much lower than in the present method. In our system the direct application of CSF (20 μl) to the column is possible without any prepurification.

The enzyme reactor contains ca. 250 μl of Sepharose 4B, to which are bound ca. 10 U of ChO and 20 U of AChE. If we take the void volume of the reactor to be 50% and the flow-rate of the solutes to be 0.6 ml/min, the residence time of the reactants in the reactor is ca. 0.22 min. The conversion of the enzyme reactor within this time can be calculated to be ca. 2200 nmol for ChO and 4400 nmol for AChE.

At a working concentration in our experiments of maximally 1 nmol per injection, the excess of the enzyme activity is at least 1000-fold. It can be safely concluded that the conversion of ACh and Ch is instantaneous, and that peak shapes are not influenced by the enzyme reactor.

In the present study we were not able to detect any endogenous ACh (detection limit 2.5 pmol/ml) in normal human CSF. It is reasonable to believe that the high activity of AChE in CSF is responsible for the absence of ACh.

When high amounts of ACh were added to CSF this ACh was converted into Ch after a short incubation period (Fig. 3a). If an inhibitor of AChE was added together with the ACh to the CSF, the ACh was protected from hydrolysis (Fig. 3b). From these data we conclude that normal human CSF does not contain detectable amounts of ACh, and that this is probably due to the activity of AChE.

The data of our study confirm and extend those of Tower and McEachern [1]. These authors found no ACh in normal CSF analysed by bioassay, but demonstrated its presence in samples taken from epileptics, as well as in those obtained from patients following head trauma. Schain [7] reviewed the results of thirteen studies, all making use of bioassay methods, which were extremely varied. Many authors found no ACh in the normal CSF, others claimed very

low concentrations (less than 1 pmol/ml) and still others concentrations of up to 600 pmol/ml.

In the presence of neurological disorders the cited ACh levels are generally above normal values. Other papers report a large range of concentrations in the normal lumbar CSF: Duvoisin and Dettbarn [3] stated values of 103 ± 33 pmol/ml by a bioassay method, Welch et al. [8] reported values of 70 ± 20 pmol/ml by a GC–MS method, and Haber and Grossmann [4] recorded values of 187 ± 84 pmol/ml by a radiometric method.

It is not easy to interpret these conflicting data. A possible explanation might be the fact that thiol reagents such as dithiothreitol [17] or imidazole-like substances such as creatinine or histamine [18] give rise to non-enzymic formation of ACh in CSF. As these substances are known to be present in normal CSF, ACh could be formed in this way during the incubation period that is included in the radiometric and bioassays (mostly in the presence of neostigmine or physostigmine) or during the isolation and derivatization steps in the GC–MS method. In our procedure no pretreatment was needed and manipulation of samples was minimal prior to the chromatographic analysis.

In conclusion, this paper describes a method for the direct determination of ACh and Ch in human CSF. The sample preparation is simple and the subsequent assay is based on chromatographic separation by ion-pair HPLC, followed by post-column enzymic derivation and subsequent electrochemical detection. The procedure is selective, sensitive and rather simple. Employing this HPLC method, we failed to detect ACh in normal human CSF.

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