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Simultaneous high-performance liquid chromatographic determination of norepinephrine, serotonin, acetylcholine and their metabolites in the cerebrospinal fluid of anaesthetized normotensive rats

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SUMMARY

A high-performance liquid chromatographic method with electrochemical detection was developed for the simultaneous determination of the levels of norepinephrine (NE), serotonin (5-HT), acetylcholine (ACh) and their metabolites in the cerebrospinal fluid (CSF) of anaesthetized rats. The response curve for each compound was linear for the concentration way of interest. The within- and between-day coefficients of variation (C.V.) for NE, 5-HT and their metabolites were less than 7 85% and 15.67%, respectively, and those for ACh and choline were less than 3.08% and 6.27%, respectively. This simultaneous determination should be useful for elucidating the noradrenergic, serotonergic and cholinergic nerve activity in the central nervous system.

INTRODUCTION

Norepinephrine (NE) and serotonin (5-hydroxytryptamine; 5-HT) are principal biogenic monoamines which play an important role in neurological disorders and cardiovascular diseases such as hypertension and/or acute stroke as neurotransmitters in the central nervous system (CNS) [1–4]. Acetylcholine (ACh), a neurotransmitter of the CNS cholinergic nerve, is believed to play a significant role in neuropsychological disorders such as Alzheimer's disease and progressive dementia [5–7].

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Histochemical studies indicate that a high density of noradrenergic nerves innervate specific regions of the brainstem and hypothalamus. As these regions of the brain are located close to the third ventricle, the NE release in this area should influence the NE levels in the cerebrospinal fluid (CSF). In fact, an NE-releasing drug, tyramine, increased the NE concentration in the CSF [8]. On the other hand, it has been demonstrated that the choroid plexus, where serotonergic nerves are located, is the major site of CSF production. Moreover, CSF acetylcholinesterase (AChE) has been shown to have a neuronal origin. Therefore, the determination of the concentration of these neurotransmitters in the CSF would be useful in elucidating the neuronal activity in the CNS and clarifying the cause of neurological disorders. In general, monoamine metabolites in the CSF are accepted as an indirect index of CNS monoamine turnover because there is a correlation between monoamine metabolite concentrations in the CSF and those in various regions of the brain [9–12]. However, the actual monoamine and ACh in the CSF provide a more direct index of central nervous activity.

Various methods, including radioenzymatic assay (REA) [1,13,14], spectrofluorimetry [15,16] and gas chromatography–mass spectrometry (GC–MS) [17] have been employed to determine NE and 5-HT in the CSF. These methods, however, are not sufficiently simple, selective or sensitive. Recently, there have been reports concerned with the determination of NE and 5-HT concentrations in the CSF that indicate that high-performance liquid chromatography (HPLC) with electrochemical detection (ED) is a simple and rapid method for determining these concentrations [18–20]. However, most animal studies have been concerned with the determination of monoamine metabolites rather than the monoamines themselves because of methodological difficulties. Moreover, there have been few reports of the determination of ACh and its precursor/metabolite choline (Ch) in the CSF.

This study was undertaken to determine simultaneously the levels of NE, 5-HT, ACh and their metabolites in the CSF of anaesthetized rats. An attempt was made to determine (1) NE, 5-HT and their metabolites, such as 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA) in the CSF using HPLC–ED and (2) ACh and Ch in the same CSF sample using HPLC–ED connected with an immobilized enzyme column. Using these methods, central noradrenergic, serotonergic and cholinergic nerve activity were elucidated.

EXPERIMENTAL

CSF sampling and preparation

Male normotensive 15–20-week-old Wistar rats were used. Rats were anaesthetized intraperitoneally with α-chloralose (50 mg/kg) and urethane (500 mg/kg). To obtain CSF, a polyethylene cannula (4 cm$\times$0.9 mm I.D.) was

inserted into the cisterna magna via the atlanto-occipital membrane, which was exposed under a microscope. After a recovery period of about 30 min, CSF was allowed to flow by gravity from the cisterna magna into an iced microhaematocrit tube (7.5 cm × 1.5 mm I.D.), then collected in polypropylene tubes in an ice–water jacket. Each fraction was examined under a microscope in a Neubauer chamber to prevent blood contamination of the CSF samples. A few minutes after sampling for 5-HT, DOPAC, HVA and 5-HIAA determination, 5–10 μl of unprocessed CSF with epinine as internal standard was injected directly into the HPLC system. The CSF for determination of catecholamines (CA) [NE, epinephrine (Ep) and dopamine (DA)] was stored in a freezer at −20 °C and assayed the next day by alumina extraction. More specifically, 70–100 μl of CSF were added to 1 ml of 0.1 *M* EDTA disodium salt solution containing 100 pg of 3,4-dihydroxybenzylamine (DHBA) as internal standard and 20 mg of acid-prepared alumina, and then adjusted at pH 8.4 with 0.1 *M* ammonium acetate buffer. This mixture was shaken for 10 min and the alumina was washed three times with distilled water. CA that was subsequently adsorbed on the alumina was eluted with 0.01 *M* hydrochloric acid. The eluate was filtered through a 0.22-μm filter (Millipore, Bedford, MA, U.S.A.) and a 25–50 μl volume was injected into the HPLC system. For ACh and Ch deter-

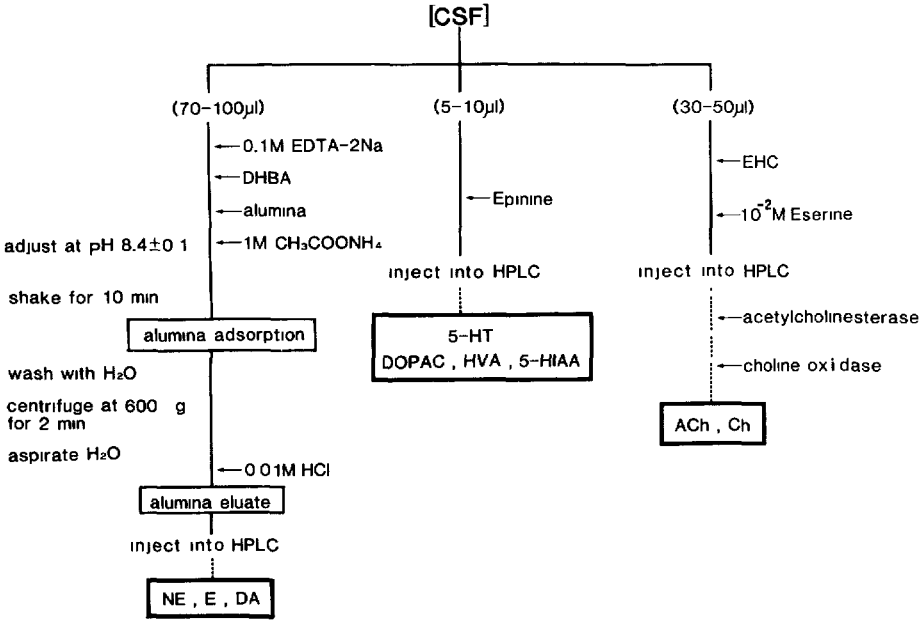

Fig. 1. Flow scheme of the procedure for the simultaneous determination of norepinephrine (NE), serotonin (5-HT), acetylcholine (ACh) and the monoamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA) in CSF using HPLC-ED.

mination, 30–50 μl of CSF added to 10^{-2} *M* eserine solution containing ethylhomocholine (EHC) as an internal standard was injected directly into the HPLC system. The total CSF sampling volume was less than 150 μl for each rat (Fig. 1).

Apparatus and chromatographic conditions

NE, 5-HT and monoamine metabolite determination. The HPLC apparatus consisted of a pump system (P-500), reversed-phase column [RP-18 octadecylsilane (ODS), 5 μm particle size, 250×4.5 mm I.D.] and electrochemical detector (E-502), all from Irika Kogyo (Kyoto, Japan). A graphite working electrode (WE-3G) (Eicom, Kyoto, Japan) was maintained at 700 mV vs. an Ag/AgCl reference electrode. The flow-rate was 0.5–0.7 ml/min, depending on the column used. The column was continuously perfused at room temperature with the mobile phase at the minimum flow-rate when not employed for actual determination. The mobile phase, consisting of 0.17 *M* monochloroacetic acid and 0.1 m*M* EDTA disodium salt, was adjusted to pH 3.22 with 1 *M* sodium hydroxide. Both 200 mg/l sodium 1-octanesulphonate as ion-pair reagent and 8% acetonitrile were added to this solution. This mobile phase was passed through a 0.22-μm filter and degassed with a water aspirator for 20 min prior to use.

ACh and Ch determination. The HPLC apparatus consisted of a pump system (EP-10), guard column (AC-ODS, 5×4 mm I.D.), separation column (AC-Gel, styrene polymer, 10 μm particle size, 150×6 mm I.D.), amine trap column (CA-Trap, strong cation-exchange column, 5×4 mm I.D.), immobilized enzyme column (AC-Enzympak, 5×4 mm I.D.) and electrochemical detector (ECD-100), all from Eicom. A platinum working electrode was maintained at 450 mV vs. an Ag/AgCl reference electrode for the detection of hydrogen peroxide. The flow-rate was a constant 1.0 ml/min. The HPLC separation and enzymatic reaction were performed at 33 °C. The mobile phase consisted of 0.1 *M* sodium phosphate buffer (pH 8.0) containing 65 mg/l tetramethylammonium chloride and 300 mg/l sodium 1-decanesulphonate as ion-pair reagent. This solution was passed through a 0.22-μm filter and degassed as in the monoamine determination. These standards were injected routinely at the beginning and end of the determination.

Reagents

The standard mixture for monoamine determination was prepared from norepinephrine bitartrate (NE), epinephrine bitartrate (Ep), dopamine hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA), 5-hydroxytryptamine creatinine sulphate (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), 3,4-dihydroxybenzylamine hydrobromide (DHBA) and N-methyldopamine hydrochloride (epinine). A stock standard solution of each compound was prepared at 1 mg/ml in 0.1 *M*

perchloric acid and stored at 4°C. The standard mixture for ACh determination was prepared from acetylcholine chloride (ACh), choline chloride (Ch) and ethylhomocholine (EHC). All compounds were purchased from Sigma (St. Louis, MO, U.S.A.). EHC was synthesized from N,N-dimethyl-3-amino-1-propanol and iodoethane and a stock standard solution was prepared at 0.1 *M* in distilled water and stored at 4°C. These standards were injected routinely at the beginning and end of the determination. Each compound in CSF samples was identified from the retentions times for the standard mixture. Concentrations of these compounds were determined by comparison with the peak heights of internal standards. The data obtained were expressed as mean ± standard error of the mean (S.E.M.).

RESULTS

Chromatographic determination of NE, 5-HT and monoamine metabolite

A typical chromatogram of a standard solution of CA, 5-HT and the monoamine metabolites DOPAC, HVA and 5-HIAA is shown in Fig. 2A. The peaks for all the compounds showed good separation. Fig. 2B shows a chromatogram of 5-HT and monoamine metabolites in CSF injected directly into the HPLC–ED system. As the monoamine metabolite concentrations were about 10–100 times greater than the 5-HT concentrations in the CSF, different intensity scales were always used. The internal standard, epinine, was not routinely detectable in CSF samples. Fig. 2C shows a chromatogram of CA in CSF obtained by injecting eluate from the alumina extraction. As regards the DOPAC concentration in CSF, a good correlation between the method based on direct injection of unprocessed CSF samples and the alumina extraction method was obtained. The mean recovery of DHBA was 60.10 ± 2.30%. The absolute detection limit of CA was 0.5 pg per injection (signal-to-noise ratio = 3). The calibration graphs for known amounts of NE, 5-HT, DOPAC, HVA and 5-HIAA which were added to pooled CSF were examined. Good linearity of the response curves for each compound was obtained over the range 0.05–1.2 ng per injection. The within-day coefficients of variation (C.V.) ($n=7$) of NE (concentration 0.25 ± 0.01 ng/ml), 5-HT (0.57 ± 0.02 ng/ml), DOPAC (10.88 ± 0.02 ng/ml), HVA (8.48 ± 0.17 ng/ml) and 5-HIAA (106.18 ± 0.02 ng/ml) were 7.28, 7.85, 0.45, 5.38 and 3.55%, respectively. The between-day C.V. ($n=9$) of NE (0.27 ± 0.01 ng/ml), 5-HT (0.53 ± 0.02 ng/ml), DOPAC (11.00 ± 0.18 ng/ml), HVA (8.14 ± 0.15 ng/ml) and 5-HIAA (95.42 ± 3.6 ng/ml) were 15.67, 9.73, 4.83, 5.42 and 11.33%, respectively.

Chromatographic determination of ACh and Ch

Fig. 3 shows typical chromatograms of ACh and Ch (A) in a standard solution and (B) in CSF. The peaks of ACh, Ch and EHC were determined within 15 min and showed a clear separation. The absolute detection limit of ACh and

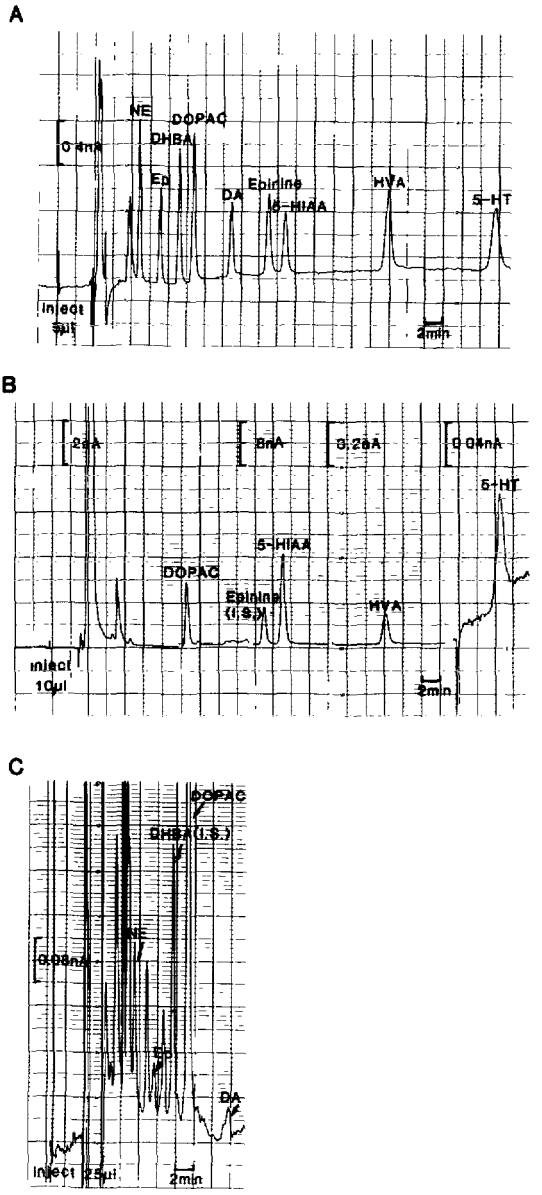

Fig. 2. Chromatograms of (A) a standard solution of 50 pg of NE, Ep, DA, 5-HIAA, 5-HT, DHBA and epinine and 100 pg of DOPAC and HVA, (B) a directly injected unprocessed rat CSF sample, with CSF DOPAC, 5-HIAA, HVA and 5-HT concentrations of 21.64, 148.15 and 31.11 ng/ml and 767.85 pg/ml, respectively (epinine internal standard) and (C) alumina extracts simultaneously determined from rat CSF samples with CSF NE, Ep and DA concentrations of 364.79, 196.85 and 184.02 pg/ml, respectively (DHBA internal standard). The applied potential was 700 mV vs. an Ag/AgCl reference electrode and the flow-rate was 0.7 ml/min.

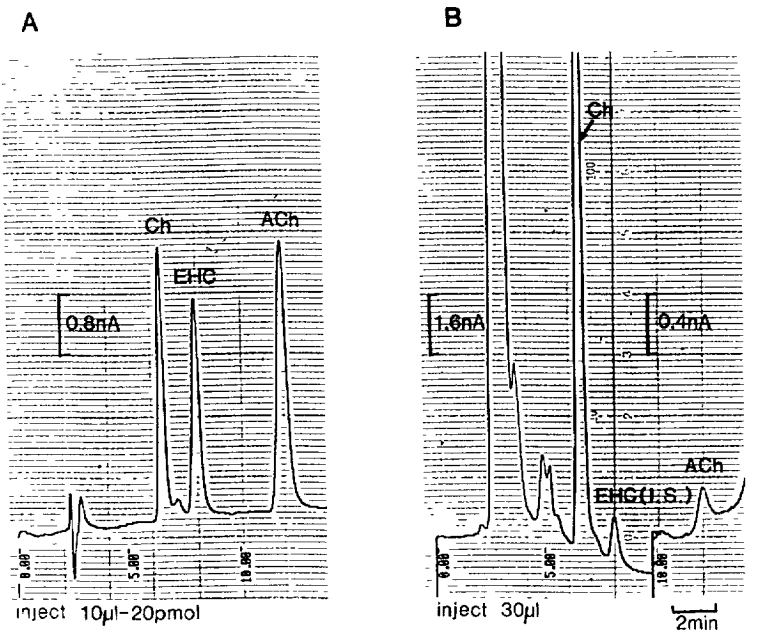

Fig. 3. Chromatograms of (A) standard solutions containing 20 pmol each of ACh, Ch and EHC and (B) a sample of directly injected rat CSF, with ACh and Ch concentrations of 75.75 pmol/ml and 8.65 nmol/ml, respectively (EHC internal standard). The applied potential was 450 mV vs. an Ag/AgCl reference electrode and the flow-rate was 1.0 ml/min.

Ch was 0.2 pmol per injection (signal-to-noise ratio = 5). The calibration graphs for ACh and Ch, which were added to the pooled CSF, showed good linearity over the ranges 1–50 and 10–800 pmol per injection, respectively. The within-day C.V. ($n=8$) for ACh (ratio to EHC = 1.19 ± 0.01) was 0.87% and for Ch (2.48 ± 0.03) it was 3.08%. The between-day C.V. ($n=8$) for ACh (1.22 ± 0.01) and Ch (2.60 ± 0.06) were 2.68 and 6.27%, respectively.

CSF concentrations of NE, 5-HT and the monoamine metabolites DOPAC, HVA and 5-HIAA

The NE concentration in the CSF ranged from 100.12 to 836.73 pg/ml with a mean ($\pm$S.E.M.) of 431.44 ± 56.34 pg/ml ($n=19$) and the 5-HT concentration in the CSF ranged from 240.00 to 1687.04 pg/ml with a mean of 808.77 ± 144.30 pg/ml ($n=10$) (Fig. 4). The CSF concentration of the DA main metabolites, DOPAC and HVA, ranged from 15.45 to 33.96 and from 26.27 to 69.81 ng/ml, respectively. The CSF concentration of 5-HT principal metabolite, 5-HIAA, ranged from 111.56 to 203.70 ng/ml. The mean values of these monoamine metabolites are given in Table I.

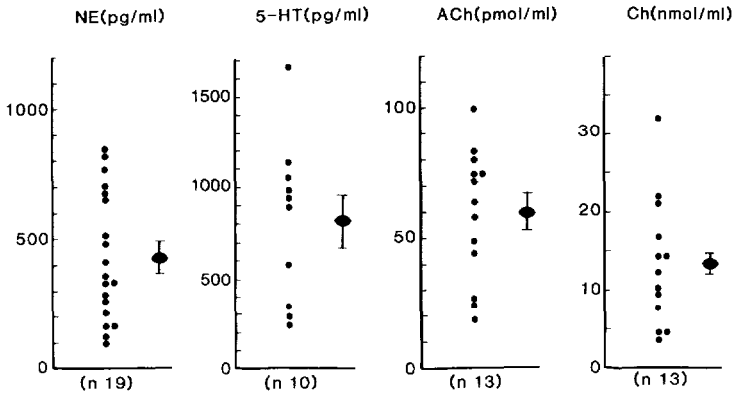

Fig. 4. Concentrations of NE, 5-HT, ACh and Ch in the CSF of normotensive Wistar rats. Each value represents the mean ± S.E.M. Numbers in parentheses indicate the number of rats.

TABLE I

CONCENTRATIONS OF MONOAMINE METABOLITES IN THE CEREBROSPINAL FLUID OF RATS

Compound[a]	*n*	Concentration (mean ± S.E.M) (ng/ml)
DOPAC	10	25.16 ± 1.72
HVA	10	38.81 ± 4.37
5-HIAA	10	169.28 ± 8.89

[a]DOPAC = 3,4-Dihydroxyphenylacetic acid; HVA = 4-hydroxy-3-methoxyphenylacetic acid; 5-HIAA = 5-hydroxyindole-3-acetic acid.

CSF concentrations of ACh and Ch

The ACh concentration in the CSF ranged from 18.56 to 100.84 pmol/ml with a mean (± S.E.M.) of 59.41 ± 7.06 pmol/ml ($n = 13$) and the CSF Ch concentration ranged from 3.63 to 32.73 nmol/ml with a mean of 13.58 ± 2.35 mmol/ml ($n = 13$) (Fig. 4).

DISCUSSION

It is well known that CSF monoamine metabolite concentrations reflect the monoaminergic nerve activity in the CNS [9–12]. However, the monoamines themselves rather than their metabolites in the CSF would provide a more direct index of neuronal activity in the CNS. For instance, in human studies, reports that CSF NE concentrations increased in hypertensive patients with cerebral infarction [3,4] or in primary hypertensive patients [1,2] suggest that increased central noradrenergic nerve activity is involved in the pathogeneses.

Meyer et al. [3] reported that the 5-HT concentration in the CSF also increased after the onset of cerebral infarction. Despite numerous reports on monoamine metabolites in the rat CSF [21,22], few have focused on the determination of the monoamines themselves because of the small CSF sample volume. In this study, NE, 5-HT and the monoamine metabolites DOPAC, HVA and 5-HIAA in the CSF were determined in rats using HPLC–ED with a newly developed and highly sensitive graphite electrode. The method showed good reproducibility, high selectivity and high sensitivity. Moreover, CSF 5-HT and monoamine metabolite levels obtained by this method concur with previous reports [16,23,24].

We simultaneously determined the ACh and Ch in the same rat CSF sample using an HPLC–ED system connected with an immobilized enzyme column. The first determination of ACh and Ch using HPLC–ED was reported by Potter et al. [25]. Since the immobilized enzyme column replaced their infused enzyme column method, many reports concerning ACh determination in the brain [26], heart [27] and blood cells [28] have appeared. However, no reports are available on the determination of ACh and Ch in the rat CSF. In this study, ACh and Ch in CSF were determined with good reproducibility and high selectivity. Moreover, the levels of ACh in the CSF of rats did not differ from those of humans [29–31]. The role of CSF ACh in various pathogeneses is still unclear. Several cardiovascular and neuropsychological studies have reported acetylcholinesterase (AChE) or choline acetyltransferase activity in the CSF. One example is that CSF AChE activity increased in patients with acute cerebral infarction [32]. In progressive dementia, CSF AChE activity decreased [7], whereas in Alzheimer's disease, CSF choline acetyltransferase activity decreased [6]. A recent report about ACh determination in human CSF demonstrated that CSF ACh concentrations in middle-aged (40–59 years) and elderly (60–80 years) normal subjects were markedly lower than that of a young group (20–39 years) [29]. These facts suggest that CSF ACh may change in various pathogeneses. Hence, the determination of ACh and Ch concentrations in the CSF may provide a direct indication of CNS cholinergic nerve activity.

In conclusion, the simultaneous determination of NE, 5-HT, ACh and their metabolites in the CSF is a useful method in neurochemical studies to investigate the activity of monoaminergic and cholinergic nervous systems.

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