Journal of Chromatography, 431 (1988) 389–394
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4327

Note

Determination of acetylcholine and choline in human cerebrospinal fluid using high-performance liquid chromatography combined with an immobilized enzyme reactor: ageing-induced change of acetylcholine level

SHINJI OKUYAMA* and YOSHIO IKEDA

Department of Neuropsychiatry, School of Medicine, Fujita-Gakuen Health University, Toyoake, Aichi 470-11 (Japan)

(First received February 2nd, 1988; revised manuscript received May 17th, 1988)

Acetylcholine (ACh), which plays a significant role as a neurotransmitter of cholinergic neurons, was identified in the central nervous system in 1929 by Dale and Dudley [1]. Recently, ACh has received renewed attention in connection with Alzheimer's disease, which is characterized by a decrease in cholinergic neurons [2]. Conventional methods for assaying ACh include bioassay, radioenzymatic assay and gas chromatography–mass spectrometry (GC–MS). In 1983, Potter et al [3] first reported a method for assaying ACh and choline (Ch) by high-performance liquid chromatography (HPLC) with electrochemical detection (ED) and determined them in brain tissue of rats. Until now ACh and Ch have not been determined by HPLC in cerebrospinal fluid (CSF) because of its very low concentration and the small volume of CSF. In this paper we describe a method for assaying ACh and Ch extracted from human CSF by a liquid cation-exchange procedure combined with HPLC–ED coupled with an immobilized enzyme reactor.

EXPERIMENTAL

Chemicals

Eserine and tetraphenylboron were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium 1-decanesulphonate, choline chloride, acetylcholine chloride and 3-heptanone were obtained from Tokyo Kasei (Tokyo, Japan). Tetramethylammonium chloride, disodium hydrogenphosphate, sodium dihydrogenphosphate

0378-4347/88/$03.50

and other chemicals were obtained commercially. Ethylhomocholine (EHC), which was used as an internal standard, was synthesized from 3-dimethylamino-1-propanol and iodoethane [2].

Samples and extraction

Samples of human CSF were obtained from 25 volunteers free from neurological and psychological diseases. The volunteers, nine males and sixteen females, were from 20 to 78 years old (mean ± S.D., 51.5 ± 18.1 years). The subjects were divided into three groups according to age. The youngest group (20–39 years old) consisted of seven subjects and the middle-aged (40–59 years old) and elderly groups (60–79 years old) consisted of nine subjects each. We collected 2 ml of human CSF by lumbar puncture in a plastic tube containing 2 μmol of eserine and kept it at 4°C until extraction. Extraction of ACh and Ch from CSF was performed within 12 h after collection according to the liquid cation-exchange method [4] as shown in Fig. 1. In brief, the samples were shaken with 1 ml of 3-heptanone containing 10 mg of tetraphenylboron for 10 min at room temperature and centrifuged at 1500 *g* for 20 min at 4°C. Then 500 μl of the supernatant were taken and added to 0.5 ml of 0.4 *M* hydrochloric acid. The mixture was shaken for 1 min and centrifuged at 1500 *g* for 5 min at 4°C. Finally, the organic layer was discarded by aspiration and the acid layer collected, lyophilized and stored at 4°C. Samples were dissolved in 100 μl of distilled water and 20 μl were injected into the HPLC system.

Apparatus and chromatographic conditions

The HPLC system consisted of an 880-50 degasser, an 880-Pu pump (Japan Spectroscopic, Tokyo, Japan), an LC-4A amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) equipped with a WE-PT platinum electrode (Eicom, Kyoto, Japan), a Rheodyne 7125 injector with a 200-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.), a Prepak guard column (5×4 mm I.D.) (Eicom), an RSpak DE613 (medium polarity methacrylate gel, 150×6 mm I.D.)

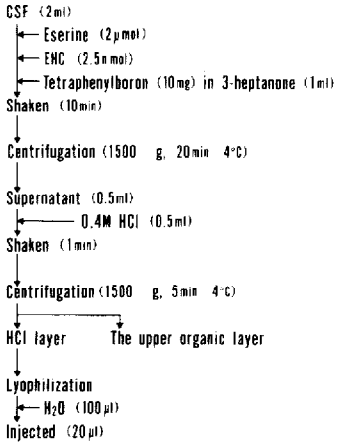

Fig. 1. Extraction of ACh and Ch from CSF.

(Shodex, Tokyo, Japan), an AC-Enzympak immobilized enzyme column (5×4 mm I.D.) (Eicom) [5] and a CA-Trap catecholamine trap column (5×4 mm I.D.) (Eicom). The mobile phase was 0.1 *M* sodium phosphate buffer (pH 8.3) containing 1.2 m*M* tetramethylammonium chloride (TMA) and 300 mg/l sodium 1-decanesulphonate, which was filtered through a 0.22-μm membrane filter (Millipore, Bedford, MA, U.S.A.). The HPLC separation and enzymatic reaction were performed at 37°C. The flow-rate was 1.0 ml/min. The electrode potential was set at +450 mV against an Ag/AgCl reference electrode for the detection of hydrogen peroxide. Under these conditions the retention times were Ch, 6.3 min, EHC 7.8 min and ACh, 11.3 min.

A statistical analysis was performed by the Wilcoxon rank-sum test.

RESULTS

This assay method is based on the separation of ACh and Ch on a Shodex RSpak column, followed by their enzymatic conversion to hydrogen peroxide through a post-column immobilized enzyme reaction with acetylcholine esterase and choline oxidase [5]. The procedure is outlined in Fig. 2. ACh, Ch and EHC could be measured with high sensitivity using this HPLC–ED system.

The peak areas increased linearly with increasing volume injected for ACh from 0.3 pmol to 5 nmol and for Ch from 1 pmol to 5 nmol. The calibration graph ($n=3$) for ACh showed linearity in the range 1–60 pmol and that for Ch in the range 10–300 pmol.

The absolute recoveries ($n=3$) of added ACh (312 pmol per 2 ml), Ch (3.1 nmol per 2 ml) and EHC (3.1 nmol per 2 ml) were ACh 91.7%, Ch 104.5% and EHC 88.5%.

Fig. 3 shows typical chromatograms of a human CSF sample (containing 156 pmol of EHC as internal standard) obtained from a 24-year-old volunteer and of an authentic standard sample (156 pmol of Ch, 156 pmol of EHC and 15.6 pmol of ACh). The peaks of Ch, EHC and ACh from the CSF sample were clearly separated and were identified by comparison with the authentic samples.

Concentrations of ACh in CSF from normal subjects ($n=25$) ranged from 0 to

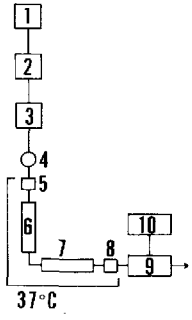

Fig. 2. Schematic diagram of the HPLC-ED system: 1=solvent reservoir; 2=degasser; 3=pump; 4=sample injector; 5=guard column; 6=analytical column; 7=immobilized enzyme column; 8=catecholamine trap column; 9=electrochemical detector; 10=recorder.

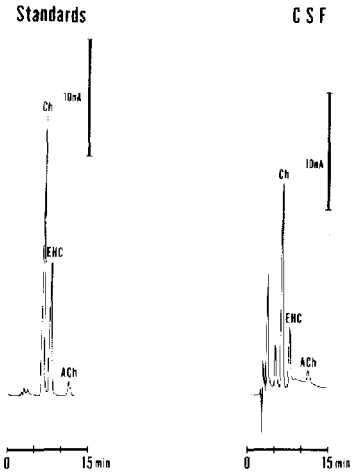

Fig. 3. Chromatograms of standards and a human CSF extract containing 2.5 nmol of EHC as an internal standard. The standards contained 15.6 pmol of ACh and 156 pmol each of Ch and EHC.

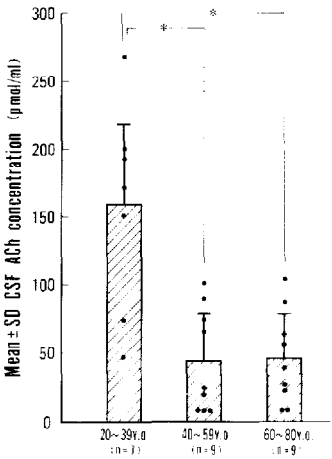

Fig. 4. Concentrations of ACh in human CSF (mean ± S.D.). (*) $P < 0.01$

268.9 pmol/ml. Five subjects had no detectable levels of ACh. For the statistical analysis, their levels were calculated as 10 pmol/ml, which was the detection limit with the present system. The mean and standard deviation for all the specimens were 78.2 ± 69.1 pmol/ml. The elderly and middle-aged groups demonstrated markedly lower concentrations of CSF ACh (47.7 ± 32.0 and 45.8 ± 36.1 pmol/ml, respectively) than the youngest group (159.0 ± 70.5 pmol/ml) as shown in Fig. 4 ($p < 0.01$).

The CSF concentrations of Ch in 25 human subjects ranged from 684.1 to 4338.0 pmol/ml (mean 2362.3 ± 991.7 pmol/ml). The elderly, middle-aged and young groups had similar levels of Ch (2791.7 ± 902.2, 2503.9 ± 959.9 and 1628.2 ± 692.3 pmol/ml, respectively). There were no significant differences in Ch concentration among the three groups.

DISCUSSION

Potter et al. [3] first developed an assay for ACh and Ch by HPLC–ED in brain tissues of rats killed by microwave irradiation. Fujimori and Yamamoto [6] later developed an immobilized enzyme reactor for assaying ACh instead of enzyme infusion as reported by Potter et al. This method is extensively used for measuring the concentration not only in the brain but in many other tissues and also in perfusate obtained from various tissues, e.g., brain slices. It is difficult, however, to use this system for measuring ACh concentrations in CSF because of its very low level. In previous reports [3,6], HPLC–ED was combined with various methods for extracting ACh from brain tissues. We found, however, that ACh was not satisfactorily extracted from CSF. The liquid cation-exchange method described here is a known procedure for extracting choline compounds [4]. By using this procedure coupled with the HPLC–ED system, we were able to measure ACh and Ch contents simultaneously in human CSF.

A method for assaying ACh and Ch in CSF by HPLC–ED combined with an immobilized enzyme reactor is reported in this paper for the first time. Until recently, ACh and Ch were routinely measured by biological methods using frog rectus abdominis muscle [7] or leech dorsal muscle, etc., or GC–MS [8]. In a report on biological methods, Duvoisin and Dettbarn [7] stated that ACh in human CSF was detectable by biological assay in all instances, but pointed out the problems concerning the biological methods for ACh published by others. They reported that the mean ACh value in normal subjects was 0.10 ± 0.03 μmol/l [mean $\pm$ standard error (S.E.)]. On the other hand, using a GC–MS method, Welch et al. [8] determined the contents of ACh and Ch in human CSF from normal subjects and from patients with Huntington's chorea and Parkinson's disease and reported a mean value in normal subjects of 0.07 ± 0.02 μmol/l (mean $\pm$ S.E.). In our work, ACh and Ch were also detected in the CSF specimens examined, except in five cases in the middle-aged and elderly groups. Our results do not differ from those of Duvoisin and Dettbarn and Welch et al. with respect to the concentration of ACh and Ch in human CSF. However, their methods are very complicated and may have suffered from problems concerning specificity and sensitivity [9]. The advantages of our system for assaying ACh and Ch are simple extraction from CSF, stability on storage after lyophilization and a short analysis time by HPLC–ED (15 min).

The HPLC–ED method combined with an immobilized enzyme reactor is highly specific, sensitive and simple compared with biological methods and GC–MS. Further, it is inexpensive, rapid and reliable for analysing ACh and Ch in CSF.

A significant ageing-induced change of ACh concentration in human CSF has been demonstrated for the first time in this work. Previously, the central cholinergic activity was evaluated only by assaying choline acetyltransferase (CAT) and acetylcholinesterase (ChE) activities in brain regions or CSF because of the extremely rapid degradation of ACh after death and the stability of CAT and ChE. Some investigators have also pointed out a significant decrease in CAT activity in the brain and CSF from Alzheimer patients [2,10]. Their results suggest that degeneration of the cholinergic system of the central nervous system

plays a significant role in some mental disorders such as senile dementia and Alzheimer's disease. Thus cholinergic activity may be assessed by directly assaying ACh itself in human CSF. The method described here for CSF may become a useful technique in studying not only normal ageing but also the pathogenesis of these forms of dementia.

REFERENCES

1 H.H. Dale and H.W. Dudley, J. Physiol., 68 (1929) 97.
2 P. Davies and A.J.F. Maloney, Lancet, ii (1976) 1403.
3 P.E. Potter, J.L. Meek and N.H. Neff, J. Neurochem, 41 (1983) 188.
4 F. Fonnum, Biochem. J., 115 (1969) 465.
5 T. Yao and M. Sato, Anal. Chim. Acta, 172 (1985) 371.
6 K. Fujimori and K. Yamamoto, J. Chromatogr., 414 1987) 167.
7 R.C. Duvoisin and W.D. Dettbarn, Neurology, 17 (1967) 1077.
8 M.J. Welch, C.H. Markham and D.J. Jenden, J. Neurol. Neurosurg. Psychiatr., 39 (1976) 367.
9 I. Hanin, in S. Spector and N. Back (Editors), Modern Methods in Pharmacology, Alan R. Liss, New York, 1982, p. 29.
10 D.M. Bowen, C.B. Smith, P. White and A.N. Davison, Brain, 99 (1976) 559.