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Note

Improved analysis of acetylcholine and choline in canine brain and blood samples by capillary gas chromatography—mass spectrometry*

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Acetylcholine (ACh) is an important neurotransmitter in the central and peripheral nervous system. Investigations of this neurotransmitter and its metabolism often require determination of ACh and choline (Ch) concentrations in biological specimens. Several gas chromatographic and gas chromatographic—mass spectrometric (GC—MS) methods have been reported for the analysis of ACh and Ch in tissue [1—5]. These methods utilize extraction of ACh and Ch by either ammonium reinecktate precipitation [6] or dipicrylamine extraction [7] followed by conversion of the extracted ACh and Ch to dimethylaminoethylacetate and dimethylaminoethanol, respectively, with sodium benzenethiol as described by Jenden et al. [8].

This paper describes the application of a sensitive capillary column for the analysis of ACh and Ch in brain and blood samples utilizing a GC—MS method. The column and chromatographic conditions used in this study improved sensitivity of the assay and separation of ACh, Ch, and butyrylcholine (BCh, internal standard). This technique was used to determine the concentrations of ACh and Ch in various regions of canine brain and in canine blood.

EXPERIMENTAL

Materials

The gas chromatograph—mass spectrometer used was a Hewlett-Packard

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Model 5993C-OP-95 with an electron-impact ionizer and a 15-m capillary HP-SE 54 bonded methyl silicon column. Dipicrylamine (2,2,4,4,6,6-hexanitrodiphenylamine) was obtained from Aldrich. ACh, Ch, BCh, and tris-(hydroxymethyl)methylaminopropane sulfonic acid (TAPS) were purchased from Sigma. Benzenethiol and silver *p*-toluenesulphonate were purchased from Kodak.

Extraction of ACh, Ch and BCh

Brain samples. Cerebral tissues were obtained from halothane (2%) anesthetized adult mongrel dogs and from canine brains isolated [9] and perfused for 90 min with heparinized dog blood as previously described [10]. (The perfused brain allows maintenance of a constant and defined perfusate composition and flow-rate.) Tissue samples were collected rapidly (within 2—4 min) after cessation of cerebral blood flow and immediately frozen in liquid nitrogen prior to storage at —70° C until analysis.

The extraction procedure of Kosh et al. [7] was implemented for the extraction of brain samples. Frozen samples obtained from various regions of canine brains (non-perfused and perfused) were weighed and mixed with 0.1 ml of BCh (0.1 μmol/ml). The mixture was homogenized in acetone—formic acid (85:15) at 0.5 g/ml. The homogenate was centrifuged, and the supernatant was collected. The supernatant was mixed with 2 vols. of diethyl ether, and the organic phase was aspirated. The aqueous phase was mixed with 0.5 ml of 2 *M* TAPS buffer (pH 9.5) and 2—4 ml of dipicrylamine (2 m*M* in dichloromethane). Each sample was vortexed for 2 min and centrifuged. The lower dipicrylamine phase was transferred into a screw-cap tube and was evaporated under a stream of nitrogen. The dry residue was redissolved in 0.5 ml of silver *p*-toluenesulphonate (5 m*M* in acetonitrile) and mixed with propionyl chloride (50 μl). After 5 min, samples were dried in nitrogen, and 0.5 ml of reaction mixture was added to each tube (reaction mixture was sodium benzenethiol in methyl ethyl ketone, 6 mg/ml, containing 25 m*M* benzenethiol). The mixture was incubated at 80° C for 30 min. The ACh, Ch, and BCh were extracted in chloroform by the method described by Kosh et al. [7].

Blood samples. Freshly collected or frozen blood samples were mixed with 0.1 ml of 0.1 μmol/ml BCh and were deproteinized with 0.6 *M* perchloric acid. After complete deproteinization, samples were centrifuged at 4° C. The supernatant was transferred into a screw-cap tube and washed with 5 ml of diethyl ether. The organic phase was aspirated. The aqueous phase was mixed with 0.5 ml of 2 *M* TAPS buffer (pH 9.5) and 2—4 ml of dipicrylamine (2 m*M* in dichloromethane). ACh, Ch, and BCh were extracted from the dipicrylamine layer by the method of Kosh et al. [7].

Preparation of standards

Standards consisted of 0.5 ml of 5 m*M* silver *p*-toluenesulphonate in acetonitrile, 20 pmol ACh, 40 pmol Ch and 20 pmol BCh. These were prepared by making up a solution of 5 m*M* silver *p*-toluenesulphonate in acetonitrile and 1.0 m*M* of these esters in methanol, followed by transfer of 500, 20, 40, and 20 μl, respectively, to reaction tubes and evaporation of the solution with dry nitrogen. These standards were esterified, demethylated and analyzed simultaneously with the tissue sample.

GC—MS conditions

The following conditions were selected to yield a short run time with clean separation of ACh, Ch, and BCh. Inlet temperature: 180°C; oven temperature: 60°C for 3 min, then increasing 25°C/min to 200°C; source chamber pressure: $8 \cdot 10^{-4}$ Pa ($6 \cdot 10^{-6}$ Torr); electron-impact voltage: 70 eV. Mass detection was initiated 2 min after sample injection to allow the elution of solvent and to protect the ion detector. To determine the mass fragmentation pattern for the demethylated products of ACh, Ch, and BCh, individual standards were processed and extracted in chloroform by the method described above, and 1 μl of the chloroform extract was injected into the GC—MS system. Ions were monitored in the range 30—300 *m/z*.

The ion at *m/z* 58 was monitored to determine the concentrations of ACh and Ch in external standards and tissue extracts.

Determination of recovery

Each brain sample was divided into two parts. ACh and Ch were extracted from one part by the method described, and the amounts of these substances were determined with appropriate external standards. The second part of each sample was spiked with known amounts of ACh and Ch before extraction. The amounts of ACh and Ch recovered were determined by subtracting the endogenous ACh or Ch amount from the amount of ACh or Ch present in the spiked samples.

RESULTS AND DISCUSSION

Selection of ions

Similar to the observations of Zsilla et al. [11] we also observed that the ion at *m/z* 58 was present in greatest abundance for all three compounds. Ions at *m/z* 71 and 72 were also present; however, the relative abundancies of these ions were < 1% of the base ion (*m/z* 58). Molecular ions were not detected. Ion *m/z* 58 was selected for monitoring ACh, Ch, and BCh because of its uniqueness and high abundance for these compounds. Ions at *m/z* 71 and 72 were also unique for these compounds and could be included in ion monitoring. However, in our experience, increasing the number of low-abundance ions monitored decreased the sensitivity of this method.

Separation of ACh, Ch, and BCh

Our capillary column provided excellent separation of ACh, Ch, and BCh extracted from tissue samples (Fig. 1). The initial solvent peak was absent since the ion monitoring did not begin until 2 min after injection. Under the chromatographic conditions described, ACh, Ch, and BCh eluted at 2.3, 3.5, and 4.7 min, respectively. These observations suggest that the performance of the capillary column used in this study was superior to glass columns [6, 7].

Recovery of ACh and Ch

Approximately 80% of the added ACh and Ch was recovered from tissue samples (Fig. 2). Fig. 2 also demonstrates the linearity of the method at concentrations ranging from 10 to 100 pmol/ml. Kosh et al. [7] have shown that

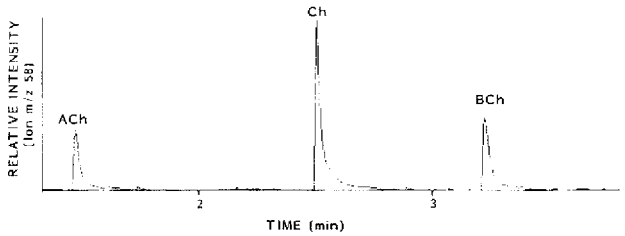

Fig. 1. Analysis of acetylcholine (ACh) and choline (Ch) in brain. Brain tissue extracts were prepared and subjected to quantitative GC—MS as described in the text using butyrylcholine (BCh) as the internal standard. Peak areas were used for quantitation.

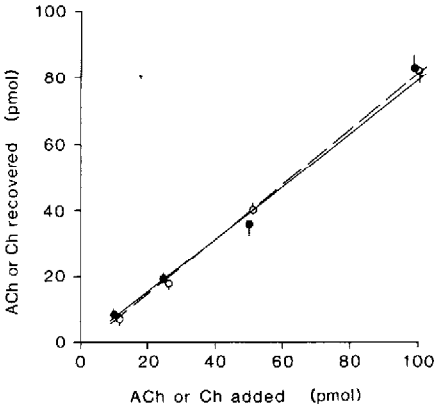

Fig. 2. Recovery of acetylcholine (ACh, •) and choline (Ch, ○) from brain samples. Brain samples were spiked with various concentrations of ACh and Ch. Samples were processed by the procedure described in the text for analysis of ACh and Ch. The amounts of ACh and Ch recovered were determined by subtracting the endogenous ACh or Ch from the amount of ACh and Ch present in the spiked samples, $n = 3$.

the linearity of the choline curve is lost at concentrations above 400 nmol/ml. The lower limit of detection using this method at 2200 electron multiplier volts (emV) was 5 pmol/ml for both ACh and Ch. At 2800 emV, however, the detection limit was approximately 0.5 pmol/ml.

ACh and Ch levels

We have analyzed various regions of canine brain for ACh and Ch. Similar to the previous observations in intact rat brain [12], we also observed differences in the concentration of ACh in various regions of canine brain (Table I). Brain cortex and cerebellum had the highest and lowest ACh values, respectively. ACh levels in various regions of canine brain were significantly lower than the previously reported ACh levels in the same regions of rat brain [7]. Unlike ACh, distribution of choline in the canine brain did not show any regional differences (Table I).

We have determined the levels of ACh and Ch also in the isolated canine brain perfused for at least 1.5 h as described previously [9, 10]. The ACh values in the various regions of non-perfused and isolated perfused canine brain did not differ significantly (Table I). The Ch values in the isolated perfused canine brain were significantly higher than the Ch values in the non-perfused brain (Table I). Although the reasons for the high Ch values in the

TABLE I

CONCENTRATIONS OF ACETYLCHOLINE AND CHOLINE IN NON-PERFUSED AND ISOLATED PERFUSED CANINE BRAIN

Values are mean ± S.D., $n = 3$

Brain region	Acetylcholine (nmol/g)		Choline (nmol/g)	
	Non-perfused brain	Isolated perfused brain	Non-perfused brain	Isolated perfused brain
Cerebral cortex	4.3 ± 0.7	7.5 ± 2.3	41.0 ± 2.1	288 ± 170
Cerebellum	0.5 ± 0.2	0.5 ± 0.1	60.9 ± 7.2	273 ± 53
Hippocampus	1.2 ± 0.3	0.8 ± 0.2	67.3 ± 5.3	250 ± 32
Brain stem	4.0 ± 0.2	2.6*	78.3 ± 2.4	254*

*$n = 1$.

isolated perfused brain are not known, they might be due either to high extraction of Ch from the perfusate blood or to the increased production of Ch in the brain from phosphatidylcholine or other choline-containing lipids. The Ch value in perfusate blood ranged from 3 to 7 μmol/l. The observations from this study suggest (1) that the capillary column used provided better separation of ACh, Ch, and BCh in blood and brain samples; (2) sensitivity of this assay was considerably higher than other reported methods utilizing glass columns; and (3) dog brain ACh concentrations exhibit significant regional differences and are substantially lower than ACh concentrations observed in rat brain.

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